國 立 交 通 大 學

生物科技研究所

碩 士 論 文

胃幽門螺旋桿菌之熱緊迫蛋白 60 藉由活化 CXCR2 訊息路徑而增加血管新生

***Helicobacter pylori*-derived Heat shock protein 60 could enhance angiogenesis through CXCR-2 signal pathway**

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中 華 民 國 九 十 八 年 六 月

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生 物 科 技 系

碩 士 論 文

A Thesis

Submitted to Institute of Biological Science and Technology

College of Biological Science and Technology

National Chiao Tung University

in partial Fulfillment of the Requirements

for the Degree of

Master

in

Biological Science and Technology

June 2009

Hsinchu, Taiwan, Republic of China

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中文摘要

胃幽門螺旋桿菌已知為可造成胃癌的微生物，此菌之熱緊迫蛋白 60 可誘發發炎反應。本篇研究目的在於探討熱緊迫蛋白 60 在胃癌發展所扮演的角色。首先，我們發現在病人血清中的抗熱緊迫蛋白 60 抗體的表現與癌症有正向的關聯, 從此發現我們推測此蛋白與癌症有關，因此我們進一步的探討熱緊迫蛋白 60 在細胞增生、抗凋亡、血管新生、轉移能力之能力。研究結果顯示熱緊迫蛋白 60 可促進胃癌細胞之血管新生與細胞遷移，但不幫助胃癌細胞之增生與抗凋亡能力。此外，研究結果也顯示熱緊迫蛋白 60 能刺激單核球細胞株 THP1、胃癌細胞株 AGS、人類臍靜脈內皮細胞分泌血管新生因子，並且可促進人類臍靜脈內皮細胞三維血管生成、細胞遷移、間接促進內皮細胞增生。另外，以抑制劑抑制 CXCR2 訊息路徑可抑制熱緊迫蛋白 60 誘發之三維血管生成。因此，從這些結果我們推測胃幽門螺旋桿菌之熱緊迫蛋白可刺激血管新生因子分泌並且藉由 CXCR2 訊息路徑來促進血管新生。

中華民國九十八年六月

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Abstract

Helicobacter pylori, the microbe has been discovered it can promote the malignant process of gastric cancer. Heat shock protein 60 of *H. pylori* (HpHSP60) was previous identified as a potent immunogen. This study aims to study the role of HpHSP60 on gastric cancer carcinogenesis. First, the results of patients' anti-HpHSP60 antibodies in sera were correlated to gastric cancer. According to these finding, this pathogen-derived component was speculated it may play a role in tumor malignant process. Sequentially, we investigated the effect of HpHsp60 on the cell proliferation, anti-death activities, angiogenesis, and metastasis. The results showed HpHSP60 could enhance migration of gastric cancer cells and promote angiogenesis it had no effect on proliferation and rescuing cell death of gastric cancer cells. Moreover, the results showed HpHSP60 could stimulate THP1 monocytic cells, AGS gastric cancer cells, and umbilical vein endothelial cells (HUVECs) to express the angiogeneic factors. In addition, HpHSP60 could also enhance tube formation, migration and indirectly promote proliferation of HUVECs. Furthermore, inhibition of CXCR2 signal decreased the tube formation. Therefore, these results propose HpHSP60 may trigger angiogeneic factor releases and enhance the angiogenesis via CXCR2-dependent pathway.

中華民國九十八年六月

Acknowledgement

時光飛逝，在交通大學兩年的碩士生涯不知不覺就到了尾聲，而我也從剛踏入科學領域漸漸的學習了對待科學所俱備的態度與能力。在這兩年的生涯中，首先我要感謝的是我的指導教授—廖光文老師，感謝老師當我在實驗上或是未來方向有困惑時都會給予非常有用的建議，支持我在研究路上的信心，也很感謝老師總是很有耐心的解決我在實驗上所遇到的難題。

再來是感謝實驗室的各位成員，感謝于鈴學姐與 DG，身為實驗室的博班領導者規化了一套完美的流程，讓我在實驗上變得非常的順利，還有同為 HP 組的成員，啾咪、維瞳、小薇，一起完成 HP 組所須的資料與生產蛋白，還有在小組討論時培養著良好的默氣，感謝阿伯、小溫、小莉、馬馬、靜敏、晨洋雖然是不同組的成員，但是每天跟各位相處都很愉快，並且提供我許多在口頭報告上實用的技巧與建議。

雖然說即將要從這裡結束開啓一段新的人生旅程，但對於自身是否以俱備十足的能力還是沒有足夠的自信，但是想起在交大這段日子陪伴著我的各位伙伴，又讓我多了一份勇氣，最後也感謝我的父母支持我並順利的完成碩士學位。

Contents

List of abbreviation

HSP60	Heat shock protein 60
TNF-α	Tumor necrosis factor-alpha
IL-1β	Interieukin-1beta
IL-6	Interieukin-6
IL-8	Interieukin-8
GRO	Growth regulated oncogene-alpha
ENA-78	Epithelial neutrophil-activating peptide-78
GCP-2	Granulocyte chemotactic protein-2
NAP-2	Neutrophil activating peptide-2
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
HUVECs	Human umbilical vein endothelial cells

Chapter 1: Introduction

1. The background of Heat shock protein 60 (HSP60)

Heat shock proteins (HSPs) are ubiquitous and evolutionary conserved proteins. The first report the fundamental role of HSPs in cellular homeostasis and cell viability was F. Ritossa in 1962. He recognized HSPs by exposing *Drosophila* to 37 ℃ and proteins of 70 and 26 kDa were highly expressed, suggesting they are indispensable to overcome heat stress (Ritossa, 1962). Since then, sequential studies have shown heat shock response is ubiquitous in all organisms from bacteria to plants and animals, and it is an essential defense mechanism for protecting cells from a wide range of damage condition, such as heat shock, alcohol, oxidative stress, fever, or inflammation (Lindquist, 1986; Morimoto, et al., 1993). According to molecular weight, HSPs are functionally related proteins classified into several families: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs. HSPs act as housekeeping function and molecular chaperones that are important for the survival of eukaryotic and prokaryotic cells (Jolly, et al., 2000). In addition, HSPs such as HSP60 from several microorganisms may elicit a strong immune response in mammalian hosts (Zugel, et al., 1999), and HSP60 have been discovered its carcinogenesis

role in *Chlamydia trachomatis* and mammalian (Felice, et al., 2005).

2. The pro-inflammatory roles of HSP60s

HSP60 from several microorganisms such as bacteria, protozoa, fungi and helminthes have been shown to induce host immune response after infection with these organisms (Zugel, et al., 1999). Antibodies specific to *Mycobacterial tuberculosis* HSP60 have been detected in patients with tuberculosis and leprosy and also in mice (Young, et al., 1988; Barrios, et al., 1994). In addition, the HSP60 of *M. tuberculosis* was demonstrated to activate secretion of pro-inflammation cytokines, IL1-β and TNF-α from human monocytes (Friedland, et al., 1992). HSP60 of *Chlamydia pneumoniae* have been demonstrated to cause acute pulmonary inflammation via TLR4 in mice and increase the level of IL-6 in the bronchoalveolar lavage fluid (Bulut, et al., 2009). Bulut and colleagues have identified the *Chlamydia trachomatis* HSP60 could activate macrophages and endothelial cells trough TLR4 to activate NF-κB and promote inflammation (Bulut, et al., 2002.). Similarly, HSP60 of *Helicobacter pylori* has been shown to induce series of pro-inflammation cytokine expression such as IL-1β, IL-8, and IL-6 through TLR2 and 4 from human monocytes and gastric epithelium cells (Zhao, et al., 2007; Lin, et al., 2005; Gobert, et al, 2004).

3. The correlations between HSP60 and cancer

In last few years, HSP60 have been recently discovered its new function. *Chlamydia trachomiatis*, an obligate intracellular bacterium, derived HSP60 has been reported to be a risk factor for women ovarian cancer (Di Felice, et al., 2005). HSP60 of *C. trachomiatis* could form a complex with cleaved caspase-3 and/or Bax and Bak to inhibit apoptosis (Di Felice, et al., 2005). Czarnecka and colleagues have shown that human HSP60 have an anti-apoptosis effect mediated by its ability to form a complex with Bax and Bak in the cytoplasm to inhibite apoptosis (Czarnecka, et al., 2006). In addition, it has been indicated that HSP60 of human could be used as a marker for patient prognosis in a variety of tumors and pre-tumoral lesions such as breast ductal carcinoma, exocervical and prostate carcinogenesis (Bini, et al., 1997; Cappello, et al., 2003).

4. Discovery of *Helicobacter pylori* and development of its role in human disease

4.1 The morphology of *Helicobacter pylori*

In 1982, Marshall and Warren were the first group for isolation of *Helicobacter pylori* organisms are spiral-shape, gram-negative bacteria

approximately 2.5 to 5.0 micrometers long and 0.5 to 1 micrometer wide. In gastric biopsy specimens, *H. pylori* organisms have four to six unipolar sheathed flagella, which are approximately 30 μm long and are essential for motility (Goodwin, et al., 1987; Goodwin, et al., 1990). When staining with tannic acid, it can be observed that the outer membrane of *H. pylori* is coated with a glycocalyx-like structure (Goodwin, et al., 1987). The surface of viable *H. pylori* cells grown on agar plates is coated with 12 to 15 nm ring-shaped aggregates of urease and HSP60 (Austin, et al., 1992). In gastric biopsy specimens, it has been shown that both urease and HSP60 are located in the cytoplasm and surface of all bacterium (Dunn, et al., 1997).

4.2 The epidemiology of *Helicobacter pylori*

H. pylori has been found in the stomach of humans in all parts of the world. Overall *H. pylori* prevalence among the many studies varied from 11% to 69% (Thjodleifssion, et al., 2007). It has been suggested that the low socioeconomic status and high densities of living are associated with high rates of infection (Bruce et al, 2008). In addition, it is well accepted that *H. pylori* infection is acquired in childhood (Malaty, et al., 2001). There have been several studies assessed the risk of gastric caner in the presence of *H. pylori*. By the several meta-analysises, it has been indicated that *H. pylori* infection is associated with

approximately a two-fold increased risk of developing gastric cancer (Huang, et al., 2003; Xue, et al., 2000; Eslick, et al., 1999). In addition, some s studies have shown virulence factors of *H. pylori* may increase the risk of gastric cancer e.g. CagA, VacA, and dupA (Wen, et al., 2008).

4.3 *Helicobacter pylori* associated diseases

It has become clear that *H. pylori* infection was strongly associated with inflammation in the gastric mucosa, and cause polymophonuclear cell infiltration (Blaser, et al., 1990). The persistent infection of *H. pylori* could lead to gastritis, gastric ulcer and duodenal ulcer. In addition, chronic gastritis that *H. pylori*-induced has been indicated to linked to the development of gastric adenocarcinoma (Correa, et al., 1992). In 1994, the International Agency for Cancer Research, an arm of the World Health Organization, declared that *H. pylori* was a carcinogen of humans (Anonymous, 1994). *H. pylori* infection also associated with the development of gastric non-Hodgkin's lymphomas and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Parsonnet, et al., 1994; Eidt, et al., 1994). It has been indicated that eradicate *H. pylori* often contributes to regression of MALT lymphoma (Bayerdorffer, et al., 1995). Together, *H. pylori* has been clearly associated with many of the most important gastroduodenal disease.

5. *H. pylori* infection and angiogenesis

Angiogenesis is the process of new blood vessel growth, and is also a critical biological process under both physiologic and pathologic conditions. Under physiologic conditions, angiogenesis contribute to organ growth with the necessary oxygen to develop in the embryo, but during adulthood, most blood vessels remain quiescent and angiogenesis occurs only in the cycling ovary and pregnancy process (Carmeliet, 2000). By contrast, pathological angiogenesis has been studies in chronic inflammation and tumor malignancy (McDonald, et al., 2003). The first model of tumor angiogenesis was proposed by Judah Folkman in 1971 (Folkman, 1971). After that, several studies have demonstrated that growing masses of tumor cells are able to release angiogeneic signal to formation of new blood vessels for tumor growth (Parangi, et al., 1996, Holmgren, et al., 1995). Besides, the chronic inflammation, which induced by infiltrating immune cells are critical for neovascularization and cancer progression (Ruegg, et al., 2006). The infection of *Helicobacter pylori* could cause to chronic inflammation which also contribute to the neoplastic condition (gastric cancer). It has been suggested that inflammation is a potential candidate to lead to angiogenesis during *H. pylori* infection (Pousa, et al, 2006). Several authors reported *H. pylori* could lead to disorder of gastric microcirculation in

human and mice *in vivo* (Kalia, et al., 2001; Elizalde, et al., 1997). By *in vivo* fluorescence or intravital microscopy, it could observe that leukocyte adhesion and platelet aggregation in the gastric mucosal microvascular which are associated with the inflammatory process after *H. pylori* infection (Kalia, et al., 2003). In addition, infection of *H. pylori* could promote invasion and angiogenesis of gastric cancer cells by up-regulating cyclooxygenase-2, which plays a role in the inflammation and tissue damage (Chang, et al., 2005). Moreover, *H. pylori* also could stimulate IL-8, IL-6, TNF-α, and GRO expression from endothelial cells and up-regulate adhesion molecule of endothelial cells (Innocenti, et al., 2001).

6. Angiogeneic ELR$^+$ CXC chemokines

Chemokines are a family of low molecular weight chemotactic cytokines, which involved in many biological processes, such as attract and activate leukocytes, embryogenesis, angiogenesis, hematopoiesis, atherosclerosis, tumor growth and metastasis (Luster, et al., 1998; Struyf, et al., 2003). There are currently four subgroups within the chemokine family: CXC, CC CX_3C, and C chemokine (X represents any amino acid) depending on the conserved cysteines in the N-terminus of this protein (Zlotnik, et al., 2000). The CXC chemokines

are subdivided into ELR^+ and ELR^- chemokines, based on the presence or absence of glutamic acid-leucine-arginine (ELR) motif to the first cysteine (Strieter, et al., 1995). It has been indicated that the CXC chemokines with the ELR motif promote angiogenesis; on the contrary, CXC chemokines which lack this motif might inhibit angiogenesis (Strieter, et al., 1995). The ELR^+ CXC chemokines family members that promote angiogenesis are CXCL1 (GRO), CXCL5 (ENA-78), CXCL6 (GCP-2), CXCL7 (NAP-2), and CXCL8 (IL-8) (Strieter, et al., 2005). Additionally, after infection of *H. pylori*, IL-8 and GRO expressed from gastric epithelium cells or endothelium cells *in vivo* and *in vitro* (Sievekin, et al., 2004; Innocenti, et al., 2002; Eck, et al., 2000).

7. Role of CXCR2 in mediating ELR^+ CXC chemokines to promote angiogenesis and tumor progression

Initially, it has been indicated that the receptors for activating angiogeneic activity of ELR^+ CXC chemokines were CXCR1 and CXCR2. In fact, only CXCL8 and CXCL6 could specifically bind CXCR1, while, all ELR^+ CXC chemokines could bind and activate CXCR2 (Sunil, et al., 1996; Murdoch, et al., 1999). Subsequent studies have demonstrated the endothelial cells expressed both CXCR1 and CXCR2, whereas, expression of CXCR2, not CXCR1, could

mediate endothelial cell chemotaxis (Addison, et al., 2000). Furthermore, it has been found that IL-8 could induce endothelial cells chemotaxis and calcium fluxes within endothelial cells (Salcedo, et al., 2000). Heidemann et al. found that IL-8 could mediate phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK), proliferation, and chemotaxis of endothelial cells by activating CXCR2 (Heidemann et al., 2003). Using the murine model of melanoma cancer with CXCR2 -/- and CXCR2 +/+ mice, it has been observed a significant inhibition of tumor growth and metastasis in CXCR2-/- mice as well as decrease inflammatory response, and angiogenesis in CXCR2-/- mice (Singh, et al., 2009). These studies established that CXCR2 is an important receptor that mediating ELR^+ CXC chemokines-dependent angiogenesis or tumor growth.

Chapter 2: Materials and Methods

Material

1. Reagent

The following reagents obtained were described as following: RPMI1640, medium 199, Fetal Bovine Serum (FBS) were from Invitrogen Inc. (Gaithersburg, MD, USA). Penicillin/ streptomycin/ amphotericin (PSA) were from Biological industries (Beithaemek, Israel). Kanoamycin and Tris were from MDBio Inc. (Rockville, MD, USA). Isopropyl-beta-D-thiogalactopyranoside, NaCl, yeast extract, agar, Tis-HCl, Triton X-100, TEMED, imidazole, glycin, and 2-mercantoethanol were from Amresco Inc. (Solon, OH, USA). Sephadex G-25 Medium was from Amersham Bioscciences (Uppsala, Sweeden). Heparin, endothelial cell growth supplement (ECGS), thiazolyl blue tetrazdium bromide (MTT), sodium dodecyl sulfate (SDS), curcumin, gelatin, 3,3′,5,5′-tetramethylbenzidine (TMB), dimethyl sulfoxide (DMSO), ammonium persulfate (APS) and propidium iodide (PI) were from Sigma-Aldrich (Steinheim, Germany). Matrigel was from BD Biosciences (Bedford, MA, USA). Mitomycin was from KYOWA (Tokyo, Japan). Goat anti-human IgG, IgA, IgM antibody was from Millipore Co. (Billerica, MA,

USA)

2. Kit

Human IL-6, IL-8, and TNF-a ELISA kit was obtained from R&D systems (Minneapolis, MN). Coomasie Plus^{TM} Protein Assay Reagent kit and Enhanced chemiluminescence (ECL) were from Pierce (Rockford, IL, USA). Human Angiogenesis Antibody Array was from RayBiotech, Inc (GA, USA).

3. Buffer

- 1× PBS

NaCl 8.18 g, KCl 0.2 g, Na2HPO4 1.41 g, KH2PO4 0.245 g in 1 L of DDW

- EDTA-trypsin

Trypsin 2.5 g, 13 ml of EDTA (0.1 M) in 1L of 1 X PBS, pH= 7.4

- LB solution

Tryptone 10 g, Yeast extract 5 g, NaCl 10 g in 1 L of DDW

- LB agar

Tryptone 10 g, Yeast extract 5 g, NaCl 10 g, Agar 15 g in 1 L of DDW

- Alkaline lysis solution I

50 mM of Tris-HCl, 10 mM of EDTA, 100 μg/ml of RNase A in 100 ml of

DDW

- Alkaline lysis solution II

0.02M of NaOH, 1% SDS in 100 ml of DDW

- Alkaline lysis solution III

2.8 M of KOAc in 100 ml of DDW, pH = 5.1

- Buffer N2

KCl 34g, Tris-base 6.057g, 75 ml of Ethanol, 0.75 ml of TritoX100, in 500 ml of DDW, pH= 6.3

- Buffer N3

Tris-HCl 6.057 g, KCl 42.86 g, 79 ml of Ethanol in 500 ml of DDW, pH= 6.3

- Buffer N5

Tris-HCl 6.057 g, KCl 37.3 g, 79 ml of Ethanol in 500 ml of DDW, pH= 8.5

- Binding buffer for protein purification

20 mM of Na2HPO4, 0.5 M of NaCl, 40 mM of Imidazole in 500 ml of DDW, pH= 7.4

- Elution buffer for protein purification

20 mM of Na2HPO4, 0.5 M of NaCl, 500mM of Imidazole in 500 ml of DDW, pH= 7.4

- SDS-PAGE running buffer

Tris-HCl 15g, SDS 5g, Glycin 72g in 500 ml of DDW

- MTT solutaion

1g of thiazolyl blue tetrazdium bromide in 200 ml of PBS

- 1% Gelatin

Gelatin 0.5 g in 50 ml of DDW

4. Instrument

HisTrapTM HP column was from GE healthcare (Uppsala, Sweeden). Sunrise remote control (TECAN). Fluorescence microscopy was from Olympus (Hicksville, NY, USA). Transwell cell culture system was from Coring Inc. (NY, USA).

5. Cell line

The human gastric cancer epithelial cell line AGS, human umbilical vein endothelial cells HUVECs, and human monocytic cell line THP1 were obtained from the Bioresourece Collection and Research Center (Hsinchu, Taiwan).

6. Other

Escherichia coli (BL21 and DH5a) were obtained from Yeastern Biotech Co. *H. pylori* genome was from Department of Internal Medicine, College of Medicine, National Taiwan University.

Method

1. Cell culture condition

AGS were cultured in RPMI 1640 medium supplemented with 2g/L sodium bicarbonate, 10% heat-inactivated fetal bovine serum (FBS), 50ug/ml of penicillin/streptomycin. HUVECs were cultured in medium M199 supplemented with 10% heat-inactivated FBS, 50μg/ml of penicillin/streptomycin, 30μg/mL of endothelial cell growth supplement, and 25 U/mL of heparin. THP1 were cultured in RPMI 1640 medium supplemented with 0.05 mM 2-mercantoethanol, 2g/L sodium bicarbonate, 50μg/ml of penicillin/streptomycin, and 10% heat-inactivated FBS.

2. Expression and purification of HpHSP60

2.1 Transformation of *E. coli*

Transformation was assayed by using competent cells of *E. coli*. DH5α was used for plasmid amplification, and BL21 was used for protein expression. First, the competent cells were mixed gently with 1 ng DNA, and then incubated on ice for 30 min. After incubation, cells were heat shock at 42 ℃ for 90 seconds, and chilled on ice for 2 min. Next, place the competent cells to 250 μl LB and incubate at 37 ℃ with shaking 225 rpm for 1 hour. Cells were plated

onto LB agar plate containing 30 μg/ml of kanamycin and incubate at 37 ℃ for 16 hours.

2.2 Midi plasmid DNA preparation

Pick up a single colony of transformed bacteria and transfer into 100 ml LB medium containing 30 μg/ml of kanamycin. Incubate the culture at 37 ℃ with vigorous shaking for 16 hours. The bacteria were recovered by centrifugation at 8000 rpm for 15 minutes. Remove the supernatant as dry as possible, and then resuspend the bacterial pellet with 8 ml of alkaline lysis solution I. Add 8 ml of Alkaline lysis solution II for lysis cells and mix the contents by inverting five times, then incubate for 3 min. Add 8 ml of ice-cold alkaline lysis solution III for neutralizing and gently invert the tube several times, then incubate on ice for 3 minutes. Centrifuge the bacterial lysates at 12000 rpm for 30 minutes. After washing NeucleoBond ion-exchange resin with 5 ml of buffer N2, the supernatant of bacteria lysates was added to the column; following by 20 ml of buffer N3. The plasmid DNA was eluted by adding 5 ml of buffer N5 and then separating the eluted mixture into microfuge tubes. Next, add 700 μl of isopropanol to each tube for precipitating DNA and place the tube on ice for 10 minutes. Collect the precipitated DNA by centrifugation at 13000 rpm for 45 min and then remove the supernatant. The precipitated DNA was

washed by adding 1 ml of 70 % ethanol, and then dissolved into DDW. Measure the absorbance at 260 and 280 nm and assay by using restriction enzyme digestion for checking the DNA quantity and quality.

2.3 rHpHSP60 protein induction

E. Coli (BL21) were transformed with 1ng of pET-HpHSP60 plasmid, and then growth on LB plates containing 30 μg/ml of kanamycin at 37 ℃ for 16 hours. After incubation, pick up five colonies from the LB plates then inoculated into 100 ml of LB medium containing 30 mg/ml of kanamycin at 37 ℃ for 16 hours with vigorous shaking. Next, transfer the 100 ml of culture broth into 900 ml of LB medium containing 30 μg/ml of kanamycin and incubate at 37 ℃ with vigorous shaking until the value of OD600 reach the range from 0.6 to 0.8. Add 1.25 ml of IPTG (800mM) and continually incubate for 4 hours.

2.4 rHpHSP60 protein purification

After HpHSP60 induction, collect the bacterial by centrifugation at 8000 rpm for 20 min and then remove the supernatant. Resuspend the bacterial pellet with 30 ml of binding buffer, then disrupted by sonicate the whole cells mixture on ice for 15 min. After centrifugation, the supernatants were harvested. The rHpHSP60 were purified by HisTrap^{TM}HP column according the manufacturer's

instructions. The purity of rHpHSP60 was examined by SDS-PAGE and confirmed with mass spectrometry analysis.

3. Measurement of serum antibody to HpHSP60

The serum samples were obtained from National Taiwan University Hospital. The serum samples derived from the patients which were diagnosis as *Helicobacter pylori* infection. According to different diagnosis results, the samples were separated to 4 groups, including gastric cancer group (HC), gastritis group (HS), duodenal ulcer group (HD), and peptic ulcer group (HU). Serum antibodies to HpHSP60 were measured by enzyme-linked immunosorbent assay (ELISA). First, 96-well plates were coated with 100μL of HpHSP60 (10 μg/mL) and in phosphate-buffer saline overnight at 4℃. After the wells were blocked with 300 μl of phosphate-buffer saline tween-20 (PBST) containing 2% skim milk for 1 hour, plates were incubated with 100 μl of sera at a dilution of 1: 100000 for anti-HpHSP60 antibody in phosphate-buffer saline (PBS) for 1 hour at room temperature and washed three times with PBST. 100 μl of Peroxidase-labeled goat anti-human IgG, IgA, IgM antibody at a dilution of 1: 10000 in PBST containing 2% skim milk was added, and the plate was incubated for 1 hour at room temperature. After plate was washed with PBST for

three times, each well was reacted with 100 μl of 3,3′,5,5′-tetramethylbenzidine solution for 20 minutes. After plate was reacted, the wells were added 100 μl of HCl to stop reaction. The optical density was measured at 450 nm on an ELISA plate reader.

4. MTT assay

The MTT assay was used for measuring cell viability. This assay is based on cleavage of the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltertrazolium bromide) to purple formazan crystals by the mitochondrial enzyme of viable cells. To determine the effect of HpHSP60 on proliferation of gastric cancer cells, the gastric tumor cells AGS or SNU1 were harvested and seeded (2×10^4 cells/ 200 μl) in 96-well plates, then incubated in different concentrations of HpHSP60 including 0, 6.25, 12.5, 25, 50, 100, and 200 μg/ml for 48 hours at 37℃. At the end of incubation time, the supernatant of each well was removed and incubated with 0.5 mg/ml of MTT solution 200μl for 4 hours. After incubation with MTT solution for 4 hours, the MTT solution was carefully aspirated. The purple fromazan crystal was dissolved by addition of 150 μl dimethyl sulfoxide (DMSO). The optical density of the solution in each well was measured at 595

nm in a multi-well spectrophotometer.

To examine the effect of HpHSP60 on cell anti-death activity, the gastric cancer cells AGS or SNU1 were collected and seeded in 96-well pates. Next, 2.5 μg/ml of mitomycin C solution was added to AGS or SNU1, and 25 μM or 100 μM of curcumin solution was respectively added to AGS (25 μM) or SNU1 (100 μM) for 4 hours. After incubation for 4 hours, the supernatant of each well were removed, and then the cells were incubated with different concentrations of HpHSP60 including 0, 6.25, 12.5, 25, 50, 100, and 200 μg/ml for 24 hours.

5. Cell migration assays

The invasion assay was performed using the transwell cell culture chambers. AGS cells or HUVECs were harvested with trypsin and resuspend in the serum free medium. The cell suspension (3×10^4 cells) with or without different concentration of HpHSP60 was added to the upper well, and the lower well was added RPMI 1640 supplemented or medium 199 with 10% of FBS. After incubation for 8 hours, the insert was removed from the well, and the cells of upper side membrane were scrubbed with a cotton swab. The bottom side was fixed with 500 μl of methanol for 10 minutes and then freezed in -20℃ for 10 minutes. After fixed, the cells were stained with 50μg/mL of propidium iodide

(PI) 200 μl for 20 minutes. The migration activity was analyzed with a phase contrast microscope and counting the PI-stained cells on the bottom side in three random microscopic fields (× 100).

6. *In vitro* capillary-like tube formation assays

Matrigel was added to a 96-well pate (50 μl/ per well) and polymerized for 30 minutes at 37℃. HUVECs, between passages 4 and 8, were plated on a 75T flask and growth to confluence. After the cells were trypsininzed and resuspended in serum free medium 199 with or without 10μg/ml of HpHSP60, the cells were seeded onto the matrigel (1.5×10^4 cells/ 200 μl), then incubated for 6 hours. Tube formation was observed with a phase contrast microscope (× 100). The ability of tube formation was quantified by counting the number of branch point.

To examine whether HpHSP60 enhance angiogenesis activity is mediated through CXCR2-dependent signal pathway, HUVECs were seeded onto matrigel-coated plate and then cells were incubated in 100 μl of serum free-medium 199 with the present or absent 50nM of SB225002, 3μM of BAPTA, 5μM of PD98059, and 10nM of wortmannin for 1 hour. Next, 100 μl of medium contained with or without 20μg/ml of HpHSP60 was added to each

well, then incubation for 6 hours.

7. ELISA for cytokine expression

THP1 and AGS cells were seeded in 24- well culture plates with 1 ml of cell suspension (10^5 cells/ ml) and incubated at 37℃, 5% CO_2 atmosphere for 2hours. After incubation, 10 μg of HpHSP60 were added into seeded cells for the remainder of the 16 hours incubation. Supernatants were harvested after 16 hours incubating period and IL-8, IL-6, and TNF-α were assayed with ELISA system, according to the manufacturer's specifications.

8. Angiogenesis antibody array

HUVECs, AGS, or THP1 cells (1×10^5 cells/ well) were seeded in 24-well plates with 1 ml grow medium and incubated at 37℃ for 2 hours, then 10 μg of HpHSP60 was added into cells for 24 hours. After incubation, supernatants were collected and assayed for angiogeneic factors profile using the Human Angiogenesis Antibody Array kit according to the manufacturer's instructions. Briefly, membranes were blocked with 2 ml blocking buffer for 30 min, and then membranes were incubated with 1 ml of supernatant from HpHSP60-treated or untreated cells culture at room temperature for 2 hours. After samples were

decanted, 1 ml of biotin-conjugated anti-cytokine antibodies were added, and then incubation for 2 hours. Next, the membranes were incubated with 2 ml of horseradish peroxidase-conjugated streptavidin for 2 hours and then developed using enhanced chemiluminescence (ECL) system.

9. Statistical analysis

All results were expressed as mean ± SEM. Comparison of titers of anti-HpHSP60 antibodies were performed by one-way ANOVA. The results of cell proliferation assay, migration assay, pro-inflammatory cytokine profiles and tube formation assay were analyzed by student's t test.

Chaper3: Results

1. Analysis for anti-HpHSP60 antibodies titers in *H. pylori*-positive patients with gastric cancer or other gastrodoudenal diseases.

The anti-HpHSP60 antibodies of *H. pylori*-infected patients in sera were measured. According the different symptom, samples were divided into four groups, including gastric cancer group (n = 45), gastritis group (n = 26), duodenal ulcer group (n = 76), and gastric ulcer group (n = 16). The titers of anti-HpHSP60 antibodies in sera of patients with gastric cancer were lower than other gastrodoudenal disease groups (Fig. 1). From the results, HpHSP60 might be associated with progression of gastric cancer. However, no direct evidence indicated the function of HpHSP60 involves in gastric cancer development.

2. The effects of HpHSP60 on the progression of gastric cancer.

To determine whether HpHSP60 involves in progression of gastric cancer, the effects of HpHSP60 on cell proliferation, anti-apoptosis activity, migration ability, and angiogenesis were investigated. First, whether HpHSP60 could enhance the proliferations of gastric cancer cell was examined and the results showed HpHSP60 didn't increase the growth of gastric cancer cells at any

additional doses (Fig 2a and b). Sequentially, two reagents, mitomycin C and curcumin were used to induce cell apoptosis. After treatment with mitomycin C or curcuimin for 4 hours, the results showed HpHSP60 couldn't increase or decrease cell viabilities of AGS or SNU1 cells (Fig 3 a-d). In addition, the migration ability of gastric cancer cells was also measured by transwell assay. Figure 4 revealed that the migration behavior of AGS cells was significantly enhanced by HpHSP60 in dose dependent manner (Fig 4a and b). Besides, the effect of Hphsp60 on angiogenesis was examined by the *in vitro* capillary-like tube formation assay. After HpHSP60 treatment for 6 hours, the branch point numbers of capillary-like tube formation were significantly increased comparing to the untreated group (Fig 5a and b).

3. Induction of pro-inflammatory cytokines by HpHSP60 in gastric tumor and monocytic cells

HpHsp60 is known as a potent immunogen of *H. pylori* since lots of researches have demonstrated HpHsp60 can induce pro-inflammatory cytokines such as IL-6 or IL-8 to augment inflammation (Gobert, et al., 2004; Zhao, et al., 2007; Takenaka, et al., 2004; Lin, et al., 2005). To elucidate contradictions between previous studies and our results, cytokine profiles of AGS cells and

monocytic THP-1 cells under HpHsp60 effecting were examined. The results showed the pro-inflammatory cytokines, IL-8, IL-6, and TNF-α were secretion in the present of HpHSP60 (Figure 6 a-c).

4. The release of angiogeneic factors after HpHSP60 treatment.

So far HpHSP60 has been proved it can enhance the activities for migration, tube formation and proinflammatory cytokine release. According to the previous reports, all the effects are involved in angiogenesis. Subsequently, the abilities of HpHSP60 to stimulate release of angiogeneic factors were determined. The HUVECs, THP-1 cells and AGS cells were treated with HpHSP60 and their expression profiles of angiogeneic factors (Fig. 7a) were showed at figure 7b, to expression to enhance angiogenesis. Comparing to the unstimulated cells, the antibody array revealed that two angiogeneic factors IL-8 and GRO were increased their releases by HpHSP60 (Table 1).

5. The effects of HpHSP60 on HUVECs proliferation and migration.

Because the proliferation and migration of endothelial cells is an important step for angiogenesis, HpHSP60 were added to the HUVECs to examine their effects on the proliferation and migration. Figure 8 revealed that

the migration behavior of HUVECs was significantly enhance in the present of HpHSP60. Sequentially using MTT assay to examine the proliferation of HUVECs, after treatment HpHSP60 for 24 or 48 hours, the cell proliferation rate didn't display significantly increase compared to untreated cells (Fig 9a). By contrast, the supernatants from THP1 cells or AGS cells pre-treated HpHSP60 could stimulate HUVECs proliferation (Fig 9b and c). These results indicated that HpHSP60 could not directly induce endothelial cells proliferation but it can indirectly increase the proliferation by stimulating gastric or monocytic cells to release growth factors (Table 1).

6. HpHSP60-enhanced angiogenesis via CXCR2-dependent signaling.

The expression profile of angiogeneic factors indicated HpHSP60 triggered high expressions of IL-8 and GRO. IL-8 and GRO are the ELR (Glu-Leu-Arg) motif-positive (ELR^+) CXC chemokines and all ELR^+ CXC chemokines have been indicated to share the common receptor, CXCR2. Therefore, the inhibitors of the CXCR2 pathway were used to verify the effects of HpHSP60 on cellular signaling (Fig 10). and the data displayed that the increasing branch point numbers of tube formation by HpHSP60 could be suppressed by SB225002 and BAPTA, but not by PD98059 and Wormannin (Fig

11).

Chaper4: Discussion

In this study, we examined the role of HpHSP60 on gastric cancer progression. We demonstrated that HpHSP60 could significantly enhance migration behavior of gastric cancer cells as well as angiogenesis, but HpHSP60 was unable to promote proliferations of gastric cancer cells and rescue gastric cancer cells from apoptosis. Since mitomycin C damages DNA by cross-linking bases in the DNA to induce apoptosis (Andera, et al., 1997), and curcumin induces apoptosis via inhibiting NF-κB pathway (Lin, et al., 2007). Furthermore, HpHSP60-induced angiogenesis are resulted from the angiogeneic factors releases and these results indicated that chemokines IL-8 and GRO play an important role to enhance angiogenesis. Suppression of CXCR2 pathway by CXCR2 inhibitor SB225002 and intracellular calcium chelator BAPTA conform to inhibition of angiogenesis. Take together, our results indicated HpHSP60 could trigger proinflammatory cytokine releases, which increases angiogeneic potential and this enhancement may be mediated via CXCR2 signal pathway.

Microbial HSP60s are though to be an immunodominant antigen since it could induce powerful immune response after infection (Habich, et al., 2003). *Chlamydial pneumoniae* HSP60 could cause the diffuse pneumonia with the

massive infiltration of neutrophils into the lung tissue and increase the IL-6 production in the bronchoalveolar lavege fluid (Yonca, et al., 2009). HSP65 of *Mycobacterial leprae* could induce release of TNF-α, IL-6, and IL-8 from human monocytic cells (Friedland, et al., 1992). Moreover, *H. pylori*-HSP60 has been reported to induce proinflammatory cytokines including IL-6, and IL-8 from human monocytic cells and/or gastric epithelium cells (Gobert, et al., 2004; Zhao, et al., 2007; Takenaka, et al., 2004; Lin, et al., 2005). Furthermore, the *H. pylori* HSP60-induced proinflammatory cytokine releases are through TLR-2 and TLR-4 pathway (Gobert, et al., 2004; Takenaka, et al., 2004). In this study, *H. pylori* HSP60 accord with the previous literatures, it could also trigger the releases of proinflammatory cytokines from THP-1 cells such as IL-8, TNF-α, IL-6 (Fig. 6) and GRO (Table 1). This verifies that *H. pylori* HSP60 indeed is a dominant factor to induce host inflammation.

Besides, some species of HSP60 are associated with carcinogenesis have been demonstrated. *Chlamydia trachomatis*, an obligate intracellular bacterium, has been accordingly associated with the development of cervical and ovarian cancer (Spiliopoulou, et al., 2005) and its HSP60 has an anti-apoptotic activity during persistent infection. During persistent infection, *C. trachomatis* could produce large quantities of HSP60 and transported them to the cytosol of the

host cells (Fields, et al., 2000), which can form a complex with cleaved caspase-3 and Bax and Bak to inhibit apoptosis (Felic, et al., 2005). In addition, human HSP60s, mostly localized in the mitochondrial matrix of normal cells, has been suggested have higher expression in neoplastic tissue of variety of tumors during carcinogenesis (Marasa, et al., 2005). Similarly, canine HSP60 has been shown as tumor marker involved in carcinogenesis of canine tumor (Chu et al., 2001). Furthermore, human HSP60 could interact with Bax and Bak to prevent apoptosis and it also involved in regulating cell cycle to influence the carcinogenesis (Czarnecka, et al., 2006). Moreover, increased expression of HpHSP60 on gastric cancer cells by stable transfection could regulate cell cycle-related protein to enhance cell proliferation (Luca, et al., 2003). In contrast to these studies, our results showed HpHSP60 didn't have the effect on proliferation and apoptosis inhibition. For elucidating the difference, we inferred that the location of HSP60 will affect its functions on carcinogenesis. Since human HSP60 or stable expression HpHSP60 were endogenous expression as well as intracellular parasitical *Chlamydia* HSP60 and these HSP60 could easily interact with the factors which involved in apoptosis or cell cycle to stop death or cause proliferation. However, HpHSP60 by extracellular addition as *H. prlori* secretion in this study might hardly cross the plasma membrane to interact with

the factors involved in proliferation and apoptosis.

Inflammatory responses are associated with cell migration and angiogenesis, which is involved in promoting carcinogenesis process (Lorusso, et al., 2008). For gastric cancer, *H. pylori*-associated gastric cancers have been proposed that they are associated with chronic inflammation (Karin, et al., 2006). It also has been suggested that *H. pylori*-induced chronic inflammation is associated with promoting angiogenesis process (Pousa et al., 2006). Ruegg and colleagues indicate that macrophage plays an important role in promoting chronic inflammation in the tumor microenvironment (Ruegg, et al., 2006). In addition, the pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IFN-γ, release from inflammatory cells could also affect tumor cell behavior (e.g. induction of motility), and vascular networks organization (Aggarwal et al, 2006). Besides, up-regulation of COX-2 could enhance gastric cells invasion and angiogenesis in the present of *H. pylori* (Chang, et al., 2005). Moreover, other studies have indicated that *H. pylori* could stimulate endothelial cells producing IL-6, IL-8, TNF-α and GRO as well as up-regulate adhesion molecule of endothelial cells (Innocenti, et al., 2001). In this study, we found HpHSP60 could stimulate angiogeneic factor IL-8 and GRO secretions from monocytic cells, gastric cancer cells, and endothelial cells. Furthermore, the results indicated HpHSP60

could enhance HUVECs migration (Fig. 8) to result in the increase in tube formation but it could not affect the proliferation *in vitro* (Fig. 9a). However, we proposed that HpHSP60 may promote the proliferations of HUVECs *in vivo* by prior activating gastric epithelial cells or macrophages (Fig. 9b and c) to secrete various of growth factors (PDGF-BB, PIGF, EGF, IGF-I, VEGF-D) (Table 1). Thus, we proposed that HpHSP60 is a potent promoter for gastric tumor by facilitating local angiogenesis at infection site. To summarize findings from our study, we propose the following model (Fig 12). This model depicts that HpHSP60 mediates with gastric cancer cell, monocytes, and endothelial cells with respect to the biological effects of factors to promote angiogenesis.

In this study, our results showed HpHSP60 could stimulate chemokines secretion including IL-8, GRO, MCP1, RANTES, and ENA-78. IL-8 and GRO are ELR^+ CXC chemokines and have high affinity to engage to CXCR2, which would cause the increase in cytoplasmic calcium concentration (Ahuja, et al., 1996). The CXCR2 inhibitor, SB225002 and intracellular calcium chelator BAPTA abolished the HpHSP60-induced increases in the tube formation of HUVEC that indicated the CXCR2 signal pathway played an important role for HSP60-enhanced tube formation.

Finally, we consider that HpHSP60 could activate proinflammtory response

to enhance tumor cell migration behavior and angiogenesis process. Together the results in this study may indicate that although HpHSP 60 could not directly transform normal cell in tumor cell by promoting the proliferation rate or inhibiting apoptosis, HpHSP60 might accelerate the tumor malignancy by promoting angiogenesis and metastasis. Thus, we believe that HpHSP60 should be further studied its effects on carcinogenesis in future.

Figure and Legends

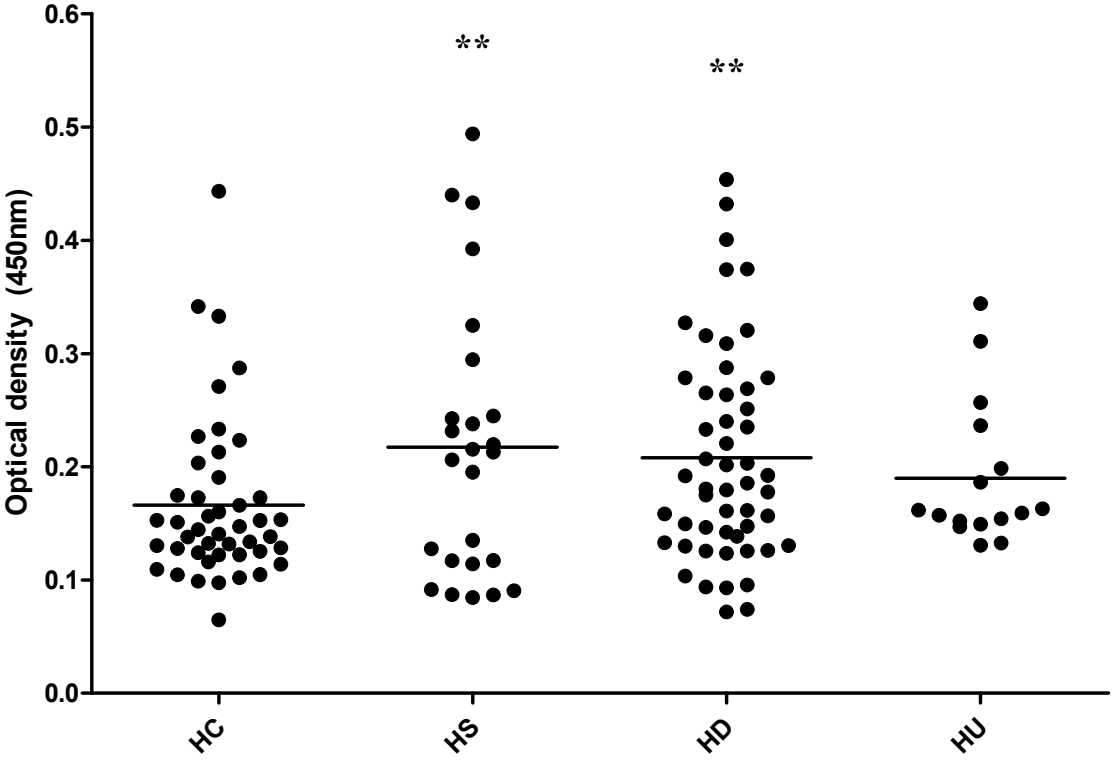
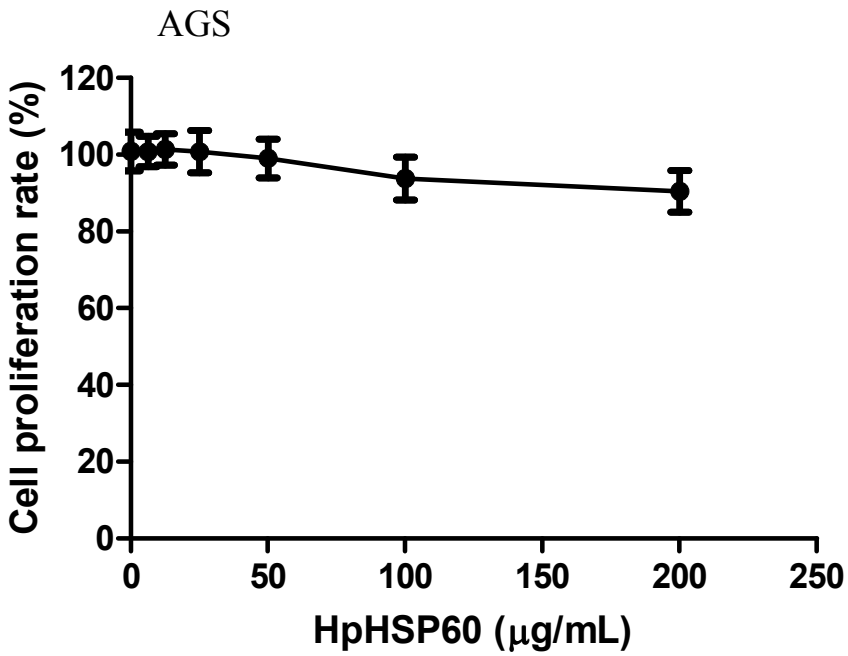


Figure 1. Serum antibodies to HpHSP60 in *H. pylori*-infected patients with gastric cancer or other gastrodoudenal disease.

The titers of anti-HpHSP60 antibodies from sera of *H. pylori*-infected patients were analyzed using ELISA. According to the different diseases, the samples were divided into four groups including gastric cancer group HC (n = 45), gastritis HS (n = 26), duodenal ulcer group HD (N=76), and gastric ulcer group HU (n=14). Multiple comparisons were performed by one-way ANOVA. The horizontal lines of the data were expressed as means. $^{**}p < 0.01$, significantly different contrast to HC group.

a.


AGS
Cell proliferation rate (%)
120
100
80
60
40
20
0
0
50
100
150
200
250
HpHSP60 (μg/mL)

b.

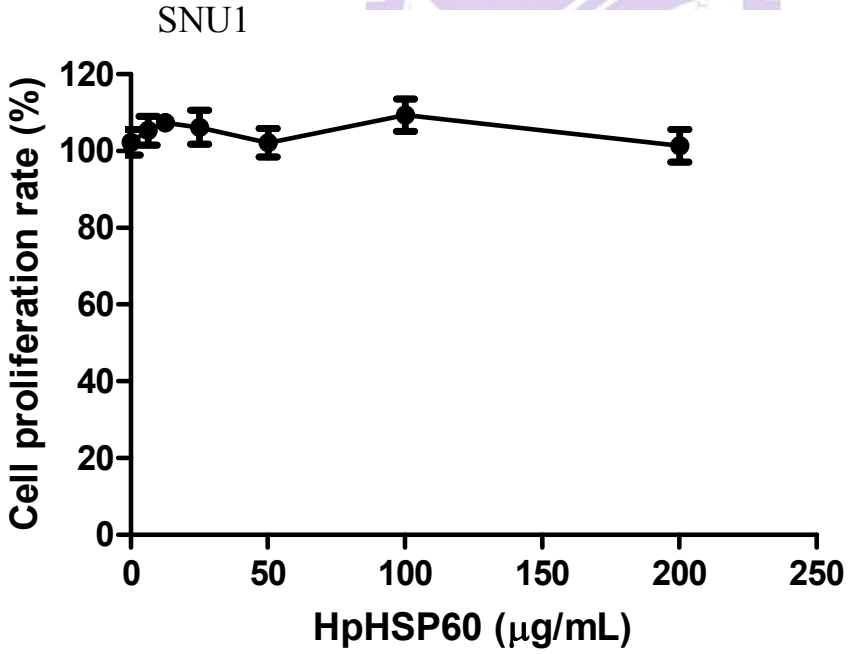
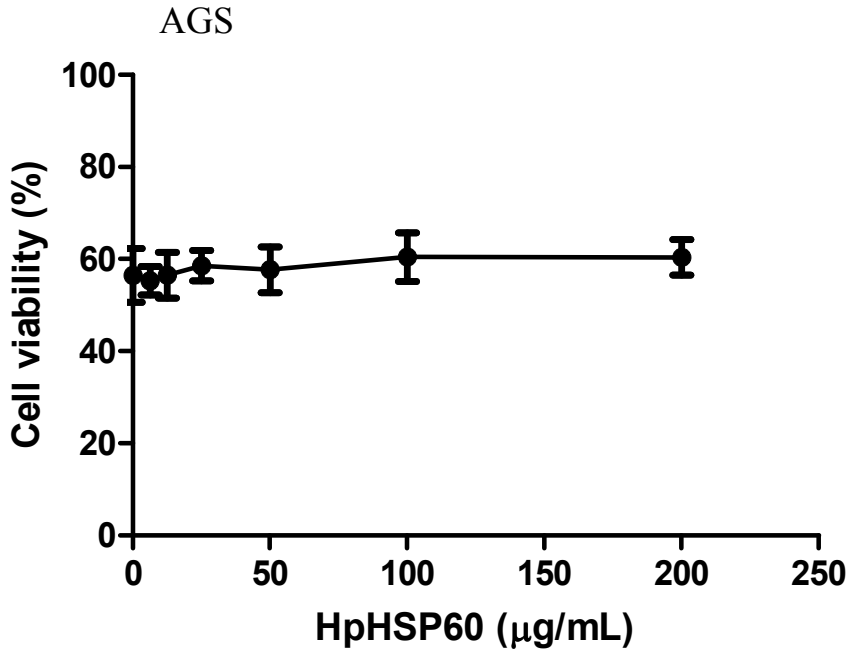

SNU1
Cell proliferation rate (%)
120
100
80
60
40
20
0
0
50
100
150
200
250
HpHSP60 (μg/mL)

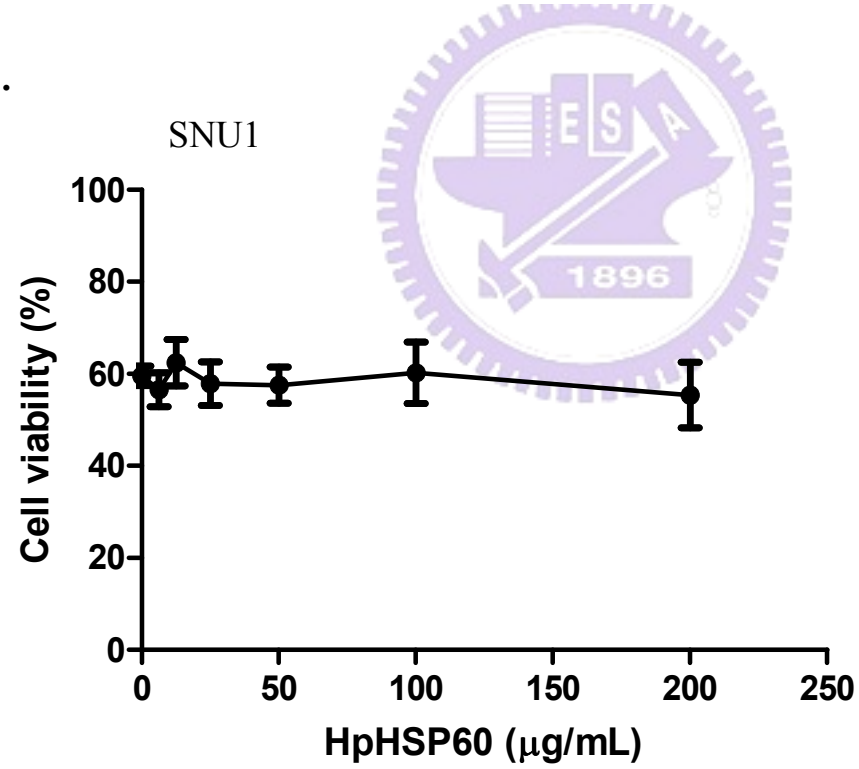
Figure 2. The effect of HpHSP60 on proliferation of AGS and SNU1 cells.

Gastric cancer cells, AGS cells (a) and SNU1 cells (b) were cultured with various concentration of HpHSP60 including 0, 6.25, 12.5, 25, 50, 100, and 200 μg/ml for 48 hours. The cell proliferation was assessed using the MTT assay as described in material and method section.. The cell proliferation rates were expressed as percentage by HpHSP60 treatment with respect to untreated cells considered as 100%. Results are shown as mean ± SEM. All experiments were performed in triplicate.

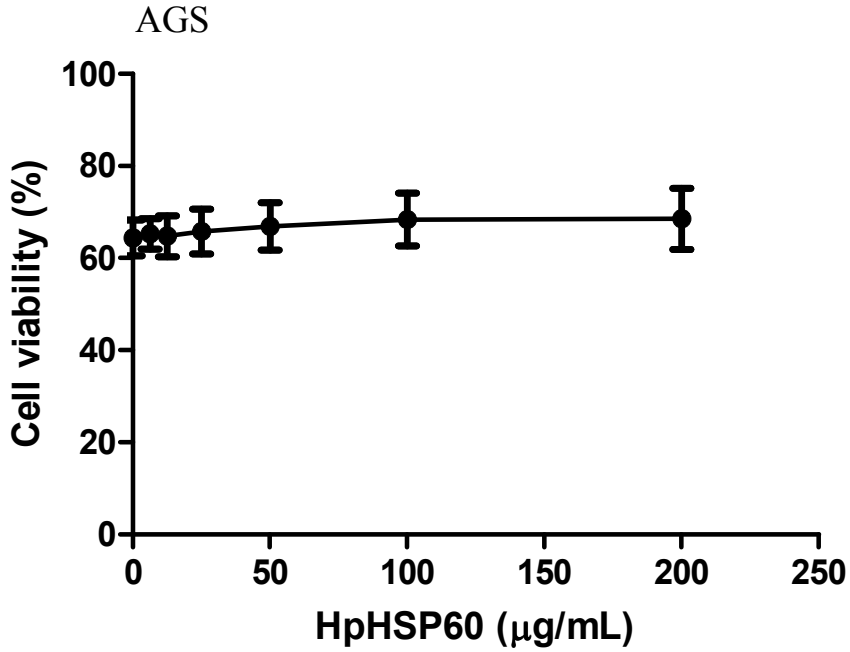
a.


AGS
Cell viability (%)
100
80
60
40
20
0
0
50
100
150
200
250
HpHSP60 (μg/mL)

b.


SNU1
Cell viability (%)
100
80
60
40
20
0
0
50
100
150
200
250
HpHSP60 (μg/mL)

c.


AGS
Cell viability (%)
100
80
60
40
20
0
0
50
100
150
200
250
HpHSP60 (μg/mL)

d.

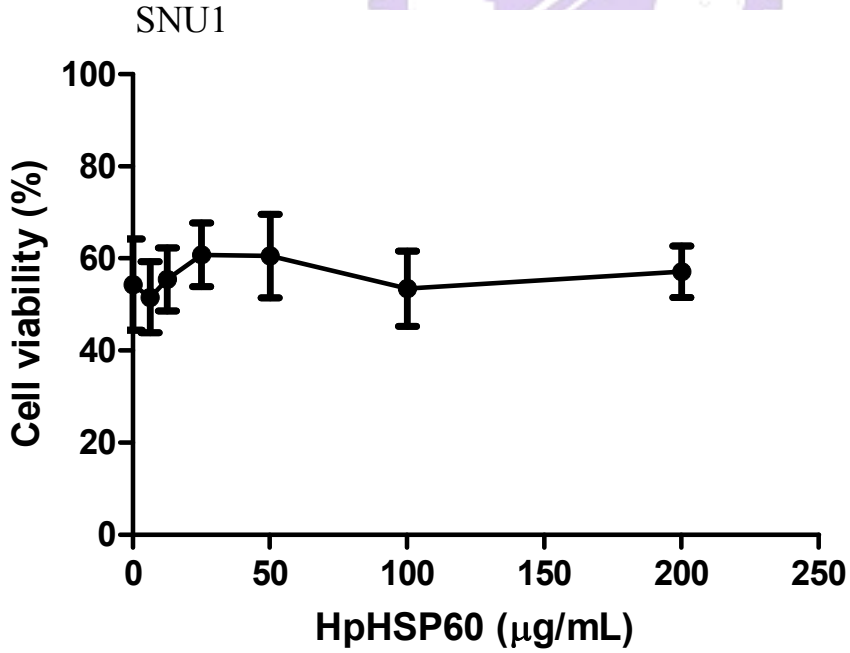
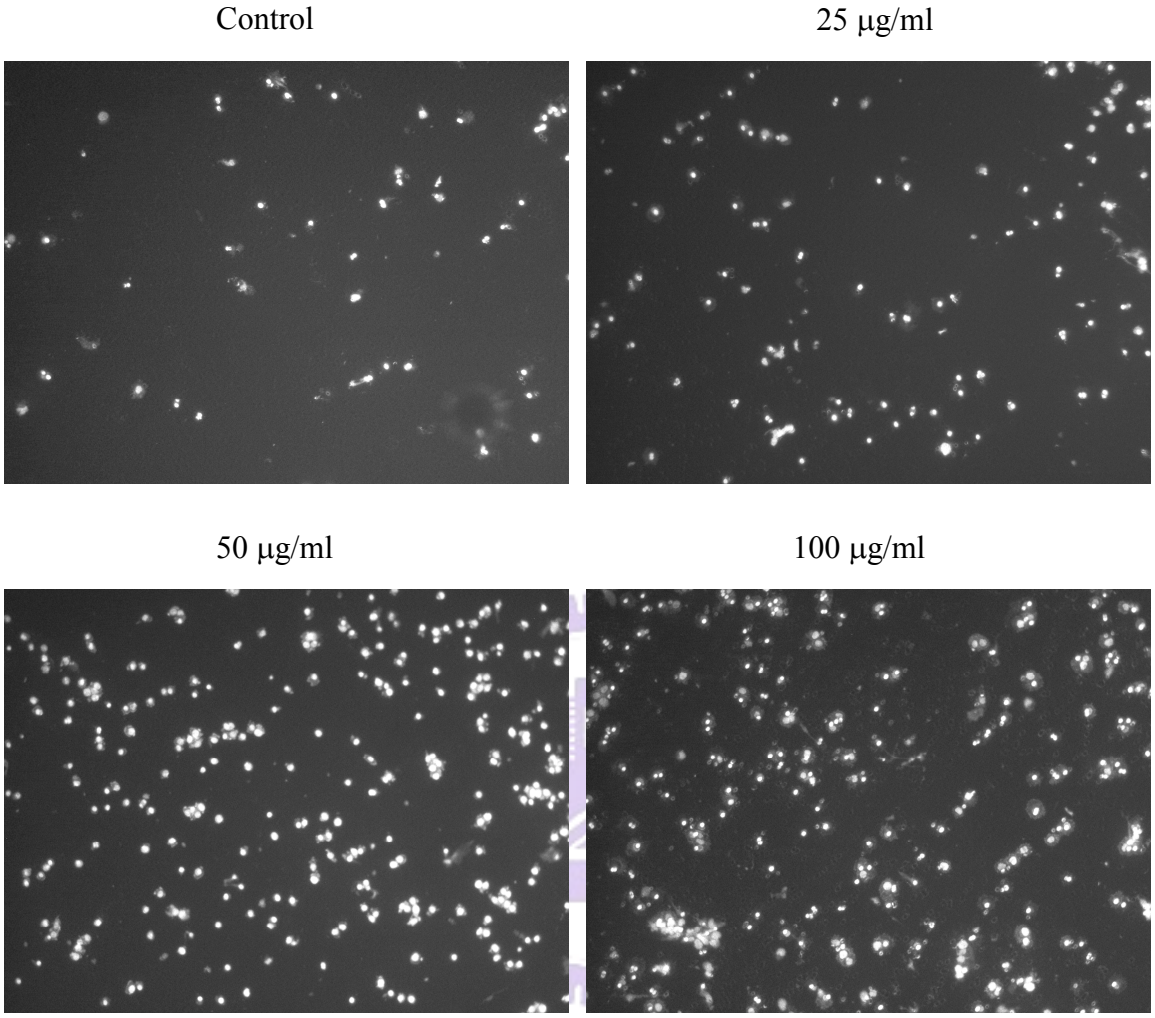

SNU1
Cell viability (%)
100
80
60
40
20
0
0
50
100
150
200
250
HpHSP60 (μg/mL)

Figure 3. The effect of HpHSP60 on anti-death activity of AGS and SNU1 cells.

The data of cell viability were assessed by MTT assay as described in material and method section. (a, b) AGS and SNU1 cells were exposed to 2.5 μg/ml of mitomycin C for 4 hours then added varying concentrations of HpHSP60 including 0, 6.25, 12.5, 25, 50, 100, and 200 μg/m and cultured for 24 hours. (c, d) AGS and SNU1 cells were pre-treated with Curcumin (AGS, 25μg/ml; SNU1, 100μg/ml) for 4 hours then cells were cultured with HpHSP60 for 24 hours. The data are expressed as percentage of cell viability with respect to the viability of drug and HpHSP60-untreated cells considered as 100 % and results are shown as mean ± SEM. All experiments were performed in triplicate.

a.


Control
25 μg/ml
50 μg/ml
100 μg/ml

b.

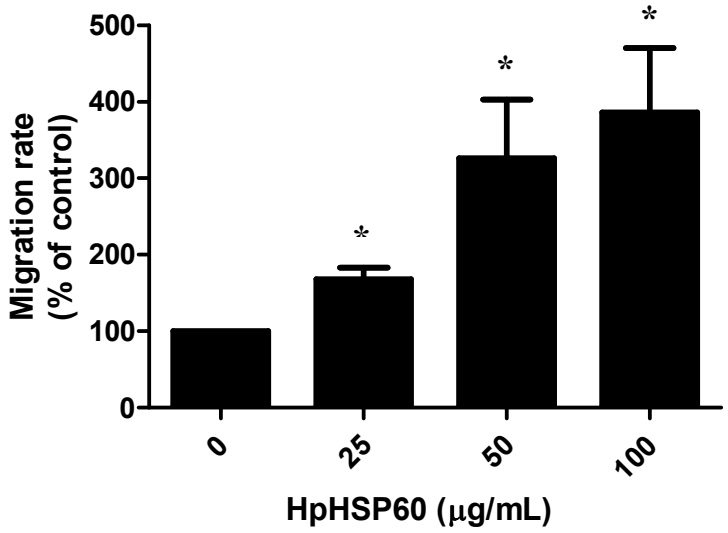
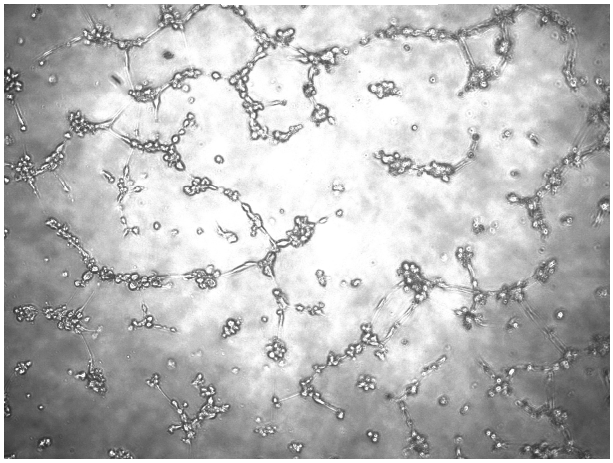

Migration rate
(% of control)
500
400
300
200
100
0
*
*
*
0
25
50
100
HpHSP60 (μg/mL)

Figure 4. HpHSP60 enhance migration ability of AGS cells.

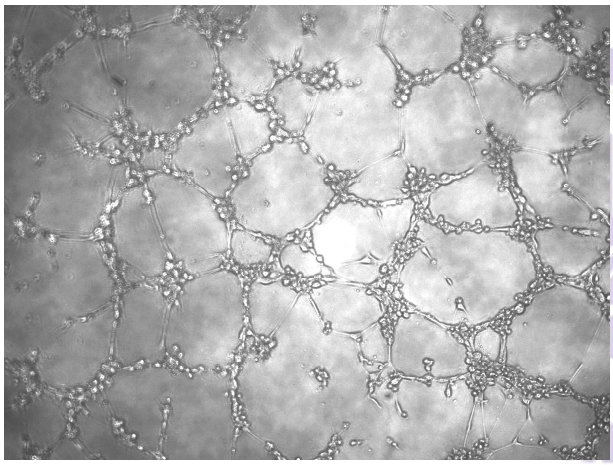
Cell migration ability was assessed using transwell assay as described in "material and method" section. (a and b) AGS cells migration assay were performed under the condition of basal medium contained with 0, 25, 50, and 100 μg/ml of HpHSP60. Migration cells were stained by propidium iodide then the cells were counted in 3 random microscopic fields (× 100). The migration rate was expressed as percentage of migration cells by HpHSP60 treatment with respect to the migration of untreated cells considered as 100 %. The results were expressed as mean ± SEM, $*p < 0.05$. The experiments were performed in triplicate.

a.

Control



HpHSP60 (10 μg/ml)



b.

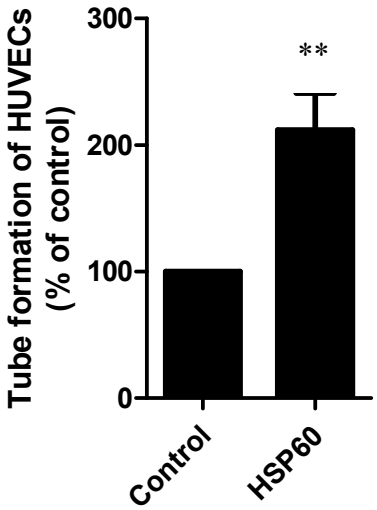
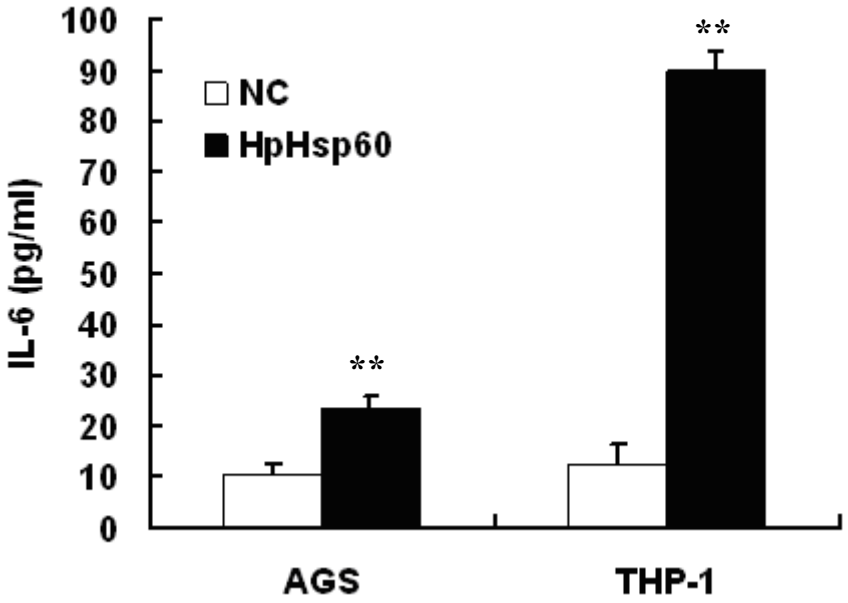


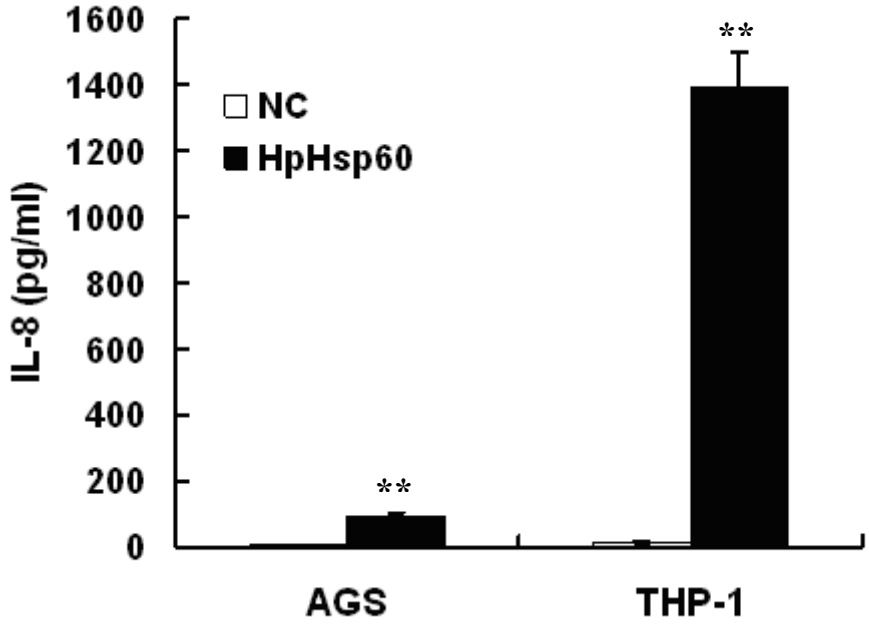
Figure 5. HpHSP60 enhance capillary-like tube formation of HUVECs.

(a) Photomicrographs (× 100) of HUVECs were treated without (control) or with 10 μg/ml of HpHSP60 for 6 hours, after cells were seeded onto matrigel-coated 96 well plates. (b) Tube branch points were counted and the tube formation of HUVECs were expressed as percentage of the branch points number of HpHSP60 treated cells contrast to the numbers of unstimulated cells regarded as 100%. The results were showed as mean ± SEM, $^{**}p < 0.01$. All experiments were repeated three times.

a.


100
90
80
70
60
50
40
30
20
10
0
IL-6 (pg/ml)
NC
HpHsp60
**
**
AGS
THP-1

b.


1600
1400
1200
1000
800
600
400
200
0
IL-8 (pg/ml)
NC
HpHsp60
**
**
AGS
THP-1

c.

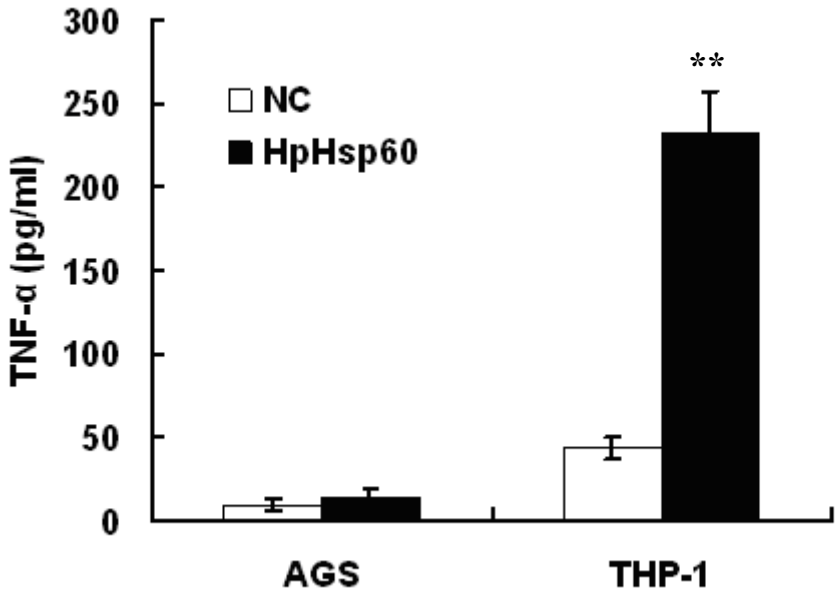


Figure 6. IL-6, TNF-α, and IL-8 expression of AGS and THP-1 cells induced by HpHSP60.

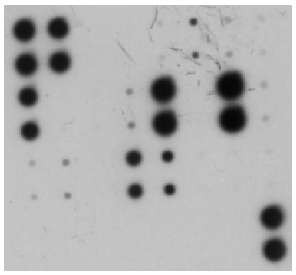
AGS or THP-1 cells were incubated without (NC) or with HpHSP60 (10 μg/ml) for 16 hours and the supernatants were harvested to be examined for the cytokine expression levels by ELISA: IL-6 (a), IL-8 (b), TNF-α (c). Data are expressed as means ± SEM for three replicates from one of three representative experiments. (**,$p < 0.01$. Conditions are compared with untreated control of each cell line.)

a.

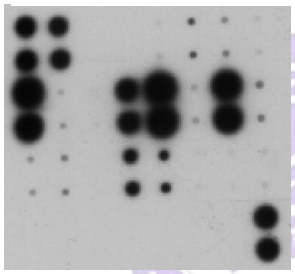
POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	b-FGF
POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	b-FGF
GRO	IFN-r	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
GRO	IFN-r	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
PIGF	RANTES	TGF-b1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
PIGF	RANTES	TGF-b1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS
BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS

b.

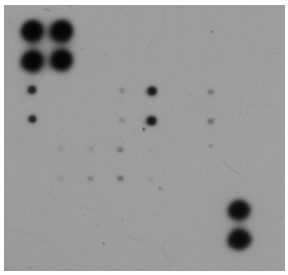
HUVECs



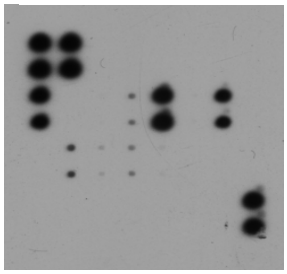
HUVECs with HpHSP60



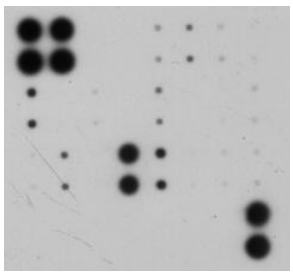
THP1



THP1 with Hpshp60



AGS



AGS with HpHSP60

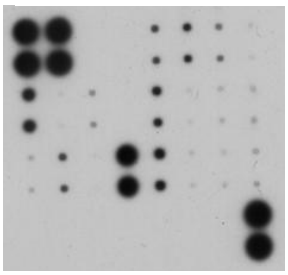


Figure 7. Angiogeneic factor profiles of HUVECs, THP1 and AGS cells after HpHSP60 treatment.

Supernatants of three kinds of cells which were stimulated with or without 10 μg/ml of HpHSP60 for 24 hours were subjected to angiogeneic factors antibody array. EGF, epidermal growth factor, ENA-78, epithelial neutrophil activating peptide, bFGF, basic fibroblast growth factor, GRO, growth-regulated oncogene, IFN-γ, interferon-gamma, IGF-I, insulin-like growth factor, IL-6, interleukin-6, IL-8, interleukin-8, MCP-1, Monocyte chemotactic protein-1, PDGF-BB, platelet-derived growth factor-BB, PIGF, placenta growth factor, RANTES, Regulated on activation normal T cell expressed and secreted, TGF-β1, Transforming growth factor-β1, TIMP, tissue inhibitors of metalloproteinases, VEGF, Vascular endothelial growth factor.

	Increase value contrast to control		
Factor	**HUVECs**	**THP1**	**AGS**
IL-8	4.453	7.185	0.387
GRO	5.318	5.811	0.566
IL-6	3.23	0.251	
MCP1	0.625	3.924	0.017
RANTES	0.002	0.477	0.055
TIMP1	-0.065	0.122	1.1314
ENA-78	0.005		0.104
PDGF-BB	0.047		0.018
PIGF	0.007		0.017
TIMP2	-0.134		0.195
EGF	-0.026		0.248
b-FGF	-0.005		
TGF-beta1		-0.007	
Angiogenin			0.186
IGF-I			0.052
VEGF-D	0.003		0.024

Table 1. The angiogeneic factors expression profile

The intensities of the relative expression level of cytokines were quantified by densitometer (GE, USA). The values from scans were normalized based on the intensity of control spots on the filter corners, and the level increase for specific cytokines were shown.

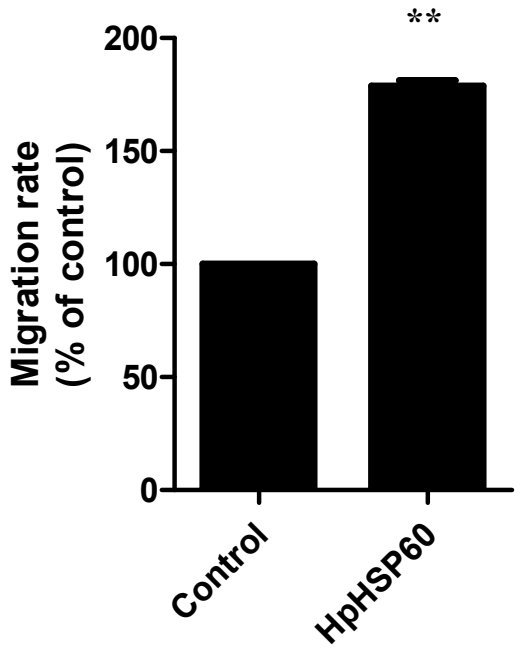
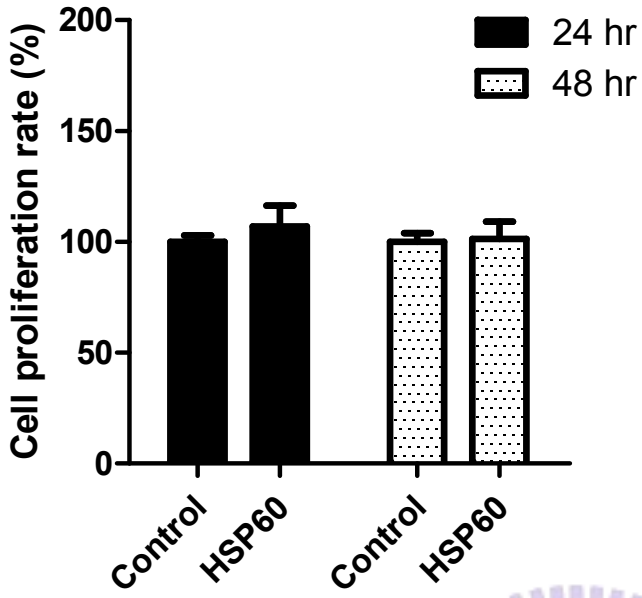


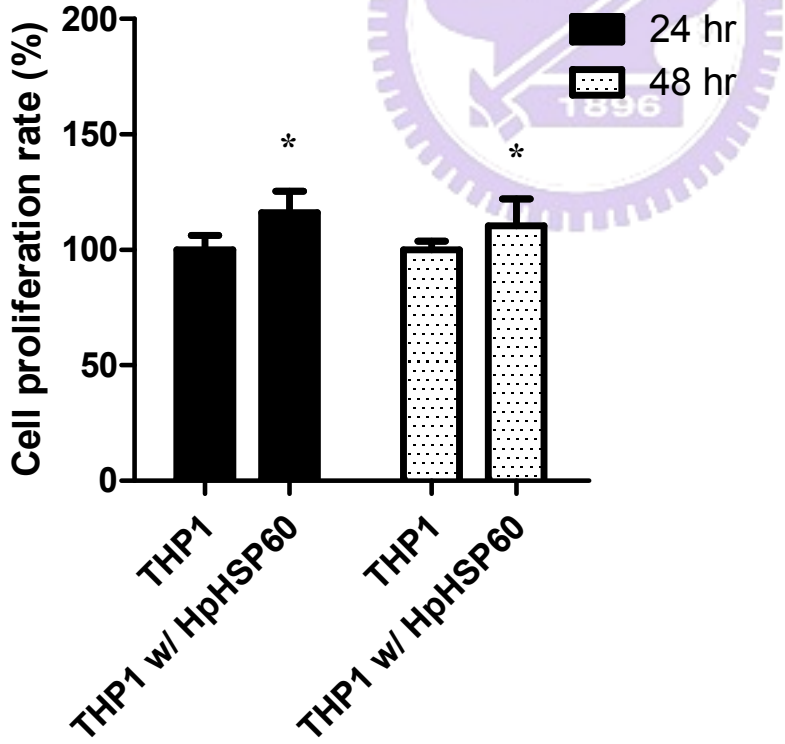
Figure 8. HpHSP60 enhance migration ability of HUVECs cells.

HUVECs cells migration assay were performed under the condition basal medium contained without (control) or with 10 μg/ml of HpHSP60. Migration cells were stained by propidium iodide then the cells were counted in 3 random microscopic fields (× 100). The migration rate was expressed as percentage of migration cells by HpHSP60 treatment with respect to the migration of untreated cells considered as 100 %. The data were expressed as mean ± SEM, $^{**}p < 0.01$. The experiments were performed in triplicate.

a.


Cell proliferation rate (%)
200
150
100
50
0
24 hr
48 hr
Control
HSP60
Control
HSP60

b.


Cell proliferation rate (%)
200
150
100
50
0
24 hr
48 hr
*
*
THP1
THP1 w/ HpHSP60
THP1
THP1 w/ HpHSP60

c.

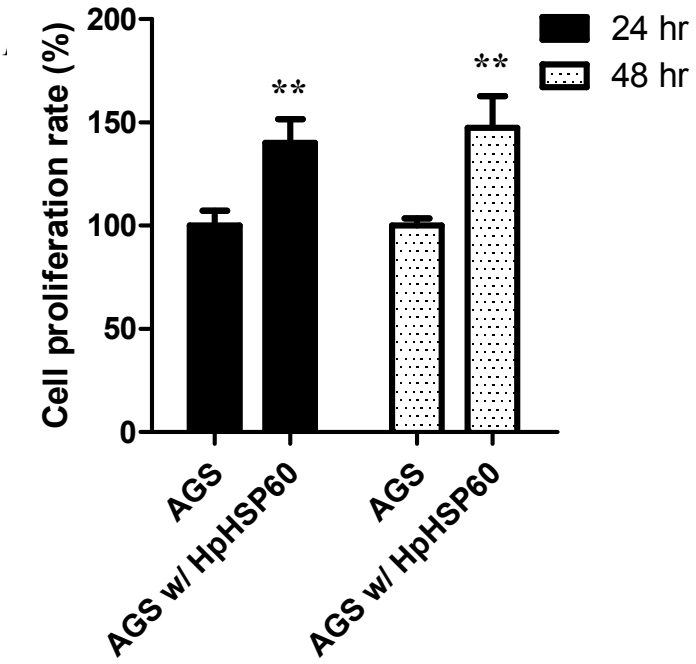


Figure 9. The supernatant from HpHSP60-treated cells could promote HUVECs proliferation.

The data of cell viability were assessed by MTT assay as described in material and method section. (a) HUVECs were treated with (control) or without 10 μg/ml of HpHSP60 for 24 hours or 48 hours. (b, c) Supernatants of THP1 or AGS cells, stimulated with or without 10 μg/ml of HpHSP60 for 24 hours, were subjected to culture with HUVECs for 24 hours or 48 hours. The cell proliferation rates were expressed as percentage by untreated HpHSP60 cells considered as 100%. The results were showed as mean ± SEM, *$p < 0.05$ and ** $p < 0.01$. All experiments were repeated three times.

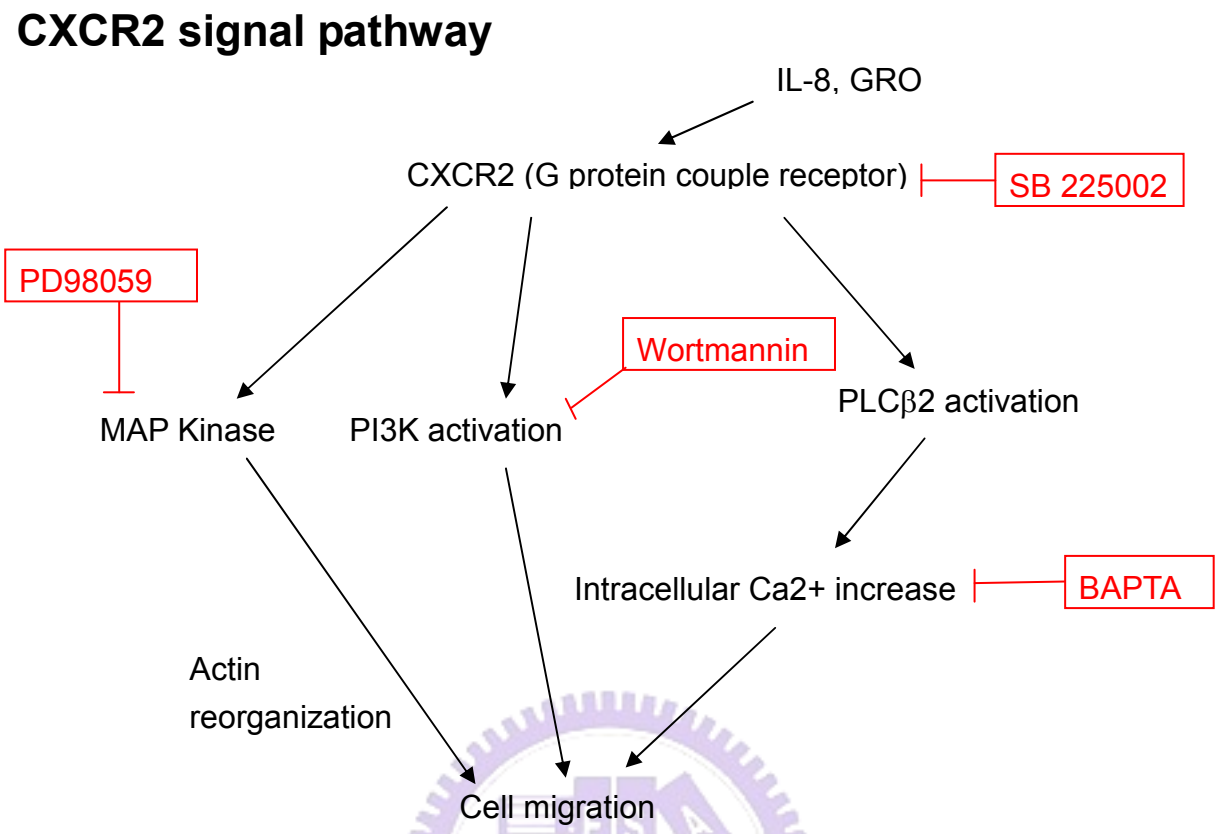


Figure 10. Characterized CXCR2 signal pathway.

After IL-8 and GRO bind to CXCR2, CXCR2 signal could activate the downstream effectors to trigger cell migration, including PI3K, MAP kinase, promoting actin reorganization, and PLCβ2, promoting calcium mobilization. MAP Kinase, mitogen-activated protein kinase, PLCβ2, phospholipase C beta 2, PI3K, phosphoinositide 3-inase.

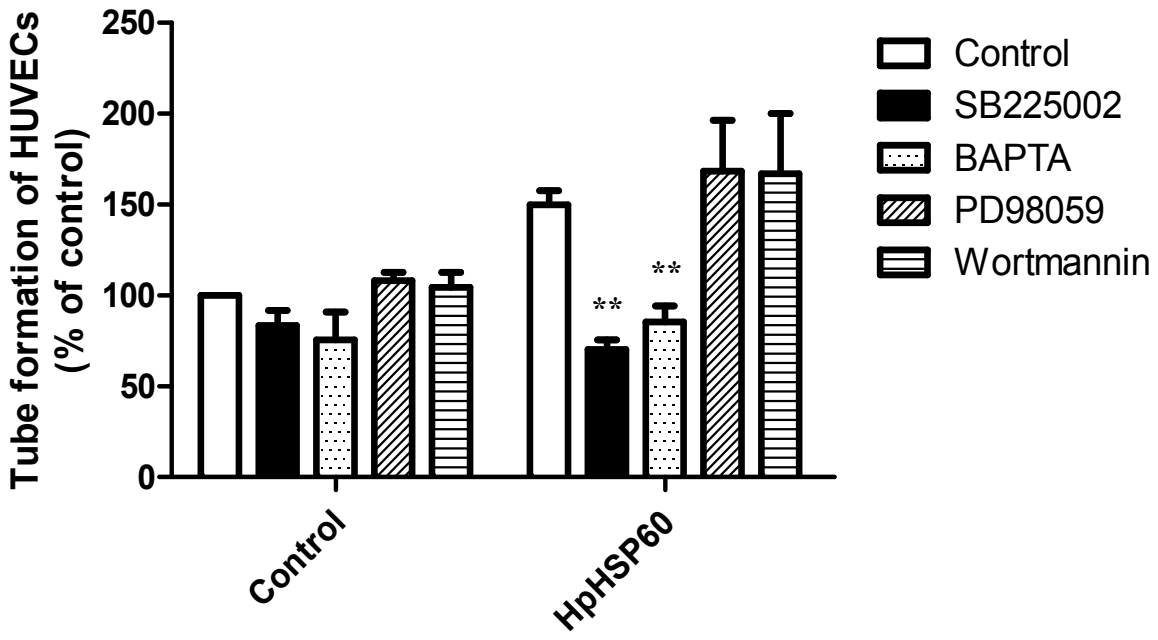


Figure 11. The role of CXCR2 in HpHSP60-mediated angiogenesis.

HUVECs were treated without or with 10 μg/ml of HpHSP60 for 6 hours before pretreatment with SB225002 (50 nM), BAPTA (3 μM), PD98059 (5 μM), Wortmannin (10nM) for 1 hour. Tube branch points were counted and the tube formation of HUVECs were expressed as percentage of the branch points number of HpHSP60 and/or inhibitors treated cells contrast to the number of unstimulated cells regarded as 100%. The results were showed as mean ± SEM, *$p < 0.01$ as compared with HpHSP60 alone. All experiments were repeated three times.

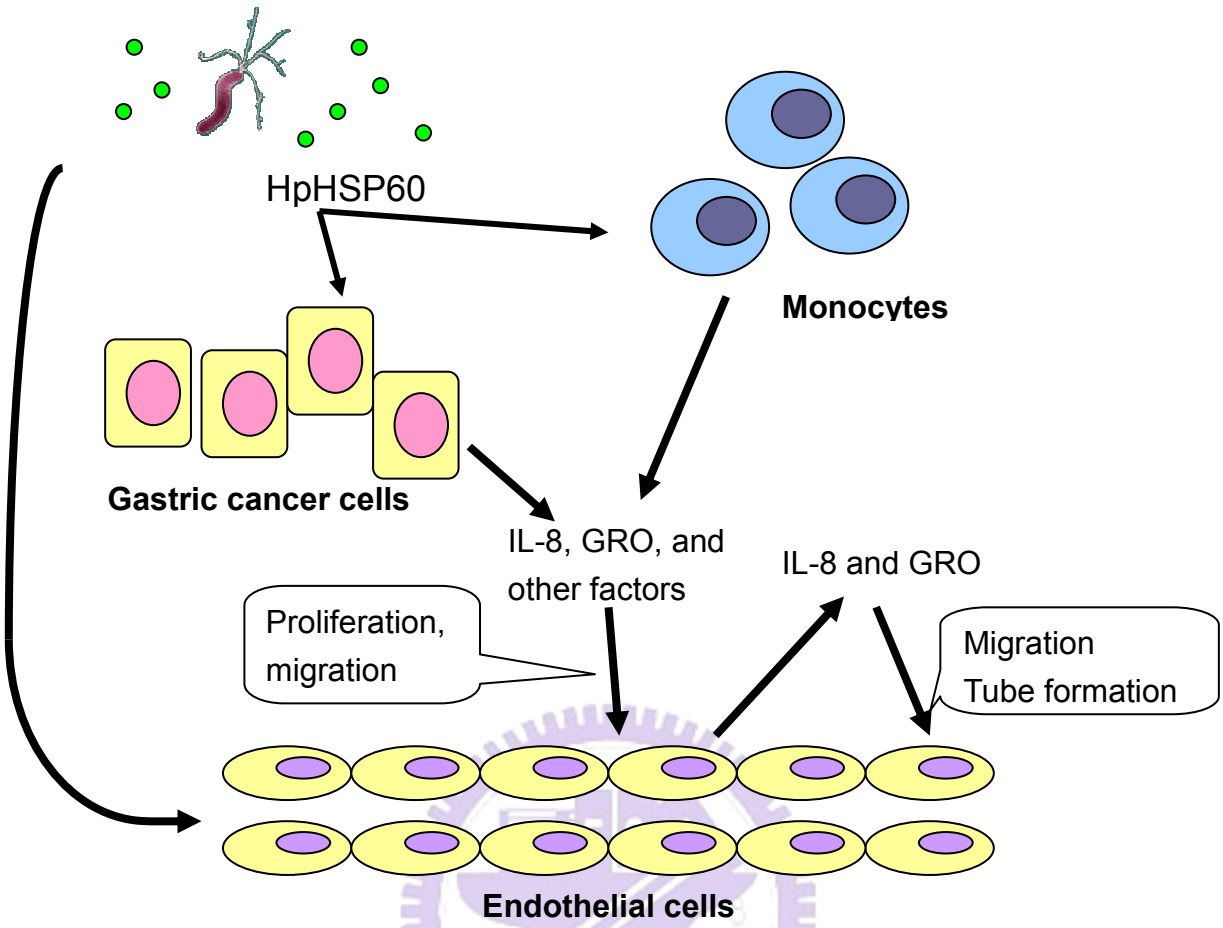


Figure 12. HpHSP60 could promote angiogenesis by stimulating gastric cancer cells, monocytes and endothelial cells.

HpHSP60 could stimulate gastric cancer cells and monocytes to secrete IL-8, GRO, and other factors. These factors could promote endothelial cell proliferation and migration. Besides, HpHSP60 could enhance endothelial cells migration by producing IL-8 and GRO. In this way, HpHSP60 could promote angiogenesis by stimulating gastric cancer cells, monocytes and endothelial cells.

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