科技部補助專題研究計畫成果報告
期末報告

檜木醇與黃連素抑制子宮頸癌細胞之侵襲轉移、上皮-間質相互轉換(EMT) 與血管新生作用之相關機制研究(第3年)

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中 華 民 國 105 年 09 月 19 日

中 文 摘 要 ： 癌細胞在轉移的過程中，癌細胞會出現上皮-間質型態相互轉換(EMT)之情形，增加較為侵襲的表型，提高轉移的能力。癌細胞的轉移往往是惡性腫瘤的重要特徵之一，並且也是在臨床治療上最難以根治的。因此，在很多研究中，大都是以針對癌細胞的轉移過程，開發新藥或是利用天然藥物處理是否會影響癌細胞的轉移。目前已有多種天然話何物已被確認具有抗發炎，抗過敏及抗癌的活性，且其中具有生物活性的有效成份，部分已被單離並經分析鑑定。目前檜木醇(β-thujaplicin)與黃連素(berberine)被發現的生物功能中包括：抗轉移、抗癌、抗發炎、抗氧化和細胞凋亡等多重功用。但是，β-thujaplicin與berberine對於人類子宮頸細胞癌的作用機制、血管新生作用與EMT現象，至今仍然不清楚。本實驗室分析研究結果發現檜木醇(β-thujaplicin)能明顯抑制人類子宮頸癌細胞的侵襲及移動能力，及VEGF或子宮頸癌細胞培養液所誘導血管內皮細胞管柱形成(tube formation)的能力，並降低由TGF-β所誘導上升MMP-9的活性。此外，β-thujaplicin能促進EMT marker E-cadherin的蛋白表達。本研究選用一株具有高度轉移能力的人類子宮頸癌細胞：SiHa HeLa CaSki來探討berberine對於癌細胞移動和侵入能力的影響。首先藉由wound healing assay與Boyden chamber assay，發現berberine具有抑制SiHa移動和侵入的能力，而且不具有細胞毒性。我們藉由cells adhesion assay也發現berberine能抑制細胞的貼附和延伸。而當癌細胞轉移時常常會伴隨著細胞外基質的分解及細胞移動能力的改變，在gelatin zymography與casein zymography assay中也發現到它們可以抑制SiHa人類 子宮頸癌細胞的MMP-2及u-PA的表現。因此我們再利Western blot發現berberine會抑制SiHa人類子宮頸癌細胞NF-kB的表現。此外，當處理berberine後會增加上皮細胞的特徵，如:E-cadherin，以及減少間質細胞的特徵，如:Snail-1和N-cadherin，使細胞的入侵和轉移降低。我們也發現berberine具有抑制細胞核內NF-kB的表現及DNA binding的活性。綜合以上結果，Berberine可能是透過抑制NF-kB蛋白的轉錄因子的活性，與抑制癌細胞蛋白水解酶來達到反轉EMT以及抑制癌細胞轉移的能力。此外在動物實驗中我們也證實berberine可抑制血管新生作用，腫瘤的生長作用以及癌細胞的轉移。

中 文 關 鍵 詞 ： 檜木醇;黃連素; 子宮頸癌; 癌症轉移; 侵襲; 上皮間質轉化

英 文 摘 要 ： Metastasis is the most common cause of cancer-related death in patients, and epithelial to mesenchymal transition (EMT) is essential for cancer metastasis, which is a multistep complicated process that includes local invasion, intravasation, extravasation, and proliferation at distant sites. When cancer cells metastasize, angiogenesis is also required for metastatic dissemination, given that an increase in vascular density will allow easier access of tumor cells to circulation and represents a rational target for therapeutic intervention. It has been showed that -thujaplicin and berberine may have potentially beneficial effects, including anti-metastatic, anti-cacinogenic, anti-inflammatory, anti-oxidant and apoptotic effects. However,

theeffects molecular mechanism of -thujaplicin and berberine in human cervical carcinoma are presently unknown. . In our study, we demonstrated that β-thujaplicin inhibited cell invasion in SiHa cervical cancer cells. β-thujaplicin was sufficient to inhibit TGF-β-induced MMP-9 expression and increase TGF-β-reduced E-cadherin (MET marker) expression in SiHa human cervical cancer cells. β-thujaplicin could reduce VEGF-induced tube formation of HUVECs. Moreover, we provided molecular evidence that is associated with the anti-metastatic effect of berberine by showing a nearly complete inhibition on invasion ($P < 0.001$) of highly metastatic SiHa cells via reduced transcriptionally activities of matrix metalloproteinase-2 and urokinasetype plasminogen activator. Berberine reversed transforming growth factor-β1-induced EMT and caused upregulation of epithelial markers such as E-cadherin and inhibited mesenchymal markers such as N-cadherin and snail-1. Selective snail-1 inhibition by snail-1-specific-siRNA also showed increased E-cadherin expression in SiHa cells. Berberine also reduced tumor-induced angiogenesis in vitro and in vivo. Importantly, in vivo BALB/c nude mice xenograft model and tail vein injection model showed that berberine treatment reduced tumor growth and lung metastasis by oral gavage, respectively. Taken together, these findings suggested that berberine could reduce metastasis and angiogenesis of cervical cancer cells, thereby constituting an adjuvant treatment for metastasis control.

英文關鍵詞： β-thujaplicin; berberine, cervical cancer, metastasis, invasion, EMT

行政院國家科學委員會補助專題研究計畫 □期中進度報告 ■期末報告

檜木醇與黃連素抑制子宮頸癌細胞之侵襲轉移、上皮-間質相互轉換(EMT) 與血管新生作用之相關機制研究表現

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中 華 民 國 105年 9月 18日

中英文摘要

癌細胞在轉移的過程中，癌細胞會出現上皮-間質型態相互轉換(EMT)之情形，增加較為侵襲的表型，提高轉移的能力。癌細胞的轉移往往是惡性腫瘤的重要特徵之一，並且也是在臨床治療上最難以根治的。因此，在很多研究中，大都是以針對癌細胞的轉移過程，開發新藥或是利用天然藥物處理是否會影響癌細胞的轉移。目前已有多種天然話何物已被確認具有抗發炎，抗過敏及抗癌的活性，且其中具有生物活性的有效成份，部分已被單離並經分析鑑定。目前檜木醇(β-thujaplicin)與黃連素(berberine)被發現的生物功能中包括：抗轉移、抗癌、抗發炎、抗氧化和細胞凋亡等多重功用。但是，β-thujaplicin 與 berberine 對於人類子宮頸細胞癌的作用機制、血管新生作用與 EMT 現象，至今仍然不清楚。本實驗室分析研究結果發現檜木醇(β-thujaplicin)能明顯抑制人類子宮頸癌細胞的侵襲及移動能力，及 VEGF 或子宮頸癌細胞培養液所誘導血管內皮細胞管柱形成(tube formation)的能力，並降低由 TGF-β 所誘導上升 MMP-9 的活性。此外，β-thujaplicin 能促進 EMT marker E-cadherin 的蛋白表達。本研究選用一株具有高度轉移能力的人類子宮頸癌細胞：SiHa HeLa CaSki 來探討 berberine 對於癌細胞移動和侵入能力的影響。首先藉由 wound healing assay 與 Boyden chamber assay，發現 berberine 具有抑制 SiHa 移動和侵入的能力，而且不具有細胞毒性。我們藉由 cells adhesion assay 也發現 berberine 能抑制細胞的貼附和延伸。而當癌細胞轉移時常常會伴隨著細胞外基質的分解及細胞移動能力的改變，在 gelatin zymography 與 casein zymography assay 中也發現到它們可以抑制 SiHa 人類 子宮頸癌細胞的 MMP-2 及 u-PA 的表現。因此我們再利 Western blot 發現 berberine 會抑制 SiHa 人類子宮頸癌細胞 NF-kB 的表現。此外，當處理 berberine 後會增加上皮細胞的特徵，如:E-cadherin，以及減少間質細胞的特徵，如:Snail-1 和 N-cadherin，使細胞的入侵和轉移降低。我們也發現 berberine 具有抑制細胞核內 NF-kB 的表現及 DNA binding 的活性。綜合以上結果，Berberine 可能是透過抑制 NF-kB 蛋白的轉錄因子的活性，與抑制癌細胞蛋白水解酶來達到反轉 EMT 以及抑制癌細胞轉移的能力。此外在動物實驗中我們也證實 berberine 可抑制血管新生作用，腫瘤的生長作用以及癌細胞的轉移。

關鍵詞：檜木醇;黃連素; 子宮頸癌; 癌症轉移; 侵襲; 上皮間質轉化

Abstract

Metastasis is the most common cause of cancer-related death in patients, and epithelial to mesenchymal transition (EMT) is essential for cancer metastasis, which is a multistep complicated process that includes local invasion, intravasation, extravasation, and proliferation at distant sites. When cancer cells metastasize, angiogenesis is also required for metastatic dissemination, given that an increase in vascular density will allow easier access of tumor cells to circulation and represents a rational target for therapeutic intervention. It has been showed that β-thujaplicin and berberine may have potentially beneficial effects, including anti-metastatic, anti-cacinogenic, anti-inflammatory, anti-oxidant and apoptotic effects. However, the effects molecular mechanism of β-thujaplicin and berberine in human cervical carcinoma are presently unknown. . In our study, we demonstrated that β-thujaplicin inhibited cell invasion in SiHa cervical cancer cells. β-thujaplicin was sufficient to inhibit TGF- β-induced MMP-9 expression and increase TGF-β-reduced E-cadherin (MET marker) expression in SiHa human cervical cancer cells. β-thujaplicin could reduce VEGF-induced tube formation of HUVECs. Moreover, we provided molecular evidence that is associated with the anti-metastatic effect of berberine by showing a nearly complete inhibition on invasion ($P < 0.001$) of highly metastatic SiHa cells via reduced transcriptionally activities of matrix metalloproteinase-2 and urokinasetype plasminogen activator. Berberine reversed transforming growth factor-β1-induced EMT and caused upregulation of epithelial markers such as E-cadherin and inhibited mesenchymal markers such as N-cadherin and snail-1. Selective snail-1 inhibition by snail-1-specific-siRNA also showed increased E-cadherin expression in SiHa cells. Berberine also reduced tumor-induced angiogenesis *in vitro* and *in vivo*. Importantly, in vivo BALB/c nude mice xenograft model and tail vein injection model showed that berberine treatment reduced tumor growth and lung metastasis by oral gavage, respectively. Taken together, these findings suggested that berberine could reduce metastasis and angiogenesis of cervical cancer cells, thereby constituting an adjuvant treatment for metastasis control.

Keywords: β-thujaplicin; berberine, cervical cancer, metastasis, invasion, EMT

報告內容

1 Introduction

Cervical cancer is the second most common female cancer worldwide, with an estimated 530,000 new cases ever year and the third greatest cause of death from cancer in women. Although cervical cytology screening has helped reduce mortality rates, managing pre-invasive and invasive cervical lesions remains a challenge (Smith et al., 2013). Cancer metastasis and resistance to treatment are two major causes for poor survival and prognosis of cervical cancer patients. Most patient deaths from cervical cancer are related to metastasis, which is a complicated and currently uncontrolled process. Therefore, reducing the metastasis of cervical tumor cells is one of the most important research areas in medicine.

Tumor malignancy consists of a series of complicated processes, including invasion, migration, adhesion, angiogenesis, and proliferation. During tumor progression, tumor cells acquire expression of mesenchymal markers, such as vimentin, N-cadherin, and fibronectin, as well as loss of epithelial markers, such as E-cadherin and α-catenin, to result in epithelial–mesenchymal transition (EMT), subsequent tumor metastasis, and proliferation at distant sites. Suppressing E-cadherin expression by its transcriptional suppressor, snail-1, is a key process in EMT (de Herreros et al., 2010). During cancer metastasis, secreting extracellular proteases is significant in cancer invasion (Rao, 2003). Among these proteases, matrix metalloproteinase-2 (MMP-2, also known as gelatinase A), which belongs to a family of structurally related zinc-dependent extracellular matrix (ECM)-degrading enzymes, is a proteolytic enzyme that is capable of degrading the structural support network for normal and malignant cells, which could serve a crucial role in invasion and angiogenesis of tumor cells (Roomi et al., 2012). In addition to matrix metalloproteinases, serine protease urokinasetype plasminogen activator (u-PA), which is secreted in cervical cancer, is a key factor to initiate a cascade of proteolytic steps that accumulates in degrading the ECM (Roomi et al., 2012; Tee et al., 2012).

Angiogenesis is a physiologic process that generates new blood vessels from pre-existing capillaries and is critical to growth and metastasis of solid tumors. In normal tissues, the vascular system is regulated by a balance of anti-angiogenic and pro-angiogenic molecules, which ensures that an efficient and orderly network

of blood vessels is maintained to meet the metabolic demands of the tissue. Unlike normal tissue vasculature, tumor vessels are generally long and highly chaotic, with numerous abnormalities that result in poor blood flow and high vascular permeability, which may lead to reduced efficiency of chemotherapy in cancer patients and elevated potential for metastasizing to distant organs (Vaupel, 2004). A fundamental step in the transition of tumors from dormant to malignant state is followed by the tumor potential for metastasis. Inhibiting the angiogenesis and metastasis of human cervical cancer cells could serve as an effective strategy against cervical cancer progression (Xie et al., 2013).

Chemopreventive approach using non-toxic botanicals could be one of the strategies for cancer management. These natural substances are of interest, as they are potential sources of anticancer compounds with minimal debilitating toxicity and side effects. Berberine, which is a naturally occurring isoquinoline alkaloid, is an active component in the roots, rhizomes, and stem barks of many medicinal plants, including *Berberis vulgaris* (barberry), *Berberis aristata* (tree turmeric), *Berberis aquifolium* (Oregon grape), and *Coptis chinensis* (Chinese goldthread). Berberine was initially used as an antibiotic because of its potent antimicrobial activity against many organisms including bacteria, fungi, protozoans, viruses, chlamydia, and helminthes (Yu et al., 2005). Berberine has a wide range of pharmacological and biochemical effects for various clinical conditions, such as diarrhea, hypertension, arrhythmias, and inflammation (Rabbani et al., 1987). Recently, berberine has been demonstrated to possess anticancer activities including DNA-modified electrodes (Tian et al., 2008), reduction of AP-1 activity to induce growth arrest and apoptosis (Mahata et al., 2011), and induction of caspase-dependent apoptosis in cervical cancer (Mantena et al., 2006a, b). Berberine inhibited invasion of human lung carcinoma A549 cells, increased expression of E-cadherin, and repressed expression of vimentin during initiation of transforming growth factor (TGF)-β1-induced EMT. Berberine inhibited the capacity of hepatocellular carcinoma to stimulate human umbilical vein endothelial cell (HUVEC) proliferation, migration, and endothelial tube formation (Jie et al., 2011). Berberine also inhibited HIF-1α expression via enhanced proteolysis in gastric adenocarcinoma cell line SC-M1 (Lin et al., 2004). However, the effects of berberine on cancer invasion, angiogenesis, and EMT of human cervical carcinoma and the underlying mechanisms of such

effects remain unclear. In the current study, we tested the hypothesis that berberine has .the anti-metastatic and reverse EMT potential for human cervical cells.

2 Materials and Methods

Materials and Chemicals.

Berberine, heparin, Giemsa, gelatin, 4'-6-Diamidino-2-phenylindole, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), Coomassie brilliant blue R-250 and crystal violet were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Matrigel was purchased from BD Biosciences (Bedford, MA, USA). The Immobilon Western Chemiluminescent HRP substrate kit was obtained from Millipore (Burlington, MA). Dulbecco's modified Eagle medium (DMEM), medium 199, penicillin, streptomycin and trypsin-EDTA were obtained from Gibco Invitrogen Corparation (Barcelona, Spain).

Cell culture

SiHa cells were obtained from the American Type Culture Collection (Manassas, VA), whereas HeLa and CaSki were obtained from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan) and were cultured in DMEM with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. HUVECs were obtained from the BCRC and cultured on gelatin-coated culture dishes in medium 199 with 10% FBS, 25 U/ml of heparin, 30 μg/ml of endothelial cell growth supplement (ECGS, Sigma, St. Louis, MO), 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin. Subcultures were performed with trypsin-EDTA. Cells from passages 5–10 were used. Media were refreshed every other day. All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Determination of cell viability (MTT assay)

Cells were incubated with 0.5 mg/mL of MTT in culture medium for an additional 4 h; the blue formazan crystals of viable cells were dissolved and measured spectrophotometrically at 570 nm (Chen et al., 2005).

Boyden chamber cell invasion and motility assays

After pre-treatment with berberine for 24 h, cells were harvested and seeded to Boyden chamber (Neuro Probe, Cabin John, MD) at 1.5×10^4 cells/well in serum free medium and then incubated for another 24 h at 37 °C. For the invasion assay, 10 μL of Matrigel (0.5 mg/1 mL) was applied to 8 μm pore-sized polycarbonate membrane filters, in which the bottom chamber of the apparatus contained standard medium. The invaded cells were fixed with methanol and stained with Giemsa. Cell numbers were counted under a light microscope, whereas motility assay was carried out as described for the invasion assay without coating of Matrigel (Chen et al., 2011).

Wound healing migration assay

Cells were seeded into a 12-well culture dish, and then wounds were introduced to the confluent monolayer of cells with a sterile 200 μL plastic pipette tip to create a denuded area. Cell movement into the wound area was photographed at 0 and 24 h under a microscope (Ho et al., 2011).

Cell matrix adhesion assay

After 24 h treatment with berberine, cells were placed on 24-well dishes that were coated with type I collagen (10 μg/mL). Non-adherent cells were removed by PBS washes. After staining with 0.1% crystal violet, fixed cells were lysed in 0.2% Triton X-100, and the absorbance was measured at 550 nm (Ho et al., 2011).

Determination of MMPs and u-PA by zymography

In gelatin zymography, collected media were subjected to 0.1% gelatin–8% SDS polyacrylamide gel electrophoresis to determine the MMPs. After electrophoresis, gels were washed with 2.5% Triton X-100 and then incubated in reaction buffer. Gel was then stained with Coomassie brilliant blue R-250. The u-PA activity was visualized by casein zymography (Chen et al., 2011).

Measurement of MMP-2 and u-PA promoter activity

A 460 bp (−218 to +243) segment from the 5'-promoter region of the MMP-2 gene and a 644 bp (−562 to +83) segment from the 5'-promoter region of the u-PA gene were cloned. The pGL3-MMP-2 and pGL3-u-PA plasmids were transfected into SiHa cells using PolyJet™ reagent (SignaGen Laboratories, Gaithersburg, MD) according to the manufacturer's instructions. After incubation with berberine, cells were collected and disrupted

by Luciferase Assay System (Promega, San Diego, CA). Firefly luciferase activities were standardized for β-galactosidase activity (Lin et al., 2010).

NF-κB binding assay

Binding of NF-κB in nuclear extracts was assessed by electrophoretic mobility shift assay (EMSA) with biotin-labeled double-stranded NF-κB oligonucleotides. EMSA was carried out with Lightshift kit. Specific binding was confirmed with a 200-fold excess of unlabeled probe as specific competitor. Gel shifts were visualized with a streptavidin-horseradish peroxidase (HRP) followed by chemiluminescent detection (Lin et al., 2010).

Immunofluorescence staining

Cells were cultured on sterile glass coverslips in six-well plates. Slides were incubated overnight at 4 °C with Texas-568 phalloidin (invitrogen, Carlsbad, CA). The slides were counterstained with 4'-6-Diamidino-2-phenylindole and analyzed by confocal microscopy.

Western blot

Samples of cell lysates were separated in 10% polyacrylamide gel and transferred onto a nitrocellulose membrane as previously described. The blot was subsequently operated with standard procedures and probed with primary and secondary antibodies. Protein expression was detected by chemiluminescence using Immoblon Western Chemiluminescent HRP Substrate kit (Ho et al., 2010).

Snail-1 siRNA

The 1:1:1 mixture of Snail-1-siRNA #5, Snail-1-siRNA #6, and Snail-1-siRNA #7 were obtained from Invitrogen. Forward transfections were performed with Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA) following the guidelines according to the manufacturer (Hsieh et al., 2013). After treatment, the cell lysates and unclear extracts were extracted and analyzed by Western blot.

Chicken chorioallantoic membrane assay

Fertilized chicken eggs were transferred into an egg incubator maintained at 37 °C and 50% humidity and allowed to grow for 9 d. For separation of chicken chorioallantoic membrane (CAM) from the shell membrane, small holes were drilled in the shell, one at the broad end of the egg where the air sac is located and the other at a position 90° halfway down the length of the egg. Gentle suction was applied at the hole at the broad end of the egg to create a false air sac directly over the CAM, and a 1 cm^2 window was removed from the eggshell immediately over the second hole. DMSO (control group) and berberine (10 μg) were placed on the CAM, and the embryos were further incubated for 48 h. Neovascular zones under the disks were photographed.

Zebrafish angiogenesis model

Transgenic Tg [*fli-1*:enhanced green fluorescent protein (EGFP)] zebrafish embryos, in which EGFP is expressed in all endothelial cells of the vasculature, were used to monitor the effects of berberine on embryonic angiogenesis (Lawson and Weinstein, 2002). Zebrafish embryos were generated by natural pairwise mating and raised at 28 °C in embryo water (0.2 g/l of Instant Ocean Salt in distilled water). Approximately 10 healthy embryos were placed in 6 cm dishes, and berberine was added into embryo water at 6 h post fertilization. The embryo water with 40 μM berberine was replaced daily. At 48 h post fertilization, the embryos were anesthetized using 0.05% 2-phenoxyethanol in the embryo water. The embryos were further observed for blood vessel development, particularly in dorsal longitudinal anastomotic vessels (DLAVs) and intersegmental arteries (ISAs) under confocal microscopy (630×).

Preparation of conditioned medium (CM)

SiHa cells were cultured in DMEM + 10% FBS until confluence for 48 h. CM was collected and centrifuged at 1000 rpm for 5 min.

Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

For reverse transcription, 2 μg of total RNA were used as templates in a 20 μl reaction with 4 μl of dNTPs (2.5 mM), 2.5 μl of Oligo dT (10 pmole/μL), and 200 U of RTase. PCR was performed using Platinum Taq polymerase (Invitrogen) as follows: 25 cycles at 94 °C for 1 min, 55 °C (u-PA and PAI-1) or 63 °C (MMP-2,

TIMP-2, and GAPDH) for 1 min, 72 °C for 2 min followed by 10 min at 72 °C.

Matrigel tube formation assay

The 96-well plates were coated with 50 µl of Matrigel (10 mg/ml) (BD Bioscience Pharmingen) by incubation at 37 °C for 1 h. HUVECs were suspended in M199 with 10% FBS and ECGS, and then plated onto a layer of Matrigel at a density of 2.5×10^4 cells/well with or without CMs of SiHa cells. The plates were then incubated for 8 h at 37 °C, and capillary-like tube formation was observed under microscope.

Tumor growth and lung metastasis

All procedures that involved animals were in accordance with the Institutional Animal Care and Use Committee (IACUC) of the institutional animal welfare guidelines of the Chung Shan Medical University (IACUC Approval Number: 1217). For nude mice xenograft model, 5 to 6 weeks old immunodeficient nude mice (BALB/c AnN.Cg*Foxn* ^{nu}/Crl Narl mice) weighing 17 g to 19 g were used. The mice were housed with a regular 12 h light/12 h dark cycle and ad libitum access to standard rodent chow diet (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO) and were kept in a pathogen-free environment at the Laboratory Animal Unit. SiHa cells (4×10^6 cells/0.1 mL/mouse) were injected subcutaneously into the right front axilla. Eight days post implantation, the mice were randomly divided into three groups (n = 5 for each group) and fed by oral gavage with placebo (control) and berberine (10 and 20 mg/day/kg). Bioluminescence imaging was performed using IVIS50 animal imaging system (Xenogen Corp., Alameda, CA) (Hu et al., 2012). Tumor growth was monitored by luciferase activity in SiHa cells, and the emitted photons from the target site penetrated through the mammalian tissue and could be externally detected and quantified using a sensitive light imaging system. For lung metastasis assay, SiHa cells (1.5×10^6 cells) that were suspended in 0.1 ml of PBS were injected into the tail vein of BALB/c nude mice. On the following day (day 1), mice were randomly divided into three groups (n = 5 for each group) to be fed by oral gavage with placebo (control) or berberine (20 mg/kg of body weight, daily). Five untreated mice were used as wild type control. After 21 d, animals were euthanized with CO_2. Lungs were isolated and weighed, and metastatic nodules on the surface of the lungs were counted under a

microscope. Lungs were fixed in neutral buffered 5% formalin, and sections were collected and stained with hematoxyline and eosine for morphological studies (Kim et al., 2009).

Immunohistochemistry analysis

Paraffin-embedded slides were deparaffinized, and antigen unmasking was carried out by microwave heating in citrate buffer for 20 min. Slides were incubated with primary anti-Ki67, anti-VEGF, and anti-CD31 antibodies, and biotinylated secondary anti-mouse antibodies were also added.

Statistical analysis

Statistical significances were analyzed by one-way analysis of variance (ANOVA) with post hoc Dunnett's test. *P* value < 0.05 was considered statistically significant (Sigma-Stat 2.0, Jandel Scientific, San Rafael, CA).

3 Results

Berberine exerts strong anti-invasion and anti-migration efficacy against SiHa cells

MTT assay results showed that berberine only slightly reduced the viability of HeLa, SiHa, and CaSki cervical cancer cell lines (**Figure 1A**). The 20 μM berberine treatment inhibited the invasion potential of CsSki, SiHa, and HeLa cells by 37%, 65%, and 22%, respectively (**Figure 1B**). Among the three cervical cancer cell lines, SiHa cells were affected at the highest extent. Berberine exhibited the strongest reduction effect on invasion and motility of SiHa cells in a dose-dependent manner (**Figure 1C**). Quantification analysis indicated that the invasiveness of SiHa cells were reduced by 69 % ($P < 0.01$) when cells were treated with 20 μM of berberine. Berberine also significantly reduced the motility ($P < 0.001$) of SiHa cells in a concentration-dependent manner (**Figure 1D**). Wound healing migration assay was performed to assess if berberine affects cell migration. Incubating SiHa with 1% FBS produced a marked cell migration in the wound area. Berberine was able to significantly inhibit SiHa cell migration by 87.7% in wound healing assays performed over a period of 24 h (**Figure 1E**). Berberine was then tested to determine its effects on cell–matrix adhesion. The results showed that berberine significantly reduced the cell–collagen interactions of SiHa cells (**Figure 1F**). To screen for preventive effectors against migration of other cervical cancer cells, the inhibitory effect of berberine on migration of HeLa and CaSki cells were examined by wound healing assay. The result showed that berberine significantly reduced the cell migration in both HeLa and CaSki cells (data not shown).

Berberine exerts an inhibitory effect on MMP-2 and u-PA, and has an increment effect on TIMP-2 and PAI-1

Given that the expression and activity of u-PA and MMP-2 are critical to cell invasion, the expression and activity of u-PA and MMP-2 of SiHa cells that were treated with different concentrations of berberine were examined by casein zymography and gelatin zymography, respectively. Berberine reduced the activities of both u-PA (**Figure 2A**) and MMP-2 (**Figure 2B**). Western blot showed that MMP-2 and u-PA protein expression were significantly decreased along with the concentration of berberine (**Figure 2C**). Physiological activity of MMP-2 is closely related to that of the specific endogenous inhibitor TIMP-2. Therefore, Western blot was

performed to determine the effects of berberine on TIMP-2 expression. The results showed that TIMP-2 protein levels were gradually increased along with the concentration of berberine in SiHa (**Figure 2C**). To evaluate the effects of berberine on MMP-2 and u-PA promoters, transient transfection was performed with pGL3-u-PA (**Figure 2D)** and pGL3-MMP-2 (**Figure 2E**) promoters. Luciferase activities of the berberine-treated transfectants were reduced in a dose-dependent manner. To examine whether the inhibitory effect of berberine on MMP-2 expression was linked to NF-κB activities, nuclear extract was analyzed by EMSA for NF-κB DNA binding activity. The result showed that a pre-treatment with berberine suppressed NF-κB binding activity **(Figure 2F)**. Subsequently, Western blot was performed to further confirm these results. The findings indicated that berberine suppressed the nuclear levels of NF-κB with C23 as the internal control. Berberine also significantly inhibited the phosphorylation of NF-κB in the cytoplasm **(Figure 2G)**. To further delineate whether the inhibition of MMP-2 secretions by berberine were mainly affected by inhibition of NF-κB signaling pathway, effects of the NF-κB inhibitor on SiHa cells were investigated. The results showed that sole treatment with the 10μM NF-κB inhibitor led to inhibition of MMP-2 activity similar to that of 10μM berberine. The combined treatment of inhibitor with 10μM berberine could also decrease the MMP-2 activity (**Figure 2H**).

Berberine targets signaling molecules that regulate EMT in SiHa cells

We examined the berberine effect on major regulators and markers of EMT. Berberine significantly elicited the upregulation of epithelial markers, such as E-cadherin. Berberine treatment slightly increased ZO-1 expression but did not affect Claudin-1 expression. Berberine also significantly decreased occludin expression, as well as mesenchymal markers, such as N-cadherin. Berberine treatment slightly decreased fibronectin and vimentin expressions (**Figure 3A**). Focal adhesion kinase (FAK) and paxillin are protein tyrosine kinases that are linked to signaling events between cells and the ECM. To understand the possible mechanism underlying berberine anti-migratory and anti-invasive efficacy on focal adhesion, we examined the effects of berberine on p-FAK, total-FAK, p-paxillin, and total-paxillin expressions. Our results showed that upon treatment of SiHa cells with berberine, phosphorylation of FAK, paxillin and Src were noticeably reduced (**Figure 3B**). Berberine

significantly decreased the transcription factor nuclear protein expression of snail-1 with C23 as the internal control sample in SiHa cells (**Figure 3C**). Cadherin-bound β-catenin is required for cell adhesion. Upon activation of wet signaling, β-catenin translocated to nucleus to induce the expression EMT-related genes. Western blot analyses clearly revealed elevated β-catenin protein levels in the cytoplasm, whereas nuclear β-catenin was decreased by berberine treatment (**Figure 3D**). Quantification of E-cadherin, Occludin, ZO-1, p-FAK, p-paxillin, and p-Src was shown in Supplemental Figure 1.

We used snail-1 siRNA to examine further the role of snail-1 in regulating E-cadherin expression. Snail -1 siRNA strongly decreased the level of snail-1 after 48 h of treatment (**Figure 3E**). Knockdown of snail-1 expression by snail-1 siRNA was accompanied by an increase in E-cadherin level in SiHa cells. We silenced the snail-1 expression by snail-1 siRNA and berberine treatment to determine if berberine increases the E-cadherin level by other pathways independent of snail-1. Western blot results showed that the combined treatment of berberine slightly increased the E-cadherin level when the snail-1 level is selectively inhibited, which suggests that part of the function of snail-1 in berberine inhibition increased the E-cadherin level (**Figure 3E**).

To clarify whether or not berberine could inhibit the expression of MAPK and PI3K/Akt pathways, Western blot analysis was conducted. Berberine significantly inhibited the phosphorylation of p38 after a treatment of 40 μM berberine, whereas it did not affect on p-ERK1/2 and p-Akt expression. Berberine treatment slightly decreased p-JNK1/2 expression. Moreover, no significant change in the total amount of ERK1/2, p38, JNK1/2, PI3K and Akt proteins were observed (**Figure 3F and 3G**).

Berberine reduced ability of TGF-β1-induced EMT, cell invasion, and MMP-2 expression

TGF-β1-mediated EMT of human cervical cancer cells may contribute to cervical cancer metastasis. To determine whether berberine could affect the TGF-β-induced scattering, SiHa cells were pretreated with berberine prior to stimulation with TGF-β1. SiHa cells were pretreated with berberine for 2 h (10 and 20 μM) prior to stimulation with TGF-β1 (5 ng/mL) for 24 h. After treatment with TGF-β1, the cells adopted a more fibroblast-like morphology and reduced their cell−cell contact. Berberine blocked TGF-β1-induced scattering in

a dose-dependent manner (**Figure 4A**). To confirm the morphology change in Fig. 4a, immunofluorescence was performed to examine the actin profile. The SiHa cells underwent morphological and compositional changes consistent with EMT following treatment with TGF-β1. These changes included loss of apical polarity with the acquisition of a more fibroblast-like spindle shape and cytoskeletal remodeling with the appearance of actin stress fibers. Berberine reversed TGF-β1-induced morphological changes (**Figure 4B**). We further examined whether berberine also affected TGF-β1-induced cell invasion. Quantitative analyses by cell invasion assay showed that the invasion of SiHa cells was increased by ~3.0-fold upon TGF-β1 treatment, and TGF-β1-induced invasion was reduced by berberine treatment in a dose-dependent manner (**Figure 4C**). Berberine also reduced TGF-β1-induced MMP-2 activity of SiHa cells in a dose-dependent manner (**Figure 4D**). To ensure that berberine-blocked TGF-β1-induced scattering was not caused by cell death or inhibition of proliferation, MTT assay revealed that berberine had no effect on cell viability (**Figure 4E**).

Anti-angiogenic effects of berberine on HUVECs in vitro and on treated chicken CAM and transgenic zebrafish embryos in vivo

MMP-2 and u-PA are key factors in degrading the ECM by invading and proliferating endothelial cells with subsequent invasion of the underlying stroma. Preventing ECM degradation by inhibiting MMP and u-PA activities could be a potential therapeutic approach to block the invasion that occurs during angiogenesis. The present study aimed to confirm whether berberine has anti-angiogenic effects on HUVECs. The HUVEC cells were treated with berberine at 0, 10, 20, 30, and 40 μM for proliferation, invasion, and expression of MMP-2 and u-PA. HUVEC cell proliferation was evaluated by MTT assay. The results showed that berberine slightly reduced the viability of HUVEC cells with 87.6% remaining after a treatment of 40 μM berberine (**Figure 5A**). Boyden chamber Matrigel invasion assay was performed to assess whether berberine affects HUVEC migration. The result showed that berberine significantly reduced the invasion ($P < 0.001$) (**Figure 5B**). Zymography showed a dose-dependent inhibition of MMP-2 and u-PA expression with virtual total inhibition at 10 μM concentration (**Figure 5C**). Western blot was performed to determine the effects of berberine on MMP-2, u-PA, TIMP-2, and PAI-1 expression, and the results showed that MMP-2 and u-PA protein expressions were

gradually decreased, whereas TIMP-2 and PAI-1 protein levels were gradually increased along with the concentration of berberine in HUVECs (**Figure 5D**). Regulatory effects of berberine on proteases and their endogenous inhibitors on mRNA levels were also validated by semi-quantitative RT-PCR analysis. With glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, mRNA levels of MMP-2 and u-PA were significantly reduced, whereas mRNA levels of TIMP-2 and PAI-1 were slightly increased in HUVEC cells (**Figure 5E**). CAM assay is an important in vivo model of microvessel formation. Anti-angiogenic activities of berberine analogs were investigated using CAM assay. A marked inhibition of angiogenesis was seen upon examination 2 d after berberine (10 μg) was placed at the vascular membrane when compared to DMSO-treated controls (**Figure 5F**). We then investigated the influence of berberine on vascular development in transgenic Tg (*flil-1*:EGFP) zebrafish. Berberine significantly decreased fluorescent intensities of DLAVs and ISAs in zebrafish (**Figure 5G**). For tube formation, HUVECs were cultured in previously polymerized Matrigel. Berberine inhibited tube formation of HUVECs (**Figure 5H**). These results together with our earlier findings suggest that berberine has anti-angiogenic effects, such as inhibiting vascular tube formation and endothelial cell invasion and protease expression.

Berberine inhibits angiogenic potential of SiHa cells by VEGF downregulation

We examined whether berberine was capable of inhibiting tumor-induced tube formation, invasion, and proliferation on HUVECs. HUVECs that were cultured with conditioned media from SiHa cells appeared in a tube-like structure or tube network form. HUVECs that were cultured with conditioned media from SiHa cells with 10 and 20 μM of berberine led to decreases in tube network compared with the control (**Figure 6A**). SiHa-induced proliferation (**Figure 6B**) and invasion (**Figure 6C**) were reduced by berberine treatment in a dose-dependent manner. Vascular endothelial growth factor (VEGF) is the most potent angiogenic factor and is associated with tumor-induced angiogenesis. To determine the effects of berberine on VEGF secretion, VEGF protein level in the conditioned medium was measured by ELISA. Berberine reduced the VEGF secretion in the culture media in a dose-dependent manner (**Figure 6D**). Western blot was performed to determine the effects of berberine on VEGF expression. The results showed that the VEGF protein expression was significantly

decreased along with the concentration of berberine in SiHa cells (**Figure 6E**). Considering that hypoxia-inducible factor-1α (HIF-1α), which is a transcription factor, has a primary role in mediating hypoxia-induced VEGF transcription, we hypothesized that berberine might downregulate the expression of HIF-1α. The results showed that HIF-1α expression was significantly decreased in SiHa cells (**Figure 6F**).

Antitumor effects of berberine *in vivo*

SiHa-bearing nude mice were treated with placebo or berberine to verify the in vivo antitumor effects of berberine. A 3.8-fold reduction in the berberine-treated (20 mg/kg) animals were observed on day 32 compared with that of the control animals (**Figure 7A**). Berberine (20 mg/kg) feeding also induced a 4.1-fold reduction in tumor weight by day 33 (**Figure 7B**) without any apparent signs of toxicity as proven by the body weight monitoring (**Figure 7C**) throughout the experiment. Consistent with the profound effect on tumor size, a significant increase in proliferation was determined by Ki-67 stain in tumors (**Figure 7D**). Histochemical analysis of the pathologic sections of these tumors showed that berberine-treated tumors had low levels of CD31 (endothelial surface marker) (**Figure 7E**) and VEGF (**Figure 7F**) compared with control SiHa tumors. These data suggested that berberine treatment reduced angiogenesis and tumor growth properties *in vivo*.

Inhibition of lung colonization of SiHa by berberine treatment

Nude mice were injected via the tail vein with SiHa cells, and administration of berberine reduced pulmonary metastasis formation of SiHa cells. Within 21 d of injection, the control mice were visibly riddled with metastatic tumor nodules compared with the lungs of SiHa-treated mice (**Figure 8A**). Mean lung weights for animals that received 20 mg/kg/day berberine (195 ± 41.3 mg; $P < 0.001$) were significantly lower than those from control animals (677 ± 119.1 mg; **Figure 8B**). Vehicle-treated control animals had massive tumor growth and were given an arbitrary-maximum countable number of about 259 ± 55.6. The number was reduced to 50 ± 67.0 (20 mg/kg/day; $P < 0.001$) countable colonies by berberine treatment (**Figure 8C**). The average body weight of berberine-treated mice and control group were not significantly affected (**Figure 8D**). Histopathology of the lungs also showed marked reduction in tumor mass in the lungs of berberine-treated animals (**Figure 8E**).

Taken together, these findings suggested that berberine is able to transcriptionally regulate MMP-2 expression via the down-regulation of NF-κB pathway, reverse EMT by modulating E-cadherin and snail-1 expression and inhibit angiogenesis, lung metastasis and tumor growth (**Figure 9**).

Figure 10 為β-thujaplicin的化學結構圖。利用MTT assay，初步實驗證實β-thujaplicin對於具有高度轉移能力的SiHa子宮頸癌細胞隨著不同濃度增加，經由24小時的處理之下在100 μM時會些微降低細胞的存活率。利用細胞侵襲實驗(Transwell invasion assay) ，證實β-thujaplicin (50與100 μM) 24小時處理之下SiHa細胞侵(invasion)能力有明顯被抑制的現象，右圖為其量化圖。証實β-thujaplicin可抑制細胞侵襲能力(**Figure 11)**。利用gelatin zymography assay，証實β-thujaplicin可抑制由TGF-β所誘導SiHa子宮頸癌細胞產生的MMP-9，右圖為其量化圖(**Figure 12)**。利用Western blot assay，証實 -thujaplicin可促進由TGF-β所降低SiHa子宮頸癌細胞產生E-cadherin的蛋白表現，右圖為其量化圖(**Figure 13)**。利用tube formation assay，証實β-thujaplicin可明顯抑制由VEGF所誘導血管內皮細胞(HUVECs :由食品工業研究所購得) tube的形成(**Figure 14)**。

5、計畫成果及自評

目前已達成計畫中預期之進度：完成分析 berberine 及β-thujaplicin 對子宮頸癌存活以及侵襲轉移的抑制效果，並進一步針對子宮頸癌細胞進行相關研究。上述之實驗結果，目前 berberine 及β-thujaplicin 抑制 SiHa 子宮頸癌細胞侵襲轉移的部份已積極整理並以發表至 Chu SC, Yu CC, Hsu LS, Chen KS, Su MY, **Chen PN***. Berberine Reverses Epithelial-to-mesenchymal Transition and Inhibits Metastasis and Tumor-induced Angiogenesis in Human Cervical Cancer Cells. **Mol Pharmacol**. 2014 Dec;86(6):609-23.，其他細胞珠的相關實驗如:血管新生作用，也正在進行及整理中。關於探討 Hinokitiol(beta-thujaplicin)的抗癌細胞轉移及抑制 EMT 及血管新生的效果及機制，也正在進行及整理準備投稿中。未來希望透過動物活體實驗我們將證實 berberine 與 beta-thujaplicin 具有抑制癌細胞轉移的功效及應用性。

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Figure Legend

Figure 1 The effects of berberine on cell viability, invasion, migration and adhesion of human cervical cancer cell lines. (A) SiHa, CaSki and HeLa cells were treated with berberine for 24 h by MTT assay. (B) SiHa, CaSki and HeLa cells were treated with berberine by invasion assay. (C) SiHa cells were treated with various concentration of Ber (berberine) for 24 h by invasion and motility assay. (D) Quantification of invasion and motility ability from C. (E) SiHa cells were subjected to analyze for cell migration by wound healing assay. (F) SiHa cells were treated with berberine for 24 h, and then subjected to analyze for cell-matrix adhesion. Results were statistically evaluated by using one-way ANOVA with post hoc Dunnett's test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$). Results from 3 repeated and separated experiments were similar.

Figure 2 Inhibitory effects of berberine on the proteinase and transcription activities of MMP-2 and u-PA. SiHa cells were treated with berberine for 24 h, and then subjected to casein zymography and gelatin zymography to analyze the activities of (A) u-PA and (B) MMP-2 respectively as described in Materials and Methods. (C) Western blot analysis of MMP-2, u-PA and TIMP-2 with β-actin being an internal control. Luciferase activity was measured in transiently transfected SiHa cells using (D) pGL3-u-PA and (E) pGL3-MMP-2. (F) Nuclear extracts were analysed for DNA binding activity of NF-κB using biotin labeled NF-κB specific oligonucleotide in EMSA. The last lane represented nuclear extracts incubated with unlabeled oligonucleotide (Comp) to confirm the specificity of binding. (G) Nuclear extracts were subjected to SDS-PAGE followed by western blotting with anti-NF-κB and C23 (nuclear internal control) antibodies. Signals of proteins were visualized with an ECL detection system. (H) Cells were treated with NF-κB inhibitor and then incubated in the presence or absence of berberine for 24 h. Condition media were subjected to gelatin zymography to analyze the activities of MMP-2. Data represented mean ± SD with that of control being 100%, and the statistical significance of results was analyzed by using one-way ANOVA with post hoc Dunnett's test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Figure 3 The effects of berberine on the cytoskeleton related protein. (A&B) Western blot analysis of cytoskeleton related protein with β-actin being an internal control in SiHa cells after 24 h of treatment with berberine. (C) Nuclear extracts were subjected to SDS-PAGE followed by Western blotting with anti-snail-1 antibodies with anti-C23 being an internal control. (D) Cytosol ectracts and nuclear extracts were subjected to SDS-PAGE followed by Western blotting with anti-β-actenin antibodies. (E) The cells were treated with snail-1 siRNA and/or 5 μM berberine. The cell lysates were then subjected to Western blot with anti-snail-1 and anti-E-cadherin antibodies. Similar results were obtained from three repeated and independent experiments.

Figure 4 The inhibitory effect of berberine on TGF-β-induced EMT, MMP-2 and cell invasion. SiHa cells were pre-treated with berberine for 1 h and then cultured in the presence of 5 ng/mL TGF-β for 24h or 48h. (A) Phase image of cells. (B) Immunofluorescence staining of SiHa cells with Texas-568 phalloidin to visualize the actin cytoskeleton. (C) Cells were then subjected to analyses for cell invasion. (D) Condition media were collected for analysis of MMP-2 by gelatin zymography. (E) Cell viability by MTT assay. The quantitative data were presented as means ± SD of three independent experiments ($^{\#}$, $P<0.001$ compared with control. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared with TGF-β1-treated group).

Figure 5 In situ inhibition of angiogenesis in the chicken chorioallantoic membrane (CAM) and zebrafish embryos. HUVECs were treated with berberine at the indicated concentration. (A) HUVECs cell viability and (B) cell invasion. (C) Conditioned media from HUVECs were run on gelatin zymography and casein zymography, and the areas of protease activities of MMP-2 and u-PA appeared as clear bands. (D) HUVECs were treated berberine for 24 h, and then subjected to western blotting to analyze the expression of MMP-2, u-PA, TIMP-2, and PAI-1 with β-actin being an internal control. (E) For mRNA levels, HUVECs total RNAs were extracted and subjected to a semi-quantitative RT-PCR for MMP-2, u-PA, TIMP-2, and PAI-1 with GAPDH being an internal control. (F) The chicken chorioallantoic membranes were treated with vehicle (control) and 10 μM berberine. (G) Lateral image of TG (flil:EGFP) zebrafish embryos 48 h (DLAV, dorsal longitudinal anastomotic vessel; ISA, intersegmental arteries; DA, dorsal aorta; PCV, posterior cardinal vein).

(H) Inhibitory effects of berberine on VEGF induced-tube formation of HUVEC cells. Data represented the mean ± SD of at least 3 independent experiments. Results were statistically evaluated by using one-way ANOVA with post hoc Dunnett's test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Figure 6 Inhibitory effects of berberine on SiHa induced-tube formation capacity of HUVEC cells and the secretion of VEGF by SiHa cells. HUVECs were cultured in conditioned media from SiHa cells, treated with berberine at the indicated concentration. (A) Phase contrast micrographs illustrating the arrangement of HUVECs into a rich meshwork of capillary-like tubular structures when cultured on Matrigel for 8 h. (B) Cell viability of HUVECs by MTT assay. (C) Cell invasion of HUVECs by invasion assay. ($^{\#}$, $P<0.01$ compared with control. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ compared with CM-treated group) (D) SiHa cells were treated with various concentrations of berberine for 24 h, and then condition media were subjected to ELISA. SiHa cells were treated with various concentrations of berberine for 24 h, and then cell lysates and nuclear extracts were subjected to SDS-PAGE followed by Western blotting with (E) anti-VEGF and (F) anti-HIF1α antibodies, respectively. Data represented the mean ± SD of at least 3 independent experiments. Results were statistically evaluated by using one-way ANOVA with post hoc Dunnett's test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Figure 7 The *in vivo* anti-tumor effects of berberine. After subcutaneous implantation of SiHa cells, BALB/c nude mice ($N = 5$ for each group) were treated with placebo or berberine and then analyzed for the growth of tumor. (A) Bioluminescence over time after s.c. inoculation of SiHa cells. (B) Average tumor weight. (C) Average mice body weight. Immunohistochemistry for (D) Ki-67 (cell proliferation marker; 200×), (E) CD31 (the endothelial surface marker; 200×), (F) VEGF in SiHa tumors (200×). Comparisons were performed by using one-way ANOVA with post hoc Dunnett's test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Figure 8 Suppression of lung metastasis of SiHa cells by berberine. SiHa cells were injected into the tail veins of 6-week-old female BALB/c nude mice. After injection of cells, berberine (20 mg/kg/day) and vehicle alone were administered oral gavage for 21 days. Mice were sacrificed and then analyzed for representative photographs of (A) lungs, (B) the weight of lung, (C) the number of lung metastasis and (D) the body weight of mice. (E) Histopathology of lung of metastatic tumor bearing animals. Lungs of the metastasis- induced animals were fixed in neutral buffered formalin, and stained with hematoxyline and eosine. Arrows showed areas of metastatic nodules (tumor). Results were statistically evaluated by using one-way ANOVA with *post-hock* Dunnett's test (***, P<0.001).

Figure 9. Proposed molecular targets in anti-invasiveness, EMT and angiogenesis efficacy of berberine.

Figure 10 為β-thujaplicin的化學結構圖。利用MTT assay，初步實驗證實β-thujaplicin對於具有高度轉移能力的SiHa子宮頸癌細胞隨著不同濃度增加，經由24小時的處理之下在100 μM時會些微降低細胞的存活率。

Figure 11利用細胞侵襲實驗(Transwell invasion assay) ，證實β-thujaplicin (50與100 μM) 24小時處理之下SiHa細胞侵(invasion)能力有明顯被抑制的現象，右圖為其量化圖。証實β-thujaplicin可抑制細胞侵襲能力。

Figure 12利用gelatin zymography assay，証實β-thujaplicin可抑制由TGF-β所誘導SiHa子宮頸癌細胞產生的MMP-9，右圖為其量化圖。

Figure 13利用Western blot assay，証實 -thujaplicin可促進由TGF-β所降低SiHa子宮頸癌細胞產生E-cadherin的蛋白表現，右圖為其量化圖。

Figure 14利用tube formation assay，証實β-thujaplicin可明顯抑制由VEGF所誘導血管內皮細胞(HUVECs：由食品工業研究所購得) tube的形成。

附表及附圖

Figure 1

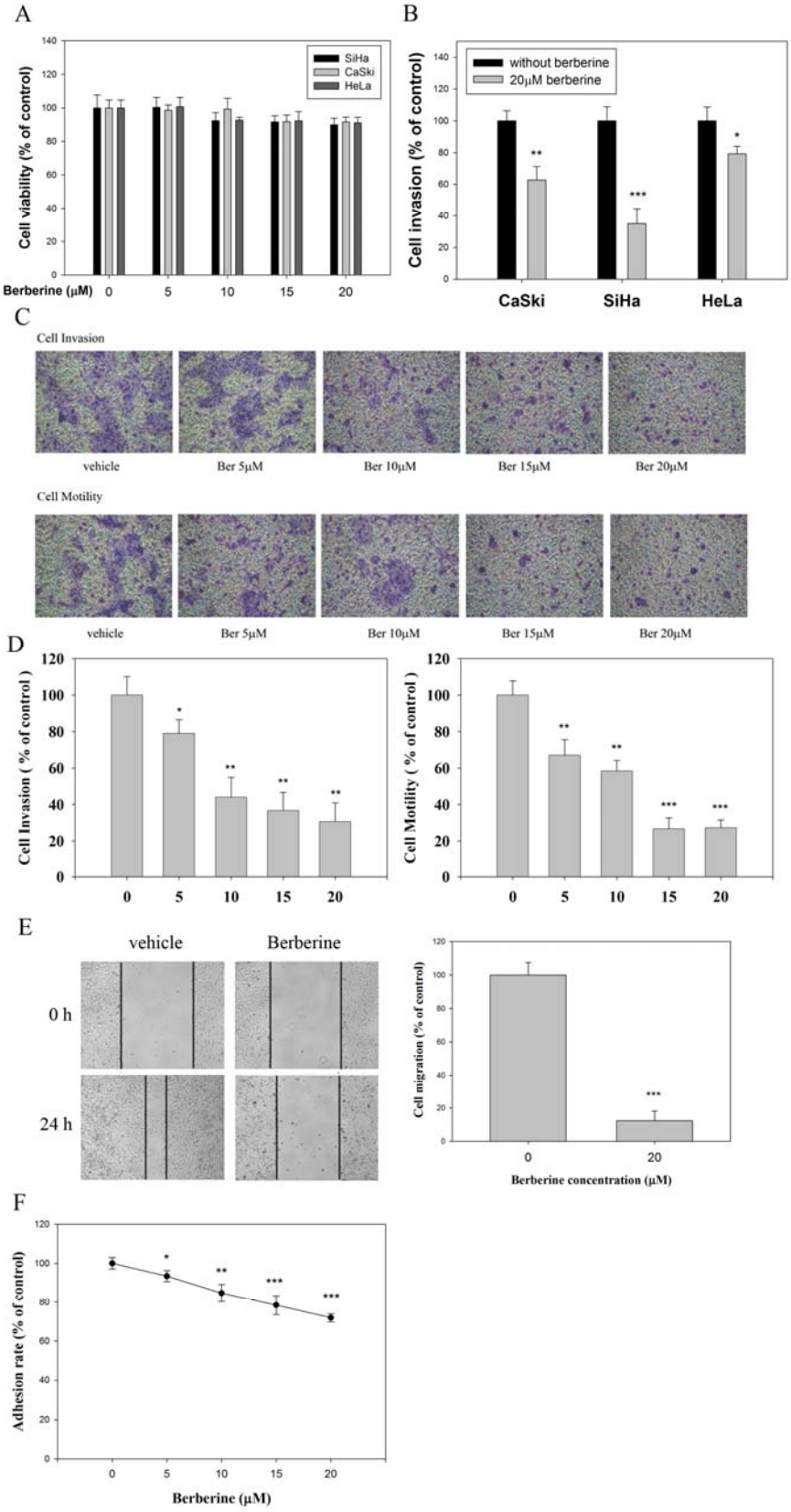


Figure 2

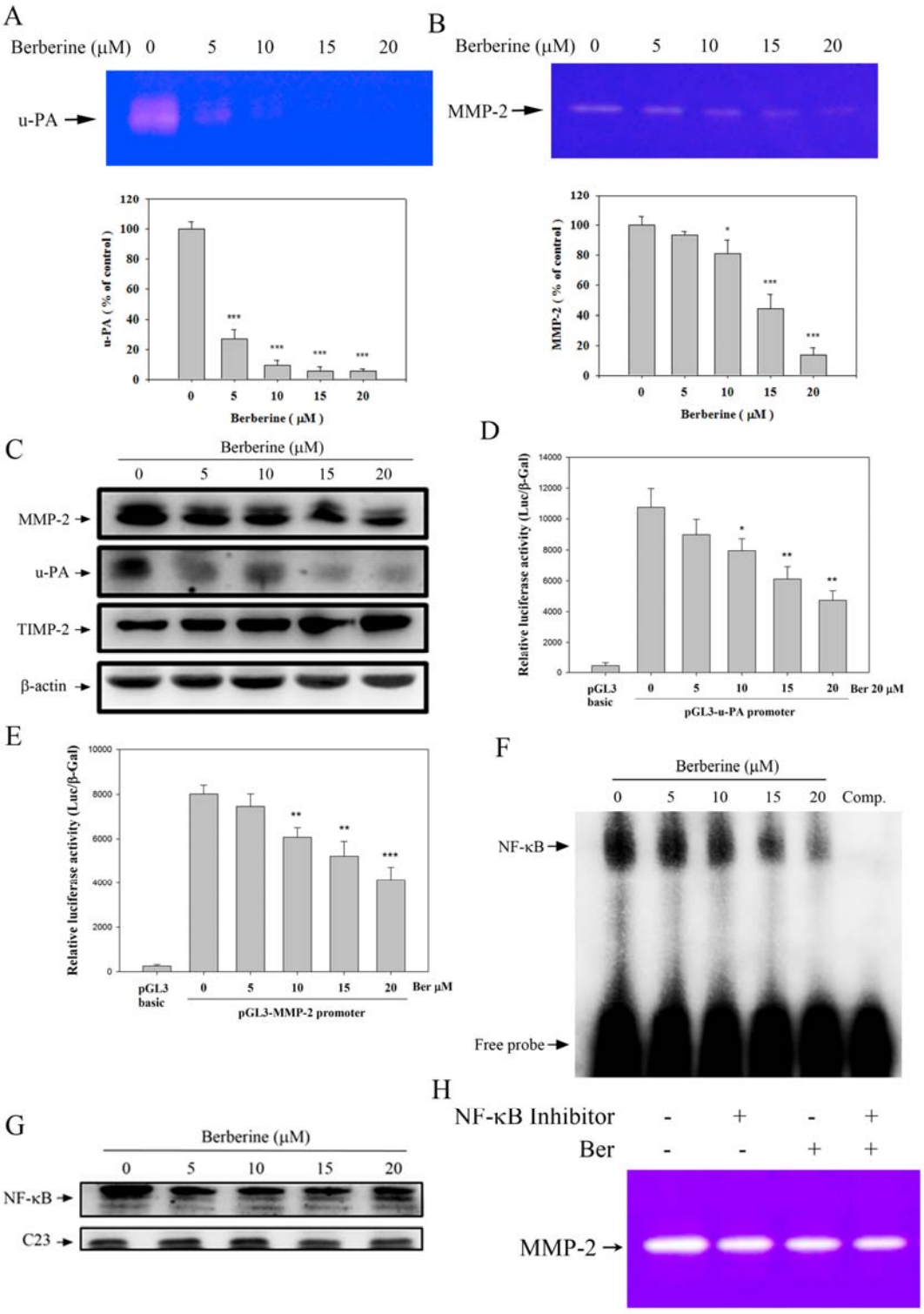

A
Berberine (μM) 0 5 10 15 20
u-PA
u-PA (% of control)
Berberine (μM)
B
Berberine (μM) 0 5 10 15 20
MMP-2
MMP-2 (% of control)
Berberine (μM)
C
Berberine (μM)
0 5 10 15 20
MMP-2
u-PA
TIMP-2
β-actin
D
Relative luciferase activity (Luc/β-Gal)
pGL3 basic
0 5 10 15 20 Ber 20 μM
pGL3-u-PA promoter
E
Relative luciferase activity (Luc/β-Gal)
pGL3 basic
0 5 10 15 20 Ber μM
pGL3-MMP-2 promoter
F
Berberine (μM)
0 5 10 15 20 Comp.
NF-κB
Free probe
G
Berberine (μM)
0 5 10 15 20
NF-κB
C23
H
NF-κB Inhibitor - + - +
Ber - - + +
MMP-2

Figure 3

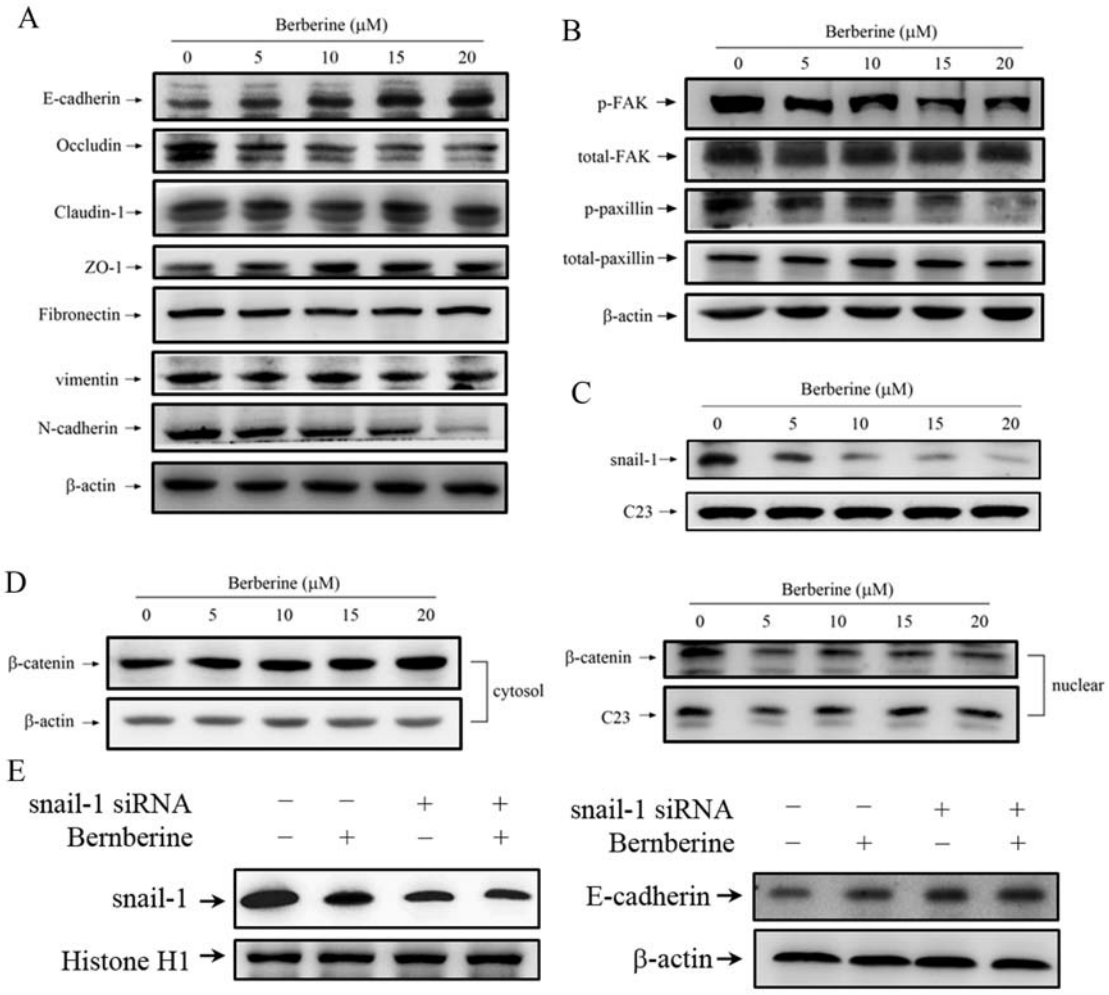

A
Berberine (μM)
0 5 10 15 20
E-cadherin
Occludin
Claudin-1
ZO-1
Fibronectin
vimentin
N-cadherin
β-actin
B
Berberine (μM)
0 5 10 15 20
p-FAK
total-FAK
p-paxillin
total-paxillin
β-actin
C
Berberine (μM)
0 5 10 15 20
snail-1
C23
D
Berberine (μM)
0 5 10 15 20
β-catenin
β-actin
cytosol
Berberine (μM)
0 5 10 15 20
β-catenin
C23
nuclear
E
snail-1 siRNA − − + +
Bernberine − + − +
snail-1
Histone H1
snail-1 siRNA − − + +
Bernberine − + − +
E-cadherin
β-actin

Figure 4

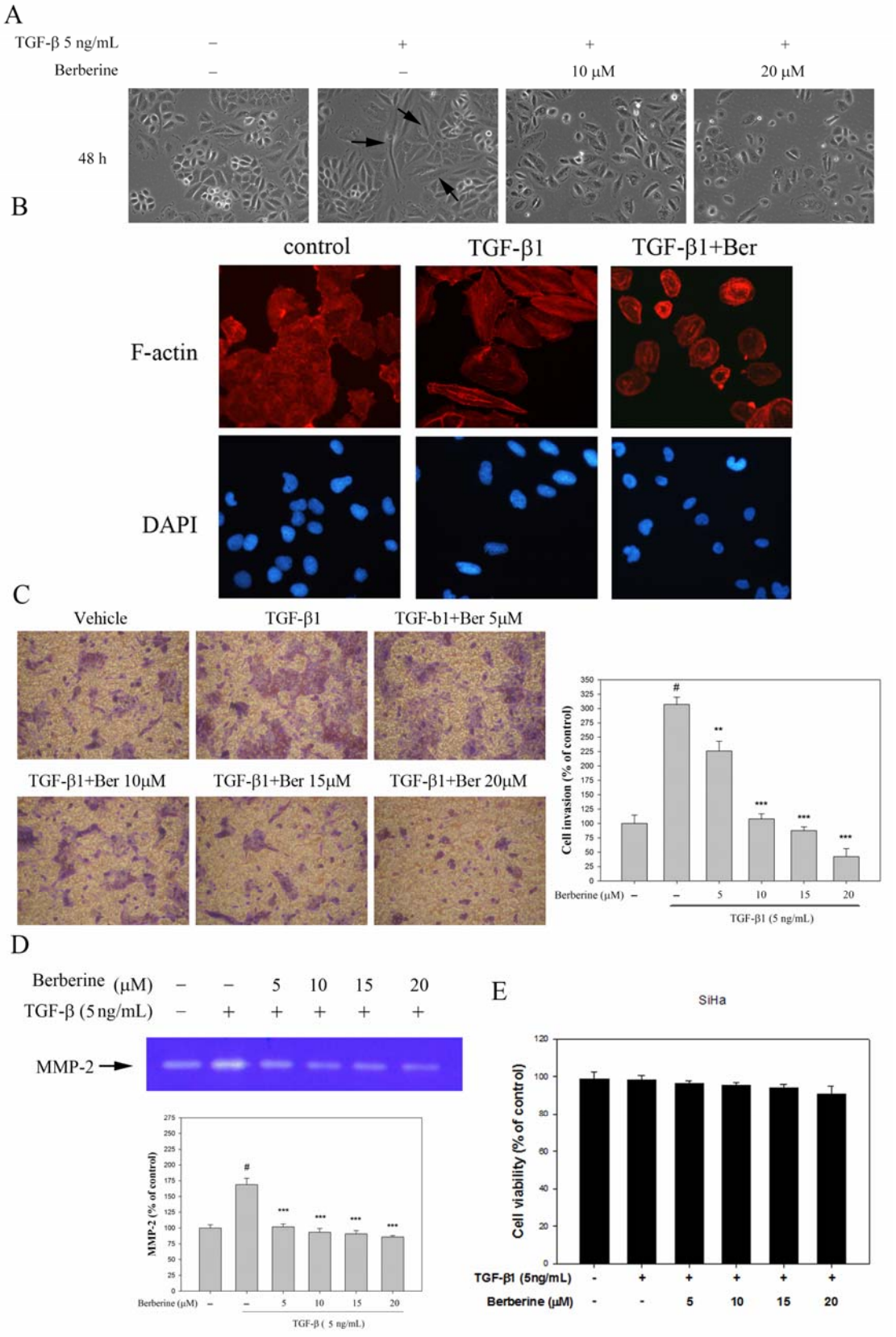

A
TGF-β 5 ng/mL
Berberine
10 μM
20 μM
48 h
B
control
TGF-β1
TGF-β1+Ber
F-actin
DAPI
C
Vehicle
TGF-β1
TGF-b1+Ber 5μM
TGF-β1+Ber 10μM
TGF-β1+Ber 15μM
TGF-β1+Ber 20μM
Cell invasion (% of control)
Berberine (μM)
TGF-β1 (5 ng/mL)
D
Berberine (μM)
TGF-β (5 ng/mL)
MMP-2
MMP-2 (% of control)
TGF-β (5 ng/mL)
E
SiHa
Cell viability (% of control)
TGF-β1 (5ng/mL)
Berberine (μM)

Figure 5

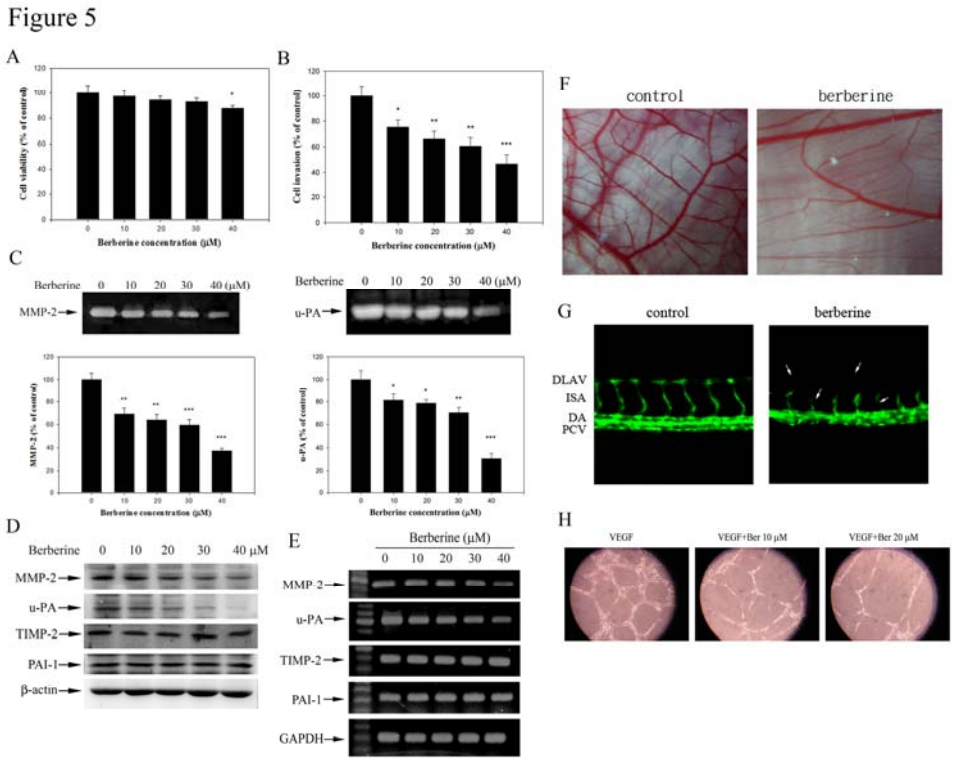


Figure 6

Figure 6

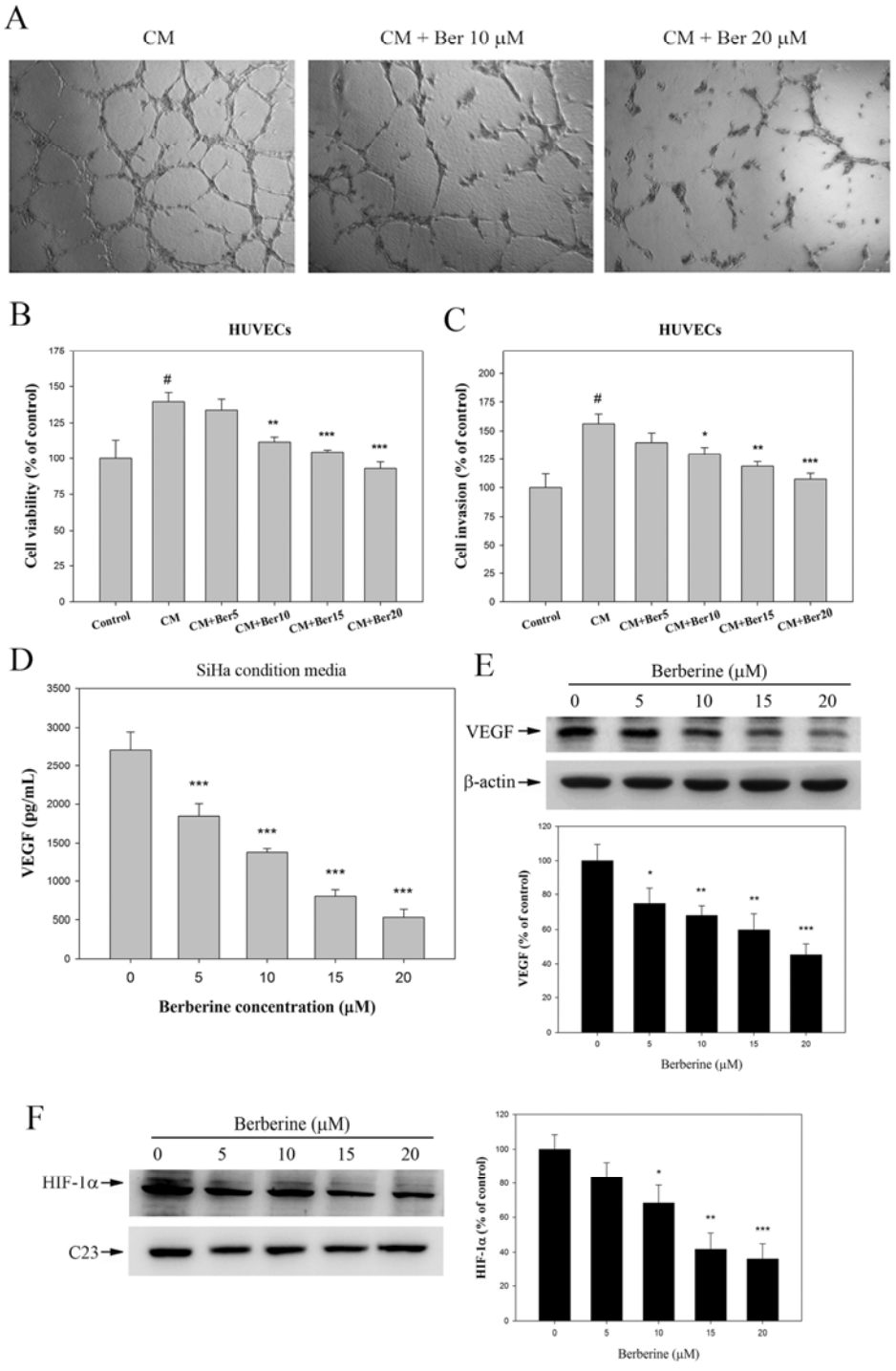


Figure 7

Figure 7

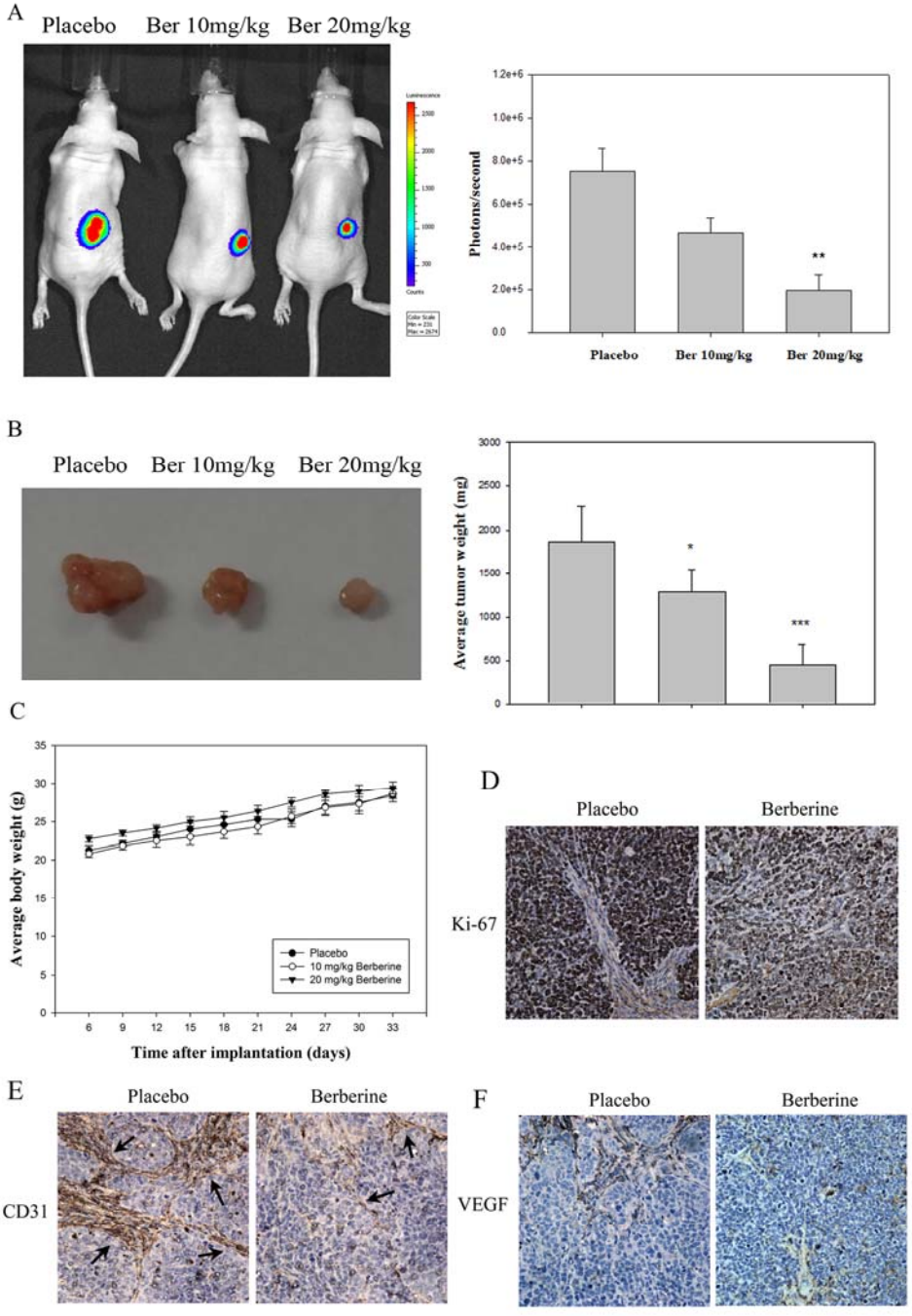


Figure 8

Figure 8

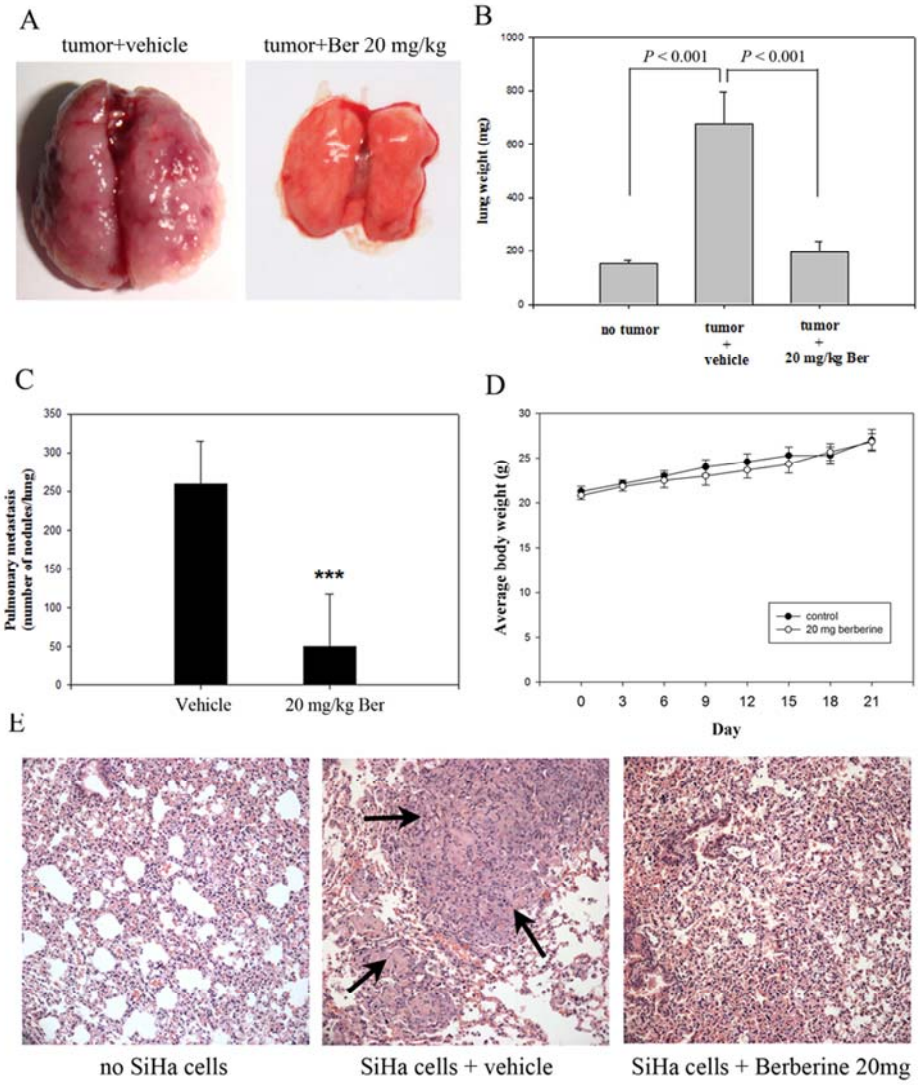


Figure 9

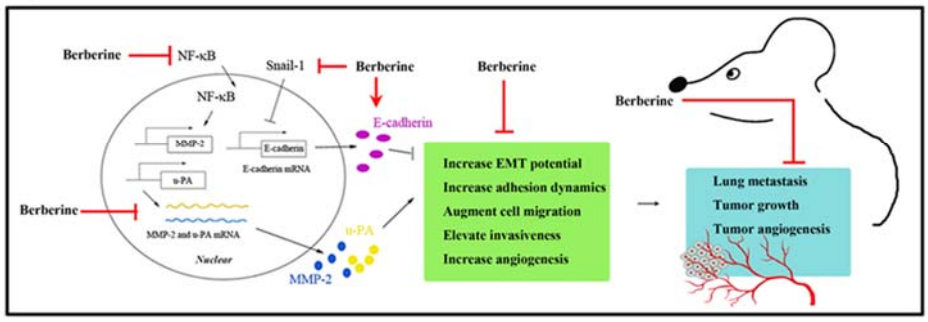


Figure 10

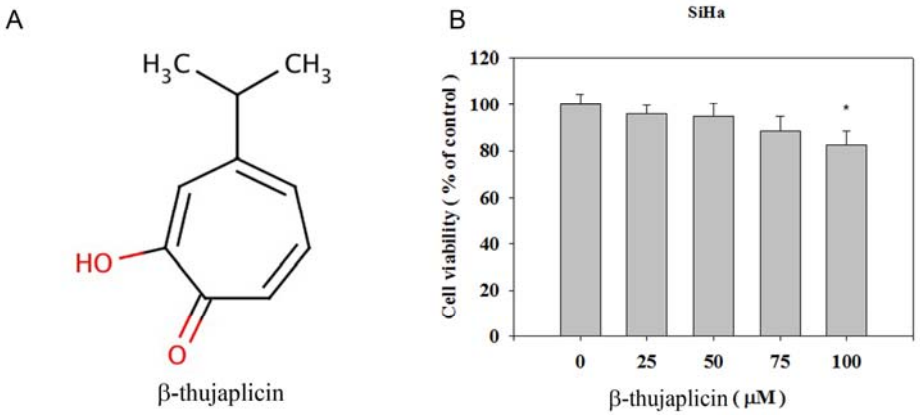


Figure11

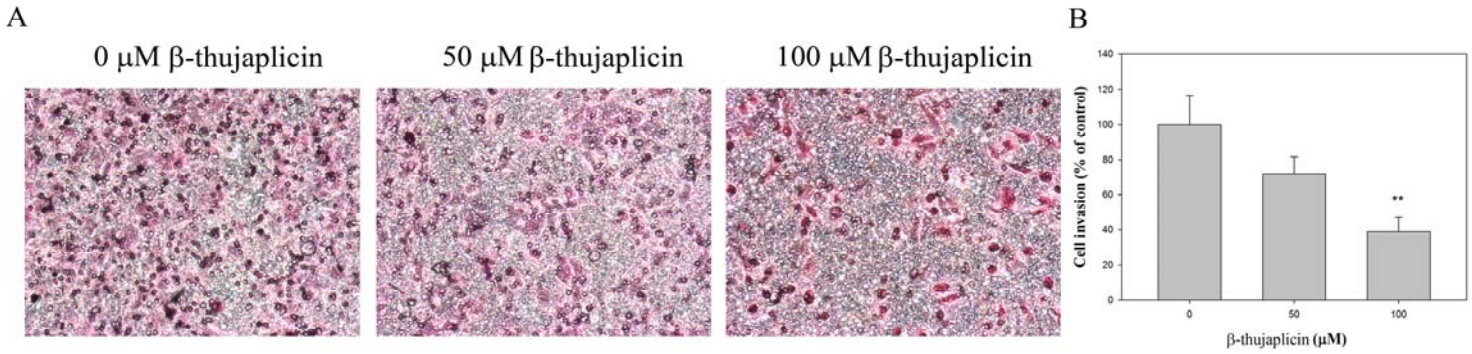


Figure 12

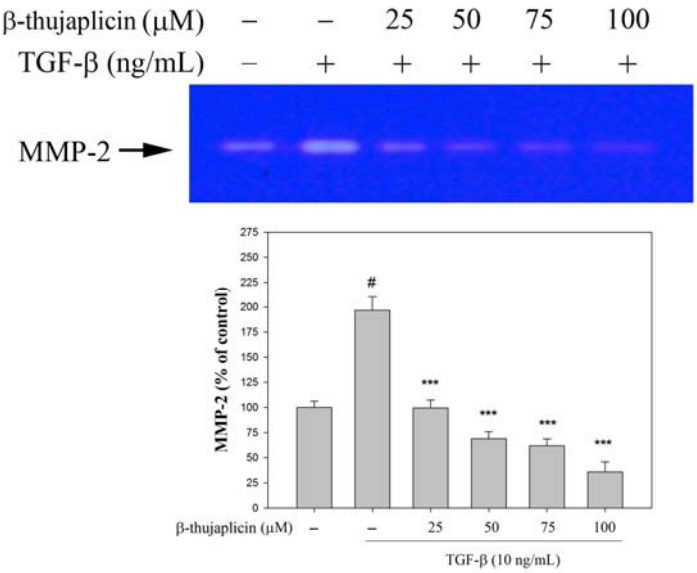


Figure13

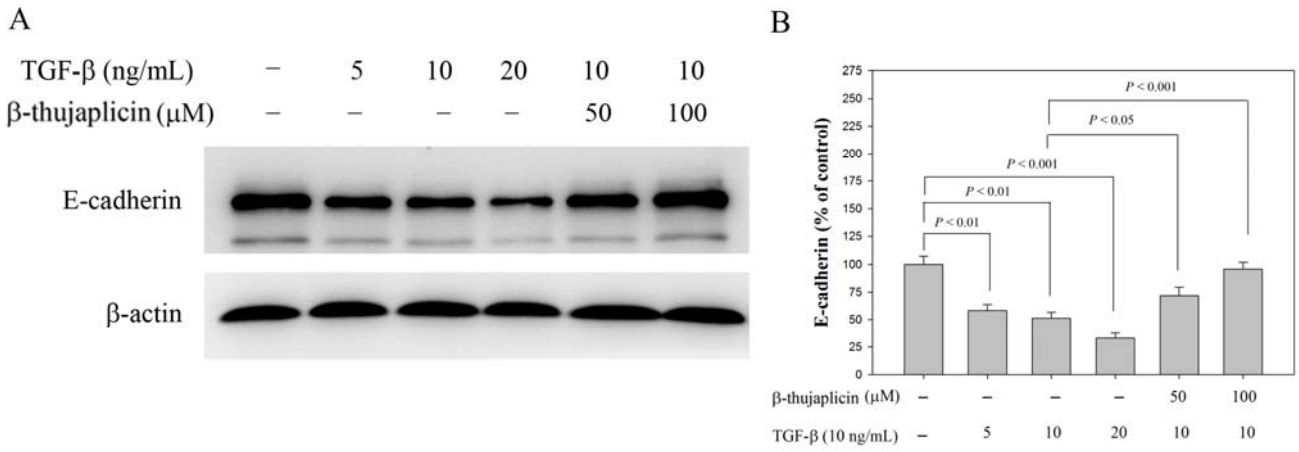
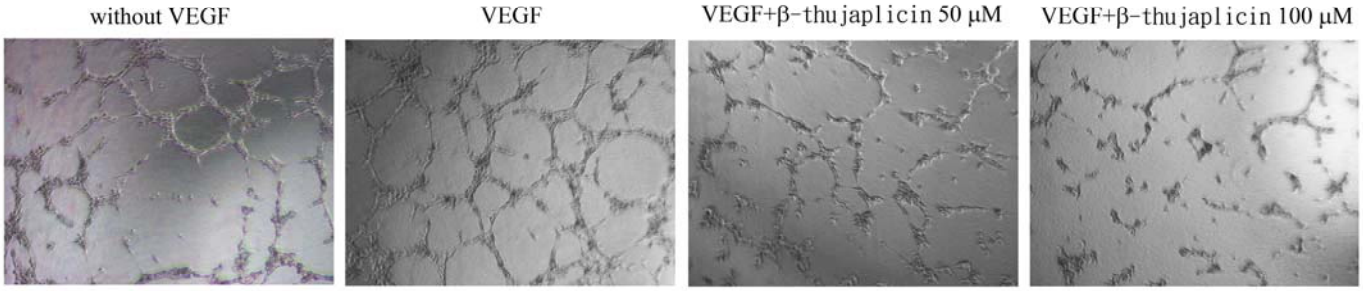

A
TGF-β (ng/mL) – 5 10 20 10 10
β-thujaplicin (μM) – – – – 50 100
E-cadherin
β-actin
B
E-cadherin (% of control)
P < 0.001
P < 0.05
P < 0.001
P < 0.01
P < 0.01
β-thujaplicin (μM) – – – – 50 100
TGF-β (10 ng/mL) – 5 10 20 10 10

Figure 14


without VEGF
VEGF
VEGF+β-thujaplicin 50 μM
VEGF+β-thujaplicin 100 μM

科技部補助計畫衍生研發成果推廣資料表

日期:2016/09/14

科技部補助計畫	計畫名稱: 檜木醇與黃連素抑制子宮頸癌細胞之侵襲轉移、上皮-間質相互轉換(EMT) 與血管新生作用之相關機制研究
	計畫主持人: 陳霈霓
	計畫編號: 102-2320-B-040-006-MY3　　學門領域: 保健營養

無研發成果推廣資料

102年度專題研究計畫成果彙整表

計畫主持人：陳霈寬	**計畫編號：**102-2320-B-040-006-MY3
計畫名稱：檜木醇與黃連素抑制子宮頸癌細胞之侵襲轉移、上皮-間質相互轉換(EMT) 與血管新生作用之相關機制研究	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			1	篇	
		研討會論文			2		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		

		其他	0		
	技術移轉	件數	0	件	
		收入	0	千元	
參與計畫人力	本國籍	大專生	1	人次	
		碩士生	1		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		

其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）	目前已達成計畫中預期之進度：完成分析berberine及β-thujaplicin對子宮頸癌存活以及侵襲轉移的抑制效果，並進一步針對子宮頸癌細胞進行相關研究。上述之實驗結果，目前 berberine及β-thujaplicin抑制SiHa子宮頸癌細胞侵襲轉移的部份已積極整理並以發表至Chu SC, Yu CC, Hsu LS, Chen KS, Su MY, Chen PN*. Berberine Reverses Epithelial-to-mesenchymal Transition and Inhibits Metastasis and Tumor-induced Angiogenesis in Human Cervical Cancer Cells. Mol Pharmacol. 2014 Dec;86(6):609-23.，其他細胞珠的相關實驗如:血管新生作用，也正在進行及整理中。關於探討Hinokitiol(beta-thujaplicin)的抗癌細胞轉移及抑制EMT及血管新生的效果及機制，也正在進行及整理準備投稿中。未來希望透過動物活體實驗我們將證實berberine與beta-thujaplicin具有抑制癌細胞轉移的功效及應用性。

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：■已發表　□未發表之文稿　□撰寫中　□無
   專利：□已獲得　□申請中　■無
   技轉：□已技轉　□洽談中　■無
   其他：（以200字為限）
   目前 berberine發表至Mol Pharmacol. 2014 Dec;86(6):609-23.，其他細胞珠的相關實驗如:血管新生作用，也正在進行及整理中

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   關於探討Hinokitiol(beta-thujaplicin)的抗癌細胞轉移及抑制EMT及血管新生的效果及機制，也正在進行及整理準備投稿中。未來希望透過動物活體實驗我們將證實berberine與beta-thujaplicin具有抑制癌細胞轉移的功效及應用性。

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：■否　□是
   說明：（以150字為限）
   本研究不具影響公共利益之重大發現