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Pyk2 and Src Mediate Signaling to CCL18-Induced Breast Cancer Metastasis

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ABSTRACT

Pyk2 and Src phosphorylation is initiated by CCL18, which promotes breast cancer metastasis via its functional G protein-coupled receptor PITPNM3. However, the function of Pyk2 and Src in CCL18-induced breast cancer metastasis is poorly understood. Quantitative reverse-transcription polymerase chain reactions (qRT-PCRs), Western blot, boyden chamber assay, and adherence assay were performed to delineate the consequences of Pyk2/Src in CCL18-induced breast cancer cells. Co-immunoprecipitation and immunofluorescence were performed to analyze the interaction of proteins. Upon the binding of CCL18 to PITPNM3, Pyk2 translocates from the cytoplasm to the plasma membrane to form a stable complex with PITPNM3, subsequently activating Src kinase. Moreover, upon stimulation with CCL18, Pyk2 and Src become essential for integrin alpha5/beta1 clustering-dependent adherence, migration, and invasion. Pyk2 and Src are important in CCL18-induced breast cancer metastasis. J. Cell. Biochem. 115: 596–603, 2014.

KEY WORDS: CCL18; INTEGRIN; METASTASIS; Pyk2; Src

Tumor metastasis is the main cause for greater than 90% of breast cancer mortalities [Hinton et al., 2008]. Chemokines in the tumor microenvironment play a pivotal role in tumor progression and metastasis. We previously reported that chemokine (C-C motif) ligand 18 (CCL18) from tumor-associated macrophages could promote breast cancer metastasis via its functional receptor PITPNM3. We also determined that CCL18 could specifically bind to PITPNM3 on the plasma membrane of breast cancer cells and significantly stimulate calcium signaling, thus inducing the migration of PITPNM3 transfectants. PITPNM3 suppression abrogates these effects, suggesting that PITPNM3 is the functional G protein-coupled receptor (GPCR) for CCL18 [Chen et al., 2011].

The human rdgB homolog PITPNM3, which encodes a protein that belongs to the phosphatidylinositol transfer protein (PITP) family [Lev, 2001], is also known as Pyk2 N-terminal domain-interacting receptor 1 (Nir1), which binds to the amino-terminal domain of proline-rich tyrosine kinase 2 (Pyk2) via a conserved carboxy-terminal sequence motif [Lev et al., 1999]. The CCL18 chemokine markedly induces Pyk2 phosphorylation at Tyr402 in breast cancer cells, while PITPNM3 silencing abrogates this phosphorylation [Chen et al., 2011].

Pyk2 (which is also known as RAFTK, CAKb, and CADTK), a member of the focal adhesion kinase (FAK) non-receptor tyrosine kinase family, is a cytoplasmic tyrosine kinase that regulates cell adhesion, morphology, migration, and invasion [Du et al., 2001;

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Lakkakorpi et al., 2003], and it is highly expressed in several types of cancers including breast cancer [Zrihan-Licht et al., 2000; Gutenberg et al., 2004; Iiizumi et al., 2008; Cao et al., 2012]. Clinical studies have revealed that Pyk2 overexpression, which leads to its activation via translocation and phosphorylation [Sun et al., 2008; Bartos et al., 2010], is strongly associated with tumor metastasis and the poor prognosis of patients [Sun et al., 2007; Iiizumi et al., 2008; Cao et al., 2012; Wendt et al., 2012]. Pyk2 is activated by a variety of GPCRs [Dikic et al., 1996; Litvak et al., 2000] and inflammatory cytokines that elevate intracellular calcium concentrations [Dikic and Schlessinger, 1998]. In its active form, Pyk2 is phosphorylated at Tyr402, translocates from the cytoplasm to the plasma membrane [Cheung et al., 2009] and forms a complex with Src by binding to the Src SH2 domain to activate Src at Tyr416 [Park et al., 2004; Sun et al., 2008]. Thus, the Pyk2/Src complex may constitute a molecular memory mechanism that is important for signaling pathway-mediated tumor metastasis.

However, it is unclear whether the binding of CCL18 and PITPNM3 can efficiently trigger Pyk2 translocation and phosphorylation to activate Src to mediate downstream signaling in breast cancer cells. In this study, we aimed to investigate the contribution of Pyk2 and Src to CCL18-induced breast cancer metastasis.

MATERIALS AND METHODS

CELL CULTURES AND TREATMENT

MDA-MB-231 and MCF-7 breast cancer cells and HEK293 embryonic kidney cells were obtained from American Type Culture Collection (ATCC). The MDA-MB-231 and HEK293 cells were maintained in DMEM (Hyclone, Beijing, China) that was supplemented with 10% fetal bovine serum (FBS, Hyclone). For chemokine treatment, breast cancer cells were exposed to 20 ng/ml CCL18 (Peprotech, Princeton, USA) or CCL20 (Peprotech) for 1 h.

TRANSFECTION WITH PLASMIDS AND SIRNA

PITPNM3 cDNA was cloned into the *Kpn*I–*Xba*I sites of the pcDNA3 vector (Invitrogen, Germany) and confirmed by DNA sequencing as previously described [Chen et al., 2011]. HEK293 cells were transfected with the pcDNA3-PITPNM3 or pcDNA3 vectors using Lipofectamine 2000 (Invitrogen) in serum-free OPTI-MEM (Invitrogen) according to the manufacturer's instructions. All siRNAs were synthesized using 2′-O-ACE phosphoramidites (Dharmacon Research, USA). Cells were transfected with specific siRNA duplexes (GenePharma, Shanghai, CH) using the NeoFX Transfection Agent (Ambion, Foster, CA) according to the manufacturer's instructions. The sense and anti-sense sequences of the siRNAs were shown in Table I.

QUANTITATIVE REAL-TIME PCR (QRT-PCR)

The total RNA was extracted using TRIzol (Invitrogen), according to the manufacturer's instructions. Real-time PCR was performed with a Light Cycler480 system (Roche Diagnostics, Switzerland) using a SYBR Premix ExTaq kit (Takara, Dalian, CH). The oligonucleotide sequences of qRT-PCR primers are listed in Table II.

WESTERN BLOT

Protein extracts were resolved by 10% SDS–PAGE, transferred to PVDF membranes (Roche, USA), and probed with antibodies directed against human Pyk2, Src, FAK and the corresponding phosphorylated proteins (1:1,000, CST, USA), E-cadherin (1:1,000, CST), vimentin (1:1,000, R&D, USA), and GAPDH (1:10,000, Invitrogen). Peroxidase-conjugated secondary antibodies (1:3,000, CST) were used, and the reactions were visualized by enhanced chemiluminescence (Thermo, Rockford, USA).

BOYDEN CHAMBER ASSAY

The migration and invasion of breast cancer cells was examined using 24-well Boyden chambers (Corning, USA) with 8-M inserts coated with fibronectin (Roche) and Matrigel (invasion only) as previously reported [Cheng et al., 2007]. Cells (10^5 cells/well) were plated on the upper cell culture inserts with 0.2% BSA in serum-free DMEM, and the same medium was added to the lower chambers in the presence of PBS, CCL20, or CCL18. After 6 h (migration) or 8 h (invasion) of culture at 37°C, the migrating or invading cells that crossed the inserts were stained with crystal violet (0.005%, Sigma, USA), captured (200×) and counted.

TABLE I. The Sense and Anti-Sense Sequences of the siRNAs

siRNA	Sense	Anti-sense
Pyk2 siRNA-1	GGAGACCUACCGCUGUGAACUCAUU	AAUGAGUUCACAGCGGUAGGUCUCC
Pyk2 siRNA-2	GAUGUGGUCCUGAAUCGUATT	UACGAUUCAGGACCACAUCTT
Pyk2 siRNA-3	GGCAAAUCCUGGACAAACATT	UGUUUGUCCAGGAUUUGCCTT
Src siRNA-1	CAAGAGCAAGCCCAAGGAUTT	AUCCUUGGGCUUGCUCUUGTT
Src siRNA-2	AGUGAGACCACGAAAGGUGTT	CACCUUUCGUGGUCUCACUTT

TABLE II. Oligonucleotide Sequences of the qRT-PCR Primers

Gene	Forward primer	Reverse primer
Pyk2	5′-GCTAGACGGCAGATGAAAGT-3′	5′-AAGCAGACCTTGAGGATACG-3′
Src	5′-CATCCA AGCCTCAGACCC A-3′	5′-TGACACCACGGCATACAGC-3′
GAPDH	5′-ATCACCATCTTCCAGGAGCGA-3′	5′-CCTTCTCCATGGTGGTGAAGAC-3′

ADHERENCE ASSAY

The adherence of breast cancer cells to fibronectin was evaluated using culture plates that were precoated with fibronectin overnight. MDA-MB-231 cells (1×10^6 cells/ml) were transfected with Pyk2 or Src siRNAs. The cells were then suspended and treated with PBS, CCL20, or CCL18 for 1 h and allowed to adhere to the bottom of the plate at 37°C for 10 min. After the non-adherent cells were removed, the adherent cells were stained with crystal violet (0.005%, Sigma), captured (100×) and counted.

CO-IMMUNOPRECIPITATION

HEK293 cells were either untransfected or transfected with vector or c-Myc-tagged PITPNM3 for 24 h and then treated with or without CCL18 for 1 h. Proteins were extracted from the plasma membrane with a Mem-PER Eukaryotic Membrane Protein Extraction Kit (Thermo) and incubated with anti-c-Myc agarose (Thermo) overnight at 4°C. Following the incubation, the protein conjugates were centrifuged and resuspended in non-reducing sample buffer (Thermo). The co-immunoprecipitates were analyzed by Western blot using primary antibodies directed against the c-Myc-tag (1:1,000, CST) or Pyk2. Each panel shows representative data from one of three experiments with similar results.

IMMUNOFLUORESCENCE

For immunofluorescence staining, cells were incubated with antibodies directed against vimentin (1:50), E-cadherin (1:100), CCL18 (1:50, R&D), PITPNM3 (1:100, Santa Cruz, USA), Pyk2 (1:50), or integrin alpha5/beta1 (1:100, Chemicon, Int., CA). This step was followed by incubation with Alexa Fluor 488/555/594/647-conjugated secondary antibodies (1:500, Invitrogen). Cells on coverslips were counterstained with DAPI and imaged by confocal laser-scanning microscopy (LSM710, Germany) with a core data acquisition system (Zen 2008 Light Edition).

STATISTICAL ANALYSIS AND IMAGE PROCESSING SOFTWARE

All statistical analyses were performed using SPSS for Windows version 16.0 (SPSS, Chicago). A *t*-test was used to analyze the differences in the cell counts with or without CCL18 treatment and RNAi. All experiments for cell culture were independently performed at least three times. A P-value < 0.05 was considered statistically significant in all cases. Canvas 14 and Photoshop 7.0 were used for image gathering and processing manipulations.

RESULTS

PYK2 IS NECESSARY FOR CCL18-MEDIATED SRC ACTIVATION IN BREAST CANCER CELLS

Our previous study showed that CCL18 markedly induced the activation of Pyk2 and Src in breast cancer cells [Chen et al., 2011]. To examine whether Pyk2 was essential for the activation of Src upon CCL18 stimulation, we employed Pyk2-siRNAs and Src-siRNAs to reduce the mRNA and protein level of Pyk2 and Src in MDA-MB-231 cells, respectively (Suppl. Fig. 1A,B). Compared with control cells treated with PBS or CCL20, the phosphorylation of Pyk2 at Tyr402 and Src at Tyr416 was remarkably induced by CCL18 in MDA-MB-231 cells without altering their total protein levels. We also found that transfection with either of the two Pyk2 siRNAs significantly abrogated the phosphorylation of Src that was induced by CCL18, whereas the total protein level of Src remained unchanged (Fig. 1A). However, the suppression of Src by either of the two Src siRNAs had no effect on the CCL18-induced phosphorylation of Pyk2 (Fig. 1B), suggesting that Pyk2 and Src were sequentially activated by CCL18 with Pyk2 acting upstream of Src.

UPON STIMULATION BY CCL18, PYK2, AND SRC ARE REQUIRED FOR THE INTEGRIN ALPHA5/BETA1 CLUSTERING-DEPENDENT ADHERENCE OF BREAST CANCER CELLS

Pyk2 and Src are associated with integrin activation, which enhances the adhesion of cancer cells to the extracellular matrix (ECM) [Duong et al., 2001; Frame, 2002; Bianchi-Smiraglia et al., 2012]. Our previous study demonstrated that CCL18 mainly affects integrin alpha5/beta1 by inducing its clustering in breast cancer cells [Chen et al., 2011]. Thus, we next analyzed the function of Pyk2 and Src in CCL18-stimulated integrin alpha5/beta1 clustering. Western blot analyses of MDA-MB-231 cells demonstrated that CCL18-dependent, CCL20-independent FAK phosphorylation was inhibited by Pyk2 or Src silencing, whereas the total protein levels of FAK and integrin-beta 1 remained unchanged (Fig. 2A,B).

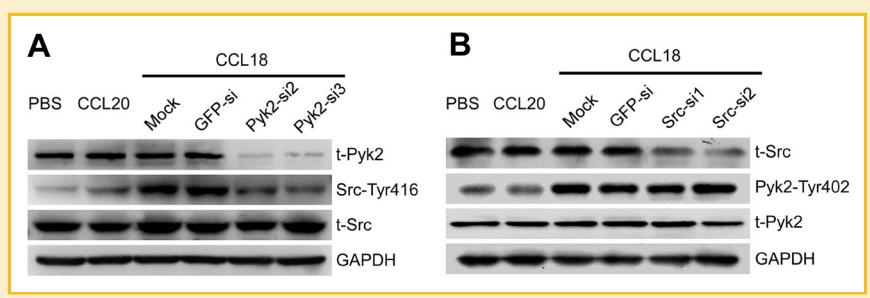

Fig. 1. Pyk2 is necessary for CCL18-mediated activation of Src in breast cancer cells. A: Western blot of total-Pyk2, Src-Tyr416, and total-Src expression in MDA-MB-231 cells transfected with Pyk2 siRNA for 48 h and treated with PBS, CCL20, or CCL18 for 1 h. B: Western blot of total-Src, Pyk2-Tyr402, and total-Pyk2 expression in MDA-MB-231 cells transfected with Src siRNA for 48 h and treated with PBS, CCL20, or CCL18 for 1 h. GAPDH was used as a loading control. The experiment was performed three times, and a representative blot is shown.

Immunofluorescence staining with an anti-integrin alpha 5/beta 1 antibody demonstrated prominent integrin alpha5/beta1 clustering on the membranes of CCL18-treated MDA-MB-231 cells but not those treated with CCL20. The loss of Pyk2 or Src expression by RNAi efficiently inhibited CCL18-induced integrin alpha5/beta1 clustering (Fig. 2C). More importantly, the adherence of breast cancer cells treated with CCL18 increased by nearly 17-fold compared with those treated with PBS (Fig. 3A, $P < 0.01$), whereas CCL20 treatment had no effect (Fig. 3A, $P > 0.05$). However, In the presence of CCL18, transfection with Pyk2 or Src siRNAs tremendously decreased the number of adherent cells to a level comparable with that of cells without CCL18 treatment (Fig. 3A, $P > 0.05$). Collectively, these data suggest that the integrin alpha5/beta1 clustering-dependent adherence mediated by CCL18-induced FAK activation requires the expression of Pyk2 and Src.

PYK2 AND SRC MEDIATE THE CCL18-INDUCED MIGRATION AND INVASION OF BREAST CANCER CELLS

Because the involvement of Pyk2 and Src in the migration, invasion and adherence of tumor cells has been reported [Sieg et al., 1998; Genua et al., 2013] together with evidence of the CCL18-dependent activation of Pyk2 and Src, we further determined whether the silencing of Pyk2 or Src could influence the biological effects of CCL18. Using Boyden chambers, the number of migrating and invading cancer cells treated with CCL18 increased sevenfold (Fig. 3B, $P < 0.01$) and sixfold (Fig. 3C, $P < 0.01$), respectively, compared with MDA-MB-231 cells treated with PBS, whereas CCL20 treatment had no effect (Fig. 3B,C, $P > 0.05$). In the presence of CCL18, the suppression of Pyk2 or Src significantly decreased the number of migrating and invading cells to a level comparable to that of cells without CCL18 treatment (Fig. 3B,C; $P > 0.05$). These results indicate that the suppression of Pyk2 or Src inhibits the CCL18-induced migration and invasion of breast cancer cells.

CCL18-MEDIATED PITPNM3 STIMULATION RESULTS IN PYK2 ACTIVATION

Because CCL4-mediated CCR5 activation could trigger Pyk2 translocation from the cytoplasm to the plasma membrane to form a multimolecular complex with PI3K and Lyn (a member of the Src kinase family) [Cheung et al., 2009], we next analyzed whether Pyk2 could translocate and cluster on the plasma membrane of breast cancer cells upon stimulation with CCL18. Confocal microscopy with triple fluorescence-labeled antibodies for Pyk2, CCL18, and PITPNM3 demonstrated that, in the absence of CCL18, Pyk2 was present in MDA-MB-231 cells in a diffuse pattern throughout the cytoplasm, while PITPNM3 was present on the plasma membrane (Fig. 4A, left). After pretreating the cells with CCL18 for 30 min, CCL18 colocalized with PITPNM3 on the plasma membrane of MDA-MB-231 cells, whereas Pyk2 translocated from the cytoplasm to the membrane and exhibited "podosome-like" structures at the periphery of the cells (Fig. 4A, middle).

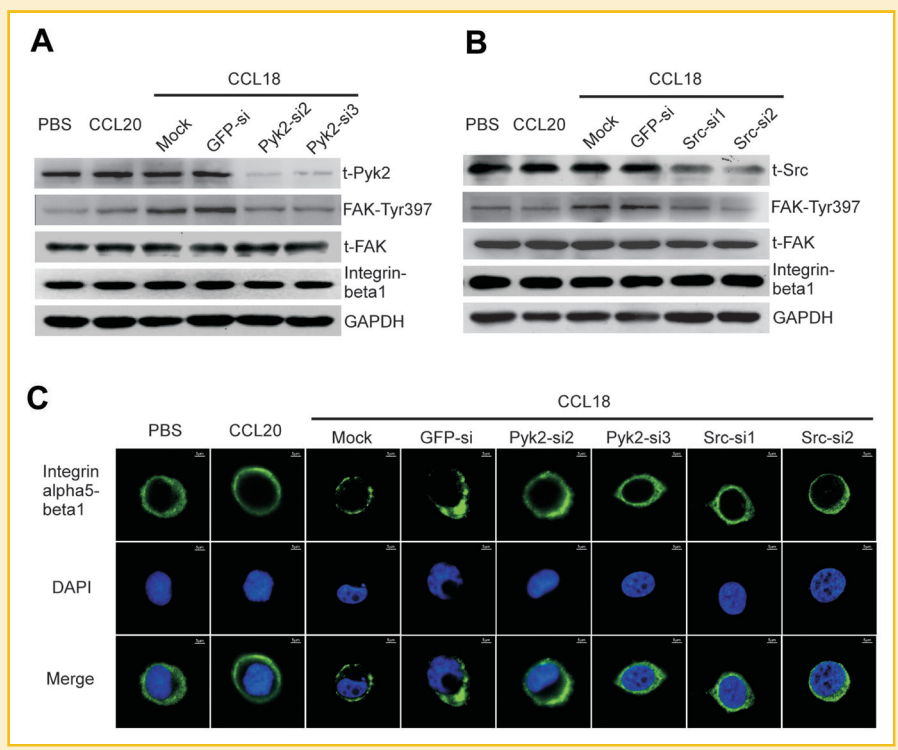

Fig. 2. Pyk2 and Src are required for CCL18-dependent integrin clustering. MDA-MB-231 cells were transfected with Pyk2 or Src siRNA for 48 h and treated with PBS, CCL20, or CCL18 for 1 h. A,B: Western blot analysis for the expression of FAK-Tyr397, total-FAK, and integrin-beta 1 in cells silenced for Pyk2 (A) or Src (B) expression. GAPDH was used as a loading control. C: Immunofluorescence analyzing integrin (green) clustering using an anti-integrin-alpha 5/beta 1 antibody. The nuclei were visualized with DAPI (blue). Bar, 5 μm. Each panel shows representative data from one of three experiments with similar results.

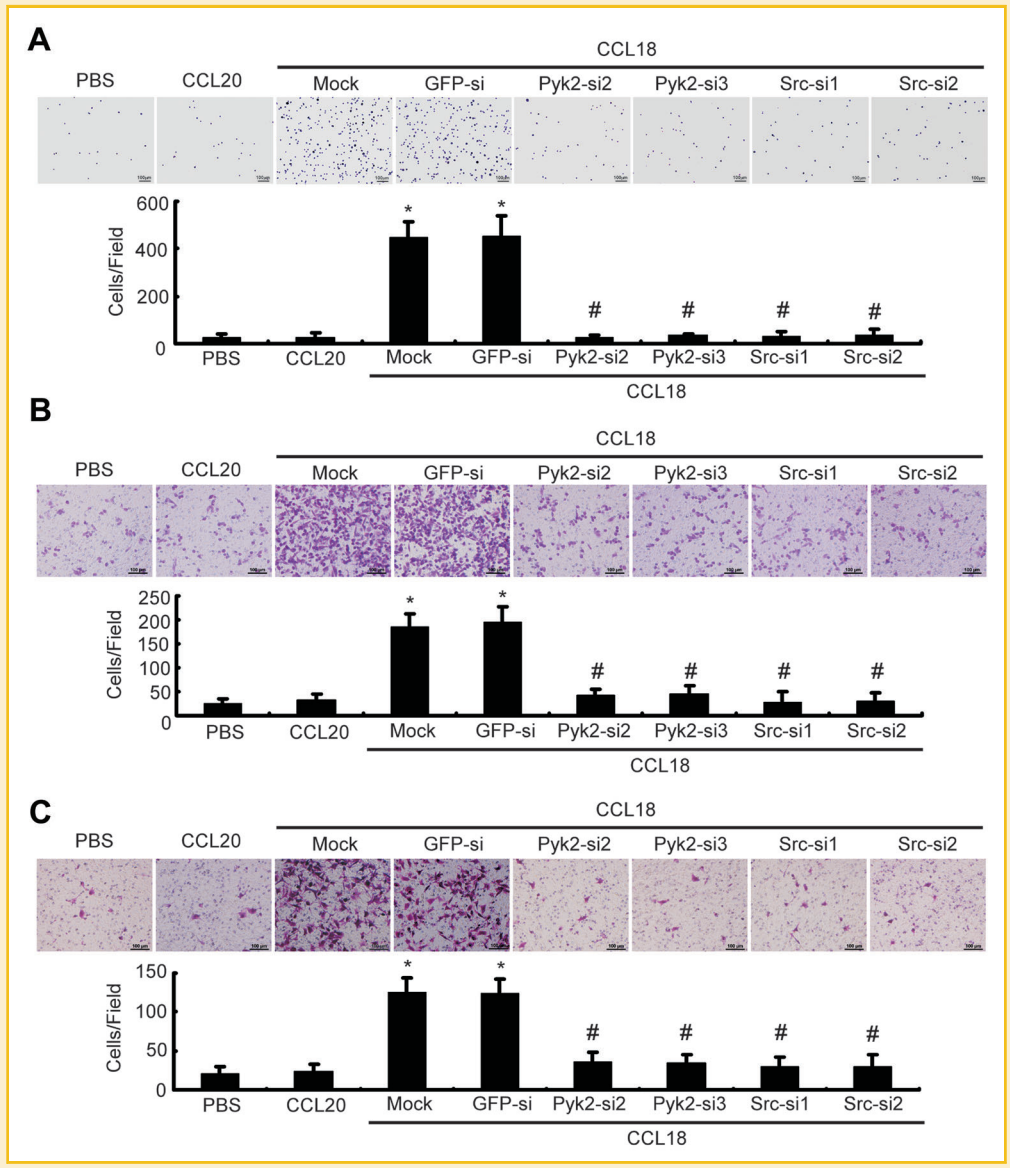

Fig. 3. Pyk2 or Src silencing inhibited the breast cancer adherence, migration and invasion promoted by CCL18. MDA-MB-231 cells were transfected with Pyk2 or Src siRNAs for 48 h. A: Adherence assay for transfected MDA-MB-231 cells with or without CCL18 treatment as indicated. The adherent cells were stained, counted and captured (100×). B: Boyden chamber assay for cells on the upper inserts with or without CCL18 in the lower chambers for 6 h. The cells that migrated were stained, counted and captured (200×). C: Boyden chamber assay for cells on the upper inserts with matrigel in the absence or presence of CCL18 in the lower chambers for 8 h. The invading cells were stained, counted and captured (200×). Bar, 100 μm. The bars correspond to the mean ± SD. Statistically significant differences were tested using a t test. $^{*}P < 0.01$ and $^{\#}P > 0.05$ compared with cells without CCL18 treatment. Each panel shows representative data from one of three experiments with similar results.

In addition, PITPNM3 suppression by siRNA resulted in the loss of Pyk2 translocation and aggregation on the cell surface (Fig. 4A, right), confirming the essential role of PITPNM3 in Pyk2 activation.

Because the activated Pyk2 clusters and colocalizes with PITPNM3 in response to CCL18, we further tested whether PITPNM3 was recruited and formed a complex with Pyk2 on the membrane of cells. A vector containing c-Myc-tagged PITPNM3 was transfected into HEK293 cells for 24 h, and empty pcDNA3 vector transfectants were used as a control. The membrane proteins were extracted from the cells, which were treated with or without CCL18 for 30 min, subjected to immunoprecipitation with c-Myc-tag antibody-coupled beads and immunoblotted with c-Myc-tag and Pyk2 antibodies. Specific c-Myc-tagged PITPNM3 bands were identified in membrane lysates from PITPNM3 transfectants but not in untransfected or empty vector transfected cells (Fig. 4B, upper), confirming that the vector transfections were specific and efficient. Interestingly, more Pyk2 was observed among the membrane proteins of CCL18-treated PITPNM3 transfectants compared with those without CCL18 treatment, confirming the translocation of Pyk2. Notably, we demonstrated that Pyk2 co-immunoprecipitated with c-Myc-tagged PITPNM3 (Fig. 4B, lower) upon CCL18 stimulation. These results suggest that the CCL18-PITPNM3 interaction promotes Pyk2

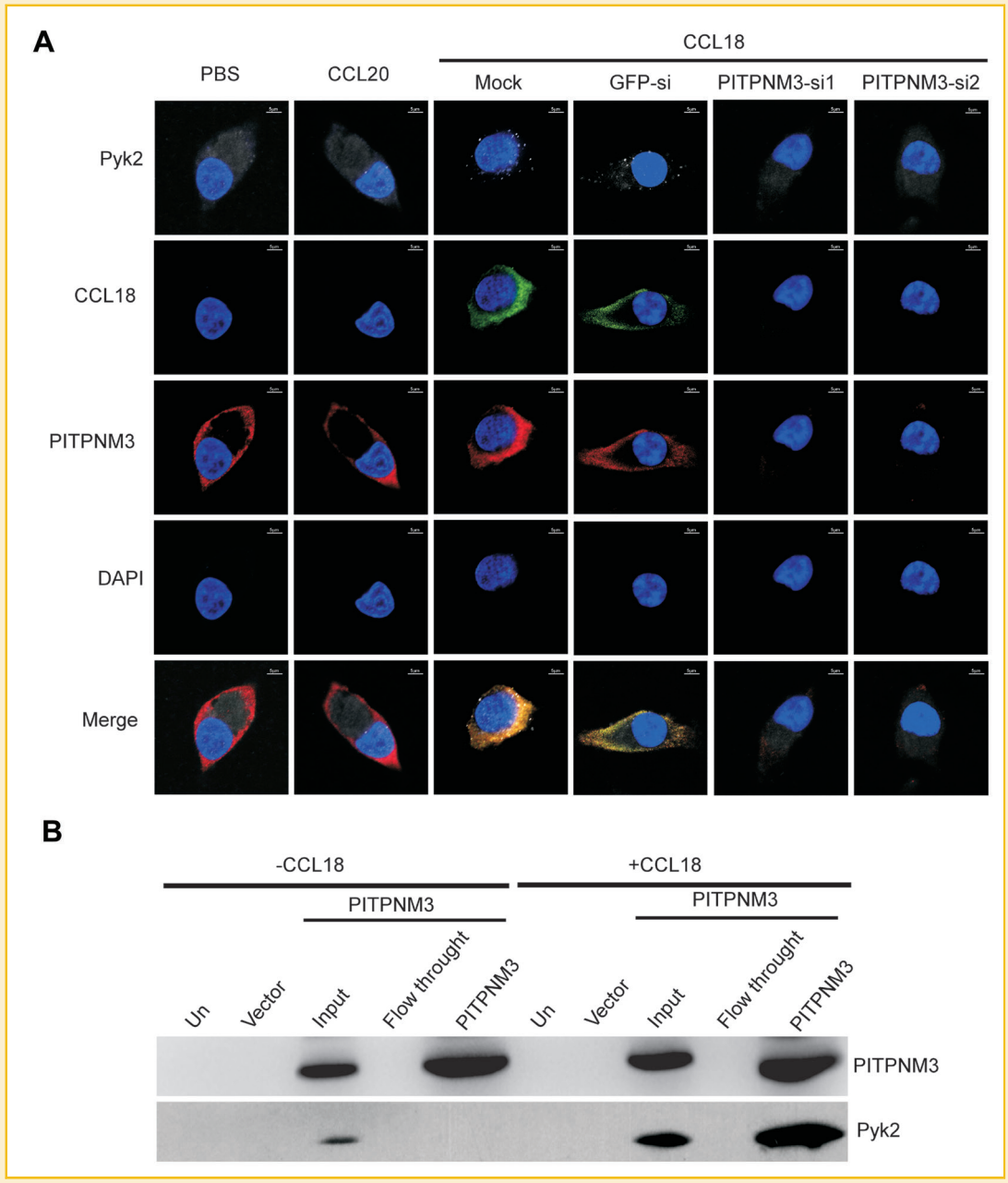

Fig. 4. CCL18-mediated PITPNM3 stimulation results in Pyk2 activation. A: Immunofluorescence of triple stained with mouse anti-CCL18, goat anti-PITPNM3, and rabbit anti-Pyk2 plus an Alexa Fluor 488/594/647-conjugated secondary Ab. Colocalization of Pyk2 (white), CCL18 (green), and PIPTNM3 (red) is indicated in the overlaid images. The nuclei were visualized with DAPI (blue). Bar, 5 μm. B: Interaction between Pyk2 and PITPNM3 in CCL18-induced PITPNM3 HEK293 transfectants. HEK293 cells were untransfected, transfected with vector or c-Myc-tagged PTIPNM3 in the absence or presence of CCL18. Membrane proteins were extracted for co-immunoprecipitation. Pyk2 was detected in the PITPNM3 immunoprecipitates upon stimulation with CCL18. Each panel shows representative data from one of three experiments with similar results.

translocated and forms a stable complex with PITPNM3, which subsequently mediates the CCL18 intracellular signaling pathway.

DISCUSSION

We previously reported that CCL18 could remarkably induce the phosphorylation of Pyk2 and Src via PITPNM3; however, the function of Pyk2 and Src in CCL18-stimulated breast cancer metastasis remained unknown. In this study, upon stimulation with CCL18, Pyk2 translocated and formed a complex with PITPNM3 and subsequently activated Src kinase. Moreover, we also provide evidence that, upon stimulation with CCL18, both Pyk2 and Src become essential to integrin alpha5/beta1 clustering-dependent adherence, migration, and invasion.

Recent studies demonstrated that Pyk2 upregulates *N*-methyl-D-aspartate receptor (NMDAR) function by activating Src upon stimulation with lysophosphatidic acid (LPA), heregulin (HRG), and long-term potentiation (LTP) [Duong et al., 1998; Zrihan-Licht

et al., 2000; Huang et al., 2001]. Consistent with these reports, we found that RNAi-induced Pyk2 knockdown resulted in the inhibition of CCL18-induced Src activation, while the suppression of Src had no effect on Pyk2. The inhibition of either Pyk2 or Src prevented the CCL18-induced activation of FAK and integrin alpha5/beta1 clustering. Our data implicate Pyk2 in the initiation of CCL18 signaling, which leads to the activation of Src and FAK and integrin alpha5/beta1 clustering.

It has been reported that the intensity of Pyk2 at Tyr402 is positively correlated with its localization, which affects its functional role as an adapter for the transduction of extracellular signals [Sun et al., 2008]. CCL4-mediated CCR5 activation triggers Pyk2 translocation from the cytoplasm to plasma membrane to form a multi-molecular complex with PI3K and Lyn, a member of the Src kinase family, leading to the activation of Pyk2 and downstream substrates [Cheung et al., 2009]. However, the suppression of CCR5 prevents Pyk2 translocation and phosphorylation [Cheung et al., 2008, 2009]. Similar findings have been reported regarding the chemokine-induced and GPCR-mediated translocation and activation of Pyk2, which results in enhanced migration and invasion [Andreev et al., 1999; McShan et al., 2002; Zrihan-Licht et al., 2000; Tse et al., 2013; Yang et al., 2013]. Consistent with these reports, our results suggest that Pyk2 translocation and the coordinated activation of Pyk2 and Src are initiated in response to CCL18 and PITPNM3 stimulation.

A previous study demonstrated that the exogenous overexpression of PITPNM3 was associated with exogenous Pyk2 expression on the surface of HEK293 cells [Lev et al., 1999], which is likely a result of autophosphorylation due to the relatively high expression of Pyk2 [Sun et al., 2008]. In our study, we focused on endogenous Pyk2 expression rather than an overexpression system to exclude the possibility of autophosphorylation induced by overexpression. The immunofluorescence staining and immunoprecipitation experiments revealed increased Pyk2 translocation to the plasma membrane and the formation of a stable complex between Pyk2 and PITPNM3 upon stimulation with CCL18, suggesting that Pyk2 translocation and activation are induced by the interaction between CCL18 and PITPNM3. This process is crucial for breast cancer metastasis.

CONCLUSION

In summary, our data suggest that Pyk2 and Src may represent novel biomarkers with diagnostic and prognostic significance for CCL18-induced breast cancer metastasis, which may lead to the development of new therapies directed against breast cancer using small-molecule inhibitors of Pyk2 or Src.

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