行政院國家科學委員會專題研究計畫 成果報告

Apigenin 對 HGF 誘發乳癌細胞侵移 生長 及血管新生之影響及其作用機制之研究
研究成果報告(精簡版)

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計 畫 主 持 人：曾翠華

計畫參與人員：博士班研究生-兼任助理：李維駿、高而仕
碩士班研究生-兼任助理：梁文海

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行政院國家科學委員會補助專題研究計畫 V成果報告 □期中進度報告

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目　錄

壹、前言

乳癌是好發於女性的惡性腫瘤，每年造成超過四十萬的婦女死亡 (Parkin et al., 2005)，儘管近年來醫界持續的對於腫瘤的檢驗與治療進行研究，然而在臨床上腫瘤的轉移仍然是造成癌症死亡的主要原因，而在轉移的過程中，癌細胞的侵犯作用扮演著關鍵性的角色，因此控制癌細胞的侵犯與腫瘤的擴散轉移，乃是成功治療癌症的重要目標。

肝臟生長因子（HGF）又被稱之為發散因子（scatter factor），在生理活性上證實對許多癌細胞具有調控所謂"侵犯生長"的腫瘤轉移關鍵步驟，包括刺激生長、擴散、移動及侵犯，同時也是很強的血管新生因子 (Jiang et al., 2005)，在臨床研究上發現乳癌組織中HGF以及其接受體c-Met有過度表現的現象 (Camp et al., 1999)，而乳癌組織中c-Met的表現量與血清HGF的含量與患者的存活率及是否形成遠端的轉移具有正相關性(Maemura et al., 1998)，例如肺部、肝臟以及骨髓是乳癌最常發生轉移的位置，然而在多發性乳癌轉移的病患中顯示血清HGF有增加的現象，而增加血清HGF的濃度與患者腫瘤大小及轉移的程度有關 (Toi et al., 1998)，顯示HGF/Met的訊息路徑在癌症轉移的發展上，扮演關鍵性的重要角色，因此在治療癌症的策略上，此路徑將可作為一個重要的標的。

HGF/Met的訊息路徑對癌細胞侵犯性生長的影響已被廣泛的研究，證實當HGF與Met結合後，Met的β次單元C端上Tyr1349以及Tyr1356的位置會被磷酸化，而c-Met上的磷酸化位置可以提供作為許多具有SH2作用區的訊息傳遞連接分子之結合位置，像是PI3-k、Ras nucleotide exchanger、Grb-2、Gab-1 (Abounader & Laterra, 2005)、Src (Schaeper et al., 2000)以及Shc (Pelicci et al., 1995)等，而這些訊息分子能更進一步的引起下游的訊息反應，包括活化ERK1/2、JNK1/2及p38 (Yao et al., 2004)，然而許多有關侵犯生長的研究也證實，至少有一部份的原因歸因於某些特定的訊息分子所引起的反應 (Giordano et al., 1997; Ponzetto et al., 1996)，例如HGF誘導Ras活化後能促進細胞的增生、癌化以及擴散 (Hartmann et al., 1994; Ridley et al., 1995)，而另一個影響HGF誘導細胞移動的主要路徑則是PI3-k的訊息路徑 (van der Voort et al., 2000)，PI3-k的訊息路徑能夠調控細胞移動時所需的細胞骨架重組以及形成集中附著點，進而在細胞邊緣產生所謂的層狀偽足（lamellipodia）或膜皺褶（membrane ruffles）的高度網狀組織結構，也可促進細胞表面形成所謂微形突出（microspikes）或絲狀偽足（filopodia）的肌動蛋白纖維 (Warn et al., 1993)，都是細胞在透過骨架重組以獲得移動

能力時的變化。

近年來醫界漸漸開始重視化學預防在抗癌上所扮演的角色，其中又以毒性及成本都較低的天然成分最被重視，目前許多的研究都朝向以天然植物所含的成分，在抗癌的活性與治療上的效果 (Birt et al., 1996)，任何一種成分只要具有抑制癌細胞侵犯的能力，就有可能成為預防腫瘤轉移的有效成分，其中類黃酮是人類飲食中常見的成分廣泛的存在餘各種的蔬菜及水果中，依照不同的結構類型可分為黃酮醇類（flavonols）、黃烷酮類（flavanones）、黃烷醇（flavanols）以及異黃酮（isoflavones）等，許多的實驗證實這些類黃酮都具有抑制癌細胞侵犯的能力 (Bigelow & Cardelli, 2006; Lee et al., 2006)。過去的研究顯示芹菜素具有低毒性且無致突變性的特性，廣泛的存在於許多的水果及蔬菜中 (Dunnick & Hailey, 1992)，在抗癌活性的研究上也證實芹菜素可以抑制紫外線引起的皮膚癌 (Birt et al., 1997) 以及細胞的增生 (Lepley & Pelling, 1997)，此外芹菜素還顯示能夠誘導癌細胞的凋亡 (Plaumann et al., 1996)，同時在其多樣性影響中也具又抑制癌細胞的侵犯過程 (Czyz et al., 2005)。

貳、研究目的

目前在癌症的治療上無論是在抗癌藥物的強大負作用上，以及龐大費用與精力的付出，對於患者與家屬都是一項沈重的負擔，因此，本研究中我們選擇性的篩選不同結構類型的類黃酮在抑制HGF誘導乳癌細胞侵犯的能力，結果發現以芹菜素（apigenin；4′,5,7,-trihydroxyflavone）的效果最強，，儘管其他的類黃酮也顯示具有類似的抗癌活性，然而對於類黃酮在影響HGF誘導的癌細胞侵犯作用則還未被研究，因此在本研究中我們將研究目標集中於篩選各種類黃酮，包括山奈酚（kaempferol）、,柚皮素（naringenin）、金雀異黃酮（genistein）以及芹菜素（apigenin）等不同結構組成的類黃酮，無論是在細胞實驗還是在動物實驗的模式下對HGF誘導乳癌細胞株MDA-MB-231侵犯性生長的影響。

參、實驗方法

1、細胞培養（cell culture）

人類乳癌細胞株 MDA-MB-231 (from *The NHRI Cell Bank*)，培養於含有10% fetal bovine serum (FBS : Gibco BRL)、1% nonessential amino acid (NEAA : Gibco)、1% Penicillin-Streptomycin (PS : Gibco)的Dulbecco's Modified eagle medium (DMEM : Gibco)，培養箱設定5% CO2，37℃恆溫環境，細胞密度維持在 $2x10^5$～$1x10^6$，每週更換培養基 2～3次。

人類臍帶內皮細胞（Human umbilical vein endothelial cells；HUVEC），培養於含有10% fetal bovine serum (FBS : Gibco BRL)、10 mM HEPES、12 units/ml heparin、1% nonessential amino acid (NEAA : Gibco)、1% Penicillin-Streptomycin (PS : Gibco)以及100 μg/ml內皮細胞補充劑的Medium 199 (Gibco)，培養箱設定5% CO2，37℃恆溫環境，細胞密度維持在 $2x10^5$～$1x10^6$，每週更換培養基 2～3次。

2、細胞活性分析（MTT assay）

利用活細胞能經由粒腺體 dehydrogenase 的作用，將(3-[4 , 5-Dimethylthiazol-2-yl]-2 , 5-diphenyl-tetrazolium bromide) [MTT]代謝還原成紫色的 formazan crystal，並在波長 570 nm 有特殊吸光，再以量化的比色法來分析細胞的存活及增殖。將細胞培養後，以 trypsin-EDTA 將細胞由培養皿打下，用 PBS 沖洗後，以培養基將細胞濃度調整至 $5x10^4$ cell / ml，各取 1 ml 的細胞液分別培養於 24-wel 的培養皿中。待細胞貼壁，更換新的培養基，同時加入 40μM 的各種不同的類黃酮，處理 12 小時後，更換新的培養基，同時加入 100 μl MTT (5mg/ml)反應 4 小時，除去培養機後，最後以 1 ml 的 DMSO 將紫色的 formazan 結晶溶解，並於波長 570 nm 下測定吸光值($O.D._{570}$)。

3、細胞侵入（invasion）與細胞移動（migration）分析

利用48 well Boyden chamber的分析方法，將具有8-μm孔徑的cellulose nitrated filters coating上 40μg/cm^2的Matrigel或10μg/cm^2的laminin-1，在laminar flow中風乾3～5小時，lower chamber中以不含HGF的DMEM作為控制組，而實驗組則以40 ng/ml的HGF作為趨

化因子，將細胞培養於100 mm-dish 24小時，細胞密度維持在 $2x10^5$/ml，再以serum-free的DMEM starvation 24小時，將細胞以0.05%的trypsin-EDTA打下，以serum-free的DMEM調整細胞密度至$5x10^5$/ml，並加入不同濃度的apigenin，於upper chamber中注入50μl的細胞液，待細胞反應12小時後，取下薄膜，以甲醇固定細胞10分鐘，風乾5分鐘之後，以Giemsa（1:20）染色1小時，最後固定住薄膜，以棉花棒擦拭掉薄膜上層細胞，在200X顯微鏡底下每個well隨機選取5個位置，共取4個well做移動細胞數之統計;細胞移動分析與細胞侵入分析類似，但cellulose nitrated filters上不coating Matrigel。

4、傷口癒合試驗（Wound Healing Assay）

將細胞培養後，以trypsin-EDTA將細胞由培養皿打下，細胞密度調整為$2x10^5$/ml培養於6-well plates，待細胞密度到達約九分滿時，以serum-free的DMEM starvation 24小時，接者以200-μl pipette tip括除細胞用以產生數條沒有細胞的區域，以PBS去除括除的細胞，在加或不加各種濃度的apigenin以及40ng/ml的HGF的情況下作用12及24小時，在100X顯微鏡底下每個well隨機選取5個位置，與最初括除細胞時(0小時)的情況做比對，計算細胞移動至劃線區域內的細胞數目。

5、細胞-基質粘附試驗（cell-matrix adhesion assay）

首先製作96-multiwell high binding tissue culture plates，將每個well分別以100μl的40μg/ml的Matrigel與type Ⅰ collagen以及2%的gelatin coating並於室溫下在laminar flow中風乾overnight，以PBS沖洗3次再以3% heat-inactivated BSA blocking 1小時，以PBS沖洗3次備用，將細胞培養於100 mm-dish 24小時，細胞密度維持在 $2x10^5$/ml，再以serum-free的DMEM starvation 24小時，將細胞以0.05%的trypsin-EDTA打下，以serum-free的DMEM調整細胞密度至$4x10^5$/ml，以不同濃度的apigene預先處理1小時，接者在加或不加40ng/ml的HGF的情況下，將每個well1注入100μl的細胞液，30分鐘後以PBS沖洗掉未貼附的細胞，以3.7% para-formaldehyde固定10分鐘，再以0.1% crystal violet染色30分鐘，以PBS沖洗3次，以10% acetic acid將crystal violet溶出，於$O.D._{595nm}$下測量吸光值顯示細胞吸附的情況。

6、細胞發散性測定（cell scattering）

將細胞培養後，以trypsin-EDTA將細胞由培養皿打下，PBS沖洗後，以1000rpm離心5分鐘，取沉澱細胞，加培養基調整細胞濃度至2x10^5/ml，將細胞培養於60 mm-dish 24小時，再以serum-free的DMEM starvation 24小時，以40 μM的apigene預先處理1小時，接者以40ng/ml的HGF作用12小時後，利用倒立式顯微鏡來觀察細胞與細胞間的黏附以及向周圍發散的情形。

7、DAPI 細胞螢光染色

利用DAPI與DNA鍵結，觀察細胞內染色質的變化，應用於apoptosis現象之定性觀察。將細胞培養後，以trypsin-EDTA將細胞由培養皿打下，PBS沖洗後，以1000 rpm離心5分鐘，取沉澱細胞，加培養基調整細胞濃度至2×10^5/ml，將細胞培養於60 mm-dish 24小時，再以serum-free的DMEM starvation 24小時，以40 μM的apigene預先處理1小時，接者以40ng/ml的HGF作用12小時後，除去培養基，以PBS清洗3次，加入固定液(3.7% formaldehyde)，於室溫下固定15分鐘，再加入新鮮配置的DAPI染劑1 ml（5μM in PBS），室溫避光反應20-30分鐘，利用倒立式顯微鏡於100～200倍下放大照相來觀察細胞核的變化。

8、細胞基質吸附試驗（Cell-matrix adhesion）

首先製作96-multiwell high binding tissue culture plates，將每個well分別以100 μl的40 μg/ml的Matrigel與type I collagen以及2%的gelatin coating並於室溫下在laminar flow中風乾overnight，以PBS沖洗3次再以3% heat-inactivated BSA blocking 1小時，以PBS沖洗3次，將細胞培養於100 mm-dish 24小時，細胞密度維持在 2x10^5/ml，再以serum-free的DMEM starvation 24小時，以不同濃度的apigenin預先處理1小時，接者以40 ng/ml的HGF或fibroblast-conditioned medium作用3小時，將細胞以0.05%的trypsin-EDTA打下，以serum-free的DMEM調整細胞密度至4x10^5/ml，並於每個well中注入100 μl的細胞液，40分鐘後以PBS沖洗掉未貼附的細胞，以 3.7 % para-formaldehyde固定10分鐘，再以0.1% crystal violet染色30分鐘，以PBS沖洗3次，以10% acetic acid將crystal violet溶出，於O.D.$_{595nm}$下測量吸光值顯示細胞吸附的情況。

9、免疫螢光染色（F-actin and integrin β4）

a、F-actin immunofluorescence microscopy

將細胞培養後，以trypsin-EDTA將細胞由培養皿打下，PBS沖洗後，以1000 rpm離心5分鐘，取沉澱細胞，加培養基調整細胞濃度至2x10^5/ml，將細胞培養於60 mm-dish 24小時，再以serum-free的DMEM starvation 24小時，以40 μM的apigene預先處理1小時，接者以40ng/ml的HGF作用12小時後，除去培養基，以PBS清洗3次，加入固定液(3.7% formaldehyde)，於室溫下固定10分鐘，接著以0.1% TritonX-100反應1分鐘，以3% heat-inactivated BSA 於37℃下blocking 15分鐘，再以TRITC-phalloidin (500ng/ml)染色1小時，以PBS沖洗3次後，以Fluorescence Microscopy觀察細胞骨架的變化。

B. integrin β4 indirect immunofluorescence microscopy

首先製作6-well high binding tissue culture plates，將每個well分別以1ml的10μg/ml的laminin-1 coating並於室溫下在laminar flow中風乾overnight，以PBS沖洗3次再以2 % heat-inactivated BSA blocking 1小時，以PBS沖洗3次備用，將細胞培養於100 mm-dish 24小時，細胞密度維持在 2x10^5/ml，再以serum-free的DMEM starvation 24小時，將細胞以0.05% 的 trypsin-EDTA 打下，以 serum-free 的 DMEM 調整細胞密度至 1x10^5/ml，以不同濃度的apigene預先處理1小時，接者在加或不加40ng/ml的HGF的情況下，將每個well注入2 ml的細胞液，45分鐘後除去培養基，以PBS清洗3次，加入固定液(3.7% formaldehyde)，於室溫下固定10分鐘，接著以0.1% TritonX-100反應1分鐘，以3% heat-inactivated BSA 於37℃下blocking 15分鐘後，分別以1 μg/ml integrin β4的primary antibodies反應30分鐘，以2 % heat-inactivated BSA沖洗3次，再分別以1 μg/ml 的 fluorescein-tagged secondary antibody (FITC-tagged for c-Met, PE-tagged for integrinβ4)作用30分鐘，以PBS沖洗3次後，以Fluorescence Microscopy觀察。

10、西方點墨法（Western blotting）

將細胞培養後，以trypsin-EDTA將細胞由培養皿打下，PBS沖洗後，以1000 rpm離心5

分鐘，取沉澱細胞，加培養基調整細胞濃度至$2x10^5$/ml，將細胞培養於60 mm-dish 24小時，再以serum-free的DMEM starvation 24小時，以實驗所需的apigene濃度預先處理1小時，接者以40ng/ml的HGF作用指定的時間後，加入RIPA buffer(150mM NaCl, 1% NP-40, 0.5% Deoxycholic acid, 0.1% SDS, 50mM Tris-base, pH7.5)，內含1mM sodium orthovanadate, 100μg PMSF, 170μg/ml leupeptin)，於冰上震盪30分鐘，在4℃下以10000g離心10分鐘，取上層液定量蛋白，將定量後之蛋白取50μg，加入等量的Sample Buffer(2ml 0.5M Tris-HCl pH6.8, 1.6ml Glycerol, 3.2ml 10% SDS, 0.8ml 2-β-mercaptoethanol, 0.4ml 0.5% bromophenol blue), 以95℃加熱5分鐘，迅速至入冰中冷卻，以離心機將Sample apin down後再loading至每個well中，上層膠以70伏特，下層以130伏特跑電泳，待電泳結束後接者進行蛋白質的轉漬至Nitrocellular paper上，以5%脫脂牛奶於室溫下進行blocking 1小時，以washing buffer(PBS with 0.5% tween-20)沖洗3次，將NC paper至於4℃冰箱中與一級抗體反應overnight，以washing buffer沖洗3次，再以Horseradish peroxidase conjugated的二級抗體反應1小時，以washing buffer沖洗3次，最後加入Western Blot Chemiluminescence Reagent Plus反應1分鐘後，以冷光螢光數位影像分析儀(LAS-1000 plus system)觀察並定量。

11、MMP-2, 9活性分析（gelatin zymography）

將細胞培養於不含胎牛血清的培養基中 24 小時後，更換新鮮的培養基並加入 40 ng/ml 的 HGF 作用 24 小時，取培養基以 1500 rpm 離心 5 分鐘，取上清液與 5 倍的染劑均勻混合，注入 0.1% gelatin-8% SDS-PAGE 電泳膠片中，置於含有電泳緩衝液的電泳槽中，以 100V 的電壓進行電泳分離，結束電泳分離後，以 washing buffer(2.5% Triton X-100 in ddH_2O)在室溫下清洗 30 分鐘 2 次，隨後加入 reaction buffer(reaction buffer : 10mM $CaCl_2$, 0.01% NaN_3, 40mM Tris-HCl pH8.0)於 37℃恆溫箱中反應 12～16 小時，反應完後以 Coomassie blue 染色液染色 30 分鐘，再以退色液退染(20% methanol, 10% acetic acid)，觀察結果。

12、u-PA活性分析（casein zymography）

將細胞培養於不含胎牛血清的培養基中24小時後，更換新鮮的培養基並加入40 ng/ml

的HGF作用24小時，取培養基以1500 rpm離心5分鐘，取上清液與5倍的染劑均勻混合，注入含有2% casein及20μg/ml plasminogen的8% SDS-PAGE電泳膠片中，置於含有電泳緩衝液的電泳槽中，以100V的電壓進行電泳分離，結束電泳分離後，以washing buffer在室溫下清洗30分鐘2次，隨後加入reaction buffer於37℃恆溫箱中反應12～16小時，反應完後以Coomassie blue染色液染色30分鐘，再以退色液退染，觀察結果。

13、裸鼠肺部移生試驗（Lung colonization assay in nude mice）

將細胞培養後，以trypsin-EDTA將細胞由培養皿打下，PBS沖洗後，以1000 rpm離心5分鐘，取沉澱細胞，加培養基調整細胞濃度至$2x10^5$/ml，將細胞培養於100 mm-dish 24小時，再以serum-free的DMEM starvation 24小時，以實驗所需的處理方式預先處理2小時，再以trypsin-EDTA將細胞由培養皿打下，PBS沖洗後，以1000 rpm離心5分鐘，取沉澱細胞，調整細胞濃度至$1×10^6$/100 μl，分別在加入及不加40 ng/ml HGF下，將細胞以尾靜脈注射方式注入六週大的母裸鼠中（100 μl/nude mice），於注射癌細胞四週後將裸鼠犧牲，分別測量肺部的大小及重量，並將肺部進行組織切片染色，以分析癌細胞肺部移生的情況。

14、組織切片之病理分析（Pathological histology-Histochemistry and H&E stain）

將裸鼠以CO_2安樂死後，取二分之一的肺葉組織，置於10%福馬林中固定，之後再依次將組織脫水，其方法如下：先用1×PBS於4℃浸泡30分鐘，然後在室溫下換為50% EtOH繼續浸泡30分鐘，二次。待組織完全脫水後，將組織於室溫中以100%的xylene浸泡30分鐘，再將組織更換至xylene：paraffin為1：1的溶液中於60℃浸泡45分鐘，再將組織置於paraffin，20分鐘三次，最後將組織固定於石蠟中，待石蠟冷卻凝固即可切片進行蘇木紫與依紅（Hematoxylin & Eosin）染色，分析觀察癌細胞在肺部組織移生的程度。染色的步驟如下：脫蠟後，以蘇木紫染液作用2～15分鐘，以水浸洗，再以0.5%溶液分別染色色度約1～5秒，進入氨水中（1000 ml的水滴入氨水2滴），於流水中清洗至少15分鐘。以0.5%依紅染液染2分鐘，再分別以80%、90%、95%酒精浸洗，依序移入絕對酒精：二甲苯（1：1）30秒，二甲苯：木餾油（4：1），二甲苯、（I）與二甲苯（II）中各半分鐘，以二甲苯使之透明，封片後以顯微鏡觀察。

15、統計分析（Statistical analysis）

實驗過程每組皆重覆2～3次以上，mean ± standard deviation， 所有數據以電腦統計軟體Sigmaplot（Jandel Scientific Software，USA），採用one-way analysis of variance（one-way ANOVA）統計分析，* P＜0.05,**P＜0.01，表示有顯著差異。

肆、結果與討論

首先我們找出幾種 flavonoids 的無毒劑量,在無毒劑量下發現 apigenin 抑制 HGF 誘發 MDA-MB-231 cell invasion and migration 作用最強(Fig.2-Fig.10)，另外發現 apigenin 對 HGF 誘發形態改變有抑制之作用(Fig.9).我們的研究顯示,當 MDA-MB-231 受到 HGF 的刺激時會引起增加癌細胞的侵犯性生長，包括增加細胞移動、發散、轉移乃至於侵犯的作用，其中的訊息機制在於 HGF 透過刺激 Met 的磷酸化後引起下游 ERK、JNK 以及 Akt 的活化(Fig.11-12)，然而芹菜素能特異性的抑制 Akt 的磷酸化(Fig.13),此外芹菜素與 PI3-k 的抑制劑 wortmannin 都可經由減少 β4 integrin 的親和力達到抑制 HGF 誘導的侵犯與吸附的作用(Fig.18-22)，在動物模式下芹菜素與 wortmannin 都顯示，能夠有效的抑制乳癌細胞在裸鼠中的肺部移生作用(Fig.23)，即使 HGF 能增強這些轉移的作用但芹菜素與 wortmannin 都能有效的降低 HGF 的影響，在細胞與細胞的吸附實驗則進一步的證實 β4 integrin 的功能也包括作為癌細胞與內皮細胞間的吸附分子。

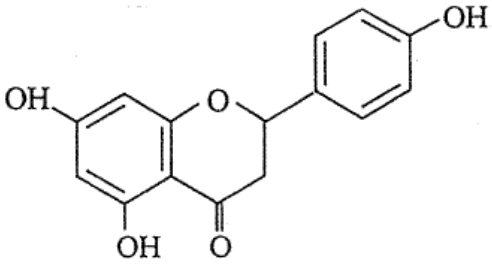
癌細胞的侵犯作用在腫瘤轉移過程中扮演關鍵性的角色，其中包含了許多重要的階段，包括癌細胞在不斷增生形成腫瘤後，會透過刺激生長因子的分泌促進血管新生，接著癌細胞必須先吸附至基質上，並利用蛋白酶將基質分解隨後移動並穿過基質，接著再穿透血管壁滲入血液循環系統中，之後癌細胞在血液循環中還必須成功的逃脫免疫系統的攻擊與失巢凋亡的機制，而進一步的與血液中的成分像是血小板等形成團塊，並吸附至血管壁上，最後穿出血管壁成功的在新的轉移位置開始生長並再次的刺激血管新生促進轉移腫瘤的生長，而這一連串的作用受到許多細胞外訊息的調控，包括生長因子例如HGF，而HGF顯示具有增加MMPs與uPA表現及分泌的能力，進而增加癌細胞侵犯的作用 (Rosenthal et al., 1998)，許多細胞外分解基質的蛋白酶都受到NFκB的調控 (Bond et al., 2001)，在人類前列腺癌細胞DU-145中NF-κB的活化必須透過PI3-k活化Akt的訊息路徑 (Fan et al., 2005)，然而儘管在

MDA-MB-231細胞中，HGF可以誘導Akt的活化，我們及其他研究的報告卻顯示，Akt的活化並無法促進IκBα的磷酸化以及隨後IκBα的蛋白質降解，同時也無法增加NFκB的DNA結合活性 (Zeng et al., 2002)，此現象顯示在不同型態的細胞中HGF活化NFκB的機制是有差異的，這也反映出各別細胞中訊息傳遞的特異性，而其中一個可能的假設就是，在MDA-MB-231細胞中，也許缺少了一些連接Akt與NFκB間的關鍵性連接分子，然而即使HGF並不能改變細胞外基質的表現及活性，然而apigenin具有抑制MDA-MB-231細胞內生性MMP-9與uPA的活性（Fig. 17B）， 顯示此抑制蛋白酶活性的作用也許提供了apigenin在裸鼠的肺部移生試驗中抑制細胞侵犯性生長的能力（Fig. 22）。

MDA-MB-231細胞表面含有integrin β1、β3、β4以及 β5 (Trusolino et al., 2000)，因此我們也評估HGF是否可刺激細胞吸附至各種不同的細胞外基質，結果證實HGF除了可以增加癌細胞與laminin-1的吸附作用外（Fig. 23），對於其他的受質像是Matrigel、 fibronectin以及 vitronectin同樣也有促進的作用（Fig. 21），而apigenin對所有基質的吸附作用也都有抑制的效果，顯示HGF也許可以影響其他的integrins進而促進細胞與各種細胞外基質的吸附作用，然而apigenin在經由PI3-K/Akt的路徑抑制HGF誘導β4 integrin的親和力降低MDA-MB-231細胞吸附及侵犯的能力，是否對其他的integrins也同樣有效，還需進一步的證實。

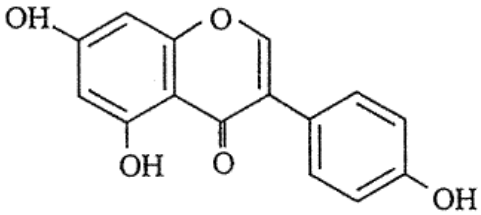
圖　表

Flavanone



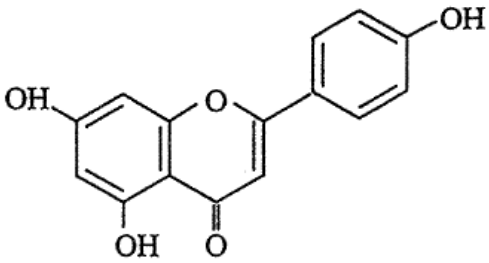
(Naringenin)

Isoflavone



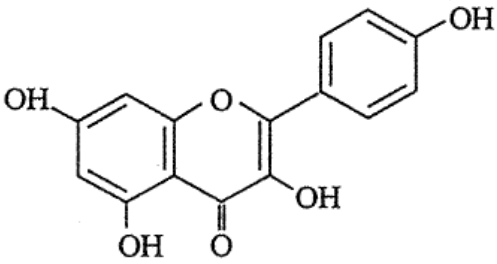
(Genistein)

Flavone



(Apigenin)

Flavonol



(Kaempferol)

Fig. 1 The chemical structures of flavonoids. Flavanones: naringenin; Flavone: apigenin; Isoflavone: genistein; Flavonol: kaempferol.

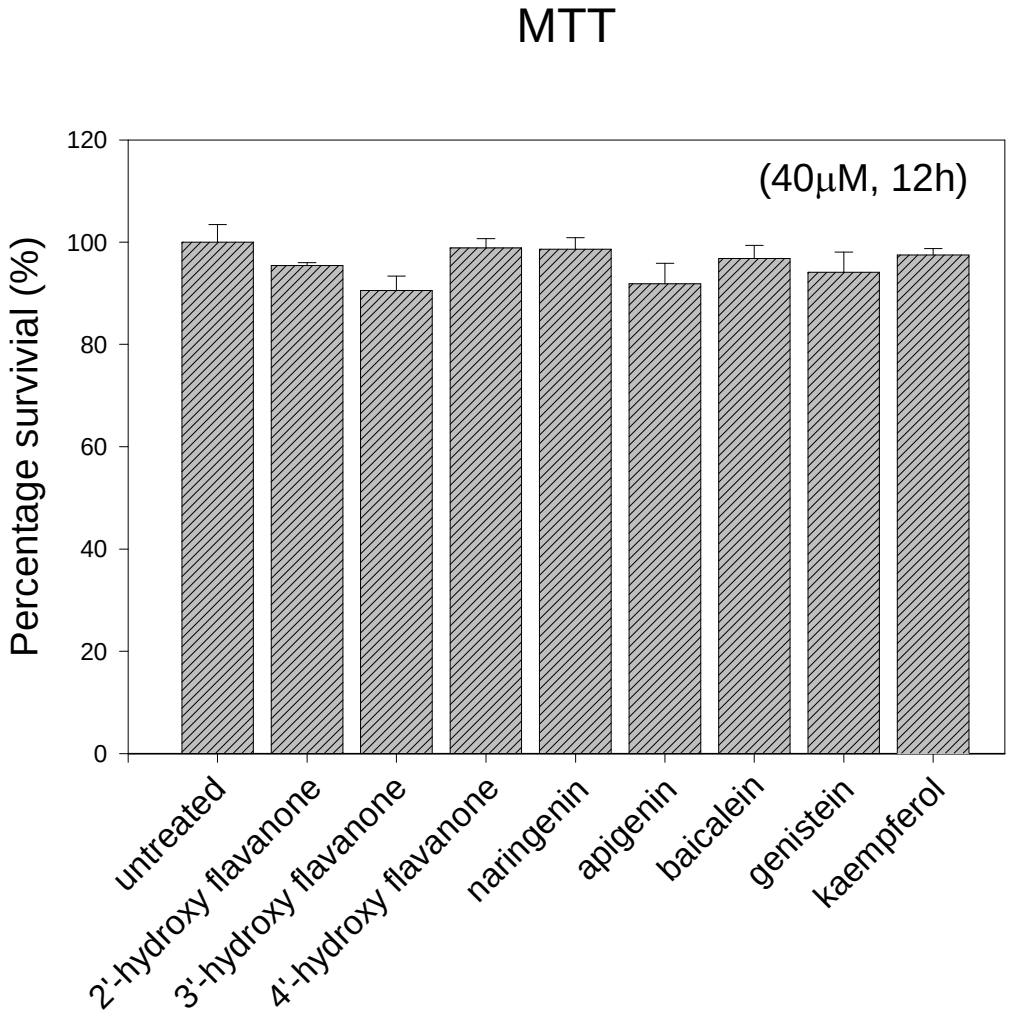


Fig. 2. Effect of flavonoids on viability of MDA-MB-231 cells. Cells were treated with 40 μM of indicated flavonoids for 12 h. Viability of MDA-MB-231 cells was measured by MTT assays as described in Materials and Methods. The results were presented as means ± SD of three independent experiments.

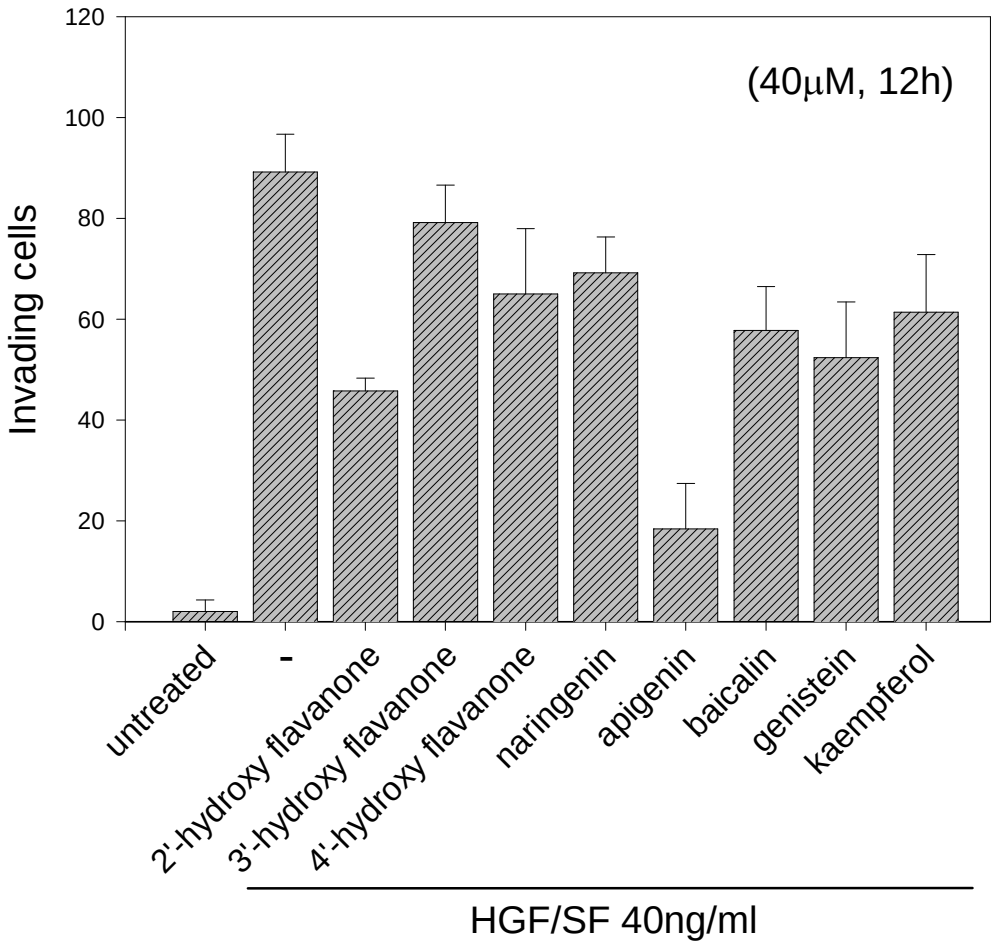


Fig. 3. Inhibition of HGF/SF-induced cell invasion by dietary flavonoids. Inhibitory effect of dietary flavonoids on invasiveness of MDA-MB-231 cells. The invasion assay was performed using the Boyden Chamber assay coated with 40 μg/cm^2 Matrigel, which mimic basement composition and then incubation with 40μM of various of flavonoids for 12 h, and with or without 40ng HGF/SF as a chemoattractive agent in the lower chamber. The lower surfaces of the membranes from the transwell units were fixed with 100% methanol and stained with Giemsa solution. Cells that had migrated to the lower surface of the membranes were counted in triplicate wells and in three identical experiments under a light microscope.

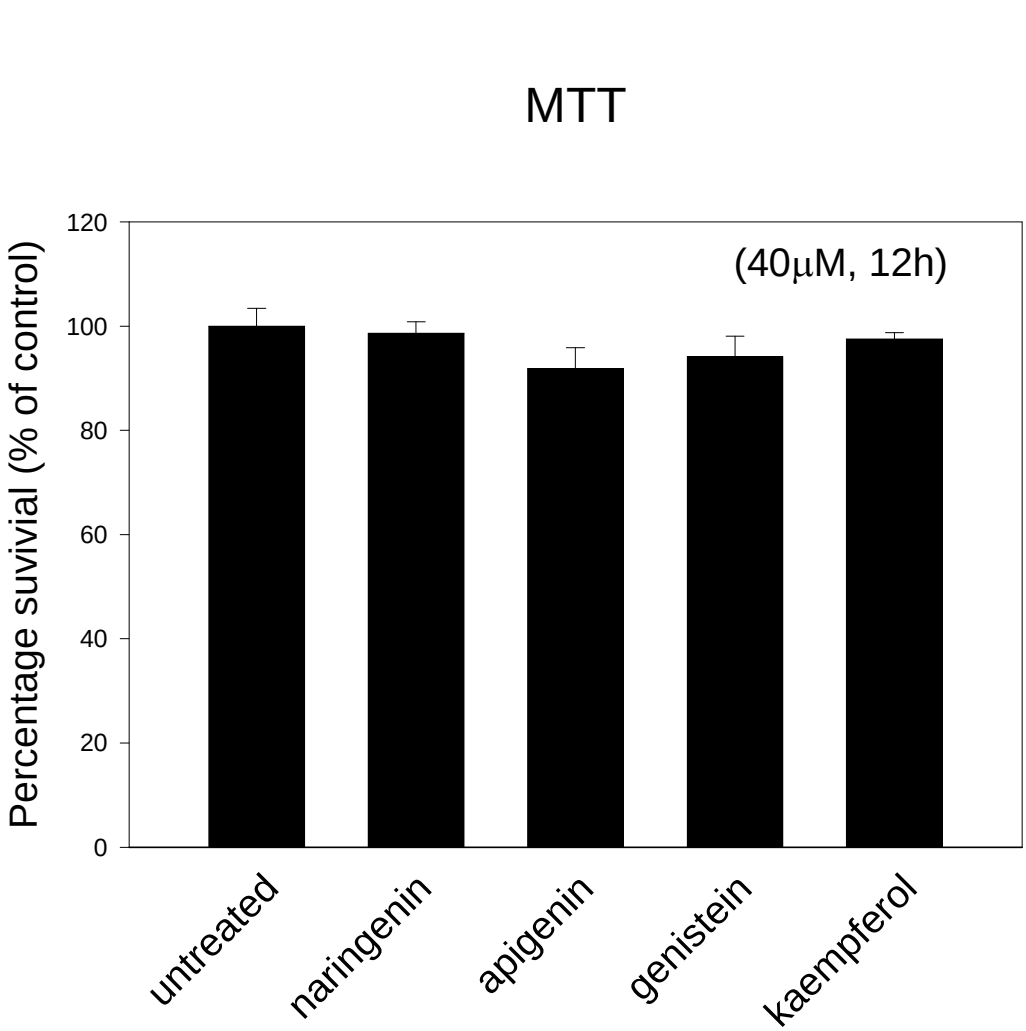


Fig. 4. Effect of flavonoids on viability of MDA-MB-231 cells. Cells were treated with 40 μM of flavonoids including naringenin, apigenin, genistein, and kaempferol for 12 h. Viability of MDA-MB-231 cells was measured by MTT assays as described in Materials and Methods. The results were presented as means ± SD of three independent experiments.

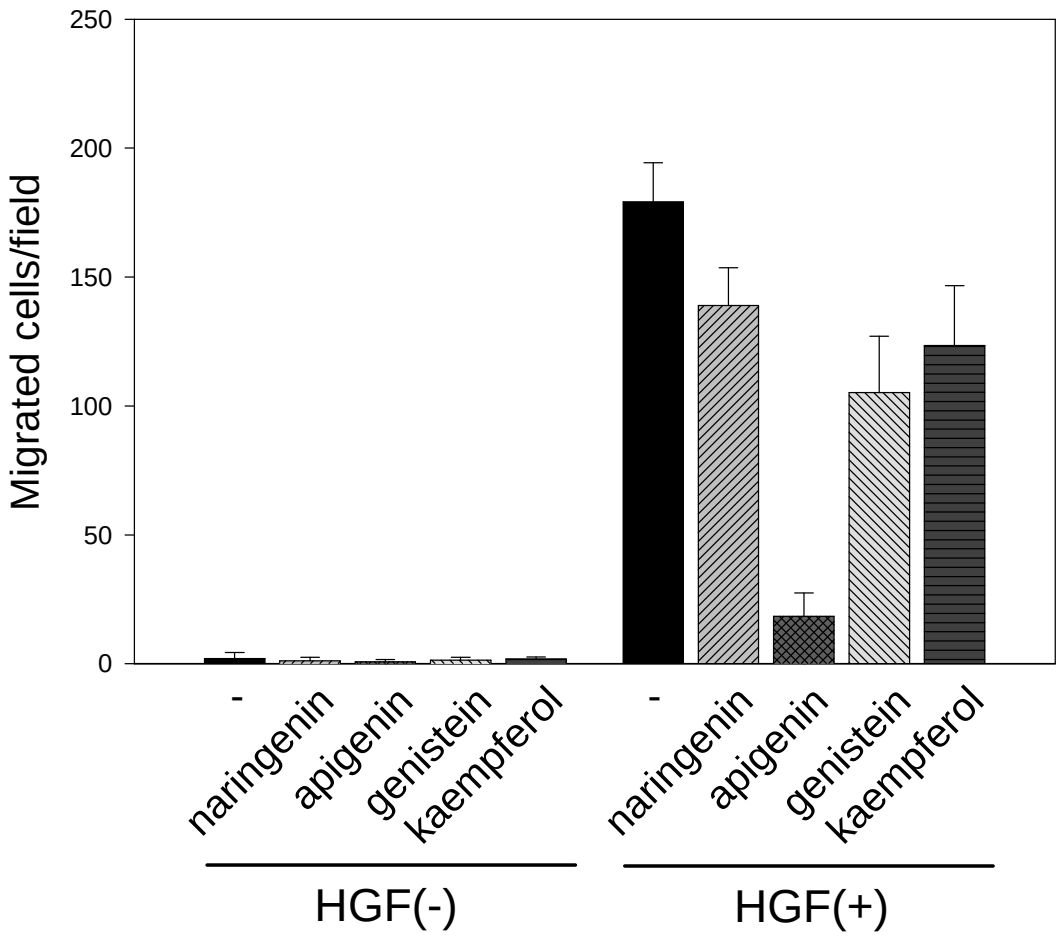


Fig. 5. Inhibition of HGF/SF-induced cell migration by dietary flavonoids. Inhibitory effect of dietary flavonoids on invasiveness of MDA-MB-231 cells. Cells (5×10^5/ml) were seeded onto transwell plates and then incubation with 40μM of various of flavonoids for 12 h, and with or without 40ng HGF/SF as a chemoattractive agent in the lower chamber. The lower surfaces of the membranes from the chamber well units were fixed with 100% methanol and stained with Giemsa solution. Cells that had migrated to the lower surface of the membranes were counted in triplicate wells and in three identical experiments under a light microscope.

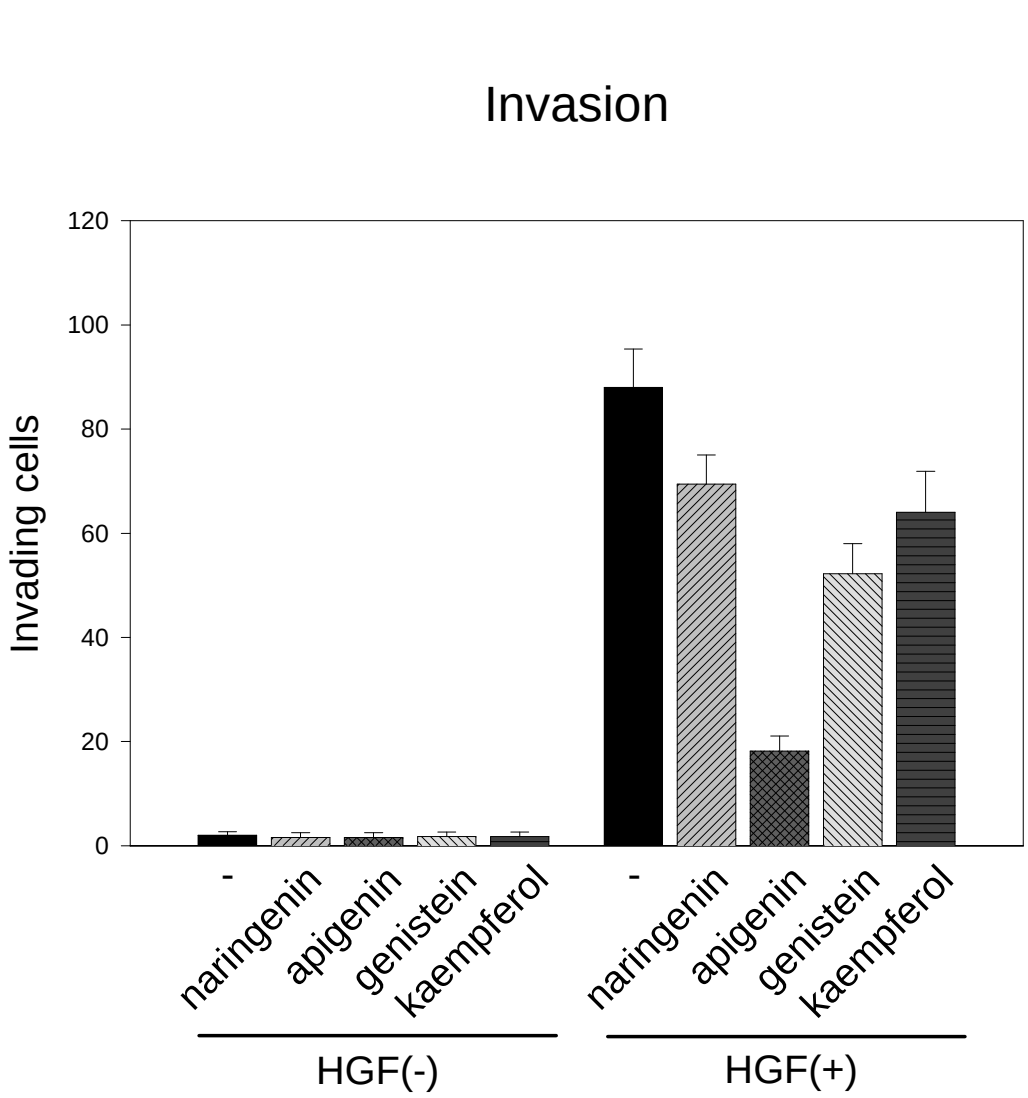
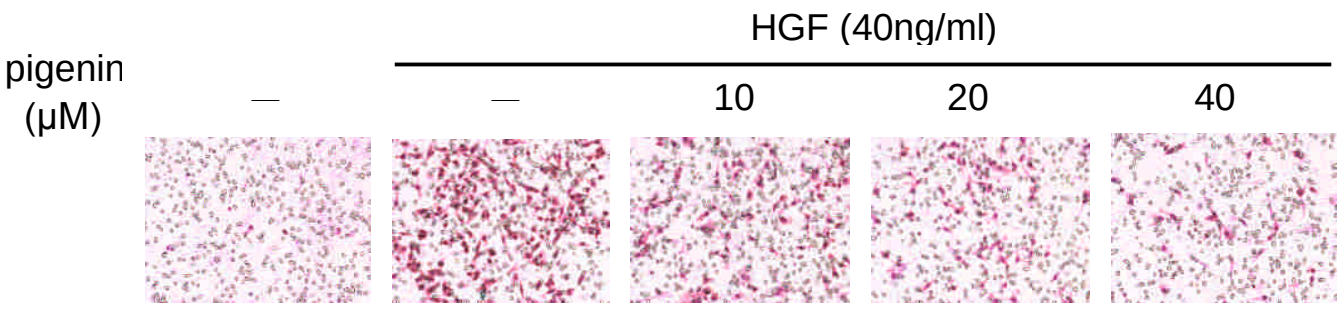


Fig. 6. Inhibition of HGF/SF-induced cell invasion by dietary flavonoids. Inhibitory effect of dietary flavonoids on invasiveness of MDA-MB-231 cells. The invasion assay was performed using the Boyden Chamber assay coated with 40 μg/cm^2 Matrigel, which mimic basement composition and then incubation with 40μM of various of flavonoids for 12 h, and with or without 40ng HGF/SF as a chemoattractive agent in the lower chamber. The lower surfaces of the membranes from the transwell units were fixed with 100% methanol and stained with Giemsa solution. Cells that had migrated to the lower surface of the membranes were counted in triplicate wells and in three identical experiments under a light microscope.

A



B

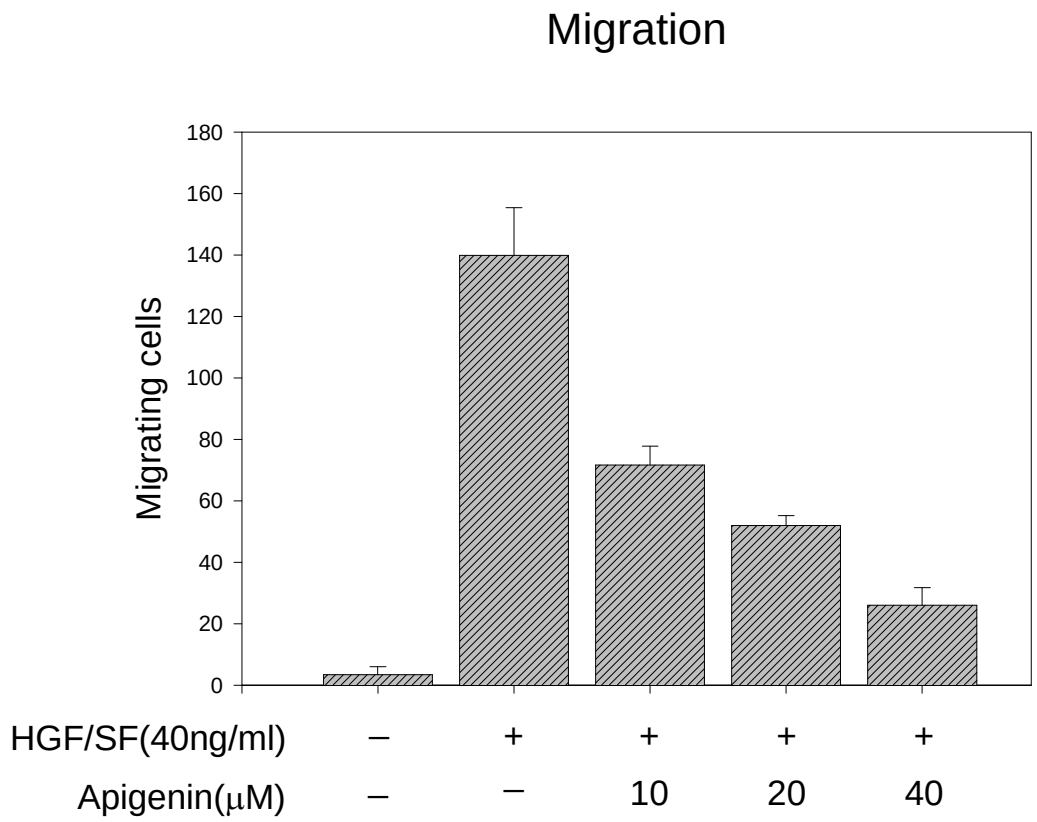
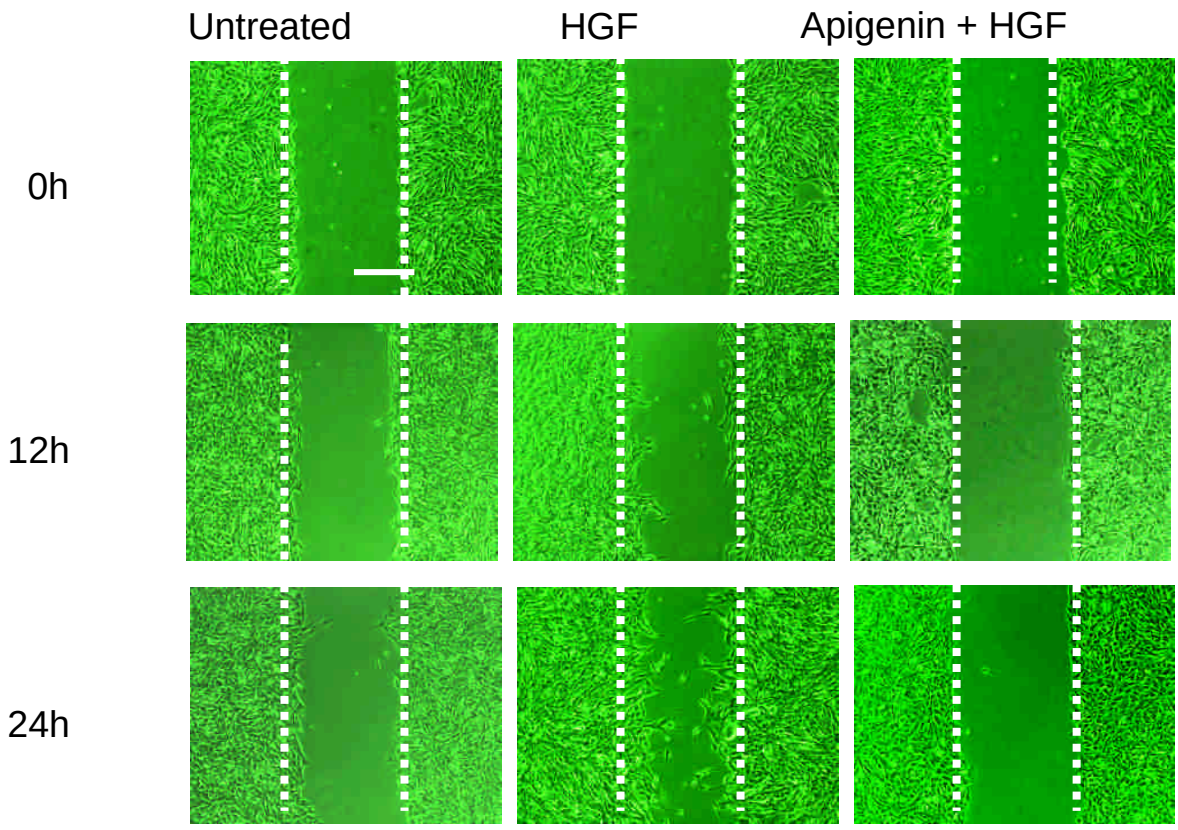


Figure 7. Apigenin inhibition of HGF/SF stimulates cell migration. The migration assay was performed using the Boyden Chamber assay, and HGF/SF(40ng/ml) as a chemoattractive agent in the lower chamber. Cells (5×10^5/ml) were seeded onto transwell plates and then incubation with or without various concentration of apigenin for 12h. The lower surfaces of the membranes from the transwell units were fixed with 100% methanol and stained with Giemsa solution. (A), Photographs of MDA-MB-231 cells after invasion.(B), Cells that had invacted to the lower surface of the membranes were counted in triplicate wells and in three identical experiments under a light microscope.

A



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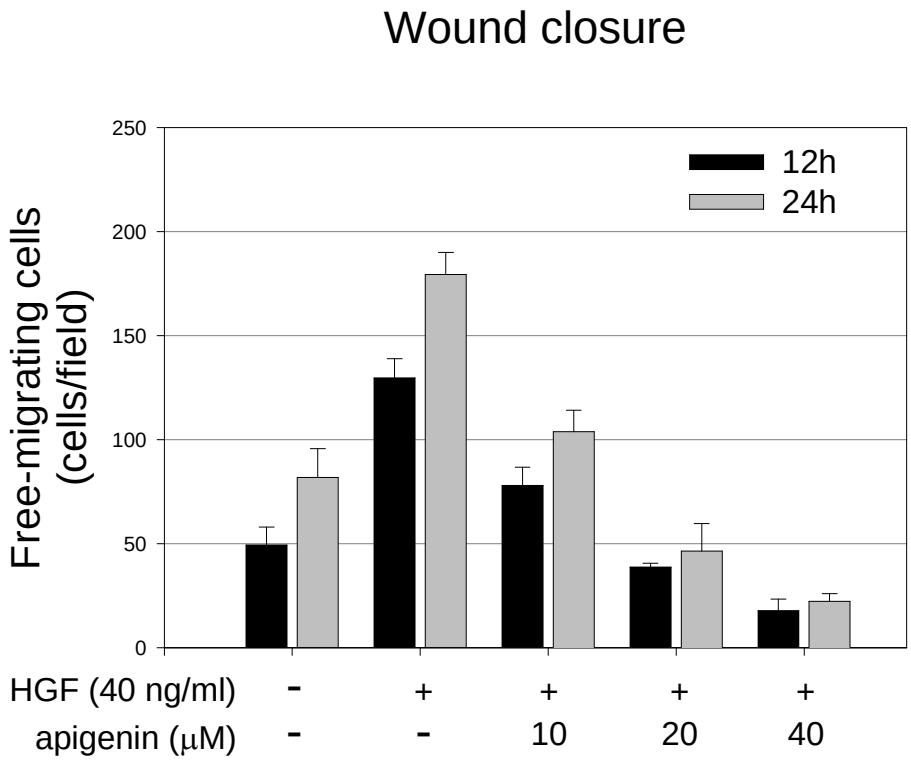


Fig. 8. Apigenin inhibits MDA-MB-231 cell *in vitro* wound healing migration. (A), cells were grown until subconfluent, serum-starved for 24h, wounded linearly with a pipette tip, and then grown in the serum-starved medium, without further treatment or with HGF/SF 40 ng alone, or HGF/SF combination with indicated concentrations of apigenin. Microphotographs of a similar randomly chosen field for each one of the wounded monolayers were taken when the wound was created (0h) as well as 12 and 24h after wounding the cells. (B), histogram representing the number of cells migrated into the wound was counted after wounding. The quantified result of the experiment were performed on 5 distinct fields along the wound at t = 12, and 24h.

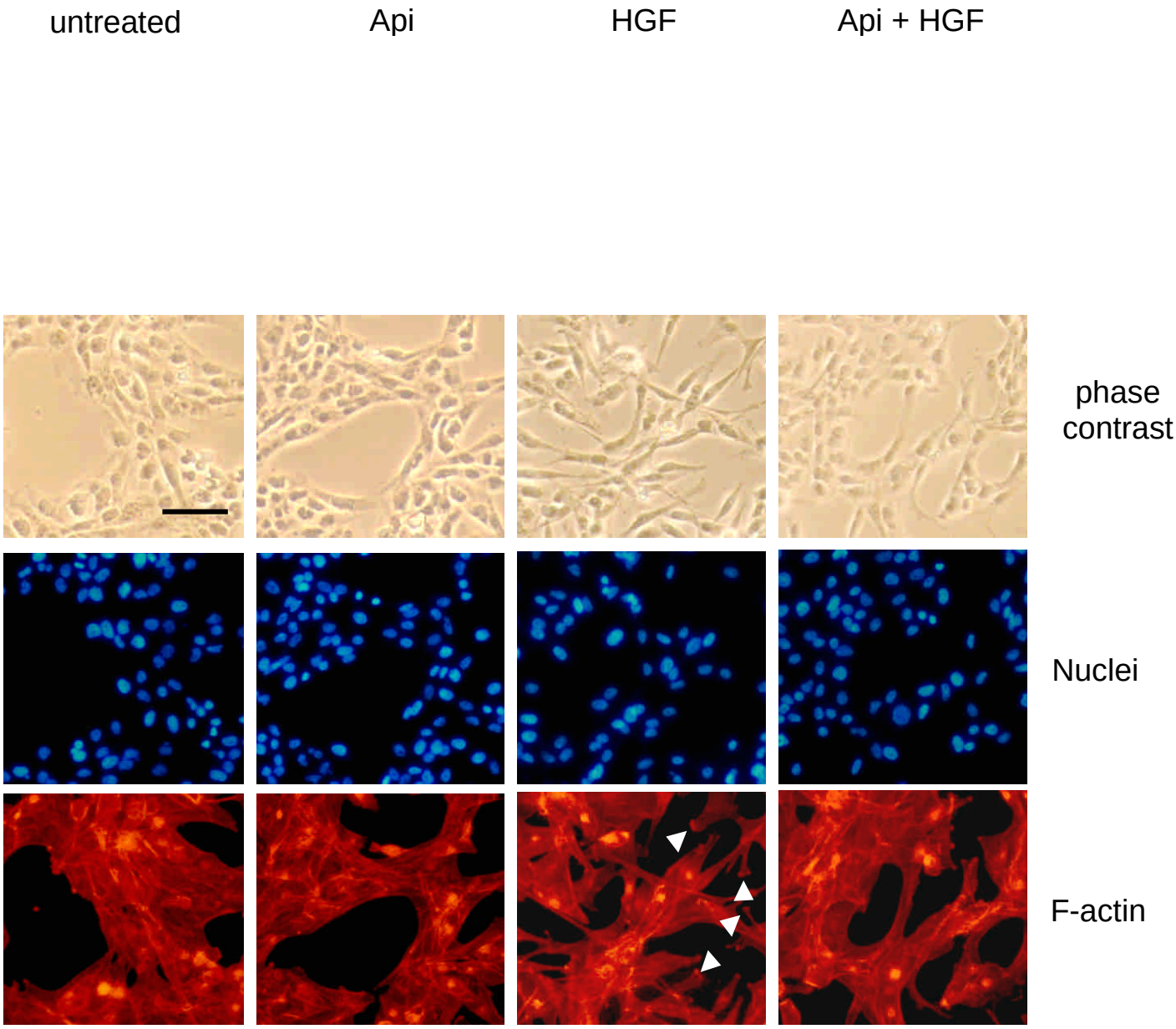
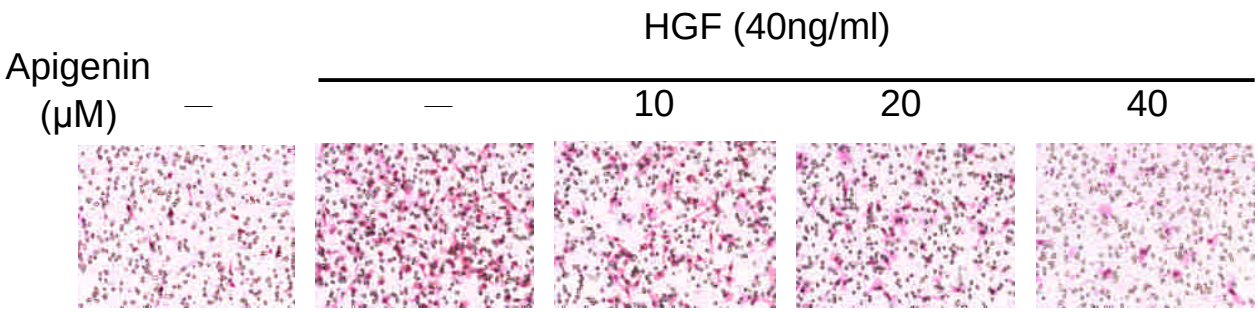


Fig. 9. Effect of apigenin on HGF/SF-induced of MDA-MB-231 cells scattering. For the scattering assay, MDA-MB-231 cells were seeded in 6-well plates at 500,000 cells/well in triplicate. After 24 h, and then grown in the serum-starved medium for another 24 h, without further treatment or with HGF/SF 40 ng/ml alone, or HGF/SF combination with 40 μM apigenin were incubated for 12 h. Representative micrographs of the variously treated MDA-MB-231 cells preparations are shown. The nuclei were stained with DAPI, which displayed *blue* fluorescence. The F-actin was stained with TRITC-conjugated phalloidin, with displayed *red* fluorescence.

A



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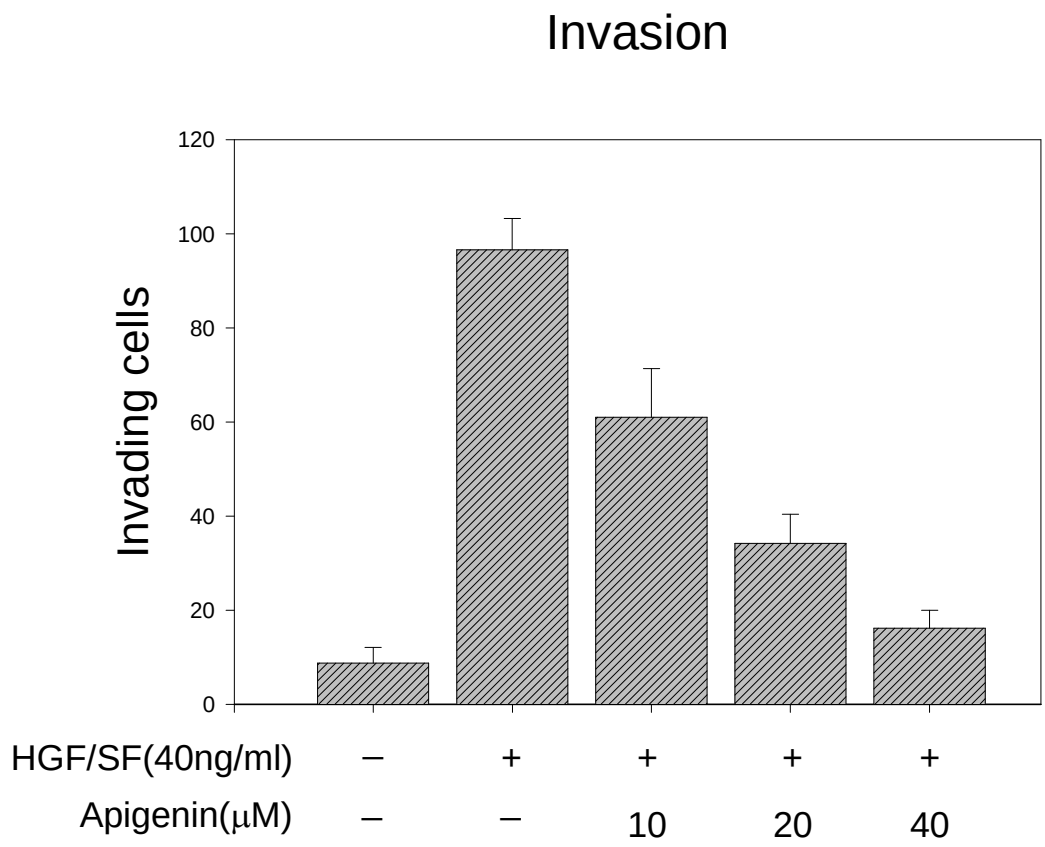
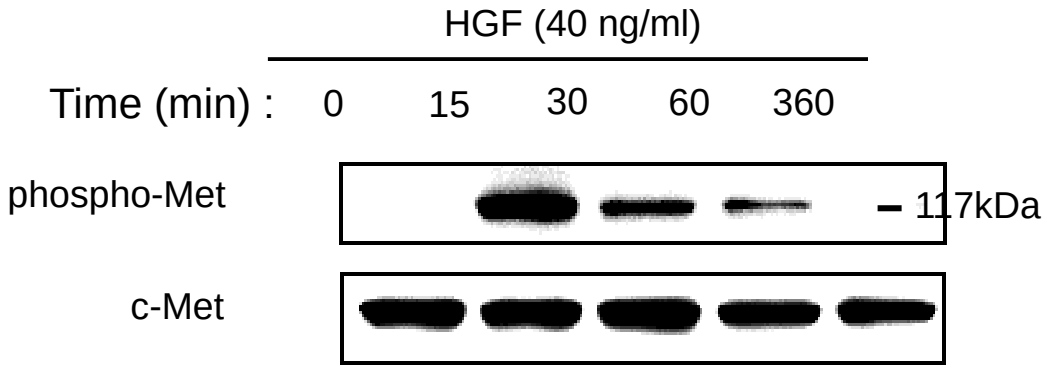


Figure 10. Apigenin inhibition of HGF/SF stimulates cell invasion. The invasion assay was performed using the Boyden Chamber assay coated with Matrigel, which mimics basement composition, and HGF/SF(40ng/ml) as a chemoattractive agent in the lower chamber. Cells (5×10^5/ml) were seeded onto transwell plates and then incubation with or without various concentration of apigenin for 12h. The lower surfaces of the membranes from the transwell units were fixed with 100% methanol and stained with Giemsa solution. (A), Photographs of MDA-MB-231 cells after invasion.(B), Cells that had invacted to the lower surface of the membranes were counted in triplicate wells and in three identical experiments under a light microscope.

A



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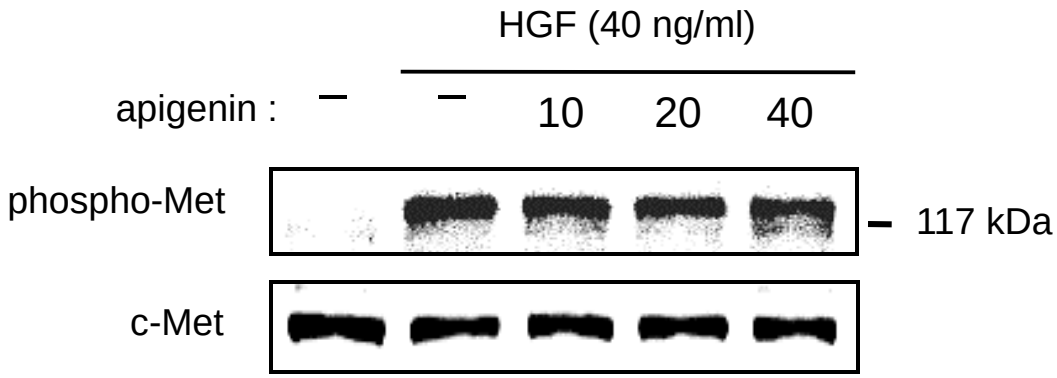


Fig. 11. Effect of apigenin on HGF-stimulated phosphorylation of Met, MAPKs and Akt. (A) Serum-starved MDA-MB-231 cells were stimulated with 40 ng/ml of HGF for the indicated time. The serum-starved cells were pretreated with the indicated concentration of apigenin for 2 h, and then stimulated with 40 ng/ml for 15 min. The cell lysates were subjected to Western blot analysis and probed with the phospho-Met and Met antibodies. (B) Serum-starved MDA-MB-231 cells were stimulated with 40 ng/ml of HGF for the indicated time. The cell lysates were subjected to Western blot analysis and probed with the indicated antibodies.

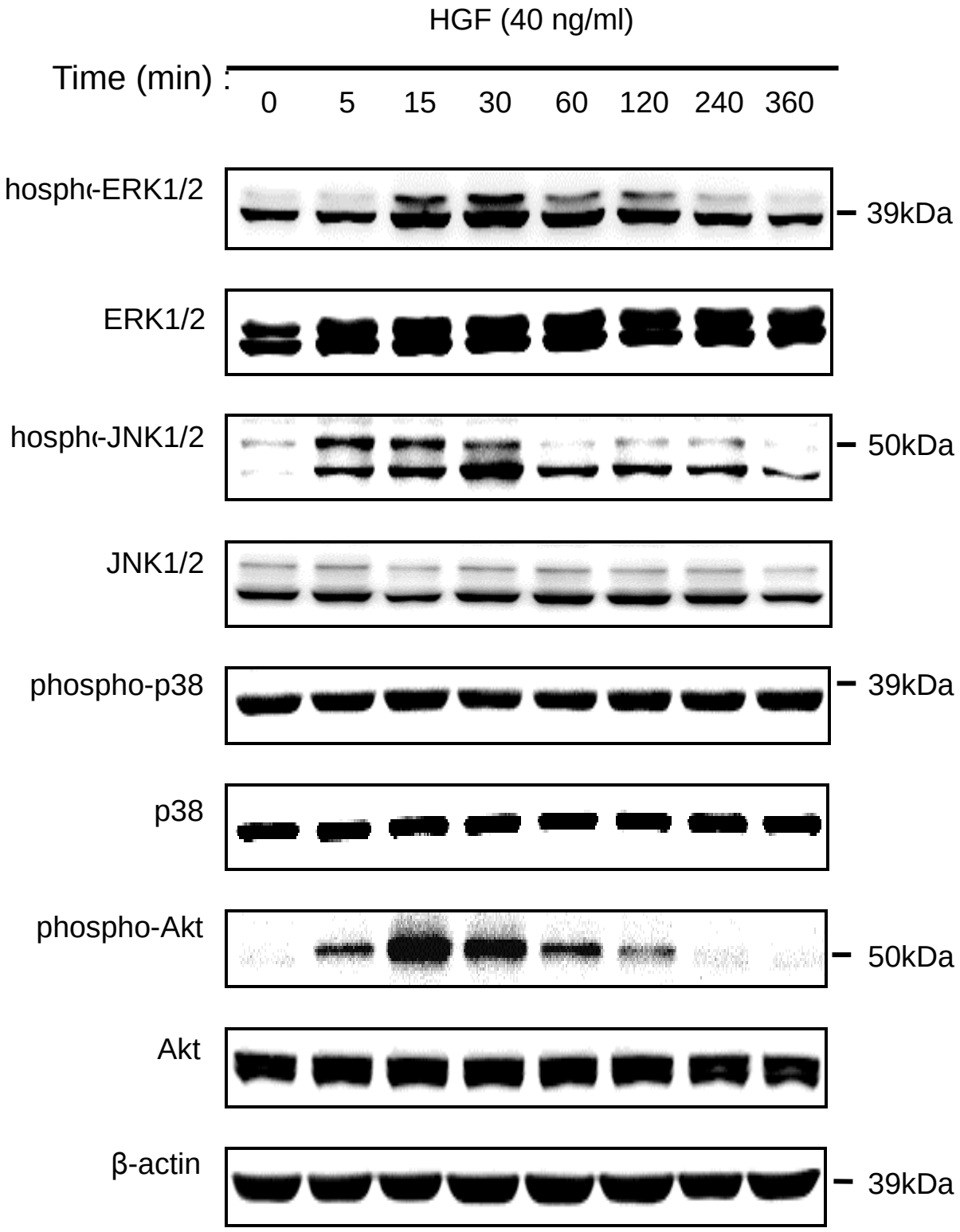


Fig. 12. The effect of HGF stimulated the activation of MAPK and Akt pathways. The serum-starved MDA-MB-231 cells were stimulated with 40 ng/mL of HGF for the indicated time. The cell lysates were subjected to Western blot analysis using either specific antibodies against the active phosphorylated forms of ERK, JNK/SAPK, p38 and Akt, or anti-ERK, JNK/SAPK, p38 and Akt antibodies, which react with both active and inactive forms and actin used for equal loading.

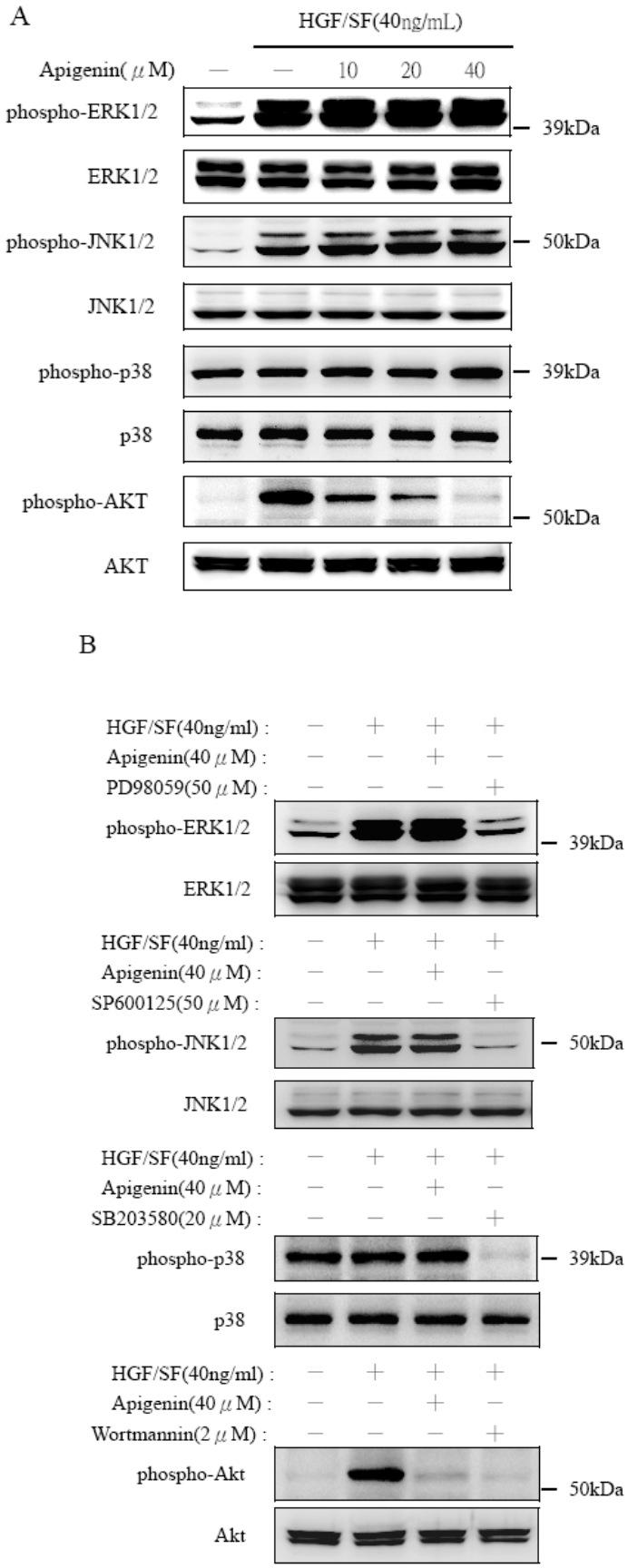


Fig 13. The effect of Apigenin in HGF/SF stimulated the activation of MAPK and PI3K/AKT pathway. (A) The serum-starved MDA-MB 231 cells were pretreated with the indicated concentration of apigenin (B) and specific MEK inhibitor PD98059

(50μM), JNK/SAPK inhibitor SP600125 (40μM), p38 inhibitor SB203580 (20μM) and Akt inhibitor wortmanin (2μM) for 1h, and then incubated in the absence or presence of 40ng/mL HGF for an additional 15 minutes. The cell lysates were subjected to Western blot analysis using either phosphospecific antibodies against phospho-ERK, JNK/SAPK, p38, and AKT, which react with active forms, and anti-ERK, JNK/SAPK, p38, and AKT antibodies, that recognized corresponding total non-phosphorylated enzymes were used as the loading control. The experiment was repeated three times with similar results.

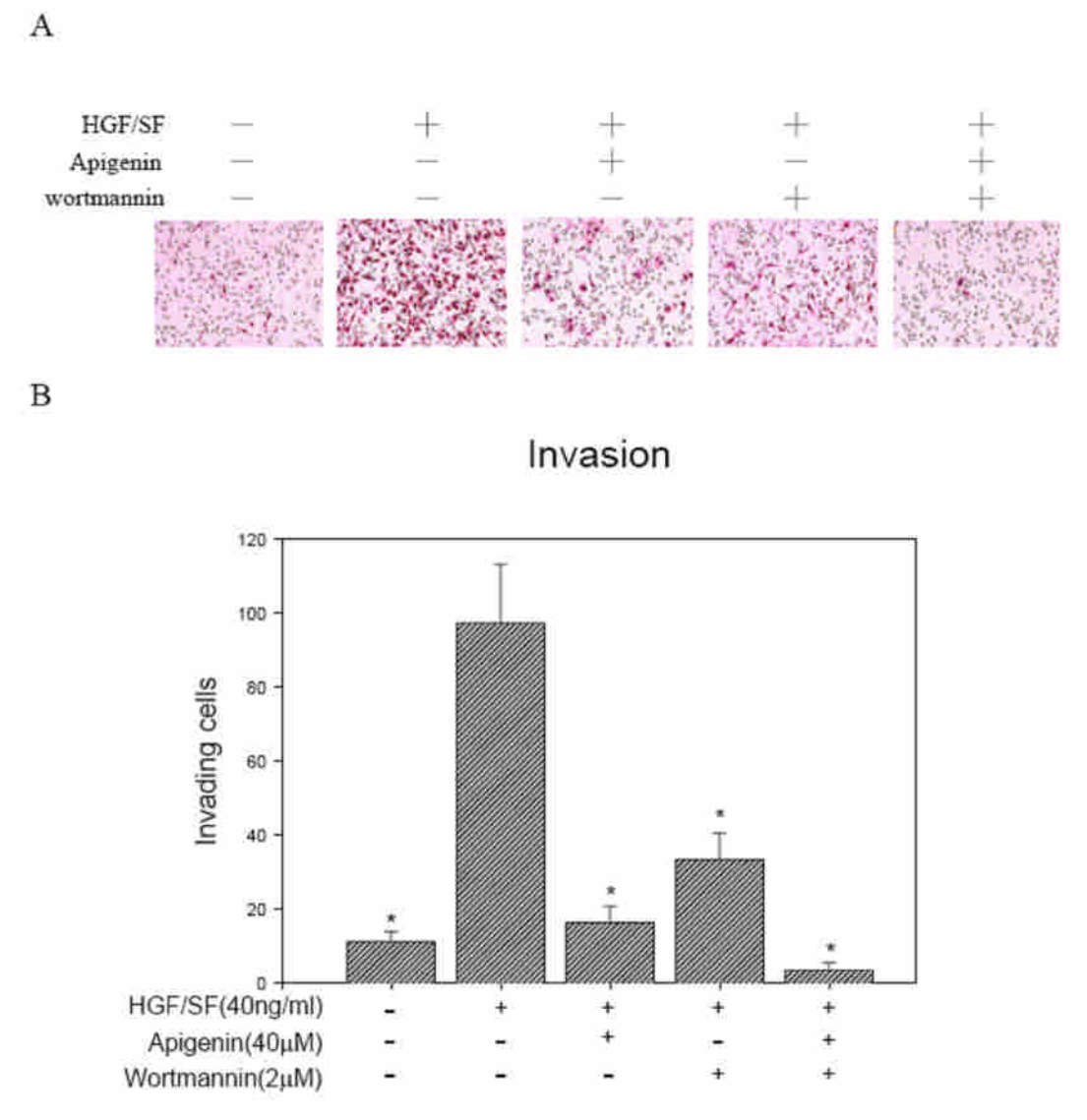


Figure 14. Effect of apigenin and PI3K inhibitors on HGF/SF stimulates cell invasion. The invasion assay was performed using the Boyden Chamber assay coated with 40μg/cm^2 Matrigel, which mimics basement composition, and 40 ng HGF/SF as a chemoattractive agent in the lower chamber. MDA-MB-231 cells (5×10^5/ml) were seeded onto transwell plates and then incubation with 40μM apigenin and 2μM wortmannin alone or combination for 12h. The lower surfaces of the membranes from the transwell units were fixed with 100% methanol and stained with Giemsa solution. (A), Photographs of MDA-MB-231 cells after invasion. (B), Cells that had invacted to the lower surface of the membranes were counted in triplicate wells and in three identical experiments under a light microscope, and the bars represent mean±S.D.*p<0.01, compared with the group treated with HGF alone.

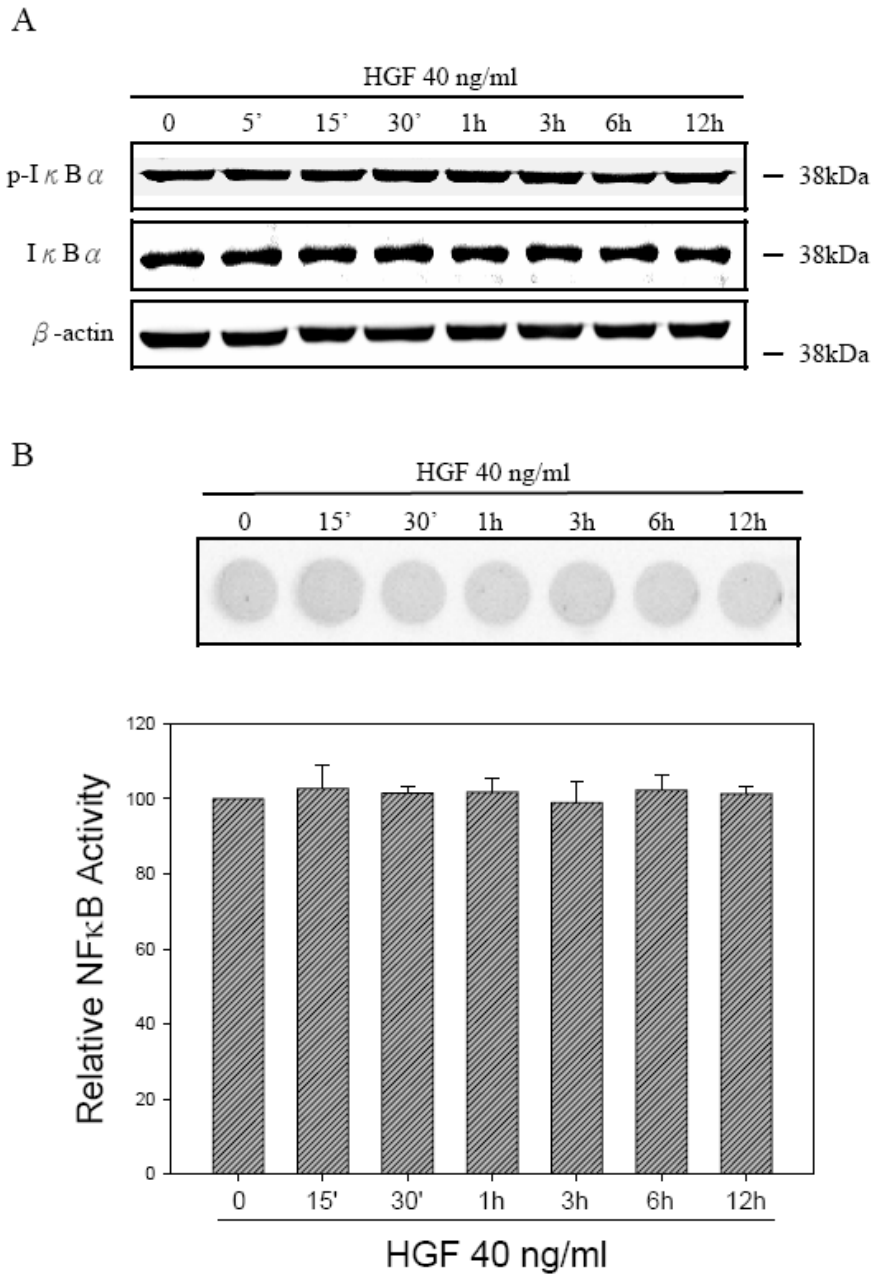
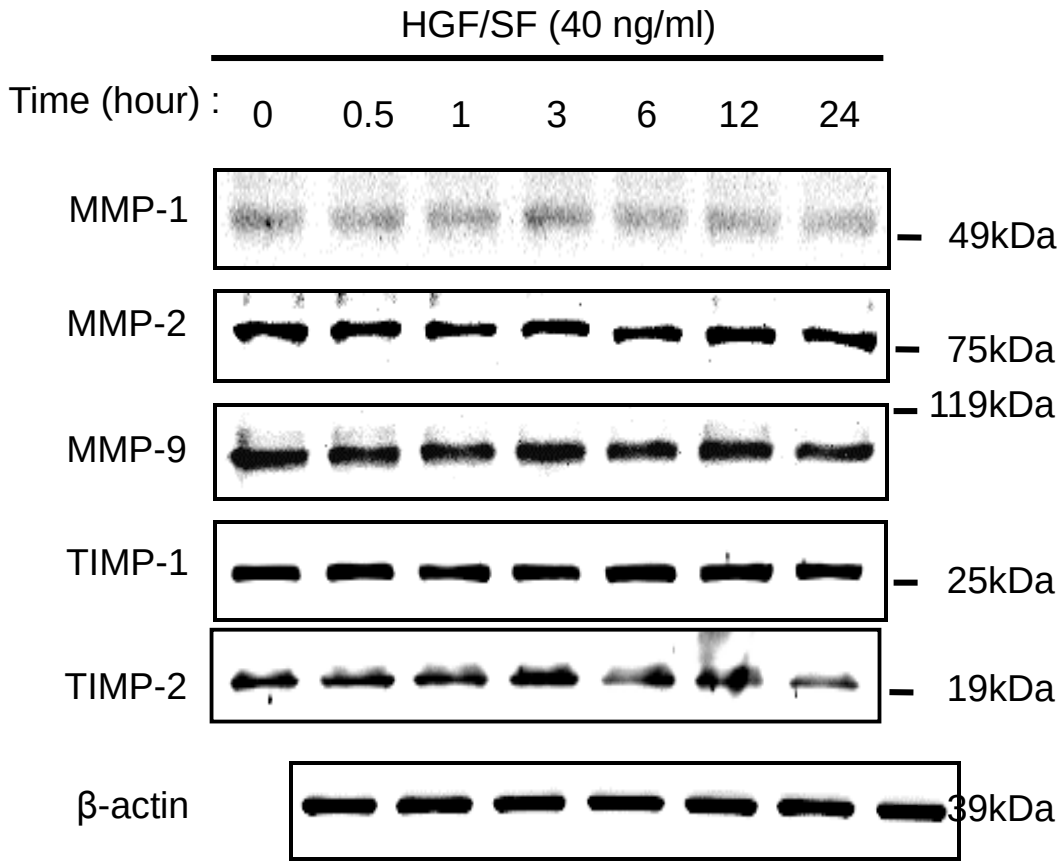


Fig. 15. The effect of HGF in the NFκB pathways. The serum-starved MDA-MB-231 cells were stimulated with 40 ng/mL of HGF for the indicated time. (A) The cell lysates were subjected to Western blot analysis using either specific antibodies against the active phosphorylated forms of c-jun, or anti-c-jun antibodies, which react with both active and inactive forms and actin used for equal loading. (B) NF-κB activity using biotin-labeled oligonucleotides containing an NFκB consensus site. The labeled DNA-protein complex was analyzed by ELISA-based DNA binding assays and detected by chemiluminescence.

A



B

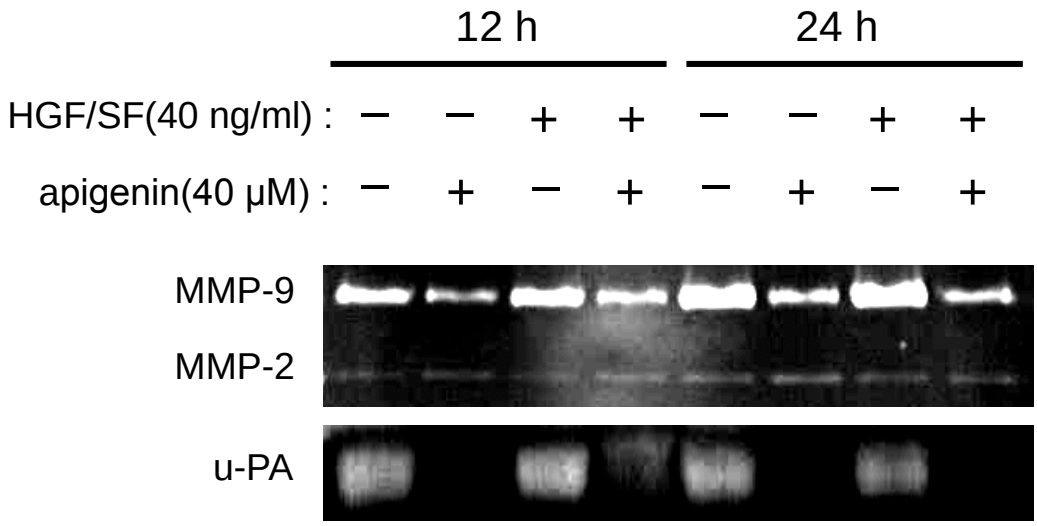


Fig. 16. Effects of HGF/SF on MMP-1, -2, -9, TIMP1 and TIMP2 and uPA expression in MDA-MB-231 cells. (A) Western blot of total extracts of MDA-MB-231 probed with antibodies to MMP-9 and MMP-2. (B) Zymograms of supernatants from MDA-MB-231 cells either unstimulated (Unst.) or treated with HGF/SF 40ng/ml for 24 h. Conditioned media were normalized to cell number prior to loading on a 8% SDS–polyacrylamide gel co-polymerized with 0.1% gelatin. Following electrophoresis, gelatin zymography was performed. uPA is detected in plasminogen-casein gels as a single band of ~55 kDa. Pro-MMP-9 is detected in gelatin gels as a single band of ~92 kDa. Pro-MMP-2 is detected in gelatin gels as a single band of ~82 kDa.

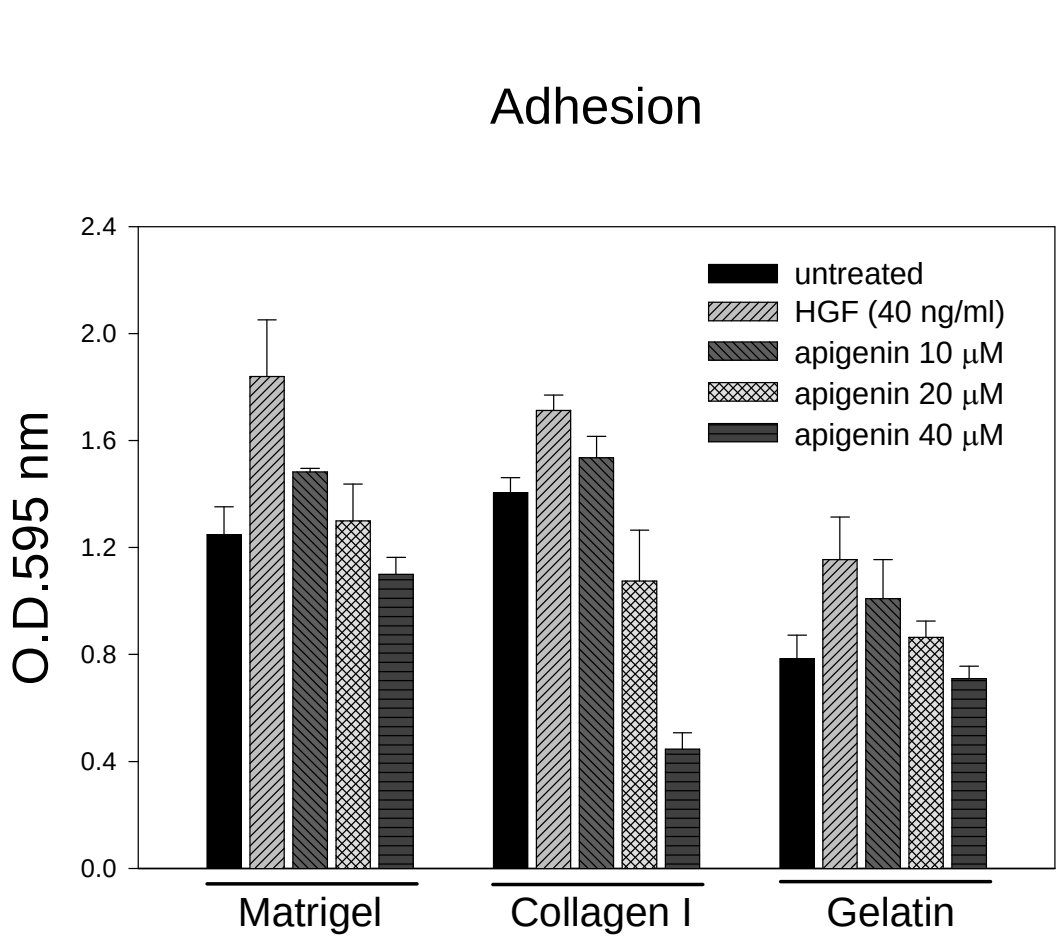


Fig. 17. Apigenin inhibit of MDA-MB-231 breast tumor cell adhesion to multiwell plates coated with matrigel, collagen I and gelatin. The serum-starved MDA-MB-231 cells were pretreated with the indicated concentration of apigenin for 1h, and then incubated in the absence or presence of 40 ng/ml HGF for an additional 2 h. The cells resuspension and plating onto 40 μg/ml of matrigel and collagen I or 2% gelatin-coated 96-well plates at a concentration of $2x10^5$ cells/ml. Histograms represent MDA-MB-231 cells attached 40 min, after the monolayers were fixed with 3.7% formaldehyde and washed extensively with PBS to remove floating tumor cells and attached tumor cells were stained with crystal violet. Ordinate represents absorbance reading of crystal violet released from adhering cells. Absorbance values of 595 were determined to directly correlate with the number of cells adhering to the multiwell plates. Error bars represent the standard deviation about the mean of three experimental values.

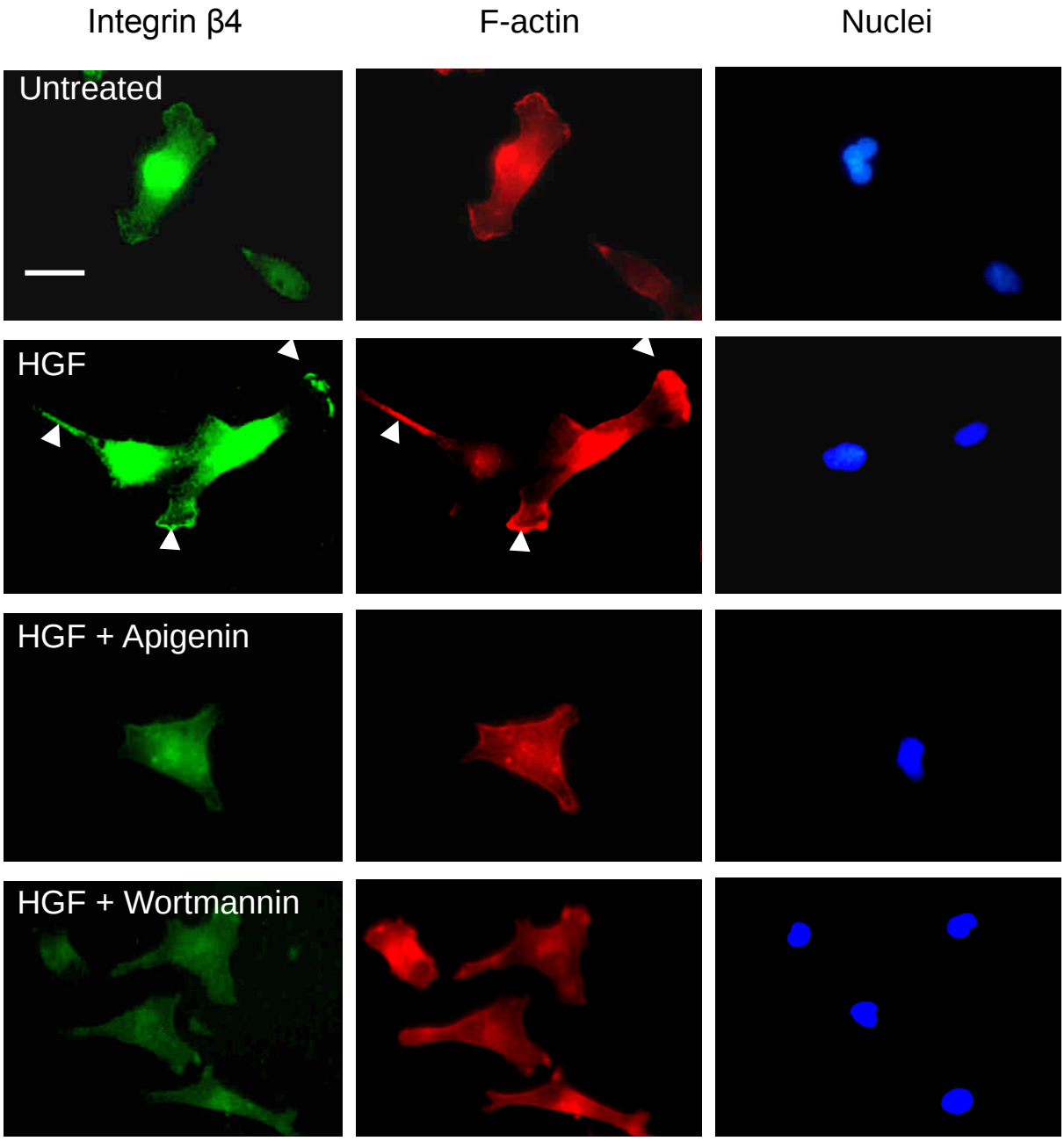


Fig. 18. Apigenin suppress PI3-K activity to prevent HGF -mediated β4 integrin clustering. MDA-MB-231 cells were seeded laminin-1-coated 24-well plates, without further treatment or with HGF 40 ng/ml alone, or pretreated with with 40 μM apigenin or 2 μM Wortmannin for 2 h then concomitant treatment with HGF for 30 min. Representative micrographs of the variously treated MDA-MB-231 cells preparations are shown. The β4 integrin displayed *green* fluorescence. The nuclei were stained with DAPI, which displayed *blue* fluorescence. The F-actin was stained with TRITC-conjugated phalloidin, with displayed *red* fluorescence. Arrowheads indicate integrin clusters (*green*) and actin rich area (*red*). Scale bar = 5 μm.

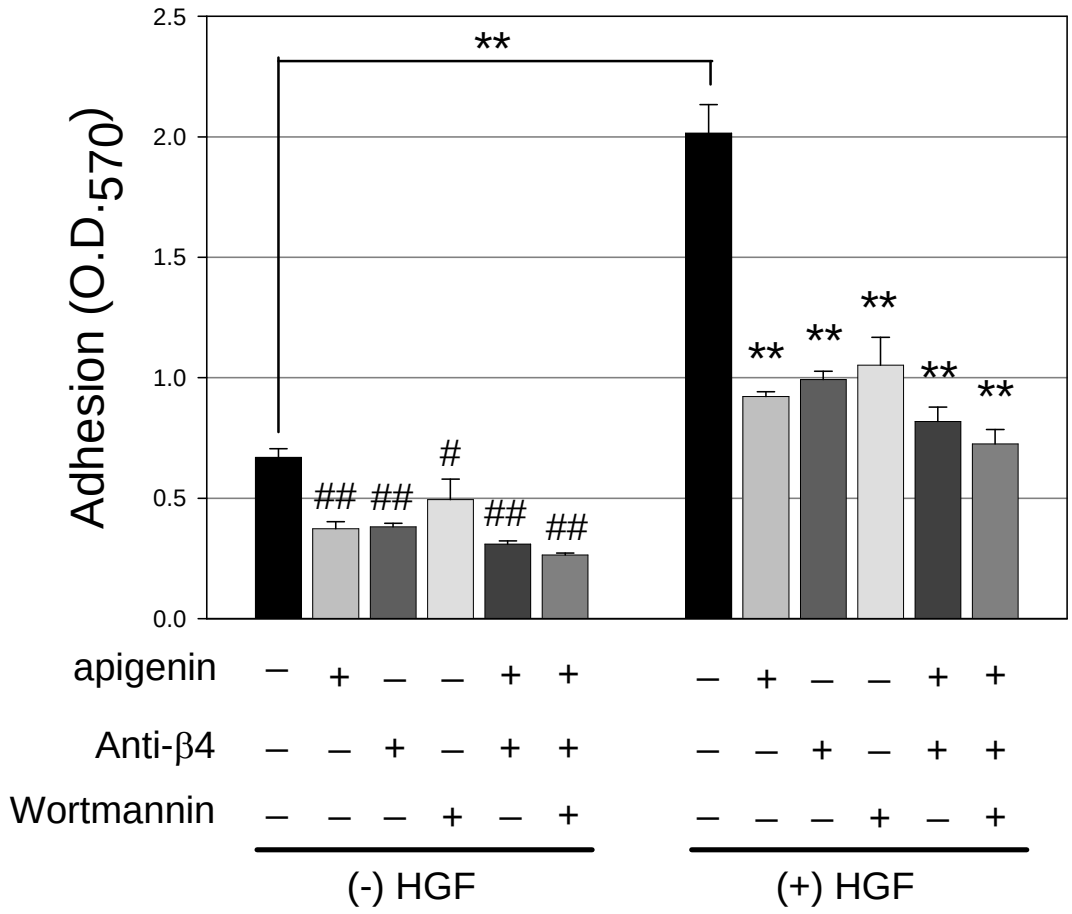


Fig. 19. Apigenin suppress PI3-K activity to prevent HGF -mediated β4 integrin clustering which reduced cell-matrix adhesion. Cell-matrix adhesion was quantified using ELISA plate reader. The pretreated cells were plate onto laminin-1-coated 96-well plates. Histograms represent attached tumor cells were stained with crystal violet. Ordinate represents absorbance reading of crystal violet released from adhering cells. Absorbance values of 570 were determined to directly correlate with the number of cells adhering to the multiwell plates. Data are mean ± SD. #$P < 0.05$; ##$P < 0.001$ *vs.* untreated cells, **$P < 0.001$ *vs.* cells treated with HGF alone.

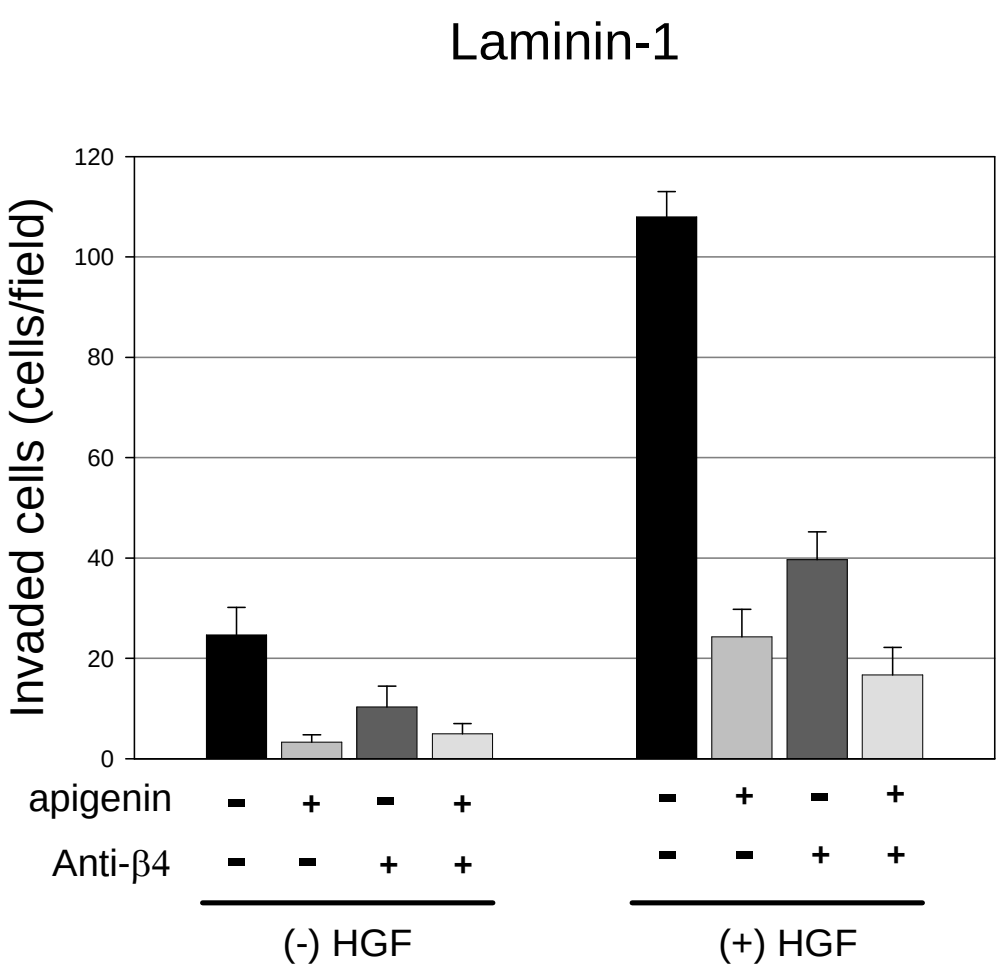


Fig. 20. Apigenin suppress PI3-K activity to prevent HGF -mediated β4 integrin clustering which reduced cell invasion. Boyden Chamber assay coated with 10μg/ml laminin-1, and 40 ng HGF as a chemoattractive agent. MDA-MB-231 cells were pretreated with indicated agents for 2 h. The lower surfaces of the membranes were counted in triplicate wells and in three indentical experiments. The bars represent mean ± S.D.

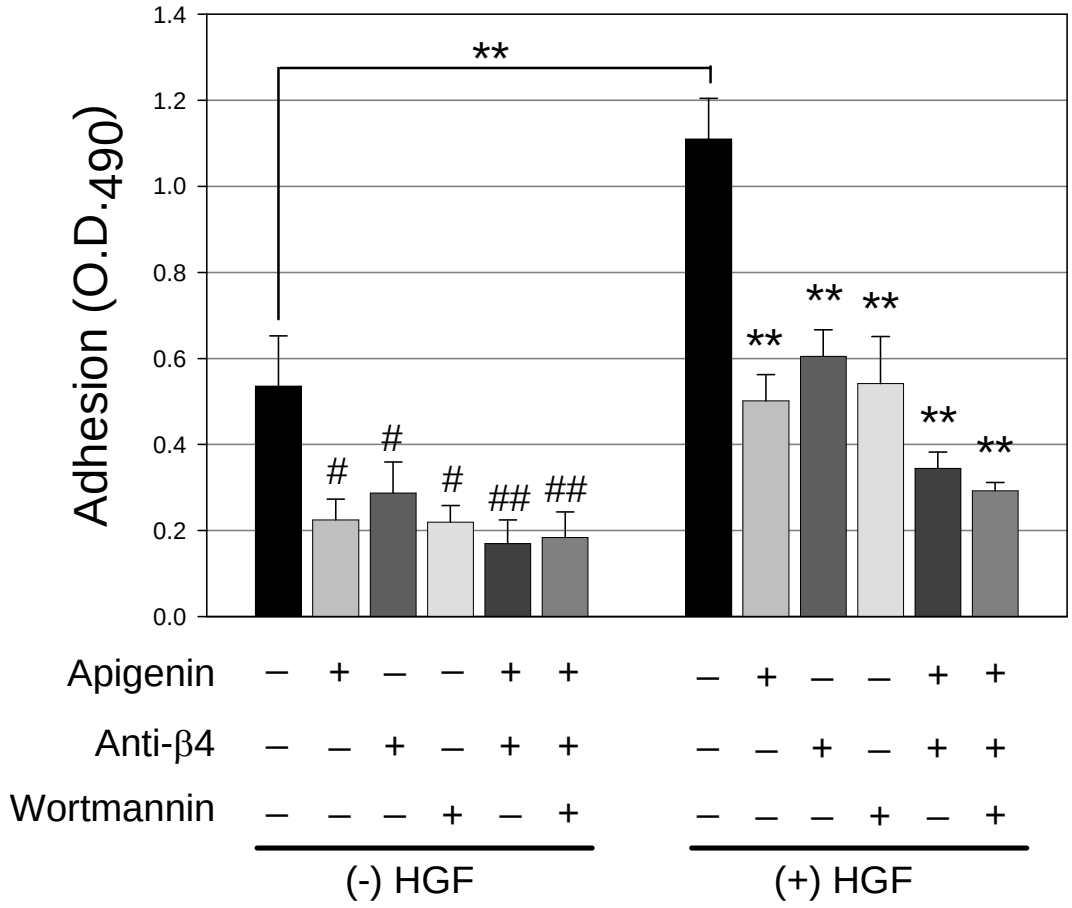


Fig. 21. Apigenin suppress PI3-K activity to prevent HGF -mediated β4 integrin clustering which reduced cell-cell adhesion. Serum-starved cells were pretreated in a manner similar to the cell-matrix adhesion assay. Cells were labeled and deposited onto HUVECs monolayer. Cell-to-cell adhesion was quantified using a fluorescent plate reader. Data are presented as mean raw fluorescent counts per well of the mean. Data are mean ± SD. #$P < 0.05$; ##$P < 0.01$ *vs.* untreated cells, **$P < 0.01$ *vs.* cells treated with HGF alone.

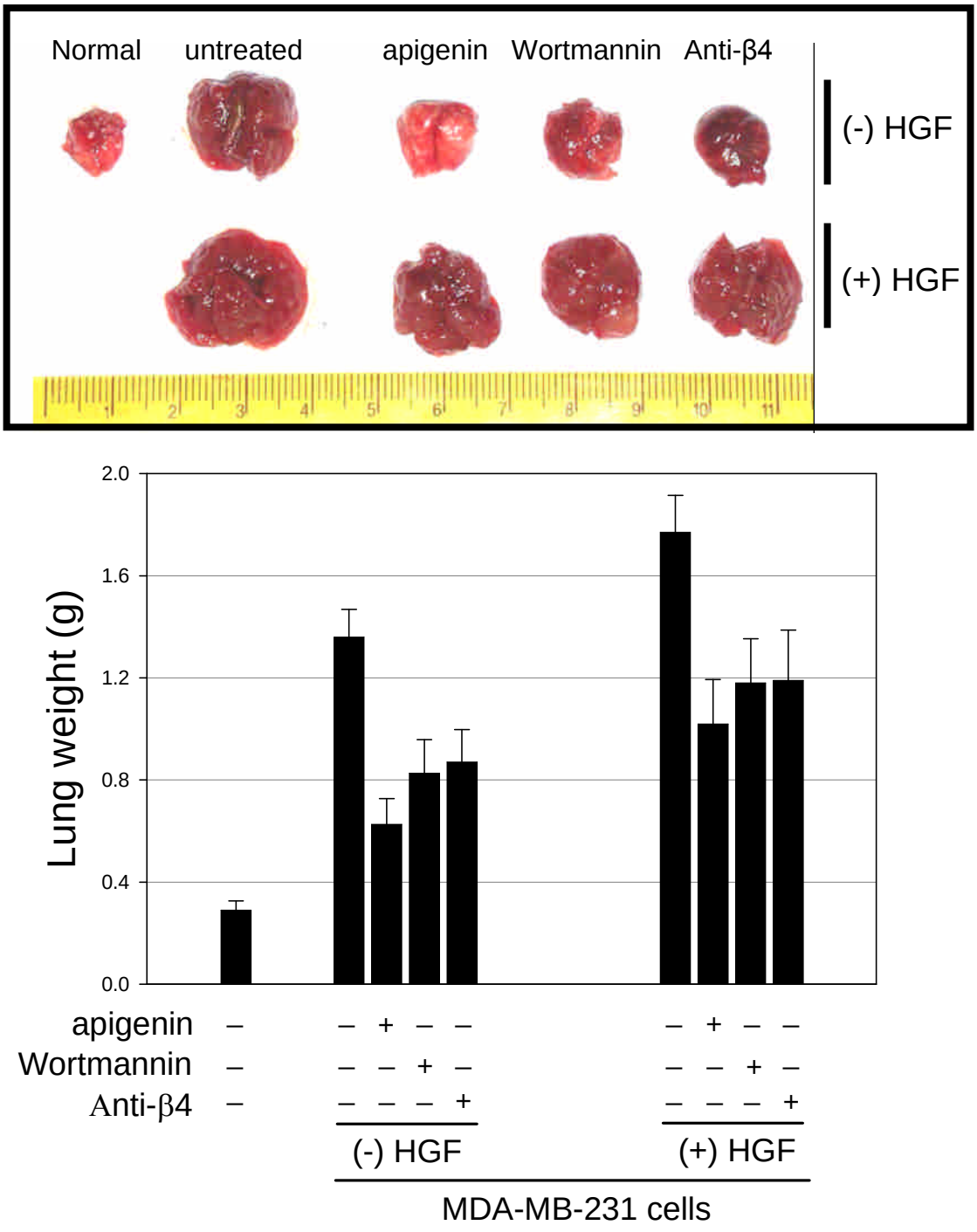


Fig. 22. Apigenin reduced HGF-induced breast cancer of lung colonization through suppression of PI3-K-mediated β4 integrin activity in nude mice. The serum-starved MDA-MB-231 cells were pretreated for 2 h with indicated agents were injected together with or without HGF into the lateral tail vein of 6-week-old, female nude mice. Total of six mice per test condition were injected. (A) Mice were sacrificed 4 weeks later. The gross view and weight of the lungs were measured.

自評

本計劃在實驗室同學一起努力下，已完成投稿並被接受,將發表於 Toxicology and Applied Pharmacology，本研究發現芹菜素具有抑制 HGF 所誘發乳癌細胞的侵犯性及轉移性, 芹菜素可由蔬果攝取到，因此其具有化學預防之作用，另外由結構的了解將有助於抑制轉移藥物之開發。而芹菜素對血管新生之影響仍繼續探討中。