Acta Medica et Biologica
Vol. 51, No.1, 31-41, 2003

Effects of EGF on Cell Migration, and the Association of FAK and Paxillin to the Cytoskeleton

Hitomi CHIBA, Takashi KAZAMA, Yoko YAMAMOTO, Takayuki SUYAMA and Masaaki ITO

Division of Dermatology, Department of Cellular Function, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

Received November 18 2002; accepted January 14 2003

Summary. **We investigated the effects of epidermal growth factor (EGF) on cell migration, and the association of focal adhesion kinase (FAK) and paxillin to cytoskeleton. A cell line of squamous cell carcinoma (SCC) of the uterine cervix, named Caski, was used. We examined the effects of different concentrations of EGF (0, 1, 10 and 100 ng/ml) on cell migration. Wound closure assay showed that cell migration within 4 h had a biphasic dose-response curve on the concentration of EGF, and that cell migration was stimulated at 1 and 10 ng/ml, with a maximum at 10 ng/ml, and suppressed at 100 ng/ml among the concentrations examined. Immunoblot and immunoprecipitation assays of whole cell lysates obtained at 20 min after the addition of EGF demonstrated that the phosphotyrosine level of FAK and paxillin had also a biphasic response curve dependent on EGF concentration, and a maximum level was observed at 10 ng/ml among the concentrations employed. To examine the amount of cytoskeleton-associated fractions of these proteins, they were extracted after a rinse in non-ionic detergent, and analyzed by immunoblot assay. At 30 min the amount of FAK and paxillin did not differ among the concentrations examined. At 90 min, however, the amount of both proteins revealed a reverse biphasic dose-response curve, with a maximal reduction at 10 ng/ml among the concentrations examined. Integrins and actinin were not changed at each concentration of EGF examined. Immunofluorescence microscopy showed that at 30 min the expression of actin stress fibers (ASF) did not differ among cells treated with each concentration of EGF. At 90 min the ratio of cells that expressed a small number of ASF and focal adhesions (FA) was significantly increased at 10 ng/ml. Although the ratio of cells that expressed large numbers of these structures was not significantly altered, ASF were gathered at the center of cells and tangled at 100 ng/ml. These data suggest that the modulation of cytoskeletal organization by EGF plays a role in the EGF-induced stimulation of cell migration.**

Key words—EGF, actin stress fibers, focal adhesion, FAK, paxillin, tyrosine-phosphorylation.

INTRODUCTION

Cell migration plays a central role in a wide variety of biological phenomena such as embryogenesis, wound healing, inflammation processes, and tumor metastasis[1-5]. Cell migration is a spatially and temporally coordinated process composed of membrane extension, the formation and stabilization of cell-substratum adhesion at the leading edge, translocation of the cell body and the nucleus, and a release of the cell-substratum adhesion at the trailing edge[1,5,6]. The strength of the adhesion between the cell front and the rear allows for directional migration, and plays a significant role in determining the

Correspondence: Takayuki Suyama, M. D, Ph D, Div. of Dermatology, Dept of Cellular Function, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Niigata 951-8510, Japan

Abbreviations—ASF, actin stress fibers, CLSM, confocal laser scanning microscope; CMF-PBS, calcium and magnesium free phosphate-buffered saline, CSK, non-ionic detergent; DMEM, Dulbecco's Modified Eagle Medium; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGTA, ethylene glycol-bis [β-aminoethyl ether]-N, N, N', N'-tetraacetic acid; FA, focal adhesions; FAK, focal adhesion kinase; FERM, band four-point-one, ezrin, radixin, moesin, MAP, mitogen-activated protein, PAGE, poly-acrylamide gel electrophoresis; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol-3-kinase; PLC-γ, phospholipase C-gamma; ptyr, phosphotyrosine, RIPA, radioimmunoprecipitation assay; SCC, squamous cell carcinoma; SDS, sodium dodecyl sulfate, SH2, Src-homology 2; tween 20, polyoxyethylenesorbitan monolaurate; Tyr, tyrosine; VEGF, vascular endothelial growth factor

rate of cell migration[1,6]. Integrin receptor-substratum ligand interaction, and organization of the adhesive complexes and the cytoskeleton modulate the strength of cell-substratum adhesion[1,2,5-8]. Therefore, each of these factors appears to regulate cell migration.

Integrins are transmembrane receptors that bind to extracellular matrixes and cytoskeletal components[6,8]. Focal adhesions (FA) are formed at the cell-substratum points of contact by clusters of integrins and cytoskeletal components comprised of focal adhesion kinase (FAK), paxillin, Crk-associate substrate, vinculin, talin and α-actinin and other signaling enzymes[6,8,9]. FAK has recently been proposed to play a central role in formation of FA and rearrangement of the actin microfilament system, which regulates cell-substratum adhesiveness and cell migration[10,11].

EGF receptor over-expression has been correlated to tumor progression and invasion[1,5], in which enhanced cell migration appears to play a role. The binding of EGF to the receptor not only leads to the transmission of mitogenic signals but also enhances cell migration[1,5,11-14]. Previous studies showed that EGF enhances the cell migration of human squamous cell carcinoma[12] (SCC), the immortalized mammary epithelial cell[13], and the human intestinal epithelial cell[14]. However, the precise mechanism of the growth factor-induced enhancement of migration has not been sufficiently investigated. Receptors of growth factors such as epidermal growth factor (EGF), nerve growth factor[15], platelet-derived growth factor[16] (PDGF), and vascular endothelial growth factor[11] (VEGF) are associated with the cytoskeleton. In addition, the molecules that function in signal transduction, such as phospholipase C-γ (PLC-γ), phosphatidylinositol-3-kinase (PI3K), diacylglycerol kinase and mitogen-activated protein (MAP) kinase, are also associated with the cytoskeleton[1,5,17-21]. These data suggest that signaling from the EGF receptor enhances tumor cell migration by a regulation of the organization of FA and actin cytoskeleton.

The present study focused on the effects of EGF on cell migration and the organization of FA and actin stress fiber (ASF). The stimulation of cell migration and tyrosine-phosphorylation of FAK and paxillin by EGF showed a biphasic dose-response, in which the maximum effect was observed at a concentration of 10 ng/ml among the concentrations employed. In addition, the amount of cytoskeleton-associated FAK and paxillin was inversely correlated with cell migration. However, the amount of either integrin or actinin associated with cytoskeleton was not changed. These findings suggested that EGF stimulates cell migration mainly by a modulation of the cytoskeletal organization.

MATERIALS AND METHODS

Cell and cell culture

A cell line of SCC from the human uterine cervix, named Caski (American Type Culture Collection, Rockville, Maryland, USA), was used in the present study. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and penicillin (100u/ml) and streptomycin (100 μg/ml) (Gibco BRL). Cells from passages 3 to 8 were allowed to become subconfluent in 3 days at 37℃, 90% humidity, and 5% CO_2. Cells were then starved for 48 h before the experiments as described below by culture in serum-free DMEM, supplemented with penicillin and streptomycin.

Wound closure assay

In the wound assay, about 7×10^5 starved cells were seeded on bovine collagen type IV (Koken, Tokyo, Japan) (20 μg/ml) pre-coated dishes (16 mm) in serum-free DMEM and allowed to become subconfluent after incubation at 37℃ for 10 h, and then wounded in the center of the dish by one scratch with a 200 μl sterile polyethylene micropipette tip. Cells were then treated with various concentrations (0, 1, 10, or 100 ng/ml) of human recombinant EGF (Upstate Biotechnology, Lake Placid, NY, USA) for 4 h at 37℃. Photographs of the wounded areas were taken immediately before and 4 h after EGF addition with an LSM 310 confocal laser scanning microscope (CLSM) (Zeiss, Oberkochen, Germany), and analyzed using NIH image 1.61 software for Macintosh. The migration index was calculated using the following formula: $[(S1-S2)/S1]\times100$ (%), in which S1 is a cell-free area in the wound prior to EGF addition, and S2 is that after EGF treatment.

Immunoprecipitation

After a washing with calcium and magnesium free phosphate-buffered saline (CMF-PBS), the starved cells were detached by 0.05% trypsin, 1 mM EDTA in CMF-PBS, followed by treatment with a 0.05% soybean trypsin inhibitor (Sigma, Saint Louis, Missouri, USA) in serum-free DMEM. About 1×10^7 cells (70-80% of subconfluence) were seeded on dishes (100

mm) that had been pre-coated with 20 μg/ml of collagen type IV overnight at 4℃, and cultured overnight in serum-free DMEM supplemented with penicillin and streptomycin at 37℃. Cells were then treated with various concentrations of EGF (1, 10, or 100 ng/ml) or without EGF for 30 or 90 min. After a gentle rinse with 0.1 mM Na_3VO_4-CMF-PBS solution on ice, proteins were extracted with a 0.1% SDS-RIPA buffer (50 mM tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA-4Na, 1% nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 0.1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1% aprotinin, 0.1 mM Na_3VO_4). To extract cytoskeleton-associated proteins, cells were rinsed with a non-ionic detergent (CSK buffer) (10 mM tris-HCl, pH 7.2, 50 mM NaCl, 10% glycerol, 0.2% triton X-100, 1 mM EGTA, 0.1 mM dithiothreitol, 1 mM $MgCl_2$, 50 mM NaF, 1% aprotinin, 0.1 mM Na_3VO_4) for 2 min before lysation with a 0.1% SDS-RIPA buffer. The CSK buffer washes out those proteins loosely bound to the cytoskeleton so that only the proteins tightly bound to the cytoskeleton remain in the dish; these were then extracted using a 0.1% SDS-RIPA buffer. Insoluble fractions were removed by centrifugation for 20 min at 15.000 x g. Protein concentrations of the supernatants were determined by BCA protein assay (Pierce, Rockford, IL, USA) and standardized. Following this, the supernatants were first mixed with anti-FAK (4 μg/500 μg cell lysate) (Upstate Biotechnology) or an anti-paxillin (9 μg/500 μg cell lysate) (Transduction Laboratories, Lexington, KY, USA) monoclonal antibody and then augmented with goat anti-mouse monoclonal IgG pre-coated magnetic beads (Dynal®, Nihon Dynal K.K., Tokyo, Japan) on a continuous sample mixer for 2 h at 4℃, each step. Immunocomplexes were collected by an immunomagnetic separation, and were boiled with SDS-reducing (6% SDS, 40% sucrose, 0.02% bromophenol blue, 0.125 M tris-HCl) buffer for 5 min at 100℃ and stored at -80℃ in a deep freezer.

Western blotting

Equal amounts of each standardized sample were boiled with an SDS-reducing buffer and separated by SDS-PAGE (25 mM tris, 192 mM glycine, 0.1% w/v SDS, pH 8.3) (BIO RAD, Hercules, CA, USA). The proteins were transferred to a nitrocellulose membrane by electrophoresis in a transfer buffer (25 mM tris, 14.4 mM glycine, and 20% v/v methanol, pH 8.3) (BIO RAD) for 2 h at 4℃. The nitrocellulose blots were blocked with 1% bovine serum albumin (Amersham Life Science, Buckinghamshire, England) in a 0.1% tris-buffered-saline-tween 20 (Sigma) buffer (10 mM tris-HCl, pH 7.4, 10 mM NaCl, 0.1% tween 20) overnight at 4℃. Blots were then incubated with anti-FAK (1 : 1000) or anti-integrin α_1 (1 : 2000) (Chemicon International Inc., Temecula, CA, USA), or anti-integrin α_2 (1 : 2000) (Chemicon), or anti-integrin α_3 (1 : 6000) (Chemicon), or anti-integrin α_5 (1 : 3000) (Chemicon) polyclonal antibody or anti-paxillin (1 : 10000), or anti-α-actinin (1 : 5000) (Upstate Biotechnology), or anti-integrin β_1 (1 : 3000) (Chemicon) monoclonal antibodies, or an anti-phosphotyrosine-horseradish-peroxidase-conjugated antibody (1 : 2000) (Amersham Life Science) overnight at 4℃, followed by incubation with a horseradish-peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit antibody (Dako, Glostrup, Denmark) for 2 h at room temperature. Proteins were detected by using ECL or ECL plus western blotting detection reagents system (Amersham Pharmacia Biotech, Buckinghamshire, England).

Fluorescence microscopy

Starved cells were detached by trypsinization (0.05% trypsin, 1 mM EDTA in CMF-PBS), followed by treatment with a 0.05% trypsin inhibitor solution. Cells were seeded at a concentration of 5×10^5/ml on dishes (15 mm) pre-coated with 20 μg/ml type IV collagen, and cultured in serum-free DMEM overnight at 37℃. They were then incubated with various concentrations of EGF (1, 10 or 100 ng/ml) for 30 or 90 min, or without EGF at 37℃. They were then rinsed with a CSK buffer for 2 min followed by fixation with 4% paraformaldehyde for 30 min at room temperature. For analysis of FA and ASF, cells were incubated with an anti-paxillin (10 μg/ml) monoclonal antibody, and incubated with a fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (Dako) and then with tetramethylrhodamine isothiocyanate-conjugated phalloidin (Molecular Probes, Eugene, Oregon, USA). For the analysis of ASF expression, cells were incubated with fluorescein isothiocyanate-conjugated phalloidin, and propidium iodide (Wako, Osaka, Japan). One hundred cells in each experimental condition were observed under a LSM 310 CLSM, and every confocal image was obtained under the same conditions (pinhole size, contrast, enhancement).

RESULTS

Effect of EGF on cell migration

The migration index was analyzed by calculating the

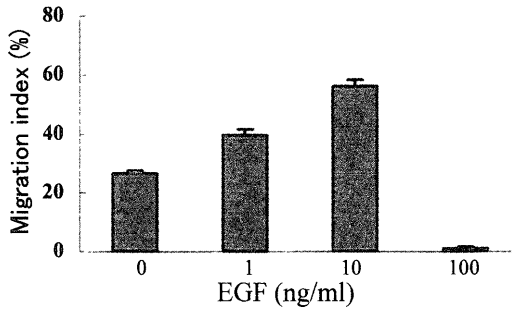

Fig. 1. Migration index examined by wound closure assay. Cells were attached in a monolayer and wounded in the center of the dish by a single scratch immediately before the addition of EGF, and photographs were taken immediately before and 4 h after the treatment. The migration index was calculated by the formula described in Materials and Methods.

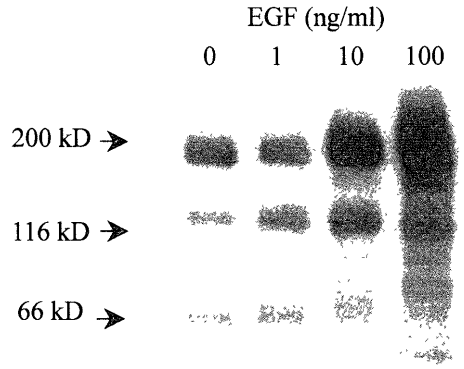

Fig. 2. Immunoblot analysis of phosphotyrosine of proteins. Whole cell lysates from cells treated with 0, 1, 10, or 100 ng/ml EGF for 20 min were separated on SDS-PAGE, transferred onto nitrocellulose, and reprobed with anti-phosphotyrosine.

ratio of a wound area covered by migrating cells for 4 h to an initial wound area. The migration index of cells treated with each concentration of EGF is shown in Fig. 1. Among the concentrations examined, cell migration was stimulated at 1 and 10 ng/ml. The migration index of the control group (cells cultured without EGF) was 26.4%, and the indices were 39.6% and 56.3% in cells treated with 1 and 10 ng/ml EGF, respectively. In contrast, among the concentrations examined, cell migration was significantly suppressed at 100 ng/ml, for which the index was only 1%.

Tyrosine-phosphorylation of FAK and paxillin

To study the tyrosine-phosphorylation of proteins in whole cell lysates, cells treated with the various concentrations of EGF for 20 min were lysed with a 0.1% SDS-RIPA buffer, and processed for immunoblot assay with an anti-phosphotyrosine antibody. The salient findings are shown in Fig. 2. In the absence of EGF proteins of 170, 125 and 68-kD were tyrosine phosphorylated. The tyrosine-phosphorylation level of 170-kD protein, which corresponds to the EGF receptor, was increased in a dose-dependent manner; was remarkably enhanced at 10 and 100 ng/ml, and reached a maximum at 100 ng/ml among the concentrations employed. In contrast, the tyrosine-phosphorylation level of 125-kD and 68-kD proteins was highest at 10 ng/ml and slightly down regulated at 100 ng/ml among the concentrations examined.

The activation of FAK plays an important role in integrin-mediated and growth factor mediated cell adhesion, spreading, and migration[8,11,14–16,21–23]. To examine whether the 125-kD and 68-kD proteins were FAK and paxillin, respectively, whole cell lysates from cells stimulated with EGF for 20 min were immunoprecipitated with a monoclonal antibody to FAK or paxillin and analyzed by immunoblot with anti-phosphotyrosine. Then these bands were reprobed with an anti-FAK or anti-paxillin antibody. The salient findings are shown in Fig. 3. Tyrosine-phosphorylation levels of FAK and paxillin exhibited a bell-shaped dose-response curve; it was highest at 10 ng/ml among the concentrations of EGF examined. Since the amount of the protein in the band of the lower lanes in Fig. 3 did not differ among the concentrations of EGF as shown by reprobing with an anti-FAK or anti-paxillin antibody, the thickness and the density of each band in the upper lanes of Fig. 3 reflected the level of tyrosine-phosphorylation.

Changes in the amount of cytoskeleton-associated FAK and paxillin

To estimate the effects of EGF on the association of FAK, paxillin, α-actinin, and integrins with the cytoskeleton, cell lysates were obtained with an RIPA buffer after cells were rinsed with a CSK buffer, and examined by immunoblot assay. The results are shown in Fig. 4 and 5.

At 30 min after the addition of EGF, the amount of cytoskeleton-associated FAK was slightly increased, whereas that of paxillin was unchanged (Fig. 4) among the concentrations employed. At 90 min after the addition of EGF, the amount of cytoskeleton-associated FAK was significantly decreased at 10 ng/ml among the concentrations examined, whereas the amount of that protein was increased at 100 ng/ml when compared with the control. The amount of paxillin associated with the cytoskeleton was de-

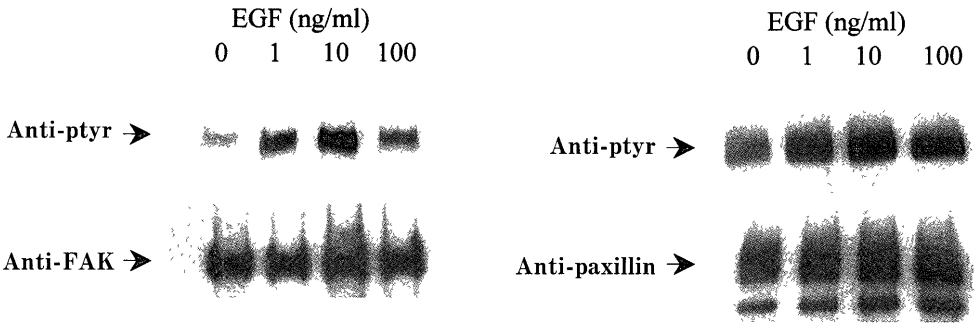

Fig. 3. Immunoblot analysis of phosphotyrosine of FAK and paxillin. Whole cell lysates from cells treated with 0, 1, 10, or 100 ng/ml EGF for 20 min were immunoprecipitated with a monoclonal antibody to FAK or paxillin. Immunocomplexes were separated on SDS-PAGE, transferred onto nitrocellulose, and probed with anti-phosphotyrosine, then reprobed with its corresponding antibody to the anti-FAK polyclonal antibody, or the anti-paxillin monoclonal antibody.

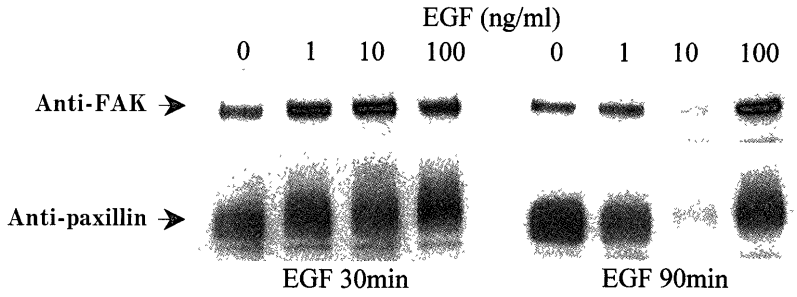

Fig. 4. Immunoblot analysis of cytoskeleton-associated FAK and paxillin. After a rinse in non-ionic detergent (CSK buffer), proteins were extracted from cells treated with 0, 1, 10 or 100 ng/ml EGF for 30 or 90 min. Equal amounts of the proteins were separated on SDS-PAGE, transferred onto nitrocellulose, and probed with the anti-FAK polyclonal antibody or anti-paxillin monoclonal antibody.

creased slightly at 1 ng/ml, and significantly so at 10 ng/ml among the concentrations employed. In contrast to these signal molecules, the amount of cytoskeleton-associated α-actinin, integrin α_1 and α_5 (data not shown), and integrin α_2, α_3 and β_1 (Fig. 5) was not changed among the concentrations of EGF examined.

Immunofluorescence micrographs of ASF and FA

To study the EGF effects on the formation of ASF and FA, cells were fixed and processed for immunohistochemical analysis. After the cells were incubated with the various concentrations of EGF for 30 or 90 min, they were fixed, permealized and immunostained with mouse monoclonal anti-paxillin and fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG, labeled with tetramethylrhodamine isothiocyanate-conjugated phalloidin, and then observed with CLSM.

FA, which was stained with anti-paxillin (green dots stained with anti-paxillin monoclonal antibody and fluorescein-conjugated anti-mouse IgG), was localized on both the edges of ASF (red bars stained with tetramethylrhodamine isothiocyanate-conjugated phalloidin) (Figs. 6a-e). Cells were divided by fluorescence microscopic findings into 4 groups by the number and thickness of ASF: cells with a large number of thick stress fibers (Fig. 6e), those with a moderate number of stress fibers (Fig. 6b and c), those with a small number of stress fibers (Fig. 6a), and those with thin and faint stress fibers (Fig. 6d). Fig. 7 shows the percentage of each type of cells at each concentration of EGF. Cells of all 4 types were observed at each concentration of EGF, although the ratio of each cell type differed. At 30 min after incubation with EGF, the expression of ASF (Fig. 7) and the intensity of the green fluorescence (data not

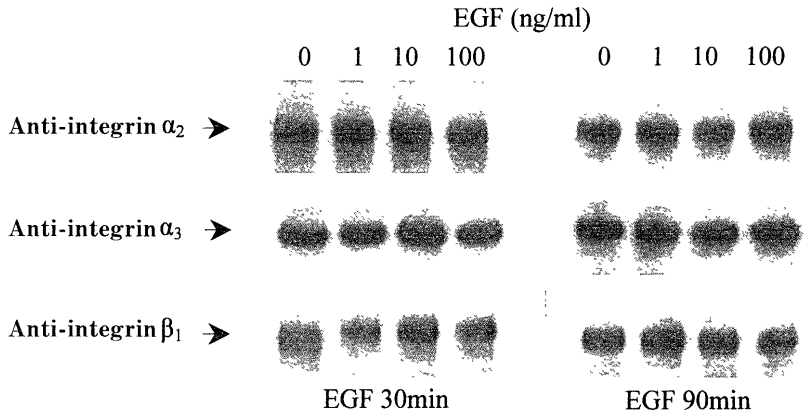

Fig. 5. Immunoblot analysis of cytoskeleton-associated integrin. After a rinse in a CSK buffer, proteins were extracted from cells treated with 0, 1, 10 or 100 ng/ml EGF for 30 or 90 min. Equal amounts of the proteins were separated on SDS-PAGE, transferred onto nitrocellulose, and probed with anti-integrins α_2, α_3, and β_1 polyclonal antibody.

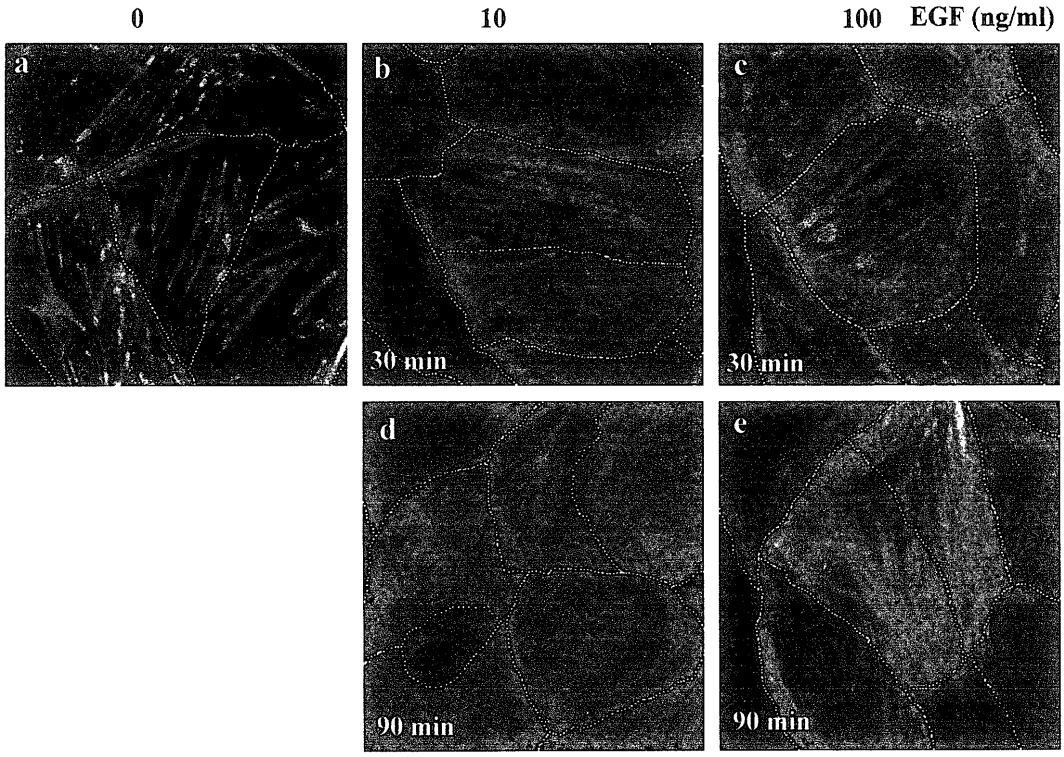

Fig. 6. Immunofluorescence micrographs of ASF and FA. Cells attached in the monolayer were treated with 0, 1, 10, or 100 mg/ml EGF for 30 or 90 min. After a rinse in a CSK buffer, they were fixed, permeabilized, and stained for paxillin and ASF. CLSM micrographs from the bottom level of the cells shows that FA (green dots stained with anti-paxillin monoclonal antibody and fluorescein-conjugated anti-mouse IgG) localizes on both edges of ASF (red bars stained with tetramethyl-rhodamine isothiocyanate-conjugated phalloidin). The intensity of green fluorescence and formation of ASF were not remarkably changed at 30 min of EGF 10 and 100 ng/ml (**b** and **c**). At 90 min after the addition of EGF 10 ng/ml, the green fluorescence of FA has been reduced or almost eliminated from the edges of ASF from the cells that are composed of thin and faint stress fibers (**d**). In the presence of EGF 100 ng/ml, in cells that are composed of thick ASF, the stress fibers gather in the center and are tangled; however, the intensity of the green fluorescence of these cells is only slightly reduced compared with the control (**e**). (**a**) EGF 0 ng/ml; (**b** and **d**) EGF 10 ng/ml; (**c** and **e**) EGF 100 ng/ml; (**b** and **c**) EGF incubation for 30 min; (**d** and **e**) EGF incubation for 90 min; (*white dotted line*) sketch line of the outer form of cell.

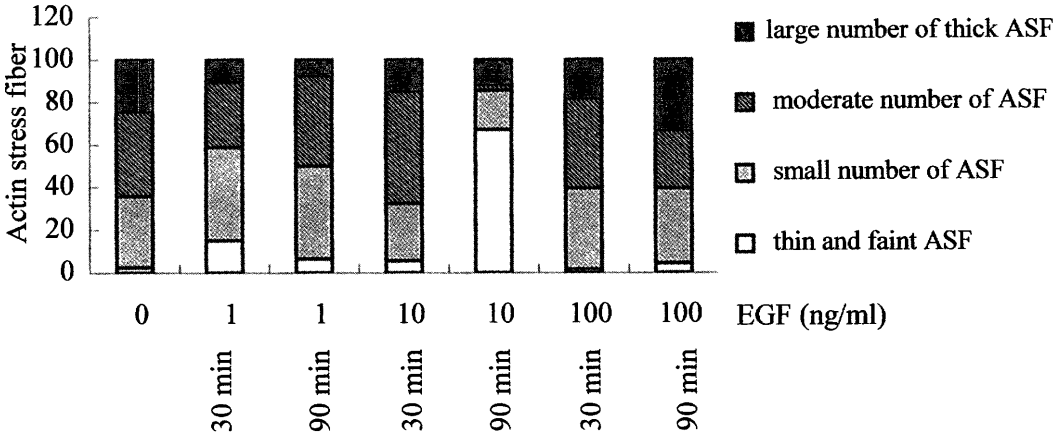

Fig. 7. Ratios of the 4 cell types classified by expression of ASF. Cells attached in the monolayer were treated with 0, 1, 10, or 100 mg/ml EGF for 30 or 90 min. After a rinse in a CSK buffer, they were fixed, permeabilized, and incubated with fluorescein-conjugated phalloidin for the visualization of ASF. Cells were divided into 4 groups by their expression of ASF: Cells with a large number of thick stress fibers (*closed area*), those with a moderate number (*hatched area*), those with a small number (*dotted area*), and those with thin and faint ones (*open area*).

shown) was slightly suppressed in cells treated with 1 ng/ml EGF, whereas these were not changed in those with 10 or 100 ng/ml EGF compared with the control cells. At 90 min after the incubation, the formation of ASF (Fig. 7) and green fluorescence intensity (data not shown) was also slightly suppressed at 1 ng/ml compared with the control. However, the formation of ASF was remarkably suppressed in cells treated with 10 ng/ml. About 70% of the cells were those with a small number of thin and faint stress fibers (Fig. 6d). The intensity of the green fluorescence on both edges of the stress fiber was reduced or had almost disappeared in cells with small numbers of thin and faint stress fibers (Fig. 6d). These findings agree with the data of Fig. 4 and indicate a reduction in paxillin amount from FA. In the presence of EGF 100 ng/ml, although the percentages of cells with a large number of thick ASF were only slightly increased, the stress fibers gathered in the center of the cells and became tangled, and the intensity of the green fluorescence of these cells did not remarkably differ from that of the control cells (Fig. 6e). Stress fibers in cells treated without EGF (Fig. 6a) or those with 1 (data not shown) or 10 ng/ml (Fig. 6b, d) of EGF were generally distributed evenly in the entire cell, were not tangled, and maintained a distance from one another.

DISCUSSION

The results of the present study demonstrate that: 1) cell migration showed a biphasic response dependent on the concentration of EGF, reaching a maximum at 10 ng/ml among the concentrations examined; 2) the tyrosine-phosphorylation of FAK and paxillin also revealed a biphasic response similar to that of cell migration; 3) 90 min after the addition of EGF, the amount of cytoskeleton-associated FAK and paxillin showed a biphasic response inverse to that of cell migration, whereas cytoskeleton-associated integrins were not changed; and 4) 90 min after the addition of the growth factor the formation of FA and ASF was maximally suppressed at 10 ng/ml among the concentrations examined. These findings indicate a close correlation among cell migration, the tyrosine-phosphorylation of FAK and paxillin, the association of these signal molecules with the cytoskeleton, and the formation of FA and ASF in the EGF-induced stimulation of cell migration.

In the present study we employed a wound closure assay to examine cell migration. Previous studies showed that wound closure is associated with both cell migration and cell proliferation[13]. Previous studies also demonstrated that EGF enhances the wound closure through the stimulation of both events, and that the latter is an important mechanism for enhanced epithelial repair, especially during the initial 48–72 h[13]. In the present study, however, we examined wound closure for 4 h. The present carcinoma cells examined did not show cell proliferation during the first 4 h in the presence or absence of EGF at the concentrations applied (data not shown). Therefore, stimulation of the wound closure induced by EGF observed in the present study was solely due to an increase in cell migration.

Cell migration speed is governed by cell-substratum adhesiveness, measured as short-term cell-substratum adhesion strength[13,24]. Cells show a biphasic migration speed dependent on the adhesion strength, and their maximum speed attainable remains unchanged as these factors vary[24]. The strength of the cell-substratum adhesiveness is determined by integrin-ligand interactions and cytoskeletal organization[24], the former being associated with the substratum ligand level, integrin expression level, and integrin-ligand affinities[25-27]. Since the substratum ligand level was fixed in the present study, the latter two factors may be associated with the present EGF-induced modulation of cell migration.

EGF stimulates α_2 integrin expression in human SCC[12] and immortalized mammary epithelial cell[13], and the selective up-regulated expression has been suggested to play a role in the EGF stimulation of cell migration. In these studies, cell migration was assayed for 18 or 24 h, and the expression level of integrin was examined after the corresponding incubation time. In the present study, however, we assayed cell migration for 4 h. Immunoblot assay demonstrated that the amount of all integrin subunits, including α_2 integrin, was unchanged at 90 min among the concentrations employed. In addition, no induction of RNA transcription or protein synthesis occurred within 4 h after stimulation[8,26]. Therefore, the expression level of integrins did not change during the present wound closure assay. Recent studies have demonstrated that the Ras-associated suppression of integrin activity could occur in association with the MAP kinase pathway or independently via a novel, undefined pathway[27-30]. Ras plays a pivotal role in the transduction of signaling events initiated by both the tyrosine kinase growth factor and G-protein-coupled receptors[27]. These data suggest the possibility that EGF weakens integrin-ligand interaction by suppressing integrin-ligand affinity, thus modulating cell migration, although this possibility remains to be studied.

The present study further suggests that EGF stimulates cell migration mainly through modulation of the cytoskeletal organization. At 90 min after the addition of EGF, immunohistochemical examination showed that ASF and paxillin-containing FA were the most significantly decreased at 10 ng/ml among the concentrations examined. Cell migration was maximally enhanced at this concentration of the growth factor among the concentrations employed. This finding is consistent with the well-known findings that the formations of FA and ASF are inversely correlated with cell adhesiveness[5,20,31], and that highly motile cells often lack easily distinguishable FA[20]. Our study also was first to reveal that the association of FAK and paxillin with the cytoskeleton was decreased slightly at 1 and most significantly at 10 ng/ml among the concentrations examined. It seems likely that the concentration in which the maximum effects were obtained would be more than 10 ng/ml if the same assays had been performed at several points between 10 and 100 ng/ml. In addition, there is a possibility that each maximum effect was obtained at a different EGF concentration. However, it can be said that the correlation among the enhancement of cell migration and tyrosine-phosphorylation of FAK and paxillin, and the suppression of the cytoskeletal association of both the proteins was observed at the lower concentration of EGF. The alteration in the formation of FA and the actin cytoskeleton induced by the activation of FAK possibly involves a myosin-based contraction via a MAP kinase-activated myosin light channel kinase function[5,21,32]. Tyrosine-phosphorylated FAK also interacts with PI3K[19] and PLC-γ[33], for which activations are also related with the actin cytoskeleton and FA. PLC-γ leads to a mobilization of actin modifying proteins that act on F-actin and G-actin filaments[5]. The role of PI3K is more complex, and this molecule plays a role in the activation of small GTPases, rac and rho[5]. The activation of the rac molecule involves actin cytoskeleton ruffling and the destabilization of FA[34], and activation of the rho molecule has been implicated in the formation of new FA[35]. How these molecules alter the actin cytoskeleton and FA formation is not clearly known, but they have been associated with a myosin-based function[36]. Immunoblot assay demonstrated that EGF did not change the cytoskeletal association of either integrin or a focal adhesion-associated non-signaling molecule, α-actinin. These findings suggest that changes in the incorporation of the signal molecules, FAK and paxillin, into FA play a primary role in changes of the cytoskeletal organization, which induces the changes in cell-substratum adhesiveness. Although we did not examine immunohistochemically whether the amount of integrins in FA was changed, the employed immunoblot assay suggests that no quantitative alteration of integrin was involved in EGF stimulation of cell migration among the concentrations examined, though changes in the integrin affinity may have participated in it. In addition, 30 min after the addition of EGF, neither immunoblot nor immunohistochemical examination showed significant changes among the concentrations of EGF examined. This can be explained by the time lag that is necessary for cells to complete the transition to

more motile phenotypes.

Tyrosine-phosphorylation of FAK has been implicated in the formation of FA[10-12,37] and rearrangements of ASF[11]. The activated EGF receptor forms a complex with FAK through its FERM homology region within the N-terminal domain, which in turn induces the tyrosine-phoshorylation of FAK-Tyr-397 site[21,22]. In the present study, the tyrosine-phosphorylation of FAK and paxillin showed a biphasic response dependent on the concentration of EGF. A similar biphasic response of tyrosine-phosphorylation of FAK was observed in Swiss 3T3 cell and endothelial cells stimulated with VEGF and PDGF, respectively[11,16]. The precise mechanism that induces the down regulation of tyrosine-phosphorylation of FAK by a high concentration of EGF is not yet known. However, over-expression of the C-terminal domain of FAK disrupts a complex formation with activated growth factor receptor and FAK, and dephosphorylates FAK at the Tyr-397 site, which reduces the propagation of downstream events and inhibits cell migration[21,22,38,39]. These data suggest that there may be a negative feedback pathway in the EGF-induced tyrosine-phosphorylation of FAK, which plays a role in the inhibition of cell migration by the high concentration of the growth factor.

In contrast to the lower concentration of EGF, the same growth factor rather significantly suppressed cell migration at 100 ng/ml among the concentrations examined. The migration index of cells at 100 ng/ml was significantly lower than that of the control cells (those treated without EGF). Although tyrosine-phosphorylation was down regulated at EGF 100 ng/ml, it did not reveal a significant difference in the tyrosine-phosphorylation level of FAK and paxillin compared with the control cells, or that also in the amount of the cytoskeleton-associated proteins, or the ratio of the 4 types of cells classified by the number and thickness of ASF. However, ASF gathered in the center, and their arrangement was disturbed in some cells treated with 100 ng/ml EGF. These findings were not observed in cells treated with a lower concentration of EGF or those without the growth factor. It has been shown that the phenotypes of cells based on the organization of the actin cytoskeleton are related to cell migration[40]. Although no definitive evidence was obtained in the present study, these findings suggest the possibility that the function of the actin cytoskeleton was disturbed by the high concentration of EGF, and cell migration was inhibited.

Acknowledgments. The author wishes to thanks Dr. Eishin Yaoita (Div. of Structural Pathology, Dept. of Nephrology, Niigata University Graduate School of Medical and Dental Sciences) for his valuable scientific suggestions. This work was supported in part by grants from Ministry of Education, Culture, Sports, Science, and Technology of Japan and by grants from the Div. of Dermatology, Niigata University Graduate School of Medical and Dental Sciences.

REFERENCES

1) Wells A, Gupta K, Chang P, Swindle S, Glading A, Shiraha H: Epidermal growth factor receptor-mediated motility in fibroblasts. *Microsc Res Tech* **43:** 395-411, 1998.
2) Burridge K, Turner CE, Romer LH: Tyrosine phosphorylation of paxillin and pp125^{FAK} accompanies cell adhesion to extracellular matrix: A role in cytoskeletal assembly. *J Cell Biol* **119:** 893-903, 1992.
3) Gross RJ: Regeneration versus repair. In: Cohen IK, Diegelmann RF, Lindblad WJ (eds) Wound healing, biochemical and clinical aspects. Saunders Company, Philadelphia 1992, p 20-39.
4) Wahl LM, Wahl Sharon: Inflammation. In: Cohen IK, Diegelmann RF, Lindblad WJ (eds) Wound healing, biochemical and clinical aspects. Saunders Company, Philadelphia 1992, p 40-55.
5) Wells A: Tumor invasion: Role of growth factor-induced cell motility. *Adv Cancer Res Acad Press* **78:** 31-101, 2000.
6) Gumbiner BM: Cell adhesion: The molecular basis of tissue architecture and morphogenesis. *Cell* **84:** 345-357, 1996.
7) Huttenlocher A, Ginsberg M, Horwitz AF: Modulation of cell migration by integrin-mediated cytoskeletal linkers and ligand-binding affinity. *J Cell Biol* **134 (6):** 1551-1562, 1996.
8) Cheresh D, Mecham R: Integrins as signaling transduction receptors. In: Molecular and biological responses to the extracellular matrix. Acad Press, California, 1994, p 34-35.
9) Albert B, Bray D, Lewis J, Raff M, Roberts R, Watson JD: The Cytoskeleton. In: Molecular biology of the cell. Third Ed. Garland Publishing, 1994, p 787-861.
10) Iiić D, Furuta K, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T, Aizawa S: Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* **377:** 539-544, 1995.
11) Abedi H, Zachary I: Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J Biol Chem* **272 (24):** 15442-15451, 1997.

12) Fujii K, Dousaka-Nakajima N, Imamura S: Epidermal growth factor enhancement of HSC-1 human cutaneous squamous carcinoma cell adhesion and migration on type I collagen involves selective up-regulation of $\alpha_2\beta_1$ integrin expression. *Exp Cell Res* **216:** 261-272, 1995.
13) Matthay MA, Thiery JP, Lafont F, Stampfer F, Boyer B: Transient effect of epidermal growth factors on the motility of an immortalized mammary epithelia cell line. *J Cell Sci* **106:** 869-878, 1993.
14) Liu Y-W, Sanders MA, Basson MD: Human caco-2 intestinal epithelial motility is associated with tyrosine kinase and focal adhesion kinase signals. *J Surg Res* **77:** 112-118, 1998.
15) Rhee S, Lee KH, Kim D, Kwon YK, Kang MS, Kwon H: Sustained formation of focal adhesions with paxillin in morphological differentiation of PC12 cells. *Mol Cells* **10:** 169-179, 1999.
16) Rankin S, Rozengurt E: Platelet-derived growth factor modulation of focal adhesion kinase (p125FAK) and paxillin tyrosine phosphorylation in Swiss 3T3 cells. Bell-shaped dose response and cross-talk with bombesin. *J Biol Chem* **269:** 704-710, 1994.
17) Payrastre B, van Bergen en Henegouwen PMP, Breton M, der Hartigh JC, Plantavid M, Verkleij AJ, Boonstra J: Phosphoinositide kinase, diacylglycerol kinase, and phospholipase C activities associated to the cytoskeleton: Effect of epidermal growth factor. *J Cell Biol* **115 (1):** 121-128, 1991.
18) Polk DB: Epidermal growth factor receptor-stimulated intestinal epithelial cell migration requires phospholipase C activity. *Gastroenterol* **114:** 493-502, 1998.
19) Chen HC, Appeddu PA, Isoda H, Guan JL: Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. *J Biol Chem* **271 (42):** 26329-26334, 1996.
20) Xie H, Pallero MA, Gupta K, Chang P, Ware MF, Witke W, Kwiatkowski DJ, Lauffenburger DA, Murphy-Ullrich JE, Wells A: EGF receptor regulation of cell motility: EGF induces disassembly of focal adhesions independently of the motility-associated PLCγ signaling pathway. *J Cell Sci* **111:** 615-624, 1998.
21) Hauck CR, Sieg DJ, Hsia DA, Loftus JC, Gaarde WA, Monia BP, Schlaepfer D: Inhibition of focal adhesion kinase expression or activity disrupts growth factor-stimulated signaling promoting the migration of invasive human carcinoma cells. *Cancer Res* **61:** 7079-7090, 2001.
22) Sieg DJ, Hauck CR, Llic D, Klingbeil CK, Schaefer E, Damsky CH, Schlaepfer DD: FAK integrates growth factor and integrin signals to promote cell migration. *Natur Cell Biol* **2:** 249-256, 2000.
23) Ueki K, Mimura T, Nakamoto T, Sasaki T, Aizawa S, Hirai H, Yano S, Naruse T, Nojima Y: Integrin-mediated signal transduction in cells lacking focal adhesion kinase p125^{FAK}. *FEBS* **432:** 197-201, 1998.
24) Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA, Horwitz AF: Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature* **385 (6616):** 537-540, 1997.
25) Friedl P, Bröcker E-B, Zänker KS: Integrins, cell matrix interactions and cell migration strategies: Fundamental differences in leukocytes and tumor cells. *Cell Adhes Commun* **6:** 225-236, 1998.
26) Danen EHJ, Lafrenie RM, Miyamoto S, Yamada K: Integrin signaling: Cytoskeletal complexes, MAP kinase activation, and regulation of gene expression. *Cell Adhes Commun* **6:** 217-224, 1998.
27) Hughes PE, Renshaw MW, Pfaff M, Forsyth J, Keivens VM, Schwartz MA, Ginsberg MH: Suppression of integrin activation: A novel function of a Ras/Raf-induced MAP kinase pathway. *Cell* **88:** 521-530, 1997.
28) Hughes PE, Oertli B, Hansen M, Chou FL, Willumsen BM, Ginsberg, MH: Suppression of integrin activation by activated Ras or Raf does not correlate with bulk activation of ERK MAP kinase. *Mol Biol Cell* **13 (7):** 2256-2265, 2002.
29) Baumann B, Weber CK, Troppmair J, Whiteside S, Israel A, Rapp UR, Wirth T: Raf induces NF-KB by membrane shuttle kinase MEKK1, a signaling pathway critical for transformation. *Proc Natl Acad Sci USA* **97 (9):** 4615-4620, 2000.
30) Pearson G, Bumeister R, Henry DO, Cobb MH, White MA: Uncoupling Raf1 from MEK1/2 impairs only a subset of cellular responses to Raf activation. *J Biol Chem* **275 (48):** 37303-37306, 2000.
31) Sondermann H, Dogic D, Pesch M, Aumailley M: Targeting of cytoskeletal linker proteins to focal adhesion complexes is reduced in fibroblasts adhering to laminin-1 when compared to fibronectin. *Cell Adhes Commun* **7:** 43-56, 1999.
32) Klemke RL, Cai S, Giannini AL, Gallagher PJ, de Lanerolle P, Cheresh DA: Regulation of cell motility by mitogen-activated protein kinase. *J Cell Biol* **137 (2):** 481-492, 1997.
33) Zhang X, Chattopadhyay A, Ji QS, Owen JD, Ruest PJ, Carpenter G, Hanks SK: Focal adhesion kinase promotes phospholipase C-gammal activity. *Proc Natl Acad Sci USA* **96 (16):** 9021-9026, 1999.
34) Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A: The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70 (3):** 401-410, 1992.
35) Ridley AJ, Hall A: The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70 (3):** 389-399, 1992.
36) Fukata Y, Kimura K, Oshiro N, Saya H, Matsuura Y, Kaibuchi K: Association of the myosin-binding subunit of myosin phosphatase and moesin: Dual regulation of moesin phosphorylation by Rho-associated kinase and myosin phosphatase. *J Cell Biol* **141 (2):** 409-418, 1998.
37) Polte TR, Hanks SK: Complexes of focal adhesion

kinase (FAK) and Crk-associated substrate ($p130^{Cas}$) are elevated in cytoskeleton-associated fractions following adhesion and Src transformation. Requirements for Src kinase activity and FAK proline-rich motifs. *J Biol Chem* **272:** 5501-5509, 1997.

38) Hauck C, Hsia DA, Schlapfer DD: Focal adhesion kinase facilitates platelet-derived growth factor-BB-stimulated ERK2 activation of vascular smooth muscle cells. *J Biol Chem* **275 (52):** 41092-41099, 2000.

39) Richardson A, Malik RK, Hildebrand JD, Parsons JT: Inhibition of cell spreading by expression of the C-terminal domain of focal adhesion kinase is rescued by coexpression of Src or catalytic inactive FAK: a role for paxillin tyrosine phosphorylation. *Mol Cell Biol* **17 (12):** 6906-6914, 1997.

40) Pokorna E, Jordan PW, O'Neil CH, Zicha D, Gilbert CS, Veseley P: Actin cytoskeleton and motility in rat sarcoma cell populations with different metastatic potential. *Cell Motil Cytoskeleton* **28(1):** 25-33, 1994.