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Focal adhesion kinase regulates intestinal epithelial barrier function via redistribution of tight junction

Yanju Ma [a,d], Shingo Semba [a], Md Rafiqul Islam Khan [a], Hiroki Bochimoto [b], Tsuyoshi Watanabe [b], Mikihiro Fujiya [c], Yutaka Kohgo [c], Yunpeng Liu [d], Takanobu Taniguchi [a,*]

[a] Division of Cellular Signal Transduction, Department of Biochemistry, Asahikawa Medical University, Asahikawa 078-8510, Japan
[b] Division of Microscopic Anatomy and Cell Biology, Department of Anatomy, Asahikawa Medical University, Asahikawa 078-8510, Japan
[c] Division of Gastroenterology and Hematology/Oncology, Department of Internal Medicine, Asahikawa Medical University, Asahikawa 078-8510, Japan
[d] Department of Medical Oncology, The First Hospital of China Medical University, Shenyang 110001, China

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ABSTRACT

Disruption of epithelial barrier function was identified as one of the pathologic mechanisms in inflammatory bowel diseases (IBD). Epithelial barrier consists of various intercellular junctions, in which the tight junction (TJ) is an important component. However, the regulatory mechanism of tight junction is still not clear. Here we examined the role of focal adhesion kinase (FAK) in the epithelial barrier function on Caco-2 monolayers using a specific FAK inhibitor, PF-573, 228 (PF-228). We found that the decrease of transepithelial resistance and the increase of paracellular permeability were accompanied with the inhibition of autophosphorylation of FAK by PF-228 treatment. In addition, PF-228 inhibited the FAK phosphorylation at Y576/577 on activation loop by Src, suggesting Src-dependent regulation of FAK in Caco-2 monolayers. In an ethanol-induced barrier injury model, PF-228 treatment also inhibited the recovery of transepithelial resistance as well as these phosphorylations of FAK. In a sucrose gradient ultracentrifugation, FAK co-localized with claudin-1, an element of the TJ complex, and they co-migrate after ethanol-induced barrier injury. Immunofluorescence imaging analysis revealed that PF-228 inhibited the FAK redistribution to the cell border and reassembly of TJ proteins in the recovery after ethanol-induced barrier injury. Finally, knockdown of FAK by siRNA resulted in the decrease of transepithelial resistance. These findings reveal that activation of FAK is necessary for maintaining and repairing epithelial barrier in Caco-2 cell monolayer via regulating TJ redistribution.
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1. Introduction

Epithelial cells lining the lumens of gastrointestinal tract act as selective physical barriers that regulate transepithelial movement of solutes, ions, water and hostile substance. A number of recent studies have suggested that defective intestinal epithelial barrier function is an important pathogenic factor contributing to the development of intestinal inflammation [1–3]. Although the pathogenesis of ulcerative colitis (UC) and Crohn's disease, which are known as inflammatory bowel diseases (IBD), remains unclear, a number of studies suggest that the destruction of the gastrointestinal tract integrity by the alteration of epithelial barrier function was identified as one of pathologic mechanisms in IBD [4].

Epithelial barrier consists of various intercellular junctions, such as adherens junction, gap junction, desmosome and tight junction (TJ). The TJ is the most apical component of the epithelial junctional complex [5], acting as gates of the paracellular pathway by controlling the extent and selectivity of diffusion and as fences that keep the identity of plasma membrane domains by forming an apical–basolateral intramembrane diffusion barrier [6]. TJ consists of transmembrane proteins, such as claudin and occludin. These proteins form the paracellular seals by interaction of extracellular domain with each other, and the cytoplasmic plaque by interaction of intercellular domain and various adaptor proteins, such as ZO-1. These adaptor proteins are thought to be a linker connecting the transmembrane proteins and the actin cytoskeletons [7]. TJ also have been linked to various signaling molecules that are regulated by extracellular stimuli. Cytokines such as TNFα and interferon-γ, which are considered playing a key role in the pathogenesis of inflammatory disorders, disrupt TJ and barrier functions to increase epithelial permeability in various epithelial monolayers [8–10]. Furthermore, TNFα and interferon-γ synergized to induce TJ disruption and intestinal epithelial barrier dysfunction [11].

Although precise regulatory mechanism of TJ barrier functions is still not clear, recent studies have demonstrated an important role for focal adhesion kinase (FAK), which is a non-receptor protein tyrosine kinase, in modulating barrier function. Zhao et al. reported that

* Corresponding author at: Cellular Signal Transduction, Department of Biochemistry, Asahikawa Medical University, Asahikawa 078-8510, Japan. Tel.: +81 166 68 2340; fax: +81 166 68 2349.
E-mail address: takotago@asahikawa-med.ac.jp (T. Taniguchi).

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FAK knockout mice exhibit increased permeability compared with wild type endothelial cell during embryonic development [12]. FAK activity is necessary for barrier enhancement in endothelia [13,14]. Siu et al. have identified that FAK is an integrated component of the occludin/ZO-1 complex that regulates cell adhesion by determining the phosphorylation status of occludin at the blood–testis barrier [15,16]. These results are suggesting that FAK is required for maintenance of TJ barrier functions via tyrosine phosphorylation of TJ protein complex. Furthermore, Golubovskaya reported that transcription of FAK was regulated by NF-κB, which is known as "first responder" against various cytokines [17]. After mucosal injury, FAK protein expression in gut epithelial cells was enhanced via Smad- and p38-mediated pathways [18]. We have also demonstrated that protein expression level of FAK increased in colonic epithelial cells by inducing chemical colitis [19]. These results suggest that FAK expression and functions may regulate by various cytokines in pathological conditions, however, the role of FAK in colonic epithelial cells remains unclear.

The purpose of this study was to examine the role of FAK on the epithelial barrier function on Caco-2 monolayers. We investigated the effect of FAK inhibitor, PF-573, 228 (PF-228) on barrier function in Caco-2 monolayers. PF-228 is a small molecule FAK inhibitor, which interacts with FAK in the ATP-binding pocket, and effectively blocks the catalytic activity of FAK [20]. This compound is a useful tool to dissect the role of FAK in regulation of cell adhesion signaling and dynamics [20], in signaling pathways in human platelet [21], and in directional motility of cells [22]. In this manuscript, we sought to find the role of FAK in intestinal epithelial barrier function by measuring changes of transepithelial electrical resistance (TEER), paracellular permeability and phosphorylation of FAK by PF-228 treatment and by knockdown with siRNA. Furthermore, we determined the effect of PF-228 on distribution of TJ proteins, ZO-1 and occludin. Our findings indicate that activation of FAK is necessary for maintaining and repairing epithelial barrier in Caco-2 cell monolayer via regulating TJ redistribution.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) and trypsin–EDTA solution were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was from Invitrogen Life Technologies Corp. (Carlsbad, CA, USA). The focal adhesion kinase (FAK) inhibitor PF-573, 228 (3,4-dihydro-6-[[4-[[[3-(methyl-sulfonyl)phenyl]methyl]amino]-5-(trifluoromethyl)-2-pyrimidinyl]amino]-2(1H)-quinolinone) was from Tocris (Bristol, UK). Antibodies against total FAK, phosphorylated FAK at Tyr397, at Tyr576/577, Src and claudin-1 were from Cell Signaling Technology (Danvers, MA). For immunofluorescence staining, anti-total FAK was purchased from Calbiochem (Billerica, MA), anti-phosphorylated FAK at Tyr397 was from Abcam (Cambridge, UK) and anti-ZO-1 and anti-occludin antibodies were from Invitrogen Life Technologies Corp.

2.2. Cell culture

Caco-2 cells were purchased from the RIKEN Cell Bank (Tsukuba, Japan). The cells were cultured in DMEM supplemented with 10% FBS, 1% nonessential amino acids, 10 mM HEPES (pH 7.2), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown in a humidified atmosphere containing 5% CO_2 at 37 °C. Culture medium was changed every 2 days. For TEER study, Caco-2 cells were seeded in cell culture inserts (Millicell-PCF, 0.4 μm pore size, 12 mm diameter, Millipore, Ireland) in 24-well plates at a density of 1×10^5 cells/cm^2. For immunofluorescence staining, Caco-2 cells were plated in 8-well Millicell EZ slide (Merck Millipore, Billerica MA, USA) at 1.5×10^5 cells/well in growth medium.

2.3. Measurement of Caco-2 monolayer epithelial resistance and paracellular permeability

Caco-2 cells were cultured for 3–4 weeks on Millicell culture inserts to form a monolayer. TEER values across the cell monolayers were measured using a Millicell ERS-2 epithelial volt-ohm meter (Merck Millipore). For resistance measurements, both apical and basolateral sides of the epithelia were bathed in culture medium. Inserts having over 400 $\Omega\cdot cm^2$ of TEER values were used for further experiments. Each experiment was repeated at least 3 times to ensure reproducibility. The modulation of Caco-2 monolayer paracellular permeability by ethanol treatment was carried out as established [23]. Briefly, Caco-2 monolayers were incubated in DMEM containing 7.5% ethanol at 37 °C for 30 min. After removal of ethanol, monolayers were incubated with culture medium and measured TEER at indicated time points. For determination of lucifer yellow permeability, Caco-2 monolayers were incubated for 4 h in the indicated concentration of PF-228. After washed with HBSS, lucifer yellow was added to the apical compartment at a final concentration of 1 mM. The monolayers were placed on a shaker in a 37 °C incubator with a humidified atmosphere containing 5% CO_2 for 1 h. Fluorescence leakage was determined for lucifer yellow by 430 nm excitation and 535 nm emission using a fluorescence spectrophotometer.

2.4. Immunoprecipitation and Western blot

Immunoprecipitation was performed as described [24] with some modifications. Caco-2 cells were lysed in immunoprecipitation buffer (20 mM Tris–HCl (pH 7.5), 0.5 M NaCl, 1% TritonX-100, 2 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 0.1% sodium dodecyl sulfate, 0.5% Nonidet P-40, 1% Triton X-100 and 10% glycerol (vol/vol)), containing protease inhibitor cocktail (Roche, Mannheim, Germany). After 4 cycles of freeze–thaw and sonication, lysates were centrifuged at 12,000 *g* for 10 min. The supernatant was incubated with anti-FAK antibody overnight. The immunocomplex was then precipitated with protein G-Sepharose beads (GE Healthcare). The immunocomplex was washed five times with the immunoprecipitation buffer, eluted by boiling in sample buffer for SDS-PAGE and Western blot was performed as described previously [19]. About 1.4 mg protein in approximately 2 ml was used per sample tube for immunoprecipitation.

2.5. Immunofluorescence and confocal microscopy

Caco-2 cells that were grown on 8-well glass slide chambers to 100% confluence for 3–4 weeks were used. After treatment as indicated in the figures, cells were rinsed once with PBS, and fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 15 min at room temperature. Then cells were rinsed three times with PBS and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. After washing, cells were blocked in Image-IT FX signal enhancer (Invitrogen Life Technologies Corp.) for 1 h at room temperature. And then cells were incubated with respective primary antibodies to total FAK (1:100), phosho-FAK at Y397 (1:100), ZO-1 (1:100), and occludin (1:100) diluted in 0.5% Triton X-100 in PBS overnight at 4 °C. Subsequently the secondary antibodies, goat anti-rabbit IgG-Alexa 555 and goat anti-mouse IgG-Alexa 488 (Invitrogen Life Technologies Corp.) were diluted at 1:100 with PBS containing 0.5% Triton X-100 and incubated with cells for 1 h at room temperature in the dark. Slides were mounted in ProLong Gold antifade reagent with 4′, 6′-diamidino-2-phenylindole (DAPI) (Invitrogen Life Technologies Corp.) to stain nucleus. Cells were observed with confocal laser scanning microscope, FV1000-D (Olympus, Tokyo, Japan) using a ×100 oil immersion objective and FV10-ASW software (Ver 3.0, Olympus).

2.6. Isolation and analysis of detergent-insoluble membrane fractions

Detergent-insoluble membrane fractions were prepared as described previously [25] with some modifications. Caco-2 cells were lysed in 2 ml of lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4 and protease inhibitors cocktail) and removed nuclei and cellular debris by a low speed centrifugation (500 *g* for 10 min). The supernatant was diluted with 80% sucrose without detergent to a final concentration of 40% sucrose. Samples were placed at the bottom of a centrifuge tube and 2 ml each of 35, 25, 15, and 5% sucrose in TBS was layered. After ultracentrifugation at 218,000 *g* for 18 h at 4 °C using Hitachi RPS40-T rotor, the buoyant was collected as eight 1 ml fractions from the top. Each fraction was diluted with 3 volumes of lysis buffer, and insoluble material was pelleted by centrifugation (12,000 *g* for 20 min at 4 °C). Resultant pellet was suspended in sample buffer for SDS-PAGE.

2.7. Knockdown of FAK by siRNA

We used predesigned siRNAs as follows: FAK, validated MISSION siRNA (SASI_Hs01_00035697 and SASI_Hs01_00035698; Sigma-Aldrich); and negative control, MISSION siRNA Universal Negative Control (SIC-001; Sigma-Aldrich). Transient transfections of siRNA into Caco-2 cells were performed using siLentFect Lipid Reagent (Bio-Rad Laboratories, Hercules, CA) as described previously [26]. After transfection, the cells were cultured for 4 days on Millicell culture insert to develop tight junction assembly and polarization before TEER measurement and Western bolt experiments.

2.8. Statistical analysis

Results are expressed as means ± SEM of n experiments. Statistical significance was evaluated using Student's *t*-test. $p<0.05$ was considered statistically significant. The results shown are representative data of at least three independent experiments.

3. Results

3.1. Inhibition of FAK activity by PF-228 impaired barrier function of Caco-2 monolayers

To investigate the function of FAK on the barrier function of Caco-2 monolayers, we used a specific FAK inhibitor, PF-228, and evaluated the monolayer permeability. We first examined the effect of PF-228 on the maintenance of epithelial barrier function. Filter-grown monolayers were incubated in culture medium containing various doses (3, 10 and 30 μM) of PF-228 and the change of TEER was measured. As shown in Fig. 1A, vehicle and 3 μM PF-228 had no effect of TEER, however, 10 and 30 μM PF-228 caused a dose-related decrease in TEER. TEER was gradually decreased through 24 h to 96.3 ± 2.6% for 3 μM, 81.8 ± 1.2% for 10 μM, 65.9 ± 1.3% for 30 μM PF-228 compared to control. Previous studies have demonstrated an inverse relationship between intestinal epithelial resistance and paracellular permeability in Caco-2 cell monolayers [27]. Therefore, we also examined the effect of PF-228 on paracellular permeability by measuring the flux rates of a paracellular marker, lucifer yellow. After incubation with various concentrations of PF-228 for 4 h, paracellular flux of lucifer yellow was significantly increased in monolayers treated with 10 μM and 30 μM PF-228 to 1.6 ± 0.2- and 2.6 ± 0.6-fold, respectively (Fig. 1B). There was a linear relationship ($r=0.99$) between decrease of

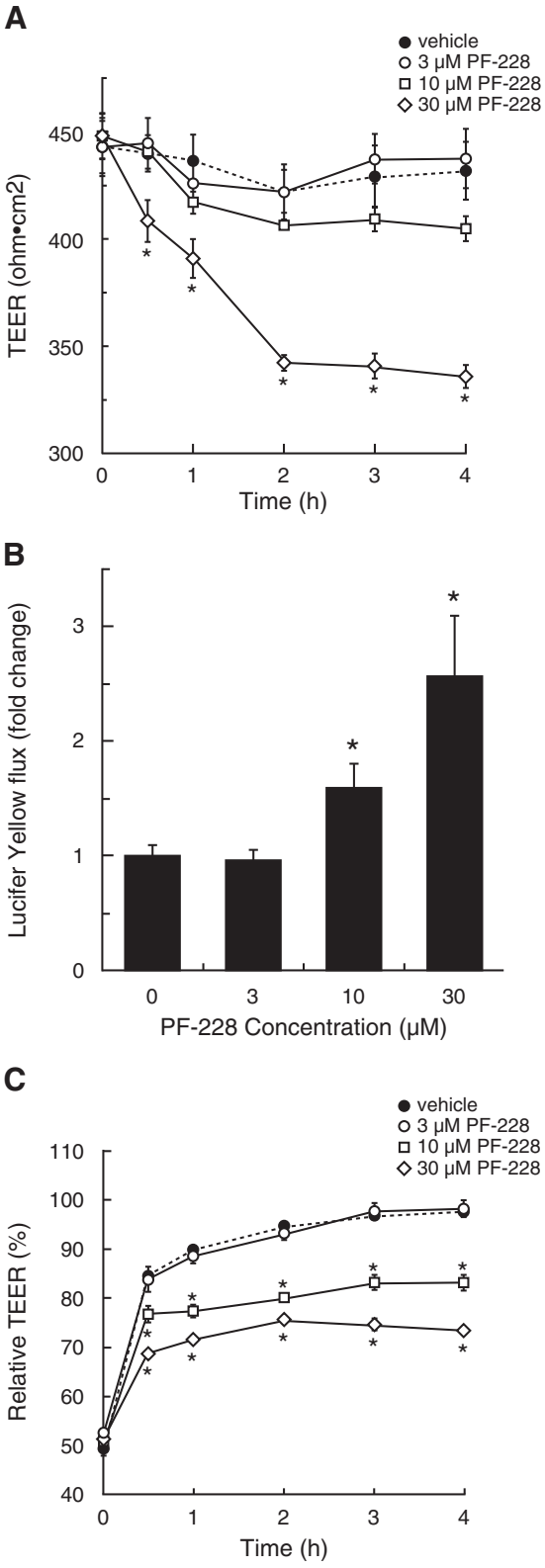


Fig. 1. Effect of PF-228 on Caco-2 monolayers permeability. (A) Time course of PF-228 effect on Caco-2 monolayer epithelial resistance. Filter-grown Caco-2 monolayers were incubated with culture medium containing DMSO (vehicle) or the indicated concentration of PF-228. (B) Effect of PF-228 on paracellular permeability of Caco-2 monolayers. Filter-grown Caco-2 monolayers were incubated with culture medium containing DMSO (vehicle) or the indicated concentration of PF-228 for 4 h and the lucifer yellow permeability was measured as described in Materials and methods. (C) Effect of PF-228 on TEER recovery after the ethanol-induced barrier disruption. Filter-grown Caco-2 monolayers were treated with 7.5% ethanol (vol/vol) for 30 min. After ethanol removal, Caco-2 monolayers were incubated with culture medium containing DMSO (vehicle) or the indicated concentration of PF-228 and TEER was measured at the time indicated. TEER values were expressed relative to TEER before ethanol treatment. Each value represents the mean ± SEM determined from four to six independent experiments. *$p<0.05$ versus control.

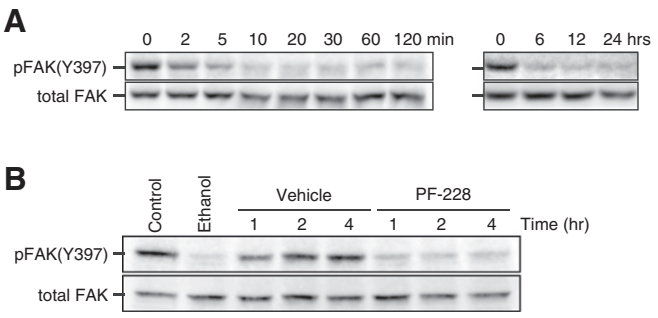


Fig. 2. Effect of PF-228 on FAK autophosphorylation in Caco-2 monolayers. (A) Time course of PF-228 effect on autophosphorylation of FAK at Y397. Caco-2 monolayers were incubated with 10 μM PF-228 for the indicated time. The autophosphorylation of FAK was assessed by Western blot. PF-228 inhibited the autophosphorylation from 2 min (left panel) to 24 h (right panel). (B) Time course of PF-228 effect on the autophosphorylation of FAK during the TEER recovery after ethanol treatment. Caco-2 monolayers were treated with ethanol as described in Fig. 1, and then incubated with culture medium containing DMSO (vehicle) or 10 μM PF-228 for 1, 2 and 4 h. We noted that the level of autophosphorylation was significantly decreased by ethanol treatment, and then increased again to original level after ethanol removal. PF-228 prevented the increase of autophosphorylation during TEER recovery.

monolayers TEER and increase of lucifer yellow flux by PF-228 treatment. We next investigated the effect of PF-228 on the recovery of epithelial barrier function after functional epithelial damage. We employed an ethanol-induced damage model, in which the treatment with low noncytotoxic dose of ethanol produced a reversible modulation of Caco-2 epithelial barrier function concomitant with the disruption of TJ [23]. A 30-min ethanol treatment caused TEER decrease to about 50% compared with the value before treatment (Fig. 1C). After ethanol removal, TEER recovered to the original level within 4 h. Although a low dose (3 μM) of PF-228 had no effect on TEER recovery, higher doses of PF-228 significantly impaired TEER recovery in a dose-dependent manner.

Next, we asked whether a decrease in autophosphorylation of FAK at Y397 was accompanied by a decrease in TEER. Filter-grown monolayers were incubated in culture medium containing 10 μM PF-228. As shown in Fig. 2A, PF-228 significantly inhibited the autophosphorylation of FAK within 10 min and this inhibition was sustained through 24 h. As shown in Fig. 2B, ethanol treatment of monolayers caused transient decrease of FAK autophosphorylation. After ethanol removal, FAK autophosphorylation was completely recovered to original level within 4 h. The incubation in culture medium containing 10 μM PF-228 after ethanol removal inhibited the recovery of FAK autophosphorylation.

3.2. Src regulates ethanol-induced TEER decrease and recovery by phosphorylation of FAK

Autophosphorylation of FAK creates a Src homology 2 (SH2)-binding site that together with a nearby proline-rich SH3-binding site can interact with Src. The activation of Src in the FAK/Src complex leads to further phosphorylation of Tyr576 and Tyr577 within the activation loop of FAK kinase domain, which is known to potentiate FAK activity [28]. Therefore, we postulated that FAK could structurally interact with Src to form a protein complex in ethanol-induced disruption and recovery of epithelial barrier function in Caco-2 monolayers. To verify this hypothesis, we performed co-immunoprecipitation assay using anti-FAK antibody. Western blot analysis showed that Src was co-immunoprecipitated with FAK in control monolayers (Fig. 3A). Ethanol treatment of monolayers caused not only the inhibition of FAK autophosphorylation but also attenuation of Src binding to FAK. By the incubation in culture medium with vehicle after removal of ethanol, the level of FAK autophosphorylation and Src binding returned to control level within 30 min. In the presence of 10 or 50 μM PF-228, FAK autophosphorylation and associating Src decreased. Western blot using specific antibody against phospho-Tyr576/577 of FAK revealed that phosphorylation of FAK activation loop was also attenuated in a parallel manner during disruption and recovery of epithelial barrier. These results suggested that the activation of FAK by Src was necessary for the recovery of epithelial barrier function. Thus, we examined the effect of Src inhibitor, PP2, on TEER recovery. As shown in Fig. 3B, incubation in culture medium containing 10 μM PP2 after ethanol treatment induced a significant decrease of TEER recovery ($p<0.003$) in the early phase (0.5 to 2 h). Western blot analysis showed that PP2 inhibited the phosphorylation of FAK on Tyr576/577 (Fig. 3C, E), but not the autophosphorylation of FAK on Tyr397 (Fig. 3C, D).

3.3. Localization of FAK in Caco-2 monolayers

To investigate the function of FAK in epithelial barrier function, we examined the localization of FAK in Caco-2 monolayers by immunofluorescent microscopy. In the control monolayers, FAK was present in the cellular periphery and appeared as a diffuse band localized at the intercellular borders (Fig. 4A). Reconstruction of confocal image in *xz* plane clearly showed the localization of FAK not only at the basement/apical side but also at the cell–cell interface. Ethanol treatment resulted in a formation of paracellular gaps and a translocation of FAK away from the cell–cell interface to the apical surface of monolayers where significant increase in fluorescent intensity was observed (Fig. 4B). After ethanol removal, the paracellular gaps closed and FAK re-localized at the cell–cell interface of monolayers (Fig. 4C). The incubation with 10 μM PF-228 after ethanol removal impaired the relocation of FAK to the cell–cell interfaces from apical surface (Fig. 4D). We also examined the localization of autophosphorylated FAK. Phospho-FAK was diffusely stained near the cellular periphery in control monolayers (Fig. 4E). Ethanol treatment caused a significant decrease of phospho-FAK staining (Fig. 4F). After ethanol removal, phospho-FAK staining increased again especially at the intercellular borders (Fig. 4G), however, the incubation with 10 μM PF-228 after ethanol removal caused a small increase of punctuate staining of phospho-FAK in cytoplasm and did not show the localization at the cell–cell interface (Fig. 4H).

3.4. FAK and claudin-1 distribution on sucrose gradients after ethanol-induced disruption

According to the results of confocal imaging, we hypothesized that FAK was one of the components of tight junction protein complex. In order to test this hypothesis, we isolated the detergent-insoluble membrane factions from Caco-2 cells. Cell lysates were loaded under 5 to 40% discontinuous sucrose gradient. After ultracentrifugation, fractions were removed and analyzed by immunoblot for total FAK, autophosphorylated FAK and claudin-1. In the control monolayers, total and phospho-FAK were detected in buoyant fractions, especially fractions 3 and 8, and load fraction 12 (Fig. 5A, B, control). A marker of tight junction, claudin-1 also was strongly detected in fraction 3 corresponding to 15% sucrose, suggesting that this fraction contained tight junction-enriched membrane (Fig. 5C, control). As shown in Fig. 5, ethanol treatment caused distribution change of total and phosho-FAK and claudin-1; both proteins were more strongly detected in fraction 8 and fractions 9 to 12 which were corresponding to loaded lysate containing 40% sucrose. By incubation in control culture media after ethanol removal, FAK and claudin-1 in fractions 8 and 9 were decreased, and in fractions 3 and 4 were increased. However, PF-228 impaired these migrations. We also found that the level of FAK autophosphorylation in fraction 3 was changed by ethanol-induced barrier damage and following barrier recovery in the absence or presence of PF-228; ethanol treatment of monolayers caused decrease of FAK autophosphorylation. After ethanol removal, FAK autophosphorylation was recovered to original level, however, PF-228 inhibited this recovery. These

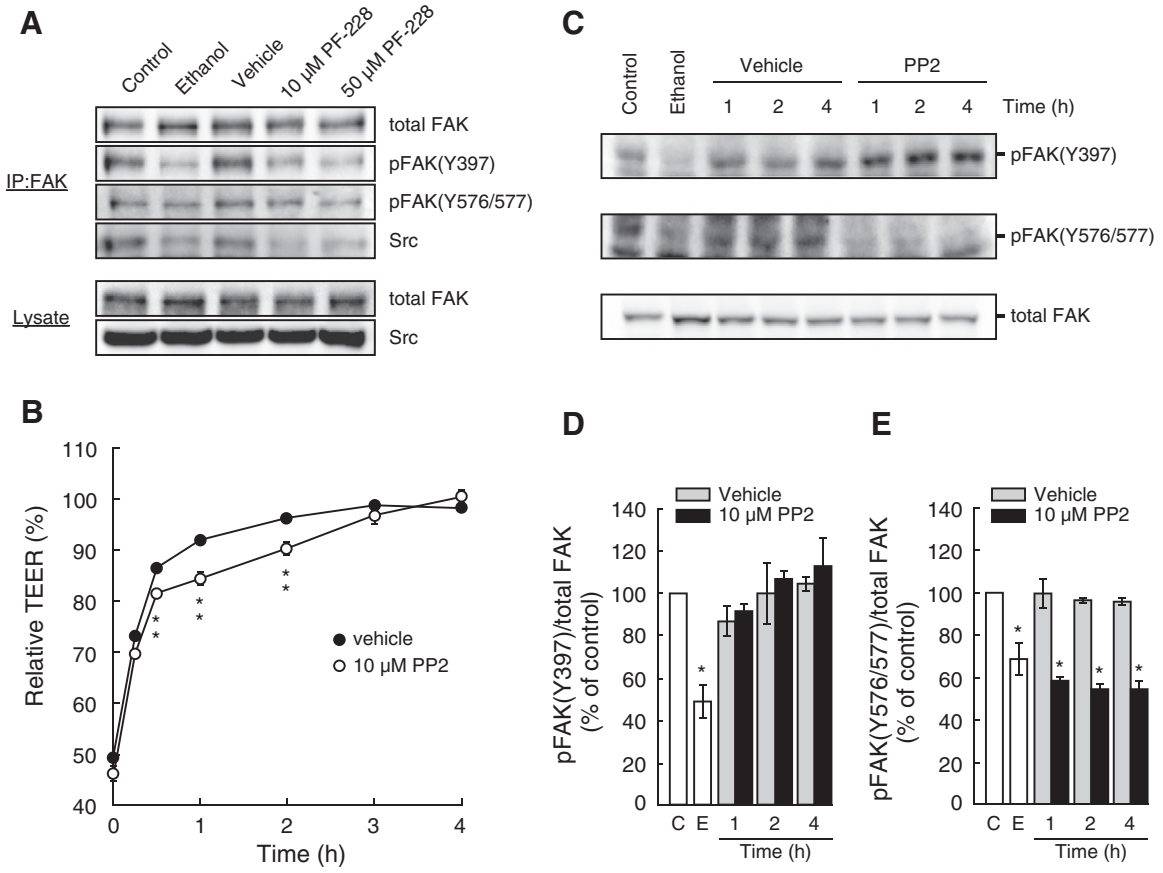


Fig. 3. Src regulates ethanol-induced TEER decrease and recovery via phosphorylation of FAK. (A) Phosphorylation-dependent interaction of FAK and Src. Caco-2 monolayers were treated with ethanol as described in Fig. 1, and then incubated with culture medium containing DMSO (vehicle), 10 or 50 μM PF-228 for 30 min. The whole cell lysates were immunoprecipitated with the FAK antibody. Total FAK, autophosphorylation of FAK (pFAK(Y397)), and phosphorylation of FAK by Src (pFAK(Y576/577)), and co-precipitated Src were detected by Western blot (upper panel). Lysate was immunoblotted for total FAK and Src (lower panel). (B) Effect of PP2 on TEER recovery after the ethanol-induced barrier disruption. After ethanol treatment of Caco-2 monolayers, cells were incubated with culture medium containing DMSO (vehicle) or 10 μM PP2, a selective inhibitor of Src-family kinases. (C) Effect of PP2 on the FAK phosphorylation during TEER recovery. After treatment of Caco-2 monolayers as described above, the phosphorylation of FAK at Y397 and at Y576/577 was assessed by Western blot. The phosphorylation levels of FAK at Y397 (D) and at Y576/577 (E) were determined by quantitative densitometric analysis. Phosphorylation levels were normalized against those of total FAK, and the resulting ratios were expressed relative to those before treatment with ethanol. The lanes C and E indicate before and after ethanol treatment, respectively. Each value represents the mean ± SEM determined from three to four independent experiments. $^{*}p<0.05$; $^{**}p<0.003$ versus control.

results indicated that FAK interacted with TJ and its distribution might drastically change during epithelial barrier recovery.

3.5. Effect of PF-228 on distribution of TJ proteins, ZO-1 and occludin

To determine whether PF-228 alters the distribution of TJ protein, Caco-2 monolayers were treated with PF-228 (10 μM) for 2 h and labeled with anti-ZO-1 or anti-occludin antibody. In the untreated cells, ZO-1 immunofluorescence staining was localized at the cell borders (Fig. 6A). Occludin was also localized at the cell borders, with some punctate staining in the cytoplasm (Fig. 6B). However, incubation with PF-228 caused a displacement of ZO-1 from cell borders, large punctate staining in cytoplasm, and ring-like staining pattern adjacent to cell–cell interface were observed (Fig. 6C). Staining of occludin became irregular, discontinuous staining at cell borders and increased punctate staining were observed (Fig. 6D). Next, we examined the effect of PF-228 on ethanol-induced disruption and recovery. In the control monolayers, ZO-1 and occludin staining were present in a continuous band at the cell borders (Fig. 7A, B). Distribution of ZO-1 became diffuse and the amount of punctate occludin increased after ethanol treatment (Fig. 7C, D). After ethanol removal, ZO-1 and occludin rapidly reassembled at the cellular borders (Fig. 7E, F). However, the addition of PF-228 delayed the reassembly of the TJ proteins. In addition, diffuse distribution and many small ring-like staining of ZO-1 were observed on cell borders (Fig. 7G). Staining of occludin became irregular, and punctate staining still remained (Fig. 7H). These findings suggest that PF-228 inhibits reassembly of ZO-1 and occludin at the cell border in the recovery after ethanol-induced barrier injury, indicating that activation of FAK is associated with TJ during barrier recovery.

3.6. Effect of FAK knockdown on Caco-2 monolayers permeability and autophosphorylation of FAK

To confirm the importance of FAK for epithelial barrier function, we knocked down the expression of FAK in Caco-2 cells using specific siRNA and examined the barrier function by measuring TEER. As shown in Fig. 8A, both predesigned siRNAs for FAK (FAK siRNA Nos. 1 and 2) were efficiently suppressed in the protein expression of FAK at 4 days post-transfection. Quantitative densitometric analysis showed that these siRNAs inhibited the expression of total FAK, which fell to 37.9 ± 1.9% and 52.0 ± 2.1% respectively. At the same time TEER of monolayers transfected with negative control siRNA was 389.9 ± 3.3 $\Omega \cdot cm^2$. The knockdown of FAK caused statistically significant decrease of TEER (379.3 ± 2.7 $\Omega \cdot cm^2$ for siRNA No. 1 and 374.4 ± 4.2 $\Omega \cdot cm^2$ for siRNA No. 2, $p = 0.037$ for both siRNA compared with negative control TEER). We also examined the effect of knockdown

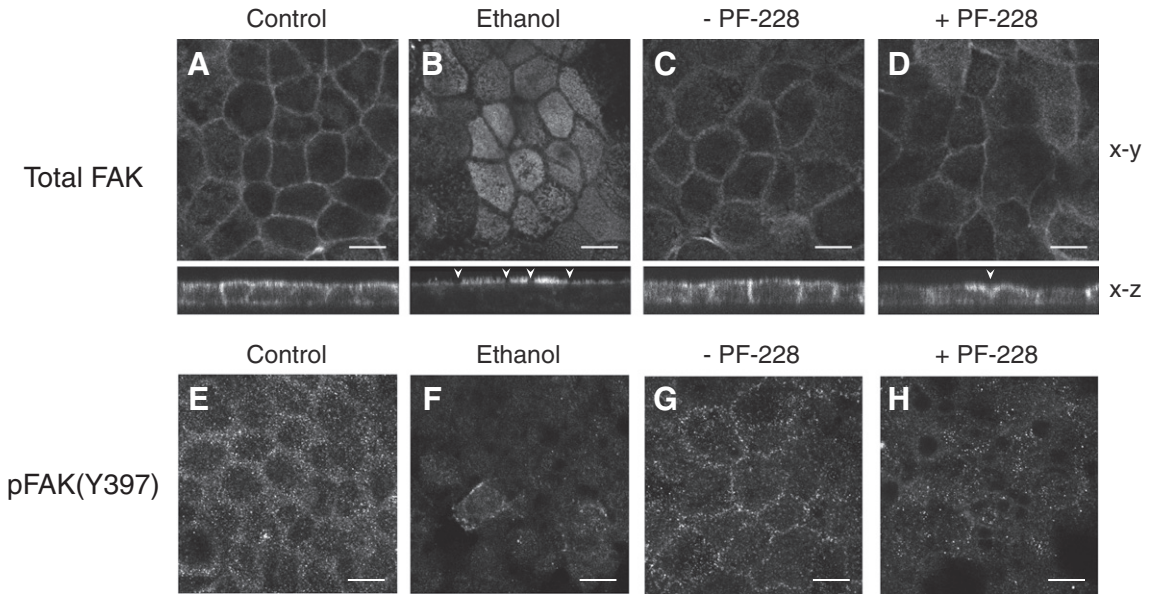


Fig. 4. Differential localization of FAK in Caco-2 monolayers during barrier disruption and recovery. Caco-2 cells were labeled with anti-total FAK antibody (A to D) or anti-phosphorylated FAK at Tyr397 (E to H) and visualized by confocal microscopy as described in Materials and methods. Caco-2 cells were untreated (A and E), or treated with 7.5% ethanol for 30 min (B and F), and followed by incubation with culture medium containing DMSO (Vehicle) for 4 h (C and G) or with culture medium containing 10 μM PF-228 for 4 h (D and H). Lateral (*xy*, upper panels) and axial (*xz*, lower panels) sections obtained from the same area are shown. These results are representative of at least three independent experiments. The arrowheads show the paracellular gaps on apical cell surface. Scale bars, 10 μm.

on FAK autophosphorylation (Fig. 8A, D). Interestingly, the ratio of autophosphorylated FAK increased by knockdown to 123.5 ± 4.0% for siRNA No. 1 and 128.6 ± 7.3% for siRNA No. 2. Each *p* value compared with negative control was 0.0026 and 0.023, respectively. These results indicate that FAK contributes to the development of tight junction assembly and the epithelial barrier formation.

4. Discussion

In this article, we examined the role of FAK in the epithelial barrier function, employing a selective FAK inhibitor, PF-228. As shown in Figs. 1 and 2, PF-228 decreased TEER and increased paracellular permeability in parallel with the inhibition of FAK autophosphorylation, in Caco-2 monolayers. In addition, in the recovery after ethanol-induced barrier injury, PF-228 treatment attenuated the recovery of TEER (Fig. 1C) and prevented the increase in FAK phosphorylation (Fig. 2B). These results suggest that FAK activity is required not only to maintain but also to recover the barrier function.

Because it has been shown that binding of Src to FAK leads to full activation of FAK [28], we examined the association of FAK with Src and FAK phosphorylations by Src as well as FAK autophosphorylation. Significant levels of Src association and FAK phosphorylations were recognized in Caco-2 monolayers. They decreased upon ethanol-induced barrier injury and then recovered within 30 min (Fig. 3A). These results suggested that the autophosphorylation of FAK, the association of FAK with Src and the further phosphorylation of FAK by Src are important events in the barrier function. In order to verify this scheme, we examined the effects of PP2, a selective inhibitor of Src family kinase. Our data showed that PP2 induced a statistically significant decrease of TEER recovery (Fig. 3B) accompanied with an attenuation of phosphorylation on FAK Tyr576/577 that is catalyzed by Src, but without affecting the autophosphorylation of FAK (Fig. 3C–E). The reduction of TEER by PP2 was approximately 10%, which was smaller than that by PF-228 that was 20–30% reduction as shown in Fig. 1. We thus speculated that the inhibition of Src prevented the tyrosine phosphorylation and the full activation of FAK, leading to the delay of TEER recovery within 4 h. There are several reports showing that the inactivation of Src reduced oxidative stress-induced epithelial barrier damage [29–31] and that PP2 attenuated ischemia-induced blood–brain barrier damage [32]. Although they suggested either that Src activity augmented the barrier damage or that Src inhibition facilitated the recovery of the barrier function after 6 h of reperfusion, we speculate that our data in the early recovery phase of barrier injury are not inconsistent with them, because there may be different mechanisms in the damage phase and the recovery phase and in the late and early phase of barrier recovery.

An interesting finding in this study is that localization of FAK in Caco-2 monolayers by immunofluorescent microscopy showing that ethanol caused a disappearance of FAK at cell–cell interface, and that PF-228 inhibited the redistribution of FAK to the cell–cell interface (Fig. 4). Because TJ is one of the most important components in the epithelial barrier at the cell–cell interface, we hypothesized that FAK correlated with barrier function via TJ. To test our hypothesis, first, we isolated the detergent-insoluble membrane fractions from Caco-2 monolayers by discontinuous sucrose density gradient ultracentrifugation (Fig. 5). FAK and claudin-1 were observed in the light density fractions in the control, suggesting that FAK co-migrated with claudin-1 in these fractions. Because membrane raft containing TJ proteins are separated in these lower density fractions, these results indicate that FAK is included in detergent-resistant microdomains. It is noteworthy that ethanol treatment causes a shift of FAK distribution into heavy density fractions corresponding to fraction numbers 9 to 12. After ethanol removal, FAK redistributed to lower density. Addition of PF-228 also prevented the redistribution and FAK remained in the fractions 9 to 12. The similar pattern was observed not only in phospho-FAK but also in claudin-1. These results also support the change of FAK localization by ethanol treatment as shown in Fig. 4. These findings suggest the dynamic regulation of FAK activation and localization during epithelial barrier recovery.

Furthermore, as shown in Fig. 6, PF-228 disrupted the assembly of both ZO-1 and occludin at the cell border. In addition, PF-228 hampered redistribution and reassembly of these TJ proteins in the recovery phase of barrier injury (Fig. 7). These results indicate that FAK regulates the epithelial barrier function by reconstructing TJ proteins, at least ZO-1 and occludin. This is consistent with the previous study that showed an occludin/ZO-1/FAK complex, in which FAK is an integrated regulatory

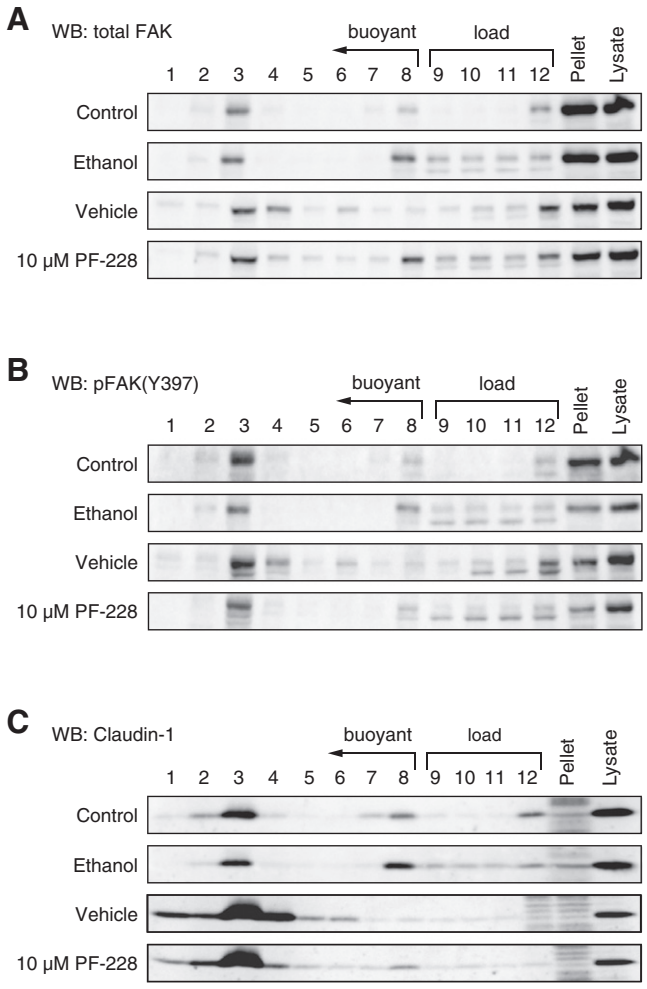


Fig. 5. Association of FAK with TJ fractions. Caco-2 monolayers were treated with ethanol for 30 min and then incubated with the culture medium containing DMSO (Vehicle) or 10 μM PF-228 for 1 h. Cell lysates that were prepared from each treated Caco-2 monolayer were fractionated by sucrose density-gradient centrifuge into 12 fractions and detergent-insoluble membrane fractions were analyzed by Western blot using anti-total FAK (A), anti-phosphorylated FAK at Tyr397 (B) and anti-claudin-1 (C) antibodies.

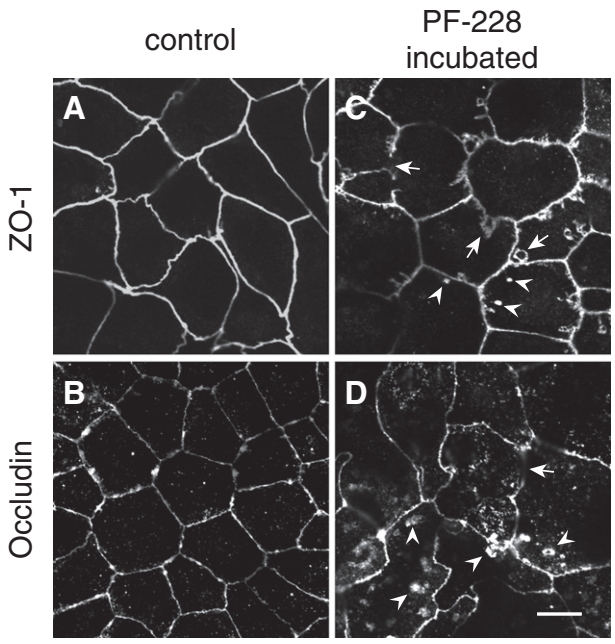


Fig. 6. Effect of PF-228 on localization of TJ proteins, ZO-1 and occludin. Caco-2 monolayers that were cultured on chamber slides were incubated in culture medium containing vehicle (A and B) or 10 μM PF-228 (C and D) for 2 h. Localization of ZO-1 (A and C) and occludin (B and D) was examined by immunofluorescence labeling and confocal microscopy. Treatment with PF-228 caused obvious disrupted and discontinuous distribution (indicated by arrowheads) and punctate cytoplasmic immunostaining of ZO-1 and occludin (indicated by arrows). Scale bar, 10 μm.

component of the occludin–ZO-1 protein complex in blood–testis barrier dynamics [15,16]. Yan et al. reported that these TJ proteins were continuously endocytosed and recycled back to the Sertoli cell surface via the clathrin-mediated pathway in blood–testis barrier [33].

Lastly, we tested siRNAs which are specific to FAK. As shown in Fig. 8, knockdown of FAK resulted in significant decrease in TEER of Caco-2 monolayers. This is in accordance with the results obtained by incubation with PF-228. The decrease was not comparable to the decrease of total FAK protein, however, the ratio of autophosphorylated FAK increased as shown in Fig. 8C. We speculated that the decrease in total FAK protein lead to an increase in the autophosphorylation of FAK in order to compensate the reduction of FAK activity.

Recently, experimental and clinical studies suggest that disruption and reassembly of TJ is an important component of diseases caused by epithelial injury including IBD. Our previous study showed a positive correlation between FAK expression level in colonic epithelial cells and the severity of chemical colitis, which suggested important functions of FAK in epithelial cells during colitis formation and/or wound repairing [19]. Lee et al. reported the important associations between epithelial barrier function and kidney diseases [34]. The proinflammatory cytokines in IBD such as TNFα and INF-γ can downregulate the expression of occludin, causing barrier disturbance [35]. TNFα also induced claudin-1 disassembly from tight junction and increased claudin-2 expression mediated via a tyrosine kinase, Akt and NF-κB pathways [36]. NF-κB also regulated the transcription of FAK gene [17]. These studies suggest that the proinflammatory cytokines alter the protein expression level of various TJ proteins including FAK, and alter the integrity of epithelial barrier under pathological conditions. Thus, further studies are needed to clarify the mechanism of the epithelial barrier disruption and to identify novel molecular target for pharmacological modulation of epithelial integrity.

It is well known that FAK also over-expressed in most human cancers, particularly in invasive and metastatic cancers. Therefore, FAK has been proposed to be a target for anticancer therapy [37–40]. Thus, as inhibitor of FAK, PF-228 may apply an approach to treat cancer through FAK inhibition. However, according to our results, gastrointestinal adverse effects may occur during the treatment.

In conclusion, our data show for the first time that PF-228 induced an increase in epithelial barrier permeability and TJ disassembly in Caco-2 monolayer. Inhibition of FAK activity is likely to be a crucial mechanism in the signaling pathway leading to the decreased TEER, increased permeability and TJ disassembly, both functionally and morphologically. Thus, our data show that FAK activity is a key element in the maintenance and repair of intestinal epithelial barrier function, regulating the assembly and distribution of TJ proteins.

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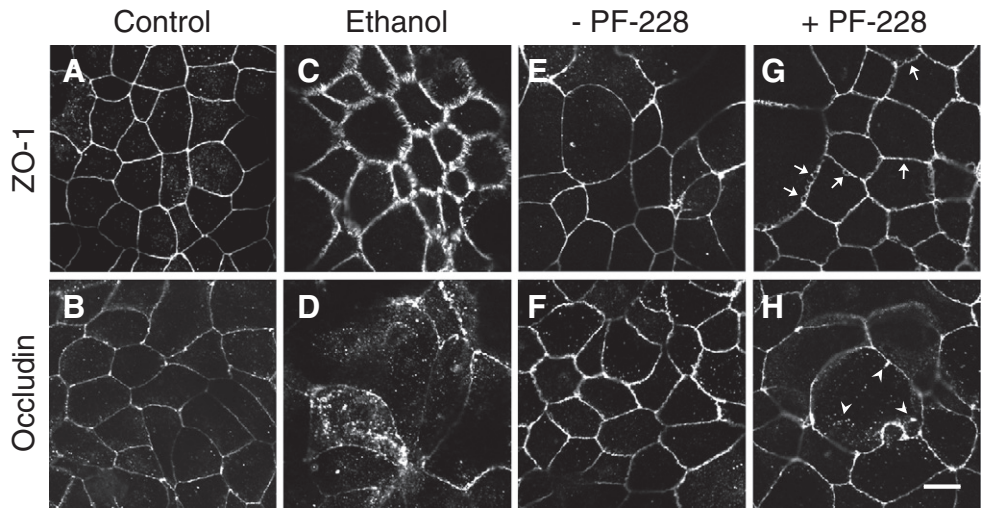


Fig. 7. Effect of PF-228 on localization change of ZO-1 and occludin during ethanol-induced disruption and recovery of TJ barrier. Localization of ZO-1 (upper) and occludin (lower) was examined by immunofluorescence labeling. Caco-2 monolayers were untreated (A, B), or treated with 7.5% ethanol for 30 min (C, D), and followed by incubation with culture medium containing DMSO for 1 h (E, F) or with culture medium containing 10 μM PF-228 for 1 h (G, H). Ethanol treatment caused diffuse distribution of ZO-1 and punctate cytoplasmic staining of occludin (C, D). Both proteins rapidly reassembled at the cellular borders after ethanol removal (E, F); however, the addition of PF-228 prevented the reassembly of TJ proteins (G, H). Ring-like staining and discontinuous distribution of ZO-1 at cell–cell interface (indicated by arrows) and punctate cytoplasmic immunostaining of occludin (indicated by arrowheads) are shown. Scale bar, 10 μm.

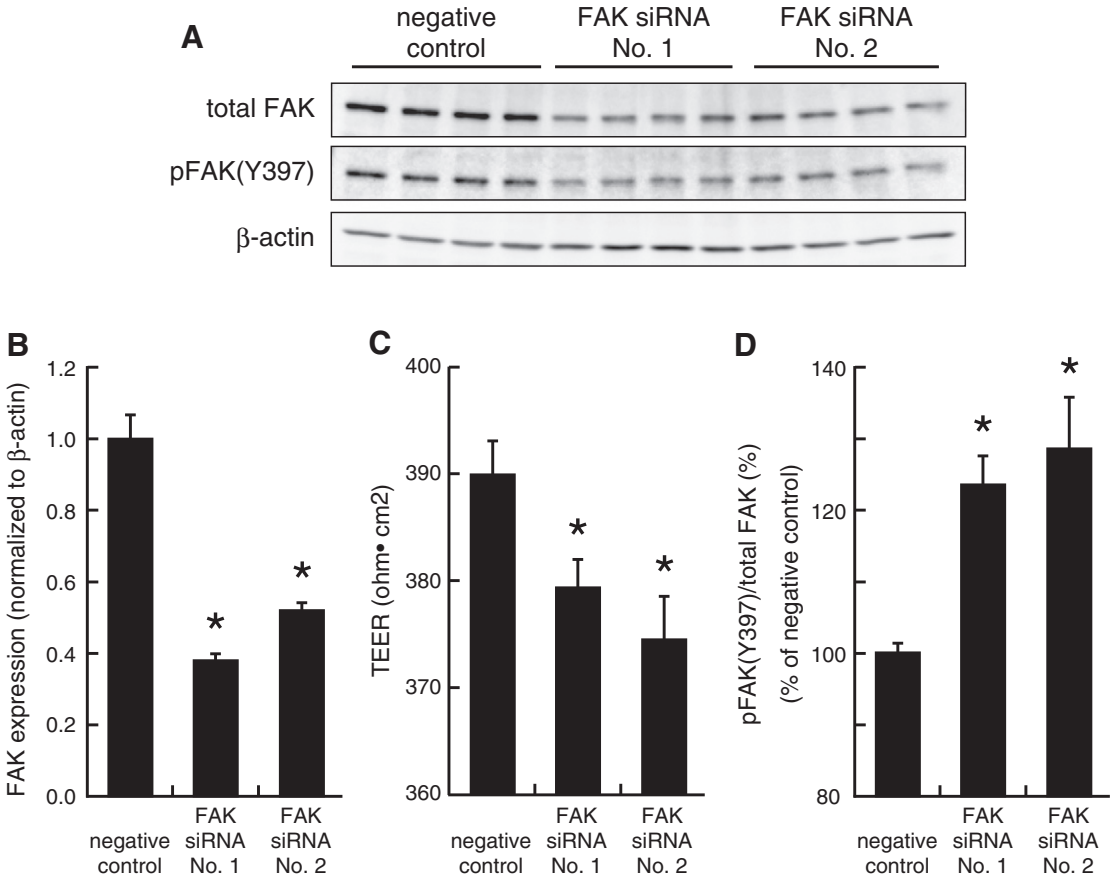


Fig. 8. Effect of FAK knockdown on Caco-2 monolayer permeability and autophosphorylation of FAK. Caco-2 cells were transfected with nonspecific siRNA as negative control or 2 siRNAs targeting FAK and cultured on Millicell inserts for 4 days. (A) Representative results of Western blot detected total FAK and phosphorylation of FAK at Y397. (B) Quantitative densitometric analysis demonstrates that siRNA significantly inhibited the expression of total FAK, which fell to 37.9 ± 1.9% and 52.0 ± 2.1%, in No. 1 and No. 2 siRNA, respectively. (C) The TEER of Caco-2 monolayer transfected with FAK siRNA was decreased significantly in either siRNA treatment. (D) The ratio of phosphorylated FAK at Y397 is increased by 123.5 ± 4.0% and 128.6 ± 7.3% respectively. Each value represents the mean ± SEM determined from four to eight independent experiments. $^{*}p<0.05$ versus negative control.

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