

Fibronectin promotes tyrosine phosphorylation of paxillin and cell invasiveness in the gastric cancer cell line AGS

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ABSTRACT

Aims and background. Paxillin is a central protein within the focal adhesion and serves as a critical transducer of signals from fibronectin. Although abnormal expression of fibronectin and paxillin is often observed during the development of human malignancies, the relationship between paxillin and cell invasion in gastric cancer is still unclear. The current study was designed to investigate the potential role and mechanisms of fibronectin in tyrosine phosphorylation of paxillin and in the invasiveness of gastric cancer cells.

Methods. Expression of paxillin in human gastric cancer samples was examined by immunohistochemical staining. A gastric cancer cell line, AGS, was stimulated by fibronectin with gradient concentrations, and expression of paxillin and phosphorylation of paxillin tyrosine 118 (tyr118) was detected by immunoprecipitation and Western blotting. The invasiveness of AGS cells was measured by the modified Boyden chamber assay. Small interfering RNA (siRNA) targeting paxillin was used to establish the role of paxillin(tyr118) in the process of cell invasion enhanced by fibronectin. siRNA targeting focal adhesion kinase (FAK) was used to verify the effect of FAK tyrosine 397 (tyr397) on phosphorylation of paxillin(tyr118).

Results. Positivity for paxillin staining in human gastric cancer was associated with tumor stage. AGS cell showed dose dependence on fibronectin for invasiveness and phosphorylation of paxillin (tyr118). Invasiveness and phosphorylation of paxillin(tyr118) in AGS cells reached their peak when the concentration of fibronectin reached 100 nmol/L. siRNA targeting paxillin decreased the phosphorylation of paxillin(tyr118) and the invasiveness of AGS cells significantly as compared with controls. Blockage of FAK(tyr397) can inhibit phosphorylation of paxillin(tyr118) stimulated by fibronectin.

Conclusions. Fibronectin promotes paxillin(tyr118) phosphorylation and invasiveness of AGS cells. Paxillin silencing by RNA interference inhibits the cell invasiveness stimulated by fibronectin. Paxillin is a key factor in the fibronectin-stimulated invasiveness of AGS cells.

Introduction

Gastric cancer is one of the most common malignancies worldwide. Its incidence rates show substantial variation internationally, with highest rates in Japan, China and eastern Asia¹⁻³. There is an urgent need to explore the mechanism of gastric cancer occurrence, progress and metastasis. Abnormalities in oncogenes contribute to the pathogenesis of this disease, but the most important factors that influence survival in resectable gastric cancer are the depth of invasion through the gastric wall and the presence or absence of regional lymph node metastases⁴. Progression and metastasis are characteristics of malignant tumors; they include cellular adhesion,

Key words: fibronectin, paxillin, focal adhesion kinase, invasiveness, gastric cancer.

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destruction of the extracellular matrix (ECM), and deregulation of the cytoskeleton. The cytoskeleton consists of multiple parts including the actin cytoskeleton, which is involved in regulation of cell shape, cell motility and adhesion⁵. It interacts with the ECM and intracellular molecules via the focal adhesion^{6,7}. An important component of the ECM is fibronectin, which effectively induces cell adhesion and migration. In addition, it regulates focal adhesion dynamics and organization of the membrane cytoskeletal structures⁸.

Fibronectin is a large glycoprotein distributed in the basement membrane of connective tissue, on the cell surface, and in plasma. It facilitates cell-connective tissue and cell-cell adhesion, helps to maintain the integrity of vessel walls, and promotes wound healing⁹⁻¹³. Recent findings have suggested that fibronectin has a stimulatory effect on the migration and proliferation of tumor cells. Researchers have also found that fibronectin can promote migration and progression of invasive human bladder cancer¹⁴, ovarian cancer¹⁵ and glioblastoma cells¹⁶. Novel methods of suppressing cell migration have been shown to be connected to the fibronectin signaling pathway; for example, intravesical bacillus Calmette-Guerin (BCG) therapy against human bladder cancer migration produces its effect through inhibition of fibronectin function¹⁷. Fibronectin exerts its effects on tumor cells through integrins on the cell surface¹⁸. Further studies have found that signaling pathways activated after binding of fibronectin to its receptor are involved in the process of tumor migration¹⁹.

Focal adhesion contains proteins such as paxillin, focal adhesion kinase (FAK), Src, Csk, Crk, growth factor receptor-bound protein (Grb-2), p130 Crk-associated substrate (p130^{cas}), and phosphatidylinositol 3-kinase (PI-3 kinase)²⁰. Paxillin is a central protein within the focal adhesion that was originally identified in Rous sarcoma virus-transformed chick embryo fibroblasts. Its primary functions are those of an integrin effector and a molecular adapter of a scaffold protein that provides multiple docking sites at the plasma membrane and binds to many proteins that are involved in the organization of the actin cytoskeleton^{21,22}. Tyrosine phosphorylation of paxillin is a prominent event in integrin activation in normal epithelial cells, and the phosphorylation of paxillin on tyr31 and tyr118 correlates with cell adhesion and migration²³. Paxillin provides a platform for many protein tyrosine kinases including FAK and Src²². Although it has been verified that paxillin can be phosphorylated by fibronectin^{23,24}, it is still unknown how fibronectin mediates the biological function of focal adhesion on gastric cancer cells, especially the motility and invasiveness of cells via paxillin.

To better define the paxillin abnormalities in gastric cancer, we examined the expression of paxillin expression in human gastric cancer samples. We also evaluated its expression in the gastric cell line, AGS, and investigated the potential role of fibronectin in the expres-

sion and tyrosine phosphorylation of paxillin and the motility of AGS. RNA interference technology was applied to silence the genes of paxillin and FAK to further explore the role of paxillin phosphorylated by fibronectin in signaling pathway affecting the invasiveness of AGS.

Materials and methods

Materials

The human gastric cancer cell line AGS was obtained from the China Center for Type Culture Collection (Shanghai, China). RPMI 1640 medium and fetal bovine serum were purchased from Gibco Corporation (Carlsbad, CA, USA). Fibronectin was from Sigma Corporation (St. Louis, MO, USA). Oligofectamine and Plus Reagent were obtained from Invitrogen Corporation (Carlsbad, CA, USA). Rabbit monoclonal anti-paxillin antibody and phospho-paxillin(tyr118) antibody were purchased from BioLegend Corporation (San Diego, CA, USA). Rabbit monoclonal anti-FAK(tyr397) antibodies were purchased from Neomaker Corporation (Fremont, CA, USA). The Boyden chamber (Millicell chamber) was manufactured by Millipore Corporation (Billerica, MA, USA). Matrigel was from BD Corporation (Franklin Lakes, NJ, USA). Double-stranded small interfering RNAs (siRNAs) were synthesized by Shenggong Biotechnology Corporation (Shanghai, China). Silencer™ negative control siRNA was purchased from Ambion Corporation (Austin, TX, USA). The electrochemiluminescence kit was purchased from Boshide Corporation (Wuhan, China). Other reagents were from Sigma Corporation (St. Louis, MO, USA).

Stock culture of human AGS cells was maintained at 37°C in RPMI 1640 medium, supplemented with 10% fetal bovine serum in a humidified atmosphere containing 5% CO₂ and 95% air.

Expression of paxillin in human gastric cancer samples and clinicopathological analyses

In this retrospective analysis, 90 primary tumors were collected from patients diagnosed with gastric cancer who underwent surgery between 2000 and 2003 at the Union Hospital of Fujian Medical University and the First Affiliated Hospital of Fujian Medical University. None of the patients had received radiotherapy, chemotherapy or hormone treatment. Histological classification (intestinal, diffuse-adherent, and diffuse-scattered types) was according to the Lauren classification system. For tumor staging the tumor-node-metastasis staging system was used. The procedure to protect the patients' privacy was in accordance with the ethical guidelines enacted by the Ministry of Public Health of China.

Monoclonal antibody against paxillin diluted 1:100 was used with the Envision immunohistochemical

staining method. Four-micron-thick sections of phosphate buffered saline/formalin-fixed and paraffin-embedded specimens were routinely dewaxed in xylene, rehydrated in a graded series of ethanol, and washed in distilled water. Antigen retrieval was obtained by placing the specimens in 0.01 mol/L citrate buffer at pH 6.0 and exposing them to repeated microwave heating for 10 minutes at 450 W. The buffer was replenished after each interval because of evaporation. The specimens were cooled at room temperature for 15 minutes and washed in sterile water for 5 minutes and then in PBS at pH 7.6 for 5 minutes. Endogenous peroxidase or phosphatase activities were quenched in 0.3% H₂O₂ for 30 minutes, followed by blocking of nonspecific antibody binding in 10% goat serum for 30 minutes at room temperature. Tissue sections were incubated at 37 °C for 1 hour with primary rabbit anti-human paxillin. Mayer's hematoxylin stain was used as a counterstain. Negative controls were performed using conjugate alone.

Assessment of immunohistochemical staining

The staining for paxillin was classified according to the percentage of stained cancer cells. Expression was considered to be negative if <50% (0, 0-10%; 1+, 10-50%) of cancer cells were stained. When at least 50% (2+, 50-80%; 3+, >80%) of cancer cells were stained, immunostaining was considered positive. The study was performed in a blind fashion, so that patient outcome was unknown to the pathologists examining the immunohistochemical stains.

Statistical analysis

Statistical analyses for Table 1 were carried out with Fisher's exact test. For all the statistical analyses, $P < 0.05$ was considered statistically significant.

Effect of fibronectin with gradient concentrations on expression and phosphorylation of paxillin(Tyr118) in AGS cells

AGS cells were grown to a confluence of 1.5×10^7 cells/mL in RPMI 1640 medium containing 10% fetal bovine serum. Before experiments, the AGS cells were cultured overnight in RPMI 1640 without fetal bovine serum. Fibronectin was added to the medium to obtain final gradient concentrations of 0, 10, 100, and 1000 nmol/L. After 5 minutes the medium was removed and cells were pelleted and lysed in ice-cold lysis buffer (Tris-HCl 25 mmol/L, EDTA 1 mmol/L, NaCl 150 mmol/L, NaF 50 mmol/L, 1% Triton-100, PMSF 1 mmol/L, leupeptin 1 mg/L, aprotinin 1 μ mol/L, pH 7.6). Equivalent amounts of protein were separated by SDS-PAGE. Protein on the SDS-PAGE was transferred to nitrocellulose using a semidry transfer apparatus (Bio-Rad Laboratories), blocked with 3% bovine serum albumin in TBST [10 mmol/L Tris (pH 8.0), 150 mmol/L Na-

Table 1 - Expression of paxillin in human gastric cancer samples and pathological analysis

	Paxillin expression		P
	Positive No. (%)	Negative No. (%)	
T stage			
T ₁	6	10	$P < 0.05$
T ₂ /T ₃ /T ₄	53	21	
N stage			
N ₀	14	13	$P > 0.05$
N ₁ /N ₂ /N ₃	45	18	
TNM stage			
I/II	23	14	$P > 0.05$
III/IV	36	17	
Histological subtype			
Scattered	16	9	$P > 0.05$
Other types	43	22	

Cl, 0.1% Tween 20], incubated at room temperature for 1 hour with rabbit monoclonal anti-paxillin antibody, rotated for 1 hour at room temperature, and washed with TBST 3 times. Protein on the nitrocellulose was then detected by electrochemiluminescence with the kit including an anti-rabbit antibody according to the manufacturer's instructions. Simultaneously, equal amounts of cell lysates were centrifuged at 4 °C and 12,000g/min, rotated with rabbit monoclonal anti-paxillin antibody for 2 hours, then precipitated by protein A Sepharose at 4 °C overnight. Beads were washed 5 times with cold wash buffer (20 mmol/L Tris, pH 7.8, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% Triton X-100, 100 μ M PMSF, and 1 mmol/L Na₃VO₄), then the bound protein was eluted with Laemmli sample buffer and separated by SDS-PAGE. Protein on the SDS-PAGE was transferred to nitrocellulose and blocked as described previously, incubated at room temperature for 1 hour with rabbit anti-human paxillin(Tyr118) antibody, rotated for 1 hour at room temperature, washed with TBST 3 times, then protein on the nitrocellulose was detected by electrochemiluminescence with the kit including a goat anti-rabbit antibody according to the manufacturer's instructions.

Effect of fibronectin with gradient concentrations on invasiveness of AGS cells

Invasion assays were performed with the modified Boyden chamber assay. The filter membrane on the bottom of the Millicell chamber was coated with human matrigel. About 1×10^5 cells were added into each Millicell chamber, 5 chambers per group. Fibronectin was added to the chambers with a gradient concentration of 0, 10, 100 and 1000 nmol/L. The chambers were put into the 24-well cell culture plate, maintained at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air for 12 hours. The cells on the lower surfaces of the filter membranes were scraped and smeared, dyed with rou-

tine HE, and then counted. Results from quintuple wells were expressed as means \pm SD.

Effect of siRNA targeting paxillin on phosphorylation of paxillin(tyr118) in AGS cells

The 2 siRNA sequences targeting human paxillin (from mRNA sequence; GeneBank™ accession number NM-002859) chosen in this study and designed by the software developed by Ambion Inc. were siRNA1: 5-CUCGU-CUAUGCCCCGCAACTt-3 and siRNA2: 5-AUCUCCUG-GUAUGUGUGGutt-3. Before transfection, cells cultured at 50% confluence in 6-well plates were washed twice with Opti-MEM (Invitrogen Inc.) without fetal bovine serum and incubated in 1.5 mL of this medium without fetal bovine serum for 1 hour. Oligofectamine in Opti-MEM was used for transfection at 10 μ g/mL according to the manufacturer's protocol. The final siRNA concentration was 100 nmol/L. After 5-8 hours of transfection, 0.5 volume of AGS medium containing 30% serum was added to the cells, and transfection was continued overnight. Silencer™ negative control 1 siRNA (Ambion Inc.) was used as negative control under similar conditions. There were 4 groups of AGS cells: pure AGS cells, cells transfected with siRNA1 targeting paxillin, cells transfected with siRNA2 targeting paxillin, and cells transfected with negative control siRNA. The 4 groups of cells were pelleted, the total cellular extracts were prepared, and the amount of phosphorylation of paxillin(tyr118) in each group was tested by immunoprecipitation and Western blotting as described previously.

Effect of paxillin silencing on phosphorylation of paxillin(tyr118) and invasiveness of AGS cells stimulated by fibronectin

After transfection for 36 hours, cells of 3 groups (AGS, AGS transfected with siRNA1 targeting paxillin, AGS transfected with negative control siRNA) were added to 100 nmol/L fibronectin for 5 minutes. Then the medium was removed, cells were pelleted, the total cellular extracts were prepared, and the phosphorylation of paxillin(tyr118) was tested by immunoprecipitation and Western blotting as described previously. Meanwhile, about 1×10^5 cells of the 3 groups were added into the Millicell chambers, 5 chambers per group. Medium with 100 nmol/L fibronectin was added into the Boyden chambers, and the chambers were put in the 24-well cell culture plate and maintained at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air for 12 hours. The cells on the lower surfaces of filter membranes were scraped and smeared, dyed with routine HE, and then counted.

Effect of siRNA targeting FAK on phosphorylation of FAK(tyr397) and paxillin(tyr118) in AGS cells stimulated by fibronectin

The siRNA sequence targeting human FAK was 5-CAAUUCUAUJUCCACUCCt-3, whose efficiency was

verified in previous studies at our laboratory²⁵. Before transfection, cells cultured at 50% confluence in 6-well plates were washed twice with Opti-MEM without fetal bovine serum and incubated in 1.5 mL of this medium without fetal bovine serum for 1 hour. Oligofectamine in Opti-MEM was used for transfection at 10 μ g/mL according to the manufacturer's protocol. The final siRNA concentration was 100 nmol/L. After 5-8 hours of transfection, 0.5 volume of AGS medium containing 30% serum was added to the cells, and transfection was continued overnight. Silencer™ negative control 1 siRNA was used as negative control under similar conditions. There were 3 groups of AGS cells: pure AGS cells, cells transfected with siRNA targeting FAK, and cells transfected with negative control siRNA. 100 nmol/L fibronectin was added to the culture media of the 3 cell groups for 5 minutes. Then the 3 groups of cells were pelleted, the total cellular extracts were prepared, and the amount of FAK(tyr397) and the phosphorylation of paxillin(tyr118) in each group were tested by immunoprecipitation and Western blotting as described previously.

Statistical analysis

Results were compared using analysis of variance (ANOVA) and Student's *t* test and considered statistically significant when $P < 0.05$. Calculations were done using SPSS for Windows, version 11.5 (SPSS, Chicago, IL, USA). All experiments were done independently at least 3 times unless indicated otherwise.

Results

Expression of paxillin in human gastric cancer samples and clinicopathological analyses

Immunohistochemical staining of paxillin was done in 90 human gastric cancer specimens. Representative results of paxillin immunostaining are shown in Figure 1 and Table 1.

Paxillin staining was observed more frequently in T2, T3 and T4 than in T1 tumors [53 of 90 (58.89%); $P < 0.05$]. Paxillin staining showed no differences between stage I/II and III/IV cases ($P > 0.05$), nor between N0 and N1/N2/N3 cases. Although it was thought that paxillin was more frequently expressed in epithelial-derived cells, in this study no statistically significant difference in paxillin expression was detected in intestinal-type and diffuse-type gastric cancer. In other words, positivity for paxillin staining was only associated with advanced T stage (depth of invasion).

Effect of fibronectin with gradient concentrations on expression and phosphorylation of paxillin(tyr118) in AGS cells

To determine whether fibronectin promotes the expression and phosphorylation of the cytoskeleton-asso-

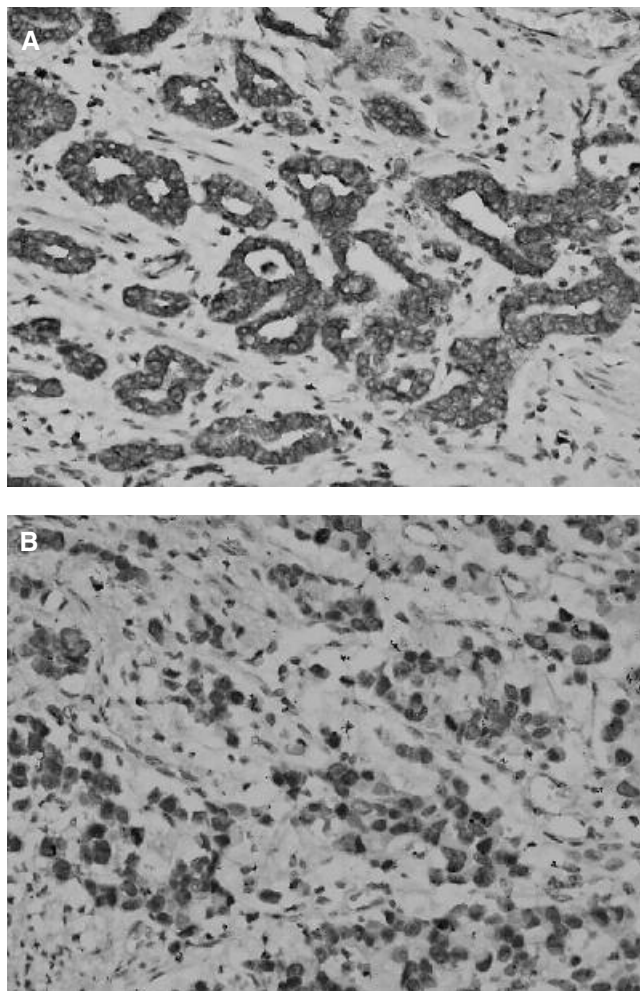
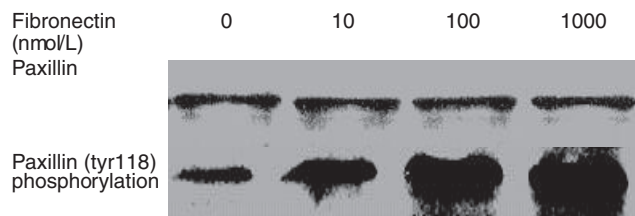
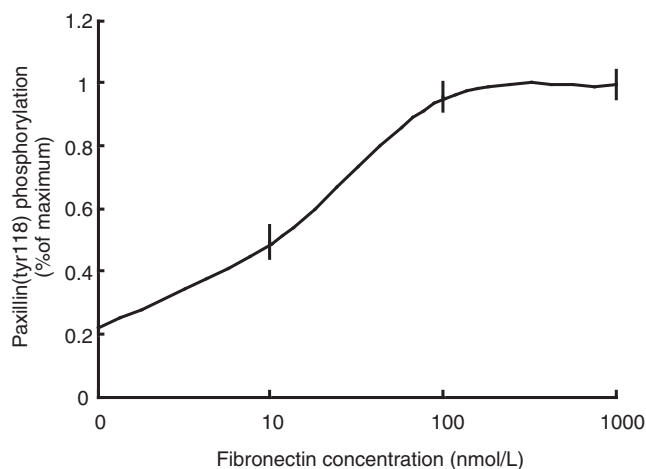


Figure 1 - Immunohistochemical staining of paxillin in a gastric cancer tissue array. A) Paxillin immunostaining. B) No immunostaining for paxillin.

ciated protein paxillin, cell lysates were immunoprecipitated with a rabbit monoclonal anti-paxillin antibody followed by immunoblotting with phosphospecific monoclonal rabbit antibodies to the tyr118 site of paxillin after stimulation by various concentrations of fibronectin. As shown in Figure 2A and 2B, fibronectin had no effect on the total expression of paxillin at concentrations of 10 nmol/L to 1000 nmol/L, whereas paxillin(tyr118) phosphorylation was significantly increased in a dose-dependent manner after stimulation by fibronectin at concentrations of 0 nmol/L to 100 nmol/L. Phosphorylation of paxillin(tyr118) reached its peak when the fibronectin concentration was 100 nmol/L. Stimulation with 1000 nmol/L fibronectin was unable to increase the phosphorylation of paxillin(tyr118) any further ($P > 0.05$). Taken together, these data suggested that fibronectin was able to increase the tyrosine phosphorylation of paxillin (tyr118), but not the total amount of paxillin *in vitro*.



A



B

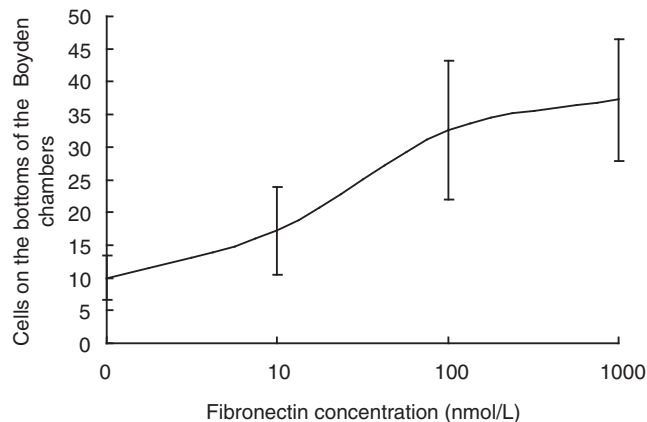
Figure 2 - Effect of fibronectin with gradient concentrations on paxillin expression and paxillin(tyr118) phosphorylation of AGS cells (mean \pm SD, n = 3).

Effect of fibronectin with gradient concentrations on invasiveness of AGS cells

A variety of *in vitro* test systems have been used to assess the cell's ability to cross tissue barriers. The basement membranes in the tissues have been assumed to create a critical barrier to the passage of cells. The modified Boyden chambers use human matrigel as a coating on the top of a filter as a barrier to the passage of cells, which is considered a convenient and objective way to measure invasiveness of different tumor cells²⁶. The AGS cells showed low invasiveness before fibronectin stimulation. After stimulation with 10 nmol/L fibronectin, the cell invasiveness increased significantly. The amplitudes of invasiveness increased considerably when the concentration of fibronectin changed from 0 nmol/L to 10 nmol/L, and from 10 nmol/L to 100 nmol/L ($P < 0.05$). However, when the fibronectin concentration changed from 100 nmol/L to 1000 nmol/L, the cell invasiveness did not increase correspondingly ($P > 0.05$) (Table 2, Figure 3).

Table 2 - Modified Boyden chamber assay to test the effect of fibronectin with gradient concentrations on invasiveness of AGS cells (mean \pm SD, n = 5)

Fibronectin (nmo/L)	0	10	100	1000
Cells on the bottoms of the Boyden chambers	10.0000 \pm 3.3912	17.2000 \pm 6.6858	32.6000 \pm 10.6542	37.2000 \pm 9.3621

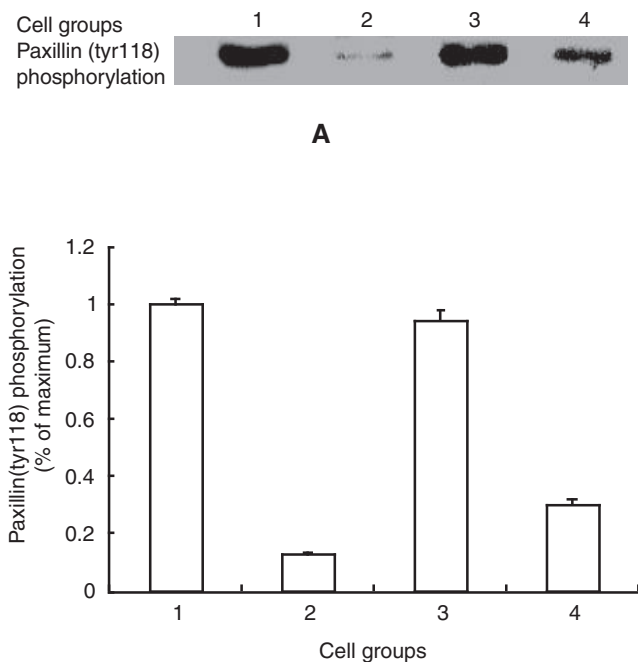
Figure 3 - Modified Boyden chamber assay to test the effect of fibronectin with gradient concentrations on the invasiveness of AGS cells (mean \pm SD, n = 5).

Effect of siRNA targeting paxillin on phosphorylation of paxillin(tyr118) in AGS cells

The transfection efficiencies in AGS cells were evaluated by immunoprecipitation and Western blotting. As shown in Figure 4, protein levels for phosphorylation of paxillin(tyr118) in AGS cells transfected with siRNA1 and siRNA2 were significantly inhibited simultaneously. However, the phosphorylation level of paxillin in the AGS cells transfected with siRNA2 decreased much less than that of cells transfected with siRNA1. To exclude the possibility that Oligofectamine mediated the expression and phosphorylation of paxillin, AGS cells were transfected temporarily with Silencer™ negative control 1 siRNA and Oligofectamine, and the cell lysates were immunoprecipitated and immunoblotted with specific antibodies as described above. As shown in Figure 4, this had no effect on the phosphorylation of paxillin at the protein level. These data indicated that the interference efficiency of siRNA1 was satisfactory and siRNA1 could therefore be employed in further studies.

Effect of paxillin silencing on phosphorylation of paxillin(tyr118) and invasiveness of AGS cells stimulated by fibronectin

As shown previously, the expression and phosphorylation of paxillin(tyr118) and the invasiveness of AGS cells reached their peak at 100 nmol/L fibronectin stim-



Cell groups: 1, AGS cells; 2, AGS cells transfected with siRNA1; 3, AGS cells transfected with Silencer™ negative control siRNA; 4, AGS cells transfected with siRNA2.

Figure 4 - Effect of 2 designed siRNAs targeting paxillin on phosphorylation of paxillin(tyr118) without fibronectin stimulation in AGS cells (mean \pm SD, n = 3).

ulation. We therefore treated 3 groups of AGS cells with 100 nmol/L fibronectin for 5 minutes in this experiment. Before fibronectin stimulation, paxillin(tyr118) phosphorylation of AGS cells transfected with siRNA1 was lower than in the other groups. There was no difference between AGS cells and AGS cells transfected with negative control siRNA ($P > 0.05$), as shown by Figure 5. After stimulation with 100 nmol/L fibronectin, the paxillin(tyr118) phosphorylation of all cell groups increased significantly ($P > 0.05$), and there were differences between the increasing amplitudes of paxillin(tyr118) phosphorylation in different cell groups. The increasing amplitude of AGS cells transfected with siRNA1 was much lower than that of AGS cells and AGS cells transfected with negative control siRNA ($P < 0.05$), while there

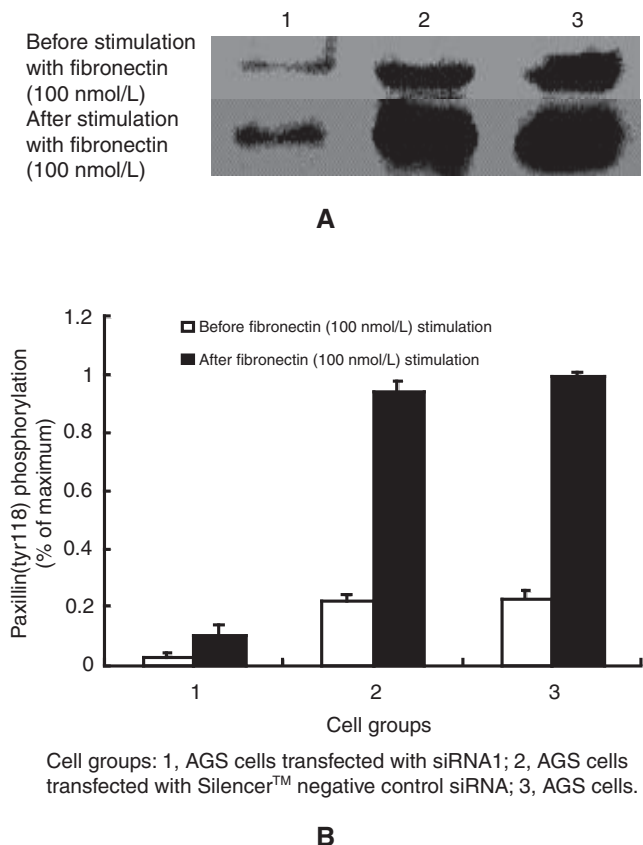


Figure 5 - Effect of siRNA targeting paxillin on phosphorylation of paxillin(tyr118) with fibronectin stimulation in AGS cells (mean ± SD, n = 3).

was no difference between the latter 2 groups ($P > 0.05$). To determine whether siRNA targeting paxillin affects the invasive ability of gastric cells stimulated by fibronectin, we used the modified Boyden chamber assay to test the invasion ability. As shown by Table 3 and Figure 6, before fibronectin stimulation there were no differences in cell invasiveness between AGS cells, AGS cells transfected with siRNA 1, and AGS cells transfected with negative control siRNA ($P > 0.05$). After stimulation by 100 nmol/L fibronectin, the invasiveness of all cell groups was significantly increased ($P < 0.05$) and there were differences in the increasing amplitude of cell in-

vasiveness between the different cell groups. The increasing amplitude of AGS cells transfected with siRNA1 was also much weaker than that of AGS cells and AGS cells transfected with negative control siRNA ($P < 0.05$), while there was no difference between the latter 2 groups ($P > 0.05$). The alteration in cell invasiveness was consistent with the phosphorylation of paxillin(tyr118) in AGS cells stimulated by fibronectin. This suggested that fibronectin induced the invasiveness of AGS cells via regulation of phosphorylation of paxillin (tyr118).

Effect of siRNA targeting FAK on phosphorylation of FAK(tyr397) and paxillin(tyr118) in AGS cells stimulated by fibronectin

The transfection efficiencies in AGS cells were evaluated by immunoprecipitation and Western blotting. Before stimulation with 100 nmol/L fibronectin, the protein level of FAK(tyr397) phosphorylation in AGS cells transfected with siRNA targeting FAK was significantly inhibited compared with that in AGS cells and cells transfected with Silencer™ negative control siRNA. The protein level for phosphorylation of paxillin(tyr118) in AGS cells transfected with siRNA targeting FAK was inhibited simultaneously, compared with that in AGS cells and cells transfected with Silencer™ negative control siRNA (Figure 7).

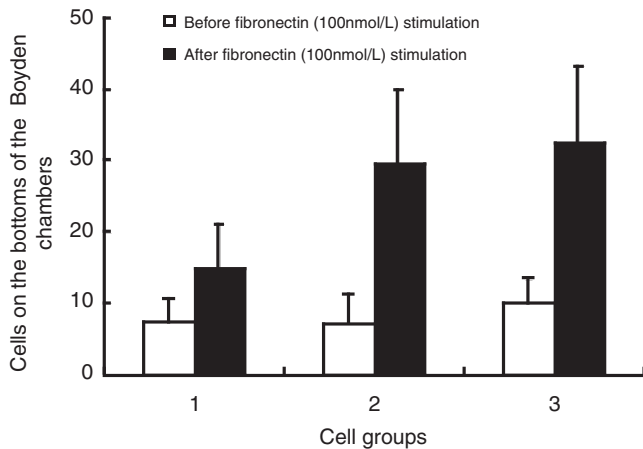
After stimulation with 100 nmol/L fibronectin, protein levels for phosphorylation of FAK(tyr397) were increased significantly in AGS cells transfected with siRNA targeting FAK, and also in AGS cells and AGS cells transfected with Silencer™ negative control siRNA. But there were significant differences in the increasing amplitudes of FAK(tyr397) phosphorylation in different cell groups. The increasing amplitude of FAK(tyr397) in AGS cells transfected with siRNA targeting FAK was much lower than that of AGS cells and AGS cells transfected with Silencer™ negative control siRNA, while there was no difference between the latter 2 groups.

After stimulation with 100 nmol/L fibronectin, protein levels for phosphorylation of paxillin(tyr118) were increased significantly in AGS cells transfected with siRNA targeting FAK, and also in AGS cells and AGS cells transfected with Silencer™ negative control siRNA. But there were significant differences in the increasing amplitudes of paxillin(tyr118) phosphorylation in different cell groups. The increasing amplitude of paxillin(tyr118)

Table 3 - Modified Boyden chamber assay to test the effect of siRNA targeting paxillin on the invasiveness of AGS cells with fibronectin stimulation (mean ± SD, n = 5)

Cell groups	1	2	3
Before stimulation with fibronectin (100nmol/L)	7.4000 ± 3.2094	7.2000 ± 4.0249	10.0000 ± 3.3912
After stimulation with fibronectin (100nmol/L)	15.0000 ± 6.1237	29.8000 ± 10.2078	32.6000 ± 10.6542

Cell groups: 1, AGS cells transfected with siRNA1; 2, AGS cells transfected with Silencer™ negative control siRNA; 3, AGS cells.



Cell groups: 1, AGS cells transfected with siRNA1; 2, AGS cells transfected with Silencer™ negative control siRNA; 3, AGS cells.

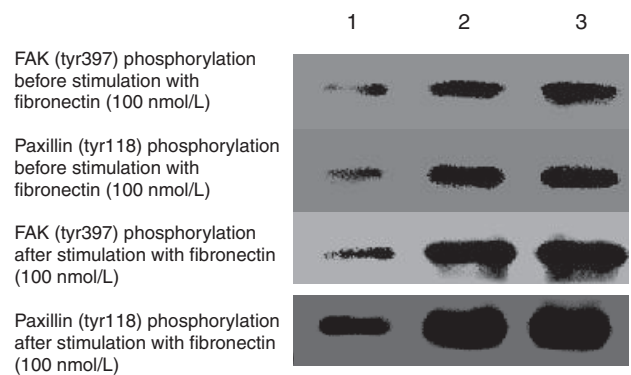
Figure 6 - Modified Boyden chamber assay to test the effect of siRNA targeting paxillin on the invasiveness of AGS cells with fibronectin stimulation (mean \pm SD, n = 5).

in AGS cells transfected with siRNA targeting FAK was much lower than that in AGS cells and AGS cells transfected with Silencer™ negative control siRNA, while there was no difference between the latter 2 groups.

Discussion

Gastric cancer is characterized by numerous abnormalities in the cytoskeleton, which is very important in cell motility and migration. The cytoskeleton of normal cells is highly stable and there is little movement of cells. When cells become cancerous, the cytoskeleton is disrupted and the motility of the cells increases as a result^{27,28}. It has not yet been fully established how cytoskeletal proteins are expressed in different tumor types. One of these proteins, the focal adhesion protein paxillin, has been suggested to be a key regulator of oncogenesis²⁹.

Paxillin is involved in focal adhesion formation during cell adhesion and migration. It contains LD motifs, LIM domains, and a Src homology 2 (SH2) and SH3 binding domain that participates in a variety of protein-protein interactions with kinases, GTPase-activating proteins, and cytoskeletal proteins³⁰. Paxillin has multiple serine/tyrosine phosphorylation sites and serves as a substrate for several tyrosine kinases such as Src, FAK, and p120BRC/ABL³¹. Tyrosine phosphorylation of paxillin occurs in response to growth factors, neuropeptides, and integrins. The major sites of tyrosine phosphorylation include tyr31 and tyr118. Phosphorylation of paxillin on tyr118 is influenced by conditions that change cell-cell adhesion³². This would confirm that paxillin is involved



Cell groups: 1, AGS cells transfected with siRNA targeting FAK; 2, AGS cells transfected with Silencer™ negative control siRNA; 3, AGS cells.

A

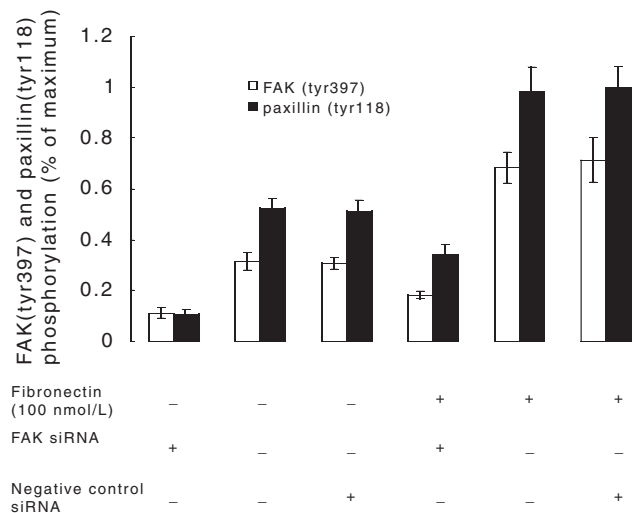


Figure 7 - Effect of siRNA targeting FAK on phosphorylation of FAK(tyr397) and paxillin(tyr118) stimulated by fibronectin in AGS cells (mean \pm SD, n = 3).

B

in the regulation of cell morphology, adhesion and migration. In addition, because of its SH3-binding domain, paxillin associates tightly with FAK and Crk in an extracellular-matrix-independent manner. Recently, tyrosine phosphorylation of paxillin and FAK has been shown to be an early response of cells to diverse signaling molecules that stimulate cell growth, differentiation and migration³³⁻³⁵. The increase in paxillin and FAK tyrosine phosphorylation is always accompanied by marked alterations in the organization of the actin cytoskeleton, which is related to invasion and metastasis of human cancer. Previous studies showed that protein kinase C (PKC) activator and 12-O-tetradecanoylphorbol-13-acetate (TPA) increased the expression of tyrosine phosphorylation in paxillin and promoted the invasion abili-

ty of human hepatocellular carcinoma cells *in vitro*³⁶. Investigators also showed that phosphorylation of paxillin at the tyr31 site increased cell motility by formation of paxillin/Crk complexes³⁷.

To get a general view of paxillin expression in human gastric cancer, we examined the expression of paxillin in gastric cancer samples. Our findings suggested that highly expressed paxillin could be generally related to the depth of tumor invasion of gastric cancer. It was interesting that paxillin expression was not correlated with TNM stage or N status, but only with T stage. So we conjecture that when gastric cancer cells connect with the extracellular matrix, paxillin is phosphorylated and its expression increased, which is a precondition for gastric tumor cell invasion. Since the effective form of paxillin is tyrosine phosphorylation of paxillin, it is thought that overexpression of paxillin results in increased paxillin phosphorylation.

In the present study we demonstrated for the first time that fibronectin caused dose-dependent tyrosine phosphorylation of paxillin(tyr118) and cell invasiveness at concentrations from 0 nmol/L to 100 nmol/L in AGS human gastric cancer cells. Experiments on paxillin silencing by RNA interference affected the motile properties of AGS cells and significantly decreased their migration toward matrigel. This finding confirmed prior results and suggested a possible cause-and-effect relationship between the 3 variables. Interestingly, while the paxillin(tyr118) phosphorylation plateaued when the concentration of fibronectin increased from 100 nm/L to 1000 nm/L, the invasiveness of AGS cells also plateaued when the concentration of fibronectin increased from 100 nm/L to 1000 nm/L. One of the possible explanations may be that when the concentration of fibronectin reaches 100 nm/L, the receptors of fibronectin (integrins) are saturated and thus cannot deliver the message of overdose fibronectin; as a result, the phosphorylation of paxillin(tyr118) and the invasiveness of AGS cells cannot augment unlimitedly. Our observation is consistent with evidence in the literature suggesting that paxillin phosphorylation is a early and necessary step in cell migration in various cell types^{38,39}.

Although the biological significance of fibronectin-mediated tyrosine phosphorylation of paxillin is not entirely clear, the fibronectin-stimulated increase in FAK activity has been verified in different laboratories⁴⁰⁻⁴². FAK binds to a number of signaling proteins via SH2 and SH3 reorganization sites and it mediates connections with proteins such as paxillin, p130^{cas}, and Src, which could link FAK to promigration signaling pathways⁴³. Previous studies in our laboratory showed that fibronectin promotes FAK tyrosine phosphorylation (tyr397) and cell invasiveness in Colo320 cells. The obtained data make it conceivable that phosphorylation of paxillin acts as a migration activator in gastric cancer downstream of FAK. However, contrary to the conclusion from the above results, other reports state that siR-

NAs knock out paxillin-enhanced spread and movement of Hela cells and stimulate activation of Rac³⁸, and that low levels of paxillin together with high levels of p130^{cas} expression may cause certain breast cancers to be more motile and possibly more aggressive⁴⁴. The reason for the discrepancy between these and our results has not been elucidated, but it might be due to different assay conditions and different histological tumor subtypes.

Elevated tyrosine 397 phosphorylation of FAK has been monitored as one of the critical factors and central processes of integrin adhesion turnover that promote cell migration⁴⁵⁻⁵⁰. Stable re-expression of epitope-tagged FAK in primary FAK-null fibroblasts promotes the reversion of a rounded FAK cell morphology to an elongated and normal fibrillar fibroblast phenotype⁵¹. Inhibition of FAK function has also been considered as a promising anticancer therapy⁴⁹. In order to further investigate the biological function of the phosphorylation of FAK(tyr397), which is mediated by paxillin, and the effect of FAK(tyr397) on the phosphorylation of paxillin(tyr118), we used siRNA targeting FAK and explored its effect on phosphorylation of paxillin(tyr118). As shown by the results, blockage of phosphorylation of FAK(tyr397) can also inhibit the phosphorylation of paxillin(tyr118) before stimulation by fibronectin, and greatly decreases the phosphorylation of paxillin(tyr118) stimulated by fibronectin. This proves that paxillin is a downstream molecule of FAK in fibronectin phosphorylated signal transduction. Inhibition of FAK(tyr397) phosphorylation is another means to inhibit paxillin(tyr118), although not as effective as direct inhibition of paxillin. This may be because there are other signaling pathways that cause phosphorylation of paxillin(tyr118) besides FAK.

In conclusion, our results clearly demonstrate that phosphorylation of paxillin(tyr118) by fibronectin induces an increase in cell invasiveness in AGS cells *in vitro*. This finding indicates that paxillin serves as a pharmacological downstream target mediated by fibronectin and FAK during tumor cell invasion. Furthermore, downregulation of paxillin by RNA interference has potential as a new anticancer strategy. For future studies, it would be useful to establish which portion of paxillin is the most important in the cellular dynamics of motility.

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