# 科技部補助專題研究計畫成果報告
# 期末報告

## 乳癌幹細胞的新血管生成潛力研究(第 2 年)

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報 告 附 件 ： 出席國際會議研究心得報告及發表論文

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中 文 摘 要 ： 新血管生成(neovascularization)為胚胎形成及發育中的自然過程，藉由血管新生(angiogenesis)以及脈管發生(vasculogenesis)兩種作用形成身體的血管網路構造，並且新血管生成對於腫瘤的維持及進展也是必須的。乳癌幹細胞是存在於乳癌細胞中一群特殊的細胞，目前認為這群細胞是乳癌的源頭，並與乳癌產生抗藥性以及轉移有密切的關係。過去我們在實驗室中從乳癌病人檢體建立了能夠在免疫不全小鼠乳腺形成腫瘤的乳癌細胞株，並以細胞表面分子 CD24、CD44 的表現或細胞內乙醛去氫酶(aldehyde dehydrogenase, ALDH)活性成功鑑定出這些乳癌細胞株中的乳癌幹細胞群。過去文獻發現具乳癌幹細胞活性的癌細胞具有血管擬態的行為，但其詳細調控之分子機制仍未完全明瞭。本研究計畫第一年首先確立乳癌幹細胞具有血管擬態活性，ALDH+細胞或形成乳腺小球之細胞相對於 ALDH-或一般培養之乳癌細胞，在 matrigel 覆蓋之微孔中能形成管狀結構。藉由不同培養基之分析，我們發現上皮生長因子在乳癌幹細胞之血管擬態活性扮演關鍵角色，而利用 gefitinib 抑制上皮細胞生長因子受體的活化，可以抑制乳癌幹細胞之血管擬態活性。我們進一步發現上皮細胞生長因子訊息可能透過熱休克蛋白 27 來誘導乳癌幹細胞之血管擬態行為，當利用槲黃素或 RNA 干擾方式抑制熱休克蛋白 27 的表現時，則能完全阻斷乳癌幹細胞之血管擬態活性。在第二年計畫中，我們進一步證明熱休克蛋白 27 的磷酸化對於上皮生長因子受體誘導之乳癌幹細胞血管擬態活性具有重要的調節角色，若讓乳癌幹細胞表現熱休克蛋白 27 之磷酸化死亡突變型，則上皮生長因子即失去其誘導乳癌幹細胞形成血管擬態的功能。我們還發現扁柏醇(hinokitiol)具有抑制乳癌幹細胞血管擬態活性的功效，其作用機轉也與上皮生長因子受體有關。當以扁柏醇處理乳癌幹細胞，其上皮生長因子受體蛋白之表現明顯受到抑制；此現象與蛋白酶體有關，當乳癌幹細胞同時處理蛋白酶體活性抑制劑 MG132，則能回復扁柏醇所抑制之上皮生長因子蛋白表現。透過本研究計畫，我們清楚證明乳癌幹細胞具有血管擬態活性，並證明此活性主要受到上皮生長因子受體與熱休克蛋白之磷酸化訊號調節，也找到可抑制乳癌幹細胞血管擬態活性之天然藥物與其作用機轉，提供以乳癌幹細胞之血管擬態活性為治療標的之藥物發展之學理基礎。

中文關鍵詞： 乳癌，癌症幹細胞，上皮生長因子，熱休克蛋白 27，血管擬態

英 文 摘 要 ：

英文關鍵詞：

計畫名稱: 乳癌幹細胞的新血管生成潛力研究

計畫編號: NSC-101-2314-B-040-015-MY2

計畫執行期間: 101/08/01-103/07/31

計畫主持人: 張文瑋

計畫參與人員: 謝鴻鈞、林程偉、吳育庭、郁芸

**中文摘要**

新血管生成(neovascularization)為胚胎形成及發育中的自然過程，藉由血管新生(angiogenesis)以及脈管發生(vasculogenesis)兩種作用形成身體的血管網路構造，並且新血管生成對於腫瘤的維持及進展也是必須的。乳癌幹細胞是存在於乳癌細胞中一群特殊的細胞，目前認為這群細胞是乳癌的源頭，並與乳癌產生抗藥性以及轉移有密切的關係。過去我們在實驗室中從乳癌病人檢體建立了能夠在免疫不全小鼠乳腺形成腫瘤的乳癌細胞株，並以細胞表面分子 CD24、CD44 的表現或細胞內乙醛去氫酶(aldehyde dehydrogenase, ALDH)活性成功鑑定出這些乳癌細胞株中的乳癌幹細胞群。過去文獻發現具乳癌幹細胞活性的癌細胞具有血管擬態的行為，但其詳細調控之分子機制仍未完全明瞭。本研究計畫第一年首先確立乳癌幹細胞具有血管擬態活性，ALDH+細胞或形成乳腺小球之細胞相對於 ALDH-或一般培養之乳癌細胞，在 matrigel 覆蓋之微孔中能形成管狀結構。藉由不同培養基之分析，我們發現上皮生長因子在乳癌幹細胞之血管擬態活性扮演關鍵角色，而利用 gefitinib 抑制上皮細胞生長因子受體的活化，可以抑制乳癌幹細胞之血管擬態活性。我們進一步發現上皮細胞生長因子訊息可能透過熱休克蛋白 27 來誘導乳癌幹細胞之血管擬態行為，當利用槲黃素或 RNA 干擾方式抑制熱休克蛋白 27 的表現時，則能完全阻斷乳癌幹細胞之血管擬態活性。在第二年計畫中，我們進一步證明熱休克蛋白 27 的磷酸化對於上皮生長因子受體誘導之乳癌幹細胞血管擬態活性具有重要的調節角色，若讓乳癌幹細胞表現熱休克蛋白27之磷酸化死亡突變型，則上皮生長因子即失去其誘導乳癌幹細胞形成血管擬態的功能。我們還發現扁柏醇(hinokitiol)具有抑制乳癌幹細胞血管擬態活性的功效，其作用機轉也與上皮生長因子受體有關。當以扁柏醇處理乳癌幹細胞，其上皮生長因子受體蛋白之表現明顯受到抑制；此現象與蛋白酶體有關，當乳癌幹細胞同時處理蛋白酶體活性抑制劑 MG132，則能回復扁柏醇所抑制之上皮生長因子蛋白表現。透過本研究計畫，我們清楚證明乳癌幹細胞具有血管擬態活性，並證明此活性主要受到上皮生長因子受體與熱休克蛋白之磷酸化訊號調節，也找到可抑制乳癌幹細胞血管擬態活性之天然藥物與其作用機轉，提供以乳癌幹細胞之血管擬態活性為治療標的之藥物發展之學理基礎。

**英文摘要**

Neovascularization is a natural process to form vascular networks during embryogenesis and development via angiogenesis and vasculogenesis and it is also necessary for tumor maintenance and progression. Breast cancer stem cells have been identified as a subpopulation of breast cancer cells with markers of CD24-CD44+ or high aldehyde dehydrogenase activity (ALDH+) and have been proved to be associated with radiation resistance and metastasis. We previously have established several xenograft human breast cancer cells from patients in immunocompromised NOD/SCID mice and the BCSC population within these cells has been identified by surface markers CD24 and CD44 and intracellular ALDH activity. Recently, it has been reported that there was a vasculogenic mimicry (VM) activity in breast cancer cells with CSC properties, but the detail molecular mechanisms are not fully understood. In the first year of this project, we successfully characterized the VM activity of BCSCs. Only ALDH+ or spheroid derived breast cancer cells displayed VM activity. Through comparison of different culture medium, we next discovered that epidermal growth factor (EGF) played a critical role in the VM activity of BCSCs. Inhibition of EGFR signaling by gefitinib suppressed the VM activity of BCSCs. We further demonstrated that Hsp27 could be a downstream effector of EGFR signaling in regulating the VM activity of BCSCs. Disruption of Hsp27 expression by quercetin or RNA interference totally abolished the EGF induced VM activity of BCSCs. In the second year, we further demonstrated that the phosphorylation of Hsp27 regulates EGF/EGFR induced VM activity of BCSCs. Forced expression of phosphor-dead mutant of Hsp27 totally abolished EGF/EGFR induced VM activity of BCSCs. We also discovered that hinokitiol, a natural monoterpenoid compound found in the heartwood cupressaceoud plants, displayed anti-VM activity of BCSCs. The mechanism of hinokitiol in inhibition of VM activity of BCSCs was mediated by the proteasome dependent down-regulation of EGFR. MG132, the proteasome inhibitor, could reverse hinokitiol-inhibited EGFR expression. Through this two-years-project, we have clearly demonstrated that BCSCs display VM activity and it is mediated by EGF/EGFR/Hsp27 signaling pathway. We have also discovered that hinokitiol could be used as a natural compound with anti-VM activity of BCSCs. These data could serve as basic mechanism studies for future development of breast cancer therapy through targeting their VM activity.

## I. Introduction

The formation of blood vessels (neovascularization) during embryogenesis and development occurs by either sprouting of pre-existing endothelial tissues (angiogenesis) or de novo production of endothelial cells from bone marrow derived circulating endothelial precursor cells (vasculogenesis) [1]. Besides the importance in development, neovascularization is also required for cancer progression. In addition to angiogenesis, tumor vasculatures could also contribute by vasculogenic mimicry (VM), a process that tumor cells mimic the pattern of embryonic vascular networks [2]. VM has been observed in many aggressive malignancies including hepatocellular carcinoma, breast and ovarian cancers [3]. In breast cancer study, the vascular-associated markers expressed in aggressive MDA-MD-231 breast cancer cell line, which was described as an embryonic-like cell type, but not nonaggressive MCF7 cells. Also using spheroid culture, the sphere-forming cells of MCF7 arranged into vascular structures in response to nutrient limitation and expressed endothelial markers CD31, angiopoietin-1 and endoglin [4]. Although these previous studies suggest that some breast cancer cells have the neovascularization potential, the involvement of BCSCs and the underlying molecular mechanisms remains unclear. Recently, CD133+ breast cancer cells have been demonstrated to display characteristics of cancer stem cells and VM activity [5]. Spheroid-derived breast cancer cells also displayed the VM activity and which was mediated by miR-299-5p regulated osteopontin [4]. In this project, we investigated the VM activity of BCSCs and tried to find out the regulating molecules in this behavior of BCSCs.

## 2. Results

### *2.1. Epidermal growth factor/heat shock protein 27 pathway regulates vasculogenic mimicry activity of breast cancer stem/progenitor cells*

#### *2.1.1 Vasculogenic mimicry activity of breast cancer stem/progenitor cells*

We have previously established two breast cancer xenografts (BC0145 and BC0244) from human breast cancer specimens and demonstrated the existence of breast cancer stem/progenitor cells (BCSCs). To investigate the contribution of BCSCs in tumor vasculogenic mimicry (VM), we first used PAS stain to

observe the VM structures within xenografted tumor tissues derived from CD24-CD44+ BC0145 cells or ALDH+ BC0244 cells. Both xenogafted BC0145 and BC0244 tumors contained PAS-positive VM structures and some of them contained erythrocytes (Fig. 1A). The H&E stain is commonly used for the demonstration of the presence of erythrocytes VM structures in several reports. In H&E stain results, erythrocytes were clearly found in VM channels which were lined by tumor cells with basement membrane in both BC0145 and BC0244 tumors (Fig. 1A). Some of erythrocytes containing VM structures in BC0244 tumors were observed to connect with endothelial vessels (Fig.1A). Both PAS and H&E stain demonstrated the presence of VM structures in BCSCs derived human breast cancer xenografts. We next separated ALDH- (non-BCSCs) or ALDH+ (BCSCs) cells from AS-B244 cells by FACS and performed in vitro VM assay by culturing on matrigel-coated wells with medium for cultivation of endothelial cells. ALDH+ AS-B244 cells, but not ALDH-, could form vessel-like structures as similar as HMEC-1, an immortalized human microvascular endothelial cell line (Fig. 1B). In addition to sorting of ALDH+ cells, we also used mammosphere cultivation to enrich BCSCs from AS-B145 cells. In comparison with normal cultured AS-B145 cells which were maintained in two dimensional cultures, mammosphere cells displayed obvious VM activity (Fig.1C). From these results, it suggests that there is a strong VM activity of BCSCs.

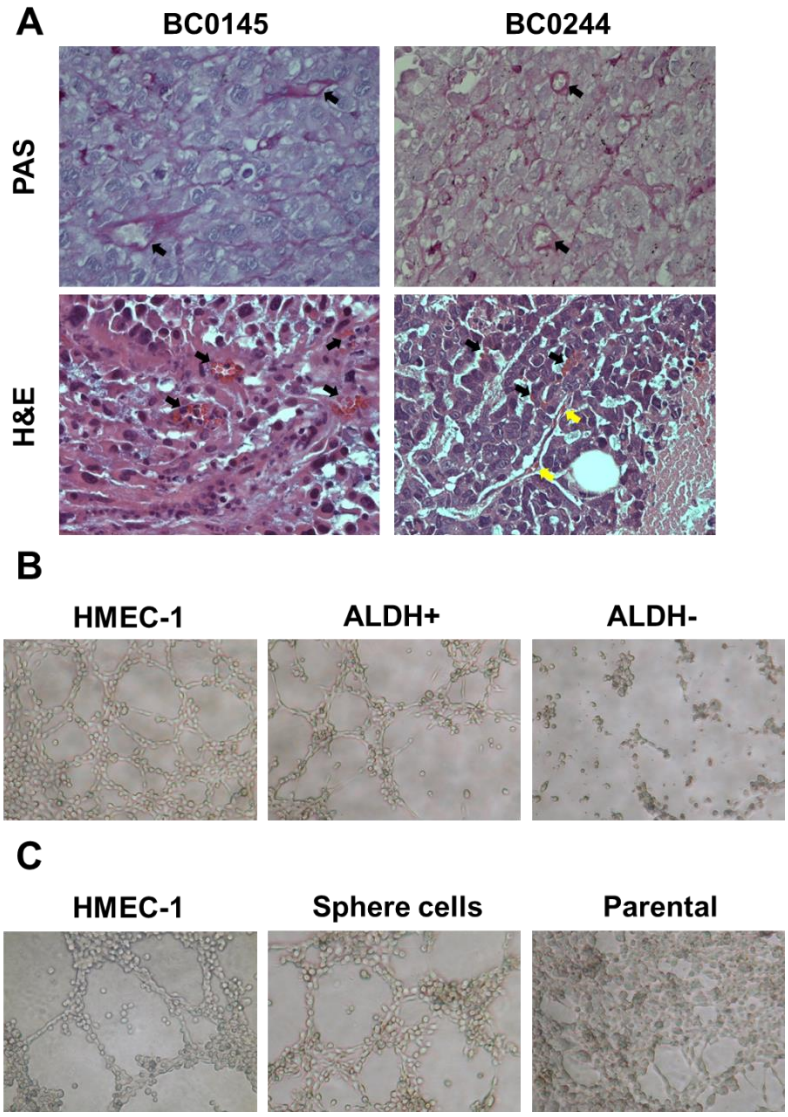


Figure 1. In vivo and in vitro observations of VM activity of BCSCs. (A) 105 of CD24-CD44+ BC0145 or ALDH+ BC0244 xenografted breast cancer cells were sorted and injected into mammary fat pads of NOD/SCID mice for 4 weeks. VM in tumor tissues were determined by PAS (upper panel) and H&E (lower panel) stain. Black arrows indicated erythrocytes containing VM structures and yellow arrows indicated erythroctyes containing endothelial vessels. (B) ALDH- or ALDH+ cells were sorted from AS-B244 cells and seeded into matrigel coated wells to observe the formation of vessel-like structures. HEMC-1 endothelial cells were used as positive control. Data were presented from one of three independent experiments. (C) Parental or mammosphere forming AS-B145 cells were collected and seeded into matrigel coated wells to observe the formation of vessel-like structures. HMEC-1 cells were used as positive control. Data were presented from one of three independent experiments.

### *2.1.2. EGF mediates VM activity of BCSCs.*

We next wanted to search for factors which could regulate VM activity of BCSCs. We first compared the VM activity of $ALDH^+$ AS-B244 cells in medium for cultivation of endothelial cells (M200 in supplemented with LSGS) or breast cancer cells (MEMα). $ALDH^+$ AS-B244 cells lost their VM activity in MEMα medium (Fig.2Aii) while there was a strong VM activity of these cells in M200/LSGS medium (Fig.2Ai). After comparison of medium used for cultivation of endothelial cells and breast cancer cells (Table 1), we hypothesized that EGF or basic fibroblast growth factor (bFGF) might involve in regulating VM activity of BCSCs. By supplementation of EGF or bFGF into MEMα medium, EGF was found to stimulate VM activity of $ALDH^+$ AS-B244 cells in MEMα medium (Fig. 2Aiii) and there was no significant difference between EGF and M200/LSGS groups ($P$=0.239, Fig. 2B). The effect of bFGF in stimulating VM activity of

ALDH$^+$ AS-B244 cells was minor (Fig. 2Aiv) but still significant ($P$=0.03, Fig. 2B). Supplementation with EGF plus bFGF also stimulated VM activity of ALDH+ AS-B244 cells in MEMα medium (Fig. 2Av) but there was no significant difference when compared with supplementation of EGF alone ($P$=0.245, Fig. 2B). To further confirm that EGF signaling is the key factor in VM activity of BCSCs, gefitinib was used to block the phosphorylation and activation of EGF receptor (EGFR). Treatment of ALDH$^+$ AS-B244 cells with gefitinib inhibited the phosphorylation of EGFR$^{Tyr1173}$ in a dose-dependent manner (Fig. 3A). 5μM of gefitinib significantly abolished VM activity of ALDH$^+$ AS-B244 cells ($P$<0.01, Fig. 3B). In addition to gefitinib treatment, we also applied lentiviral transduction of EGFR specific short hairpin RNA (shRNA) to knockdown EGFR in ALDH$^+$ AS-B244 cells to examine the role of EGFR signaling in the VM activity of BCSCs. In comparison with control sh-LacZ transduced cells, transduction with EGFR specific shRNA lentivirus (sh-EGFR#1 or sh-EGFR#2) in ALDH$^+$ AS-B244 cells completely abolished their *in vitro* VM activity (Fig. 3C). These results demonstrate that EGFR signaling is the key regulator in VM activity of BCSCs.

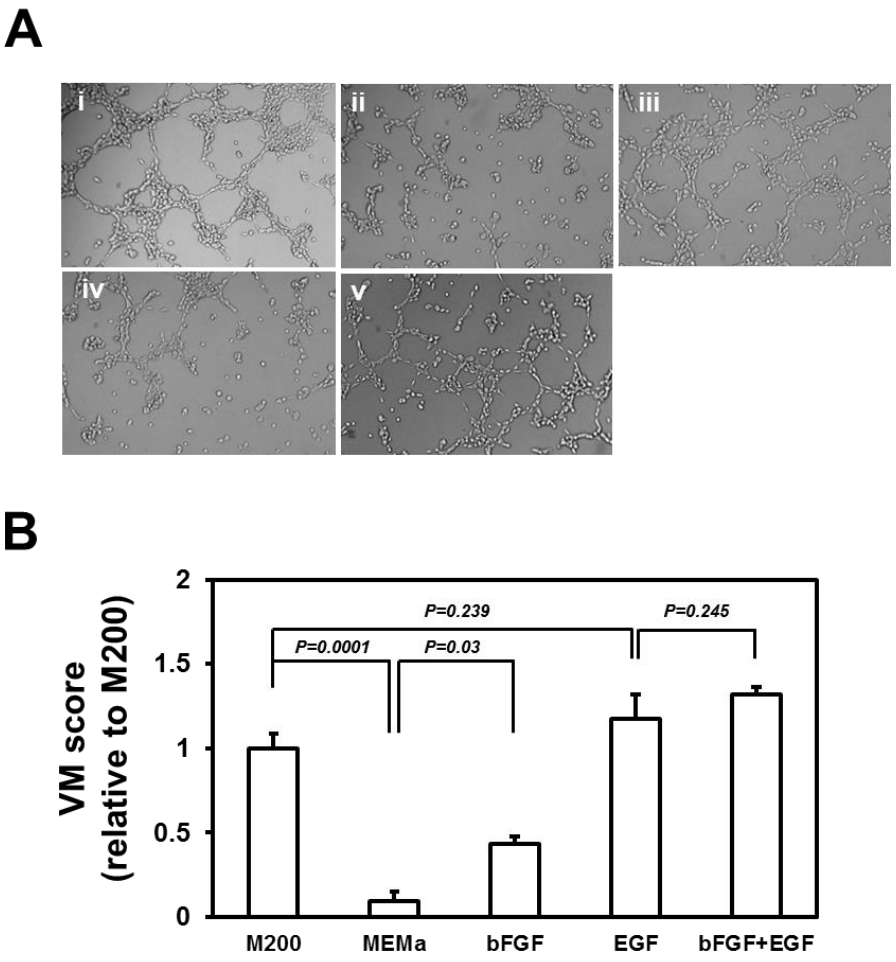


**Figure 2. EGF stimulates *in vitro* VM activity of ALDH$^+$ BCSCs.** (A) ALDH$^+$ AS-B244 cells were sorted and seeded into matrigel coated wells to observe the formation of vessel-like structures in different conditions. i, M200+LSGS; ii, MEMα; iii, MEMα+EGF (10 ng/ml); iv, MEMα+bFGF (3 ng/ml); v, MEMα+EGF (10 ng/ml)+bFGF (3ng/ml). Data were presented from one of three independent experiments. (B) Images from three wells of each condition in (A) were used for analysis of VM scores and data were presented from one of three independent experiments as the relative value to M200 group.

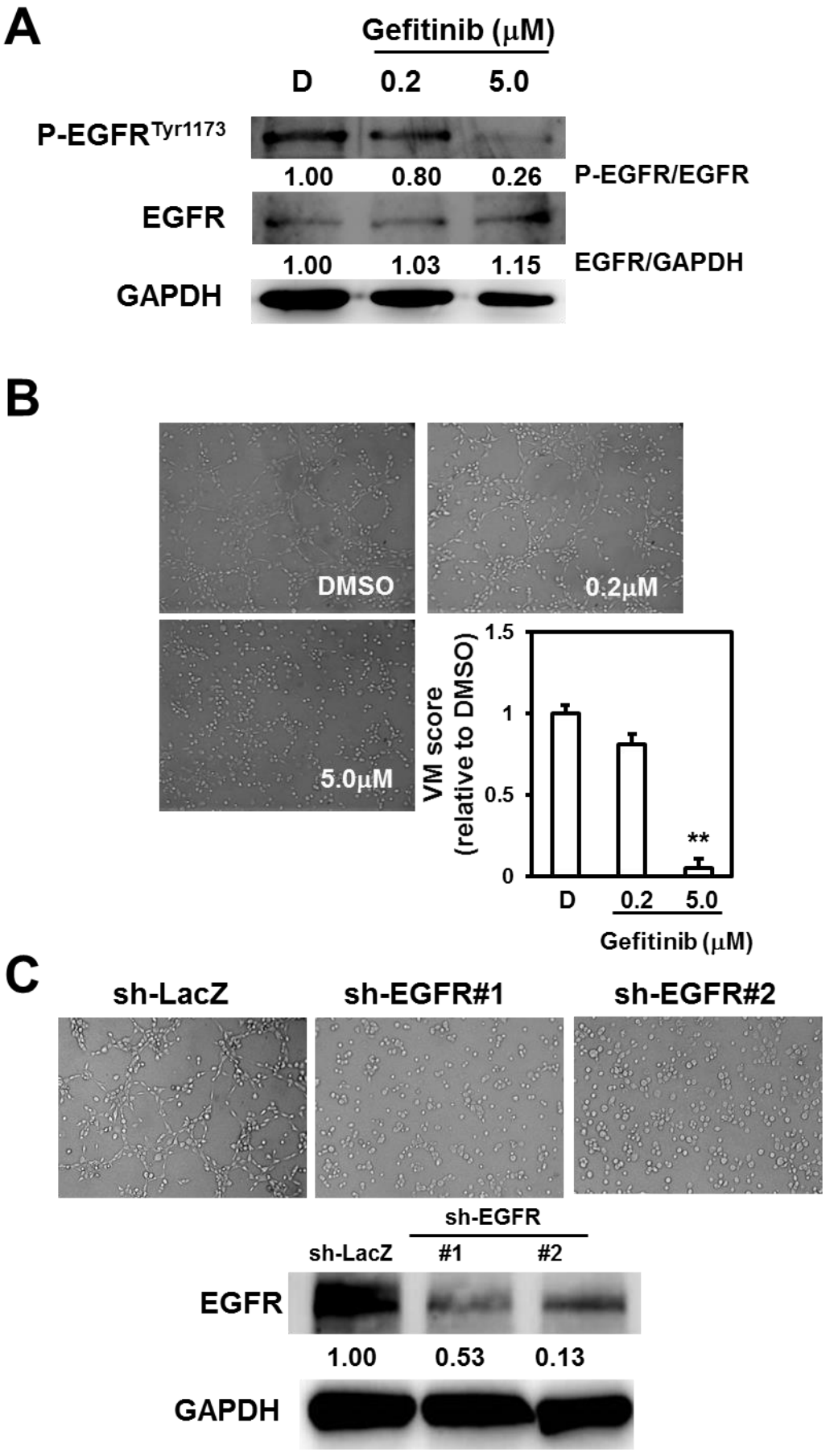


**Figure 3. Disruption of EGFR activation or expression suppresses VM activity of ALDH$^+$ BCSCs.** (A) ALDH$^+$ AS-B244

cells were suspended in M200 medium containing LSGS and treated with 0.1% DMSO or indicated concentration of gefitinib for 1 hour. The activation of EGFR was determined by western blot of tyrosine 1173 phosphorylation. The inserted numbers indicated the relative protein expression in comparison with 0.1% DMSO. Data were presented from one of three independent experiments. (B) DMSO or gefitinib treated cells were further seeded into matrigel coated wells to observe the formation of vessel-like structures. Images from three wells of each condition were used for analysis of VM scores and data were presented one of three independent experiments as the relative value to 0.1% DMSO group. D, 0.1% DMSO; **, $p<0.01$. (C) $ALDH^+$ AS-B244 cells were transduced with lentivirus carrying control shRNA (sh-LacZ) or EGFR specific shRNA (sh-EGFR#1 or sh-EGFR#2) for 24 hours and selected for successfully transduced cells by 2 μg/ml puromycin for further 3 days. Selected cells were plated on matrigel-coated microwells for *in vitro* VM determination. Knockdown efficiency was determined by western blot analysis of EGFR expression. The inserted numbers indicated the relative protein expression in comparison with sh-LacZ transduced cells.

### *2.1.3 Hsp27 is the downstream molecule in EGF mediated VM activity of BCSCs*

Recently, it has been reported that knockdown of Hsp27 suppressed EGF-induced endothelial cord formation within tumors[6]. We have also demonstrated Hsp27 controls the maintenance of BCSCs[7]. We next wanted to investigate the role of Hsp27 in VM activity of breast cancer stem/progenitor cells. Quercetin, an Hsp27 inhibitor, decreased Hsp27 expression in $ALDH^+$ AS-B244 cells in a dose-dependent manner (Fig. 4A). VM activity of $ALDH^+$ AS-B244 cells was significantly inhibited by quercetin at 25 μM or 50 μM ($P<0.01$, Fig.4B). In addition to quercetin, we also applied siRNA-mediated gene silencing to decrease the Hsp27 expression. Specific Hsp27 siRNA could effectively decrease Hsp27 expression in both AS-B145 sphere cells and $ALDH^+$ AS-B244 cells (Fig. 4C). Knockdown of Hsp27 in AS-B145 sphere cells or $ALDH^+$ AS-B244 cells significantly suppressed their VM activity in M200+ LSGS medium ($P<0.01$, Fig. 4D). We further examined if Hsp27 could be a downstream molecule of EGF mediated VM activity of BCSCs. Supplementation of EGF in MEMα medium significantly stimulated VM activity of $ALDH^+$ AS-B244 cells transfected with negative control siRNA ($P<0.05$, Fig. 5Aiii and 5B) while knockdown of Hsp27 completely blocked EGF-induced VM activity of $ALDH^+$ AS-B244 cells ($P<0.01$, Fig. 5Aiv and 5B). In order to investigate the involvement of Hsp27 activation in EGF-induced VM activity of BCSCs, we constructed phosphor-dead (Hsp27A) and phosphor-mimic (Hsp27D) form of Hsp27 in tagged with DsRed protein and

overexpressed them in ALDH$^+$ AS-B244 cells. Overexpression of Hsp27A significantly suppressed the VM activity of BCSCs in presence of EGF (*P*=0.048, Fig.6A and 6B). Forced expression of Hsp27D in BCSCs did not enhance VM activity under EGF stimulation (*P*=0.103, Fig. 6A and 6B) but it could overcome the inhibitory effect of gefitinib in VM structures (*P*=0.005, Fig.6A and Fig. 6B). These results suggest that Hsp27 is the key downstream molecule in EGF-mediated VM activity of BCSCs.

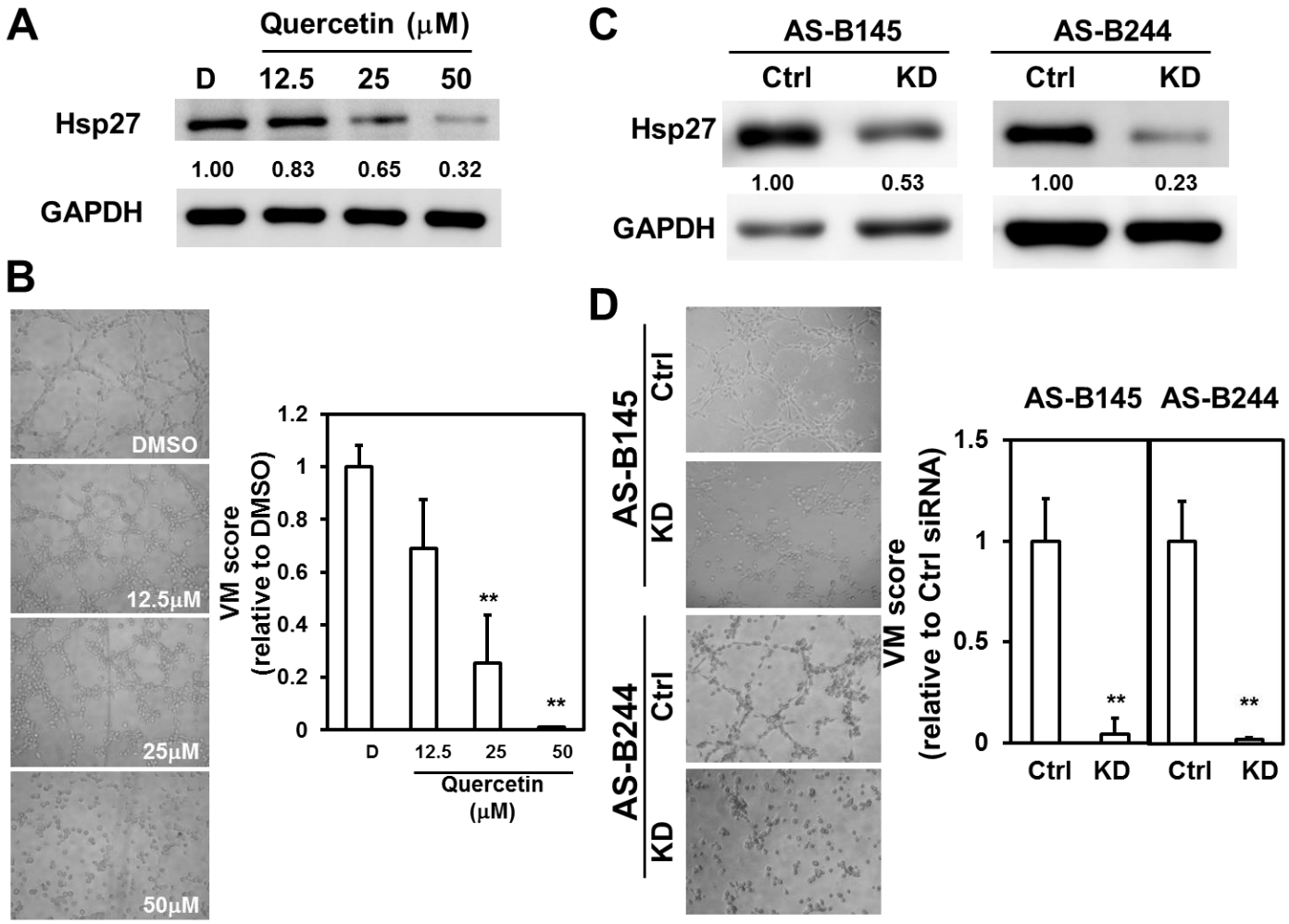


**Figure 4. Disruption of Hsp27 expression suppresses EGF-induced VM activity of ALDH$^+$ BCSCs.** (A) ALDH$^+$ AS-B244 cells were sorted and treated with 0.1% DMSO (D) or indicated concentration of quercetin for 1 hour. Expression of Hsp27 was determined by western blot. The inserted numbers indicated the relative Hsp27 expression in comparison with 0.1% DMSO. Data were presented from one of three independent experiments. (B) DMSO or quercetin treated cells were further seeded into matrigel coated wells to observe the formation of vessel-like structures. Images from three wells of each condition were used for analysis of VM scores and data were presented as the relative value to 0.1% DMSO group. D, 0.1% DMSO; **, p<0.01. (C) Mammosphere-forming AS-B145 or ALDH$^+$ AS-B244 cells were transfected with negative control (ctrl) or Hsp27 specific (KD)

siRNA oligos for 48 hours. Hsp27 expression as determined by western blot. The inserted numbers indicated the relative Hsp27 expression in comparison with negative control siRNA transfected cells. Data were presented from one of two independent experiments. (D) Negative control (ctrl) or Hsp27 specific (KD) oligos transfected cells were further seeded into matrigel coated wells to observe the formation of vessel-like structures. Images from three wells of each condition were used for analysis of VM scores and data were presented from one of two independent experiments as the relative value to negative control siRNA transfected group. **, p<0.01.

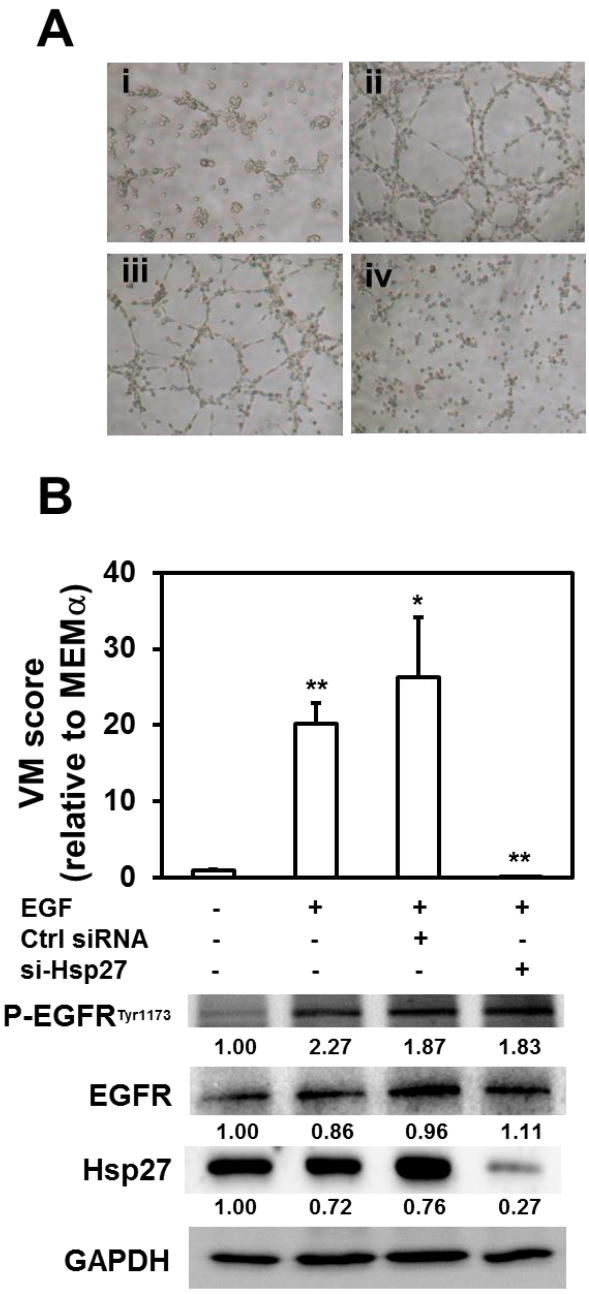


**Figure 5. Knockdown of Hsp27 inhibits EGF-induced VM activity of ALDH$^+$ BCSCs.** (A) ALDH$^+$ AS-B244 cells were transfected with negative control (ctrl siRNA) or Hsp27 specific (si-Hsp27) siRNA oligos for 48 hours, suspended in MEMα medium and seeded into matrigel coated wells for observation of the formation of vessel-like structures. i, non-tranfected cells; ii, non-transfected cells with EGF; iii, ctrl-siRNA transfected cells with EGF, iv, si-Hsp27 transfected cells with EGF. (B) Images from three wells of each condition in (A) were used for analysis of VM scores and data were presented from one of two independent experiments as the relative value to non-transfected cells and without EGF group. *, p<0.05; **, p<0.01. The expression of p-EGFR$^{tyr1173}$, EGFR or Hsp27 was determined by western blot. Data were presented from one of two independent experiments.

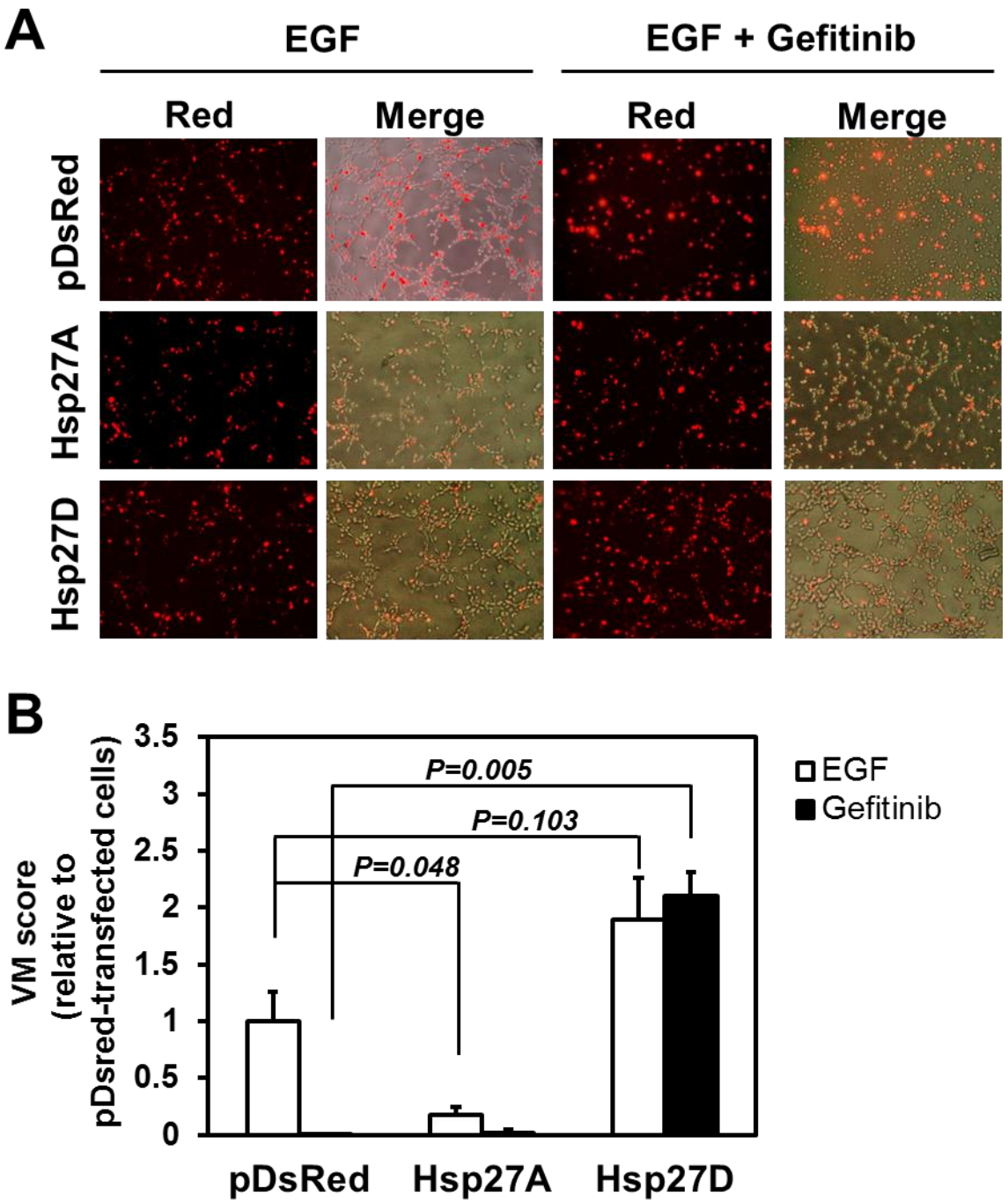


**Figure 6. Phosphorylation status of Hsp27 determines EGF-stimulated VM activity of ALDH$^+$ BCSCs.** (A) ALDH$^+$ AS-B244 cells were transfected with indicated plasmids for 48 hours and harvested for *in vitro* VM analysis. The concentration of EGF or gefitinib was 10 ng/ml or 5 μM, respectively. (B) Images from three wells of each condition in (A) were used for analysis of VM scores and data were presented from one of two independent experiments as the relative value to pDsRed-transfected group. A *p* value less than 0.05 was considered as significant difference.

### *2.2. Hinokitiol inhibits VM activity of BCSCs through proteasome-mediated degradation of EGFR*

#### *2.2.1. Hinokitiol dose-dependently inhibits VM activity of ALDH+ breast cancer cells*

Hinokitiol (also known as β-thujaplicin) is a tropolone-related natural compound with antimicrobial, anti-inflammatory or antitumor activity[8]. We first examine the $IC_{50}$ value of hinokitiol to sphere cells derived from AS-B244 or MDA-MB-231 human breast cancer cells and results showed that $IC_{50}$ value of hinokitiol to AS-B244 or MDA-MB-231 cells was (33.6 ± 8.6) μM or (33.8 ± 4.1) μM, respectively (Fig. 7A and 7B). We next determined if hinokitiol displayed an inhibitory effect in VM activity of BCSCs. The sphere cells derived from AS-B244 or MDA-MB-231 cells were treated with 1 or 10 μM hinokitiol for 24 hours and seeded into matrigel-coated microwells for analyzing in vitro VM activity in presence of hinokitiol. From Figure 7C and 7D, hinokitiol could dose-dependently inhibit the VM activity of breast cancer stem-like cells.

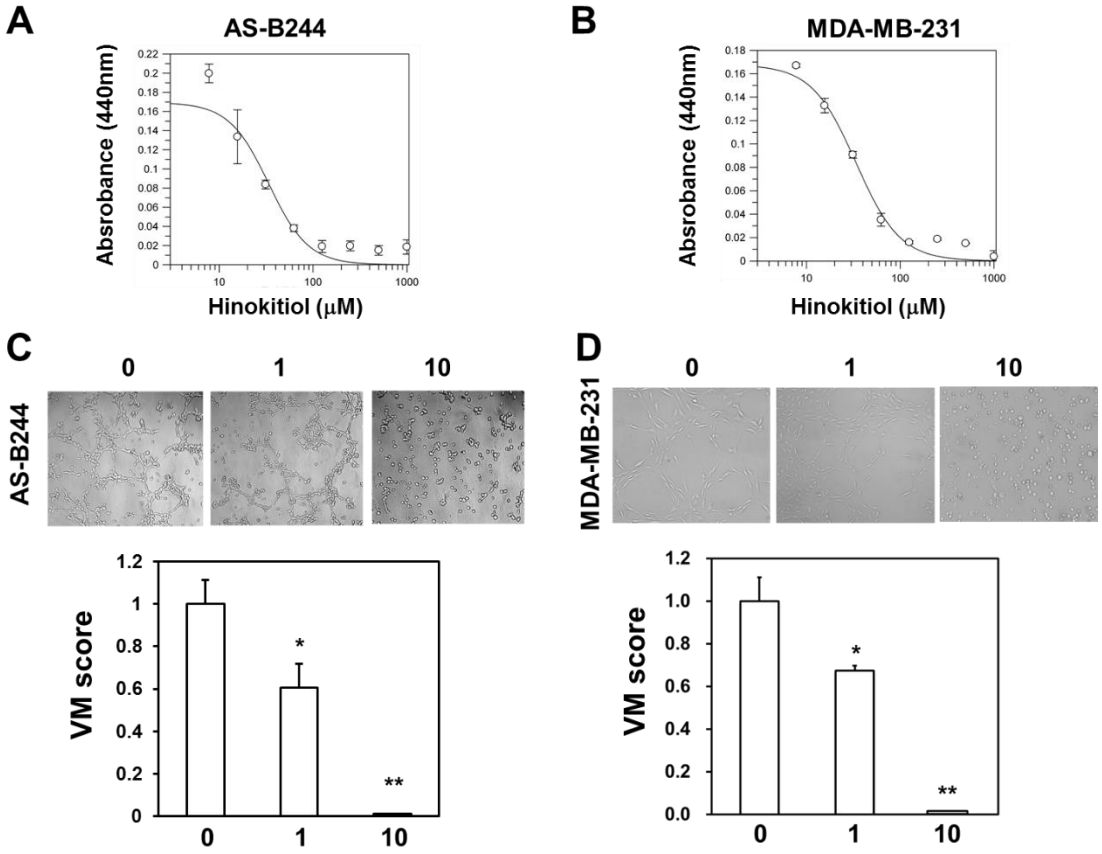


**Figure 7. Hinokitiol inhibits VM activity of breast cancer stem-like cells.** (A, B) Mammosphere cells derived from AS-B244 or MDA-MB-231 human breast cancer cells were treated with different

concentration of hinokitiol for 48 hours and cell viability was determined by WST-1. IC50 value was calculated with GraFit software. (C, D) Mammosphere cells derived from AS-B244 or MDA-MB-231 cells were treated with 0.1% EtOH (0 μM) or hinokitiol (1 or 10 μM) for 24 hours and then seeded on matrigel coated microwells for VM activity assay. Images were captured at 4 hours and used for analyzing VM scores. *, p< 0.05; **, p< 0.01.

### *2.2.2. Hinoikitiol inhibits EGFR expression through proteasome-mediated degradation*

We have previously demonstrated that the VM activity of BCSCs is mediated by EGF/EGFR activation (). We next examined the effect of hinokitiol in EGFR expression. With western blot analysis, the protein level of EGFR was dose-dependently decreased by hinokitiol (Fig. 8A). We next examined if hinokitiol affected the mRNA expression of EGFR. With quantitative RT-PCR analysis, the mRNA level of EGFR did not inhibit by hinokitiol (Fig. 8B).

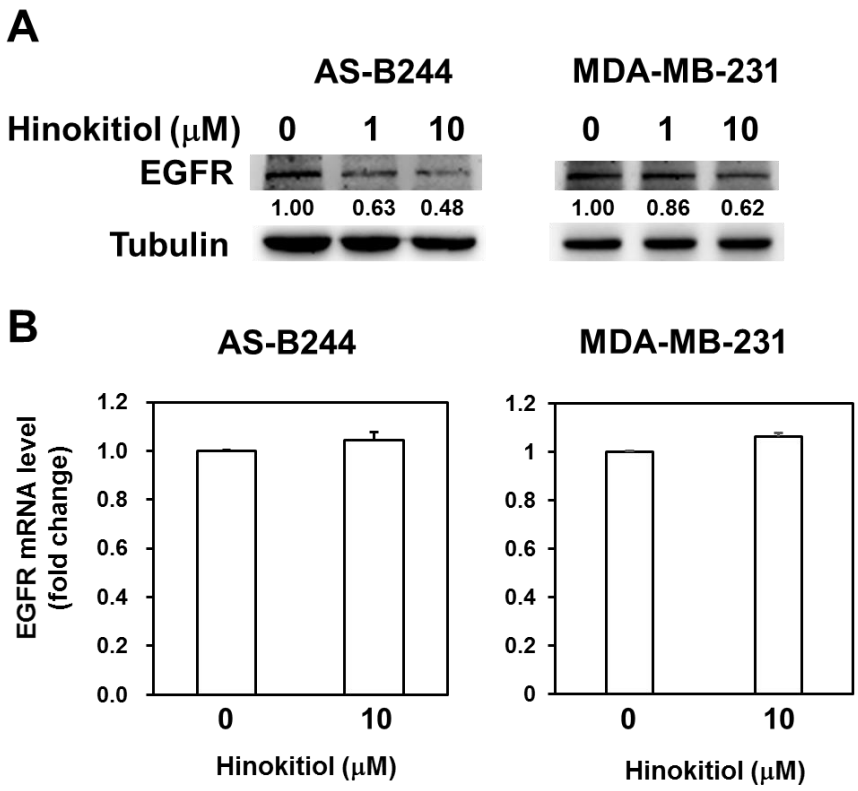


**Figure 8. Hinokitiol suppresses the expression of EGFR protein in breast cancer stem-like cells without affecting its mRNA.** AS-B244 or MDA-MB231 sphere cells were treated with indicated concentration of hinokitiol for 24 hours. (A) The expression of EGFR or tubulin protein was detected by western blot. The insert number represented as relative expression level in comparison with 0.1% EtOH control. (B) The expression of EGFR mRNA was determined by quantitative RT-PCR.

It has been reported that surface EGFR expression could be regulated by proteasomal protein degradation pathway [9]. We next examined the protein stability of EGFR under hinokitiol treatment and results indicated

that hinokitiol decreased EGFR protein stability in AS-B244 sphere cells (Fig. 9).

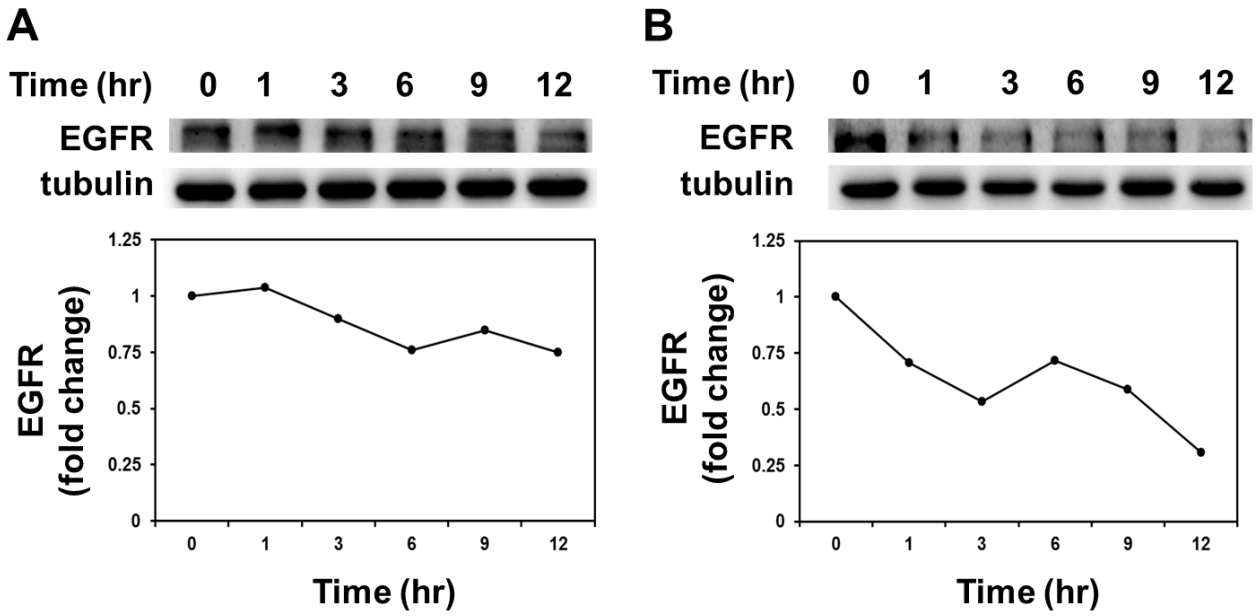


**Figure 9. Hinokitiol decreases EGFR protein stability in AS-B244 sphere cells.** AS-B244 sphere cells were treated with 0.1% EtOH (A) or 10 μM hinokitiol (B) in presence of 100 μg/ml cyclohexamide and total cell lysates were collected at indicated time point. The expression of EGFR protein was determined by western blot and data was presented fold change to start point (0 hour).

We next determined if the down-regulation of EGFR protein expression by hinokitiol in breast cabcer stem-like cells was mediated by proteasomal degradation pathway. By co-treatment of MG132, a proteasome inhibitor, hinokitiol lost the activity in inhibiting EGFR protein expression in AS-B244 or MDA-MB-231 sphere cells (Fig. 10A). The VM inhibition activity of hinokitiol in AS-B244 sphere cells could also be reversed by MG132 treatment (Fig. 10B). From these results, the VM inhibition activity of hinokitiol in breast cancer stem-like cells is mediated by proteasomal degradation of EGFR.

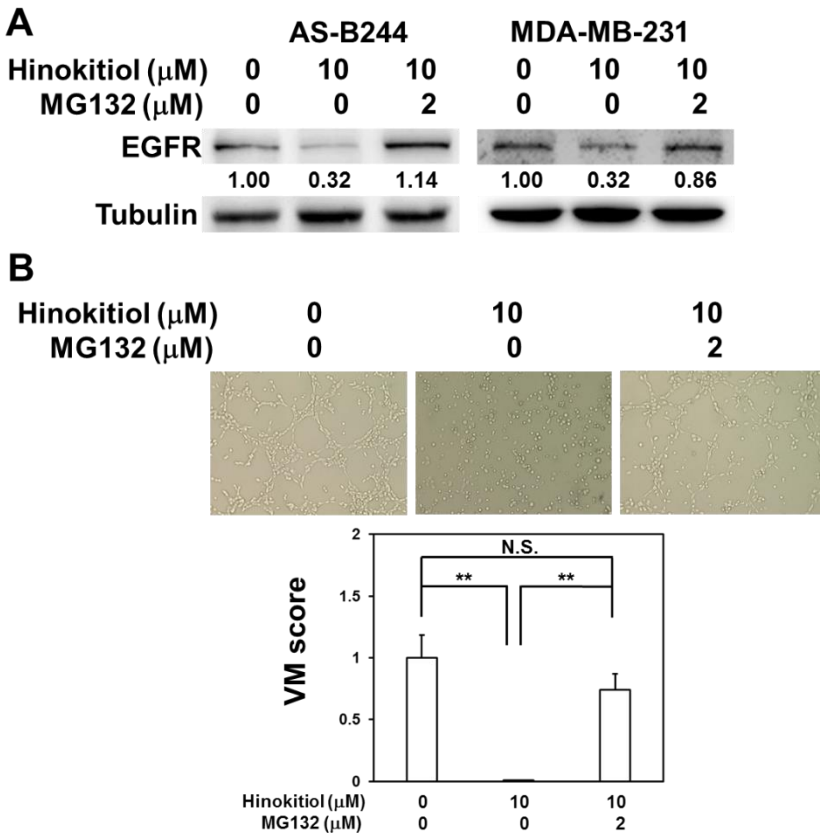


**Figure 10. Hinokitiol inhibits EGFR expression in breast cancer stem-like cells through proteasomal degradation.** (A) AS-B244 or MDA-MB231 sphere cells were treated as indicated conditions for 24 hours. The expression of EGFR or tubulin protein was detected by western blot. The insert number represented as relative expression level in comparison with 0.1% EtOH control. (B) AS-B244 sphere cells were treated as indicated conditions for 24 hours and cells were then harvested for VM activity determination by matrigel-caoted microwells. Images were captured at 4 h post-seeding and VM scores were calculated with ImageJ software. N.S., not significant; **, $p<0.01$.

## Conclusion

Our results demonstrate that breast cancer stem/progenitor cells (BCSCs) display vasculogenic mimicry (VM) activity which is regulated by EGF/Hsp27 signal transduction pathway. We also provide evidence to demonstrate that EGFR inhibitor could suppress VM activity of BCSCs. In addition, we also discover that hinokitiol, a natural monoterpenoid compound, could inhibit VM activity of BCSCs through proteasome mediated EGFR degradation. These data provide a new insight to develop novel therapeutic strategy in treatment of breast cancer by suppression of vasculogenic mimicry.

## References

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**Publications contributed by the fund of this project**

1. **Chang WW**, Lin RJ, Yu J, Chang WY, Fu CH, Lai ACY, Yu JC, Yu AL.,(2013), The expression and significance of insulin-like growth factor-1 receptor and its pathway on breast cancer stem/progenitors. *Breast Cancer Research*. 15:R39. (First author, IF=5.881, 12.4% (25/202) in Oncology)

2. Peng CY, Fong PC, Yu CC, Tsai WC, Tzeng YM and **Chang WW**,(2013), Methyl Antcinate A Suppresses the Population of Cancer Stem-Like Cells in MCF7 Human Breast Cancer Cell Line. *Molecules.* 18, 2539-2548. (Corresponding author, IF=2.095, 30/58 in Chemistry, Organic)

3. Lee CH, Wu YT, Hsieh HC, Yu Y, Yu AL, Chang WW. Epidermal growth factor/heat shock protein 27 pathway regulates vasculogenic mimicry activity of breast cancer stem-like/progenitor cells. Biochimie. 2014. 104: 117-126. (Corresponding author, IF=3.123, 122/291 in Biochemistry & Molecular Biology)

# 科技部補助專題研究計畫項下出席國際學術會議心得報告

日期：103 年 10 月 30 日

| | | | |
|---|---|---|---|
| 計畫編號 | NSC101－2314－B－040－015－MY2 | | |
| 計畫名稱 | 乳癌幹細胞的新血管生成潛力研究 | | |
| 出國人員姓名 | 張文瑋 | 服務機構及職稱 | 中山醫學大學生物醫學科學系<br>副教授 |
| 會議時間 | 103 年 04 月 14 日<br>至<br>103 年 04 月 17 日 | 會議地點 | 日本京都 |
| 會議名稱 | (中文)2014 第 18 屆國際血管生物學會議<br>(英文)2014 The 18th International Vascular Biology Meeting | | |
| 發表論文題目 | (中文)上皮生長因子/熱休克蛋白 27 路徑調控乳癌幹細胞的血管擬態活性<br>(英文) Epidermal growth factor/heat shock protein 27 pathway regulates vasculogenic mimicry activity of breast cancer stem-like/progenitor cells | | |

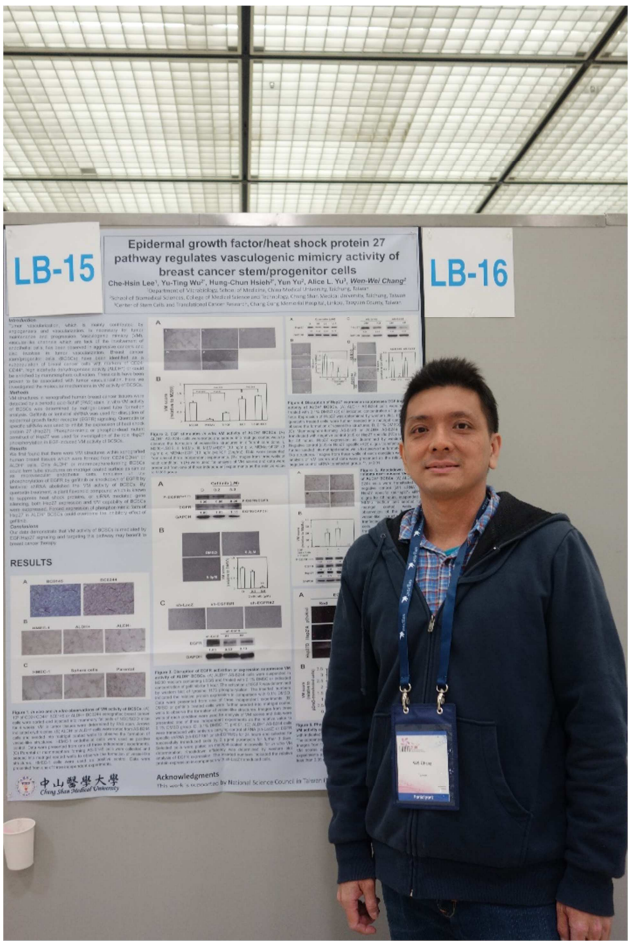
一、參加會議經過

本人大會網頁上傳英文摘要，2014.1.14 收到大會接受函，於 2014.04.13 出發前往京都 Miyakomesse 會議中心參加會議，並於 2011.04.18 日返抵台灣

二、與會心得

本會議為 Japan Convention Service, Inc.舉辦之 2014 第 18 屆國際血管生物學會議(IVBM2014)，主要由日本京都大學與大阪大學心血管疾病、分子病理及臨床基因

治療的學者主辦，台灣則有成功大學醫學院吳華林特聘教授為會議委員，本次會議分成3場Memorial lectures、12個plenary lectures及28個scientific sessions，總共有636個海報(包含29個late breaking abstracts)，整體而言，會議學術性質十分濃厚，內容涵蓋訊息傳遞、基礎血管生物學研究(包含胚胎發育之血管新生、血管老化、平滑肌細胞生物學等)、疾病血管生物學研究(包含粥狀動脈硬化、腫瘤血管、肺動脈高壓)、幹細胞研究、腫瘤研究、轉譯醫學研究，雖名為血管生物學會議，但實際上涵蓋學科廣，能使若非為專門血管生物學研究學者，也能獲得有興趣領域的新發展近況。當然主題為血管生物學，其研究發展迅速，尤其在影像技術上，從中也了解到要做尖端的血管生物學研究，高端的影像技術設備是必要的。而來自國防大學/中央研究院細胞與個體生物研究所的吳孟穎研究員同時獲得兩項獎項，也讓身為台灣的與會者與有榮焉。會中也遇到幾位來自台灣的學者，包含成功大學江美治教授、中國醫藥大學李哲欣教授，在海報報告時間中有了充分的交流。另外，京都是日本著名的文化之都，趁會議空檔就近參觀鴨川、祇園、清水寺等著名景點，體會日本當地的文化。




海報展示報告留影

三、考察參觀活動(無是項活動者略)

無

四、建議

無

五、攜回資料名稱及內容

1. 會議資料袋一只(內含會議議程手冊、個人識別名牌)

2. 大會摘要隨身碟一只

六、其他

# 科技部補助計畫衍生研發成果推廣資料表

日期:2014/10/30

| | |
|---|---|
| 科技部補助計畫 | 計畫名稱: 乳癌幹細胞的新血管生成潛力研究 |
| | 計畫主持人: 張文瑋 |
| | 計畫編號: 101-2314-B-040-015-MY2　　學門領域: 血液科腫瘤科風濕免疫及感染 |

無研發成果推廣資料

# 101 年度專題研究計畫研究成果彙整表

| 計畫主持人：張文瑋 | 計畫編號：101-2314-B-040-015-MY2 |
|---|---|
| 計畫名稱：乳癌幹細胞的新血管生成潛力研究 | |

| 成果項目 | | | 量化 | | | 單位 | 備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等） |
|---|---|---|---|---|---|---|---|
| | | | 實際已達成數（被接受或已發表） | 預期總達成數(含實際已達成數) | 本計畫實際貢獻百分比 | | |
| 國內 | 論文著作 | 期刊論文 | 0 | 0 | 100% | 篇 | |
| | | 研究報告/技術報告 | 0 | 0 | 100% | | |
| | | 研討會論文 | 1 | 1 | 100% | | |
| | | 專書 | 0 | 0 | 100% | | |
| | 專利 | 申請中件數 | 0 | 0 | 100% | 件 | |
| | | 已獲得件數 | 0 | 0 | 100% | | |
| | 技術移轉 | 件數 | 0 | 0 | 100% | 件 | |
| | | 權利金 | 0 | 0 | 100% | 千元 | |
| | 參與計畫人力（本國籍） | 碩士生 | 2 | 2 | 100% | 人次 | |
| | | 博士生 | 0 | 0 | 100% | | |
| | | 博士後研究員 | 0 | 0 | 100% | | |
| | | 專任助理 | 0 | 0 | 100% | | |
| 國外 | 論文著作 | 期刊論文 | 3 | 2 | 100% | 篇 | |
| | | 研究報告/技術報告 | 0 | 0 | 100% | | |
| | | 研討會論文 | 1 | 1 | 100% | | |
| | | 專書 | 0 | 0 | 100% | 章/本 | |
| | 專利 | 申請中件數 | 0 | 0 | 100% | 件 | |
| | | 已獲得件數 | 0 | 0 | 100% | | |
| | 技術移轉 | 件數 | 0 | 0 | 100% | 件 | |
| | | 權利金 | 0 | 0 | 100% | 千元 | |
| | 參與計畫人力（外國籍） | 碩士生 | 0 | 0 | 100% | 人次 | |
| | | 博士生 | 0 | 0 | 100% | | |
| | | 博士後研究員 | 0 | 0 | 100% | | |
| | | 專任助理 | 0 | 0 | 100% | | |

| | |
|---|---|
| 其他成果<br>(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。) | 無 |

| | 成果項目 | 量化 | 名稱或內容性質簡述 |
|---|---|---|---|
| 科教處計畫加填項目 | 測驗工具(含質性與量性) | 0 | |
| | 課程/模組 | 0 | |
| | 電腦及網路系統或工具 | 0 | |
| | 教材 | 0 | |
| | 舉辦之活動/競賽 | 0 | |
| | 研討會/工作坊 | 0 | |
| | 電子報、網站 | 0 | |
| | 計畫成果推廣之參與（閱聽）人數 | 0 | |

# 科技部補助專題研究計畫成果報告自評表

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：

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 100 字為限）

Lee CH, Wu YT, Hsieh HC, Yu Y, Yu AL, Chang WW. Epidermal growth factor/heat shock protein 27 pathway regulates vasculogenic mimicry activity of breast cancer stem-like/progenitor cells. Biochimie. 2014. 104: 117-126. (Corresponding author, IF=3.123, 122/291 in Biochemistry & Molecular Biology)

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）

We demonstrate that breast cancer stem/progenitor cells (BCSCs) display vasculogenic mimicry (VM) activity which is regulated by EGF/Hsp27 signal transduction pathway. We also provide evidence to demonstrate that EGFR inhibitor could suppress VM activity of BCSCs. In addition, we also discover that hinokitiol, a natural monoterpenoid compound, could inhibit VM activity of BCSCs through proteasome mediated EGFR degradation. These data provide a new insight to develop novel therapeutic strategy in treatment of breast cancer by suppression of vasculogenic mimicry.