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HB-EGF Promotes Cell Migration and Adhesion via Focal Adhesion Kinase

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Abstract

Background—Cell migration and adhesion are essential in intestinal epithelial wound healing and recovery from injury. Focal adhesion kinase (FAK) plays an important role in cell-extra cellular matrix (ECM) signal transduction. We have previously shown that heparin-binding epidermal growth factor-like growth factor (HB-EGF) promotes intestinal epithelial cell (IEC) migration and adhesion *in vitro*. The current study was designed to determine whether FAK is involved in HB-EGF-induced IEC migration and adhesion.

Materials and Methods—A scrape wound healing model of rat intestinal epithelial cells (RIE-1 cells) was used to examine the effect of HB-EGF on FAK-dependent cell migration *in vitro*. Immunofluorescence and Western blot analyses were performed to evaluate the effect of HB-EGF on the expression of phosphorylated FAK (p-FAK). Cell adhesion assays were performed to determine the role of FAK in HB-EGF-induced cell adhesion on fibronectin (FN).

Results—HB-EGF significantly increased healing after scrape wounding, an effect that was reversed in the presence of a FAK inhibitor (FAK I-14) (both with $p < 0.05$). HB-EGF increased p-FAK expression, and induced p-FAK redistribution and actin reorganization in migrating RIE-1 cells. Cell adhesion and spreading on FN were significantly increased by HB-EGF ($p < 0.05$). FAK I-14 significantly inhibited both intrinsic and HB-EGF-induced cell adhesion and spreading on FN (both with $p < 0.05$).

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Author contributions:

Y Su: Designed methods and experiments, carried out the laboratory experiments, analyzed the data, interpreted the results and wrote the paper.

G Besner: Defined the research theme, designed experiments, and critically proofread and revised the paper.

Conclusions—FAK phosphorylation and FAK-mediated signal transduction play essential roles in HB-EGF-mediated IEC migration and adhesion.

Keywords

HB-EGF; cell migration; cell adhesion; focal adhesion kinase; wound healing

INTRODUCTION

The gastrointestinal epithelium functions as an important barrier that separates luminal contents from the underlying tissue compartment, and is vital in maintaining mucosal homeostasis.^{1–3} Mucosal wounds associated with inflammatory disorders compromise this critical epithelial barrier, undermine gut barrier function and allow bacterial translocation and absorption of toxins, antigens, proteases and other macromolecules, leading to local infection followed by distant organ pathology. In response to injury, rapid re-establishment of epithelial barrier function by intestinal epithelial cells (IEC) depends on migration of uninjured IEC to cover denuded sections of basement membrane.^{1, 4, 5} This process is known as intestinal restitution.^{6, 7}

IEC migration is a mitosis independent process that starts as early as a few minutes after injury and is completed within 6–12 hours.⁸ To initiate migration, cells polarize, extend protrusions (lamellopodia) in a particular direction, and form adhesions in the direction of migration. Focal adhesions (FA) are composed of various structural proteins and represent sites where a number of intra- and extracellular signaling events regulating cell migration take place.^{9, 10} A key player involved in the control of the interaction between cells and the extracellular matrix (ECM) is focal adhesion kinase (FAK), a tyrosine kinase that is localized to cellular focal contact sites, and is the pivotal molecule that controls FA formation.^{11–13} Upon stimulation, FAK is phosphorylated at Tyr397, a step that is necessary for the full activation of the kinase domain.¹⁴ Activated FAK partners with cell membrane integrins with the assistance of other proteins such as paxillin and vinculin, resulting in FA formation, cell adhesion and cell migration.^{10, 15}

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a member of the epidermal growth factor (EGF) family that was initially identified in the conditioned medium of cultured human macrophages,¹⁶ and then found to be a member of the EGF family.¹⁷ During the past 20 years, we have investigated the roles of HB-EGF in protecting the intestines in various models of intestinal injury including NEC,^{18–22} ischemia/reperfusion injury,^{23, 24} and hemorrhagic shock and resuscitation.^{25–27} Our previously studies have demonstrated that HB-EGF promotes intestinal restitution in murine NEC and intestinal ischemia/reperfusion models *in vivo*, and in an IEC scrape wound healing model *in vitro*.^{28, 24, 29} We have also demonstrated that HB-EGF promotes enterocyte migration, in part, by affecting integrin-extracellular matrix (ECM) interactions and intercellular adhesions.²⁸ Numerous studies in the last 20 years have established FAK as a central mediator of integrin signaling, as well as an important component of signaling by other cell surface receptors in the regulation of cell migration in many cell types.^{10, 14, 30, 31} The

current study was designed to examine the effect of HB-EGF on FAK in IEC, and to explore the role of FAK in HB-EGF-induced IEC migration and adhesion.

MATERIALS and METHODS

Materials

Tyr-397 Phosphorylated FAK (p-FAK) antibody and the FAK inhibitor 14 (FAK I-14) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rhodamine Phalloidin was from Cytoskeleton Inc. (Denver, CO, USA). HRP-conjugated goat anti-rabbit antibody, Cy3 and Alexa-488 secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Human plasma fibronectin (FN) was from Millipore (Billerica, MA, USA). Mounting medium for fluorescence with DAPI was from Vector Laboratories Inc. (Burlingame, CA, USA). Radio Immuno Precipitation Assay (RIPA) buffer, protease inhibitor cocktail and the BCA protein assay kit were from Thermo Scientific (Rockford, IL, USA). Phosphatase inhibitor cocktail was Sigma-Aldrich Corp (St Louis, MO, USA). The ECL Plus system was from Amersham Biosciences (Piscataway, NJ, USA). All experiments used human recombinant HB-EGF corresponding to amino acids 74–148 of the mature HB-EGF protein that was produced using a *Pichia pastoris* expression system (Trillium Therapeutics, Toronto, Canada). The EGF receptor (EGFR) inhibitor AG1478 [4-(3-chloroanilino)-6, 7-dimethoxyquinazoline] was from Calbiochem (San Diego, CA, USA). The doses of HB-EGF and AG 1478 chosen were based on our previous studies.^{24, 28, 29, 32}

Cell Culture

The RIE-1 cell line was kindly provided by Dr. John Barnard (Columbus, OH, USA). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100µg/ml streptomycin at 37°C in 5% CO₂. All experiments were performed on cells between passages 4–10.

Scrape Wound Healing Assay

The scrape wound healing assay was performed as we have previously described.^{24, 28, 29} Briefly, cells were grown in 24-well plates until confluent and then serum-starved overnight. The bottom of each plate was marked by drawing two crossed lines across the plate diameter, and then a 100 µL sterile pipette tip was used to create a scrape wound perpendicular to the marked lines. After scraping, cells were immediately treated with either: 1) regular control culture medium, 2) HB-EGF (10 ng/ml), 3) the epithelial growth factor receptor (EGFR) inhibitor AG1478 (500 nmol/L), or 4) AG1478 followed 30 min later by HB-EGF. To assess the effect of FAK activation on HB-EGF-induced cell migration, scraped cells were treated with FAK I-14 (2µM), with some cells receiving HB-EGF (10 ng/ml) 60 min later. Non-treated cells served as a negative control. Cell migration was quantified at various time points (6h, 12h, 18h, 24h, 28h). Photographs were taken immediately after wounding (0 h) or at the indicated time points after wounding (IT). The width of the wound was measured across each of the two marked lines in each well (2 fields/well) using Axiovision 3.1 software (Carl Zeiss Inc., Thornwood, NY). Each experiment was performed 3 times in duplicate. The percent of wound healing was calculated as: Wound healing % = (W0h) – (WIT) where W0h and WIT represent the average wound

width/well (measured across the pre-marked lines) at 0h and at the indicated time points respectively. The cells were then fixed for immunofluorescent staining or harvested for protein analysis.

Western Blot Analysis

Cells were lysed in RIPA buffer supplemented with phosphatase inhibitor cocktail and protease inhibitor cocktail. Protein concentration was measured using a BCA protein assay kit. Protein extracts were subjected to SDS-PAGE and proteins transferred to nitrocellulose membranes using transfer buffer (25 mM Tris, pH 8.8, 192 mM glycine). Nitrocellulose membranes were incubated in PBS with 0.1% Tween-20 (PBST) containing 5% skim milk for 30min at 37°C to block non-specific binding. Membranes were incubated with p-FAK (Tyr-397) antibodies at 4°C overnight followed by HRP conjugated secondary antibody at room temperature (RT) for 1 h. Blots were developed using the ECL Plus system with quantification performed using Image J software. Values were normalized to β -actin and expressed as mean \pm SD.

Fluorescent Immunohistochemistry and F-actin Filament Staining

RIE-1 cells were seeded in 8-well culture slide chambers until confluent, maintained in serum-free medium overnight, and then subjected to scrape wounding. After wounding, cells were treated with HB-EGF (10 ng/ml), AG1478 (500 nM), or with AG1478 (500 nM) followed 30 min later by HB-EGF (10 ng/ml) for 18 h, and were then fixed with 4% paraformaldehyde in PBS for 20 min. Cells were permeabilized by incubating in 0.1% Triton X-100 for 10 min and blocked with 1% bovine serum albumin (BSA) in PBST for 30min. Cells were incubated with p-FAK (Tyr-397) antibody at 4°C overnight, rinsed with PBST, and incubated with secondary antibody labeled with Alexa-488 at RT for 1 h. For labeling of actin filaments, cells were incubated with RhodaminePhalloidin (100nM) in 1% BSA at RT for 30 min. Mounting medium containing DAPI was used to visualize nuclei. Fluorescent staining was imaged with a Zeiss LSM 710 confocal imaging system (Carl Zeiss Inc., Thornwood, NY) across the pre-marked lines as mentioned previously.²⁸

Cell Adhesion and Spreading Assay

96 well flat bottom microwell plates were pre-coated with fibronectin (FN) (10 μ g/ml) or BSA (10 μ g/ml) at 4°C overnight, and blocked with 1% BSA at RT for 30 min. Serum-starved cells were detached for 5 min with 0.25% trypsin to prepare single cell suspensions in DMEM with 2% FBS. Cells were seeded into the wells (5 \times 10⁴ cells per well) and incubated with the following reagents at 37°C in 5% CO₂: 1) no addition (control), 2) HB-EGF (10 ng/ml), 3) AG1478 (500 nM), 4) AG1478 (500 nM) followed 30 min later by HB-EGF (10 ng/ml), or 4) FAK I-14 (2 μ M), 5) FAK I-14 (2 μ M) followed 60 min later by HB-EGF (10 ng/ml). After 15, 30, or 60 min, wells were gently washed 3 times with PBS to remove non-adherent cells. Adherent cells were fixed with 95% ethanol for 10 min, stained with 0.1% crystal violet, and photographed using a Zeiss AxioSkop 2 Plus microscope (Carl Zeiss Inc., Thornwood, New York). The dye absorbed by adherent nuclei was extracted with 0.2% Triton X-100, and absorbance was measured using a plate reader with a 570 nm filter set. Values were normalized to untreated control cells and expressed as mean \pm SD. Adherent cells were also fixed with 4% paraformaldehyde for immunofluorescent staining

using p-FAK (Tyr- 397) antibodies and RhodaminePhalloidin. To quantify cell spreading, cells were stained with RhodaminePhalloidin, three separate fields were photographed, and cells that showed a clear extension of lamellipodia were counted. Each experiment was performed in triplicate wells and was repeated three times.

RESULTS

HB-EGF promotes RIE-1 cell migration via activation of EGFR

RIE-1 cells subjected to scrape wounding were used to examine the role of HB-EGF in cell migration. Addition of HB-EGF significantly increased RIE-1 cell migration after scraping at all time points: $23.52\% \pm 4.75\%$ vs. $10.53\% \pm 4.21\%$, $p=0.036$ at 6 h; $61.92\% \pm 2.57\%$ vs. $25.78\% \pm 3.71\%$, $p=0.023$ at 12 h; $79.01\% \pm 4.04\%$ vs. $46.15\% \pm 2.48\%$, $p=0.031$ at 18 h; $89.92\% \pm 2.38\%$ vs. $54.30\% \pm 1.14\%$, $p=0.011$ at 24 h; $99.37\% \pm 1.09\%$ vs. $58.25\% \pm 0.84\%$, $p=0.002$ at 28 h) (Figure 1A). Eighteen hours after scraping, the addition of AG 1478 significantly inhibited both intrinsic ($18.67\% \pm 9.74\%$ vs. $32.98\% \pm 15.42\%$, $p=0.031$) and HB-EGF-induced cell migration ($20.88\% \pm 8.22\%$ vs. $64.24\% \pm 16.61\%$, $p=0.021$) (Figure 1B,C).

HB-EGF promotes cell migration of RIE-1 cells after scrape wounding via activation of FAK

The scrape wounding assay was also used to investigate the role of p-FAK in HB-EGF-induced cell migration. Eighteen hours after scraping, wound healing was significantly increased in the presence of HB-EGF ($70.91\% \pm 3.47\%$ vs. $34.38\% \pm 2.43\%$, $p=0.007$), and significantly decreased in the presence of FAK- I 14 ($28.25\% \pm 6.28\%$ vs. $34.38\% \pm 2.43\%$, $p=0.047$) (Figure 2). Compared to cells treated with HB-EGF alone, treatment of cells with FAK 1–14 prevented HB-EGF-induced cell migration ($30.39\% \pm 7.71\%$ vs. $70.91\% \pm 3.47\%$, $p=0.011$).

HB-EGF induces F-actin filament reorganization and increases the expression and polarization of p-FAK in migrating RIE-1 cells in an ErbB-1 dependent fashion

To investigate the role of HB-EGF in regulating actin remodeling and p-FAK expression during cell migration, we examined the effect of HB-EGF on the distribution and localization of p-FAK and actin cytoskeleton rearrangements in migrating RIE-1 cells after scrape wounding. Eighteen hours after scrape wounding, at the foremost edge of migrating RIE-1 cells, control RIE-1 cells had intrinsic organization of F-actin and formed a few lamellipodia, with p-FAK distributed equally throughout the cytoplasm (Figure 3). Addition of HB-EGF increased the number of lamellipodia and resulted in significantly increased accumulation of p-FAK at the foremost edge of the migrating cells. Cells exposed to AG 1478 had significantly reduced numbers of lamellipodia and reduced staining of p-FAK compared with control cells. Treatment of cells with AG 1478 inhibited both intrinsic and HB-EGF-induced F-actin reorganization and p-FAK redistribution.

We next examined changes in p-FAK protein levels in RIE-1 cells after scrape wounding as determined by Western blotting. Eighteen hours after scrape wounding, addition of HB-EGF significantly increased relative protein levels of p-FAK (1.42 ± 0.05 vs. 1.00 ± 0.01 , $p=0.019$), whereas blocking of EGFR activity with AG1478 inhibited the expression of p-

FAK (0.83 ± 0.08 vs. 1.00 ± 0.01 , $p=0.046$) and also abolished HB-EGF-induced phosphorylation of FAK (0.93 ± 0.07 vs. 1.42 ± 0.05 , $p=0.025$) (Figure 4).

HB-EGF promotes cell adhesion and spreading of RIE-1 cells on fibronectin (FN) via activation of FAK

We next determined whether inhibition of p-FAK affected HB-EGF-mediated cell adhesion and spreading on extracellular substrates. First we investigated the role of FN on RIE-1 cell adhesion. Compared with control cells plated in the absence of FN, RIE-1 cell adhesion was significantly increased at 30 min ($172.84\% \pm 5.25\%$ vs. $117.16\% \pm 3.70\%$, $p=0.015$) and 60 min ($229.02\% \pm 10.43\%$ vs. $160.39\% \pm 6.79\%$, $p=0.028$) after incubation on FN coated plates, and was further enhanced when cells were treated with HB-EGF at 30 min ($209.31\% \pm 6.12\%$ vs. $172.84\% \pm 5.25\%$, $p=0.031$) and 60 min ($283.53\% \pm 10.29\%$ vs. $229.02\% \pm 10.43\%$, $p=0.030$) (Figure 5A,B). After 60 min of incubation, HB-EGF treatment significantly increased not only cell adhesion on FN ($132.37\% \pm 7.83\%$ vs. $100.00\% \pm 3.54\%$, $p=0.033$) (Figure 6A) but also significantly increased cell spreading on FN ($79.92\% \pm 8.11\%$ vs. $53.50\% \pm 7.59\%$, $p=0.041$) (Figure 6B). Inhibition of EGFR activation with AG 1478, or inhibition of p-FAK with FAK I-14, significantly decreased intrinsic cell adhesion (AG1478: $70.42\% \pm 5.72\%$ vs. $100.00\% \pm 3.54\%$, $p=0.035$; FAK I-14: $80.53\% \pm 10.68\%$ vs. $100.00\% \pm 3.54\%$, $p=0.038$), as well as cell spreading on FN (AG1478: $28.74\% \pm 7.20\%$ vs. $53.50\% \pm 7.59\%$, $p=0.039$; FAK I-14: $40.58\% \pm 3.52\%$ vs. $53.50\% \pm 7.59\%$, $p=0.041$) compared to control cells, and also decreased HB-EGF-induced cell adhesion (AG1478: $79.51\% \pm 2.47\%$ vs. $132.37\% \pm 7.83\%$, $p=0.020$; FAK I-14: $85.71\% \pm 5.15\%$ vs. $132.37\% \pm 7.83\%$, $p=0.030$) and spreading on FN (AG1478: $34.43\% \pm 5.41\%$ vs. $79.92\% \pm 8.11\%$, $p=0.023$; FAK I-14: $51.35\% \pm 7.11\%$ vs. $79.92\% \pm 8.11\%$, $p=0.038$) (Figure 6A-C).

HB-EGF induces p-FAK redistribution in RIE-1 cells grown on FN

We next examined p-FAK localization and actin skeleton organization in RIE-1 cells adhering to FN. In control RIE-1 cells, p-FAK was distributed equally throughout the cytoplasm, with actin fibers forming lamellipodia (Figure 7). Addition of HB-EGF resulted polarized p-FAK at the cell periphery, and promoted cell spreading on FN. Addition of AG 1478 to cells treated with or without HB-EGF significantly inhibited cell spreading on FN and prevented the redistribution of p-FAK to the cell periphery.

DISCUSSION

Cell migration and adhesion are essential components of many biological processes that are critically involved in cell growth, embryonic development, immunity, angiogenesis, and wound healing.^{10, 33, 34} These processes are mediated by key cytoskeletal structures and are controlled by signal transductions that are organized in lamellipodial extensions. These signals are received by cells through contact with the extracellular matrix, in combination with exposure to soluble molecules, lipids, or growth factors.^{30, 35, 36} Cell recognition of the ECM is initiated by the membrane receptor integrin, accompanied by a series of complex molecular events regulating intracellular signaling processes.³⁷⁻³⁹ One of the key elements in intracellular signaling is FAK.^{39, 40} FAK is a nonreceptor protein-tyrosine kinase that is one component of the focal adhesion complex that plays a major role in signaling pathways,

especially integrin-mediated signal transduction.^{14, 31, 41} FAK also participates in signal transduction by G-protein-coupled receptors, such as angiotensin II, receptor tyrosine kinases, and the platelet-derived growth factor (PDGF) receptor.^{42, 43} Many studies have demonstrated that FAK may function as a convergence point for signaling pathways triggered by integrins and other humoral factors that are important in the regulation of cell function.^{10, 44, 45} Our previous studies have confirmed that HB-EGF promotes cell migration by affecting integrin-extracellular matrix interactions, suggesting that FAK may play a crucial role in HB-EGF-induced intestinal restitution. However, evidence confirming a relationship between HB-EGF-induced intestinal restitution and FAK was previously lacking.

The current study used a scrape wounding model to provide direct evidence that FAK activation is an essential factor in HB-EGF-induced cell migration. We found that HB-EGF promotes enterocyte migration in an experimental model of wound healing. During enterocyte migration, HB-EGF induced F-actin reorganization associated with increased protein levels and cell peripheral polarization of phosphorylated FAK. In addition, inhibition of FAK activity significantly reversed HB-EGF-mediated IEC migration, consistent with the findings of others that FAK is a critical regulator of cell migration in other cell types.^{14, 34, 43, 46} We have previously shown that the ability of HB-EGF to affect enterocyte migration is EGFR (ErbB-1)-dependent.²⁸ Our current results confirmed that EGFR blockade not only inhibited HB-EGF-induced cell migration, but also reversed HB-EGF-induced F-actin reorganization and FAK activation. Taken together, these findings demonstrate that HB-EGF promotes cell migration through an EGFR-dependent pathway via activation of FAK.

The interaction of cells with various ECM molecules plays a critical role in both tissue construction and regulation of cellular functions, including cell adhesion, migration, proliferation, and differentiation.^{10, 31, 34} FN is one of the signals in the ECM that is essential for embryonic development, and is associated with wound healing and angiogenesis.⁴⁷ The importance of FAK on signal transduction has been emphasized in many earlier studies.^{10, 15, 31, 45} In addition, we have previously confirmed that HB-EGF promotes RIE-1 cell adhesion and spreading on FN.²⁸ However, evidence confirming a relationship between FAK and HB-EGF-induced cell-ECM interactions is still lacking. We showed in this study that HB-EGF promoted IEC adhesion and spreading on FN, and induced phosphorylated FAK redistribution to the cell periphery, which were dramatically reversed by EGFR blockade or FAK inhibition, indicating that HB-EGF promotes IEC adhesion and spreading on FN through EGFR and via activation of FAK. Multiple downstream signaling pathways including paxillin and members of the Rho family have been identified that mediate FAK regulation of migration and adhesion in various cells,¹⁰ and these downstream molecules may be investigated in our future studies of HB-EGF-mediated intestinal restitution.

In summary, we have demonstrated the critical role of FAK activation in HB-EGF-induced cell migration and adhesion. These findings, in combination with our previous studies demonstrating that HB-EGF promotes intestinal restitution *in vivo*,^{48, 49} support a potential

future therapeutic role for HB-EGF in the promotion of cell migration and adhesion in processes such as wound healing and recovery from injury.

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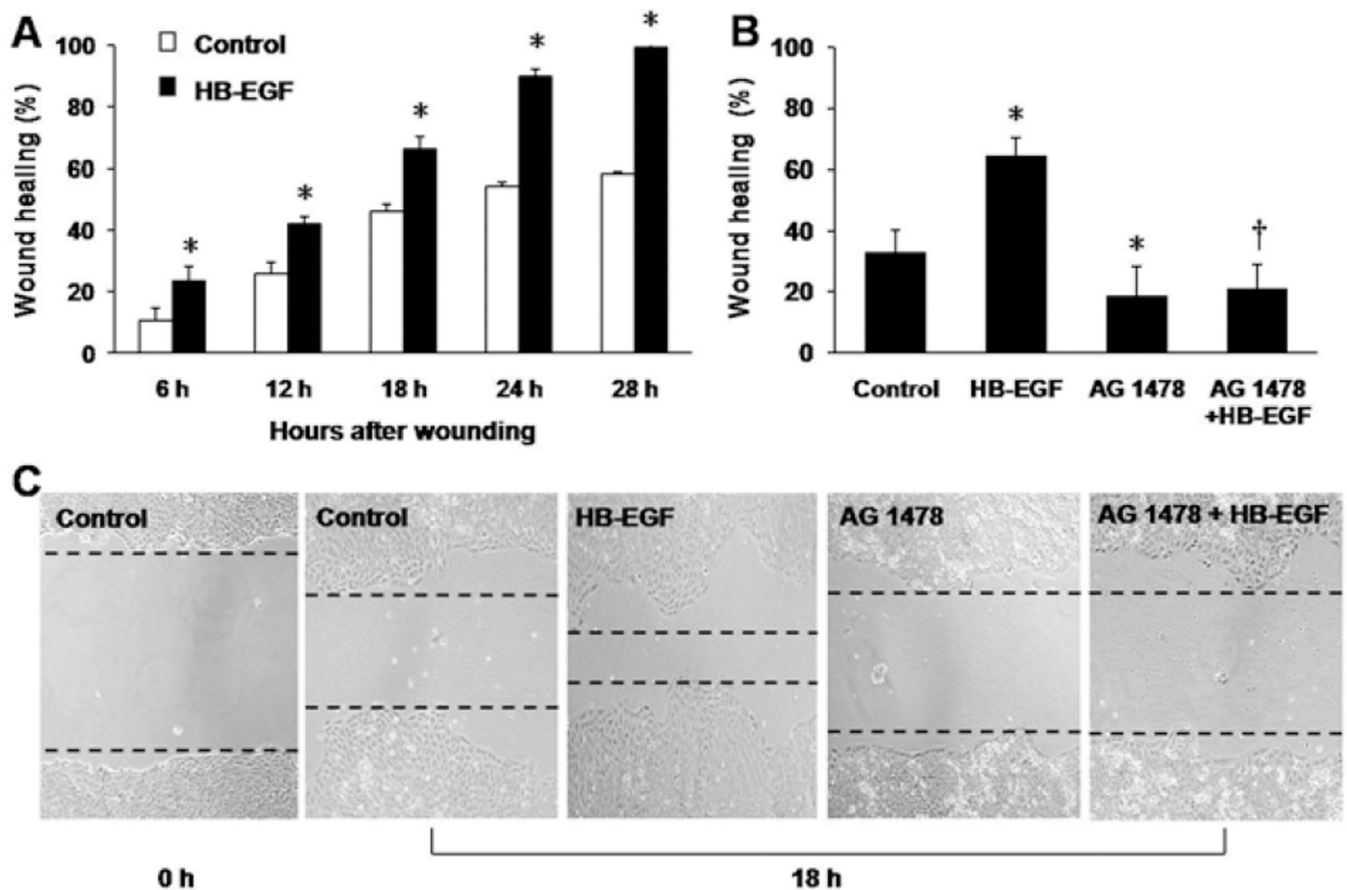


Figure 1. RIE-1 cell migration after scrape wounding in the presence of EGFR inhibition. **(A)** Time course of RIE-1 cell wound healing in the presence or absence of HB-EGF *in vitro*. * $p < 0.05$, vs. control. **(B)** Effect of HB-EGF on wound healing of RIE-1 cells 18 h after scraping. * $p < 0.05$, vs. control; † $p < 0.01$, vs. HB-EGF. **(C)** Representative images of RIE-1 cells 18 h after scrape wounding. From left to right: 0 h after scrape wounding of untreated RIE-1 cells; 18 h after wounding of untreated cells, HB-EGF-treated cells, AG1478-treated cells and cells treated with AG1478 plus HB-EGF. The black dotted lines present the wound edges at 18 h. Scale bar = 200 μ m.

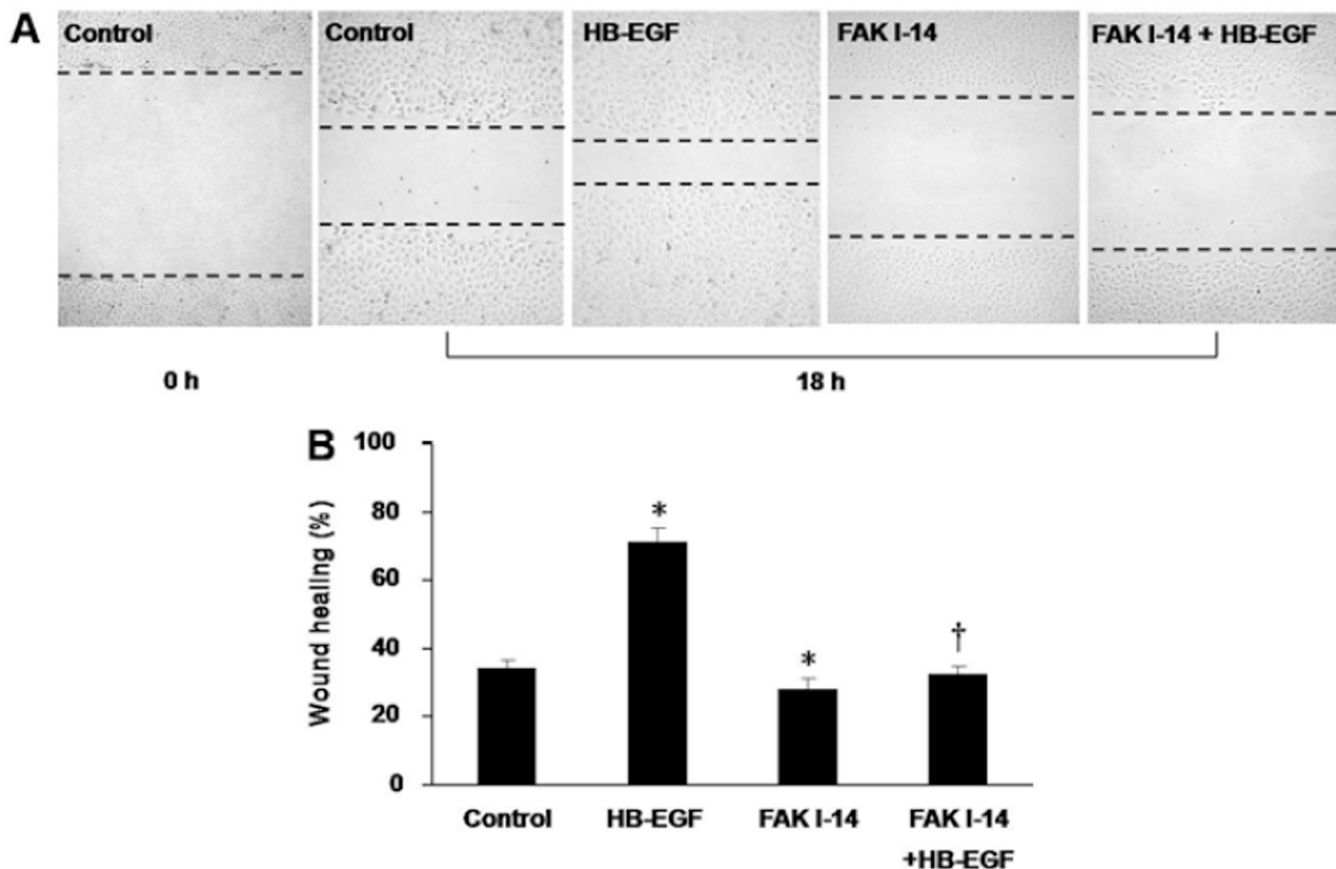


Figure 2.

RIE-1 cell migration after scrape wounding in the presence of p-FAK inhibition. **(A)** Representative images of RIE-1 cells 18 h after scrape wounding. From left to right: 0 h after scrape wounding of untreated RIE-1 cells; 18 h after wounding of untreated cells, HB-EGF-treated cells, FAK1-14-treated cells and cells treated with FAK1-14 plus HB-EGF. The black dotted lines present the wound edges at 18 h. Scale bar = 200 μ m. **(B)** Effect of p-FAK inhibition on RIE-1 cell migration 18 h after scrape wounding. * $p < 0.05$, vs. control; † $p < 0.05$ vs. HB-EGF.

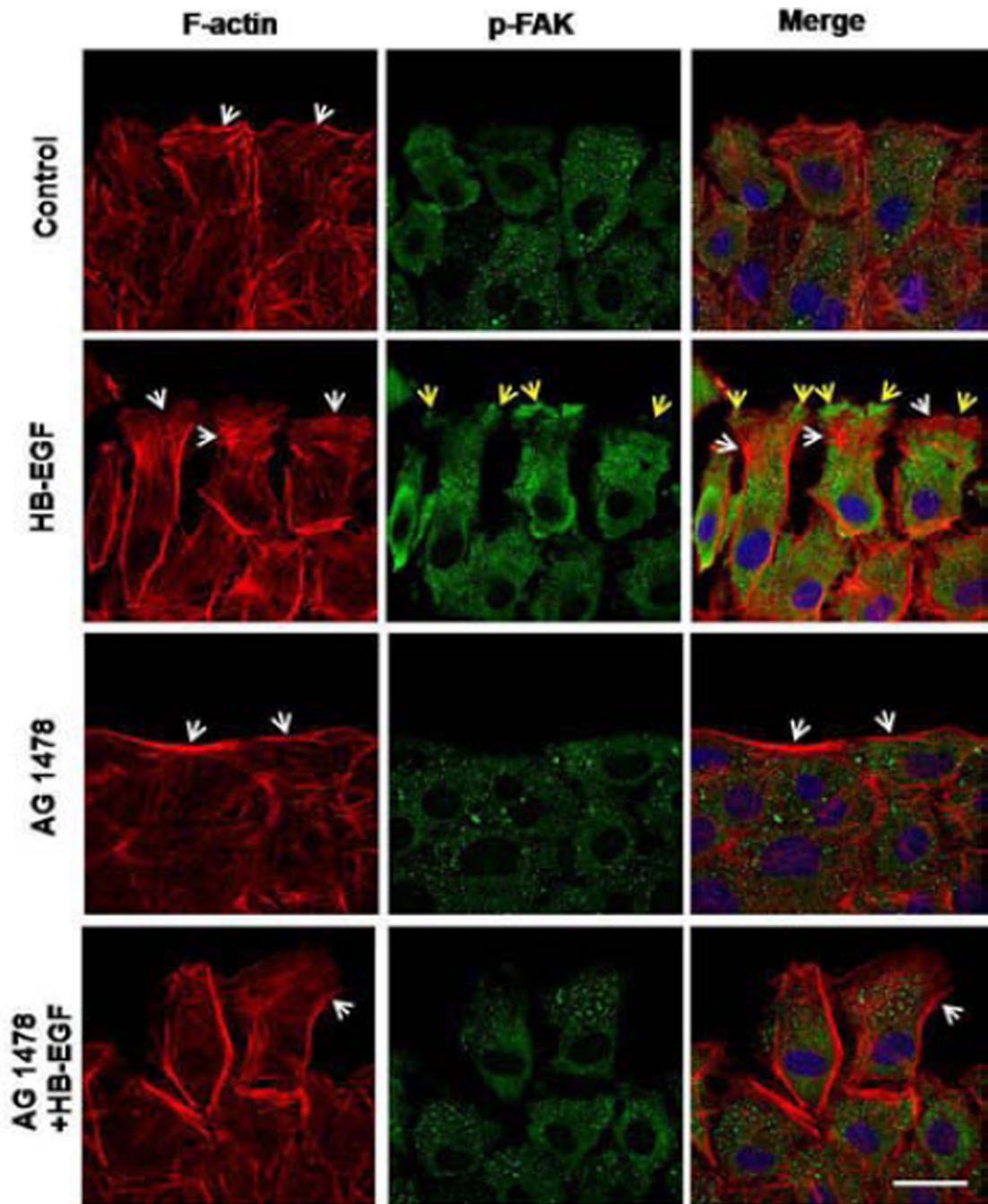


Figure 3.

Distribution of F-actin filaments and p-FAK after scrape wounding. Shown are representative immunofluorescent images of F-actin filaments (red) and p-FAK (green) in migrating RIE-1 cells 18 h after scrape wounding. At the wound edges, the white arrows indicate lamellipodia, and the yellow arrows indicate polarization of p-FAK to the foremost edge of the migrating RIE-1 cells. Scale bar = 20 μ m.

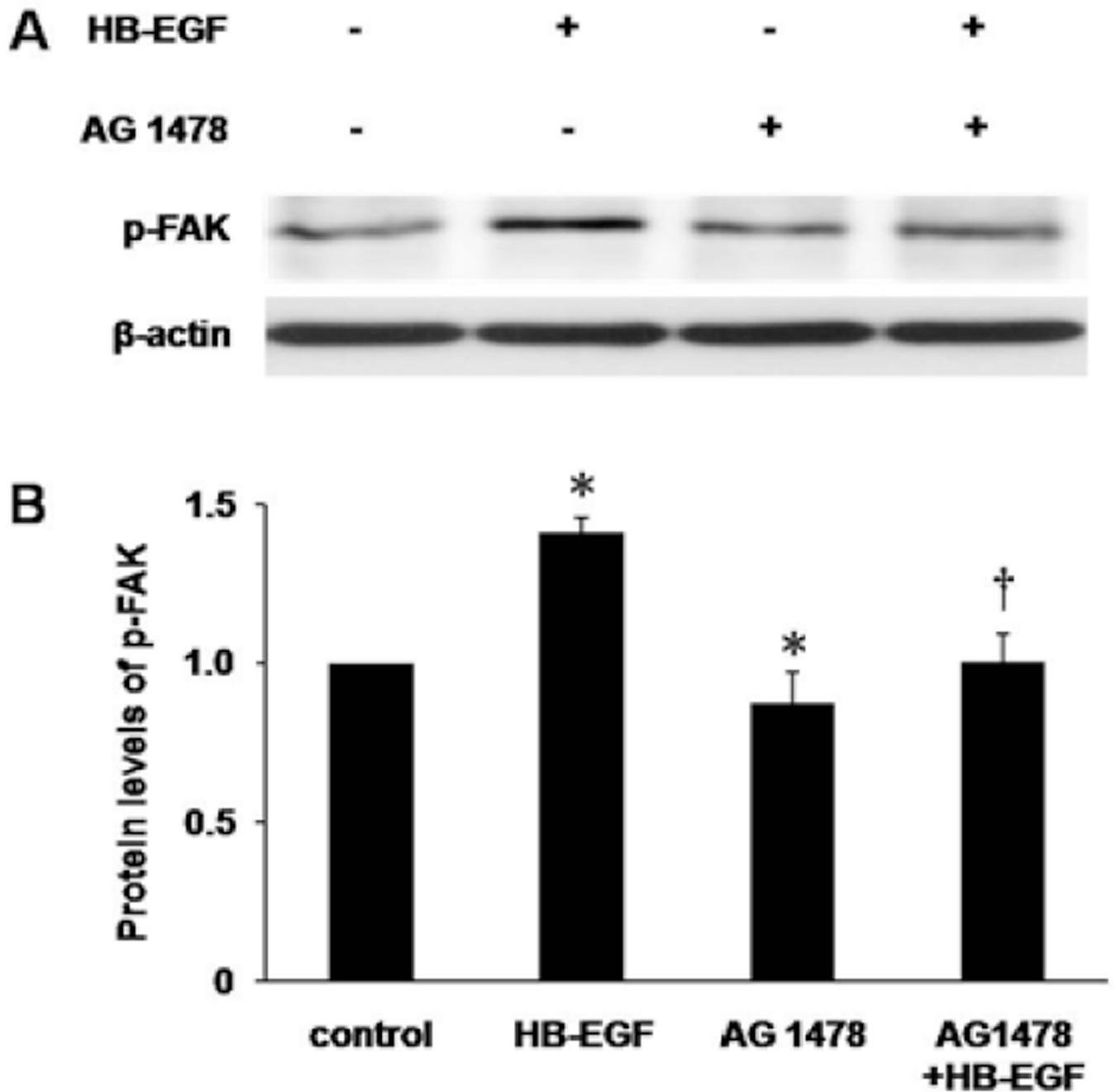


Figure 4. p-FAK protein levels in RIE-1 cells as determined by Western blotting. **(A)** Western blotting of p-FAK 18 h after scrape wounding. **(B)** Densitometric quantification of protein levels of p-FAK. The protein level of p-FAK in control group was defined as 1.0. * $p < 0.05$ vs. control; † $p < 0.05$ vs. HB-EGF.

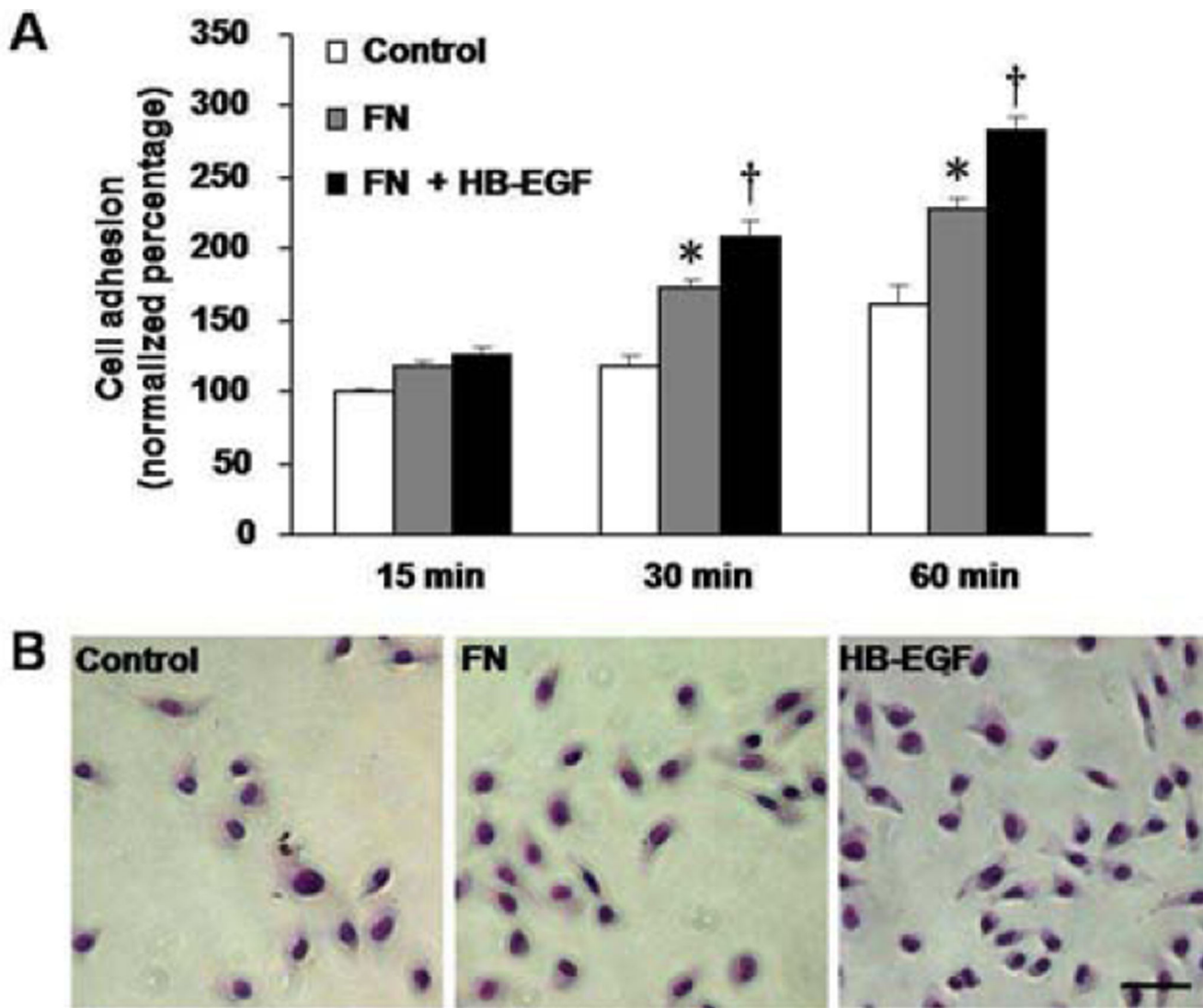


Figure 5. RIE-1 cell adhesion on fibronectin (FN). **(A)** RIE-1 cells adhesion on FN is shown at different time points. * $p < 0.05$ vs. control cells plated in the absence of FN; † $p < 0.05$ vs. cells grown on FN in the absence of HB-EGF. **(B)** Representative images of RIE-1 cell adhesion after 60 min of incubation. Scale bar = 30 μ m.

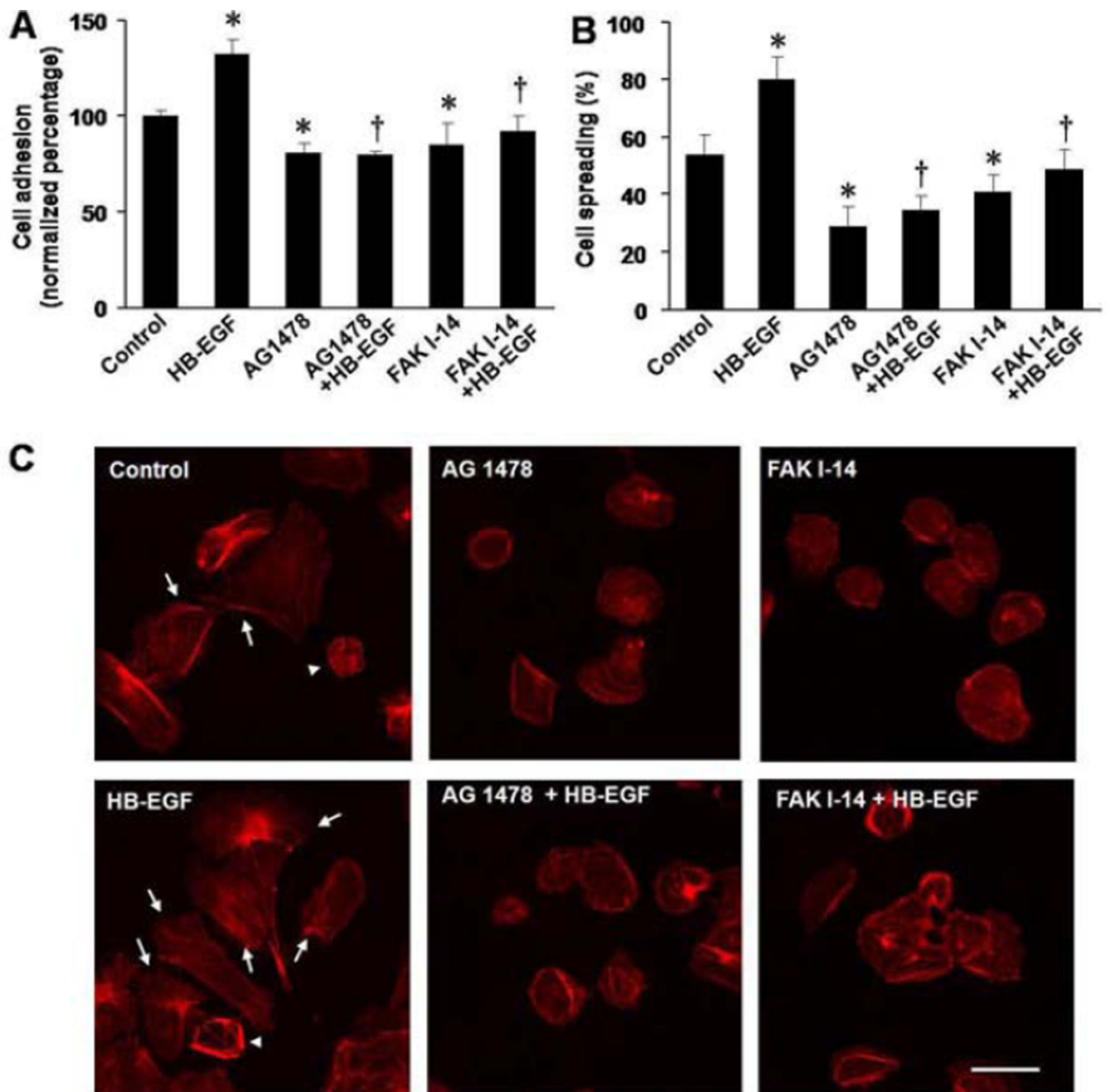


Figure 6.

RIE-1 cell adhesion and spreading on FN in the presence of EGFR and p-FAK inhibition. (A) Effect of EGFR and p-FAK inhibition on RIE-1 cell adherence to FN-coated plates after 60min of incubation. * $p < 0.05$ vs. control cells; † $p < 0.05$ vs. HB-EGF-treated cells. (B) Effect of EGFR and p-FAK inhibition on RIE-1 cell spreading on FN after 60 min after incubation. * $p < 0.05$ vs. control cells; † $p < 0.05$ vs. HB-EGF-treated cells. (C) Representative images of the RIE-1 cells grown on FN. Cell spreading was defined by formation and extension of lamellipodia, where F-actin (red staining) formed thick bundles (arrows). Cells without lamellipodia did not spread (arrowheads). Scale bar = 25 μ m.

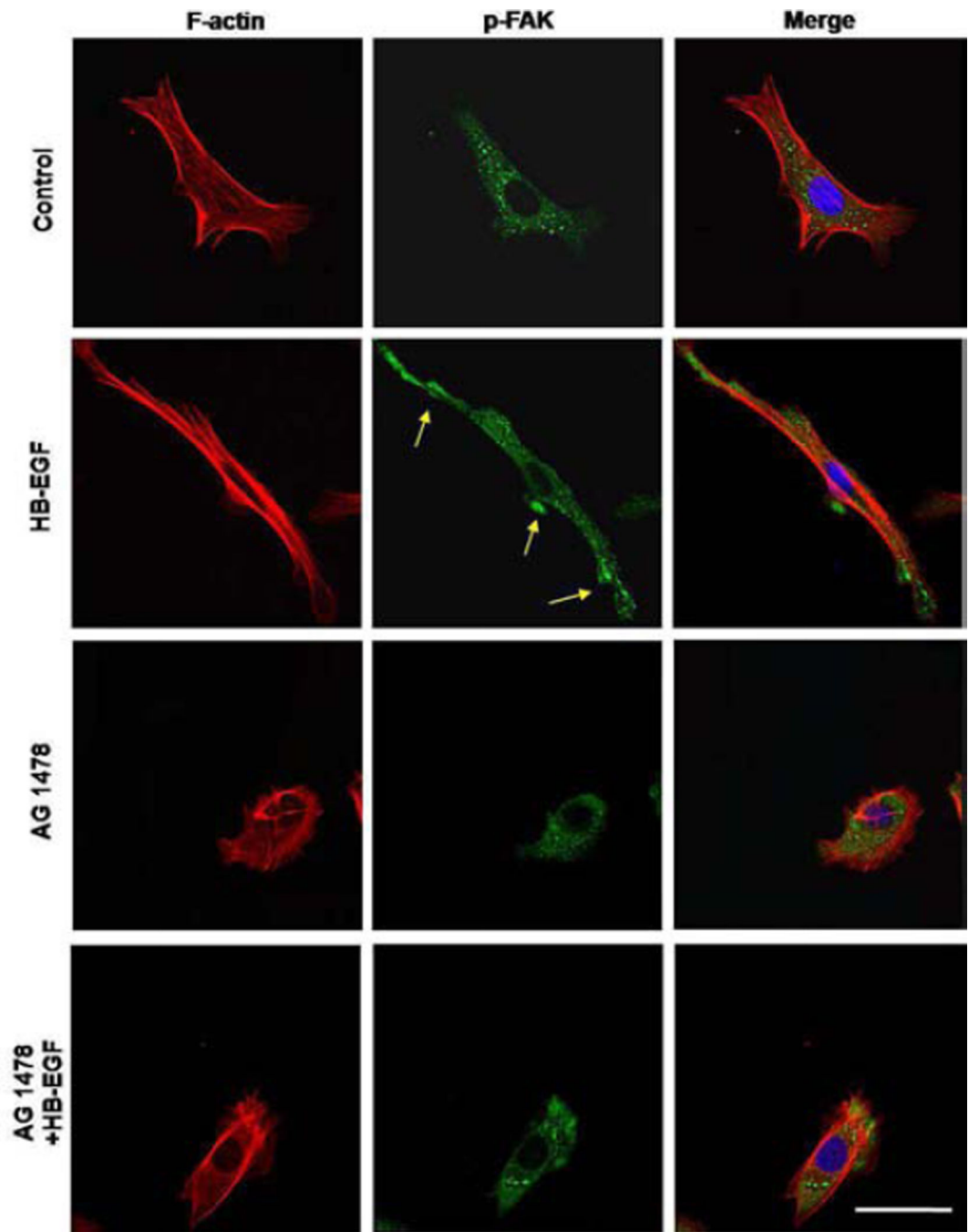


Figure 7. Distribution of F-actin filaments and p-FAK in RIE-1 cells grown on FN. Shown are representative immunofluorescent images of F-actin filaments (red) and p-FAK (green) in migrating RIE-1 cells grown on FN. Yellow arrows indicate polarized p-FAK at the cell periphery. Scale bar = 30 μ m.