THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 7, pp. 4511–4519, February 12, 2010

Cyclic Mechanical Stretch Decreases Cell Migration by Inhibiting Phosphatidylinositol 3-Kinase- and Focal Adhesion Kinase-mediated JNK1 Activation*◆

Received for publication, November 11, 2009, and in revised form, November 30, 2009 Published, JBC Papers in Press, December 14, 2009, DOI 10.1074/jbc.M109.084335

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Epithelial cell migration during wound healing requires coordinated signaling pathways that direct polarization of the leading and trailing ends of the cells, cytoskeletal organization, and remodeling of focal adhesions. These inherently mechanical processes are disrupted by cyclic stretch (CS), but the specific signaling molecules involved in this disruption are not well understood. In this study, we demonstrate that inhibition of phosphatidylinositol 3-kinase (PI3K) or expression of a dominant-negative form of PI3K caused inhibition of airway epithelial cell wound closure. CS caused a sustained decrease in activation of PI3K and inhibited wound healing. Expression of constitutively active PI3K stimulated translocation of Tiam1 to the membrane, increased Rac1 activity, and increased wound healing of airway epithelial cells. Increased Rac1 activity resulted in increased phosphorylation of JNK1. PI3K activation was not regulated by association with focal adhesion kinase. Restoration of efficient cell migration during CS required coexpression of constitutively active PI3K, focal adhesion kinase, and JIP3.

Migration of cells into an injury site is a primary component of repair after injury (1). Epithelial cells in airways have an important role both in defining the physical barrier between the host and external environment and in regulating the response to inflammation in airways diseases such as asthma. Airway epithelial cells (AECs)[2] migrate in response to injury along a basement membrane containing several extracellular matrix proteins using cell-surface receptors in the integrin family (2, 3). Binding of extracellular matrix proteins such as collagen IV and laminin to appropriate integrins signals to several key pathways via focal adhesion kinase (FAK), a non-receptor tyrosine kinase residing within the focal adhesion complex (4, 5). FAK mediates both epithelial (3) and non-epithelial (6, 7) cell migration via several signaling pathways mediated by c-Jun N-terminal kinase (JNK).

AEC migration after injury occurs during a state of cyclic mechanical distention of the underlying substrate in the airways during respiration. Conditions such as asthma may alter these mechanical stresses due to changes in bronchomotor tone, volume trapping, or structural remodeling (8–11). We demonstrated previously that cyclic stretch (CS) of AECs causes inhibition of wound healing primarily through mechanisms that decrease cell migration (12–14).

Activation of JNK from FAK is one such signaling pathway critical to epithelial cell migration after injury. We recently demonstrated that CS mediates this pathway through decreased phosphorylation of FAK at Tyr^{397}, leading to dissociation of the JNK-interacting protein JIP3 and subsequent decreased kinase activation of JNK (12).

Several signaling pathways modulate JNK activation, and some of these have known effects on cell migration. One such pathway is mediated by phosphatidylinositol 3-kinase (PI3K), part of a family of kinases that phosphorylate the membrane lipid phosphatidylinositol (15). Although the role of PI3K in cell motility is well documented, the interactions of PI3K with other signaling pathways involved in cell migration vary markedly in different types of cells (15–20), and no clear interaction between the PI3K and JNK pathways has been demonstrated in the early steps in cell migration. Furthermore, to our knowledge, no studies have examined how mechanical forces regulate PI3K signaling during cell migration.

Therefore, we investigated PI3K signaling in wounded AECs exposed to CS. We found that wounding of AECs activated PI3K and that CS inhibited this activation, leading to decreased activation of Rac1. Because FAK has been shown to serve as a scaffold for interactions with other signaling molecules involved in cell migration (21, 22) and because Tyr^{397} has been identified as a binding site for PI3K (23), we also investigated whether PI3K activity is affected by changes in FAK signaling and further investigated the potential interaction in signaling via PI3K and JNK.

EXPERIMENTAL PROCEDURES

Cell Culture—A human AEC line, 16HBE14o$^-$ (provided by D. Gruenert, California Pacific Institute), was used in all studies. These cells were transformed by SV40, maintain the capability to form polarized monolayers and tight junc-

* This work was supported, in whole or in part, by National Institutes of Health Grants HL-094366 (to C. M. W.) and HL-080417 (to S. R. W.).

◆ This article was selected as a Paper of the Week.

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[2] The abbreviations used are: AEC, airway epithelial cell; FAK, focal adhesion kinase; JNK, c-Jun N-terminal kinase; CS, cyclic stretch; PI3K, phosphatidylinositol 3-kinase; EGFP, enhanced green fluorescent protein; WT, wild-type; FRNK, FAK-related non-kinase; DN, dominant-negative; CA, constitutively active.

tions, and retain other characteristics of differentiated native epithelium (reviewed in Ref. 24). Cells were grown either on collagen IV-coated Silastic membranes (Flexcell International Corp., Hillsborough, NC) or on collagen IV-coated membranes upon which laminin-5-rich matrix was deposited by 804G cells (rat bladder carcinoma cells known to lay down a laminin-5 matrix in culture (25)) as described previously (12). Cyclic strain of 20% with a frequency of 30 cycles/min was applied using the Flexercell FX-4000T™ tension unit. In some experiments, cells were infected with an adenovirus expressing enhanced green fluorescent protein (EGFP) as a control (26), wild-type (WT) FAK (27), kinase-inactive FAK (FAK-related non-kinase (FRNK)) (28), WT-Rac1 (26), dominant-negative (DN) Rac1 (26), JIP3 (29), constitutively active (CA) PI3K (30), or DN-PI3K (31) (multiplicity of infection of 6–8) as described previously (26). Experiments were conducted 48 h after adenoviral infection.

Migration—Multiple wounds were scraped through confluent monolayers grown on either collagen IV or laminin-5 matrix using a fine-toothed comb in all cell migration assays. Each well contained ~17 wounds of ~475 μm, and migration was followed for up to 10 h in serum-free medium as described previously (26).

PI3K Activity Assay—PI3K activity was determined in either multiple wounded or unwounded cells using an activity kit from Echelon Biosciences Inc. (Salt Lake City, UT). Briefly, the assay involves isolation of PI3K from cell lysates (75 μg) using antibodies bound to agarose beads, incubation with substrate (phosphatidylinositol 3,4,5-bisphosphate), and a competitive enzyme-linked immunosorbent assay in which the signal is inversely proportional to the amount of phosphatidylinositol 3,4,5-trisphosphate produced from phosphatidylinositol 3,4,5-bisphosphate by PI3K.

Immunoprecipitations and Immunoblotting—Multiple wounds were applied to confluent monolayers, and cells were immediately exposed to static or CS conditions for 2 or 6 h. Cells were lysed with radioimmune precipitation assay buffer (150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and 50 mM Tris, pH 7.2) containing 1 mM sodium vanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin at 4 °C. Equal amounts of cell lysates were subjected to immunoprecipitation with either anti-FAK (BD Transduction Laboratories) or anti-JIP3 (Santa Cruz Biotechnology) antibody. Immunoblotting of the SDS-PAGE-separated protein complex was done using anti-PI3K (Upstate) or anti-JNK1 (Santa Cruz Biotechnology) antibody. For immunoblotting experiments to detect phosphorylated JNK1, 50 μg of the radioimmune precipitation assay cell lysates were loaded on gels for SDS-PAGE, transferred onto nitrocellulose membranes, and probed with anti-phospho-JNK1 (Abcam) and anti-JNK1 antibodies. For detection of expression of WT-FAK and CA-PI3K, blots were probed with anti-EGFP antibody (Santa Cruz Biotechnology), whereas JIP3 was detected using anti-FLAG antibody (Sigma). The secondary antibody used was either anti-rabbit (Amersham Biosciences) or anti-mouse (Jackson ImmunoResearch Laboratories) horseradish peroxidase.

Rac1 Activation Assay—Activated Rac1 (GTP-Rac1) levels in multiple wounded 16HBE14o⁻ cells, transfected with either CA-PI3K or DN-PI3K and exposed to static or CS conditions, were determined using the Rac1 activation assay kit from Upstate as described previously (32) using anti-Rac1 antibody (BD Transduction Laboratories).

Cytosolic and Membrane Fraction Preparations—Multiple wounded 16HBE14o⁻ cells expressing EGFP, CA-PI3K, or DN-PI3K were lysed, and the total, cytosolic, and membrane fractions for Tiam1 localization were prepared as described previously (32).

Statistical Analysis—All values are presented as means ± S.E. Statistical analysis was performed with the SigmaStat statistical package (Version 3.5, Jandel Scientific, San Rafael, CA). One-way analysis of variance with the Holm-Sidak method was performed for comparisons of multiple treatments to determine significant differences between individual or two different conditions. Significant differences were determined based on a threshold of $p < 0.05$.

RESULTS

PI3K Regulates Wound Closure of AECs—To investigate the role of PI3K in AEC wound healing, we first measured the closure of scrape wounds in 16HBE14o⁻ cells treated with the PI3K inhibitor LY294002. As shown in Fig. 1*A*, LY294002 (20 μM) slowed wound closure significantly, and the extent of inhibition was comparable with that caused by CS (20% linear strain, 30 cycles/min). Adenoviral expression of DN-PI3K also slowed wound closure comparable with that caused by CS, and expression of CA-PI3K stimulated cell migration (Fig. 1*B*). Because measurements were made over the initial 9 h following wounding, cell spreading and migration were the major mechanisms involved in wound closure. Adenoviral expression of EGFP as a control had no effect on wound closure.

Wounding Activates PI3K—To determine whether PI3K was activated by wounding of AECs, we measured PI3K activity in multiple wounded and unwounded monolayers grown either on collagen IV or on laminin-5 deposited upon collagen IV. Fig. 2*A* shows that there was a 4-fold increase in PI3K activity 2 h after multiple scrape wounds were applied to cells grown on either collagen or laminin, and the activity continued to increase 6 h after wounding. PI3K activity was higher in both wounded and unwounded cells grown on laminin-5 compared with collagen IV. We showed previously that cell migration of 16HBE14o⁻ cells is significantly faster on laminin-5 compared with collagen IV (12), and the results in Fig. 2 suggest that this difference may be due in part to enhanced activation of PI3K.

PI3K Activity Is Inhibited by CS—Because our previous studies demonstrated that CS inhibits wound closure of 16HBE14o⁻ cells (12, 13), we examined whether CS affected PI3K activation during wound closure. As shown in Fig. 2*B*, CS caused a significant decrease in PI3K activity in monolayers with multiple wounds on both collagen and laminin at 2 and 6 h after injury.

To investigate whether we could restore the rate of wound closure in cells exposed to CS by expression of CA-PI3K, we

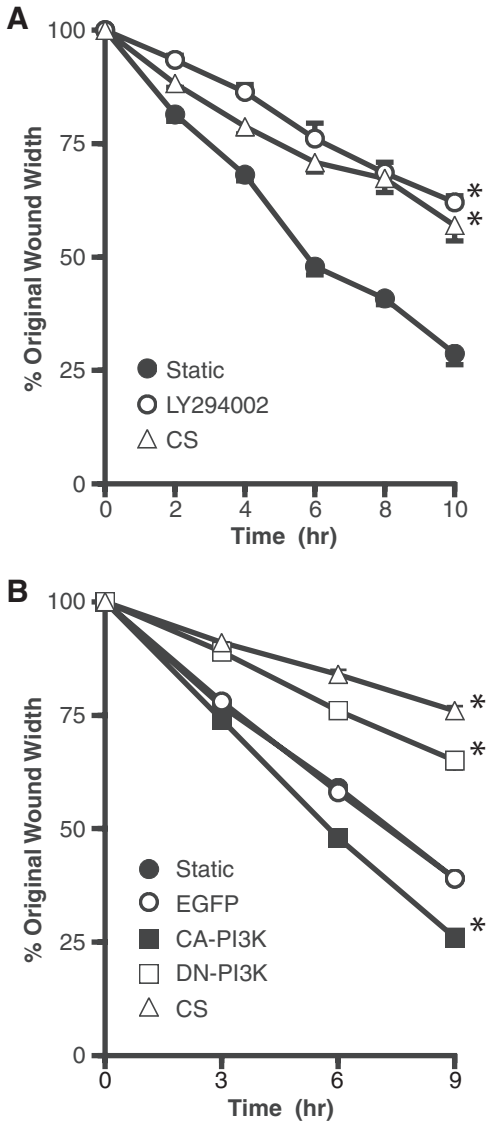


FIGURE 1. **PI3K regulates migration of 16HBE14o⁻ cells.** Scrape-wounded 16HBE14o⁻ cells grown on collagen IV (*A*) or laminin-5 (*B*) matrix were exposed to static conditions or cyclic mechanical stretch (CS) for 9 h. Multiple wounds were created in each well, and measurements were made on three wounds/well and averaged. *A*, treatment of static cells with the PI3K inhibitor LY294002 (20 μM) inhibited cell migration to the same extent as cells exposed to CS. *B*, cells were infected with adenovirus expressing EGFP, CA-PI3K, or DN-PI3K, and wound widths were measured. CA-PI3K stimulated cell migration, and DN-PI3K inhibited cell migration to the same extent as CS. Data are expressed as means ± S.E. (n = three independent experiments). *, $p < 0.05$, significant differences from static untreated cells.

first measured PI3K activity. Fig. 3*A* shows that CA-PI3K caused increased PI3K activity in cells under static conditions and in cells subjected to CS. Likewise, DN-PI3K significantly decreased PI3K activity both in cells under static conditions and in cells subjected to CS. Expression of CA-PI3K in cells exposed to CS enhanced wound closure, but closure was not completely restored in comparison with static cells (Fig. 3*B*). Adenoviral expression of EGFP as a control had no effect on either PI3K activity or cell migration (data not shown).

PI3K Regulates Rac1 Activity through Tiam1—We next investigated whether the loss of PI3K activity caused by CS also caused decreased Rac1 activity. Our previous studies demonstrated that CS inhibits wound healing of rat alveolar epithelial cells by causing decreased Rac1 activity in rat alveolar type II cells (32). Fig. 4 shows that 6 h of CS caused a significant decrease in GTP-Rac1 levels in wounded 16HBE14o⁻, and this effect was also observed after 2 h (data not shown). Fig. 4 also shows that expression of CA-PI3K caused a significant increase in Rac1 activity, and this increase was maintained under conditions of CS. DN-PI3K caused decreased Rac1 activity similar to the levels caused by CS. Expression of a control adenovirus (EGFP) had no effect on Rac1 activity.

PI3K has been shown to regulate Rac1 activity through the guanosine exchange factor Tiam1 in Madin-Darby canine kidney cells (33), and increased membrane localization of Tiam1 has been shown to increase Rac1 activity (34). To determine whether loss of PI3K activity in stretched cells caused decreased Rac1 activity by affecting Tiam1, we examined the cytosolic and membrane localization of Tiam1. CS caused increased cytosolic localization of Tiam1 (Fig. 5*A*) and decreased membrane localization (Fig. 5*B*) compared with static cells. Expression of CA-PI3K caused a significant increase in membrane-associated Tiam1 (Fig. 5*B*), whereas expression of DN-PI3K caused increased cytosolic Tiam1 (Fig. 5*A*). CS did not affect the distribution of Tiam1 when either CA-PI3K or DN-PI3K was expressed. We also found that treatment with the Tiam1 inhibitor NSC23766 caused increased cytosolic localization of Tiam1, decreased Rac1 activity, and decreased cell migration (data not shown). These results suggest that the decrease in PI3K activity caused by CS leads to increased cytosolic localization of Tiam1 and decreased Rac1 activity.

Rac1 Activation Causes Increased Phosphorylation of JNK1—JNK1 has been implicated as a downstream target of Rac1 in other cell types (35–37), and our previous studies have shown that inhibition of JNK1 decreases migration of AECs (38). In addition, we have shown that CS causes decreased phosphorylation of JNK1 and its downstream target, c-Jun (12). Therefore, we examined whether expression of DN-Rac1 induced decreased phosphorylation of JNK1. Fig. 6 shows that expression of DN-Rac1 caused decreased phosphorylation of JNK1, whereas overexpression of WT-Rac1 caused increased phosphorylation of JNK1. These results demonstrate that JNK1 phosphorylation can be modulated by Rac1 in AECs.

PI3K Activation in AECs Is Independent of FAK Phosphorylation—PI3K-mediated stimulation of cell migration has been shown to be dependent upon binding to FAK (at Tyr^{397} (23)) in Chinese hamster ovary cells (39), malignant breast epithelial cells (MCF-7) (21), and cardiomyocytes (40). To determine whether association with FAK affected PI3K activation in 16HBE14o⁻ cells, we expressed WT-FAK and a kinase-inactive form of FAK (FRNK) and measured PI3K activity. Previous studies have shown that WT-FAK expression increases and FRNK expression decreases FAK phosphorylation at Tyr^{397} (12). Fig. 7*A* shows that PI3K activity was not affected by expression of either vector under static or stretched conditions. In addition, immunoprecipitation of FAK-associated compounds showed that PI3K was not bound to FAK when cells were grown on either collagen or laminin (Fig. 7*B*).

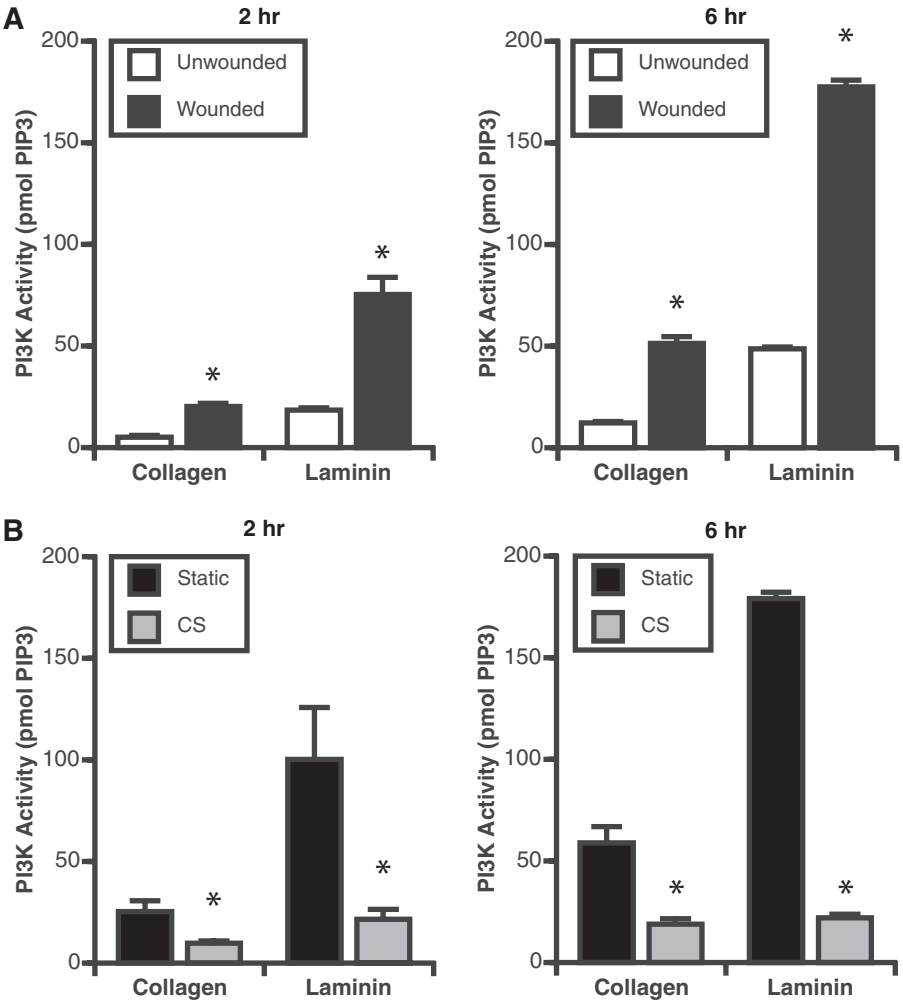


FIGURE 2. **Wounding stimulates PI3K activation, and CS inhibits activation.** *A*, wounding of 16HBE14o⁻ cells caused a sustained increase in PI3K activation, and activity was increased in cells grown on laminin-5 relative to collagen IV. Multiple wounds were applied to confluent cells, and cell lysates were collected after 2 and 6 h. *, $p < 0.05$, *versus* unwounded cells on the same matrix. *B*, CS inhibited the activation of PI3K in wounded cells on both collagen IV and laminin-5. Multiple wounds were applied to confluent cells, and cell lysates were collected after 2 and 6 h of CS or static conditions. *, $p < 0.05$, *versus* static cells on the same matrix. PI3K activity was estimated based on the formation of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) as described under "Experimental Procedures."

We also found recently that JIP3 associates with FAK to facilitate interaction with JNK and to regulate migration of cyclically stretched 16HBE14o⁻ cells (12). JIP3 is a scaffold protein, and to examine its possible interaction with PI3K, we determined whether PI3K co-immunoprecipitated with JIP3. As shown in Fig. 7*C*, we could not detect an association between JIP3 and PI3K.

Recovery of Cell Migration during CS Requires Restoration of PI3K, FAK, and JIP3—As shown in Fig. 3, expression of CA-PI3K, which prevents loss of PI3K activity during CS, accelerated cell migration during CS but did not restore the migration to the same level as in cells under static conditions. We found a similar result in a previous study when we coexpressed WT-FAK and JIP3 in 16HBE14o⁻ cells (12). These findings suggest that multiple pathways are affected by CS. We therefore coexpressed CA-PI3K, WT-FAK, and JIP3 to determine whether that would be sufficient to restore cell migration in stretched cells. Fig. 8 shows that the coexpression in cells exposed to CS restored cell migration to the level in cells under static conditions. These results indicate that restoration of FAK, JIP3, and PI3K signaling pathways is necessary for efficient migration of AECs during CS.

DISCUSSION

Our results demonstrate for the first time that wounding of AECs causes activation of PI3K and that CS both inhibits this activation and decreases cell migration. We also found that activation of PI3K stimulates Rac1 activation through Tiam1 and that Rac1 activation leads to phosphorylation of JNK1. However, restoration of cell migration in cells exposed to CS requires both stimulation of PI3K activity and recovery of FAK and JIP3 interactions.

PI3K activation has been shown to be an essential component of leukocyte migration, involved in both motility and directional sensing (reviewed in Ref. 15). PI3K is involved in similar processes in adherent cells, but the specific role of PI3K and the interactions with parallel pathways appear to vary markedly depending upon the type of cell or tissue (20). Wounding of retinal epithelial cells (ARPE-19) causes activation of PI3K through transactivation of the epidermal growth factor receptor (41), and inhibition of PI3K impairs cell migration of cultured intestinal epithelial cells (42, 43), Madin-Darby canine kidney cells (44), and corneal epithelial cells (45). Zhang *et al.* (46) suggested that the time-dependent increase in PI3K in wounded rabbit corneal epithelial cells *in vivo* primarily affects cell proliferation. Others have suggested that cell migration is independent of PI3K-mediated Rac activation in human keratinocytes (47), that oxidant-mediated inhibition of IEC-6 (intestinal epithelial) cell wound closure is not regulated by PI3K (48), and that ACK-2-mediated inhibition of HeLa cell migration is independent of PI3K (49). In our studies, PI3K activity was significantly increased 2 h after wounding and continued to increase after 6 h (Fig. 2). We found that inhibition of PI3K, both pharmacologically and through expression of DN-PI3K, caused decreased cell migration comparable with the levels of migration measured in cells exposed to CS (Fig. 1).

The activation of PI3K was significantly higher in cells grown on laminin-5 matrix compared with cells grown on collagen IV (Fig. 2). Airway wall remodeling is known to

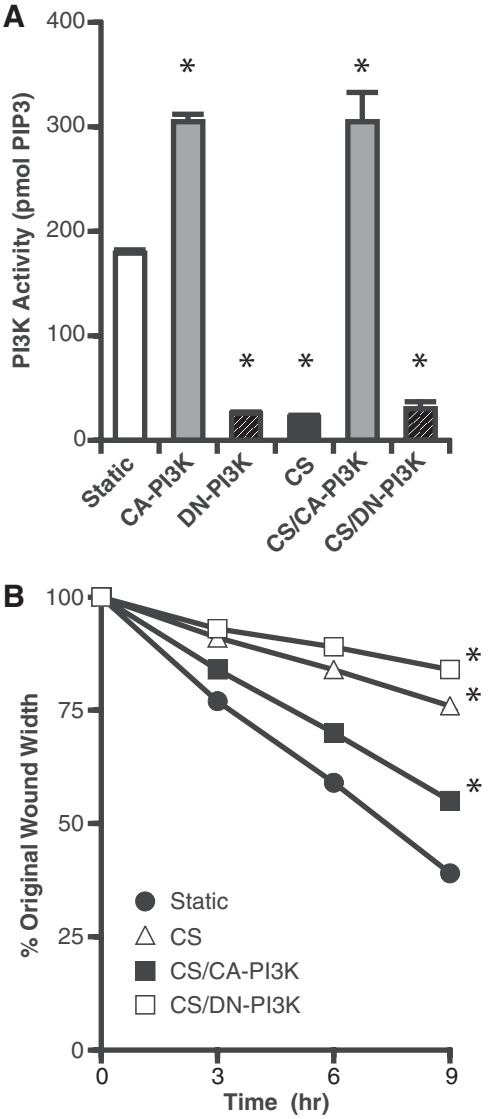


FIGURE 3. **Expression of CA-PI3K prevents CS-induced loss of PI3K activity and partially restores cell migration.** Cells were grown on laminin-5 matrix and treated with adenovirus expressing CA-PI3K, DN-PI3K, or EGFP (not shown) for 48 h prior to wounding. *A*, multiple wounds were applied, and cell lysates were collected at 6 h for PI3K activity. *B*, wound closure was measured over 9 h. (Similar results were obtained using cells grown on collagen IV.) *, $p < 0.05$, significant difference from static untreated cells. PIP_3, phosphatidylinositol 3,4,5-trisphosphate.

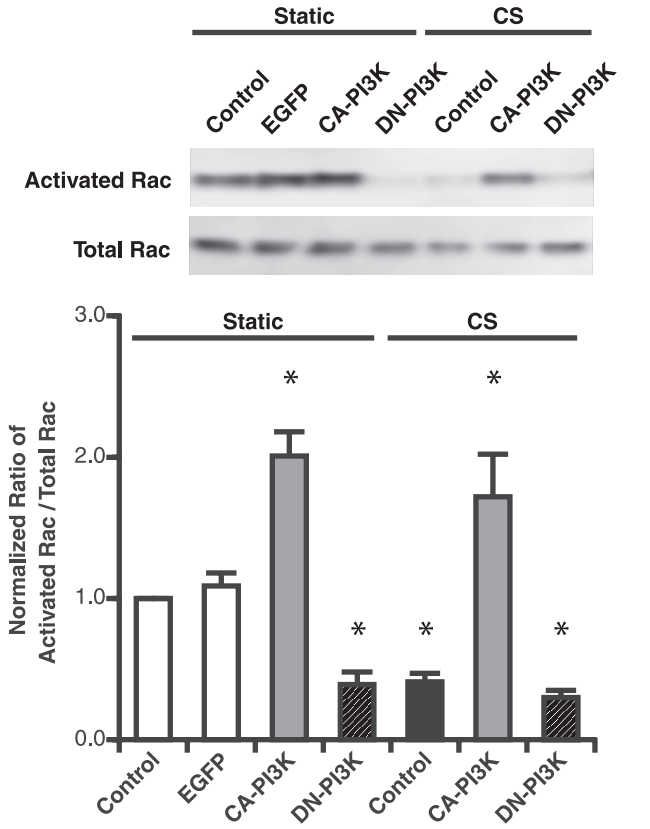


FIGURE 4. **PI3K regulates Rac1 activation.** Cells were grown on laminin-5 matrix and treated with adenovirus expressing CA-PI3K, DN-PI3K, or EGFP for 48 h prior to wounding. Multiple wounds were applied, cells were exposed to CS or static conditions for 6 h, and Rac1 activity (GTP-Rac1) was assessed via a pulldown assay. The *blot* is representative of four independent experiments, and the *bar graph* summarizes the densitometry data expressed as means ± S.E. Values are expressed as the ratio of active GTP-bound Rac1 to total Rac1. *, $p < 0.05$, significant difference from the static control. (Similar results (not shown) were obtained after 2 h.)

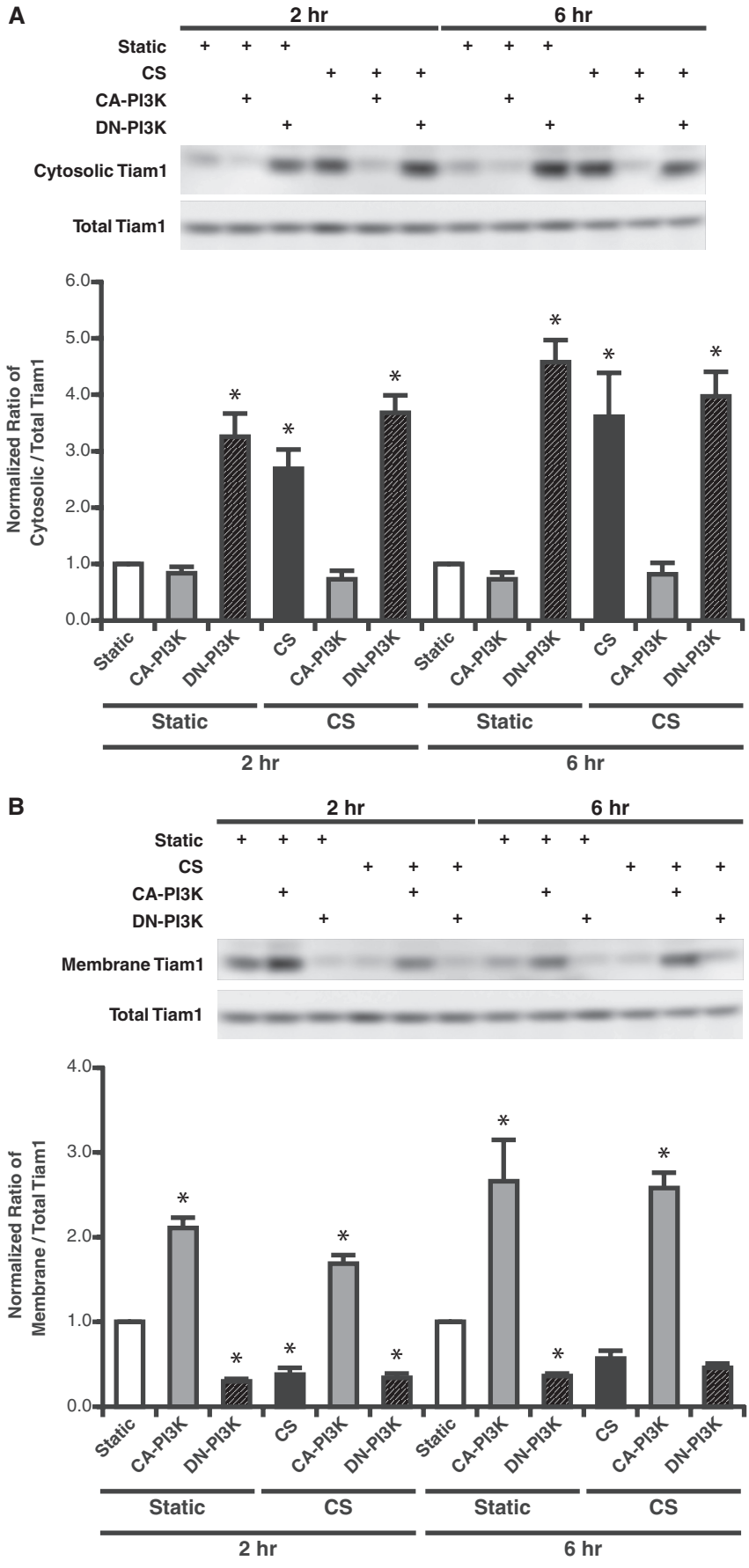
occur in asthmatics (50–53) with changes in both the basement membrane and the subepithelial layer (54–56). Laminin-5 is a major component of non-pathological basement membrane and has been demonstrated to play an important role in cell migration in other cell types (57–59). It has been reported that adhesion of human colon adenocarcinoma cells to laminin-5 induces PI3K-dependent activation of Rac1 (60), and activation of β-catenin in colon cancer cells is inhibited by fluid shear stress through a pathway involving laminin-5, PI3K, and Rac1 (61). Migration of human corneal epithelial cells is significantly increased through expression of laminin-5 by activation of the PI3K/Akt pathway (62), whereas deposition of laminin-5 and ligation by integrins activate PI3K and promote adhesion and spreading of keratinocytes (58). We and others have shown that laminin-5 matrix up-regulates FAK Tyr^{397} phosphorylation and increases migration of A549 cells (63) and cyclically stretched 16HBE14o⁻ cells (12). We also demonstrated that laminin-5 matrix activates a signaling pathway involving FAK, JIP3, and JNK that accelerates migration of cyclically stretched AECs (12).

Airways in asthmatics are subject to increased mechanical stimulation due to both increased base-line tone of the airways and changes in airway wall structure. We showed that CS caused decreased PI3K activity and that the effect persisted for up to 6 h (Fig. 2). In contrast with our findings with longer duration of stretch, short-term (<5 min) CS has been shown previously to stimulate PI3K activity in endothelial cells (64, 65), fibroblasts (66), osteoblasts (67), bladder smooth muscle cells (68), vascular smooth muscle cells (69, 70), and epithelial cell lines (71). Although we did not measure PI3K activation in response to short-term CS, our previous studies showed that FAK phosphorylation is initially increased in response to CS (<30 min) but then is significantly decreased with time relative to unstretched cells (12). We speculate that an initial increase in PI3K activity also occurred in our cells but that adaptation to CS with time resulted in decreased PI3K activity. Hammerschmidt *et al.* (72) found that PI3K activity is decreased in rat alveolar epi-

thelial cells after 24 h of CS. Our results support those findings in AECs and demonstrate for the first time that CS inhibits PI3K activation during wound healing.

Previous studies from our laboratory have demonstrated that cyclic mechanical strain inhibits migration of AECs after injury (13, 14), as shown in Fig. 1, and we showed recently that the inhibition is due in part to decreased FAK phosphorylation, which results in decreased association of JIP3 and downstream JNK signaling (12). Because previous studies have suggested that PI3K activation may be dependent upon interactions with FAK (21, 23, 39, 40), we hypothesized that the decrease in PI3K we observed in response to CS was due to diminished interaction with FAK. However, we could not detect an association between PI3K and FAK in our cells (Fig. 7). Furthermore, manipulation of FAK phosphorylation using expression of WT-FAK and FRNK had no effect on PI3K activation.

In this study, we identified a parallel pathway involved in cell migration that was inhibited by CS. We showed that CS caused decreased Rac1 activation (Fig. 4) and that this decrease was dependent upon CS-induced localization of Tiam1 to the cytosol (Fig. 5). It has been demonstrated previously that Tiam1 requires membrane localization via its N-terminal pleckstrin homology domain to activate endogenous Rac1 (73, 74). In Madin-Darby canine kidney cells, Tiam1-mediated Rac1 activation is dependent on PI3K activity on both a collagen I and fibronectin matrix, suggesting that PI3K is required for Tiam1/Rac1-induced cell migration. Similarly, Shaw *et al.* (19) demonstrated that PI3K acts upstream of Rac1 in PI3K-induced motility of colon carcinoma cells. However, using T47D mammary carcinoma cells, Keely *et al.* (18) found that PI3K is located downstream of Rac1, and others have suggested a negative feedback loop for the regulation of Rac1 through PI3K (75, 76).



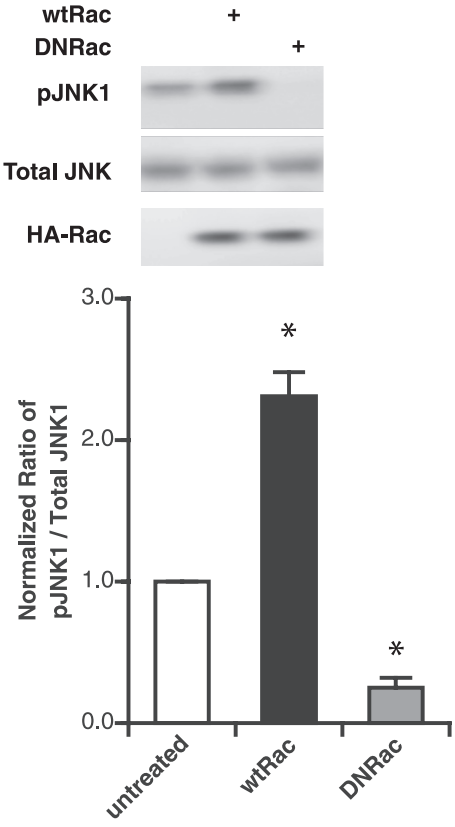


FIGURE 6. **Activated Rac induces phosphorylation of JNK1.** Representative immunoblots of equal amounts of cell lysates from multiple wounded monolayers grown on laminin-5 matrix expressing WT-Rac1 or DN-Rac1 were probed with anti-phosphorylated and anti-total JNK1 antibodies. The *bar graph* summarizes the densitometry data expressed as means ± S.E. (n = four independent experiments). *, $p < 0.05$, significant difference from the untreated control. *HA*, hemagglutinin.

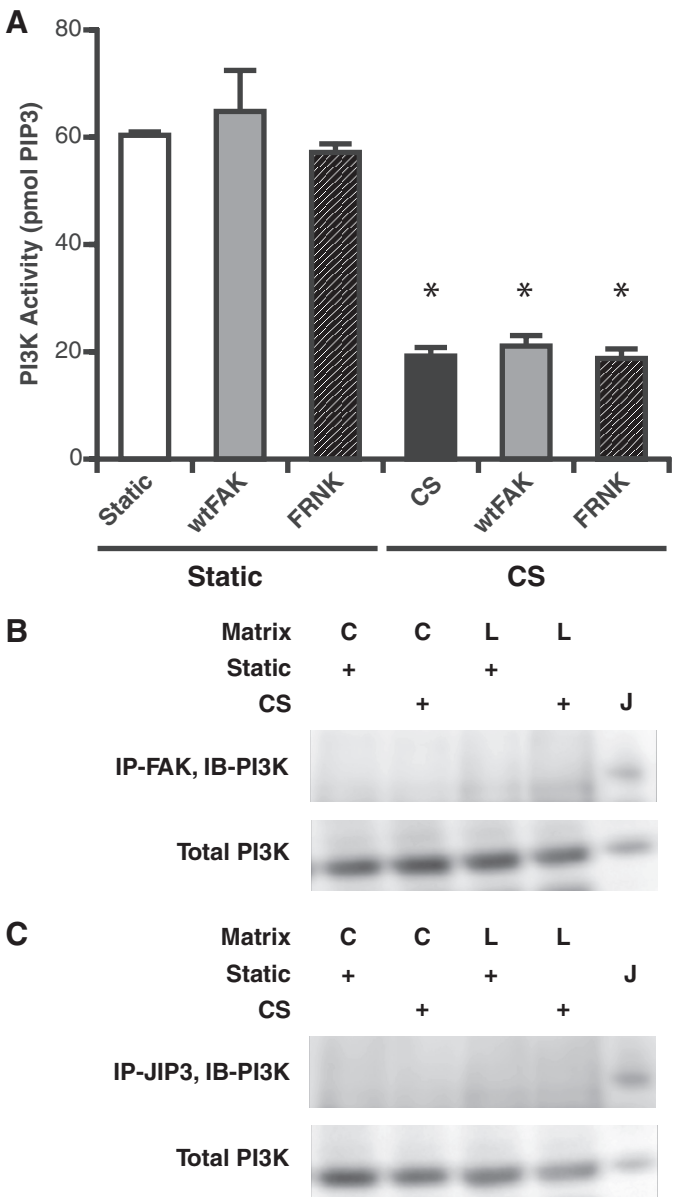


FIGURE 7. **PI3K activation is independent of FAK phosphorylation.** Confluent cells grown on collagen IV (*C*) or laminin-5 (*L*) matrix were treated with adenovirus expressing WT-FAK or FRNK for 48 h prior to application of multiple wounds. Cells were exposed to static or CS conditions for 6 h. *A*, PI3K activity was not affected by expression of WT-FAK or FRNK. *, $p < 0.05$, significant difference from the static control. PIP_3, phosphatidylinositol 3,4,5-trisphosphate. *B* and *C*, cells were lysed and immunoprecipitated (*IP*) with anti-FAK (*B*) or anti-JIP3 (*C*) antibody and then immunoblotted (*IB*) with anti-PI3K antibody. Representative Western blots from four different experiments are shown. Jurkat (*J*) cell lysate was used as a positive control.

Our findings suggest that PI3K acts upstream of Tiam1/Rac1 in the response to wounding and that CS-induced loss of PI3K activity results in downstream loss of Rac1 activity (Figs. 4 and 5).

In our previous study, we were able to stimulate cell migration in cells exposed to CS by coexpression of WT-FAK and JIP3, but we were unable to fully restore migration to the level in static cells (12). We observed the same response in this study using CA-PI3K (Fig. 3). When we coexpressed WT-FAK, JIP3, and CA-PI3K, we restored the migration of cells exposed to CS. These results suggest that parallel pathways are involved in the regulation of cell migration in AECs, as shown in Fig. 9. In this scheme, wounding induces integrin-mediated activation of both FAK and PI3K. Activation of FAK leads to association with JIP3, phosphorylation of JNK1, and activation of other pathways that regulate cell migration. Activation of PI3K causes translocation of Tiam1 to the cell membrane and subsequent Rac1 activation that leads to regulation of F-actin at the leading edge of the cell and to phosphorylation of JNK1. CS causes disruption of association between FAK and JIP3 and independently decreases PI3K-mediated activation of Rac1. Inoue and Meyer (76) recently proposed an

FIGURE 5. **PI3K regulates localization of the Rac1 guanosine exchange factor Tiam1.** Cells were grown on laminin-5 matrix and treated with adenovirus expressing CA-PI3K, DN-PI3K, or EGFP for 48 h prior to wounding. Multiple wounds were applied, and cells were exposed to CS or static conditions for 2 or 6 h. Cells were lysed, aliquots of the total fraction were withdrawn, and cell lysates were further subjected to high speed centrifugation to prepare cytosolic and membrane fractions. The total, cytosolic (*A*), and membrane (*B*) levels of Tiam1 were assessed by Western blotting. The ratio of cytosolic or membrane-associated Tiam1 to total Tiam1 was then normalized to the ratio for static control cells at that time. The *blots* are representative of four different experiments, and the *bar graphs* summarize the densitometry data expressed as means ± S.E. (n = four independent experiments). *, $p < 0.05$, significant difference from the static control.

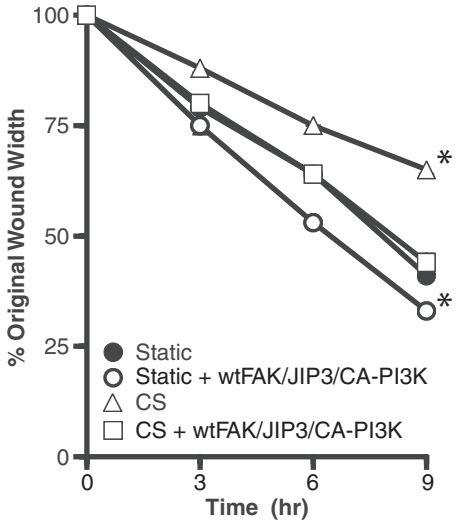


FIGURE 8. **Coexpression of WT-FAK, JIP3, and CA-PI3K restores migration of cells exposed to CS to the level in static cells.** Cells were grown on laminin-5 matrix and treated with adenovirus expressing WT-FAK, JIP3, and CA-PI3K for 48 h prior to wounding. Multiple wounds were applied, and cells were exposed to CS or static conditions for 9 h. *, $p < 0.05$, significant difference from the static control.

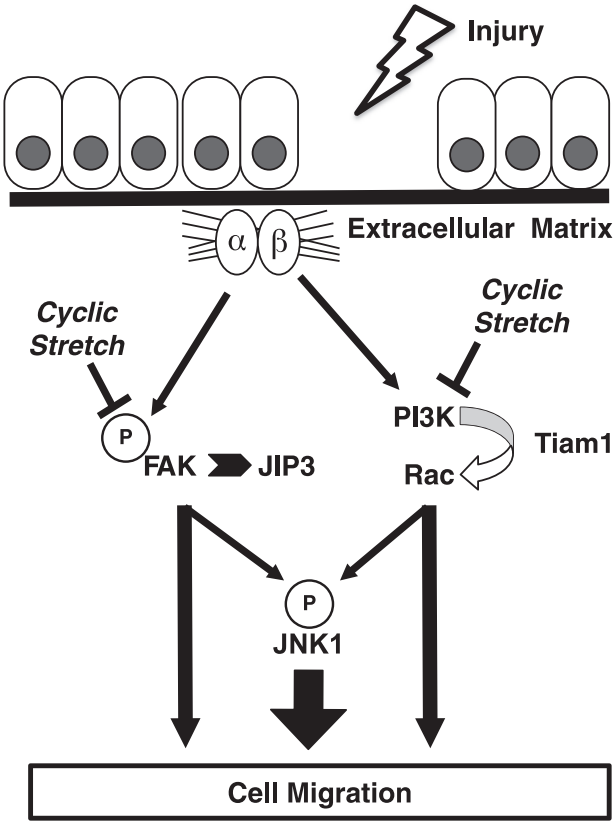


FIGURE 9. **Schematic representation of pathways affected by cyclic strain causing inhibition of migration of AECs.** After injury, signals from integrin receptors in the extracellular matrix activate two signaling pathways, one that leads to phosphorylation of FAK and one that leads to activation of Rac via activation of PI3K. Both lead to phosphorylation of JNK and thus to cell migration. Both pathways may be down-regulated by cyclic mechanical stretch, and loss of either pathway slows cell migration.

"AND-gate" control mechanism for PI3K-dependent and PI3K-independent pathways in the regulation of cell polarization and migration, and this mechanism supports our findings. In summary, we have defined a unique signaling mechanism that shows that both a phosphorylated FAK/JIP3 scaffold and PI3K-induced Rac activation are required for increased JNK1 phosphorylation and efficient cell migration in cyclically stretched cells.

Acknowledgment—We thank Dr. Meena P. Desai for suggestions and help with the enzyme-linked immunosorbent assays.

REFERENCES

1. Erjefält, J. S., Erjefält, I., Sundler, F., and Persson, C. G. (1995) *Cell Tissue Res.* **281,** 305–316
2. Rickard, K. A., Taylor, J., Rennard, S. I., and Spurzem, J. R. (1993) *Am. J. Respir. Cell Mol. Biol.* **8,** 63–68
3. White, S. R., Dorscheid, D. R., Rabe, K. F., Wojcik, K. R., and Hamann, K. J. (1999) *Am. J. Respir. Cell Mol. Biol.* **20,** 787–796
4. Cox, B. D., Natarajan, M., Stettner, M. R., and Gladson, C. L. (2006) *J. Cell. Biochem.* **99,** 35–52
5. Schlaepfer, D. D., and Mitra, S. K. (2004) *Curr. Opin. Genet. Dev.* **14,** 92–101
6. Huang, C., Jacobson, K., and Schaller, M. D. (2004) *J. Cell Sci.* **117,** 4619–4628
7. Xia, Y., and Karin, M. (2004) *Trends Cell Biol.* **14,** 94–101
8. An, S. S., Bai, T. R., Bates, J. H., Black, J. L., Brown, R. H., Brusasco, V., Chitano, P., Deng, L., Dowell, M., Eidelman, D. H., Fabry, B., Fairbank, N. J., Ford, L. E., Fredberg, J. J., Gerthoffer, W. T., Gilbert, S. H., Gosens, R., Gunst, S. J., Halayko, A. J., Ingram, R. H., Irvin, C. G., James, A. L., Janssen, L. J., King, G. G., Knight, D. A., Lauzon, A. M., Lakser, O. J., Ludwig, M. S., Lutchen, K. R., Maksym, G. N., Martin, J. G., Mauad, T., McParland, B. E., Mijailovich, S. M., Mitchell, H. W., Mitchell, R. W., Mitzner, W., Murphy, T. M., Paré, P. D., Pellegrino, R., Sanderson, M. J., Schellenberg, R. R., Seow, C. Y., Silveira, P. S., Smith, P. G., Solway, J., Stephens, N. L., Sterk, P. J., Stewart, A. G., Tang, D. D., Tepper, R. S., Tran, T., and Wang, L. (2007) *Eur. Respir. J.* **29,** 834–860
9. Fredberg, J. J., and Kamm, R. D. (2006) *Annu. Rev. Physiol.* **68,** 507–541
10. Wiggs, B. R., Hrousis, C. A., Drazen, J. M., and Kamm, R. D. (1997) *J. Appl. Physiol.* **83,** 1814–1821
11. Tschumperlin, D. J., and Drazen, J. M. (2006) *Annu. Rev. Physiol.* **68,** 563–583
12. Desai, L. P., White, S. R., and Waters, C. M. (2009) *Am. J. Physiol. Lung Cell. Mol. Physiol.* **297,** L520–L529
13. Savla, U., and Waters, C. M. (1998) *Am. J. Physiol. Lung Cell. Mol. Physiol.* **274,** L883–L892
14. Savla, U., Olson, L. E., and Waters, C. M. (2004) *J. Appl. Physiol.* **96,** 566–574
15. Procko, E., and McColl, S. R. (2005) *BioEssays* **27,** 153–163
16. Royal, I., and Park, M. (1995) *J. Biol. Chem.* **270,** 27780–27787
17. Wennström, S., Hawkins, P., Cooke, F., Hara, K., Yonezawa, K., Kasuga, M., Jackson, T., Claesson-Welsh, L., and Stephens, L. (1994) *Curr. Biol.* **4,** 385–393
18. Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J., and Parise, L. V. (1997) *Nature* **390,** 632–636
19. Shaw, L. M., Rabinovitz, I., Wang, H. H., Toker, A., and Mercurio, A. M. (1997) *Cell* **91,** 949–960
20. Kölsch, V., Charest, P. G., and Firtel, R. A. (2008) *J. Cell Sci.* **121,** 551–559
21. Kallergi, G., Tsapara, A., Kampa, M., Papakonstanti, E. A., Krasagakis, K., Castanas, E., and Stournaras, C. (2003) *Exp. Cell Res.* **288,** 94–109
22. Kaczmarek, E., Erb, L., Koziak, K., Jarzyna, R., Wink, M. R., Guckelberger, O., Blusztajn, J. K., Trinkaus-Randall, V., Weisman, G. A., and Robson, S. C. (2005) *Thromb. Haemost.* **93,** 735–742
23. Chen, H. C., Appeddu, P. A., Isoda, H., and Guan, J. L. (1996) *J. Biol. Chem.* **271,** 26329–26334
24. Gruenert, D. C., Finkbeiner, W. E., and Widdicombe, J. H. (1995) *Am. J. Physiol. Lung Cell. Mol. Physiol.* **268,** L347–L360
25. Tsuruta, D., Hopkinson, S. B., Lane, K. D., Werner, M. E., Cryns, V. L., and

Jones, J. C. (2003) *J. Biol. Chem.* **278,** 38707–38714
26. Desai, L. P., Aryal, A. M., Ceacareanu, B., Hassid, A., and Waters, C. M. (2004) *Am. J. Physiol. Lung Cell. Mol. Physiol.* **287,** L1134–L1144
27. Bryant, P., Zheng, Q., and Pumiglia, K. (2006) *Mol. Cell. Biol.* **26,** 4201–4213
28. Taylor, J. M., Mack, C. P., Nolan, K., Regan, C. P., Owens, G. K., and Parsons, J. T. (2001) *Mol. Cell. Biol.* **21,** 1565–1572
29. Song, J. J., and Lee, Y. J. (2005) *J. Biol. Chem.* **280,** 26845–26855
30. Matsui, T., Li, L., del Monte, F., Fukui, Y., Franke, T. F., Hajjar, R. J., and Rosenzweig, A. (1999) *Circulation* **100,** 2373–2379
31. Kong, M., Mounier, C., Wu, J., and Posner, B. I. (2000) *J. Biol. Chem.* **275,** 36035–36042
32. Desai, L. P., Chapman, K. E., and Waters, C. M. (2008) *Am. J. Physiol. Lung Cell. Mol. Physiol.* **295,** L958–L965
33. Sander, E. E., van Delft, S., ten Klooster, J. P., Reid, T., van der Kammen, R. A., Michiels, F., and Collard, J. G. (1998) *J. Cell Biol.* **143,** 1385–1398
34. Moissoglu, K., Slepchenko, B. M., Meller, N., Horwitz, A. F., and Schwartz, M. A. (2006) *Mol. Biol. Cell* **17,** 2770–2779
35. Coso, O. A., Chiariello, M., Yu, J. C., Teramoto, H., Crespo, P., Xu, N., Miki, T., and Gutkind, J. S. (1995) *Cell* **81,** 1137–1146
36. Chan, A., Akhtar, M., Brenner, M., Zheng, Y., Gulko, P. S., and Symons, M. (2007) *Mol. Med.* **13,** 297–304
37. Hwang, S. Y., Jung, J. W., Jeong, J. S., Kim, Y. J., Oh, E. S., Kim, T. H., Kim, J. Y., Cho, K. H., and Han, I. O. (2006) *Int. J. Cancer* **118,** 2056–2063
38. White, S. R., Tse, R., and Marroquin, B. A. (2005) *Am. J. Respir. Cell Mol. Biol.* **32,** 301–310
39. Reiske, H. R., Kao, S. C., Cary, L. A., Guan, J. L., Lai, J. F., and Chen, H. C. (1999) *J. Biol. Chem.* **274,** 12361–12366
40. Del Re, D. P., Miyamoto, S., and Brown, J. H. (2008) *J. Biol. Chem.* **283,** 35622–35629
41. Xu, K. P., and Yu, F. S. (2007) *Invest. Ophthalmol. Vis. Sci.* **48,** 2242–2248
42. El-Assal, O. N., and Besner, G. E. (2005) *Gastroenterology* **129,** 609–625
43. Tétreault, M. P., Chailler, P., Beaulieu, J. F., Rivard, N., and Ménard, D. (2008) *J. Cell. Physiol.* **214,** 545–557
44. Boca, M., D'Amato, L., Distefano, G., Polishchuk, R. S., Germino, G. G., and Boletta, A. (2007) *Mol. Biol. Cell* **18,** 4050–4061
45. Xu, K. P., Riggs, A., Ding, Y., and Yu, F. S. (2004) *Invest. Ophthalmol. Vis. Sci.* **45,** 4277–4283
46. Zhang, Y., Liou, G. I., Gulati, A. K., and Akhtar, R. A. (1999) *Invest. Ophthalmol. Vis. Sci.* **40,** 2819–2826
47. Tscharntke, M., Pofahl, R., Krieg, T., and Haase, I. (2005) *FASEB J.* **19,** 1836–1838
48. Zou, L., Sato, N., and Kone, B. C. (2004) *Shock* **22,** 453–459
49. Coon, M., and Herrera, R. (2002) *J. Cell. Biochem.* **84,** 655–665
50. Bousquet, J., Jeffery, P. K., Busse, W. W., Johnson, M., and Vignola, A. M. (2000) *Am. J. Respir. Crit. Care Med.* **161,** 1720–1745
51. Jeffery, P. K. (2001) *Am. J. Respir. Crit. Care Med.* **164,** S28–S38
52. Benayoun, L., Druilhe, A., Dombret, M. C., Aubier, M., and Pretolani, M. (2003) *Am. J. Respir. Crit. Care Med.* **167,** 1360–1368
53. Homer, R. J., and Elias, J. A. (2005) *Physiology* **20,** 28–35
54. Roche, W. R., Beasley, R., Williams, J. H., and Holgate, S. T. (1989) *Lancet* **1,** 520–524
55. Chu, H. W., Halliday, J. L., Martin, R. J., Leung, D. Y., Szefler, S. J., and Wenzel, S. E. (1998) *Am. J. Respir. Crit. Care Med.* **158,** 1936–1944
56. Laitinen, A., Altraja, A., Kämpe, M., Linden, M., Virtanen, I., and Laitinen, L. A. (1997) *Am. J. Respir. Crit. Care Med.* **156,** 951–958
57. Kariya, Y., and Miyazaki, K. (2004) *Exp. Cell Res.* **297,** 508–520
58. Nguyen, B. P., Gil, S. G., and Carter, W. G. (2000) *J. Biol. Chem.* **275,** 31896–31907
59. Baba, Y., Iyama, K. I., Hirashima, K., Nagai, Y., Yoshida, N., Hayashi, N., Miyanari, N., and Baba, H. (2008) *Br. J. Cancer* **98,** 974–980
60. Chartier, N. T., Lainé, M., Gout, S., Pawlak, G., Marie, C. A., Matos, P., Block, M. R., and Jacquier-Sarlin, M. R. (2006) *J. Cell Sci.* **119,** 31–46
61. Avvisato, C. L., Yang, X., Shah, S., Hoxter, B., Li, W., Gaynor, R., Pestell, R., Tozeren, A., and Byers, S. W. (2007) *J. Cell Sci.* **120,** 2672–2682
62. Lee, H. K., Lee, J. H., Kim, M., Kariya, Y., Miyazaki, K., and Kim, E. K. (2006) *Invest. Ophthalmol. Vis. Sci.* **47,** 873–882
63. Kodama, K., Ishii, G., Miyamoto, S., Goya, M., Zhang, S. C., Sangai, T., Yoshikawa, T., Hasebe, T., Hitomi, Y., Izumi, K., and Ochiai, A. (2005) *Int. J. Cancer* **116,** 876–884
64. Thodeti, C. K., Matthews, B., Ravi, A., Mammoto, A., Ghosh, K., Bracha, A. L., and Ingber, D. E. (2009) *Circ. Res.* **104,** 1123–1130
65. Ikeda, M., Kito, H., and Sumpio, B. E. (1999) *Biochem. Biophys. Res. Commun.* **257,** 668–671
66. Katsumi, A., Naoe, T., Matsushita, T., Kaibuchi, K., and Schwartz, M. A. (2005) *J. Biol. Chem.* **280,** 16546–16549
67. Danciu, T. E., Adam, R. M., Naruse, K., Freeman, M. R., and Hauschka, P. V. (2003) *FEBS Lett.* **536,** 193–197
68. Adam, R. M., Roth, J. A., Cheng, H. L., Rice, D. C., Khoury, J., Bauer, S. B., Peters, C. A., and Freeman, M. R. (2003) *J. Urol.* **169,** 2388–2393
69. Sedding, D. G., Hermsen, J., Seay, U., Eickelberg, O., Kummer, W., Schwencke, C., Strasser, R. H., Tillmanns, H., and Braun-Dullaeus, R. C. (2005) *Circ. Res.* **96,** 635–642
70. Sedding, D. G., Seay, U., Fink, L., Heil, M., Kummer, W., Tillmanns, H., and Braun-Dullaeus, R. C. (2003) *Circulation* **108,** 616–622
71. Kippenberger, S., Loitsch, S., Guschel, M., Müller, J., Knies, Y., Kaufmann, R., and Bernd, A. (2005) *J. Biol. Chem.* **280,** 3060–3067
72. Hammerschmidt, S., Kuhn, H., Gessner, C., Seyfarth, H. J., and Wirtz, H. (2007) *Am. J. Respir. Cell Mol. Biol.* **37,** 699–705
73. Michiels, F., Stam, J. C., Hordijk, P. L., van der Kammen, R. A., Ruuls-Van Stalle, L., Feltkamp, C. A., and Collard, J. G. (1997) *J. Cell Biol.* **137,** 387–398
74. Stam, J. C., Sander, E. E., Michiels, F., van Leeuwen, F. N., Kain, H. E., van der Kammen, R. A., and Collard, J. G. (1997) *J. Biol. Chem.* **272,** 28447–28454
75. Costa, C., Barberis, L., Ambrogio, C., Manazza, A. D., Patrucco, E., Azzolino, O., Neilsen, P. O., Ciraolo, E., Altruda, F., Prestwich, G. D., Chiarle, R., Wymann, M., Ridley, A., and Hirsch, E. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104,** 14354–14359
76. Inoue, T., and Meyer, T. (2008) *PloS One* **3,** e3068