pISSN 1229-845X, eISSN 1976-555X
J. Vet. Sci. (2014), **15**(1), 111-115
http://dx.doi.org/10.4142/jvs.2014.15.1.111
Received: 2 Sep. 2013, Revised: 25 Oct. 2013, Accepted: 23 Nov. 2013

JOURNAL OF
Veterinary Science

Original Article

Mouse Fyn induces pseudopodium formation in Chinese hamster ovary cells

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Molecular mechanisms underlying the effects of Fyn on cell morphology, pseudopodium movement, and cell migration were investigated. The Fyn gene was subcloned into pEGFP-N1 to produce pEGFP-N1-Fyn. Chinese hamster ovary (CHO) cells were transfected with pEGFP-N1-Fyn. The expression of Fyn mRNA and proteins was monitored by reverse transcription-PCR and Western blotting. Additionally, transfected cells were stained with 4′,6-diamidino-2-phenylindole and a series of time-lapse images was taken. Sequences of the recombinant plasmids pMD18-T-Fyn and pEGFP-N1-Fyn were confirmed by sequence identification using National Center for Biotechnology Information in USA, and Fyn expression was detected by RT-PCR and Western blotting. The morphology of CHO cells transfected with the recombinant vector was significantly altered. Fyn expression induced filopodia and lamellipodia formation. Based on these results, we concluded that overexpression of mouse Fyn induces the formation of filopodia and lamellipodia in CHO cells, and promotes cell movement.

Keywords: filopodia, Fyn, lamellipodia, time-lapse

Introduction

To adapt to the extracellular environment, cells change shape and form membrane extensions. Regulation of the cytoskeleton plays an important role in cancer progression and cell migration [6]. Most neurons must participate in the formation of synaptic connections after migration [1,5]. Fyn, a member of the Src tyrosine family kinases (SFKs), is associated with cell migration and movement, and interacts with other nuclear receptors. Previous studies showed that activator SFKs were considered to involve in cell migration and cell movement during the process of human disease, especially during cancer progression [8,9]. Therefore, it is crucial to elucidate the mechanism underlying the effect of Fyn on cell migration, cell motility, and pseudopodium movement.

SFKs are widely found in tissues and cells, and interact with major molecules involved in cell pseudopodium formation and migration. Pseudopodium-enriched atypical kinase 1 (PEAK1), a potential target for anticancer therapy, regulates Fyn-induced tyrosine phosphorylation in migrating cells [2,15]. Absence of Fyn results in an abnormal phenotype in mice, indicating that Fyn is an indispensable factor for cellular migration. During cell migration, phosphorylation of cofilin combined with Fyn, Akt, and Rho-family GTPases regulate cell adhesion [7]. Fyn-induced phosphorylation of Dab1 exhibited that its distinguishing expression in different tissues and organs, which resulted to different intracellular factors entranced into negative feedback and regulated corresponding physiological functions, respectively [10]. Fyn serine phosphorylation regulates the activity of tyrosine kinase and target spot adhesion [16] necessary for cell movement. These data indicated that Fyn is involved in cell migration and mobility, but molecular mechanisms underlying cellular movement promoted by Fyn remain unclear.

In the present study, mouse Fyn induced pseudopodium formation *via* filopodia and lamellipodia generation. Little information has been published about real-time changes of lamellipodia during pseudopodium formation induced by Fyn. In the present study, we constructed a recombinant vector encoding the Fyn gene to investigate the impact of Fyn activity on filopodia and lamellipodia formation using fluorescence staining and time-lapse image analysis. Results of our investigation indicate that Fyn helps regulate cell morphology, cell migration, and cancer progression.

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Materials and Methods

Reagents and antibodies

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and F12K medium were purchased from Gibco (USA). X-tremeGENE HP was purchased from Roche (Switzerland). Restriction enzymes *EcoR* I and *Sma* I were purchased from New England Biolabs (USA). RevertAidTM First Strand cDNA Synthesis Kit and ECL substrate kit were purchased from Fermentas (USA). Mouse polyclonal anti-Fyn antibody, anti-GAPDH antibody, and anti-mouse secondary antibody conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology (USA).

Construction of the overexpression vector

Total mRNA of brain (embryo 18 days) was isolated from Kunming mice (Xi'an Jiao Tong university, China) using a Trizol kit according to the manufacturer's instructions from the Invitrogen (USA). The Fyn gene was isolated from mouse mRNA by reverse transcription PCR (RT-PCR) using Revert Aid First Strand cDNA Synthesis Kit according to the manufacturer's instructions from the Fermentas (USA). The PCR product was inserted into a pMD18-T-simple vector (Takara Bio, Japan). Sequences of the PCR primers used in the experiment were (restriction enzyme sites are underlined) Fyn-F: 5′-CGGAATTCATG GGCTGTGTGCAA-3′, Fyn-R: 5′-TCCCCCGGGCCAG GTTTTCACCGG-3′, GAPDH-F: 5′-AGCGAGACCCCA CTAACA-3′, and GAPDH-R: 5′-ATGAGCCCTTCCAC AATG-3′. The PCR product was digested with *EcoR* I and *Sma* I, and ligated into a pEGFP-N1 vector (Clontech; Takara Bio) to produce the recombinant expression vector pEGFP-N1-Fyn.

Cell culture and transfection

Chinese hamster ovary (CHO) cells (Shanghai Institute of Cell Bank, China) were cultured in DMEM/F12K medium containing 10% FBS supplemented with penicillin (50 U/mL, Gibco) and streptomycin (50 U/mL, Gibco) at 37°C in a humidified atmosphere with 5% CO_2. Transient transfection was carried out using X-tremeGENE HP according to the manufacturer's instructions. The transfected cells were cultured in DMEM/F12K medium at 37°C in a humidified atmosphere with 5% CO_2.

RT-PCR and Western blot analysis

The CHO Cells were collected 24 h after transfection for RT-PCR or after 48 h for Western blotting using the RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS). The level of mRNA was normalized to that of GAPDH (RNA measure was preformatted using spectrophotometer; Shimadzu, Japan). PCR was conducted at 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 57°C for 30 sec (Fyn, 90 sec), and 72°C for 1 min. Cellular protein was extracted in lysis buffer (50 mM Tris-HCl, 0.5% Triton X-100, 2 mM EDTA, and 150 mM NaCl; pH 7.3) with 1 mM phenylmethanesulfonyl fluoride. Western blotting for Fyn and GAPDH was performed as previously described [4].

Immunohistochemistry and time-lapse imaging

The CHO Cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and counterstained with 4′,6-diamidino-2-phenylindole, (1 : 500, Millipore, USA). We used a strategy that allowed us to monitor active pseudopodia extension. Using a live cell culture system (Nikon A1; Nikon, Japan), we evaluated pseudopodia movement 12 h after transfection. The cell were continuously photographed up to 22 times per 10 min.

Results

The recombinant plasmid pEGFP-N1-Fyn was successfully generated

Using RT-PCR, the GAPDH gene was cloned from

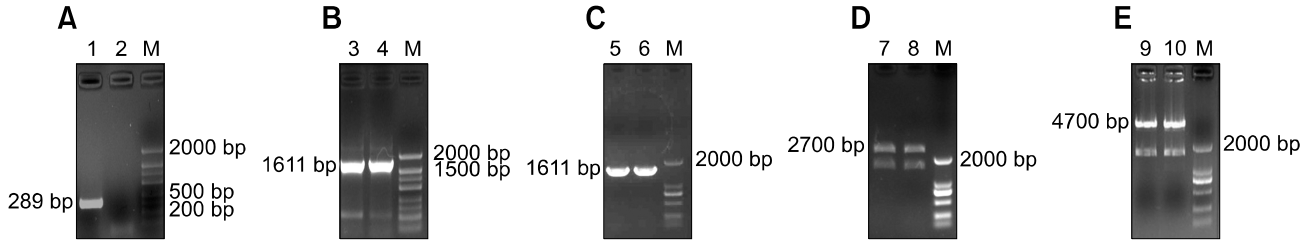

Fig. 1. Identification of the recombinant plasmid. (A) Detection of the housekeeping gene GAPDH. (B) Identification of the target gene by RT-PCR. (C) PCR product of the Fyn gene was subcloned into pMD18-T-Fyn and pEGFP-N1-Fyn. (D) Identification of pMD18-T-Fyn fragments produced by restriction enzyme digestion with *Eco*R I and *Sma* I. (E) Identification of pEGFP-N1-Fyn fragments generated by restriction enzyme digestion with *Eco*R I and *Sma* I. Lane 1, RT-PCR GAPDH product; Lane 2, negative control; Lane 3, RT-PCR Fyn product from brain; Lane 4, positive control; Lane 5, PCR product of pMD18-T-Fyn; Lane 6, PCR product of pEGFP-N1-Fyn; Lanes 7 and 9, positive control; Lanes 8 and 10, recombinant plasmid identification by digestion with *EcoR* I and *Sma* I (pMD18-T-Fyn, 2700 bp; pEGFP-N1, 4700 bp; Fyn, 1611 bp); Lane M, DNA marker.

mouse brain and appeared as a 289 bp band (Fig. 1A). The gene encoding Fyn was also cloned and generated a 1611 bp band (Fig. 1B). The Fyn gene was inserted into the pMD18-T-simple and pEGFP-N1 plasmids. The plasmids were digested with *EcoR* I and *Sma* I (Figs. 1D and E) to verify that pEGFP-N1-Fyn was successfully constructed. Furthermore, BLAST (National Center for Biotechnology Information, USA) searches of sequencing results demonstrated that the PCR product sequence perfectly matched (100%) that of Fyn (GenBank accession No. 001122893.1).

Transfection with pEGFP-N1-Fyn leads to Fyn overexpression

Transcription and expression of Fyn in CHO cells were identified with RT-PCR and Western blotting. RT-PCR was used to detect the mRNA expression of the target gene. As shown in Fig. 2A, the mRNA transcription of Fyn in cells transfected with pEGFP-N1-Fyn was significantly enhanced. In contrast, no Fyn mRNA expression was detected in wild-type cells or ones transfected with pEGFP-N1 (Fig. 2B). These results indicated that transfection with pEGFP-N1-Fyn led to the overexpression of Fyn compared to the control cells.

Overexpression of Fyn influences cell morphology

To identify changes in cell morphology spatially regulated by Fyn, we monitored filopodia and lamellipodia formation. Fig. 2C presents the morphological change of CHO cells transfected with the recombinant plasmid pEGFP-N1-Fyn. Results of this experiment showed that green fluorescent protein (GFP) was expressed in the control group (GFP group) and Fyn group (GFP-Fyn, fusion protein group). Interestingly, Fyn induced the formation of filopodia and lamellipodia in the cell membrane. Hundreds of filament-like protrusions from the membrane to the outside radial were observed with fluorescence microscopy (Figs. 2A and B). These findings indicated that Fyn induced the formation of filopodia and lamellipodia in CHO cells.

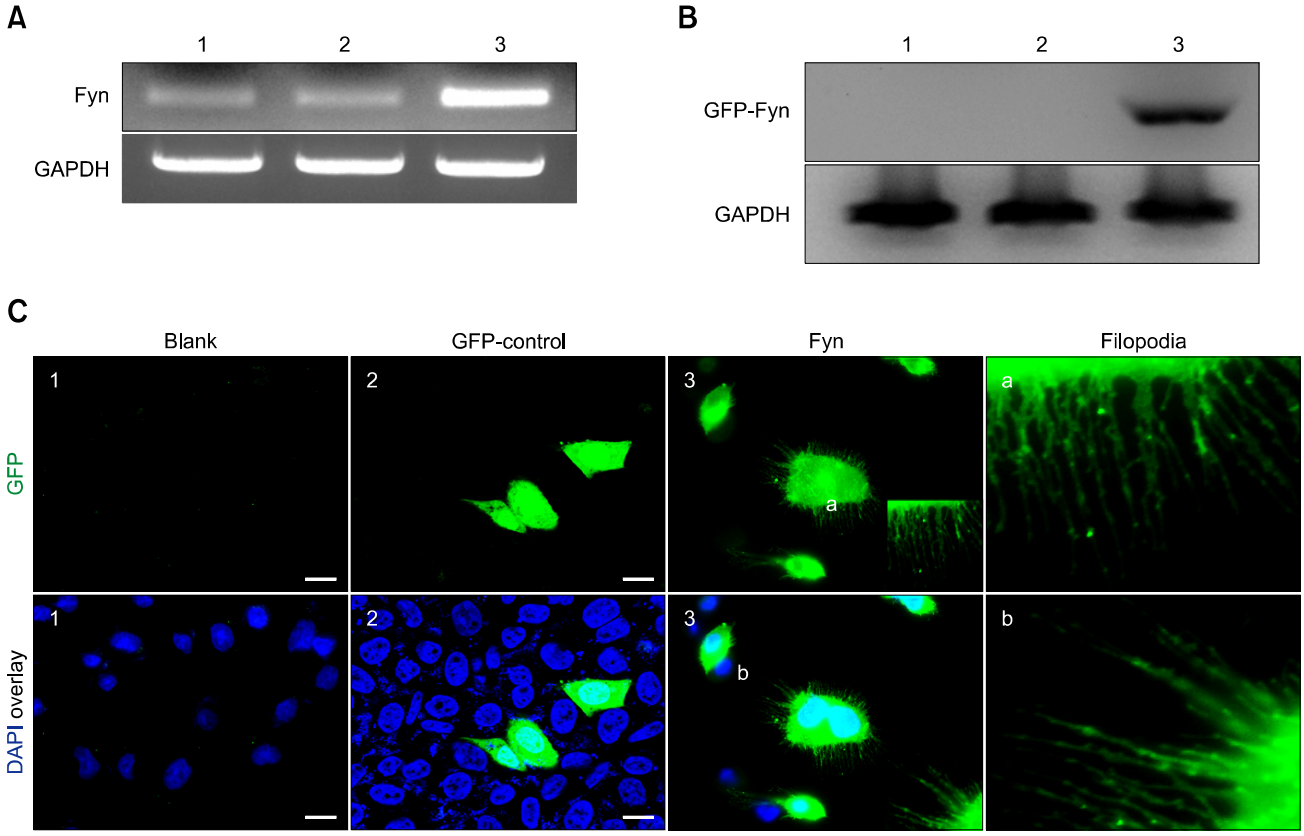

Fig. 2. Overexpression of pEGFP-N1-Fyn in CHO cells. (A) Detection of Fyn mRNA in CHO cells by RT-PCR. (B) Expression of Fyn protein in CHO cells identified by Western blotting. (C) Immunohistochemical staining and effects of Fyn expression on cell morphology. Fyn induced filopodia and lamellipodia formation in CHO cells. Lanes 1, 2, and 3 were the blank group (dealt with DMEM/F12K), control group (transfected with pEGFP-N1), and Fyn group (transfected with pEGFP-N1-Fyn), respectively. Magnified fields are labeled as a and b. Scale bars = 10 μm.

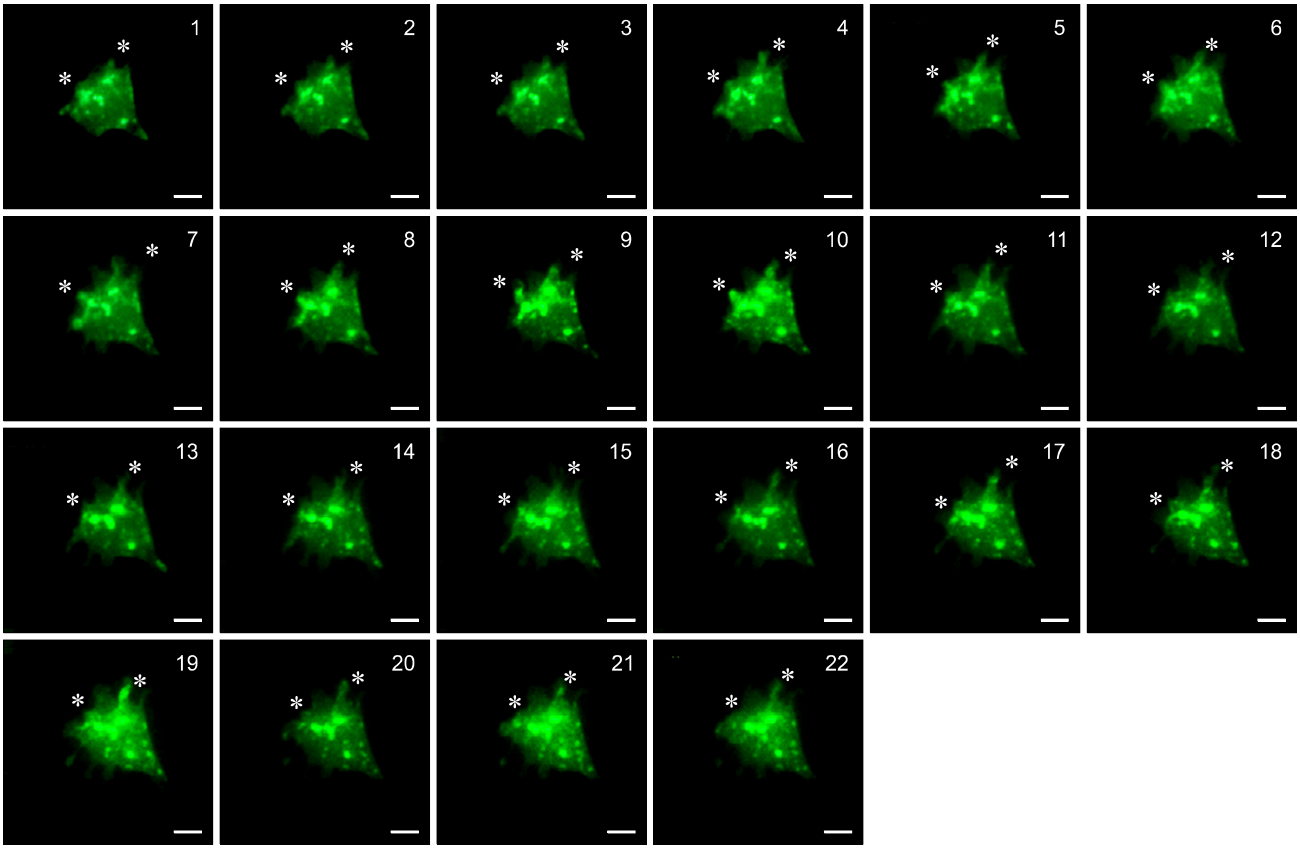

Fig. 3. Movement of a transfected CHO cell observed in a time-lapse series of images. Time-lapse confocal microscopy images show the dynamics of pseudopodium change. The 22 figures are a time-lapse series showing pseudopodium formation 12 h after transfection. Asterisks indicate the real-time location of the pseudopodium. Cells were photographed and cultured within a live cell station. Scale bars = 10 μm.

Fyn affects CHO cell movement

To gain further insight into the relationship between active pseudopodia extension and Fyn expression, we viewed the transfected cells with time-lapse microscopy to document cell migration (Fig. 3). This analysis revealed that Fyn expression altered pseudopodia movement as well as the elongating and branching of filopodia and lamellipodia. Interestingly, the transfected cell displayed a striking feature in the outgrowth of pseudopodia. Our results demonstrated that Fyn expression led to a significant increase in cell translocation.

Discussion

The ability of a cell to respond to changes in the intra/extracellular environment depends on the information transmitted across the plasma membrane through a process mediated by the actin-myosin cytoskeleton [15]. Therefore, the cell membrane is critical for sensing and adapting to these changes. In the present study, we determined whether Fyn overexpression altered cell morphology. Fyn regulates cell growth, development, migration, and differentiation, and binds to MAP-2c, tau, and WASP (Wiscott-Aldrich syndrome protein, also known as WASL) [11-12,14]. Src family members have similar or identical functional domains, and participate in the transformation of cells and intracellular signaling process.

Fyn is necessary for cell invasion. Yadav and Denning [17] demonstrated that the overexpression of Fyn improves invasion *via* the Ras/PI3K/Akt/Fyn signaling pathway. In addition, PI3K, Rap, mTor, Akt, cofilin, and Limk are involved in the cell migration process during the course of cerebral development [4]. It has been hypothesized that Fyn is associated with pseudopodia formation controlled by cytoskeleton arrangement *via* Fyn phosphorylation, thus directing cell migration to the correct position. It has also been suggested that Fyn is most abundant during active pseudopodia formation [18], but stabilization of the pseudopodia diminishes during the process. MiR-125a-3p, a tumor-suppressing miRNA molecule, has an key role in the down-regulation of Fyn expression, cell proliferation reduction, and cell migration [13]. In addition, Fyn promotes phosphorylation of collapsin response mediator proteins 1 at Tyr504 [3]. These finding indicated that Fyn

influences cell invasion and adhesion, triggers actin polymerization, and affects filopodia/lamellipodia formation. Our results demonstrated that the relationship between Fyn expression and pseudopodia formation enabled cells to sense their surroundings and respond with filopodia/lamellipodia production in an appropriate manner. Fyn significantly increased cell migration and the resulting changes of pseudopodium movement dynamics showed that filopodia and lamellipodia came into this forming, thus providing a tractable system for studying the effect of Fyn on migration.

In summary, we succeeded in expressing recombinant pEGFP-N1-Fyn, which showed high bioactivity *in vitro*, and induced filopodia and lamellipodia formation. Fyn-induced pseudopodia production was observed by time-lapse imaging in live cells, and allowed us to monitor this dynamic process. Additional experiments will be needed to further elucidate the molecular mechanism underlying the association between SFKs and downstream factors in the process governing cell migration.

Acknowledgments

This work was supported by the Foundation for Special Talent of Northwest A&F University (No. Z111021101) and the National High Technology Research and Development Program of China (863 Program; No. 2011AA10A208).

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