Phosphorylation of GIT1 tyrosine 321 is required for association with FAK at focal adhesions and for PDGF-activated migration of osteoblasts

ARTICLE *in* MOLECULAR AND CELLULAR BIOCHEMISTRY · FEBRUARY 2012

Impact Factor: 2.39 · DOI: 10.1007/s11010-012-1249-3 · Source: PubMed

8 AUTHORS, INCLUDING:

Yongxin Ren

15 PUBLICATIONS **87** CITATIONS

SEE PROFILE

Jin Fan

Nanjing Medical University

15 PUBLICATIONS **110** CITATIONS

SEE PROFILE

Mol Cell Biochem (2012) 365:109–118
DOI 10.1007/s11010-012-1249-3

Phosphorylation of GIT1 tyrosine 321 is required for association with FAK at focal adhesions and for PDGF-activated migration of osteoblasts

Yongxin Ren · Lipeng Yu · Jin Fan · Ze Rui · Zhengzhe Hua · Zitao Zhang · Ning Zhang · Guoyong Yin

Received: 10 November 2011 / Accepted: 14 January 2012 / Published online: 3 February 2012

Abstract Osteoblast migration and proliferation are fundamental processes in bone healing. We demonstrated that the G-protein-coupled receptor kinase interacting protein 1(GIT1) is a key regulator of bone mass and osteoblast cell migration, but little is known about GIT1 regulation by upstream signaling systems or the impact of GIT1 on downstream effectors. We found that platelet-derived growth factor (PDGF) stimulated the GIT1 tyrosine phosphorylation in osteoblast cells and increased the association of GIT1 with focal adhesion kinase (FAK) at osteoblast focal adhesions. The Src inhibitor PP2 and FAK siRNA inhibited GIT1 tyrosine phosphorylation and the increased association between GIT1 and FAK following stimulation with PDGF. The spa2 homology domain (SHD) of GIT1 was required for association with FAK. Furthermore, phosphorylation of tyrosine 321 of GIT1, which is localized within the SHD, was critical for association with FAK. Mutagenesis analysis revealed that GIT1Y321F inhibited the increased association between GIT1 and FAK. Immunofluorescent staining revealed that GIT1Y321F inhibited FAK activation in focal adhesions after PGDF stimulation. A cell spreading assay demonstrated that GIT1Y321F also inhibited osteoblast cell motility, while the Boyden chamber assay demonstrated that the GIT1Y321F mutation inhibited PDGF-induced osteoblastic cell migration. Phosphorylation of tyrosine 321 of GIT1 is necessary for PDGF-induced association with FAK, FAK activation in focal adhesions, and for osteoblastic cell migration.

Keywords GIT1 · FAK · Tyrosine phosphorylation

Introduction

Fracture healing is still poorly understood. It requires complex spatiotemporal interactions between different cell types and multiple molecular signaling events. Initial hematoma formation is followed by inflammation, repair, and finally, remodeling. The inflammatory phase is a critical period characterized by impaired perfusion and the migration of a wide array of osteoprogenitor cells, mesenchymal cells, and osteoblast cells to the site of injury [1]. In addition, the development and maintenance of a healthy skeleton depends on the migration of cells to areas of new bone formation [2]. Many growth factors, including PDGF and transforming growth factor-β (TGF-β), induce the migration of various cell types that contribute to bone healing [3]. Osteoblastic migration in response to growth factors is essential for skeletal development, bone remodeling, and fracture repair, as well as pathological processes like metastasis [4]. Osteoblast migration is a dynamic process that requires the coordinated formation and disassembly of focal adhesions (FAs) [5]. Proteins such as paxillin, focal adhesion kinase (FAK), and GIT1 are known to play a regulatory role in FA disassembly and turnover [6].

GIT1 is a signaling adaptor protein that localizes to adhesions, cytoplasmic complexes, and the leading edges of cells to regulate migration, protrusions, adhesion dynamics, and cytoskeletal organization [7]. Structurally,

Yongxin Ren and Lipeng Yu contributed equally to this study.

Y. Ren · L. Yu · J. Fan · Z. Rui · Z. Hua · Z. Zhang · N. Zhang · G. Yin (✉)
The First Affiliated Hospital of Nanjing Medical University, 300 Guang Zhou Road, Jiangsu 210029, China
e-mail: Guoyong_Yin2005nanjing@Yahoo.com

GIT1 consists of an N-terminal ARF-GAP domain, Spa homology domain (SHD), ankyrin repeats, synaptic localization domain (SLD), and paxillin-binding site (PBS) [8]. The biological functions of GIT1 are diverse. We have previously shown that GIT1 participates in postnatal lung development by regulating lung vasculature and is also involved in endothelial cell (EC) and vascular smooth muscle cell (VSMC) migration [9]. A major function of GIT1 is to regulate cytoskeletal dynamics to facilitate cell spreading and spatial targeting [9, 10]. We also reported that GIT1 is a key regulator of bone mass [11] and osteoblast cell migration [12], but little is known about GIT1 regulation during bone healing.

The role of FAK in bone formation, remodeling, and repair is similarly unclear because deletion of FAK in the mouse germ line results in embryonic death at E8.5–E9.0 [13]. Recent reports showed that the phosphorylation of FAK was critical for bone formation and osteoblast migration induced by bone morphogenetic protein (BMP) [14]. Several studies have demonstrated that GIT1 interacts with FAK and paxillin [15, 16], and we have previously shown that GIT1 colocalized with FAK at focal adhesions in thrombin-stimulated endothelial cells [15]. FAK is a non-receptor tyrosine kinase activated by autophosphorylation at Y397. Phosphorylated FAK binds to the Src SH2 domain. In turn, Src phosphorylates FAK on additional tyrosine residues to increase FAK activity and to facilitate downstream binding to Src/FAK phosphorylation targets, including the focal adhesion protein paxillin [17, 18]. FAK and Src cooperate to phosphorylate paxillin kinase linker (PKL), a member of PKL/GIT/CAT family, to promote the localization of FAK to focal adhesions and regulate cell spreading and migration [19]. We demonstrated that GIT1 was tyrosine phosphorylated by c-Src in response to angiotensin II and epidermal growth factor (EGF) stimulation [8] and also documented an important role for GIT1 tyrosine phosphorylation in signal transduction, especially in FAK, MEK1-ERK1/2, and PLCγ activation [8–10]. Phosphorylated GIT1 was required for the activation and localization of ERK1/2 to focal adhesions and for enhanced cell migration [10]. The tyrosine phosphorylation sites in GIT1 critical for association with FAK are not known. Furthermore, the function of GIT1 tyrosine phosphorylation and the downstream signaling pathways engaged by phosphorylated GIT1 and that regulate cytoskeletal dynamics at focal adhesions have not been determined.

In this study, we examined the role of GIT1 tyrosine phosphorylation in osteoblast cells. The GIT1 protein associated with FAK at focal adhesions in osteoblast cells, and this association increases after stimulation with PDGF. Furthermore, the tyrosine 321 (Y321) of GIT1 was critical for association with FAK; mutagenesis analysis revealed that GIT1Y321F inhibited the increased association between GIT1 and FAK and suppressed osteoblast migration.

Materials and methods

Ethics statement

All procedures of the care and treatment of these mouses were maintained in accordance with Nanjing Medical University Animal Care Committee guidelines. The protocol was approved by the Experimental Animal Ethics Committee of Nanjing Medical University (20100385), China. Experimental animal practitioners post qualification certificates provided by JiangSu Province Science and Technology Office.

Cells culture

Primary murine osteoblastic cells were obtained from mouse skull as previously described [20] and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37°C in 5% CO_2.

DNA expression plasmids and regents

All constructs, including Flag-GIT1, Flag-GIT1 (del SHD), GST-GIT1, GST-GIT1 (del SHD), HA-FAK, Flag-GIT1(Y293F), and Flag-GIT1(Y321F) were kind gifts from Dr. Bradford C. Berk (University of Rochester). Monoclonal antibodies against GIT1, Src, and GST antibody were purchased from BD Transduction Laboratories (Lexington, KY). Antibodies specific for Flag M2 and HA were obtained from Sigma (St. Louis, MO) and FAK antibodies were obtained from Promega (Madison, WI). The antibody against Src (pY416), the non-specific phosphotyrosine antibody 4G10, and that against FAK (pFAK Y397) were purchased from Upstate Biotech. (Waltham, MA).

Transient transfection of siRNA and lentivirus infection

Osteoblasts at 80% confluence were transiently transfected with 100 nM control siRNA or FAK siRNA using lipofectamine 2000 reagent in OptiMEM medium (Dharmacon, Chicago, IL). Flag-GIT1, Flag-GIT1 (del SHD), Flag-GIT1(Y293F), Flag-GIT1(Y321F), and HA-FAK were ligated into the lentiviral vector PLJM. Lentiviruses were produced by cotransfection of HEK293T cells (purchased from ATCC Company, USA) with shuttle vectors, Delta891, and envelope plasmid pVSVG, followed by concentration of lentivirus-containing media by centrifugation. All these procedures and determination of viral titers were described previously [21].

Cell fractionation

Osteoblasts infected with viruses including different cDNAs were harvested and homogenized in TEEN D buffer (25 mM Tris–HCl, 5 mM EGTA, 2 mM EDTA, 100 mM NaF, 5 mM DTT, and 0.2 mM PMSF) with protease inhibitors. Cell fractions were prepared as described previously [10]. Protein concentrations of each fraction were determined using a BCA reagent (Pierce).

Immunoprecipitation and immunoblotting assays

Osteoblastic cells were infected with Flag-GIT1 and HA-FAK lentivirus. After infection for 8 h, cells were incubated with normal medium for 12 h, serum-starved for 6 h and then stimulated with PDGF. Cells fractions were prepared as described [10]. Cytoskeletal fractions were immunoprecipitated with 2 μg anti-Flag antibody, electrophoretically separated, transferred to nitrocellulose membranes, and probed with HA and Flag antibodies. Blots were then labeled with an HRP-conjugated secondary antibody (Amersham Biosciences UK limited) and visualized by enhanced chemiluminescence (ECL). Densitometric analysis of blots was performed using NIH image software.

Immunofluorescence

Osteoblastic cells infected with a specific lentivirus were starved with serum-free DMEM for 6 h and then stimulated with PDGF as indicated. Cells were fixed with 4% formaldehyde for 5 min, washed three times in PBS, permeabilized with 0.05% Triton for 5 min, and blocked with 10% normal goat serum for 1 h. Cells were incubated with pFAKY397 antibody diluted in PBS followed by Alexa Fluor 546 goat anti-mouse or rabbit IgG (H + L) for red fluorescence (Molecular Probes, Inc) at final concentrations of 1.5–2.0 μg/ml.

Spreading and chamber assays

For the cell spreading assay, osteoblastic cells were infected for 8 h, incubated with normal medium for 12 h, serum-starved for 6 h, and then suspended in DMEM containing trypsin inhibitor (0.5 μg/ml). Cells were washed three times with serum-free DMEM and incubated at 37°C under 5% CO_2 for 30 min and allowed to adhere to 10 μg/ml fibronectin-coated cover slips for the indicated times. The cell areas were calculated by ImageJ software. Statistical analysis was performed using Student's *t* tests. The Boyden chamber assay was performed as described previously [22]. Scanning density was performed with NIH image software and statistical analysis was performed using Student's *t* tests.

Results

PDGF increases the interaction of FAK with GIT1 in osteoblastic cells

We previously demonstrated that GIT1 colocalized with FAK at endothelial cell focal adhesions following treatment with thrombin [15]. To directly assess the interaction between the GIT1 and FAK in osteoblastic cells, we stimulated cells with 10 ng/ml PDGF for different durations (Fig. 1). Cell lysates were prepared and the cytoskeletal fractions analyzed. Endogenous GIT1 was immunoprecipitated and the endogenous GIT1-associated FAK was detected by western blotting. There was a very large increase in the GIT1–FAK interactions after PDGF stimulation (Fig. 1A). To confirm direct binding between FAK and GIT1, we used a GST pull down assay. Osteoblastic cells were treated with PDGF for 5 min and then GST-GIT1 and GST were used to co-precipitate FAK. FAK was pulled down by GST-GIT1, but not by GST (Fig. 1B). To provide further evidence for GIT1 and FAK localization in osteoblastic cells, we performed immunofluorescence staining for GIT1 and activated FAK (FAK phosphorylated at Y397 or pFAKY397). Under basal conditions, pFAKY397 was diffusely distributed with no accumulation at the plasma membrane (Fig. 1C-a). In contrast, GIT1 was located in discrete membrane-associated structures resembling typical focal adhesions (Fig. 1C-c). Stimulation with PDGF induced the translocation of pFAKY397 to the focal adhesions (Fig. 1C-b) such that the fluorescence staining pattern strongly resembled that of GIT1 (Fig. 1C-c, d). These results are consistent with a recent report indicating that GIT1 association with FAK in focal adhesions was increased in endothelial cells after endothelin-1 stimulation [16]. In order to determine whether GIT1 and activated FAK (FAK phosphorylated at Y397 or PFAKY397) co-localizes to the same adhesions when stimulation with PDGF, we performed immunofluorescence co-staining for GIT1 and activated FAK. As shown in Fig. 1D, after treated with PDGF for 5 min, immunofluorescence of FAKY397 and GIT1 were both dramatically increased, which means they could co-accumulate and co-localize to the same adhesions after stimulation.

The SHD of GIT1 is required for association with FAK

The SHD domain is a tandem repeat present only in GIT1 and GIT2 orthologs and yeast Spa2 and Sph1 proteins. It mediates protein–protein interactions that are essential for the promotion of cell migration [23]. To determine the importance of SHD in GIT1–FAK association, we co-infected osteoblast cells with Flag–GIT1 (del SHD) lentivirus and HA-FAK lentivirus. The association between GIT1 and FAK was increased following PDGF stimulation

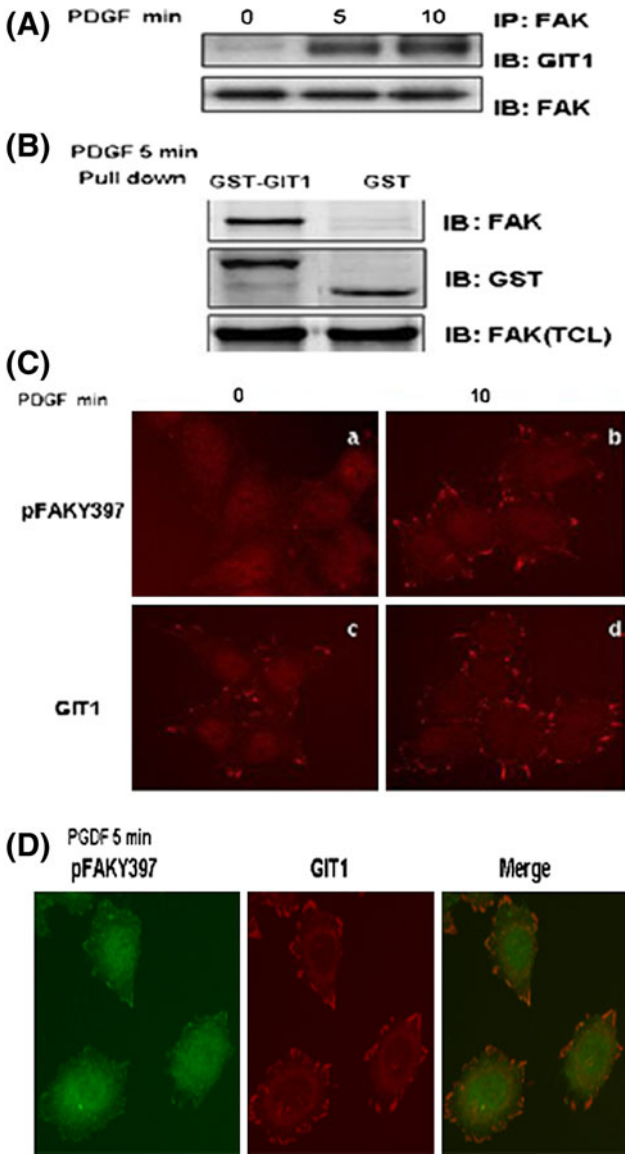


Fig. 1 FAK interaction with GIT1 is increased after PDGF stimulation in osteoblastic cells. **a** Osteoblastic cells were serum-starved for 6 h and then stimulated with 10 ng/ml PDGF. Endogenous GIT1 was immunoprecipitated and the association of FAK (*upper panel*) with GIT1 (*bottom panel*) were detected by western blot. **b** Osteoblastic cells were serum-starved for 6 h and then treated with PDGF for 5 min. GST–GIT1 and GST were immobilized on glutathione-conjugated beads and incubated with cell lysates. Beads were washed extensively and then the isolated proteins immunoblotted for FAK (*top panel*) and reprobed with GST antibody to confirm equal loading (*middle panel*). To confirm equal protein expression, cytoskeletal fractions were blotted with FAK antibody in total cell lysates (TCL) (*bottom panel*). **c** Osteoblastic cells were serum-starved for 6 h and stimulated with saline (*a*, *c*) or 10 ng/ml PDGF for 5 min (*b*, *d*). Cells were fixed with 4% formaldehyde and stained with a pFAKY397 antibody (*a*, *b*) or a GIT1 antibody (*c*, *d*). **d** Osteoblastic cells were serum-starved for 6 h and then treated with PDGF for 5 min. Cells were fixed with 4% formaldehyde and stained with pFAKY397 and GIT1 antibodies

(Fig. 2a), but this PDFG-mediated enhanced association was markedly lower in cells co-infected with GIT1 (del SHD) and FAK lentivirus (Fig. 2b). To demonstrate the importance of SHD in direct binding between FAK and GIT1, we used a GST pull down assay. Osteoblastic cells were treated with PDGF for 5 min and then GST–GIT1 and GST–GIT1 (del SHD) proteins were used to co-precipitate FAK. Indeed, FAK was pulled down by GST-GIT1, but not by GST-GIT1 (del SHD) (Fig. 2c), indicating that the SHD domain of GIT1 is essential for association with FAK.

PDGF-induced GIT1–FAK association is dependent on FAK and Src kinase activation

Previous reports revealed that Src and FAK cooperate to regulate the function of focal adhesion proteins [19]. To demonstrate the importance of Src and FAK activation in the regulation of the GIT1–FAK association, we first examined the effect of the Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyramidine (PP2) or its vehicle (DMSO) on the PDGF-induced GIT1–FAK association in osteoblastic cells infected with the GIT1 lentivirus. As expected from results presented in Fig. 1, the association between GIT1 and FAK was increased after pretreatment with DMSO, while this increased association was dramatically inhibited after 30 min pretreatment with PP2 (Fig. 3a). The association between GIT1 and FAK also was inhibited after suppression of FAK expression by transfection with FAK siRNA (Fig. 3b). These data indicated that the FAK and Src kinase activation are necessary for PDGF-induced GIT1–FAK association.

PDGF-induced GIT1 tyrosine phosphorylation is FAK- and Src-dependent

The tyrosine phosphorylation of GIT1 is a critical event in GIT1-associated signal transduction [8–10]. To determine whether GIT1 tyrosine phosphorylation is necessary for Src-mediated GIT1–FAK association in osteoblastic cells, we first examined GIT1 tyrosine phosphorylation in response to PDGF (Fig. 4a). Indeed, PDGF increased GIT1 tyrosine phosphorylation at 5 and 10 min, and enhanced phospho-GIT1 was maintained for 30 min (data not shown). In contrast, pretreatment with the Src inhibitor PP2 significantly decreased PDGF-induced tyrosine phosphorylation of GIT1 (Fig. 4a), suggesting that the phosphorylation of GIT1 induced by PDGF is dependent on Src. We previously demonstrated that Src activates MEK1 and ERK1/2 by inducing GIT1 tyrosine phosphorylation [8, 10]. FAK and Src cooperate to phosphorylate paxillin kinase linker (PKL), a member of PKL/GIT/CAT family,

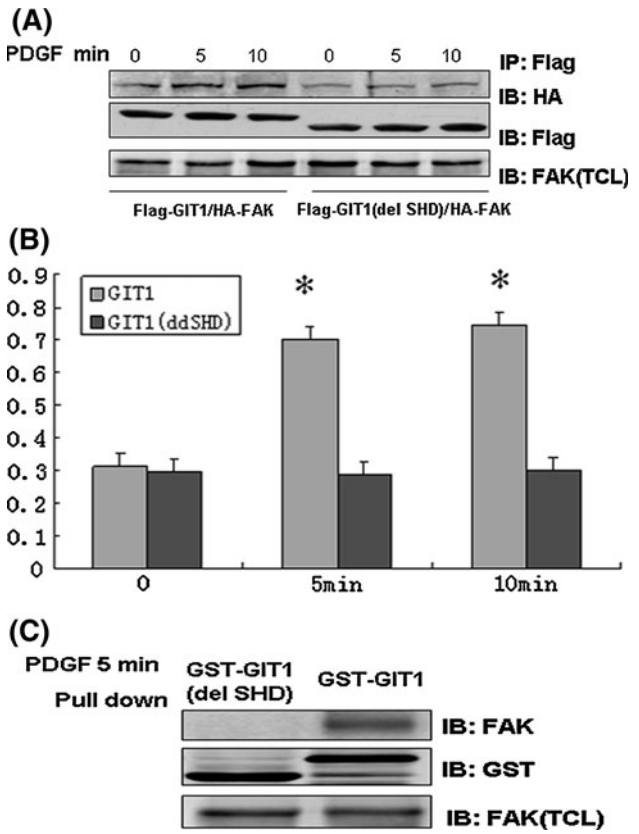


Fig. 2 The SHD domain of GIT1 is required for association with FAK. **a** Osteoblastic cells were co-infected with HA-FAK lentivirus and either Flag-GIT1 or Flag-GIT1 (del SHD) lentivirus for 8 h and incubated with normal medium for 12 h. Cells were serum-starved for 6 h and then stimulated with PDGF. The cell membrane, cytoplasm, and cytoskeleton were separated as described in "Materials and methods". Cytoskeletal fractions were immunoprecipitated with Flag antibody and probed with an HA antibody (*top panel*). To confirm equal protein immunoprecipitation, the blot was reprobed with Flag antibody (*middle panel*). HA-FAK protein expression was detected by HA in total cell lysate (*bottom panel*). **b** The relative increases were determined by quantitative densitometry ($*p < 0.01$). **c** Osteoblastic cells were serum-starved for 6 h and then treated with PDGF for 5 min. GST–GIT1 and GST–GIT1 (del SHD) were immobilized on glutathione-conjugated beads and incubated with cell lysates. Beads were washed extensively and then immunoblotted for FAK (*top panel*) and reprobed with GST antibody to confirm equal loading (*middle panel*). To confirm equal protein expression, cytoskeleton fractions were blotted with FAK antibody in total cell lysates (TCL) (*bottom panel*)

which then stimulates FAK localization to focal adhesions and thereby regulates cell spreading and migration. The role of FAK in GIT1 tyrosine phosphorylation was examined by transfecting osteoblastic cells with FAK siRNA for 24 h followed by starvation for 6 h and subsequent PDGF stimulation. Transfection with FAK siRNA markedly reduced FAK expression (Fig. 4b) and decreased the GIT1 tyrosine phosphorylation without changing GIT1 expression (Fig. 4b, c). These data suggested that the GIT1 tyrosine phosphorylation induced by PGDF in osteoblastic cells was dependent on both Src and FAK activation.

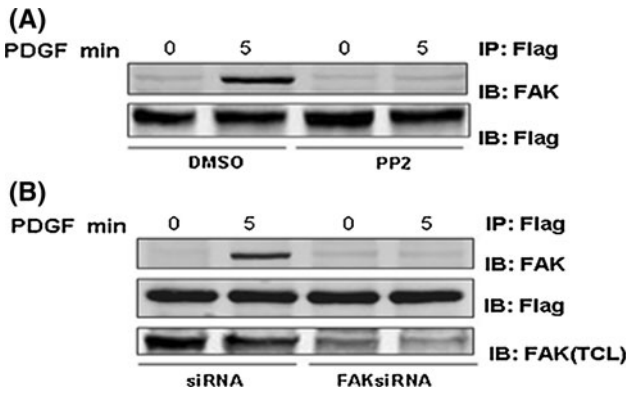


Fig. 3 PDGF-induced GIT1 tyrosine phosphorylation is FAK- and Src-dependent. **a** Osteoblastic cells were infected with Flag-GIT1 lentivirus for 8 h and incubated with normal medium for 12 h. Cells were serum-starved for 6 h, pretreated with DMSO or PP2 for 30 min, and then stimulated with PDGF. Cytoskeletal fractions were immunoprecipitated with Flag antibody and probed with a FAK antibody (*top panel*). To confirm equal protein immunoprecipitation, the blot was reprobed with Flag antibody (*bottom panel*). **b** Osteoblastic cells were infected with Flag-GIT1 lentivirus for 8 h and then transfected with siRNA or FAKsiRNA for 24 h. Cells were serum-starved for 6 h and then stimulated with PDGF. Cytoskeletal fractions were immunoprecipitated with Flag antibody and probed with a FAK antibody (*top panel*). To confirm equal protein immunoprecipitation, the blot was reprobed with Flag antibody (*middle panel*). Total cell lysates (TCL) were probed with FAK antibody to confirm FAK expression after transfection (*bottom panel*)

GIT1 tyrosine 321 phosphorylation is required for increased association with FAK

A recent study identified eight possible tyrosine phosphorylation sites on GIT1 that are conserved among different species [24]. Among these tyrosines, tyrosine 293 (Y293) and tyrosine 321 (Y321) were suggested as possible Src phosphorylation sites, and are localized in the SHD domain. In addition, our previous data demonstrated that Y321 in SHD domain was required for EGF- and AngII-stimulated, Src-dependent ERK1/2 activation [8]. Therefore, these tyrosines (Y293, Y321) are likely Src phosphorylation sites that may regulate GIT1–FAK association and osteobalstic cell migration. To define specific roles for Y293 and Y321, we mutated them individually to phenylalanine and encorporated the mutant constructs into lentivirus vectors (GIT1Y293F and GIT1Y321F). Tyrosine phosphorylation was significantly increased in osteoblasts infected with GIT1 and GIT1Y293F lentivirus after PDGF stimulation, while this increased phosphorylation was partly inhibited in cells infection with GIT1Y321F lentivirus. To confirm the importance of Y321 of GIT1, we compared FAK binding to GIT1 with binding to GIT1Y321F protein (Fig. 5a). As expected, PDGF increased the association between GIT1 and FAK, but not between GIT1Y321F and FAK. To demonstrate that GIT1 Y321 was necessary for FAK association, we co-infected osteoblastic cells with HA-FAK lentivirus

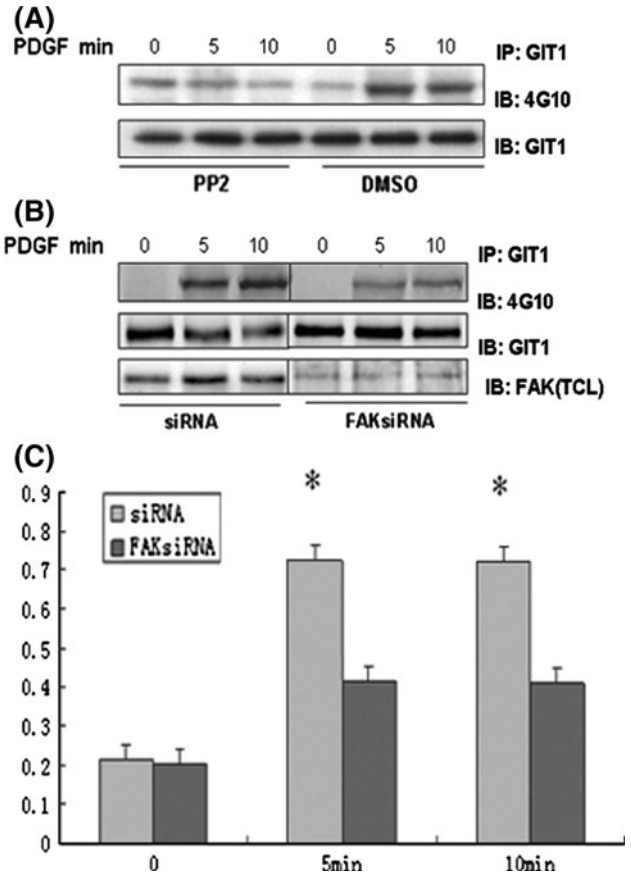


Fig. 4 PDGF-induced GIT1 tyrosine phosphorylation is FAK- and Src-dependent. **a** Osteoblastic cells were serum-starved for 6 h, pretreated with DMSO or PP2 for 30 min, and then stimulated with PDGF. Cytoskeletal fractions were immunoprecipitated with GIT1 antibody and probed with 4G10 antibody (*top panel*). To confirm equal protein immunoprecipitation, the blot was reprobed with GIT1 antibody (*bottom panel*). **b** Osteoblastic cells were transfected with siRNA or FAK siRNA for 24 h. Cells were serum-starved for 6 h and then stimulated with PDGF. Cytoskeletal fractions were immunoprecipitated with GIT1 antibody and probed with 4G10 antibody (*top panel*). To confirm equal protein immunoprecipitation, the blot was reprobed with GIT1 antibody (*middle panel*). Total cell lysates (TCL) were probed with FAK antibody to confirm FAK expression after transfection (*bottom panel*). **c** The relative increases were determined by quantitative densitometry (*$p < 0.01$)

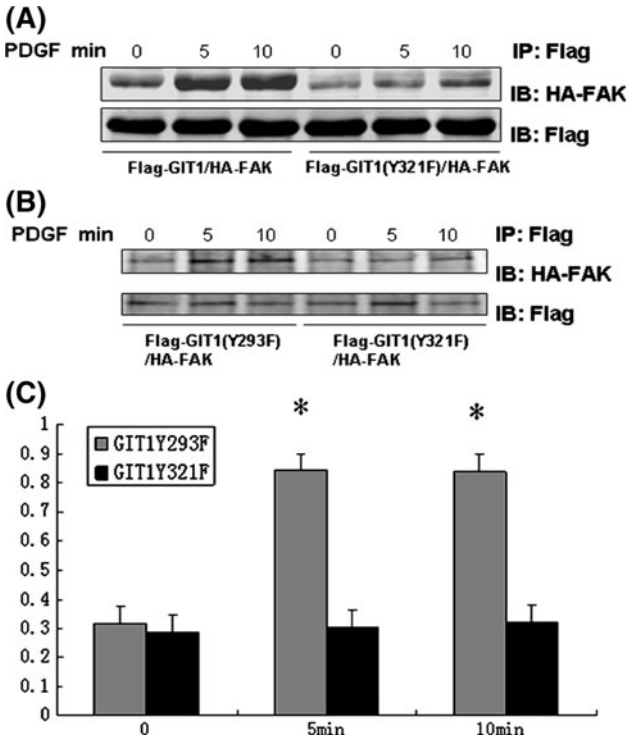
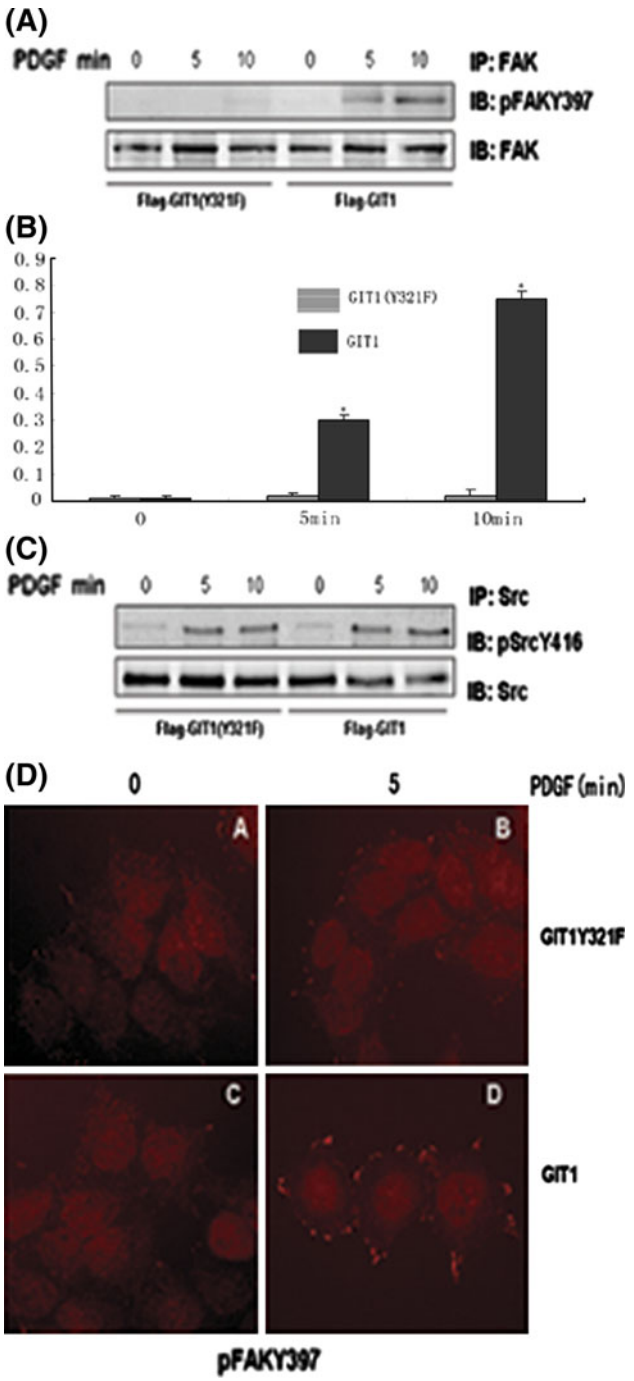


Fig. 5 GIT1 tyrosine 321 phosphorylation is required for association with FAK A) Osteoblastic cells were co-infected with HA-FAK lentivirus and either Flag-GIT1 or Flag-GIT1Y321F lentivirus for 8 h and incubated with normal medium for 12 h. Cells were serum-starved for 6 h and then stimulated with PDGF. Cytoskeletal fractions were immunoprecipitated with Flag antibody and probed with HA antibody (*top panel*). To confirm equal protein immunoprecipitation, the blot was reprobed with Flag antibody (*bottom panel*). **b** Osteoblastic cells were co-infected with HA-FAK lentivirus and either Flag-GIT1Y293F or Flag-GIT1Y321F lentivirus for 8 h and incubated with normal medium for 12 h. Cell were serum-starved for 6 h and then stimulated with PDGF as indicated. Cytoskeletal fractions were immunoprecipitated with Flag antibody and probed with HA antibody (*top panel*). To confirm equal protein immunoprecipitation, the blot was reprobed with Flag antibody (*bottom panel*). **c** The relative increases were determined by quantitative densitometry (*$p < 0.01$)

and either GIT1Y293F or GIT1Y321F lentivirus; again, GIT1Y293F association with FAK was dramatically increased after PDGF stimulation, while this association was not changed in osteoblastic cells infected with GIT1Y321F lentivirus (Fig. 5b). Together, these results suggested that the Y321 in GIT1 was the critical site for PDGF-induced assocaition with FAK in osteoblastic cells.

Tyrosine 321 of GIT1 is required for FAK activation

We previously reported that the GIT1 interaction with ERK1/2 is required for ERK1/2 activation in focal adhesion and cell migration [10]. To investigate the specific role of Y321 of GIT1 in FAK activation at focal adhesions, we infected osteoblastic cells with GIT1 or GIT1Y321F lentivirus. Activation of FAK is known to be assocaited with phosphorylation on specific tyrosine residues Y397. Indeed, PDGF rapidly induced phosphorylation of FAK on Y397 in cytoskeletal fractions of osteoblastic cells infected with GIT1 lentivirus (Fig. 6A), while FAK Y397 phosphorylation was dramatically inhibited in osteoblastic cells infected with GIT1Y321F lentivirus(Fig. 6B). To determine the specific role of GIT1Y321 in FAK activation, we measured Src phosphorylation at Y416, the critical event in Src activation. Phosphorylation of Y416 of Src was increased after PDGF stimulation (Fig. 6C), but there was no difference in Src Y416 phosphorylation in osteoblasts infected with GIT1Y321F lentivirus. To provide further



◀**Fig. 6** Tyrosine 321 of GIT1 is required for FAK activation in focal adhesion. **a** Osteoblastic cells were infected with Flag-GIT1 or Flag-GIT1Y321F lentivirus for 8 h and incubated with normal medium for 12 h. Cells were serum-starved for 6 h and then stimulated with PDGF. Cytoskeletal fractions were immunoprecipitated with FAK antibody and probed with pFAKY397 antibody (*top panel*). To confirm equal protein immunoprecipitation, the blot was reprobed with FAK antibody (*bottom panel*). **b** The relative increases were determined by quantitative densitometry (*$p < 0.01$). **c** Osteoblastic cells were infected with Flag-GIT1 or Flag-GIT1Y321F lentivirus for 8 h and incubated with normal medium for 12 h. Cells were serum-starved for 6 h and then stimulated with PDGF as indicated. Cytoskeletal fractions were immunoprecipitated with Src antibody and probed with pSrcY416 antibody (*top panel*). To confirm equal protein immunoprecipitation, the blot was reprobed with Src antibody (*bottom panel*). **d** Osteoblastic cells were infected with Flag-GIT1 (*c*, *d*) or Flag-GIT1Y321F (*a*, *b*) lentivirus for 8 h and incubated with normal medium for 12 h. Cells were serum-starved for 6 h and then stimulated with saline (*a*, *c*) or 10 ng/ml PDGF for 5 min (*b*, *d*). Cells were fixed with 4% formaldehyde and stained with pFAKY397 antibody

evidence for the importance of GIT1Y321 in FAK activation at focal adhesions, we infected osteoblastic cells with GIT1 or GIT1Y321F lentivirus and then examined changed in immunohistochemical staining in response to PDGF stimulation (Fig. 6D). There was a dramatic increase in FAK Y397 immunofluoresence at focal adhesion in osteoblastic cells infected with GIT1 lentivirus after 5 min PGDF stimulation. There was no significant increase in FAK Y397 immunofluoresence staining at focal adhesion, however, in osteoblastic cells infected with GIT1Y321F lentivirus following 5 min PGDF stimulation (Fig. 6D). These findings indicate that GIT1 Y321 is necessary for FAK activation at focal adhesions.

FAK activation is dependent on Src activation in osteoblastic cells

A recent report demonstrated that Src phosphorylates FAK on multiple tyrosine residues to increase FAK activity, and that several downstream binding partners for Src/FAK are then targeted for phosphorylation at focal adhesions [19]. To understand the relationship between Src and FAK in osteoblastic cell migration, we pretreated osteoblasts with PP2 (or DMSO) for 30 min before PDGF stimulation (Fig. 7a). The PDGF-induced phosphorylation of FAK Y397 was dramatically decreased after pretreatment with PP2 compared to vehicle pretreatment. There was no difference in the phosphorylation level of Src Y416 in osteoblasts infect with either FAK siRNA or control siRNA after PDGF stimulation (Fig. 7b). Thus, Src phospho-activation was upstream of FAK phosphorylation; Src activation by PDGF phosphorylates FAK Y397. In turn, activated FAK and Src cooperate to phosphorylate GIT1 (Fig. 4). This result is consistent with recent reports that Src and FAK kinase cooperate to phosphorylate the PKL/GIT/CAT family member PKL. Phosphorylation of PKL leads to localization at focal adhesions and regulation of cell spreading and migration [19].

The function of GIT1 tyrosine 321 phosphorylation

Recent reports revealed that the phosphorylation FAK is critical for bone formation and osteoblast migration induced by bone morphogenetic protein (BMP) [14]. In order to demonstrate the function of GIT1Y321 in

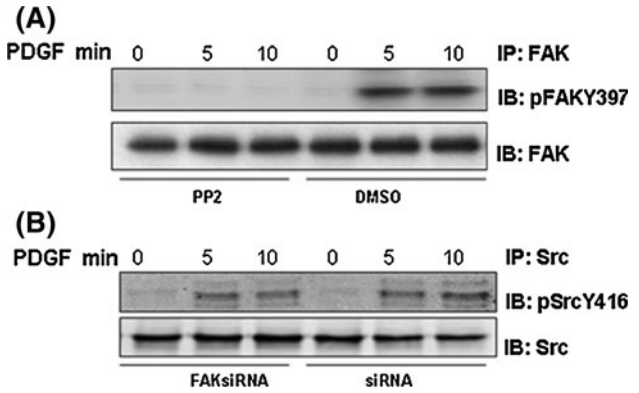


Fig. 7 FAK activation is dependent on Src activation. **a** Osteoblastic cells were serum-starved for 6 h, pretreated with DMSO or PP2 for 30 min, and then stimulated with PDGF. Cytoskeletal fractions were immunoprecipitated with FAK antibody and probed with pFAKY397 antibody (*top panel*). To confirm equal protein immunoprecipitation, the blot was reprobed with FAK antibody (*bottom panel*). **b** Osteoblastic cells were transfected with siRNA or FAK siRNA for 24 h. Cells were serum-starved for 6 h and then stimulated with PDGF as indicated. Cytoskeletal fractions were immunoprecipitated with Src antibody and probed with pSrcY416 antibody (*top panel*). To confirm equal protein immunoprecipitation, the blot was reprobed with Src antibody (*middle panel*)

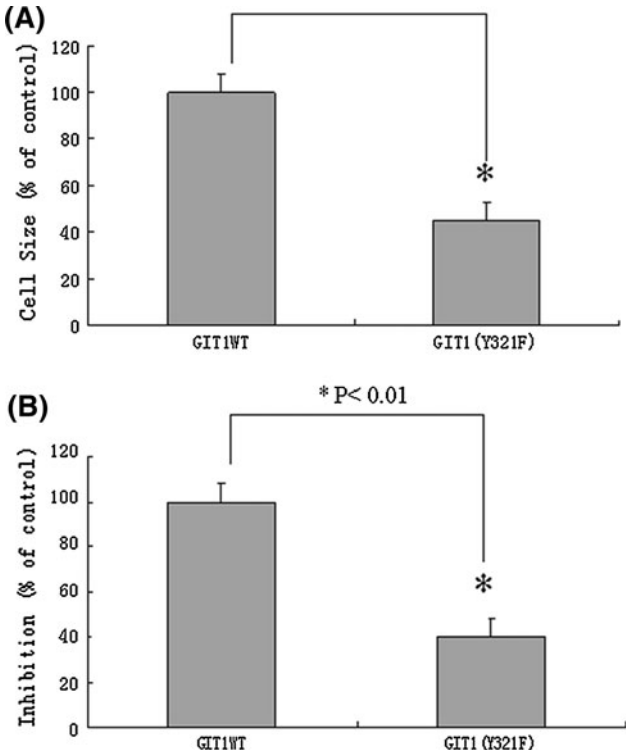


Fig. 8 Functional effect of tyrosine 321 of GIT1. **a** Osteoblastic cells were infected with Flag-GIT1 or Flag-GIT1Y321F lentivirus for 8 h and then incubated with normal medium for 12 h. Cells were starved for 6 h, stimulated with PDGF for 10 min, washed three times, and then seeded on fibronectin-coated dishes (10 μg/ml). Cell spreading was measured at 10 min. Cells were then fixed with 4% formaldehyde. One hundred attached cells were randomly selected to measure the cell area using ImageJ software ($*p < 0.01$, mean ± SE, $n = 6$). The cell area after infection with Flag-GIT1 lentivirus was set to 100. **b** Osteoblastic cells were infected with Flag-GIT1 or Flag-GIT1Y321F lentivirus for 8 h and then incubated with normal medium for 12 h. Cells were starved for 6 h and then seeded in the upper portion of a Boyden chamber on collagen-precoated PVP-free polycarbonate membranes. PDGF was added to the lower chamber. Cells were incubated for 6 h at 37°C in a 5% CO_2 humidified incubator. The membranes were removed from the chamber and adherent cells were stained. The relative increases in cell density on the two sides of the membrane were determined by quantitative densitometry ($*p < 0.01$, mean ± SE, $n = 6$)

osteoblastic cell migration, we infected osteoblastic cells with GIT1 or GIT1Y321F lentivirus and then measured cell spreading. Infection with GIT1 Y321F lentivirus inhibited cell spreading by 45% compared to GIT1 lentivirus (Fig. 8a). To further demonstrate the functional role of tyrosine 321 phosphorylation, we performed a Boyden chamber migration assay in cells expressing mutant or GIT1. PDGF significantly increased cell migration in osteoblasts infected with GIT1 lentivirus, while cell migration was dramatically reduced in osteoblasts infected with GIT1Y321F lentivirus (Fig. 8b). These results suggest an important role for GIT1 Y321 in agonist-stimulated osteoblastic cell spreading and migration.

Discussion

Fracture healing is a complex physiological process that involves the coordinated participation of several cell types [1]. Osteoblast migration and proliferation play a fundamental role in the process of fracture healing [25, 26]. Osteoblast migration in response to growth factors is essential for skeletal development, bone remodeling, and fracture repair, as well as pathological processes like metastasis [4]. Osteoblast migration is a dynamic process that requires the coordinated formation and disassembly of focal adhesions [5, 27]. FAK and GIT1 are localized to focal adhesion during migration and are known to play an important regulatory role in focal adhesion disassembly and turn over.

We have previously shown that GIT1 is a key regulator of bone mass and osteoblast cell migration [11, 12], but the molecular mechanisms are unknown. Focal adhesion kinase is a 125-kDa non-receptor and -membrane protein tyrosine kinase. Protein expression and phospho-activation at Y397 (FAK pY397) or phosphor-inhibition at Y407 regulate osteoblast substrate attachment [5, 27]. Dynamic regulation of attachment to extracellular matrix proteins results in FAK tyrosine phosphorylation, which creates a docking site for the non-receptor cytoplasmic tyrosine kinase Src. Binding of Src to phosphorylated FAK leads to the further tyrosine phosphorylation of FAK at Y397, which is essential for FAK interaction with several

additional binding partners at focal adhesions [28]. Previous studies demonstrated that GIT1 interacts with FAK and paxillin, and we have previously shown that GIT1 colocalized with FAK at focal adhesions in thrombin-treated endothelial cells [15]. In this study, we demonstrated that FAK was activated in osteoblastic cells stimulated by PDGF and co-localized with GIT1 to focal adhesions. In response to PDGF, GIT1 directly associated with FAK, and this association was critical for osteoblastic cell spreading and migration.

Recently, we demonstrated an important role for GIT1 tyrosine phosphorylation in FAK, MEK1-ERK1/2, and PLCγ activation [10–12]. Specifically, we showed that GIT1 was a substrate for c-Src tyrosine phosphorylation in response to angiotensin II (AngII) and epidermal growth factor (EGF). Phosphorylated GIT1 was required for the activation and localization of ERK1/2 to focal adhesions and enhanced cell migration in response to EGF [10]. Webb et al. [24] used mass spectrometry to generate a phosphorylation map of GIT1; among potential tyrosine residues, Y293 and Y321 were suggested to be possible Src phosphorylation sites. The SHD domain of GIT1 is named for the yeast SpaII homology domain. It spans amino acid 255–365 and encompasses Y293 and Y321 [10]. It is known that GIT1 interacts with a number of signaling molecules through this domain and regulates their subcellular localization [7]. In this study, we demonstrated that GIT1 was phosphorylated after PDGF stimulation and that both GIT1 tyrosine phosphorylation and association with FAK were prevented by the Src inhibitor PP2 and by FAK siRNA (Fig. 4). Furthermore, PDGF-induced GIT1 tyrosine phosphorylation was dramatically decreased in osteoblasts over-expressing the GIT1Y321F mutant (Fig. 5a). Furthermore, tyrosine 321 in GIT1 was required for increased association with FAK after PDGF stimulation. Infection with GIT1Y321F, but not GIT1Y293F, inhibited PDGF-mediated GIT1–FAK association, indicating that GIT1 Y321 (but not Y293) is critical for the association of GIT1 to FAK in addition to other GIT1-binding proteins [7] (Fig. 5b, c). Infection with GIT1Y321F inhibited FAK immunoreactivity at focal adhesion, indicating that GIT1 Y321 phosphorylation was a critical event mediating FAK function in PDGF-stimulated osteoblastic cells (Fig. 6). Indeed, GIT1Y321F expression inhibited osteoblastic cell migration and spreading induced by PDGF (Fig. 8).

We propose a model to explain the role of GIT1Y321 in PDGF-mediated activation of FAK, cell spreading, and migration. In serum-starved, unstimulated osteoblasts, GIT1 exists in a pre-assembled complex. Upon stimulation, Src and/or FAK are activated and phosphorylate the critical GIT1 tyrosine residues 321, which induces a conformation change in GIT1 that allows protein–protein interactions with FAK. This interaction is important for FAK activation and localization to focal adhesions, and is fundamental to cell migration. Deletion of Y321 in GIT1 (GIT1Y321F) resulted in decreased FAK activation at focal adhesions, with a concomitant reduction in cell spreading and migration (Fig. 8). Over-expression of GIT1Y321F, a mutant that can not interact with FAK, appeared to acts as a dominant negative to block activated FAK localization at focal adhesions. Additional components of the GIT1 signaling complex in focal adhesions likely include PIX, paxillin, and PKL [7].

In summary, we found that Y321 of GIT1 plays an important role in PDGF-induced association with FAK, FAK localization to focal adhesions, and stimulation of osteoblastic cell migration. These findings suggest that GIT1 is an important molecule regulating osteoblastic cell migration and that further research is warranted to elucidate the detailed molecular mechanisms of GIT1–FAK-mediated functions in cytoskeletal dynamics at focal adhesions during osteoblast migration.

Acknowledgment This study was supported by National Natural and Science Foundation (81071481), Talent Person in Medicine Foundation (RC2007059) (all foundations to GY).

References

1. Tsiridis E, Upadhyay N, Giannoudis P (2007) Molecular aspects of fracture healing: which are the important molecular? Injury 38(suppl 1):S11–S25
2. Roelofsen T, Akkers R, Beumer W, Apotheker M, Steeghs I, Van de Ven J, Gelderblom C, Garritsen A, Dechering K (2008) Sphingosine-1-phosphate acts as a developmental stage specific inhibitor of PDGF-induced chemotaxis of osteoblasts. J Cell Biochem 105:1128–1138
3. Gerstenfeld LC, Cullinane DM, Barnes GL et al (2003) Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. J Cell Biochem 88:873–884
4. Mehrotra M, Krane SM, Walters K, Pilbeam C (2004) Differential regulation of platelet-derived growth factor stimulated migration and proliferation in osteoblastic cells. J Cell Biochem 93:741–752
5. Saldana L, Vilaboa N (2010) Effect of micrometric titanium particles on osteoblast attachment and cytoskeleton architecture. Acta Biomater 6:1649–1660
6. Bertolucci CM, Guibao CD, Zheng JJ (2008) Phosphorylation of paxillin LD4 destabilizes helix formation and inhibits binding to focal adhesion kinase. Biochemistry 147:548–554
7. Hoefen R, Berk B (2006) The multifunctional GIT family of proteins. J Cell Sci 119:1469–1475
8. Yin G, Haendeler J, Yan C, Berk BC (2004) GIT1 functions as a scaffold for MEK1-extracellular signal-regulated kinase 1 and 2 activation by angiotensin II and epidermal growth factor. Mol Cell Biol 24:875–885
9. Pang J, Hoefen R, Pryhuber G, Wang J, Yin G, White R, Xu X, O'Dell M, Mohan A, Michaloski H, Massett M, Yan C, Berk B (2009) G-protein-coupled receptor kinase interacting protein-1 is required for pulmonary vascular development. Circulation 119:1524–1532

10. Yin G, Zheng QL, Yan Chen, Bradford CBerk (2005) GIT1 is a scaffold for ERK1/2 activation in focal adhesion. J Biol Chem 280:27705–27712
11. Menon P, Yin G, Smolock EM, Zuscik MJ, Yan C, Berk BC (2010) GPCR kinase 2 interacting protein 1 (GIT1) regulates osteoclast function and bone mass. J Cell Physiol 225:77–85
12. Hu Z, Zhang N, Yin G (2007) Src activation required fir pERK1/2 activation in focal adhesions in osteoblasts induced by platelet-derived growth factor. Zhongguo Xiu Fu chong Jian Wai Ke Za Zhi 21:1179–1183
13. Ilic D, Furuta Y, Kanazawa S, Take N, Sobue K, Nakatsuji N et al (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature 377:539–544
14. Sotobori T, Ueda T, Myoui A, Yoshioka K, Nakasaki M, Yoshikawa H, Itoh K (2006) BMP promotes the hyptotactic migration of murine osteoblastic and osteosarcoma cells by enhancing incorporation of integrin beta 1 into lipid rafts. Exp Cell Res 312:27–38
15. Van Nieuw Amerongen GP, Natarajan K, Yin G (2004) GIT1 mediates thrombin signaling in endothelial cells: role in turnover of RhoA-type focal adhesion. Cir Res 94:1041–1049
16. Daher Zeinab, Noel Josette, Claing Audrey (2008) Endothelin-1 promotes migration of endothelial cells through the activation of Arf6 and the regulation of FAK activity. Cell Signal 20:56–65
17. Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, Parsons JT (1994) Autophosphorylation of the focal adhesion kinase, pp 125FAK, directs SH2-dependent binding of pp60src. Mol Cell Biol 14:1680–1688
18. Schlaepfer DD, Hanks SK, Hunter T, van der Geer P (1994) Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature 372:786–791
19. Brown MC, Cary LA, Jamieson JS, Cooper JA, Turner CE (2005) Src and FAK kinases cooperate to phosphorylate paxillin kinase linker, stimulate its focal adhesion localization, and regulate cell spreading and protrusiveness. Mol Biol Cell 16:4316–4328
20. Zhang Ning Hu, Zhiyi YinGuoyong (2007) Mechanism of G protein coupled receptor kinase interacting protein 1 RNA hairpin inhibiting osteoblasts migration. Chin J Reparative Reconstr Surg 21:1–5
21. Tiscornia G, Singer O, Verma IM et al (2006) Production and purification of lentiviral vectors. J Nat Protoc 1:241–245
22. Walter C, Pabst A, Ziebart T et al (2011) Bisphosphonates affect migration ability and cell viability of HUVEC, fibroblasts and osteoblasts in vitro. Oral Dis 17:194–199
23. Arkowitz RA, Lowe N (1997) A small conserved domain in the yeast Spa2p is necessary and sufficient for its polarized localization. J Cell Biol 138:17–36
24. Webb DJ, Mayhew MW, Kovalenko M, Schroeder MJ, Jeffery ED, Whitmore L, Shabanowitz J, Hunt DF, Horwitz AF (2006) Identification of phosphorylation sites in GIT1. J Cell Sci 15: 2847–2850
25. Nakamura M, Nagai A, Tanaka Y, Sekijima Y, Yamashita K (2010) Polarized hydroxyapatite promotes spread and motility of osteoblastic cells. J Biomed Mater Res A 92:783–790
26. Saura M, Tarin C, Zaragoza C (2010) Recent insights into the implication of nitric oxide in osteoblast differentiation and proliferation during bone development. Sci W J 13:624–632
27. Kim J-B, Leucht P, Cynthia A et al (2007) Reconciling the role of FAK in osteoblast differentiation, osteoclast remodeling, and bone regeneration. Bone 41:39–51
28. Smilenov LB, Mikhailov A, Marcantonio EE (1999) Focal adhesion motility revealed in stationary fibroblasts. Science 286: 1172–1174