

Targeting of Focal Adhesion Kinase by Flavonoids and Small-interfering RNAs Reduces Tumor Cell Migration Ability

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Abstract. Focal adhesion kinase (FAK), a member of a growing family of structurally distinct protein tyrosine kinases (PTK), has been linked to specific phosphorylation events, and the elevation of FAK activity in human carcinoma cells is associated with increased invasive potential. In the present study, therefore, we developed two experiments to test the hypothesis that FAK is a determinant of, and plays an important role in, regulating tumor cell migration. In the first, the biological functions of FAK were examined using flavonoid inhibition. MiaPaCa-2 cells, treated with luteolin (Lu) and quercetin (Qu), were used to dampen FAK phosphorylation and protein expression by parallel suppression of cell migration ability. The second experiment involved suppression of FAK expression using small-interfering RNAs (siRNA) specific to FAK. While not affecting cellular proliferation or apoptosis, the siRNA targeting FAK almost completely inhibited FAK protein expression in MiaPaCa-2 cells and potently blocked the cell migration mediated by FAK. Our results show that FAK functions as a key regulator of cell migration, and that FAK activity can be suppressed by specific FAK siRNA, and by luteolin and quercetin. It appears reasonable to conclude, therefore, that suppression of FAK protein has a significant impact on tumor cell invasiveness.

Abbreviations: DMSO, dimethyl sulphoxide; EGF, epidermal growth factor; ECL, enhanced chemiluminescence; FAK, focal adhesion kinase; MMP, matrix metalloproteinase; PTK, protein tyrosine kinase; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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The flavonoids, which are primarily benzo- γ -pyrone (phenylchromone) derivatives, comprise a very large class of naturally occurring, low molecular weight polyphenolic plant compounds. Accumulating evidence indicates that certain flavonoids not only inhibit the growth of tumor cells (1-5), but also suppress their invasive potential and cell migration *in vitro* (1, 2). Thus, the inhibitory effects of flavonoids on tumor cell growth could be a consequence of their interference with protein kinase activity (1, 2, 5, 6). For example, flavonoids inhibit signal-transduction pathways mediated by epidermal growth factor receptor, favorably affecting downstream signaling molecules including phospholipase C γ , the ERK/MAPK cascade and focal adhesion kinase (FAK) (7, 8). Our earlier results indicate that the flavonoids, luteolin (Lu) and quercetin (Qu), can inhibit protein tyrosine kinase (PTK) activity, which is also involved in cell proliferation, apoptosis and matrix metalloproteinase (MMP) secretion. In addition, both Lu and Qu variants exhibit the ability to suppress tumor cell migration (1, 5).

FAK is a non-receptor and non-membrane-associated PTK, originally isolated from v-src-transformed chick-embryo fibroblasts (9, 10). The human FAK, which does not contain Src homology 2 (SH2) or SH3 protein interaction domains, is an important regulator of cellular signaling pathways essential to cell survival and cell cycle, as well as for cell motility (9, 10). Overexpression of FAK occurs in a great number of human tumors, however, and it is reportedly an important determinant of tumorigenesis, cellular invasiveness and metastasis (10). The characteristics of FAK reveal an association between elevated FAK expression in human tumor cells and increased cell invasiveness (7, 8, 11, 12), whereas inactivation of FAK suppressed cell migration and proliferation *in vitro*. In addition, tumor invasiveness is associated with increased cell migration (9), and inhibition of FAK expression decreases cell motility. In growing, integrin-

stimulated, or migrating cells, FAK is highly phosphorylated in a number of tyrosine residues, *in vivo* (13, 14). Mechanistically, the stimulated phosphorylation of FAK at Tyr-397 creates an SH2 binding motif that is required for FAK function in promoting cell motility (9, 10). Sustained tyrosine-phosphorylation of FAK is essential for cancer cell migration (10, 15). The expression of PTEN, a tumor suppressor, leads to FAK dephosphorylation and inhibition of cell motility (13, 14). Furthermore, FAK overexpression and epidermal growth factor receptor (EGFR) tyrosine-kinase activity are often amplified in tumor cells (16).

More recently, developed RNA interference (RNAi) methodologies have been emerging, promising specific inhibition of gene expression in mammals (17). Currently, the potential of RNAi technology, especially with chemically synthesized siRNA, is being intensively evaluated in the development of highly specific gene-silencing therapeutics (18). Because of its ability to silence any gene once the sequence is known, RNAi has been adopted as a tool for discrimination of gene function (19). There is compelling evidence to suggest that FAK plays an important role in both tumor migration and metastasis (7-10). Since siRNA does not affect cellular proliferation or apoptosis, the transfection of FAK siRNA into tumor cells is particularly useful for the study of FAK-mediated cell migration and invasion. Previously, our results indicated that FAK phosphorylation was dampened in tumor cells treated with Lu and Qu, with parallel reduction of the secreted matrix metalloproteinase (MMP), potentially leading to suppression of invasive potential and cell migration *in vitro* (5). It has been demonstrated that inhibition of the activity or disabling the function of FAK inhibits both growth and metastasis in a variety of tumors (5, 8-16, 20). In a previous study, we elaborated the importance of FAK function with respect to promotion of EGF-stimulated cell motility through flavonoid treatment to reduce FAK expression and phosphorylation (5). In this study, therefore, special emphasis was placed on further investigation of the effect of suppressing FAK activity in MiaPaCa-2, a human pancreatic tumor cell line, using both siRNA and flavonoids to evaluate their therapeutic significance.

Materials and Methods

Materials. Quercetin was purchased from Nacalai Tesque (Kyoto, Japan). Luteolin was purchased from Extrasynthese (Genay, France). Wogonin (Wo) was obtained from Sigma (St. Louis, MO, USA). The flavonoids were dissolved in DMSO and stored in the dark at a concentration of 100 mM. RPMI-1640, Dulbecco's Modified Eagle Medium, and fetal bovine serum were obtained from GIBCO (Grand Island, NY, USA). EGF, anti-phosphotyrosine and anti-EGFR antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit anti-FAK was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

The mouse anti-FAK-p-Y397 was purchased from BD Biosciences (San Diego, CA, USA). ECL was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Smart pool FAK siRNA and non-specific siRNA were purchased from Dharmacon (Lafayette, CO, USA).

Cell growth experiments. The MiaPaCa-2 (pancreas) tumor cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained at 37°C in a humidified atmosphere (95% air and 5% CO₂) and grown as monolayers in plastic tissue-culture flasks containing Dulbecco's Modified Eagle Medium or RPMI-1640, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO), according to the recommendations of the American Type Culture Collection. Cells growing in log phase were harvested by suspension in 0.25% trypsin-EDTA solution for 5 min. The cells were then washed once with RPMI-1640 medium and resuspended at a density of 1 x 10⁴ cells/ml in a volume of 1.0 mL RPMI-1640 medium in 24-well plates. The cells were incubated at 37°C for 24 h to allow attachment to the plates, then flavonoids were added to obtain final concentrations of 10, 20, 50 and 100 µM. The cells were also treated with EGF (10 nM) to check the stimulating effects on growth and protein tyrosine kinase activity. Control wells contained DMSO at a final concentration of 0.1%.

For growth determination, cells from triplicate wells representing each treatment condition were harvested with 0.25% trypsin-EDTA and counted in a Coulter Counter (Coulter Electronics, Luton, UK), with cell viability determined using the dye exclusion method. Cell numbers were also determined using colorimetric assay with the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide as the assessable end-point, as previously described (21).

Preparation of cell lysates. EGF- and flavonoid-treated cells were harvested and washed three times with PBS. The cells were then lysed in gold lysis buffer, as previously described (22). Insoluble material was collected by centrifugation at 12,000 x g for 10 min at 4°C. The protein concentration was determined according to the method of Bradford (23), and then adjusted to 2.0 µg/ml. The samples were then divided into 50 µl aliquots and stored at -70°C for further study.

Gel electrophoresis, Western blotting and autoradiography. The kinase assay reaction mixtures described above were also subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to examine further changes in cellular protein phosphorylation in response to EGF and flavonoids. The kinase reactions were terminated by the addition of 50 µl 2X sample buffer (0.5-M Tris-HCl, pH 6.9; 2% SDS; 20% glycerol; 0.01% bromophenol), followed by boiling in water for 3 min. The reaction products were then electrophoresed on 3-18% linear gradient SDS-polyacrylamide gels according to the method of Laemmli (24), and were dot-blotted on nitrocellulose paper. The proteins were then electrophoretically transferred to nitrocellulose membranes, as previously described (25), and then autoradiographed using Kodak X-Omat AR film (Kodak, Rochester, NY, USA). The intensities of the phosphoproteins were determined using a densitometer.

Immunoblot analysis of EGFR, phosphotyrosine protein and FAK. The proteins separated on SDS-PAGE were electrophoretically

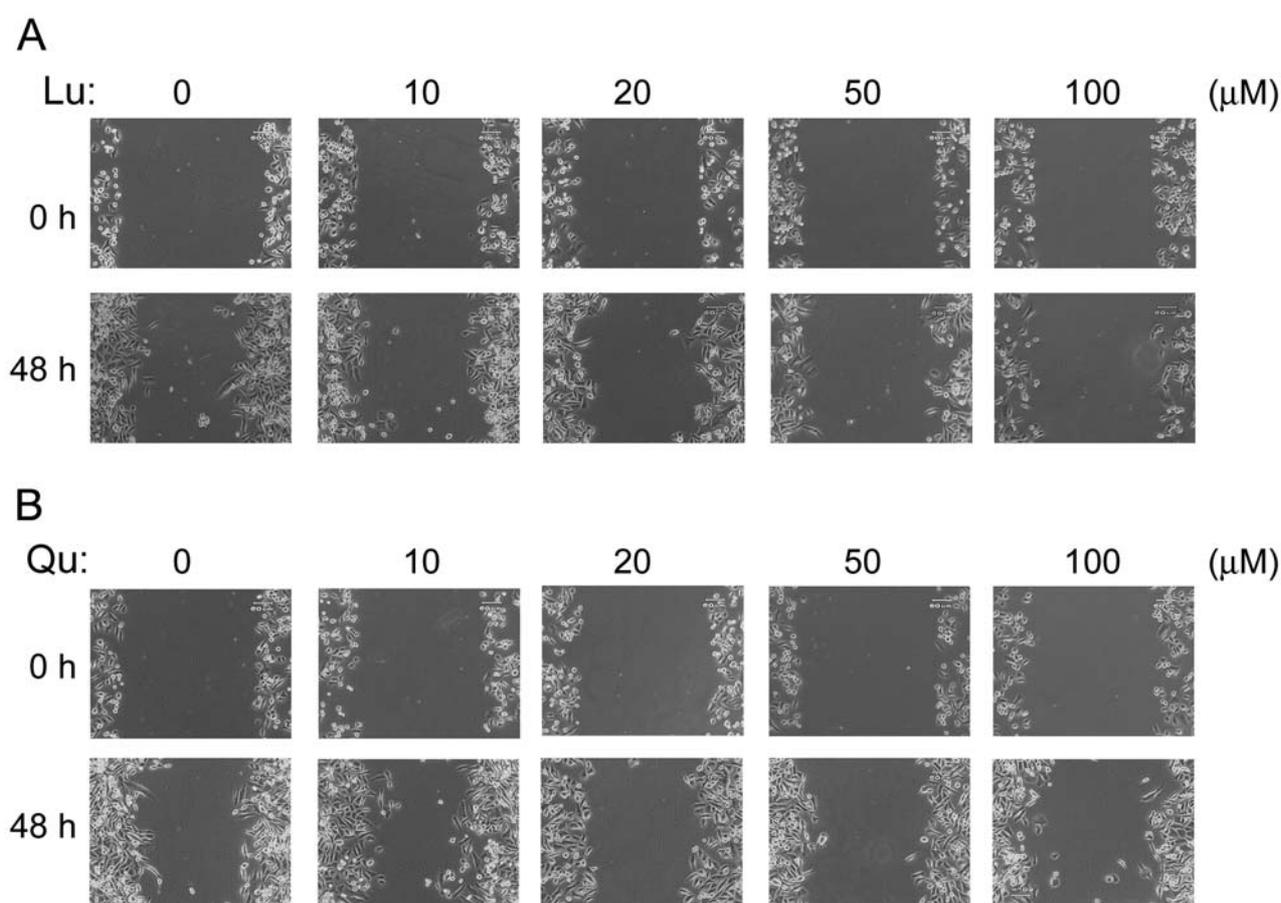


Figure 1. Effect of Lu and Qu on wound healing in MiaPaCa-2 cells. The MiaPaCa-2 cells (1×10^5) were plated onto 6-well plates and allowed to grow in the presence of 10% fetal bovine serum. After 24 h, a wound was created by scratching with a pipette tip (panels A & B, 0 h) and the cells were then allowed to migrate into the wound area in the presence of 10% fetal bovine serum. Phase contrast images were taken to assess cell migration after the cells had been treated without (panel A & B) or with 10, 20, 50, and 100 mM of Lu (panel A) and Qu (panel B) for an additional 48 h.

transferred to nitrocellulose membrane, according to the method of Towbin (25). The electrophoretic blots were blocked in PBS containing 5% BSA for 2 h at room temperature, and then rinsed in PBS three times and incubated for 2 h at room temperature with primary antibody appropriately diluted in PBS/1% BSA. The primary antibodies used in this study included: mouse monoclonal IgG-anti-phosphotyrosine, monoclonal IgG-anti-human FAK and anti-FAK-p-Y397. The nitrocellulose membranes were then thoroughly washed three times (10 min each) in TBST, and incubated with the secondary antibody (goat anti-rabbit or rabbit anti-mouse IgG) conjugated with horseradish peroxidase. The membranes were subsequently washed three times with TBST and twice with triethanolamine-buffered saline (TBS). Bands of FAK and p-FAK were detected with ECL reagents on Kodak BioMax film. To ascertain whether the phosphotyrosyl-125 kDa protein was indeed a FAK, anti-FAK polyclonal antibody was used to immunoprecipitate the FAK protein. EGF and/or flavonoids-treated and untreated cell lysates with equivalent amounts of proteins were incubated with anti-FAK polyclonal antibodies (1 μg/mL) for 4 h at 4°C with gentle agitation, according to the

manufacturer's recommendations. The FAK/anti-FAK antibody complexes were removed by centrifugation at 14,000 x g at 4°C for 20 min. The immunoprecipitates were then washed three times in PBS containing 1% Triton-100 and subjected to FAK immunoblotting analysis. In addition, the same immunoprecipitates were subjected to total kinase assay, as described above.

Transfection of siRNAs. SMART pool FAK siRNA and non-specific RNA were dissolved in 250 μl 1X universal buffer provided by the manufacturers to a concentration of 20 μM. MiaPaCa-2 cells (2×10^5) were plated into 35-mm 6-well trays and allowed to adhere for 24 h. Five microliters of Lipofectamine 2000 (GIBCO) per well was added into 250 ml Opti-MEM, thoroughly mixed, and incubated at room temperature for 5 min. Five microliters of SMART pool FAK siRNA per well was added to 250 μl Opti-MEM, thoroughly mixed and then combined with the diluted Lipofectamine 2000. The siRNA/Lipofectamine mixture was gently mixed and incubated at room temperature for 15 min, and then added into 6-well plate containing 1.5 ml DMEM with 10% FBS for 48 h. All assays were performed 48 h post-transfection.

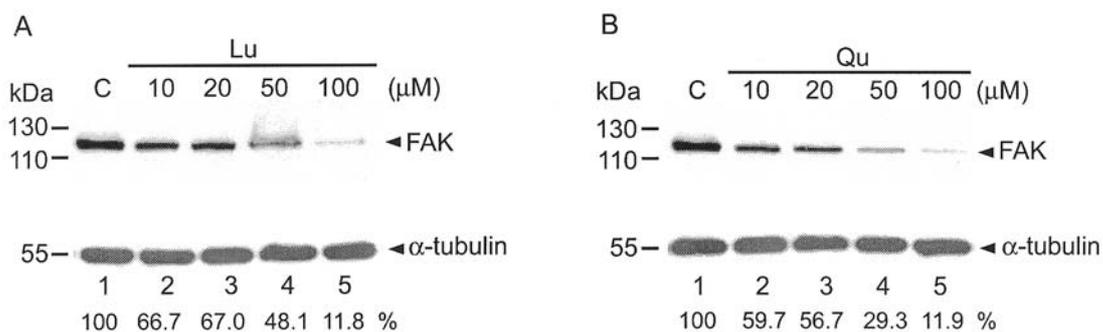


Figure 2. Effect of Lu and Qu on basal FAK expression in MiaPaCa-2 cells. Cells were treated with 10, 20, 50 and 100 μM of Lu (panel A) and Qu (panel B) for 48 h, respectively. At the end of culture, approximately 4×10^6 cells were harvested. Cellular lysates of 50 μg protein were subjected to immunoblotting analysis using anti-FAK antibody. The equal quantity of α-tubulin indicates similar protein levels in each lane.

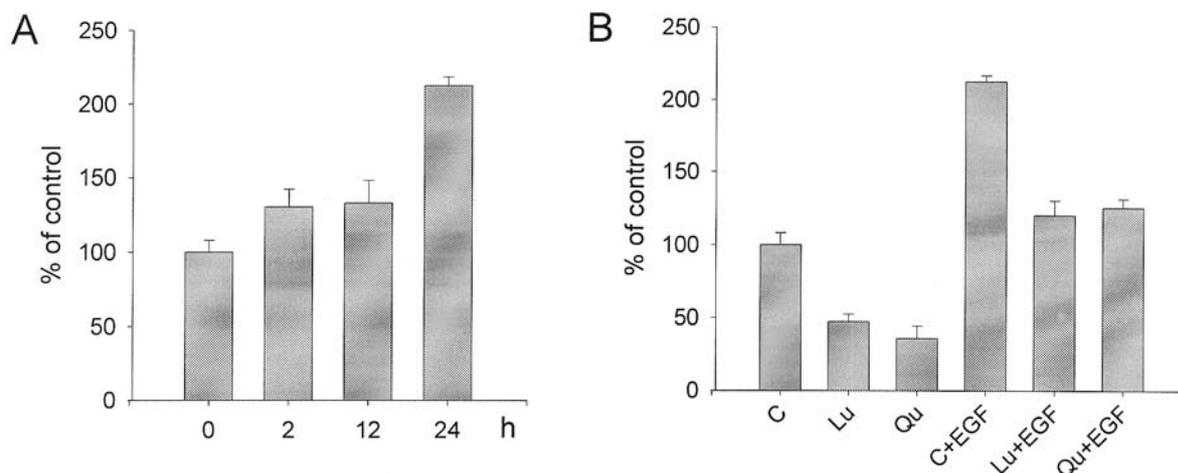


Figure 3. Effect of Lu and Qu on basal and EGF-induced tyrosine phosphorylation of FAK in MiaPaCa-2 cells. (A) Cells were treated with 10 nM EGF for 0-24 h. (B) Cells were treated for 24 h in the absence (lane a, control) or presence of 20 μM Lu (lane b), 20 μM Qu (lane c), or 10 nM EGF (lane d). MiaPaCa-2 cells were first (separately) treated with, respectively, 20 μM Lu and Qu for 24 h, and then with 10 nM EGF for an additional 24 h (lanes e & f). Cellular lysates of 50 μg protein were subjected to immunoblotting analysis using anti-FAK-p-Y397. An equal quantity of α-Tubulin was measured (data not shown). Scanning densitometry was used for quantitative analysis, and the percentage optical autoradiographic density was compared to controls. Values are mean percentage-density change for samples from three separate experiments.

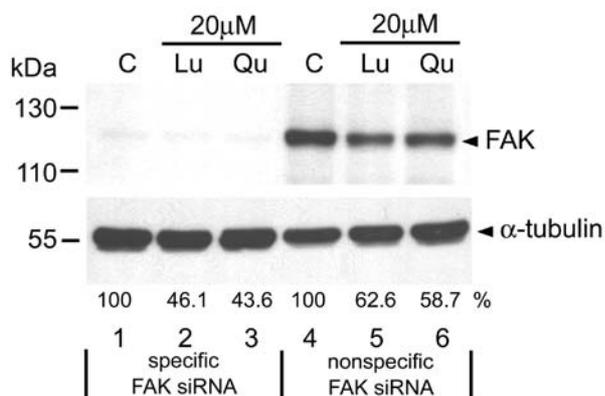


Figure 4. Effect of Lu and Qu on specific FAK siRNA and non-specific siRNA-transfected MiaPaCa-2 cells. Specific FAK siRNA (lanes 1-3) and non-specific FAK siRNA (lanes 4-6)-transfected cells were treated with 20 μM Lu and Qu for 48 h, respectively. At the end of culture, approximately 4×10^6 cells were harvested. The cellular lysates were subjected to immunoblotting analysis using anti-FAK antibody. Scanning densitometry was used for quantitative analysis. The equivalent quantity of α-tubulin indicates the similar protein levels in each lane.

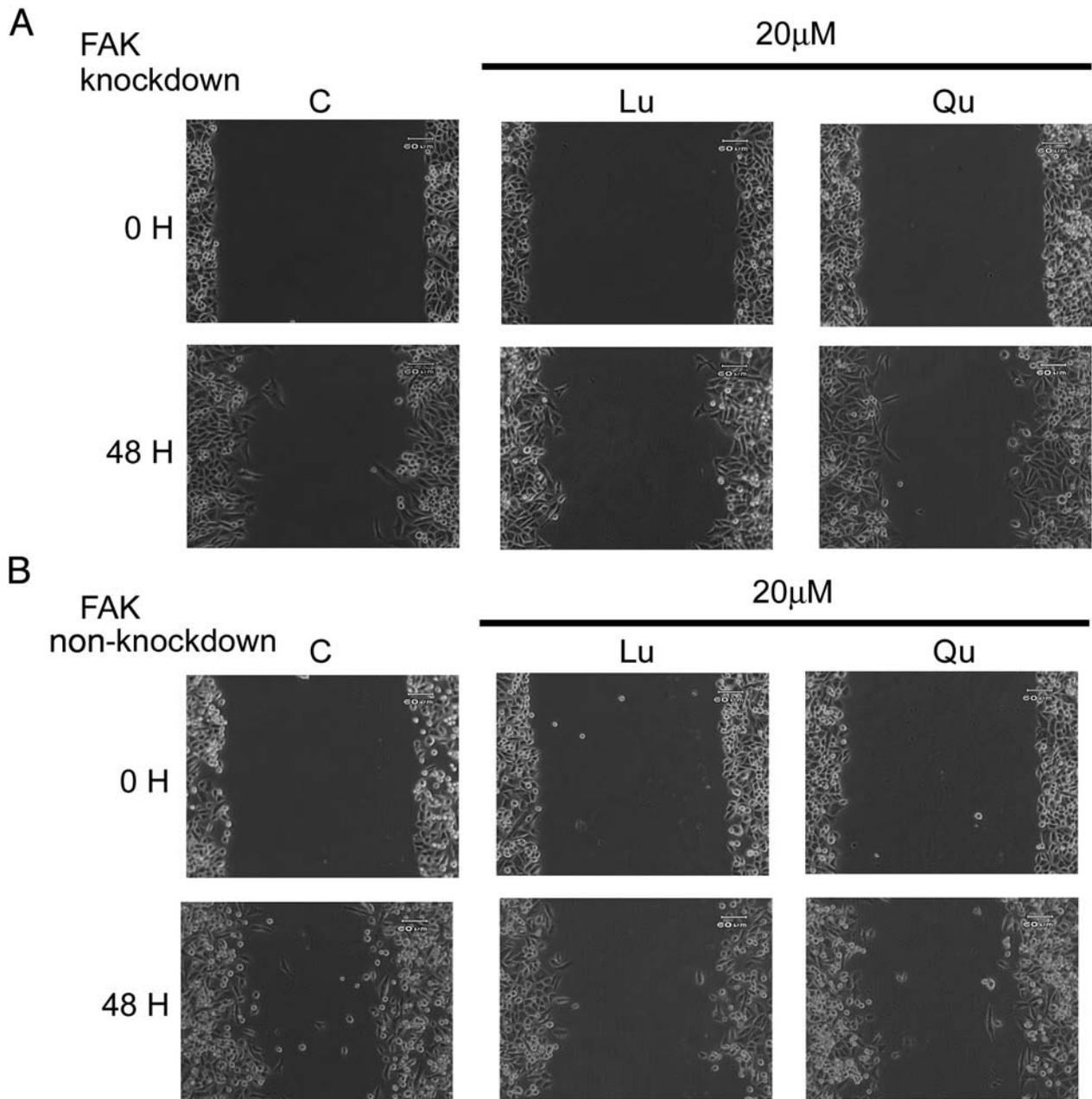


Figure 5. Effect of Lu and Qu on wound healing in FAK knockdown and non-knockdown MiaPaCa-2 cells. FAK knockdown (specific FAK siRNA) and non-knockdown MiaPaCa-2 cells (1×10^5) were plated onto 6-well plates and allowed to grow in the presence of 10% fetal bovine serum. After 24 h, a wound was created by scratching with a pipette tip (panels A & B, 0 h) and the cells were then allowed to migrate into the wound area in the presence of 10% fetal bovine serum. Both FAK knockdown and non-knockdown cells were treated without (panels A & B, control) or with 20 μ M of Lu (panel A) and Qu (panel B) for an additional 48 h, then phase contrast images were taken to assess cell migration.

In vitro chemo-invasion assay. *In vitro* invasiveness was investigated according to a previously described procedure (26), with modification. In brief, 24-well Transwell units with polycarbonate filters (8 mm pore size; Becton Dickinson, Franklin Lake, NJ, USA) were coated (1 h at room temperature) with 0.1 mL of 0.8-mg/mL Englebreth-Holm-Swarm (EHS) sarcoma tumor

extract consisting of reconstituted basement-membrane substances (EHS Matrigel; Sigma, St. Louis, MO, USA). These filters were then air-dried at room temperature to form a thin continuous layer on the filter surface. The lower compartment contained 0.6 mL laminin (20 mg/ml) as a chemoattractant or RPMI-1640 medium as a negative control. MiaPaCa-2 cells (1×10^5 cells/0.2 mL

of RPMI-1640 containing 0.1% BSA) were placed in the upper compartment and incubated with or without either flavonoids or EGF at 37°C for 72 h in a humidified atmosphere of 95% air/5% CO₂. Following incubation, the filters were fixed with 3% glutaraldehyde in PBS and stained with crystal violet. Cells on the upper surface of the filter were removed by wiping with a cotton swab, while those that had penetrated through the Matrigel to the lower surface of the filter were counted under a microscope (200x). Each treatment was assayed in triplicate, and two independent experiments were performed.

Wound-healing assay. Both siRNA-transfected and control MiaPaCa-2 cells (2x10⁵) were plated onto fibronectin-coated (5 µg/ml) 6-well culture plates in complete growth media. After 24 h, the cell monolayer was wounded by manual scratching with a pipette tip (27), washed with PBS, photographed in phase contrast using an Olympus IX70 camera (Tokyo, Japan), and then placed into complete medium, with or without either flavonoids or EGF, at 37°C in a humidified atmosphere of 95% air/5% CO₂. Matched-pair marked wound regions by manual scratching with a pipette tip were photographed again after 48 h of treatment.

Statistical analysis. The statistical significance between groups was determined by means of unpaired Student's *t*-tests. A probability of *p*<0.05 was considered significant for all tests. Results (from three-to-six independent experiments) are expressed as mean±SEM.

Results

Effects of luteolin and quercetin on MiaPaCa-2 cell migration. To investigate the effect of flavonoids on cell migration, *in vitro* wound-healing assays were performed. The MiaPaCa-2 cells (2x10⁵) were grown to the same density in culture medium on 6-well culture plates. The cell monolayer was wounded with a pipette tip and the cells allowed to migrate into the clear area (Figure 1; panels A & B, 0 h). After 48 h, the control sets exhibited a cell-reorientation response along the wound margin and had migrated into the wound area. Cells treated with 10, 20, 50 and 100 µM of Lu (Figure 1; panel A, 48 h) and Qu (Figure 1; panel B, 48 h) exhibited only limited cell-reorientation response along the wound edge in a dose-dependent manner, and they did not efficiently repopulate the open space in comparison to the controls.

Effects of luteolin and quercetin on FAK protein expression and phosphorylation. To study the association between Lu- and Qu-mediated reduction of FAK expression and phosphorylation, we examined the levels of FAK expression and relative levels of FAK tyrosine phosphorylation with the inhibition of EGF-stimulated signal events in MiaPaCa-2 cells. Two approaches were employed: (i) The cell lysates obtained from MiaPaCa-2 cells treated with 10, 20, 50, 100 µM Lu and Qu for 48 h were subjected to immunoblotting analysis. The FAK protein expression, expressed as a

percentage of the control value, was reduced by both Lu and Qu in a dose-dependent manner (Figure 2A and 2B). (ii) MiaPaCa-2 cells were treated with 10 nM EGF in culture medium for 2, 12 and 24 h, after which the cell lysates were analyzed. Using immunoblotting and scanning densitometry with anti-FAK-p-Y397 antibodies, we observed that EGF produced a time-dependent increase in FAK phosphotyrosine levels (Figure 3A). As the results of our previous studies suggest that both Lu and Qu can block approximately 50% of the growth and kinase activity of MiaPaCa-2 cells at 20 µM, this concentration was used throughout the present phosphorylation study. The cell lysates obtained from MiaPaCa-2 cells treated for 24 h with 20 µM Lu and Qu and/or 10 nM EGF were also subjected to immunoblotting and scanning densitometry with anti-FAK-p-Y397 antibodies. Lu and Qu significantly reduced FAK tyrosine phosphorylation (53% and 60%, respectively; Figure 3B). To determine if FAK phosphorylation was reversible using EGF, MiaPaCa-2 cells treated for 24 h with 20 µM Lu and Qu were then placed in fresh medium containing 10 nM EGF for an additional 24 h, with the FAK protein phosphorylation elevated to about 120% and 125% of that of controls, respectively (Figure 3B). The results suggest that EGF may counteract the inhibitory effect of Lu and Qu treatment on tyrosine phosphorylation of FAK.

Effect of FAK siRNAs on the expression of FAK from MiaPaCa-2 cells. To investigate whether FAK siRNAs could suppress FAK synthesis and secretion in human pancreatic MiaPaCa-2 tumor cells, five FAK siRNAs mixtures were examined for their ability to target human FAK in tumor cells. In order to confirm inhibition of FAK expression by siRNA transfection, the cell lysates were subjected to immunoblot analysis for FAK. After transfection with Lipofectamine 2000, FAK-specific siRNA exhibited potent ability to knockdown FAK production (Figure 4, lane 1). Up to 95% suppression of FAK protein expression was observed after a 48-h transfection (Figure 4, lanes 1-3). No effect was demonstrated on FAK expression using non-specific FAK siRNA (Figure 4, lanes 4-6), with α -tubulin expression also unaffected by both specific and non-specific FAK siRNA treatments (Figure 4, lanes 1-6), suggesting that non-specific down-regulation of FAK expression did not occur upon transfection. It is worth noting that suppression of FAK expression on the growth of MiaPaCa-2 cells, using specific or non-specific FAK siRNA, neither induced detectable changes in doubling time, nor caused apoptosis in these cells (data not shown). We also sought to determine the effect of Lu and Qu on the expression of FAK proteins of post-transfection cells. Control siRNA and specific FAK siRNA-transfected cells were treated with 20 µM Lu and Qu for 24 h, and then harvested. Lu and Qu treatment of FAK siRNA-

transfected MiaPaCa-2 cells resulted in up to 95% suppression of FAK production (Figure 4, lanes 1-3). Lu and Qu, at the concentration of 20 μ M, further suppressed FAK expression in specific FAK siRNA-transfected cells (Figure 4, lanes 1-3). For non-specific FAK siRNA-transfected MiaPaCa-2 cells, marked decreases in the production of FAK protein were exhibited for Lu and Qu (37% and 41%, respectively; Figure 4, lanes 4-6).

Effects of luteolin and quercetin on FAK knockdown MiaPaCa-2 cell migration. It is widely believed that FAK plays an important role in cell migration events. To study the relationship between FAK expression and cell migration, wound-healing assays were performed to determine whether decreased FAK expression could result in reduced cell migration ability. FAK-specific siRNA (FAK knockdown, FAK⁻) MiaPaCa-2 cells and FAK non-specific siRNA (FAK non-knockdown, FAK⁺) cells were grown to the same density in culture medium in 6-well culture plates. The cell monolayer was then wounded with a pipette tip and the cells allowed to migrate into the clear area, as described above (Figure 5; panels A & B, 0 h). At 48 h after the scratch, the FAK⁻ cells had become quite dense along the wound edge due to continued cell growth, however, they did not significantly migrate into the open area or show changes in cell morphology (Figure 5; panel A, 48 h) as compared to the FAK⁺ control cells (Figure 5, panel B, 48 h). In addition, within the same 48-h period, Lu and Qu (20 μ M) were found to slightly suppress the FAK⁻ cell migration activity (Figure 5, panel B, 48 h). It is worth noting that the time-courses for total wound closure for MiaPaCa-2 FAK⁺ and FAK⁻ were identical (60 h; data not shown).

Discussion

It is well known that the process of tumor cell invasion relies on several cell properties, including proteolysis, actin dynamics, adhesion and motility. In our earlier studies, we showed that, of the investigated flavonoids, Lu and Qu were the most potent inhibitors of tumor cell growth and EGFR tyrosine kinase activity (1, 5). We observed that both Lu and Qu exhibited strong inhibition of EGFR autophosphorylation, as well as FAK transphosphorylation (5). Additionally, the tumor cells responded to Lu and Qu exposure by parallel reductions in the levels of phosphorylated FAK and secreted MMPs, which may lead to suppression of invasive potential and cell migration *in vitro* (1, 5). It has been determined that FAK is overexpressed in a variety of tumors (10). High levels of FAK expression have also been found in preinvasive and invasive cancers (28). In addition, a direct relationship has been demonstrated between increased FAK expression and elevated cell motility (29). Taken together, these findings suggest that FAK is a logical target for therapeutic intervention.

The mechanism(s) by which flavonoids affect cell adhesion and migration is not yet clear, however, it appears reasonable to suggest that their determination and elaboration would aid in identification of potential applications for these agents against tumor proliferation and metastasis. In the present study, therefore, we examined FAK protein expression after treatment of MiaPaCa-2 cells with Lu, Qu or EGF. A dose-dependent decrease in FAK protein expression was demonstrated for both Lu- and Qu-treated cells (Figure 2), with a limited cell-reorientation response along the wound edge (Figure 1). Further, EGF stimulation also led to elevated FAK tyrosine phosphorylation (Figure 3) and FAK expression (5). Interestingly, Lu and Qu were both found to counteract the stimulatory effect of EGF on FAK expression (Figure 3, lanes e & f), and also phosphorylation (5). Furthermore, Lu and Qu partially suppressed the EGF-induced migration activity of MiaPaCa-2 cells (5). These findings suggest that Lu and Qu may act partly through inactivation of EGFR tyrosine kinase activity which, in turn, mediates FAK expression. Moreover, expression of basal and EGF-induced phosphotyrosyl-FAK (FAK-p-Y397) levels in MiaPaCa-2 cells is also inhibited by Lu and Qu (5). Our finding that EGF stimulates FAK phosphorylation in MiaPaCa-2 cells is also in agreement with the report of Hsia *et al.* (16).

Since FAK functions as a key regulator of extracellular matrix-dependent cell migration, and as endogenous levels of FAK protein expression also limit cell motility, it seems likely that reducing FAK expression may inhibit cell migration. Recently, RNA interference (RNAi) has emerged as a powerful tool for elaboration and elucidation of gene function (30). Tuschl's group found that transfection of synthetic 21-nucleotide small-interfering RNA into mammalian cells exhibits high gene specificity, without secondary effects (31). For this study, therefore, we chose specific siRNA to knockdown FAK expression in MiaPaCa-2 cells and investigate cell migration ability in response to treatment with Lu and Qu. Analysis of our data indicates that transfected specific FAK siRNA successfully inhibits the expression of FAK protein in MiaPaCa-2, human pancreatic carcinoma cells (Figure 4), leading to potent suppression of cell migration (Figure 5). These results clearly demonstrate that the design of a novel FAK blockade system using RNAi may be a powerful technique for controlling tumor cell motility and invasiveness. Other factors, such as the expression or the activity of MMP, are involved in tumor cell migration and invasion. MMP activity has been associated with elevated cell migration and invasion levels (5, 32). Our previous report indicates that *in vitro* inhibition of FAK expression and phosphorylation by Lu and Qu results in decreased MMP-9 secretion and the inhibition of MiaPaCa-2 cells through a reconstituted basement

membrane (5). Others studies also support this contention that decreased FAK expression and signaling in tumor cells inhibits migration and invasion, and that it is associated with a decrease in MMP secretion (32-34). Furthermore, these results suggest that cell invasion is linked to phosphotyrosyl-FAK accumulation in the lamellipodia of tumor cells (5).

Interestingly, FAK is an important mediator of cell survival and apoptosis (16, 27, 28). In the present study, however, we observed that siRNA FAK suppression does not affect MiaPaCa-2 cellular proliferation or induce apoptosis (data not shown). Taken together, these findings indicate that transfected specific FAK siRNA to tumor cells is, therefore, not a simple pro-apoptotic or directly cytotoxic process. This is consistent with other research, which has demonstrated that FAK suppression does not necessarily induce apoptosis (35).

In conclusion, we have demonstrated that utilization of siRNA oligonucleotides targeting FAK provides valuable information with respect to the efficacy of RNAi in terms of reducing cell migration ability. As the antioxidant flavonoids, Lu and Qu, are also able to block the EGFR-signaling pathway, leading to growth inhibition and reduced cell invasion/metastasis, suppression of FAK activity using a combination of siRNA and these flavonoids may be a reasonable approach to the development of more effective anticancer strategies although, clearly, much remains to be elaborated in *in vivo* systems.

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References

- Lee LT, Huang YT, Hwang JJ, Lee HPP, Ke FC, Nair MP, Kandaswain C and Lee MT: Blockade of the epidermal growth factor receptor tyrosine kinase activity by quercetin and luteolin leads to growth inhibition and apoptosis of pancreatic tumor cells. *Anticancer Res* 22: 1615-1628, 2002.
- Huang YT, Hwang JJ, Lee PPH, Ke FC, Huang JH, Huang CJ, Kandaswami C, Middleton E Jr and Lee MT: Effects of luteolin and quercetin, inhibitors of tyrosine kinase, on growth and metastasis-associated properties in A431 cells overexpressing epidermal growth factor receptor. *Br J Pharmacol* 128: 999-1010, 1999.
- Kandaswami C, Perkins E, Soloniuk DS, Drzewiecki G and Middleton E Jr: Antiproliferative effects of citrus flavonoids on a human squamous cell carcinoma *in vitro*. *Cancer Lett* 56: 147-152, 1991.
- Constantinou A, Kiguchi K and Huberman E: Induction of differentiation and DNA strand breakage in human HL-60 and K-562 leukemia cells by genistein. *Cancer Res* 50: 2618-2624, 1990.
- Lee LT, Huang YT, Hwang JJ, Lee AYL, Ke FC, Huang CJ, Kandaswami C, Lee PPH and Lee MT: Transinactivation of the epidermal growth factor receptor tyrosine kinase and focal adhesion kinase phosphorylation by dietary flavonoids: effect on invasive potential of human carcinoma cells. *Biochem Pharmacol* 67: 2103-2114, 2004.
- Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M and Fukami Y: Genistein, a specific inhibitor of tyrosine kinases. *J Biol Chem* 262: 5592-5595, 1987.
- Owens LV, Xu L, Craven RJ, Dent GA, Weiner TM, Kornberg L, Liu ET and Cance WG: Over expression of the focal adhesion kinase (p125^{FAK}) in invasive human tumors. *Cancer Res* 55: 2752-2755, 1995.
- Parsons JT, Martin KH, Slack JK, Taylor JM and Weed SA: Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. *Oncogene* 19: 5606-5613, 2000.
- Sieg DJ, Hauck CR, Ilic D, Klingbeil CK, Schlaepfer E, Damsky CH and Schlaepfer DD: FAK integrates growth-factor and integrin signals to promote cell migration. *Nat Cell Biol* 2: 249-256, 2000.
- Schlaepfer DD, Hauck CR and Sieg DJ: Signaling through focal adhesion kinase. *Prog Biophys Mol Biol* 71: 435-478, 1999.
- Kornberg L and Fleigel: The effects of inducible overexpression of FAK-related non-kinase (FRNK) on a transformed epithelial cell line. *Anticancer Res* 23: 91-98, 2003.
- Gabriel B, Mildenerger S, Weissner CW, Metzger E, Gitsch G, Schule R and Muller JM: Focal adhesion kinase interacts with the transcriptional coactivator FHL2 and both are overexpressed in epithelial ovarian cancer. *Anticancer Res* 24: 921-928, 2004.
- Tamura M, Gu J, Matsumoto K, Aota SI, Parsons R and Yamada KM: Inhibition of cell migration, spreading, and focal adhesion by tumor suppressor PTEN. *Science* 280: 1614-1617, 1998.
- Gu J, Tamura M, Pankov R, Danen EJK, Takino T, Matsumoto K and Yamada KM: Shc and FAK differentially regulate cell motility and directionality modulated by PTEN. *J Cell Biol* 146: 389-404, 1999.
- Mukai M, Togawa A, Imamura F, Iwasaki T, Ayaki M, Mammoto T, Nakamura H, Tatsuta M and Inoue M: Sustained tyrosine-phosphorylation of FAK through Rho-dependent adhesion fibronectin is essential for cancer cell migration. *Anticancer Res* 22: 3175-3184, 2002.
- Hsia DA, Mitra SK, Hauck CR, Streblov DN, Nelson JA, Ilic D, Huang S, Li E, Nemerow GR, Leng J, Spencer KSR, Cheresch DA and Schlaepfer DD: Differential regulation of cell motility and invasion by FAK. *J Cell Biol* 160: 753-767, 2003.
- Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K and Tuschl T: Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411: 494-498, 2001.
- Takei Y, Kadomatsu K, Yuzawa Y, Matsuo S and Muramatsu T: A small interfering RNA targeting endothelial growth factor as cancer therapeutics. *Cancer Res* 64: 3365-3370, 2004.
- Caplen NJ, Parrish S, Imani F *et al*: Specific inhibition of gene expression by small double stranded RNAs in invertebrate and vertebrate systems. *Proc Natl Acad Sci USA* 98: 9742-9747, 2001.
- Han EKH, Mcgonigal T, Wang J, Giranda VL and Luo Y: Functional analysis of focal adhesion kinase reduction by small inhibitory RNAs. *Anticancer Res* 24: 3899-3906, 2004.

- 21 Mosmann TJ: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity. *J Immunol Methods* 65: 55-63, 1993.
- 22 Samuels ML, Weber MJ, Bishop JM and McMahon M: Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human raf-1 protein kinase. *Mol Cell Biol* 13: 6241-6252, 1993.
- 23 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the protein-dye binding. *Anal Biochem* 72: 248-254, 1976.
- 24 Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4a. *Nature* 227: 680-685, 1970.
- 25 Towbin H, Staehelin T and Gordon J: Electrophoretic transfer of proteins from poly-acrylamide gels to nitrocellular sheets: procedure and some application. *Proc Natl Acad Sci USA* 76: 4350-4354, 1976.
- 26 Saiki H, Fuji J, Yoneda F, Abe M, Nakajima M and Tsuruo T: Role of aminopeptidase N(CD13) in tumor-cell invasion and extracellular matrix degradation. *Int J Cancer* 54: 137-143, 1993.
- 27 Sieg DJ, Hauck CR and Schlaepfer DD: Required role of focal adhesion kinases (FAK) for integrin-stimulated cell migration. *J Cell Sci* 112: 2677-2691, 1999.
- 28 Gabarra-Niecko V, Schaller MD and Dunty JM: FAK regulates biological processes important for the pathogenesis of cancer. *Cancer Metastasis Rev* 22: 359-374, 2003.
- 29 Akasaka T, Leeuwen RL, Yoshinaga IG, Mihm MC Jr and Byers HRJ: Focal adhesion kinase (p125FAK) expression correlates with motility of human melanoma cell lines. *J Invest Dermatol* 105: 104-108, 1995.
- 30 Elbashir SM, Harborth J, Lendeckel W, Yalein A, Weber K and Tuschl T: Duplexes of 21-nucleotide RNAs mediate interference in cultured mammalian cells. *Nature (Lond)* 411: 494-498, 2001.
- 31 Zamore PD, Tuschl T, Sharp PA and Bartel DP: RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101: 25-33, 2000.
- 32 Legrand C, Gilles C, Zahm JM, Polette M, Buisson AC, Kaplan H, Birembaut P and Tournier JM: Airway epithelial cell migration dynamics: MMP-9 role in cell-extracellular matrix remodeling. *J Cell Biol* 146: 517-529, 1999.
- 33 Shibata K, Kikkawa F, Nawa A, Thant AA, Naruse K, Mizutani S and Hamaguchi M: Both focal adhesion kinase and c-Ras are required for the enhanced matrix metalloproteinase 9 secretion by fibronectin in ovarian cancer cells. *Cancer Res* 58: 900-903, 1998.
- 34 Sein TT, Thant AA, Hiraiwa Y, Amin AR, Sohara Y, Liu Y, Matsuda S, Yamamoto T and Hamaguchi M: A role for FAK in the Concanavalin A-dependent secretion of matrix metalloproteinase-2 and -9. *Oncogene* 19: 5539-5542, 2000.
- 35 Duxbury MS, Ito H, Benoit E, Zinner MJ, Ashley SW and Whang EE: RNA interference targeting focal adhesion kinase enhances pancreatic adenocarcinoma gemcitabine chemosensitivity. *Biochem Biophys Res Commun* 311: 786-792, 2003.

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