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Integrin-linked kinase: A multi-functional regulator modulating extracellular pressure-stimulated cancer cell adhesion through focal adhesion kinase and AKT

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Abstract. Cell adhesion is important in cancer metastasis. Malignant cells in cancer patients may be exposed to physical forces such as extracellular pressure and shear, that stimulate their adhesion to matrix proteins, endothelium and surgical wounds. Pressure induces phosphorylation of AKT and focal adhesion kinase (FAK), which are required for pressure-stimulated cancer cell adhesion, but what mediates this effect is unknown. ILK may influence cell adhesion and FAK and AKT phosphorylation in other settings. We therefore hypothesized that ILK might also regulate pressure-stimulated cancer cell adhesion through AKT and FAK phosphorylation. Silencing ILK by siRNA reduced basal cancer cell adhesion and prevented the stimulation of adhesion by pressure. ILK mediated pressure-stimulated adhesion through specifically regulating phosphorylation of AKT at Ser473 and FAK at Tyr397 and 576 as well as ILK association with FAK and AKT. The siRNA-mediated loss of function of ILK in regulating increase in adhesion by pressure was not rescued by overexpression of α-parvin, an important ILK binding partner, although pressure promoted ILK–α-parvin association and translocated both ILK and α-parvin from cytosol to membrane/cytoskeleton. ILK may be a key mediator of mechanotransduced signals in cancer cells and an important therapeutic target to inhibit metastatic cancer cell adhesion.

Keywords: Cell adhesion, integrin-linked kinase (ILK), alpha-parvin/actopaxin/CH-ILKBP, AKT/protein kinase B, focal adhesion kinase (FAK), extracellular pressure, phosphorylation

1. Introduction

Cell adhesion, an important biological process, plays a pivotal role in cancer metastasis. Cancer cells in cancer patients may be exposed various physical forces, such as increased extracellular pressure and shear stress during vascular and lymphatic transit, perioperatively during laparoscopic insufflation of the abdomen, irrigation of the surgical site or surgical manipulation, or after surgery when intraabdominal pressure increases due to third spacing and bowel edema. Modest 15 mmHg increases in extracellular pressure consistent with these pathophysiologic effects activate an intracellular signaling pathway in cancer cells that stimulates adhesion to matrix proteins and endothelial cells *in vitro* [1–3] and significantly promotes tumor development and impairs tumor-free survival in a transplantable murine tumor model [4]. Therefore, forces such as pressure may promote cancer metastasis in shed tumor cells by stimulating the adhesiveness of viable metastasizing tumor cells to distant sites. A more precise understanding of the underlying mechanisms by which physical forces such as extracellular pressure stimulate cancer cells to increase their adhesiveness, therefore, would be of benefit not only to the delineation of this novel mechanotransduced cell signaling pathway, but also to the design of rational intervention to inhibit cancer metastasis, the major cause for cancer reoccurrence after potentially cu-

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rative surgery. AKT/protein kinase B, consisting of AKT1, AKT2 and AKT3 isoforms, is a ubiquitously expressed Ser/Thr kinase regulating various pathological and physiological processes [5–7]. Phosphorylation of AKT at Thr308 and Ser473 is required for its full activation. Extracellular pressure specifically promotes the phosphorylation of AKT1 and this phosphorylation is required for pressure-stimulated cancer cell adhesion [5].

It is now clear that phosphorylation of AKT at T308 is regulated by phosphoinositide-dependent kinase 1 (PDK1) [6,7]. However, it is still controversial which Ser473 kinase (also called PDK2) mediates the phosphorylation of AKT at Ser473, in particular, in response to physical force stimulation. Several candidates have been proposed, including integrin-linked kinase (ILK) [8], DNA-dependent protein kinase, AKT itself, mitogen activated protein kinase activated protein kinase 2, protein kinase C βII [7], and, more recently, the mTOR complex TORC2 [9]. The Ser/Thr kinase ILK is a particularly intriguing candidate for an upstream mediator of the pressure-activated cancer cell adhesive signal pathway. In response to other stimuli, ILK influences a wide range of biological processes including cell adhesion, migration and motility [10], cell proliferation [11] and mitotic spindle organization [12]. Proteins such as α-parvin/actopaxin/CH-ILKBP and PINCH bind directly to ILK and help ILK localize to focal adhesion plaques [10,13,14]. In addition, α-parvin regulates AKT (S473) phosphorylation in HeLa cells through facilitating the membrane translocation of AKT [15]. We hypothesized that ILK, working with its binding partner, α-parvin, is the upstream regulator of AKT that mediates pressure-induced cancer cell adhesion. We tested our hypothesis in human Caco-2 colon cancer cells and confirmed our key findings in another human colon cancer SW620 cells and primary human colon cancer cells isolated from surgical specimens.

In addition, focal adhesion kinase (FAK) and paxillin are two important components of focal adhesion complexes that are phosphorylated in cancer cells in response to increased extracellular pressure, and that are also required for pressure-stimulated cancer cell adhesion [1,2,19]. The phosphorylation of FAK and paxillin can be regulated by ILK in cells and mice [20,21]. Therefore, we also investigated the potential relationship between ILK and the phosphorylation of FAK and paxillin under ambient and increased pressure conditions. Our present results demonstrated that ILK, associating with AKT and FAK directly or indirectly, mediates pressure-stimulated cancer cell adhesion by regulating the phosphorylation of AKT specifically at Ser473 and FAK, specifically at Tyr397 and 576. The function of ILK in mediating the pressure-induced cell adhesion was not replaceable by α-parvin which we found to form a complex with ILK in colon cancer cells.

2. Materials and methods

2.1. Antibodies and reagents

Horseradish peroxidase (HRP)-conjugated anti-mouse or rabbit IgG, polyclonal anti-AKT, monoclonal anti-AKT1, polyclonal anti-phospho-AKT (Thr308), monoclonal anti-phospho-AKT (Ser473), polyclonal anti-α-parvin, polyclonal anti-ILK1, polyclonal anti-phospho-paxillin (Tyr118), polyclonal anti-FAK antibodies and 10x Cell lysis and kinase buffer and 10 mM ATP for the ILK kinase assay were all from Cell Signaling Technology (Beverly, MA). Anti-Rho-GDI, monoclonal anti-FAK (clone 4.47) antibodies and the purified 6His tagged recombinant full-length human AKT1, expressed in insect cells were purchased from Millipore (Lake Placid, NY). Phospho-FAK antibodies directed to tyrosine 397, 576 or 925 and Lipofectamine 2000 and other transfection supplies as well as Calcein-AM (calcein-acetoxymethyl ester) were purchased from Invitrogen (Camarillo or Carlsbad, CA). Polyclonal anti-E-cadherin and mouse monoclonal anti-ILK (65.1) antibodies and ExactaCruz™ C kit were from Santa Cruz Biotechnology (Santa Cruz, CA). GAPDH (glyceraldehyde-3-PDH) antibody was obtained from Biodesign International (Saco, ME).

pEGFP-C2 plasmid encoding human α-parvin was a kind gift (see Acknowledgments) and used previously [16]. pEGFP-C1 vector was obtained from Clontech (Mountain View, CA). QIAprep spin Miniprep and QIAfilter Plasmid Maxi kits were purchased from Qiagen (Valencia, CA). The SMARTpool siRNA specifically targeting human ILK and a non-specific siRNA, siCONTOL non-Targeting siRNA #1 (NT1 siRNA) were purchased from Dharmacon (Lafayette, CO). The ILK siRNA, a pool of four ILK siRNA duplexes, were synthesized with symmetric 3′-UU overhangs. The sense sequences of these four ILK siRNA duplexes were AACAAACACUCUGGCAUU, UGACGAAGCUCAACGAGAA, CAGCAGACAUGUGGA-

GUUU, CCGAAGCUCUGCAGAAGAA. PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3, 4d] pyrimidine), LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) were from Calbiochem (San Diego, CA). Bicinchoninic acid (BCA) protein assay reagents were from Pierce (Rockford, IL). Fibrillar collagen I and all other chemical reagents were from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

Caco-2 and SW620 colon cancer cells were cultured as described previously [22]. Single-cell suspensions of primary human colon cancer cells were obtained from surgically resected tumors by mincing and collagenase digestion as previously described [23]. More than 90% of cells excluded trypan blue at each isolation. The use of human samples was approved by the Wayne State University Human Investigation Committee.

2.3. Transfection

For transfection with siRNA, Caco-2 or SW620 cells were plated on p100 dishes at 20–25% confluence on the day before transfection. siRNAs were transfected using the Lipofectamine 2000 according to the manufacturer's instructions. The final concentrations for siRNA and Lipofectamine 2000 were 1 nM and 2.5 μg/ml, respectively. Five hours after transfection, the medium was replaced with 15 ml of pre-warmed medium without antibiotics. 72 hours after siRNA transfection, the ILK siRNA-treated cells were used for adhesion and expression studies.

For transfection with plasmids, Caco-2 cells were plated on 100 mm^2 dishes at 30–35% confluence on the day before transfection. For the gain of function experiments, the Caco-2 were first transfected by 1 nM siILK to reduce the expression of endogenous ILK (see results) and then, 24 hours later, transfected by pEGFP-C2 plasmid encoding human α-parvin or a non-insertion pEGFP-C1 plasmid to determine whether α-parvin can rescue the loss of function engendered by reducing ILK. Briefly, pEGFP-C2 plasmid encoding human α-parvin or non-insertion pEGFP-C1 plasmid as control were transfected into Caco-2 cells at final concentrations of 2 μg/ml plasmid and 5 μg/ml Lipofectamine 2000. Five hours after transfection, the medium was replaced with 15 ml pre-warmed Caco-2 medium without antibiotics. Forty eight hours after DNA transfection, the transfected cells were lysed for western analysis or used for adhesion assays.

2.4. Pressure regulation

15 mmHg pressure was applied as previously described [1,26] within an airtight box with inlet and outlet valves, thumb screws, a pressure gauge and an O-ring for an airtight seal. Temperature was maintained within $\pm 2^{\circ}$C and pressure within ± 1.5 mmHg.

2.5. Cell adhesion assay

We utilized a fluorescence-labeling based cell adhesion assay adapted from that described previously by Haier and colleagues [24]. 24-well plates were precoated with fibrillar collagen I (12.5 μg/ml) in ELISA buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.4) overnight at 4°C and the wells were rinsed three times with sterile phosphate buffered saline (PBS) prior to cell seeding. The trypsinized Caco-2 or SW620 cells in suspension were then labeled with 1 μM Calcein-AM for 15 min at 37°C. Equal number (2.0×10^5) of labeled cells were seeded to collagen I coated 24-well plates under ambient or increased pressure conditions for 30 min. Non-adherent cells were washed away, and adherent cells were gently washed twice with PBS at room temperature. The fluorescent cells were immediately quantified by microplate fluorescent reader with FITC filter.

For Caco-2 cells transfected with the GFP-tagged α-parvin construct, the fluorescent cells in 6 well plates were counted directly by fluorescence microscopy using our previously reported method [5].

2.6. Pressure-induced signaling

Because adhesion itself induces intracellular signaling, it was critical to be able to investigate the effects of increased extracellular pressure in the absence of cell adhesion. For all cell signaling studies, Caco-2 cells in suspension were subjected to ambient pressure or 15 mmHg increased pressure for 30 min in bacteriological plastic plates pretreated with 1% heat inactivated bovine serum albumin in PBS to prevent all adhesion and consequent adhesion-induced signaling. In some studies, cells were pretreated with PI-3K inhibitor LY294002 (20 μM) or Src inhibitor PP2 (20 μM) for 1.5 h. After pressure treatment, the suspended cells were collected by centrifugation. The cell pellet was washed with ice-cold PBS and lysed in lysis buffer as described below.

2.7. Western blotting and immunoprecipitation

Transfected or untransfected cells in suspension were exposed to 15 mmHg pressure for 30 min in plates pretreated as above to prevent adhesion and consequent adhesion-induced signals. Cells were then lysed and subjected to Western blotting as described [25]. Immunoprecipitation and co-immunoprecipitation were performed as previously described [3,26] with modification, except that we used the ExactaCruz™ C kit according to the manufacturer's guide in order to eliminate the IgG heavy chain which may mask ILK. Prior to separation by SDS-PAGE, immunoprecipitated samples were completely reduced and denatured to facilitate the performance of the ExactaCruz kit. Mouse monoclonal anti-ILK, FAK and AKT1 antibodies were used for immunoprecipitation.

2.8. ILK kinase assays

ILK activity was assayed using purified AKT1 as substrate assay [8,27]. Caco-2 or primary human colon cancer cells in suspension exposed to ambient or 15 mmHg pressure for 30 min were lysed in 20 mM Tris-HCl buffer, pH 7.5 containing 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF. Equivalent amounts of protein (450 μg) were, as described above, immunoprecipitated with mouse ILK monoclonal antibody or mouse IgG control. ILK kinase assays were performed in 50 μl kinase reaction buffer (25 mM Tris-HCl pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$) containing 0.2 mM ATP and 1 μg inactive recombinant human AKT1 as substrate. Reactions were incubated at 30°C for 30 min, and stopped by the addition of SDS-PAGE sample buffer. Phosphorylated AKT1 and total AKT and ILK were then detected by Western blotting as described above by using rabbit polyclonal specific anti-phospho-AKT (S473), AKT and ILK antibodies, respectively. The relative ILK kinase activity was calculated as the ratio of p-AKT(S473)/ILK with normalization.

2.9. Subcellular fractionation and translocation assay

Soluble and particulate cell fractions were prepared as previously described, by sonication and serial ultracentrifugation [2]. Briefly, the cells were lysed in first extraction buffer (20 mM HEPES, pH 7.6, containing 5 mM EGTA, 5 mm Na pyrophosphate, 1 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin and aprotinin, 5 mM NaF). The lysates were then centrifuged at $(1 \times 10^5)g$ for 60 min, and the soluble supernatants were collected as the "cytosolic fraction". The resulting pellets were then washed three times with the above first extraction buffer and resuspended in a second extraction buffer of 20 mM HEPES, pH 7.6, containing 150 mM NaCl, 1 mM $MgCl_2$, and 1% Triton X-100 with the same concentrations of the above protease inhibitors. After incubation on ice for 15 min, the lysates were briefly sonicated at 20% duty cycles 3 times and then centrifuged at $(1 \times 10^4)g$ for 15 min. The resulting supernatant of Triton X-100 soluble fractions were collected as the "membrane/cytoskeleton" fraction. Equal protein aliquots of the cytosolic fraction and membrane/cytoskeleton fractions were either directly resolved by SDS-PAGE, or subjected to immunoprecipitation prior to SDS-PAGE/Western blot detection. Rho-GDI and E-cadherin antibodies were used as protein loading controls for the cytosolic and plasma membrane/cytoskeletal fractions respectively. Although pharmacologic inhibition of ILK in SW-480 colon cancer cells has been reported to upregulate the expression of E-cadherin [28], siILK did not alter E-cadherin levels, compared with actin, consistent with previous observations and ours [29]. Therefore, we used E-cadherin as a loading control for the membrane-enriched fraction for Caco-2 cells transfected with NT1 or siILK.

2.10. Statistical analysis

Statistical analysis was performed using Student's t-tests or ANOVA as appropriate. Bonferroni correction was used for multiple comparisons, seeking 95% confidence. All experiments were done independently at least three times unless indicated otherwise. All blots were within the linear range of exposure. All data were expressed as mean ± SEM.

3. Results

Suppression of ILK expression by ILK siRNA blocked the pressure-stimulated colon cell adhesion and significantly reduced the basal cell adhesion.

To investigate the role of ILK in pressure-stimulated cell adhesion, we examined the effect on pressure-

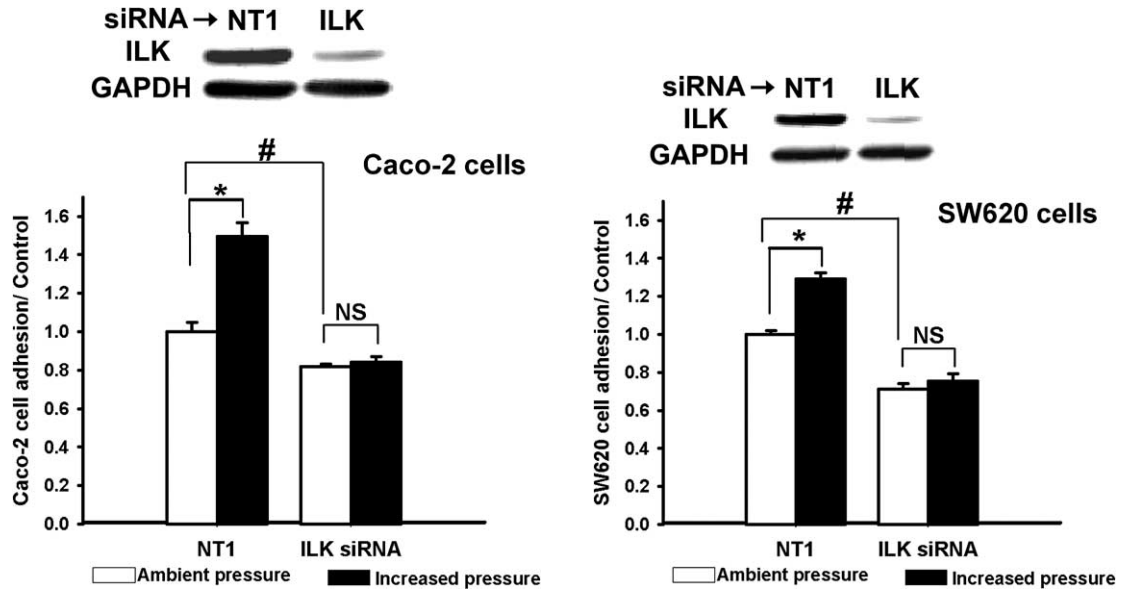

Fig. 1. Suppression of ILK expression by siILK blocked the pressure-stimulated Caco-2 and SW620 cell adhesion and significantly reduced the basal cell adhesion. All data were normalized against the NT1-transfected controls ($^{*}p < 0.05$, NS: not significant, $^{\#}p < 0.05$). ILK expression at the presence of NT1 or siILK was detected by WB. GAPDH was used as a loading control. The top panels in (A) and (B) represent typical blots.

stimulated adhesion of reducing ILK by ILK siRNA (siILK) which is a pool of four siILK duplexes. The 1 nM siILK functionally reduced endogenous ILK protein (>75% knockdown) in Caco-2 and SW620 colon cancer cells (Fig. 1(A and B), top panels).

ILK reduction by siILK statistically significantly reduced cell adhesion of Caco-2 and SW620 to collagen I matrix at ambient pressure (Fig. 1(A and B), lower panels, compare open bars for NT and ILK siRNA, $n = 4$, $p < 0.01$). More importantly, the $49 \pm 7\%$ increase in Caco-2 cell adhesion and the $29 \pm 3\%$ increase in SW620 cell adhesion induced by a 30-min treatment with 15 mmHg increased extracellular pressure ($p < 0.001$) were each prevented by siILK (Fig. 1(A and B), lower panels $n = 4$). Since the response of each cancer cell line to extracellular pressure appeared similarly dependent upon ILK, we utilized Caco-2 cells for our subsequent studies of intracellular signaling.

Pressure-induced endogenous ILK translocation from cytosol to plasma membrane/cytoskeleton and also activated ILK in a PI-3K and Src dependent manner.

We sought to determine whether ILK translocates to the membrane/cytoskeleton with pressure. The purities of cytosolic and membrane/cytoskeletal fractions were confirmed by immunoblotting these fractions with anti-Rho-GDI or anti-E-cadherin antibodies (Fig. 2(A), upper panel). In cells maintained in suspension in bacteriological plastic plates pretreated with 1% heat inactivated bovine serum albumin in PBS, so that cell-matrix interactions did not occur, pressure statistically significantly increased ILK in the membrane/cytoskeleton fraction in concert with decreased ILK in the cytosolic fraction (Fig. 2(A), $n = 4$, $p < 0.05$), suggesting that pressure induces ILK translocation from the cytosol to the plasma membrane/cytoskeleton.

We sought to determine whether pressure also activates ILK kinase activity in suspended Caco-2 cells prior to cell adhesion and whether Src or PI-3K might regulate this effect. Caco-2 cells were pretreated with PP2, a potent inhibitor of Src family tyrosine kinases, or with LY294002, which competes with ATP binding to PI-3K. Assessed by phosphor-AKT(S473), ILK activity was increased by extracellular pressure in DMSO-pretreated control cells by $39 \pm 7\%$ (Fig. 2(B), $n = 4$, $p < 0.05$). 20 μM PP2 or LY294002 prevented pressure-induced activation of ILK kinase activity with no statistically significant effect on basal ILK activity under ambient pressure (Fig. 2(B), $n = 4$), suggesting that pressure-induced ILK activation requires upstream activation of Src and PI-3K although these re-

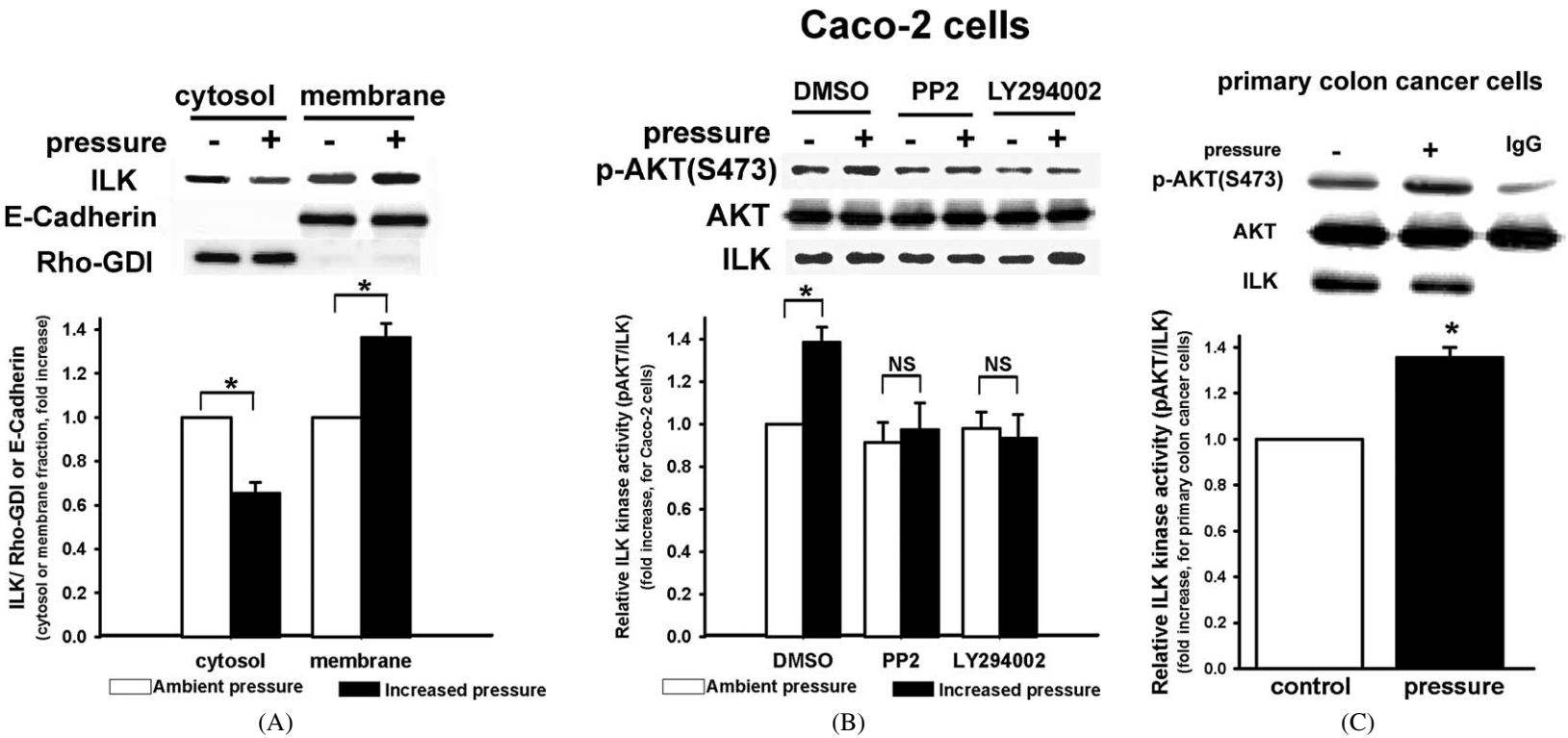

Fig. 2. Pressure-induced endogenous ILK translocation from cytosol to plasma membrane/cytoskeleton and also activated ILK in the PI-3K and Src dependent manner. (A) Extracellular pressure significantly promoted subcellular translocation of endogenous ILK from cytosol fraction to plasma membrane/cytoskeleton fraction. Typical blots are shown in the upper panel and densitometric analysis of the ratios of ILK to Rho-GDI (cytosol) or ILK to E-cadherin (membrane) in the lower graph ($n = 4$, $^*p < 0.05$). (B) Pressure activated ILK in a PI-3K and Src dependent manner in Caco-2 cells ($n = 4$, $^*p < 0.05$, NS: not significant). (C) Pressure activated ILK in primary cells isolated from human colon cancers ($n = 4$, $^*p < 0.05$).

sults do not imply direct interaction between Src or PI-3K and ILK. Furthermore, extracellular pressure increased ILK kinase activity in suspended cells prior to adhesion not only in Caco-2 cells, but also in primary human colon cancer cells isolated directly from surgical specimens (Fig. 2(C), $n = 4$, $p < 0.05$). Microscopic observations of the cell culture dishes prior to and after removing the suspended cells for immunoprecipitation confirmed that there were no adherent cells in these experiments.

SiILK prevented the increase in AKT phosphorylation induced by extracellular pressure specifically in Ser473 with no effect on the basal level.

Consistent with our previous reports [2,5], extracellular pressure significantly stimulated phosphorylation of AKT at Ser473 in NT1-treated Caco-2 control cells (Fig. 3(A), left panel, $n = 4$, $p < 0.05$). This increased phosphorylation of AKT was prevented by siILK with no effect on total AKT expression (Fig. 3(A), $n = 4$). The pressure-induced phosphorylation of AKT seemed specific for Ser473 since pressure did not measurably affect AKT phosphorylation at Thr308 (Fig. 3(B), left panel, $n = 4$). Transfection of siILK did not sensitize Caco-2 cells so that pressure would also affect AKT phosphorylation at Thr308 (Fig. 3(B), right panel, $n = 4$). In addition, siILK treatment did not statistically significantly affect the basal level of phosphorylation of AKT at Thr308 under ambient pressure (Fig. 3(B), compare row 1, lines 1 and 3).

Pressure-induced translocation of FAK, AKT and ILK to the plasma membrane/cytoskeleton and siILK prevented FAK or AKT translocation.

Unlike ILK protein which distributes roughly evenly in the cytosolic and membrane/cytoskeleton fractions in Caco-2 cells (or even more in the membrane/cytoskeletal fraction, Fig. 2(A)), the proteins FAK and AKT are predominantly present in the cytosol ([30] and data not shown). Changes in the relatively small pools of FAK and AKT in the membrane/cytoskeletal fraction may, therefore, not necessarily produce measurable shifts in the much larger cytosolic pools of these proteins. We, therefore, studied the protein changes of FAK and AKT only in the membrane-enriched fraction in response to increased extracellular pressure. Consistent with previous observations [2,5], pressure statistically significantly increased FAK (Fig. 4(B), left panel, $n = 8$, $p < 0.05$) and AKT (Fig. 4(C), left panel, $n = 8$, $p < 0.05$) in the membrane-enriched

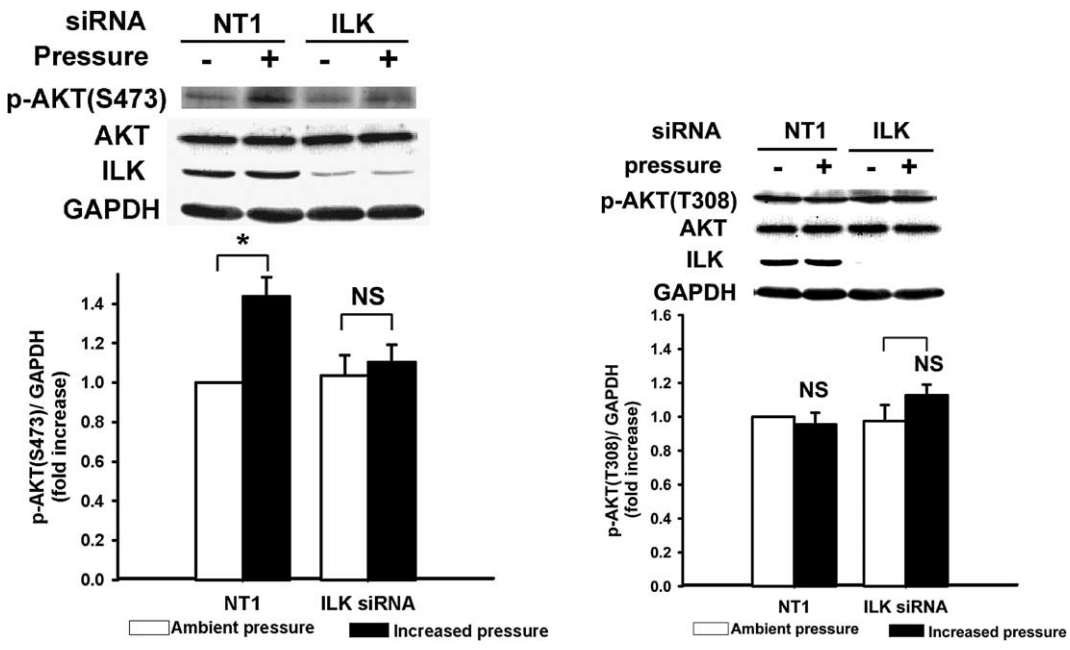

Fig. 3. SiILK prevented the increase in AKT phosphorylation induced by extracellular pressure specifically in Ser473 with no effect on basal phosphorylation. GAPDH was used as loading control. The top panel represents typical blots, and the graph summarizes densitometric analysis of the ratios of p-AKT(S473) (A) or p-AKT(T308) (B) to GAPDH in each experiment ($n = 4$, $^*p < 0.05$, NS: not significant).

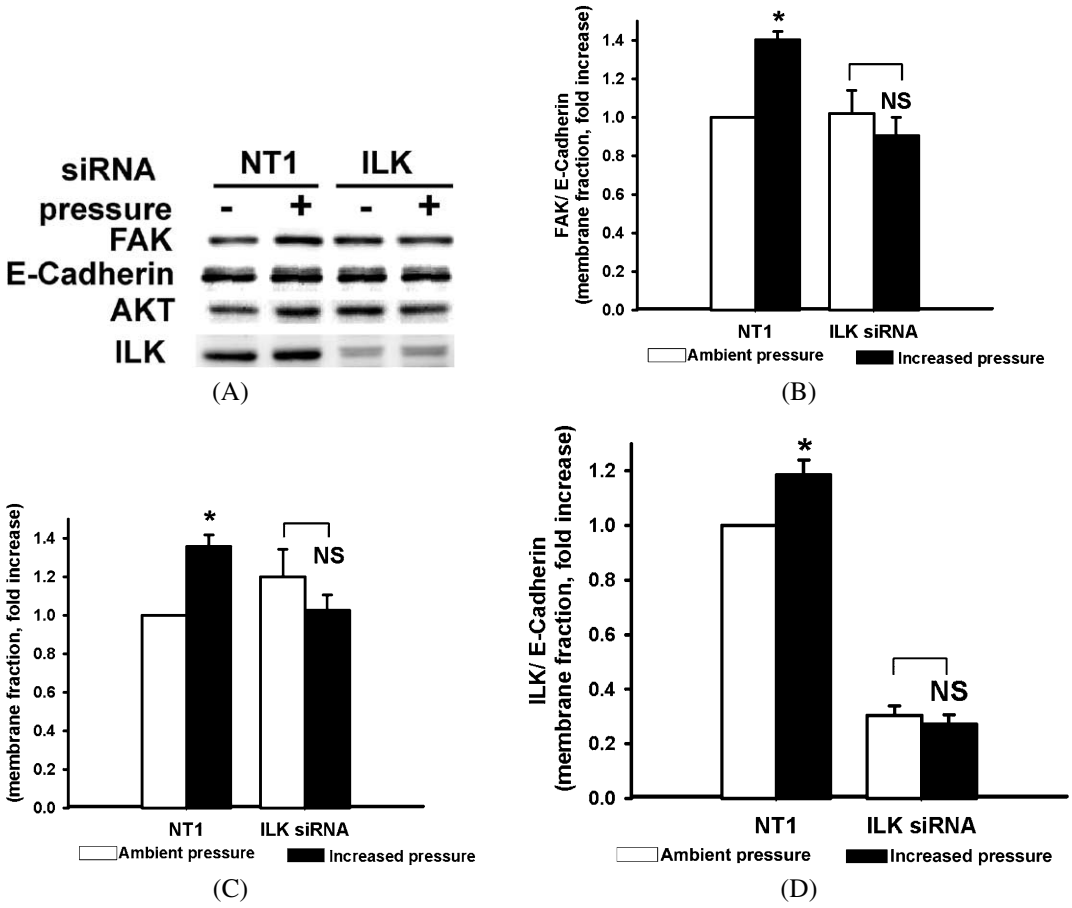

Fig. 4. Pressure-induced translocation of FAK, AKT and ILK protein levels in a membrane-enriched subcellular fraction in NT1-treated Caco-2 cells and siILK prevented FAK or AKT translocation. E-cadherin served as a loading control for the membrane-enriched fraction. Typical blots are shown in the upper panel (A) and densitometric analysis of the ratios of either FAK (B), AKT (C) or ILK (D) to GAPDH in each experiment ($n = 8$, $^*p < 0.05$, NS: not significant).

fraction in NT1-treated Caco-2 control cells. These shifts were prevented by siILK treatment (Fig. 4(B, C), right panels). In comparison, pressure also translocated ILK to the membrane/cytoskeleton (Fig. 4(D), $n = 8$, $p < 0.05$) in NT1-treated control cells, consistent with observations in non-treated Caco-2 cells (Fig. 2(A)). ILK in the membrane-enriched fraction was not detectably increased under pressure in the cells in which ILK expression had been markedly reduced by siILK (Fig. 4(D), right panel, $n = 8$).

Reducing ILK expression in Caco-2 cells inhibited pressure-stimulated phosphorylation of FAK at Y397 and Y576, but not paxillin at Y118.

We next sought to investigate whether ILK regulates the phosphorylation of FAK and paxillin. Consistent with previous observations, extracellular pressure modestly but statistically significantly stimulated phosphorylation of FAK at Y397 (Fig. 5(A), left panel, $n = 4$, $p < 0.05$) and Y576 (Fig. 5(B) left panel, $n = 4$, $p < 0.05$) in the NT1-treated control cells. The increased phosphorylation at these two sites may specifically mediate the pressure effect since increased pressure did not alter the phosphorylation of FAK-Y925 (Fig. 5(C), left panel). SiILK significantly reduced basal levels of phosphorylation of FAK at Y397, Y576 and Y925 (Fig. 5(A–C), compare row 1, lanes 1 and 3, $n = 4$, $p < 0.05$). The pressure-associated increases in phosphorylation of FAK at Y397 and Y576 were prevented by transfection with siILK (Fig. 5(A, B), right panel and compare row 1, lanes 3 and 4). Although silencing ILK reduced the basal phosphorylation of FAK-Y925 under ambient pressure, this reduction by siILK did not make FAK-Y925 phosphorylation sen-

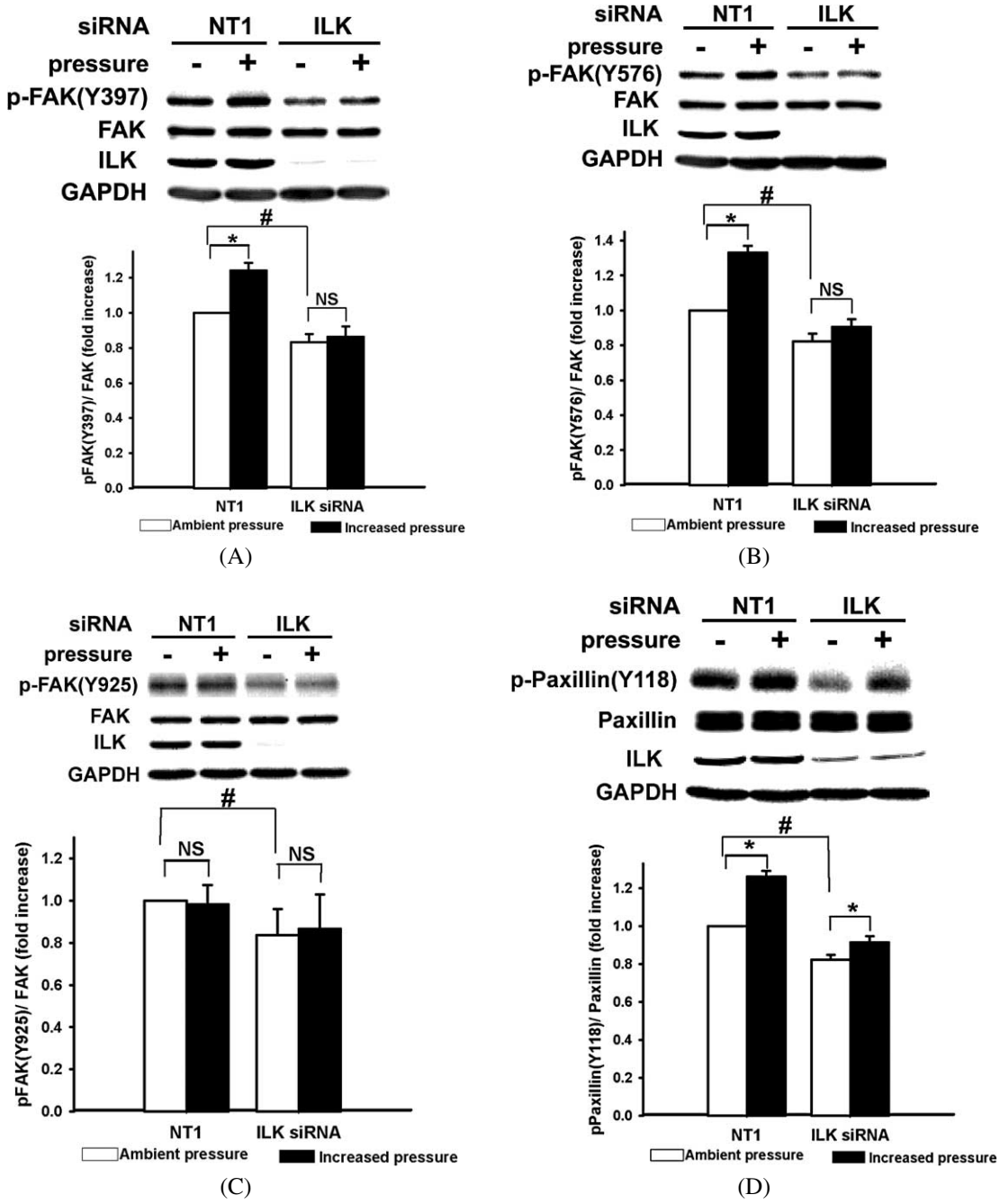

Fig. 5. Effects of siILK on the phosphorylation of FAK at Y397, 576 and 925 and paxillin at Y118. E-cadherin and total FAK or paxillin served as loading controls for the membrane-enriched fraction. Typical blots are shown in the upper panels and the graphs summarize densitometric analysis of the ratios of either pFAK (Y397) (A), pFAK (Y576) (B), pFAK (Y925) (C) to total FAK or p-paxillin (Y118) to total paxillin (D) in each experiment ($n = 4$, $^*p < 0.05$, NS: not significant).

sitive to increased pressure (Fig. 5(C), right panel, compare row 1, lanes 3 and 4). Unlike the increased phosphorylation of FAK-Y397-Y576 under pressure, pressure-stimulated phosphorylation of paxillin-Y118 was not blocked by siILK although the basal phosphorylation of paxillin was significantly reduced by siILK under ambient pressure (Fig. 5(D), compare row 1, lanes 1 and 3, $n = 4$, $p < 0.05$). Neither total FAK nor

total paxillin protein expression was affected by silencing ILK (Fig. 5(A–D), compare row 2 in each upper panel).

Overexpression of α-parvin did not rescue the siILK-induced function loss of ILK in pressure-stimulated cell adhesion.

We sought to determine if α-parvin, like ILK, also translocated to membrane/cytoskeleton in response to pressure. We used the same technique as the one used above for translocation of ILK study to investigate the translocation of α-parvin. Pressure statistically significantly increased α-parvin in the membrane/cytoskeleton fraction and decreased α-parvin in the cytosolic fraction (Fig. 6(A), $n = 4$, $p < 0.05$), suggesting that pressure also induces α-parvin translocation from the cytosol to the plasma membrane/cytoskeleton. In addition, α-parvin, similar to ILK, was roughly evenly distributed in cytosol and plasma membrane/cytoskeleton fractions (Fig. 6(A), top panel, first row).

SiILK has been reported to inhibit α-parvin expression in HeLa cells [31]. We confirmed in Caco-2 cells that α-parvin expression was also reduced by siILK (Fig. 6(B), rows 2 and 3). Accordingly, it became important to determine whether the effects of siILK might actually reflect a reduction of α-parvin. Therefore, we next studied whether overexpression of exogenous α-parvin could restore loss of adhesive responsiveness to pressure induced by ILK reduction. For these studies, Caco-2 cells were first transfected with siILK or NT1 and then with plasmids encoding GFP protein or GFP-tagged α-parvin fusion protein. SiILK dramatically inhibited the expression of endogenous ILK as well as of endogenous α-parvin, but not the expression of the exogenous GFP-α-parvin fusion protein (Fig. 6(B), compare rows 1–3). Pressure increased cell adhesion in the control Caco-2 cells transfected with NT1 and the GFP plasmid (Fig. 6(C), $n = 4$, $p < 0.05$). Neither GFP nor GFP-α-parvin could rescue the function of ILK in mediating the pressure-associated increased adhesion (Fig. 6(C), $n = 4$).

Effect of pressure on interaction of ILK with FAK, AKT, α-parvin.

To further investigate the ILK-mediated signaling mechanism of pressure-induced cell adhesion, we evaluated the effect of pressure on the potential interactions between ILK and FAK, AKT and α-parvin, each important in the focal adhesion complex. Increased extracellular pressure significantly inhibited FAK co-immunoprecipitation with ILK (Fig. 7(A), $n = 4$, $p < 0.05$). Because the apparent size of ILK is approximately 50 kDa, almost identical to the size of IgG heavy chain, a commercial kit was used to eliminate the IgG heavy chain. We used a parallel immunoprecipitated sample from siILK-treated cells to confirm that we were evaluating the ILK band rather than IgG heavy chain (Fig. 7(A), up and right panel). In contrast to FAK and ILK interaction, ILK co-immunoprecipitation with AKT or α-parvin was promoted under conditions of elevated pressure (Fig. 7(B, C), $n = 4$, $p < 0.05$). Consistent with above western blot results (Figs 3(A, B) and 5(A–C), row 2 in each upper panel), ILK siRNA did not affect the expression of FAK (Fig. 7(A), upper panel, lane 2) or AKT (Fig. 7(B), upper panel, row 2) in the co-immunoprecipitation studies. The effect of pressure on FAK–ILK interaction depended on FAK and ILK subcellular localization as pressure statistically significantly promoted FAK–ILK interaction in the plasma membrane-enriched fraction (Fig. 7(D), right panel, $n = 5$, $p < 0.001$) while reducing FAK–ILK interaction in cytosol fraction (Fig. 7(D), left panel, $n = 5$, $p < 0.05$).

4. Discussion

This study demonstrates that pressure activates ILK in a PI-3K and Src-dependent manner. ILK, in turn, working with its partner α-parvin, is the kinase that regulates the pressure-induced phosphorylation of AKT, specifically at Ser473, and FAK at Y397 and Y576, but not at Y925. Extracellular pressure increased the direct interaction between ILK and AKT or α-parvin, but inhibited the indirect association of ILK with FAK. These new findings, taken together with our previous reports [2,5], suggest an ILK-mediated mechanism for pressure-induced regulation of cancer cell adhesion (Fig. 8). Although the overall effect of pressure on cancer cell adhesion *in vitro* and phosphorylation of various proteins in our study might seem relatively modest, these seemingly modest effects are sufficient to cause substantial and highly statistically significant increases in the adhesion of tumor cells to murine surgical wounds, the subsequent development of murine tumors in surgical wounds or the peritoneal cavity, and dramatic impairment of tumor free survival in murine transplantable tumor models [4,32,33].

The central role for ILK in mediating pressure-stimulated adhesion is consistent with previous obser-

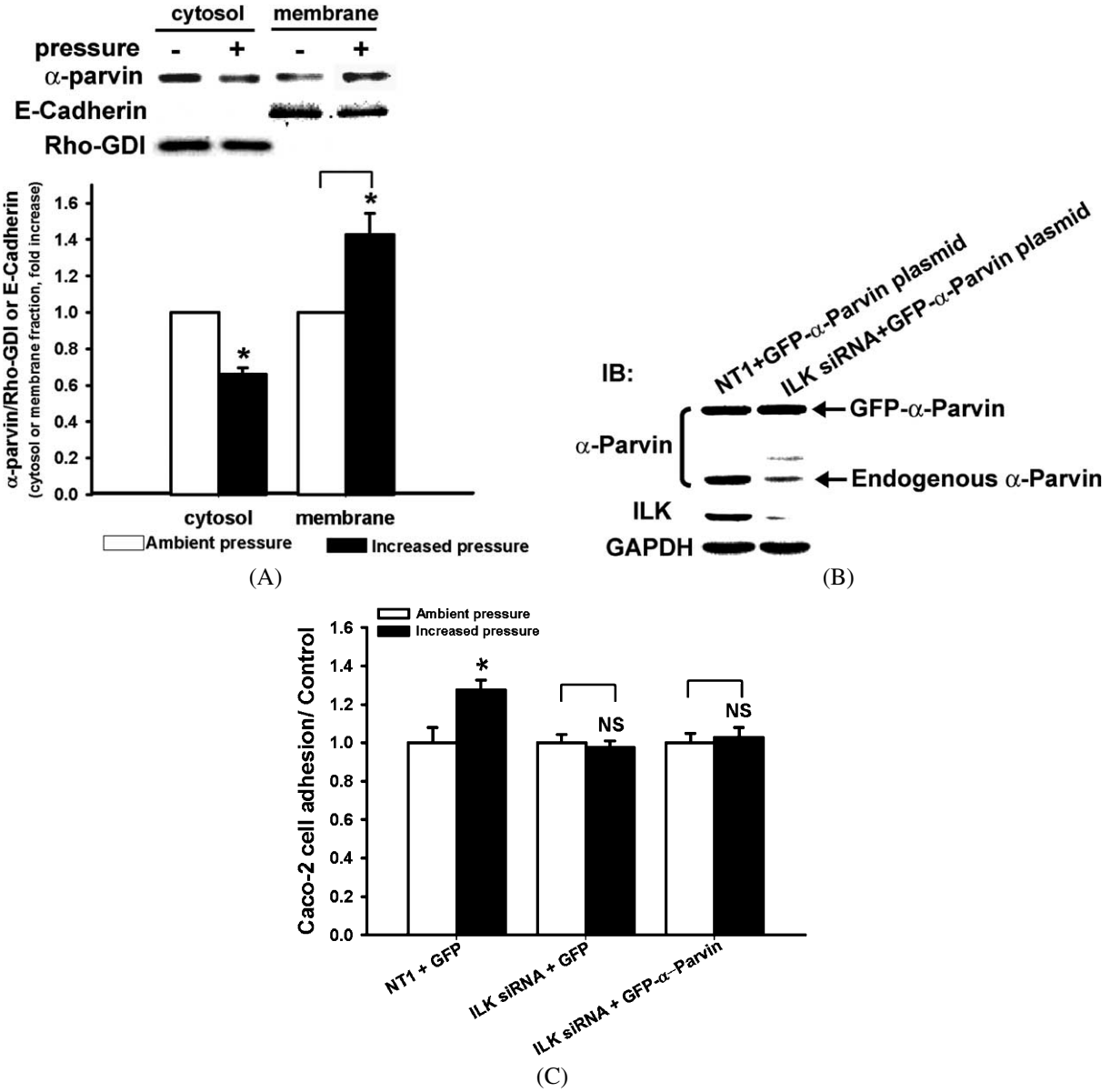

Fig. 6. Pressure-induced endogenous α-parvin translocation from cytosol to plasma membrane/cytoskeleton and overexpression of α-parvin did not rescue the siILK-induced function loss of ILK in pressure-stimulated cell adhesion. (A) Extracellular pressure significantly promoted subcellular translocation of endogenous α-parvin from the cytosolic fraction to the plasma membrane/cytoskeleton fraction. E-cadherin and Rho-GDI served as controls for the membrane/cytoskeletal and cytosolic fractions respectively. Typical blots are shown in the upper panel and densitometric analysis of the ratios of α-parvin to Rho-GDI (cytosol) or α-parvin to E-cadherin (membrane) in the lower graph ($n = 4$, $^*p < 0.05$). (B) Expression of endogenous α-parvin or exogenous GFP-tagged α-parvin was detected by specific anti-α-parvin antibody. GAPDH was used as loading control. (C) Caco-2 cells were co-transfected by siILK and plasmids as in (B). The non-inserting pEGFP-C1 plasmid was used for transfection as a negative control. All data were normalized against control cell number under ambient pressure for each transfection conditions. Neither overexpression of GFP nor GFP-tagged α-parvin restored the increase in pressure-induced cell adhesion after blockade by siILK ($n = 4$, $^*p < 0.05$, NS: not significant).

vations that ILK can influence cell adhesion at ambient pressures and can be activated by integrin-mediated cell adhesion itself in a PI-3K dependent manner [10,34]. It is likely that ILK regulated pressure-induced cell adhesion, at least in part, through regulating the pressure-induced phosphorylation of AKT at Ser473

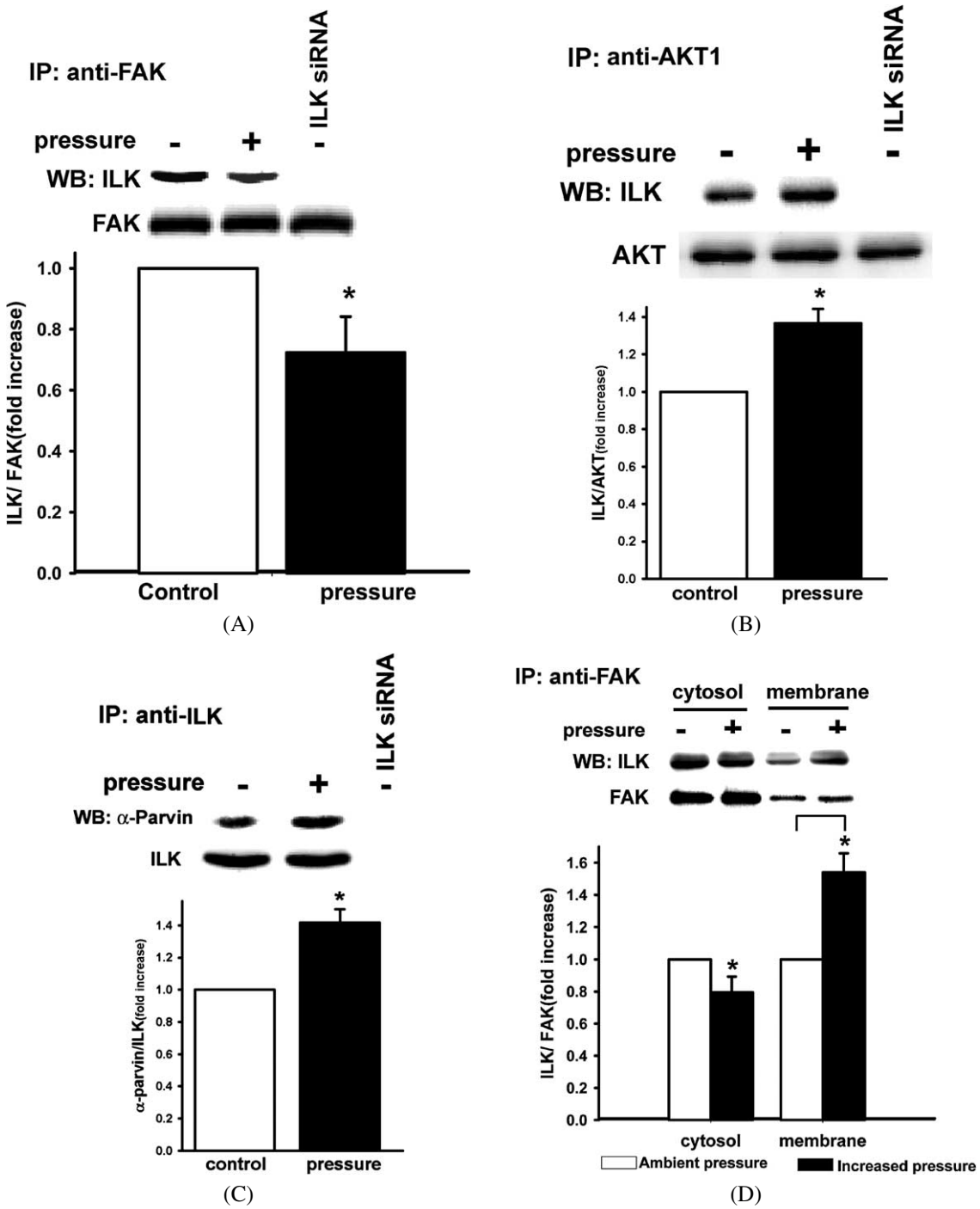

Fig. 7. Effect of pressure on interaction of ILK with FAK, AKT and α-parvin. (A–C) Protein samples from the cell lysates were subjected to co-immunoprecipitation and analyzed by Western blot. Parallel siILK-treated lysates were also subjected to immunoprecipitation with ILK antibody to confirm that the band under study represented ILK rather than IgG heavy chain. All observations were normalized against the respective ambient pressure controls ($n = 4$, $^*p < 0.05$). (D) Pressure significantly reduced ILK–FAK interaction in the subcellular cytosol fraction while promoting ILK–FAK interaction in the membrane/cytoskeletally enriched fraction ($n = 5$, $^*p < 0.05$). Data for Fig. 7(D) were normalized to ambient pressure controls for each subcellular fraction individually because of the differences in FAK levels between the two fractions.

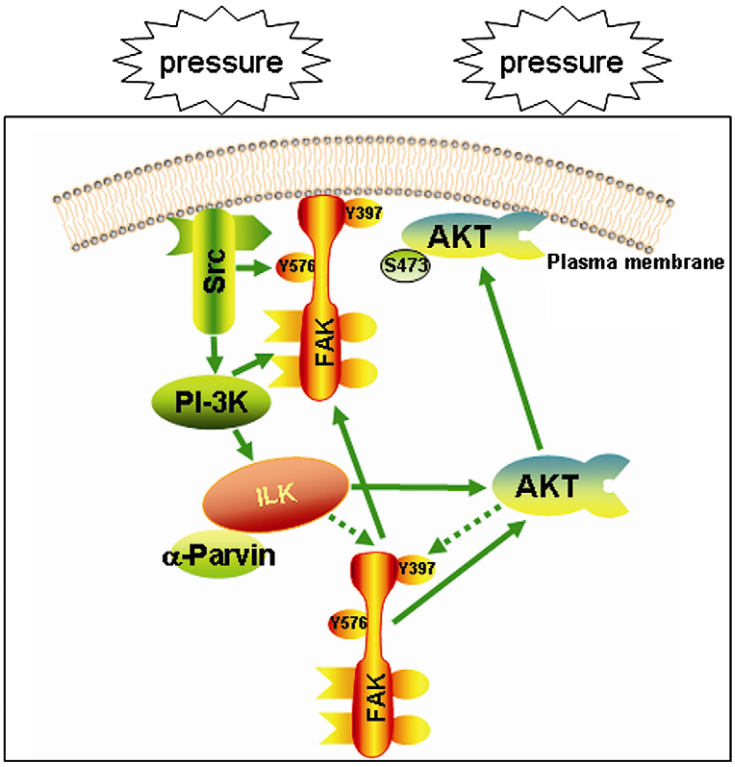

Fig. 8. Potential mechanism for pressure-induced regulation of cancer cell adhesion. This cartoon summarizes our present observations as well as our previous reports on pressure-induced signaling pathway. Pressure activates ILK by a Src and PI-3K dependent pathway. The activated ILK in turn associates with FAK and AKT through binding its co-factor, α-parvin. The cytosolic ILK/FAK/AKT complex then translocates to plasma membrane where AKT is phosphorylated at Ser473 for its full activation. The phosphorylation of FAK at Y397 and 576 appear specifically regulated by ILK directly or indirectly through their association.

because siILK treatment prevented the pressure-stimulated phosphorylation of AKT (S473). Furthermore, AKT itself and AKT (S473) phosphorylation are required for this pathway [2,5]. ILK-dependent pressure activated phosphorylation of AKT Ser473 may be specific to the force-activated pathway described here, however, since another kind of mechanical force, cyclic strain, can activate phosphorylation of AKT Ser473 independently of ILK in mouse kidney fibroblasts [35]. The exact mechanism underlying this distinct AKT phosphorylation at Ser473, but not Thr308 in response to pressure awaits identification. However, similar dissociation of AKT phosphorylation at Ser473 and Thr308 has been described in cultured neurons exposed to glutamate [36].

In our hands, siILK treatment did not reduce the already very low basal level of phosphorylation of AKT Ser473 in unstimulated Caco-2 cells in suspension prior to adhesion and adhesion-related signaling. Certainly, the balance between other kinases and phosphatases may maintain this tonically low AKT Ser473 phosphorylation level in these cells independently of ILK. Among the potential candidates that might influence this basal level of AKT phosphorylation are a novel plasma membrane raft-associated Ser473 kinase that is distinct from ILK [37]; rictor and ILK complex [38] and PHLPP, a recently identified phosphatase that specifically dephosphorylates AKT at Ser473 [39]. Nevertheless, our results suggest that the stimulation of AKT Ser473 phosphorylation in cancer cells by increased pressure does require ILK, because reducing ILK by siRNA prevents this effect.

Increasing evidence suggests that ILK can sometimes directly phosphorylate AKT at Ser473 [8,40]. Indeed, AKT may be a direct substrate of ILK in response to extracellular pressure, because siILK prevented pressure-stimulated translocation of AKT to the plasma membrane; because extracellular pressure promoted the association of AKT with ILK in suspended Caco-2 cells prior to the localization of these proteins to focal adhesion; and because immunoprecipitated ILK from Caco-2 and primary hu-

man colon cancer cells directly phosphorylated inactive/nonphosphorylated recombinant AKT1 at Ser473. Indeed, the *in vitro* kinase assay results suggest not only that ILK can phosphorylate AKT1 but also that increased pressure stimulates this ILK activity. The relatively strong baseline phosphorylation of AKT in our *in vitro* kinase assays by ILK derived from unstimulated Caco-2 cells under ambient pressure is probably because recombinant, rather than endogenous AKT phosphorylation was studied, independent of the effects of other signals that may also influence AKT phosphorylation within the cell as discussed above. The stoichiometry of the ILK–AKT ratio was also likely radically enhanced by the enrichment of ILK levels by immunoprecipitation and the addition of a large quantity of purified AKT in the absence of subcellular compartmentalization of these molecules which might influence their *in vivo* interaction.

Like endogenous AKT and FAK in colon cancer cells [2,5], ILK also translocated from the cytosol to the plasma membrane/cytoskeleton in response to pressure. This is consistent with a recent report indicating that ILK is recruited to the cell membrane during contractile stimulation of smooth muscle cells with acetylcholine [41].

We have previously reported that extracellular pressure increases phosphorylation of FAK at Y397, an autophosphorylation site, and at Y576, a Src phosphorylation site. Phosphorylation of FAK at both Y397 and Y576 is required for pressure-induced cell adhesion [2,25], and FAK can be phosphorylated at Y925 in Caco-2 cells in response to cyclic strain [42]. The reduction of phosphorylation of FAK Y397 and 576 by siILK under ambient pressure may contribute, at least in part, to the reduced basal level of cancer cell adhesion with siILK treatment. However, the influence of ILK on the phosphorylation of FAK Y397 and 576 may vary with the cell type and tissue studied and the stimulus applied. For instance, phosphorylation of FAK Y397 and 576 is significantly reduced in the ILK-knockout murine heart and skin [20,43], but phosphorylation of FAK(Y397) is elevated in ILK-knockout mouse kidney [44]. Although the phosphorylation of FAK at Y925 site may mediate Caco-2 cell migration in response to cyclic strain, FAK-Y925 phosphorylation does not seem important in the response of these cells to extracellular pressure.

Further evidence of ILK–FAK interaction is that ILK co-immunoprecipitated with FAK. ILK–FAK interaction was reported most recently in vascular smooth muscle cells, in which their interaction is reduced by osteopontin [17]. However, this ILK–FAK interaction may also be indirect because FAK binds proteins such as PI-3K and Src [45] which could then associate with ILK.

Y397 and Y576 in FAK are autophosphorylated or phosphorylated by Src, but ILK is a serine/threonine kinase, so the effects of ILK reduction on the basal and pressure-induced phosphorylation of FAK Y397 and 576 may require ILK activation of some other intermediary kinase. Alternatively, ILK might phosphorylate Ser/Thr residue(s) in FAK directly, which in turn could then modulate FAK Y397 and/or Y576 phosphorylation. A recent report suggests that FAK phosphorylation at Ser843 inhibits Tyr397 phosphorylation [46], so the model of a serine/threonine phosphorylation affecting FAK tyrosine phosphorylation seems plausible although Ser843 phosphorylation would probably not be the key event unless ILK activation leads to dissociation from FAK and thus decreased Ser843 phosphorylation.

The reduction in ILK–FAK association that we observed in response to pressure in whole cell lysates could be explained if ILK serine/threonine phosphorylation of FAK reduces pressure-induced phosphorylation of FAK (Y397 and Y576) in a negative feedback loop. However, ILK–FAK interaction is likely more complex, and may depend upon the subcellular localization of ILK and FAK. Pressure significantly reduced ILK–FAK association in the cytosol but increased ILK–FAK interaction at the plasma membrane/cytoskeleton, an effect probably not apparent in our initial studies of ILK–FAK binding in whole cell lysates because most FAK is cytosolic. Therefore, one model consistent with our observations would be that pressure translocates both ILK and FAK to the plasma membrane/cytoskeleton where they interact more strongly, but then dissociate or associate more weakly after recycling into the cytosol.

Paxillin phosphorylation at Y118 is also important for pressure-stimulated cell adhesion [47,48]. Although the ambient pressure decrease in paxillin (Y118) phosphorylation with ILK reduction might contribute to the reduced basal level of cell adhesion that we observed at ambient pressure, unblocked pressure-promoted paxillin phosphorylation in the presence of siILK suggests that in contrast to FAK phosphorylation, paxillin phosphorylation in response to increased pressure is independent of ILK.

α-parvin is an important cofactor for ILK since α-parvin-depleted cells exhibit lower ILK kinase activity [49]. α-parvin also regulates AKT S473 phos-

phorylation by facilitating the membrane translocation of AKT [15] and forming an α-parvin–ILK complex [49]. Although ILK siRNA also inhibited expression of endogenous α-parvin, overexpression of GFP–parvin fusion protein did not rescue the function of ILK in mediating the pressure-induced cell adhesion, highlighting the importance of ILK in pressure-stimulated cell adhesion. However, these results do not exclude a subsidiary role for α-parvin in this pathway since pressure promoted translocation of both α-parvin and ILK from the cytosolic fraction to the plasma membrane/cytoskeletal fraction as well as α-parvin–ILK association in suspended Caco-2 cells. This is consistent with previous observation that the formation of α-parvin–ILK–PINCH complexes still occurs in suspended HeLa cells [50]. Therefore, our results support a model that pressure strengthens α-parvin–ILK complex prior to their location to focal adhesion.

Consistent with a previous report [31], our results suggest that ILK and α-parvin are mutually dependent for their expression. Reducing one reduces the other. This coordinated down-regulation of ILK and α-parvin proteins has been theorized to be mediated at least in part by proteasomes [31]. Exogenous GFP–α-parvin may not have been affected by reducing ILK because exogenous GFP–α-parvin expression, in contrast to endogenous α-parvin, is overdriven by a different promoter. Alternatively, the fused GFP may have changed the confirmation of α-parvin in such a way as to preserve its function but render it unrecognizable by proteasomal degradation systems. Finally, the GFP–α-parvin might be stabilized and recruited by binding to remaining small amount of endogenous ILK in ILK reduced cells. These competing possibilities await exploration, but exceed the scope of the present study which simply took advantage of the fact that the GFP–α-parvin was still well expressed to demonstrate that α-parvin overexpression does not reconstitute pressure-stimulated cell adhesion lost in siILK-treated cells.

In summary, our observations suggest that ILK is an important mediator in regulating pressure-stimulated colon cancer cell adhesion through regulating the phosphorylation of AKT at S473 and FAK at Y397 and 576, as well as association of ILK with AKT and FAK. Furthermore, we identified ILK as a pivotal upstream regulator, upon stimulation of cancer cells by extracellular pressure, of FAK and AKT which are important focal adhesion proteins. These results raise the possibility that ILK may be a key mediator of force-associated signaling in cancer cells and a potentially important therapeutic target for preventing metastasis.

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