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Osteopontin stimulates vascular smooth muscle cell migration by inducing FAK phosphorylation and ILK dephosphorylation

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Abstract

Focal adhesion kinase (FAK) and integrin-linked kinase (ILK) are both involved in integrin-mediated cell migration. However, the molecular mechanism, and the relationship between FAK and ILK activity in signaling transduction for the osteopontin (OPN)-induced migration of vascular smooth muscle cells (VSMCs) remain unclear. Here, we show that treating VSMCs with OPN could result in the dissociation of FAK with ILK by inducing phosphorylation of the former and dephosphorylation of the latter. Furthermore, we demonstrate that FAK phosphorylation induced by OPN is coupled with ILK dephosphorylation. We also provide evidence that ILK acts downstream of FAK in the signaling pathways that mediate OPN-induced VSMC migration. These findings suggest that FAK phosphorylation and ILK dephosphorylation play important roles in VSMC migration induced by OPN.

Keywords: Focal adhesion kinase; Integrin-linked kinase; Migration; Osteopontin; Signal transduction; VSMC

Cell migration plays a pivotal role in many biological processes that are essential for development, repair, and pathogenesis. The cell–extracellular matrix (ECM) interaction is necessary for cell migration. At the molecular level, the cell–ECM interaction is mediated by transmembrane cell-adhesion receptors (e.g., integrins) and a number of highly selective receptor-proximal cytoplasmic proteins [1]. Determining the functions of each of the focal adhesion proteins is therefore a prerequisite for understanding the molecular basis of cell–ECM interaction-mediated signaling and the regulation of cell migration. The cytoplasmic domains of the integrin subunits do not show kinase activities; thus, upon ligand interactions, integrins are thought to recruit intracellular signaling molecules to their cytoplasmic domains (e.g., focal adhesion kinase (FAK) [2], integrin-linked kinase (ILK) [3], and paxillin [2]) in order to transduce signaling through tyrosine or serine/threonine phosphorylation of these proteins [4]. FAK, which is a non-receptor cytosolic tyrosine kinase, is tyrosine phosphorylated in response to β1 and β3 integrin-mediated cell adhesion with ECM proteins. Clustering of integrins results in rapid phosphorylation of FAK at Tyr397, as well as at several additional sites with kinase and carboxy (C)-terminal domains [5]. Phosphorylation of these tyrosine residues is important to maximize the adhesion induced by FAK activation and signaling to downstream effectors. FAK activation has been shown to promote cell proliferation, survival, and migration [6]. ILK, which also mediates integrin signal transduction, is a serine/threonine kinase that can be activated by ECM and growth factor stimulation. ILK was recently reported to regulate numerous aspects of cellular signaling, including cell survival, differentiation, and migration, by mediating the phosphorylation of a variety of intracellular substrates [7]. Most research has focused on the effects of ILK on carcinoma development and metastasis, and there are few reports on ILK physiological function in vascular smooth muscle cells (VSMCs), especially the role of its phosphorylation, and the relationship between FAK and ILK in signaling transduction during VSMC adhesion and migration.

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In this paper, we investigated the relationship between the phosphorylation of FAK and ILK and VSMC migration, and analyzed the interaction between FAK and ILK in osteopontin (OPN)-induced VSMC migration. Our results revealed that OPN could induce FAK phosphorylation and ILK dephosphorylation, and disrupt the association of FAK with ILK. Furthermore, we found that the phosphorylation of FAK regulated ILK phosphothreonine levels, whereas silencing the ILK gene did not affect FAK; this suggested that ILK acts downstream of FAK in the signaling pathways that lead to OPN-induced VSMC migration.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM) and phosphate-free DMEM were purchased from Gibco (Life Technologies, Grand Island, USA). Anti-mouse FAK, anti-mouse ILK, anti-phosphotyrosine (PY-99) monoclonal antibodies, protein A–agarose for immunoprecipitation, and ECL detection system were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphothreonine antibody was obtained from Sigma (St. Louis, MO). The non-receptor protein–tyrosine kinase Src inhibitor, PP2, was from Calbiochem (LaJolla, CA). OPN was purchased from Chemicon (Temecula, CA). Mammalian expression plasmids, pGZ21δxZ-FRNK that encodes FAK-relative non-kinase (FRNK, endogenous FAK inhibitor), pIL2R-FAK (constitutively activated FAK) and pIL2R-FAK^{Y397F} constructs were kindly provided by Dr. K.M. Yamada (NIH/NIDCR, Bethesda, Maryland, USA).

Cell culture and transfections. VSMCs were isolated from the thoracic aorta of 90–110 g male Sprague–Dawley rats as described previously [8]. Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified 95% air and 5% CO_2 atmosphere. VSMCs were grown up to 50% confluence and transiently transfected with individual DNA constructs as described above using ESCORT™ Transfection Reagent (Sigma) according to manufacturer's protocols. After transfection for 24–48 h, the phosphorylation level of FAK and ILK, and the interaction of FAK with ILK were detected by immunoprecipitation and Western blotting in the presence or absence of OPN (20 μg/ml).

Small interfering RNA. The mammalian expression vector, pENTR™/H1/TO (Invitrogen), was used for expression of siRNA in VSMCs. The specific nucleotide sequences (IACGCACTCAATAGCCGTAGTGT AAT, IIGCCCACGTGTGTAAGCTCATGAAGAT) corresponding to ILK were selected and inserted into above vector, respectively, to construct short hairpin (shRNA) constructs according to the Invitrogen Block-IT RNAi designer (http://www.invitrogen.com/rnai) online and the manufacturer's protocols of BLOCK-iT™ Inducible H1 RNAi Entry Vector kit (Invitrogen). These two constructs were referred to as ILK siRNAI and II. They were transformed into TOP 10 One shot™ competent cells (Invitrogen). Successful ligation was tested by sequence (data not shown). After VSMCs were transfected with ILK siRNAI or II for 24 h, the expression level of ILK was detected by Western blot analysis.

Immunoprecipitations and Western blotting. VSMC lysates were prepared with lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP-40). Equal amounts of proteins (80–100 μg) were separated by SDS–PAGE, and electrotransferred to a PVDF membrane. Membranes were blocked in 5% BSA for 2 h, and incubated with the primary antibodies, PY-99 (1:1000), anti-phosphothreonine antibody (1:400), anti-FAK (1:800) or anti-ILK (1:500), overnight and then 2 h with the respective secondary antibody. Thereafter, proteins were visualized using the ECL detection system, and protein abundance was quantified by densitometry analysis (Kodak-Imager 1D). Immunoprecipitations were performed as described previously [9]. Briefly, cell extract was first pre-cleared with 20 μl of protein A–agarose (50% v/v) in 200 μl reaction buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin). The supernatant was incubated with 1–2 μg of anti-FAK, or anti-ILK antibodies for 1 h, and subsequently incubated with protein A–agarose overnight at 4 °C. Protein A–agarose–antigen–antibody complexes were collected by centrifugation at 12,000 rpm for 20 s at 4 °C. The pellets were washed three times with 500 μl NET-washing buffer I (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% NP-40, 1 mM EDTA, and 0.25% gelatin), buffer II (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 0.1% SDS, 1 mM EDTA, and 0.25% gelatin), buffer III (10 mM Tris–HCl, pH 7.5, 0.1% NP-40) respectively, for 20 min each time at 4 °C. Immunoprecipitated proteins were separated by SDS–PAGE and immunoblotted with antibodies as indicated. The experiments were replicated three times at least.

In vivo ILK phosphorylation and dephosphorylation analysis. The levels of [^{32}P] incorporation into ILK *in vivo* were analyzed as described previously [10]. Briefly, VSMCs were cultured with phosphate-free DMEM for 3 h, and then [γ-^{32}P]ATP (500 μCi/ml) was added to the media. After incubation for 12 h, the cells were treated with or without OPN (20 μg/ml) for 30 min, and then were lysed with the lysis buffer. The cell lysates were immunoprecipitated with anti-ILK antibodies. Immunoprecipitates were separated by SDS–PAGE, and phosphorylated ILK bands were visualized by radioautography. After that the proteins were electrotransferred to a PVDF membrane, and total ILK protein was analyzed by immunoblotting with an anti-ILK antibody.

Wound healing assay. VSMCs grown on coverslips to 100% confluence were scraped with a sterile pipette tip to create a wound, thereafter, cells were treated with or without OPN (20 μg/ml) for 24 h at 37 °C, in 5% CO_2. Three different fields of the wound were photographed at the beginning and at the end of the experiment. The migration activity was expressed as the number of cells migrating into the wound in each field.

Statistics. Data are given as means ± SEM. Statistical analyses were carried out with the SPSS 10.0 statistical software package. Our primary statistical test was MANOVA. Differences in mean values due to main effects and their interactions were tested. $P \leqslant 0.05$ was considered statistically significant.

Results

Treating VSMCs with OPN results in dissociation of FAK with ILK by inducing FAK phosphorylation and ILK dephosphorylation

To determine the effect of OPN on the intercellular signaling response, we examined the tyrosine phosphorylation of FAK and the threonine phosphorylation of ILK. The cell lysates were immunoprecipitated with anti-FAK mAb or anti-ILK mAb, and immunoblotted using anti-Tyr (p) or anti-Thr (p), respectively. As shown in Fig. 1A, the level of phosphorylated FAK was lower in VSMCs without OPN treatment. After the cells were stimulated by OPN for 10 min, the phosphorylated FAK levels increased 2.4 ± 0.1-fold ($P \leqslant 0.05$, $n = 3$) compared with the control, and this effect persisted for up to 60 min of OPN treatment (Fig. 1A). Nevertheless, the level of phosphorylated ILK was higher in the untreated cells than in OPN-treated VSMCs. After the VSMCs were stimulated by OPN for 30 min, the phosphorylated ILK level was reduced to 44.6 ± 1.9% ($P \leqslant 0.05$, $n = 3$) of the control level, but recovered after 60 min (Fig. 1D). However, as the level of ILK protein was unchanged after OPN treatment, we supposed that the decrease in phosphorylated ILK level resulted from the dephosphorylation of ILK and not its

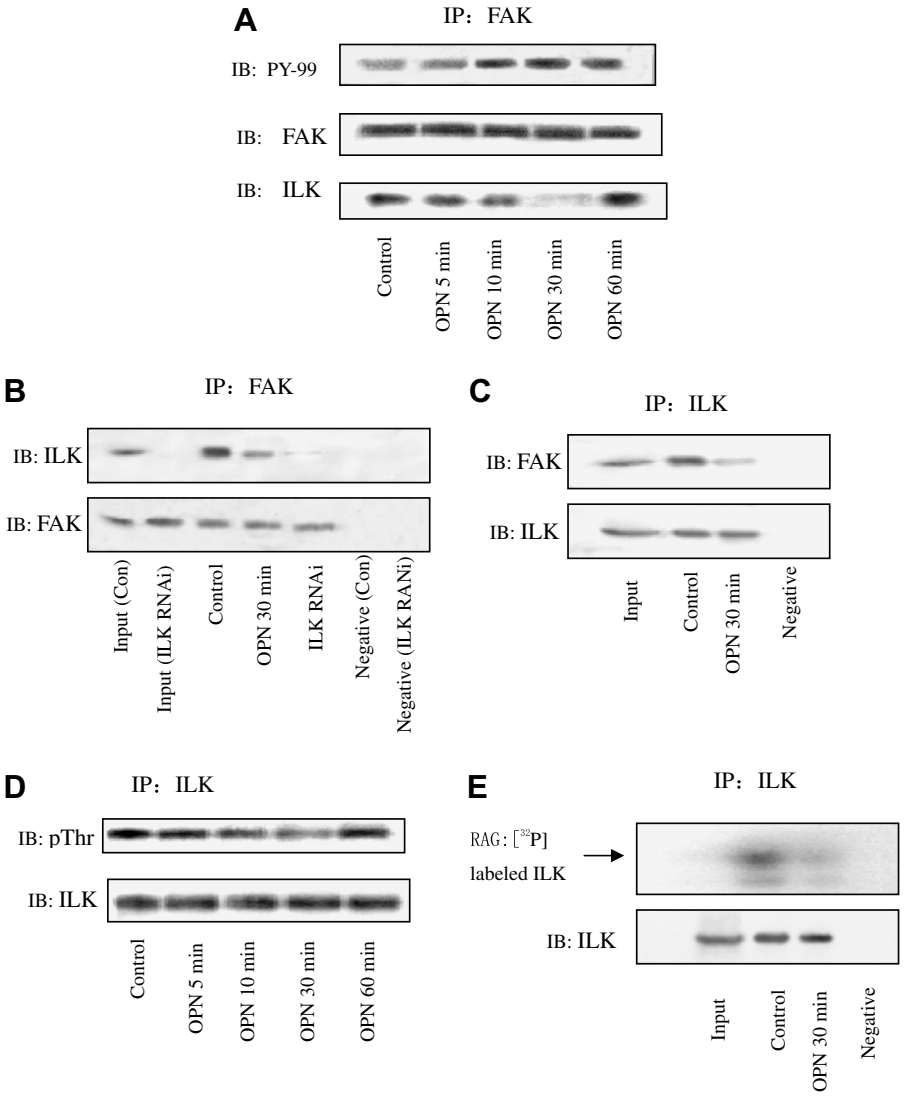

Fig. 1. OPN induces FAK phosphorylation and ILK dephosphorylation, and decreases the association of FAK with ILK. (A) VSMCs were stimulated with OPN (20 μg/ml) for different time (0, 5, 10, 30, or 60 min) and then lysed by lysis buffer. The lysates were immunoprecipitated with monoclonal anti-FAK antibody. Pellets were separated by SDS–PAGE, and then analyzed by immunoblotting with anti-PY-99 antibody, anti-ILK antibody or anti-FAK antibody. (B) The cell lysates of control, OPN-stimulated (30 min), and ILK RNAi VSMCs were immunoprecipitated with monoclonal anti-FAK antibody. The pellets were separated by SDS–PAGE, and then analyzed by immunoblotting with anti-ILK antibody or anti-FAK antibody. The lysates were presented as input, and the supernatants were presented as negative control. (C) VSMCs were stimulated with OPN (20 μg/ml) for 0 or 30 min and then lysed by lysis buffer. The lysates were immunoprecipitated with monoclonal anti-ILK antibody. Pellets were separated by SDS–PAGE, and then analyzed by immunoblotting with anti-FAK antibody and anti-ILK antibody. (D) The cell lysates were immunoprecipitated with monoclonal anti-ILK antibody. Pellets were separated by SDS–PAGE, and then analyzed by immunoblotting with anti-phosphothreonine antibody or anti-ILK antibody. (E) VSMCs were loaded with [γ-^{32}P]ATP for 12 h, and then stimulated with or without OPN (20 μg/ml) for 30 min. The cells were lysed by lysis buffer, and the lysates were immunoprecipitated with anti-ILK antibody. Pellets were separated by SDS–PAGE, and analyzed by radioautography (RAG) to detect the ILK labeled by [γ-^{32}P]. Then the total ILK protein level was detected by immunoblotting with anti-ILK antibody on the same blots.

degradation. To confirm this, we detected the ILK phosphorylation and the dephosphorylation *in vivo* following loading the VSMCs with [γ-^{32}P]ATP and then stimulating the cells with OPN. The results showed that the level of ^{32}P-labeled ILK markedly decreased in OPN-stimulated VSMCs, compared with that of control cells (Fig. 1E). These data provide a direct evidence that ILK dephosphorylation can be induced by OPN stimulation. In addition, in order to clarify the specificity of ILK antibody used and the specificity of the band detected in the co-immunoprecipitation assay, the lysates from control and ILK RNAi cells were immunoprecipitated with anti-FAK mAb, and then ILK was detected by Western blotting with anti-ILK mAb. As shown in Fig. 1B, the ILK in the immunoprecipitates of ILK RNAi cells was rarely detected, which confirmed the specificity of ILK antibody and the specificity of the band detected in the co-immunoprecipitation assay.

A band corresponding to the ILK protein (59 kDa) was found in the pellets immunoprecipitated with anti-FAK

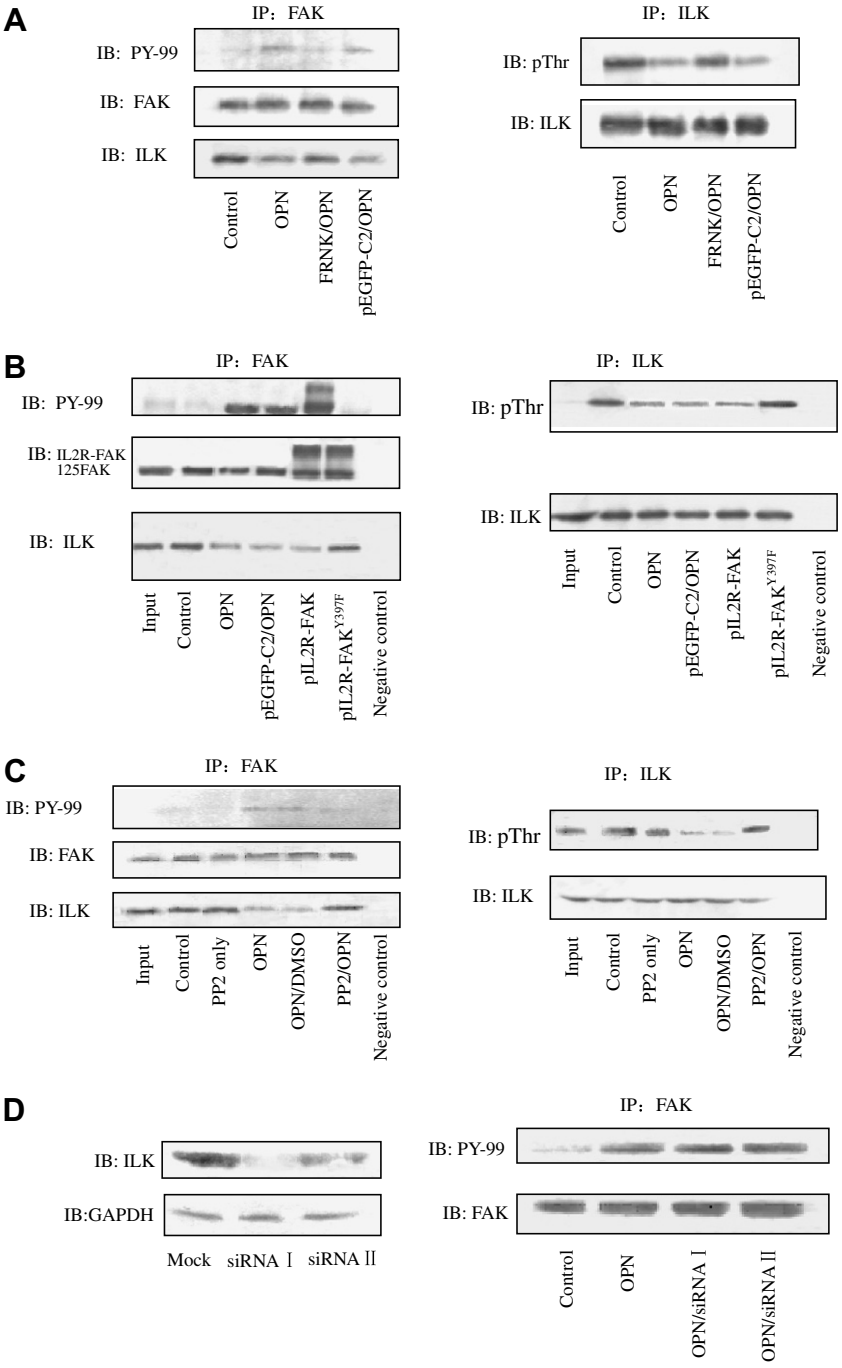

Fig. 2. FAK phosphorylation is coupled with ILK dephosphorylation and suppresses the interaction between FAK and ILK. (A) VSMCs transfected by pGZ21δxZ-FRNK were stimulated with OPN (20 μg/ml) for 30 min and lysed by lysis buffer. The lysates were immunoprecipitated with monoclonal anti-FAK antibody. Pellets were analyzed by SDS–PAGE and immunoblotting with anti-PY 99 antibody or anti-ILK antibody. Other 300 μg of lysates were immunoprecipitated with monoclonal anti-ILK antibody and analyzed by SDS–PAGE and immunoblotting with anti-phosphothreonine antibody. (B) After VSMCs were transfected by pIL2R-FAK or pIL2R-FAK^{Y393F} for 24 h, the phosphorylation levels of FAK and ILK and the interaction of them were detected. Immunoprecipitation and co-immunoprecipitation were performed as described above. (C) VSMCs were preincubated with PP2 (10 μM) for 1 h, and then were treated with OPN (20 μg/ml) for 30 min. Subsequently, FAK tyrosine phosphorylation, ILK threonine phosphorylation and the interaction between FAK and ILK were assayed by above methods. (D) VSMCs were transfected with ILK siRNAIorII plasmid expressing siRNA targeting ILK gene. The expression of ILK was analyzed by Western blotting. The level of FAK phosphorylation was evaluated by immunoprecipitating with monoclonal anti-FAK antibody and immunoblotting with anti-PY99 antibody. All above experiments were replicated three times.

antibody (Fig. 1A and B), suggesting that FAK interacts with ILK. Treatment of VSMCs with OPN resulted in a time-dependent reduction in the interaction of FAK with ILK, which reached a minimum after 30 min of OPN treatment. Furthermore, the interaction of FAK with ILK was also found in the immunoprecipitates of anti-ILK antibody, and OPN stimulation (30 min) reduced their interaction (Fig. 1C). These findings indicated that the association

of FAK with ILK depends on the phosphorylation of FAK and/or the dephosphorylation of ILK.

FAK phosphorylation induced by OPN is coupled with ILK dephosphorylation

The level of FAK phosphorylation peaked 10 min after the VSMCs were treated with OPN, which was earlier than the peak of ILK dephosphorylation at 30 min. To determine whether ILK dephosphorylation is mediated by the tyrosine phosphorylation of FAK, eukaryotic expression plasmids harboring FRNK, pIL2R-FAK, pIL2R-FAK^{Y397F} or pEGFP-C2 (control vector) were transiently transfected into VSMCs. As shown in Fig. 2A, FRNK overexpression in the VSMCs reduced the level of FAK phosphorylation by $64.5 \pm 2.7\%$ ($P \leqslant 0.05$, $n = 3$) compared with non-transfected cells, and attenuated the suppression of the FAK association with ILK induced by OPN (Fig. 2A). At the same time, the OPN-induced ILK dephosphorylation was partly suppressed by FRNK overexpression. These data indicate that the tyrosine phosphorylation of FAK might act upstream of ILK dephosphorylation in the OPN-triggered signal-transduction pathway. To test this hypothesis, active FAK (pIL2R-FAK) plasmids, the product of which was heavily tyrosine phosphorylated in its membrane-targeted form [11], were transfected into VSMCs. The results showed that, in contrast to the endogenously expressed FAK, the expression product pIL2R-FAK was phosphorylated at high levels, even without OPN treatment, which was abolished by the point mutation of Tyr^{397} to phenylalanine (Fig. 2B). Thus, Tyr^{397} is the major tyrosine phosphorylation site of FAK in VSMCs. Importantly, we found that constitutively active FAK could induce ILK dephosphorylation and reduce the interaction of FAK with ILK, similar to the effects of OPN treatment. However, pIL2R-FAK^{Y397F} abolished the effects of active FAK (Fig. 2B).

FAK is also a substrate of the protein tyrosine kinase in v-Src-transformed fibroblasts. The v-Src-associated phosphorylation of FAK is additive to that induced by integrins [12]. We therefore determined the role of FAK phosphorylation in modulating ILK by inhibiting the Src-associated phosphorylation of FAK using 10 μM of a specific inhibitor of Src, PP2. Pretreatment of the VSMCs with PP2 suppressed the FAK phosphorylation induced by OPN (Fig. 2C). Co-immunoprecipitation results showed that the association between FAK and ILK increased significantly after the VSMCs were pretreated with PP2 for 1 h. The dephosphorylation of ILK induced by OPN was inhibited by PP2 pretreatment, and the level of phosphorylated ILK increased 2.3 ± 1.4-fold ($P \leqslant 0.05$, $n = 3$) compared with that of the OPN-treated cells (Fig. 2C). These results confirm that FAK tyrosine phosphorylation plays an important role in the OPN-integrin–FAK–ILK signal pathway.

Does ILK regulate FAK phosphorylation? Western blotting results showed that after the VSMCs were transfected by above ILK siRNAI or II plasmids for 48 h, the expression of ILK was significantly inhibited, especially by ILK siRNAI. The level of FAK phosphorylation was not influenced by ILK gene silencing (Fig. 2D). The above mentioned results suggest that FAK tyrosine phosphorylation acts upstream of ILK dephosphorylation in OPN-induced signaling and the regulation of cell behavior.

FAK phosphorylation regulates the activity of VSMC migration

OPN promotes the migration of VSMCs [13]. However, it is not known whether OPN-induced migration is related to FAK tyrosine phosphorylation. We therefore investigated the activity of VSMC migration through wound-healing assays after VSMCs were transfected by the above mentioned plasmids or pretreated with PP2. The results revealed that OPN-promoted healing by stimulating VSMC migration (data not shown). After the VSMCs were transfected by pGZ21δxZ-FRNK, ILK siRNAI or II, or pretreated with PP2, the number of migratory cells was reduced to $37.1 \pm 4.3\%$ ($P \leqslant 0.05$, $n = 3$), $18.2 \pm 3.3\%$ ($P \leqslant 0.05$, $n = 3$), $38.8 \pm 9.1\%$ ($P \leqslant 0.05$, $n = 3$), and $17.4 \pm 4.5\%$ ($P \leqslant 0.05$, $n = 3$), respectively, of the control value (Fig. 3). The number of migratory cells transfected by pIL2R-FAK was similar to that of the OPN-treated group, even without OPN stimulation, but the mutation of Y397F abolished the effect of active FAK on VSMC migration (Fig. 3). These data indicated that tyrosine phos-

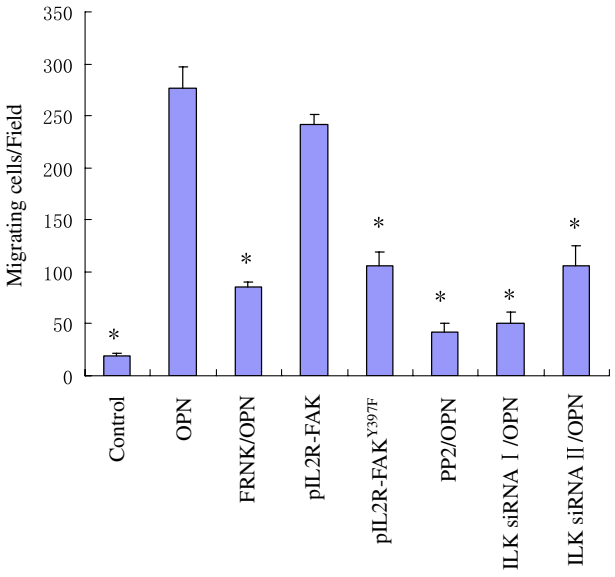

Fig. 3. Effect of FAK phosphorylation on VSMC migration. VSMCs transfected with plasmids as indicated were grown to 100% confluence and were scratched to form a 100 μm wound. After incubation for 24 h in serum-free media with (FRNK, PP2, ILK siRNAIorII) or without (pIL2R-FAK, pIL2R-FAK^{Y397F}) OPN, the cells migrating into the wound were photographed at the beginning and at the end of the experiment (data not shown) and quantitated from three replicate experiments. $^{*}P \leqslant 0.05$, compared with OPN group.

phorylation of FAK is necessary for VSMC migration induced by OPN.

Discussion

VSMC adhesion and migration are important pathological mechanisms of some vascular remodeling diseases, such as hypertension, atherosclerosis and vascular restenosis following angioplasty. Many growth factors and ECM proteins (e.g., OPN and fibronectin) are involved in the regulation of VSMC adhesion and migration. As ECM receptors, integrins not only mediate the interaction of cells and the ECM, but also transduce signals from both the outside and inside of cells, so that they can regulate cell adhesion, migration, differentiation, proliferation, and apoptosis. Therefore, clarifying the signal-transduction pathways of the integrin–ECM interaction and their effects on cell biological behaviors are helpful in understanding the molecular mechanisms of those remodeling cardiovascular diseases. FAK and ILK are important cellular signal proteins that play key roles in ECM–integrin signal pathways [13,14]. In the current study, we investigated the relationship between the phosphorylation of FAK and ILK and OPN-induced VSMC migration. The results showed that OPN could induce FAK tyrosine phosphorylation and ILK threonine dephosphorylation and disrupt the interaction of FAK with ILK (Fig. 1A–D). Furthermore, the radioautography experiments using γ-^{32}P-labeling ILK demonstrated that the decrease of phosphorylated ILK resulted from the dephosphorylation of ILK rather than its degradation (Fig. 1E). The peak of FAK phosphorylation at 10 min was earlier than the trough of ILK phosphorylation at 30 min. So, does FAK tyrosine phosphorylation regulate the threonine dephosphorylation of ILK? Some recent reports have indicated that the loss of ILK impacts β1-integrin/FAK signaling [7,15], which was not consistent with our observations in VSMCs. In order to investigate this further, the effects of FAK tyrosine phosphorylation on the level of ILK threonine phosphorylation and the interactions of the two proteins were evaluated through overexpression of the endogenous inhibitor of FAK inhibiting FAK phosphorylation (FRNK), constitutively activated FAK with a high basal level of tyrosine phosphorylation (pIL2R-FAK), dominant negative FAK (pIL2R-FAK^{Y397F}) or treatment with specific inhibitor of Src (PP2). The results showed that after OPN-induced FAK phosphorylation was inhibited by different pathways (FRNK, pIL2R-FAK^{Y397F} or PP2), the reduction of ILK phosphorylation was reversed (Fig. 2A–C); this indicated that FAK tyrosine phosphorylation, especially at Tyr397 in the NH_2-terminal non-catalytic domain, might act upstream of ILK dephosphorylation. One possible explanation is that FAK tyrosine phosphorylation induces ILK dephosphorylation by activating protein phosphatases (e.g., ILK-associated phosphatase, which is a protein serine/threonine (S/T) phosphatase of the PP2C family that associated with ILK [16]); these will need to be identified in future experiments. Meanwhile, the overexpression of pIL2R-FAK directly reduced the level of ILK phosphorylation without OPN treatment (Fig. 2B), which confirmed the importance of FAK tyrosine phosphorylation for ILK dephosphorylation. By contrast, the inhibition of ILK expression by ILK siRNAI or II did not influence FAK phosphorylation (Fig. 2D).

Tyrosine-phosphorylated residues are often sites of specific interactions with intracellular proteins that are involved in signal-transduction cascades via SH2 domains. Some SH2 domain-containing Src family members (e.g., Src, fyn [17] and tensin [18]) interact with phosphorylated FAK *in vivo*. Although there is no SH2 domain in the ILK molecule, a band corresponding to the ILK protein was found in anti-FAK immunoprecipitates, meanwhile, a FAK band was also detected in the immunoprecipitates of anti-ILK antibody according to a cross-immunoprecipitation assay (Fig. 1A and C). We propose that this interaction might occur indirectly through adapter proteins. In *Drosophila*, the proteins that associate with integrins include ILK, PINCH, tensin, and phosphorylated FAK [19–22]. However, Tanentzapf et al. reported that when the integrin β tail was placed on a heterologous transmembrane protein, it only recruited some integrin-associated proteins. In particular, talin and FAK were recruited, but not ILK, PINCH or tensin [23]. In the present study, we found that the interaction between FAK and ILK was reduced by OPN treatment, which suggested that the regulation mechanism of FAK and ILK could differ in the OPN-integrin signaling pathway in VSMCs, similar to the findings of Tanentzapf and colleagues.

We examined whether the effects of FAK and ILK were similar to those on VSMC migration induced by OPN using wound-healing assays after FAK phosphorylation or ILK expression was inhibited by the overexpression of appropriate plasmids. The results indicated that FAK-phosphorylation inhibition (FRNK, pIL2R-FAK^{Y397F} or PP2) and ILK gene silencing (ILK siRNAI or II) interfered with VSMC migration, while pIL2R-FAK reversed these effects (Fig. 3). This confirmed the importance of Y397 phosphorylation. We propose that although the regulation mechanisms of FAK and ILK are different, they both modulate the VSMC migration induced by OPN, the signaling pathways of which remain to be identified. Further analysis will be required to clarify the molecular mechanism of ILK dephosphorylation, the adapter proteins that link FAK to ILK and their downstream signal molecules in the OPN-integrin signaling pathways in VSMCs.

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