RESEARCH ARTICLE

Glabridin, an isoflavan from licorice root, inhibits migration, invasion and angiogenesis of MDA-MB-231 human breast adenocarcinoma cells by inhibiting focal adhesion kinase/Rho signaling pathway

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Received: March 26, 2010
Revised: May 3, 2010
Accepted: May 25, 2010

Scope: In this study we first report the antimigration, antiinvasive effect of glabridin, a flavonoid obtained from licorice, in MDA-MB-231 human breast adenocarcinoma cells.
Methods and results: Glabridin exhibited effective inhibition of cell metastasis by decreasing cancer cell migration and invasion of MDA-MB-231 cells. In addition, glabridin also blocked human umbilical vein endothelial cells (HUVEC) migration and decreased MDA-MB-231-mediated angiogenesis. Further investigation revealed that the inhibition of cancer angiogenesis by glabridin was also evident in a nude mice model. Blockade of MDA-MB-231 cells and HUVEC migration was associated with an increase of $\alpha\gamma\beta3$ integrin proteosome degradation. Glabridin also decreased the active forms of FAK and Src, and enhanced levels of inactivated phosphorylated Src (Tyr 416), decreasing the interaction of FAK and Src. Inhibition of the FAK/Src complex by glabridin also blocked AKT and ERK1/2 activation, resulting in reduced activation of RhoA as well as myosin light chain phosphorylation.
Conclusion: This study demonstrates that glabridin may be a novel anticancer agent for the treatment of breast cancer in three different ways: inhibition of migration, invasion and angiogenesis.

Keywords:
Angiogenesis / Breast cancer / Glabridin / Invasion / Migration

1 Introduction

Breast cancer is one of the most common human malignancies and the second leading cause of cancer-related deaths in women, and its incidence in the developing world is on the rise [1]. About 30–40% of women with breast cancer will develop metastasis, which is defined as breast tumor cells' migration to and invasion of other organs, such as the lungs, liver and bones [2]. Metastasized breast cancer is particularly challenging because it is highly resistant to radiation and conventional chemotherapeutic agents [2]. Consequently, novel therapeutic agents are needed to deal with the increasing incidence of human breast cancer.

Focal adhesion kinase (FAK)/Src signaling has been demonstrated to play a role in various cellular processes,

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Abbreviations: EMT, epithelial-mesenchymal transition; **ERK,** extracellular signal-regulated kinase; **FAK,** focal adhesion kinase; **FBS,** fetal bovine serum; **HUVEC,** human umbilical vein endothelial cell; **MLC,** myosin light chain

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including the immune function and cell differentiation, survival and motility [3, 4]. Abnormal activation of FAK/Src signaling has been seen in invasive tumors, including breast cancers [5]. Clustering of integrin by extracellular matrix ligand can initiate intracellular signaling events, leading to changes of the intercellular parts of receptors and subsequent recruitment and autophosphorylation of FAK, followed by Src docking and phosphorylation [6, 7]. Src is activated by phosphorylation at Tyr 416 and inhibited by phosphorylation at Tyr 527. Phosphorylated FAK/Src can activate several signaling cascades, such as extracellular signal-regulated kinases (ERKs) or Akt, resulting in the promotion of cell motility [8].

The root of *Glycyrrhiza glabra* (licorice) has been used for many centuries in Asia and Europe as an antioxidant, antidote, demulcent, expectorant and a remedy for allergic inflammation, as well as a flavoring and sweetening agent [9]. Glabridin, an active isoflavan in the hydrophobic fraction of licorice root [10, 11], is easily incorporated into the gut cells and released to the basolateral side by aglycone form in human and mice [12, 13]. It has shown multiple biological activities, such as antibacterial, neuroprotective, antiatherosclerotic, anti-osteoporosis and immunomodulatory activities [10–18]. Studies have shown that glabridin exhibits growth inhibition properties against many human cancers [18]. It also enhances the efficacy of cancer chemotherapy by inhibiting P-glycoprotein and multidrug resistance protein 1 synthesis [19]. However, the precise antitumorigenic mechanisms of glabridin in cancer migration and invasion still remain largely unknown.

2 Materials and methods

2.1 Test compound

Glabridin was obtained from Wako Pure Chemical Industries (Osaka, Japan), dissolved in DMSO and stored at $-20^{\circ}C$. The purity was >97%, as assessed by HPLC. Control cultures received the carrier solvent (0.1% DMSO).

2.2 Cell culture and cell proliferation assay

MDA-MB-231 human breast adenocarcinoma cells (ATCC HTB-26) were cultured in MEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 0.1 mg/mL streptomycin, and 100 units/mL penicillin (Life Technologies). Human umbilical vein endothelial cell (HUVEC) culture was grown in EGM-2 medium (Lonza, Switzerland) at 37°C in an atmosphere of 5% CO_2. To obtain the conditioned medium, cells were seeded 2×10^6 cells/100 mm dish. The following day, the medium was replaced and the supernatants harvested after 24 h of incubation. Cell proliferation was assessed by Premixed WST-1 Cell Proliferation Reagent (Clontech Laboratories, Mountain View, CA, USA) according to the manufacturer's instructions.

2.3 Cell migration and invasion assay

Cell migration and invasion assay were conducted using QCMTM 24-well Cell Migration Assay and Invasion System as described previously [6, 7]. Briefly, 3×10^4 cells were seeded into the top chamber and treated with different concentrations of glabridin. In total, 10% FBS was added to the bottom wells for 24 h as chemoattractant. At the end of the treatment, cells were post-stained with CyQuant GR dye in cell lysis buffer for 15 min at room temperature. Then, fluorescence of the invaded cells was read using a fluorescence plate reader at excitation/emission wavelengths of 485/530 nm.

2.4 Scratch wound-healing assay

MDA-MB-231 cells and HUVEC culture were allowed to grow into full confluence in 24-well plates. The following day, a uniform scratch was made down the center of the well using a micropipette tip, followed by washing once with PBS. Vehicle control and various concentrations of glabridin were added to the respective wells for the indicated times. Photographic imaging was performed using the Olympus 1×50 inverted microscope.

2.5 Tube formation assay

In vitro angiogenesis assay was performed using BD BioCoatTM Angiogenesis System according to the manufacturer's instructions. Briefly, HUVEC (2×10^4 cells/mL) was seeded onto the Matrigel-precoated well present with or without glabridin in MDA-MB-231 condition medium. Tube formation was assessed after 18 h and photographic imaging was performed using a fluorescent microscope (Nikon Eclipse TE 300, Germany).

2.6 MMP-9, MMP-2 and VEGF assays

The culture medium for these assays was grown in 6-well plates and treated with various concentrations of glabridin for 12 h. After collection, the medium was centrifuged at $800 \times g$ for 3 min at 4°C to remove cell debris. The supernatant was immediately assayed using commercially available MMP-9 and VEGF ELISA kits (R&D Systems, Minneapolis, MN, USA).

2.7 Immunoblot/immunoprecipitation

Cells (8×10^6/dish) were seeded in a 10 cm dish. After 24 h of incubation, the cells were treated with various concentrations of glabridin for the indicated times. Total cell extracts were prepared in lysis buffer (50 mM Tris-HCl,

150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 5 μg/mL Aprotinin and 5 μg/mL Leupetin). Equivalent amounts of protein were resolved by SDS-PAGE and transferred to PVDF membranes. After the membrane was blocked in Tris-buffer saline containing 0.05% Tween-20 and 5% nonfat powdered milk, the membranes were incubated with primary antibodies at 4°C for 1–16 h. After washing three times with Tris-buffer saline containing 0.05% Tween-20 for 10 min each, the membranes were incubated with horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed again, and detection performed using an enhanced chemiluminescence blotting detection system (Amersham, USA).

For immunoprecipitation, cell lysates (200 μg of total protein) were incubated with 2 μg of anti-FAK overnight, then 20 μL of protein A-agarose beads (Millipore, Bedford, MA, USA) for 2 h at 4°C. Association of FAK with Src was detected by incubating the blots with anti-Src antibodies (Cell Signaling).

2.8 Rho activity assay

Rho activity was analyzed by a Rho Activation Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA) according to the protocol supplied by the manufacturer. Cells were lysed in 500 μL of $1 \times Mg^{2+}$ lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA and 2% glycerol) and centrifuged at 12 000 rpm for 15 min at 4°C. Active (GTP/GTPγS-bound) Rho was isolated by pull-down assay: 50 μL (30 μg) of rhotekin Rho binding domain agarose bead slurry (50 mM Tris, pH 7.5, 0.5% Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM PMSF and 1 μg/mL each of aprotinin and leupeptin) were added to each sample containing 500 μg total protein, and incubated for 45 min at 4°C with gentle agitation. The rhotekin-Rho binding domain agarose bead–Rho complex was then pulled down by centrifugation (10 s, 14 000 × *g*, 4°C). The supernatant was removed, and the beads were washed three times with 0.5 mL lysis buffer. Samples were subjected to immunoblot assay performed with anti-Rho antibody.

2.9 Matrigel plug angiogenesis assay

Female nude mice (6 wk old; BALB/cA-nu (nu/nu)) were purchased from the National Science Council Animal Center (Taipei, Taiwan) and maintained in pathogen-free conditions. Ten mice were randomly divided into two groups. MDA-MB-231 cells were trypsinized and resuspended at 3×10^7 cells/mL in serum-free medium. Aliquots of cells (3×10^6 cells) were mixed with 0.4 mL of phenol red-free Matrigel (BD Biosciences) and injected into both flanks of each nude mouse. For the glabridin-treated group, 10 μM glabridin was added to the cell suspensions, whereas Matrigel mixed with the medium alone was used as a negative control. Matrigel plugs were removed 15 days after implantation, weighed and was determined content hemoglobin using a Drabkin's reagent kit containing sodium bicarbonate, potassium ferricyanide and potassium cyanide. Hemoglobin level measurements indicated blood vessel formation.

2.10 Statistical analyses

Data were expressed as means ± SD of three determinations. Statistical comparisons of the results were made using analysis of variance. Significant differences ($p < 0.05$) between the means of the two test groups were analyzed by Dunnett's test.

3 Results

3.1 Glabridin inhibits migration, invasion and epithelial-mesenchymal transition, but does not affect the proliferation of MDA-MB-231 cells

We first assessed the effect of glabridin on the viability of MDA-MB-231 cells. As shown in Fig. 1A, glabridin did not affect the cell viability of MDA-MB-231 cells at concentrations ranging from 1 to 10 μM.

To examine the effect of glabridin on human breast cancer cell migration, we employed transwell migration and wound-healing assay to characterize the cells' migration response to glabridin. As shown in Fig. 1B, culture medium increased the migration of MDA-MB-231 cells after 24 h incubation, whereas glabridin treatment decreased the migration of MDA-MB-231 cells in a dose-dependent manner. Furthermore, scratch wound-healing assay also showed that glabridin decreased MDA-MB-231 cells' migration ability after treatment for 24 h (Fig. 1C).

Next, we also assessed the effect of glabridin on breast cancer cell invasion. Compared with vehicle-treated cells, culture medium increased the invasion capability of MDA-MB-231 cells. However, glabridin treatment attenuated cell invasion in a dose-dependent manner (Fig. 1D).

Epithelial-mesenchymal transition (EMT) is a crucial progression in the development of invasive cancer cells [20]. We assessed the effect of glabridin on EMT markers. Glabridin treatment increased E-cadherin levels and decreased vimentin and *N*-cadherin (Fig. 1E).

3.2 Glabridin inhibits angiogenesis *in vitro* and *in vivo*

Because angiogenesis has been proven to be involved in cancer invasion and metastasis [20], we also tested whether

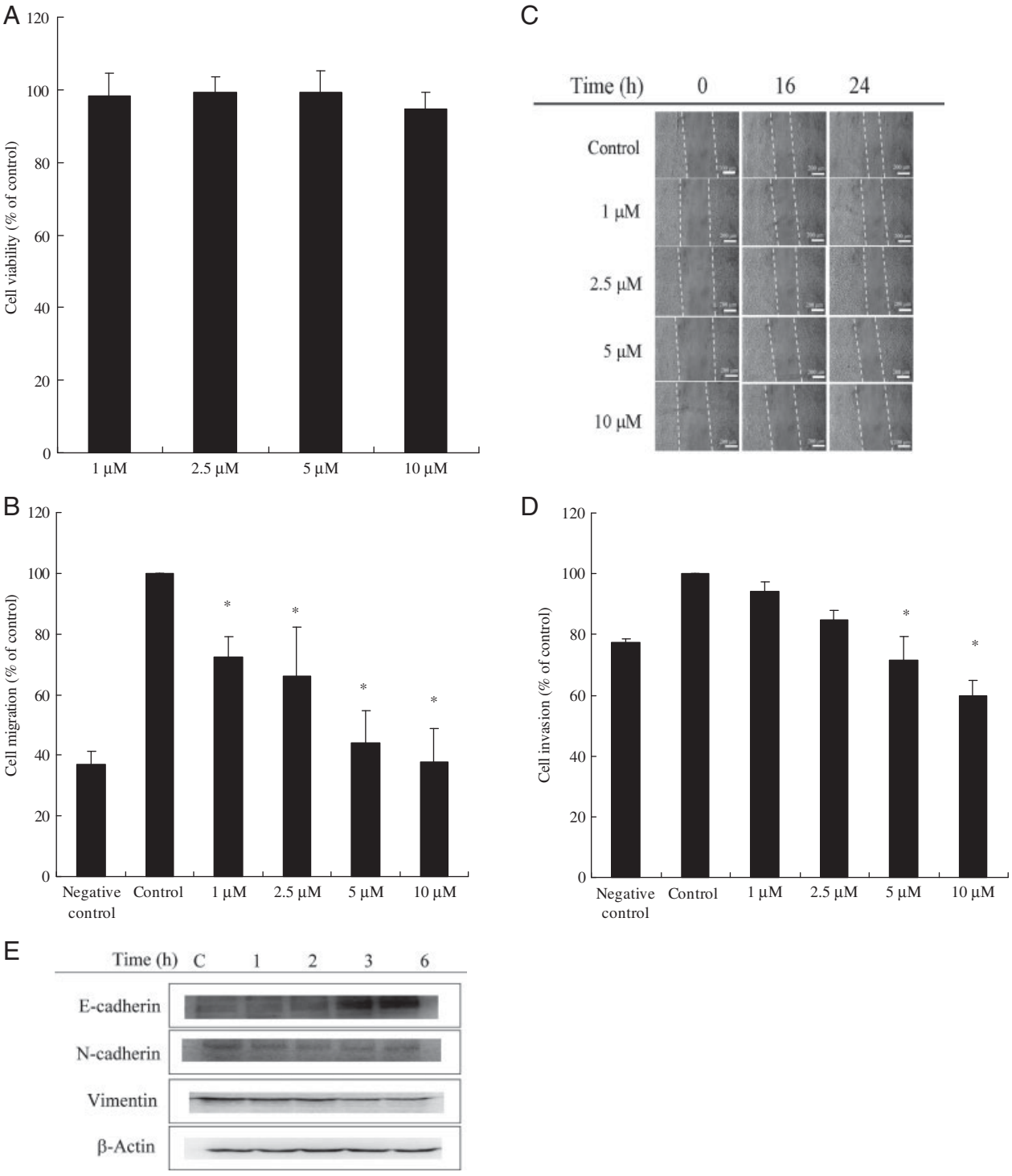

Figure 1. Glabridin inhibits breast cancer cell migration and invasion. (A) Glabridin did not affect proliferation of MDA-MB-231 cells. Glabridin attenuated the migration ability of MDA-MB-231 cells, as determined by transwell (B) and scratch wound-healing assay (C). (D) Glabridin reduced the invasiveness of MDA-MB-231 cells. (E) Glabridin (10 μM) decreased the EMT of MDA-MB-231 cells. Cell proliferation was assessed by WST-1 after 48 h treatment. The invasiveness and migration ability of MDA-MB-231 cells were quantified by BD BioCoat tumor invasion system and QCMTM 24-well Cell Migration Assay, in accord with the description in Section 2. In total, 10% FBS acts as a chemoattractant of cancer migration and invasion. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between the two test groups, as analyzed by Dunnett's test ($p < 0.05$).

glabridin inhibits angiogenesis in *in vitro* and *in vivo* models. As shown in Fig. 2A, the condition medium of MDA-MB-231 cells caused the formation of the capillary-like HUVEC structures, but this phenomenon was blocked by glabridin treatment.

Next, we assessed the effect of glabridin on cancer angiogenesis by an *in vivo* model. MDA-MB-231 cells were mixed with Matrigel and injected into the flank of nude mice. Tissue sections showed that glabridin decreased the formation of functional blood vessels, characterized as red blood cell-containing capillary structures within the Matrigel plug (Fig. 2B). In addition, relative angiogenesis was assayed by the hemoglobin content of the Matrigel plug. Compared with the Matrigel mixed with medium alone, angiogenesis of MDA-MB-231 cells was greatly increased, and hemoglobin levels in the Matrigel plugs containing MDA-MB-231 cells were 1.5-fold higher than in Matrigel alone. Glabridin treatment inhibited MDA-MB-231 cell-induced angiogenesis, and hemoglobin levels in the glabridin-treated plugs were significantly lower than those of the solvent-treated plugs (Fig. 2C). These results suggest that glabridin treatment decreases angiogenesis of MDA-MB-231 cells in an *in vivo* model.

3.3 Glabridin blocks angiogenesis by direct inhibition of HUVEC

Inhibition of angiogenesis can be achieved by indirectly influencing tumor cells to produce angiogenic factor, or by directly blocking vessel endothelial cell migration [21]. We therefore assessed the effect of glabridin on the proliferation and migration of HUVEC. As shown in Fig. 3A, glabridin failed to affect the proliferation of HUVEC after 48 h treatment. However, transwell assay data showed that glabridin did decrease HUVEC migration (Fig. 3B). Similarly, scratch wound-healing results also revealed that glabridin treatment reduced cell migration to the wounded area (Fig. 3C).

Next, we assessed whether glabridin inhibits cancer's ability to secrete angiogenic factors, which play a key role in tumor-derived angiogenesis. However, glabridin did not

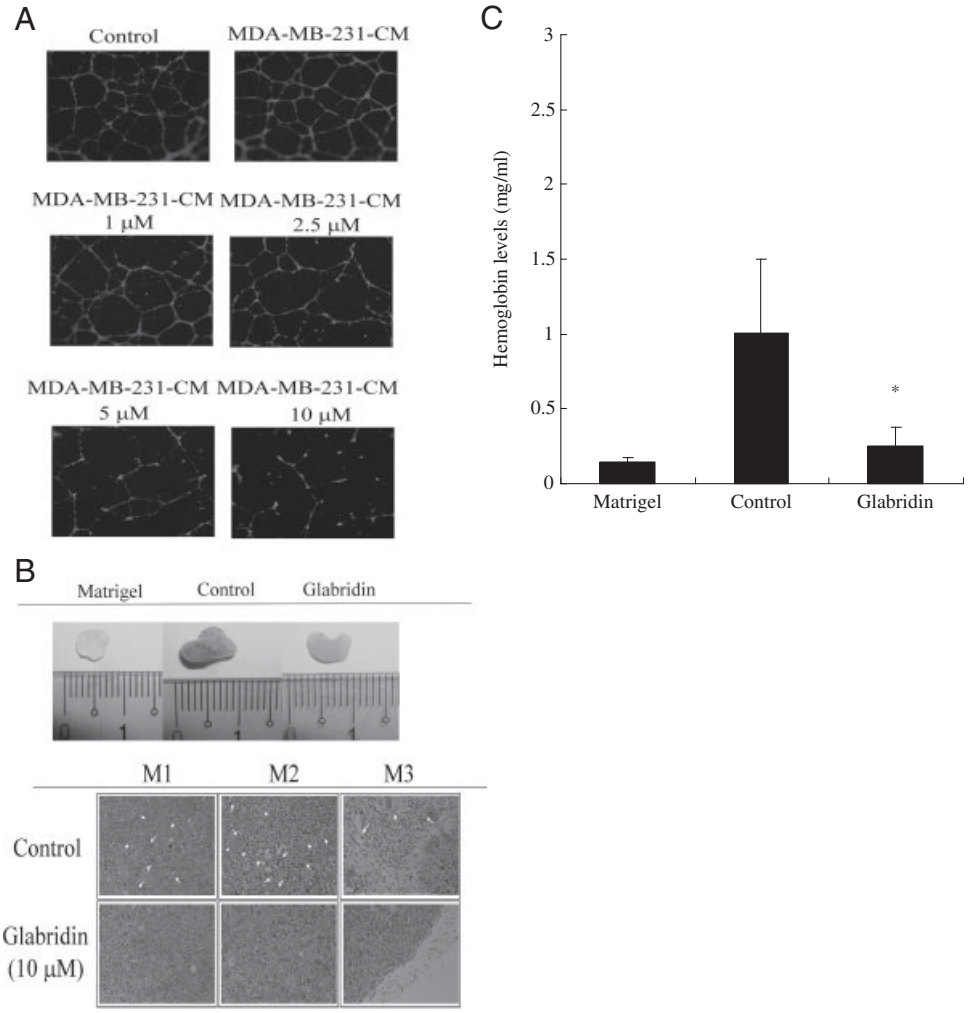

Figure 2. Glabridin inhibits angiogenesis *in vitro* and *in vivo*. (A) Glabridin inhibited the growth of capillary-like structures of HUVEC. (B) Glabridin inhibited angiogenesis *in vivo*. (C) The hemoglobin level of Matrigel plug. HUVEC was seeded into BioCoated angiogenesis 96-well plate and treated with various concentrations of glabridin presented with or without MDA-MB-231 CM for 18 h. HUVEC was stained by Claein-AM and photographic imaging was performed using a fluorescent microscope. For the *in vivo* study, MDA-MB-231 cells were mixed with Matrigel, injected into the flanks of nude mice, and allowed to develop for 15 days. The degree of angiogenesis was determined by the levels of RBC hemoglobin in the Matrigel plugs. Hemoglobin levels were determined by the Drabkin method. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between two test groups, as analyzed by Dunnett's test ($p < 0.05$).

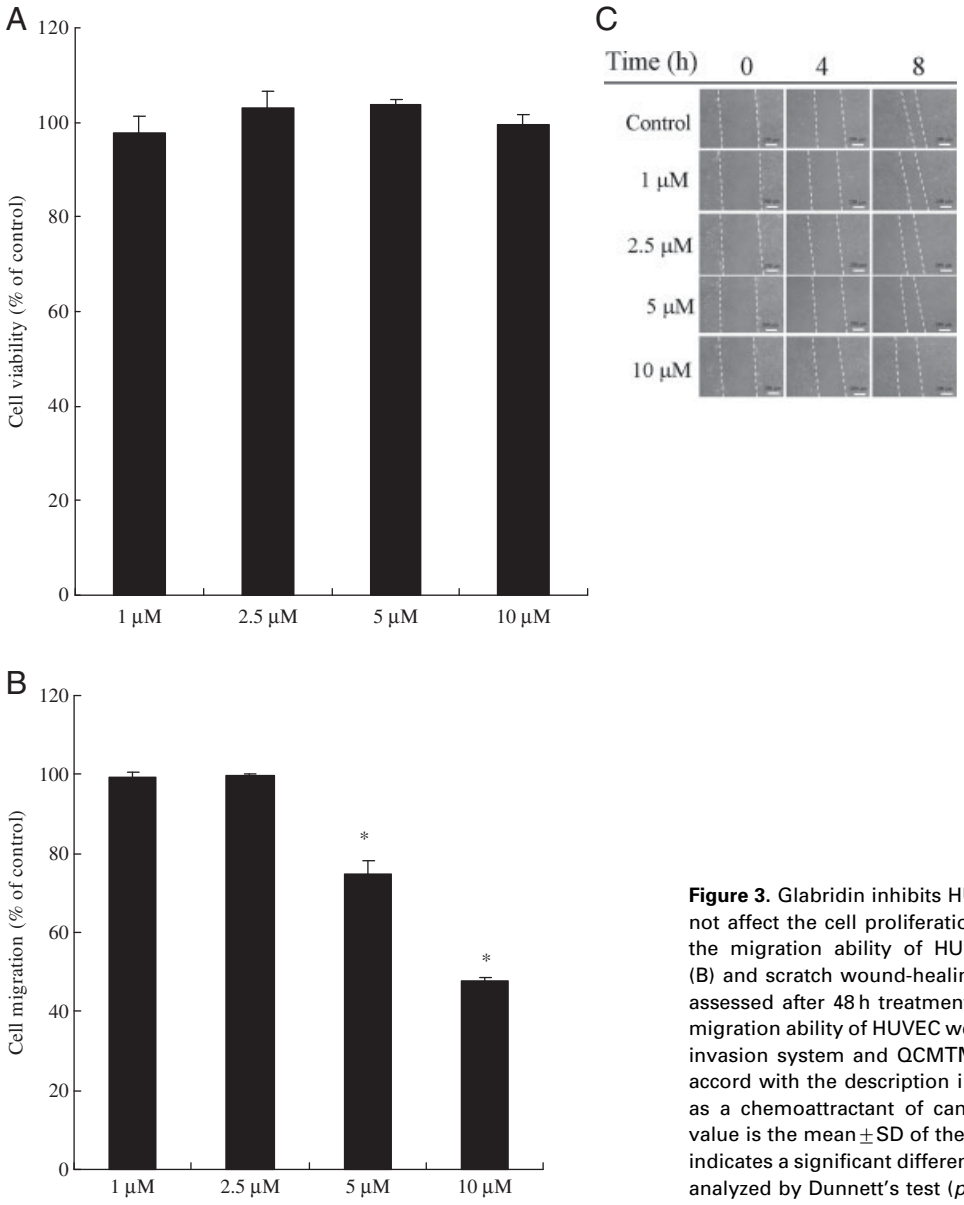

Figure 3. Glabridin inhibits HUVEC movement. (A) Glabridin did not affect the cell proliferation of HUVEC. Glabridin attenuated the migration ability of HUVEC, as determined by transwell (B) and scratch wound-healing assay (C). Cell proliferation was assessed after 48 h treatment by WST-1. The invasiveness and migration ability of HUVEC were quantified by BD BioCoat tumor invasion system and QCMTM 24-well Cell Migration Assay, in accord with the description in Section 2. MDA-MB-231 CM acts as a chemoattractant of cancer migration and invasion. Each value is the mean ± SD of the three determinations. The asterisk indicates a significant difference between the two test groups, as analyzed by Dunnett's test ($p < 0.05$).

effectively suppress the production of several angiogenic factors (VEGF, IL-8 and CXCL5) in MDA-MB-231 cells (data not shown). These data suggest that glabridin inhibits angiogenesis by inhibiting HUVEC migration, but does not affect the production of tumor-derived angiogenic factors.

3.4 Glabridin inhibits integrin levels by increasing degradation in MDA-MB-231 and HUVEC

Because integrins play an important role in cell migration [3], we assessed the intergrin expression in MDA-MB-231 cells after glabridin treatment. As shown in Fig. 4A, glabridin decreased the expression of α-type (2, 4, 5 and ν) and β-type (1, 3, 4 and 5) in MDA-MB-231 cells. Additionally, flow cytometry data also showed that glabridin decreased the expression of integrin αν (Fig. 4B). Glabridin also decreased the expression of αν and β3 integrin in HUVEC culture (Fig. 4C).

Next, we assessed whether glabridin decreased the amount of integrin by increasing protein degradation. Cells were pretreated with proteasomal inhibitor MG-132 (10 μmol/L) and then treated them with glabridin for 3 h. The results showed that glabridin decreased the levels of integrins (α5, αν and β3). This effect was restored by MG-132 (Fig. 4D), suggesting that glabridin increased integrin degradation in both MDA-MB-231 cells and HUVEC.

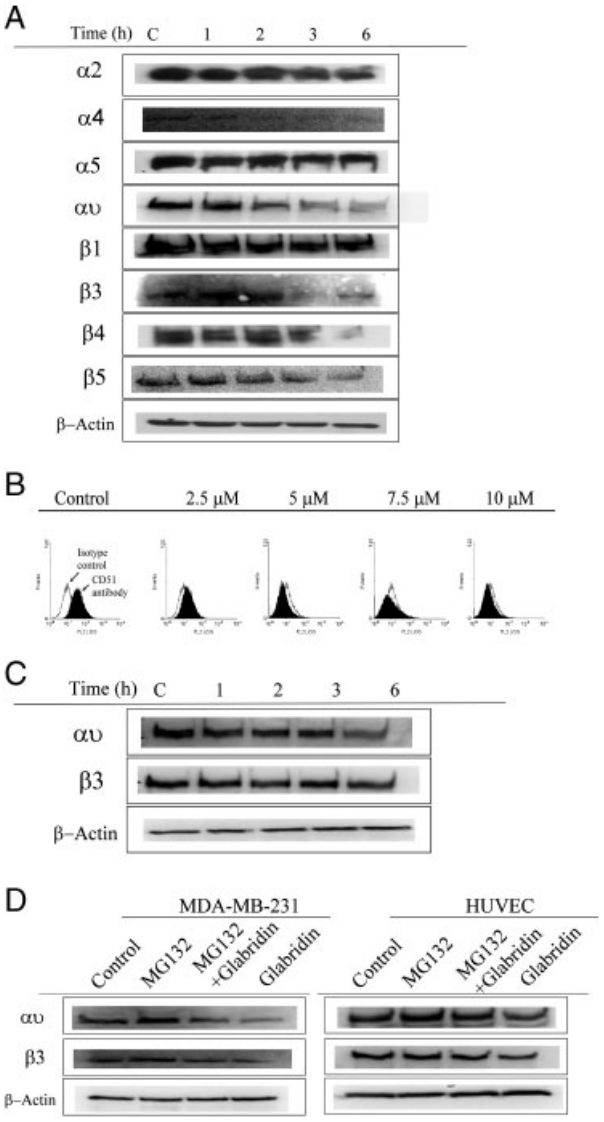

Figure 4. Glabridin increases the degradation of integrins. The expression of integrins in MDA-MB-231 cells as determined by immunoblot (A) and flow cytometry (B). (C) Integrin levels in HUVEC (B). (D) The effect of MG-132 on the expression of integrin. Cells were treated with glabridin (10 μM) for the indicated times. For blocking study, cells were pretreated with MG-132 (10 μM) for 1 h and then 10 μM glabridin was added for 3 h. The levels of integrins were assessed by immunoblot assay.

3.5 Glabridin inhibits FAK/Src signaling in MDA-MB-231 cells and HUVEC

FAK is essential for the regulation of integrin-mediated cell adhesion and migration of cancer cells [3]. We therefore assessed the effect of glabridin on FAK signaling. As shown in Fig. 5A, glabridin decreased the phosphorylation of FAK at Tyr 397, 576 and 925 sites in both MDA-MB-231 cells and HUVEC. However, glabridin did not cause any change in the protein levels of total FAK. Exposure of MDA-MB-231 cells and HUVEC to glabridin decreased active form Src (Tyr 416 phosphorylation) and decreased inactive form Src (Tyr 527). Similar responses were observed for the phosphorylated forms of FAK downstream targets, AKT (Thr 308 and Ser 473) (Fig. 5A). In addition, the association of FAK and Src increased in a time-dependent manner in glabridin-treated MDA-MB-231 cells and HUVEC, as detected by immunoprecipitation assay (Fig. 5B).

MAPK family is also a target of FAK/Src signaling, and hence we next investigated the phosphorylation of JNK, p38 and ERK1/2 in MDA-MB-231 cells. The data showed that glabridin decreased the phosphorylation of ERK1/2, but not JNK and p38 in MDA-MB-231 cells (Fig. 5C). Glabridin's inhibition of ERK1/2 phosphorylation was also found in HUVEC culture (Fig. 5D).

3.6 Glabridin inhibits RhoA expression and activity, and decreases the phosphorylation of myosin light chain in MDA-MB-231 cells and HUVEC

We assessed the expression and activity of RhoA in MDA-MB231 and HUVEC. As shown in Fig. 6A, glabridin decreased RhoA expression in a time-dependent manner. In addition, glabridin also decreased the activity of Rho in MDA-MB-231 cells (Fig. 6B). Finally, we assessed the effect of glabridin on the phosphorylation of myosin light chain (MLC), which is involved in controlling cell migration. Glabridin decreased the phosphorylation of MLC in both MDA-MB-231 and HUVEC (Fig. 6A).

4 Discussion

Breast cancer is the most common of all lethal malignancies and is also one of the four most prevalent malignant diseases of women in the word [1, 2]. In our study, we have found that glabridin effectively inhibits breast cancer and the migration and invasion of blood endothelial cells concomitant with inhibition of integrin/FAK/Src pathway, and effectively inhibits *in vivo* tumor cell angiogenesis.

Overexpression of integrins in malignancy cells contributes to cancer progression and metastasis by increasing tumor cell survival proliferation, migration and invasion [3, 22]. The integrin levels of normal and neoplasm tissues differ considerably. Several types of integrins, such as αvβ3, αvβ6 and α5β1, are usually present at low or undetectable levels in most epithelial tissue, whereas they are highly upregulated in some cancers, including breast cancer [3]. In contrast to quiescent endothelial cells, integrin $\alpha v\beta_3$ is highly expressed in tumor angiogenic endothelial cells [23]. Moreover, the expression of integrins such as αvβ3, αvβ5, α5β1, α6β4, α4β1 and αvβ6 is correlated with cancer progression, patient's survival and metastasis [24]. Our results show that glabridin decreased

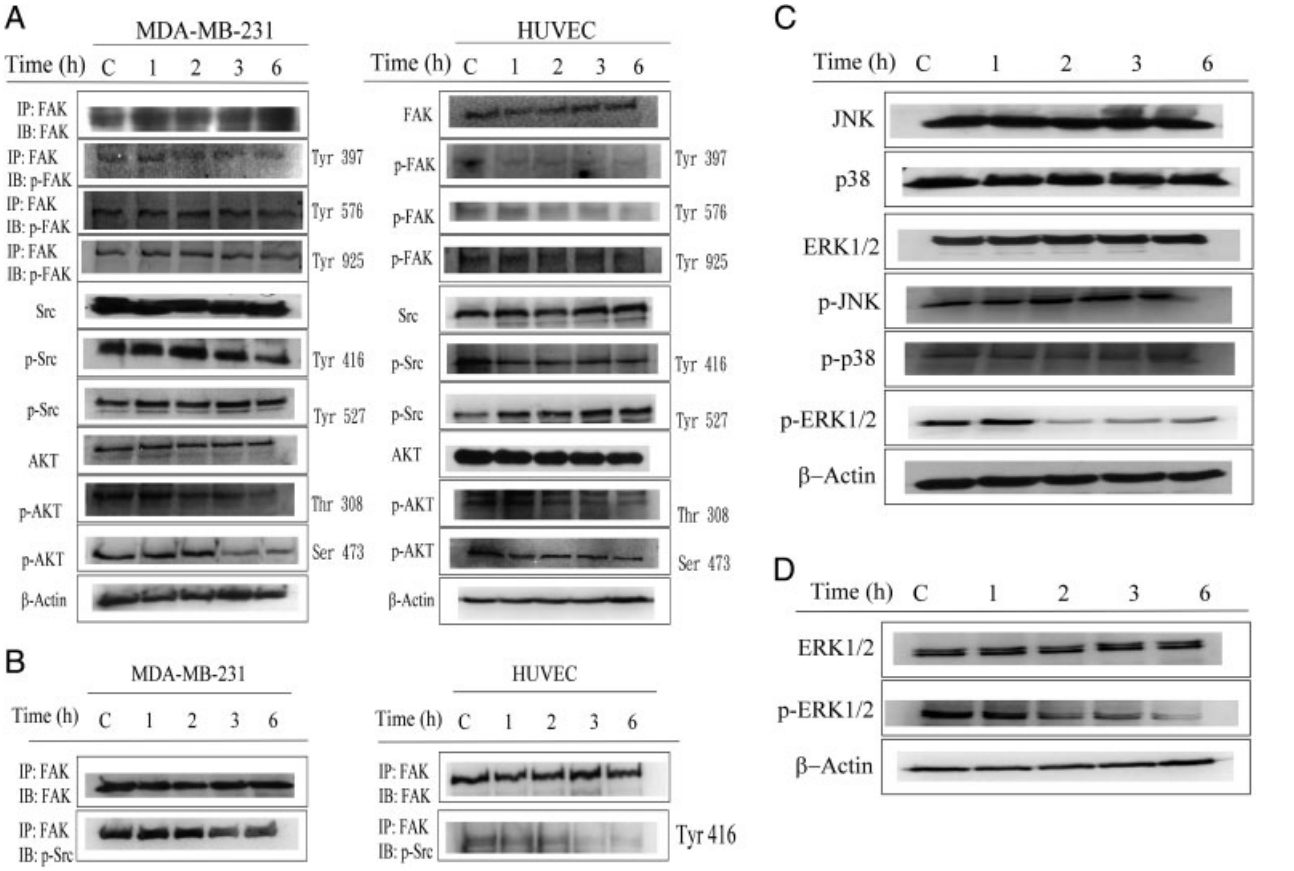

Figure 5. Glabridin inhibits FAK/Src signaling pathway. (A) The effect of glabridin in FAK/Src/AKT signaling pathway in MDA-MB-231 cells. (B) The interaction of FAK and Src. The effect of glabridin on ERK1/2 in MDA-MB-231 cells (C) and HUVEC (D). Cells were treated with glabridin (10 μM) for the indicated times. The levels of various proteins were assessed by immunoblot assay.

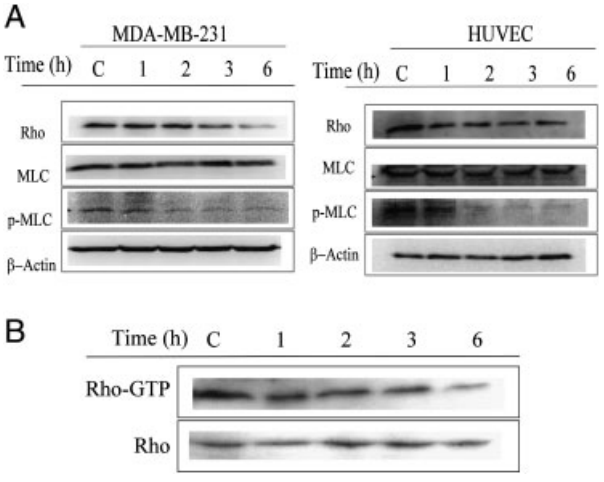

Figure 6. Glabridin decreases the activation of RhoA. (A) Glabridin decreased RhoA expression and MCL phosphorylation. (B) Glabridin reduced the activation of RhoA. Cells were treated with glabridin (10 μM) for the indicated times. The levels of various proteins were assessed by immunoblot assay. RhoA activity was assessed by Rho Activation Assay Kit.

the amount of integrin αv, β3, β4 and β5 in MDA-MB-231 cells, and αv, β3, β4 and β5 in HUVEC. In addition, proteosome inhibitor MG-132 reversed glabridin-mediated downregulation of αv and β3 integrins in MDA-MB-231 and HUVEC, suggesting that glabridin can inhibit integrins, at least for αvβ3, by increasing the degradation of these integrins.

FAK/Src signaling has been implicated in extracellular matrix/integrin-mediated signaling pathways, and plays an important role in tumor metastasis by increasing cell migration and invasiveness [6, 25]. Autophosphorylated FAK (Tyr 397) binds to the SH2 domain of Src, relieving inhibitory interaction and leading to activation of Src. Conversely, activated Src phosphorylates additional sites on FAK, including residues Tyr 576 and Tyr 577, resulting in further increased activity of FAK [26]. Activated FAK (Tyr 397)/Src (Tyr 416) transducts signaling through multiple downstream targets, such as PI3K/AKT and Ras/ERK1/2 cascades in cancer cells [7]. FAK also binds SH domain of PI3K, which in turn activates AKT kinase, thereby promoting cell migration by regulating various cell movement proteins [7, 25].

The formation of FAK/Src complex allows Src to phosphorylate Tyr 925 on FAK to mediate its interaction with Grb2 (growth factor receptor-bound protein 2), leading to the activation of Ras-ERK signaling pathway [26]. In our study, we found that glabridin decreased the phosphorylation of FAK at 397, 576 and 925, and increased inactive phosphorylation Src at Tyr 527. Glabridin also caused that release of Src from FAK, resulting in the inactivation of FAK/Src complex. In addition, glabridin also decreased the phosphorylation of two FAK/Src downstream kinases, AKT and ERK1/2, in both breast cancer and blood endothelial cells. These data suggest that the cooperation of FAK/Src with AKT and ERK1/2 plays a crucial role in glabridin-mediated cell migration in human breast cancer and HUVEC.

Activation of AKT and ERK1/2 promotes cytoskeletal rearrangements and cell movement by the activation of guanine exchange factors P-Rex1 (phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1), Vav (CG7893 gene product from transcript CG7893-RB), Tiam1 (T-cell lymphoma invasion and metastasis 1) and Sos (Son of sevenless), which in turn activate GTPase protein Rho family protein [27, 28]. Rho family GTPases are important regulators of the cytoskeleton, and affect cell adhesion and migration. Activation of RhoA promotes actin stress fiber formation, focal adhesions-related protein clusters, cell body movement and cell rear detachment [29, 30]. Upregulation of RhoA in HUVEC significantly enhanced morphogenetic changes and cytoskeletal reorganization, resulting in enhanced cell migration and angiogenic capacity [31]. Aberrant regulation of Rho proteins is associated with metastasis by promoting cancer angiogenesis and tumor cell motility [31]. One of the major downstream effectors of RhoA with regard to the cell migration is Rho kinase (ROCK), which can phosphorylate MLC at Thr 18/Ser 19 and subsequently trigger myosin contraction, leading to cell migration [32]. Our results show that glabridin treatment decreases the activation of AKT and ERK1/2, followed by a decrease of Rho expression and activity. In addition, exposure to glabridin also decreased the phosphorylation of MLC at Thr 18/Ser 19. These findings indicate that glabridin can be an effective inhibitor of AKT/Rho/MLC or ERK/Rho/MLC signaling in both breast cancer and blood vessel endothelial cells. FAK has been demonstrated to promote cell migration by decreasing RhoA activity [33]. On the contrary, FAK has also reported to stimulate RhoA activation by an increase of p190RhoGEF phosphorylation. It remains unknown why both FAK and loss of FAK can stimulate RhoA activation. This may be due to the fact that FAK may regulate RhoA *via* multiple mechanisms in cell migration [34].

Tumor angiogenesis, the central process of new blood vessels forming a pre-existing blood supply system in response to angiogenic stimuli, promotes tumor progression by supporting cancer cell survival, invasion and ultimately metastasis [21]. A growing body of evidence indicates blockade of cancer angiogenesis can decrease cancer progression by inhibiting tumor growth and metastasis [21]. There are two types of angiogenesis inhibitors: direct and indirect. Direct inhibitors prevent vascular endothelial cells from proliferating and migrating in response to a spectrum of pro-angiogenic proteins, whereas indirect inhibitors generally block the expression of cancer-derived angiogenic factors. Direct angiogenesis inhibitors are regarded as the preferred course to develop because they are least likely to induce acquired drug resistance by targeting stable endothelial cells rather than unstable mutating cancer cells [21, 35]. We found that glabridin inhibits HUVEC migration and angiogenesis by targeting the integrin/FAK/Src/Rho system. Importantly, these *in vitro* data were also confirmed by *in vivo* angiogenesis assay. Therefore, we suggest that glabridin may prove to be a valuable tool for inhibition of cancer angiogenesis.

In conclusion, we have provided evidence demonstrating that glabridin inhibits cancer cell migration and invasion as well as angiogenesis *in vivo*. Glabridin inhibits the ability of MDA-MB-231 and HUVEC to migrate and invade by decreasing integrin levels and sequentially reducing the activation of FAK/Src/AKT/Rho in these cells. Therefore, glabridin is a potentially useful anti-invasive agent in the treatment of human breast carcinoma.

This study was supported by grants from the National Science Council of Taiwan (NSC 96-2628-B-037-042-MY3), Center of Excellence for Environmental Medicine, Kaohsiung Medical University (KMU-EM-98-3), and Excellence for Cancer Research Center Grant, Department of Health, Executive Yuan, Taipei, Taiwan (DOH99-TD-C-111-002).

The authors have declared no conflict of interest.

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