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RESEARCH ARTICLE

Overexpression of Fyn Induces Formation of filopodia and lamellipodia in CHO Cells via Rearrangement of F-Actin

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ABSTRACT

As a main ancillary molecule involved in the regulation of proliferation, differentiation and migration of cortical neurons, Fyn has also been identified as a signal factor in motility and growth involved with cytoskeleton. However, the molecular mechanism of Fyn on cytoskeleton remains unclear. The results showed that the morphology of the CHO cells transfected with the recombination vector changed remarkably. The numbers of stress fibers may be crunched to transformation for generating and supporting the formation of filopodia and lamellipodia, but the changes of vinculin and tubulin are unremarkable. In addition, high concentrations of Fyn cause formation of lamellipodia as well as filopodia. From this research we can draw a conclusion that overexpression of mouse Fyn induces the F-actin cytoskeleton rearrangement and both vinculin and tubulin are absent in the process of Fyn-mediated F-actin reorganization.

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INTRODUCTION

During cell process extension, actin and myosin, the important cytoskeleton unit, are crucial for relying on associated proteins to initiate outgrowth. Actin stress fiber is one of the major cytoskeleton structures in fibroblasts and plays important roles in various cellular functions, including cell motility and morphogenesis (Bianchi *et al*., 2013). Vinculin, a membrane cytoskeleton protein in focal adhesion, is involved in the linkage of focal adhesion molecules. Fyn, a member of the Src tyrosine kinase family (SFKs), has widely been expressed in organism and was involved in stress fiber formation (Xu *et al*., 2012). Fyn was reported to take part in cell invasion (Yang *et al*., 2011; Minami *et al*., 2012) and was involved in cell motility and process outgrowth (Zamora-Leon and Shafit-Zagardo, 2005). As a main ancillary molecule, Fyn has been involved in cell metabolism, cell growth (Nicholson-Dykstra *et al*., 2005), development, differentiation and neuronal migration (Ninio-Many *et al*., 2013). It is reported that Fyn participated in Ras/PI3K/Akt signaling pathway and induced phosphorylation of collapsin response mediator proteins (CRMPs) and focal adhesion kinase (FAK) (Buel *et al*., 2010; Yadav and Denning, 2011; Yeo *et al*., 2011). Moreover, actin filaments, involved in cell structure changes as well as in cancer process, may be relevant to interact with epithelial integrin α5β6 (Lewin *et al*., 2010). As a novel signaling factor, Fyn acts in actin morphology as a mediator of the effects of tau on actin rearrangements (Sharma *et al*., 2007).

Despite the functional importance of Fyn on actin cytoskeleton, no information is available on the temporal and spatial patterns of its expression during lamellipodia and filopodia formation. To investigate this issue, we investigated the relationship between actin cytoskeleton and morphology. Fyn could induce cytoskeleton reorganization in CHO cells and had positive effects on distribution of vinculin in CHO cells. Interestingly, concentration of Fyn was not distributed in transfected cells homogeneously, and a high concentration of Fyn was present in lamellipodia/filopodia. The results showed that over-expression of mouse Fyn induced the formation of filopodia and lamellipodia via F-actin reorganization while microtubule and vinculin might not be involved in the event.

MATERIALS AND METHODS

Construction of over-expression vector: The Fyn gene was isolated from mouse cDNA by reverse transcription PCR (RT-PCR). The total mRNA was isolated from the cerebral cortex of Kunming strain mice using Trizol Kit (Invitrogen, USA). The PCR product of Fyn was inserted

into vector pMD18-T-simple (TaKaRa, Japan). The PCR primers used in the experiment were (restriction enzyme sites are underlined) Fyn-F: 5'-CGGAATTCATGGGCTG TGTGCAA-3' and Fyn-R: 5'-TCCCCCGGGCCAGGTT TTCACCGG-3'; GAPDH-F: 5'-AGCGAGACCCCACT AACA-3' and GAPDH-R: 5'-ATGAGCCCTTCCACAA TG-3'. PCR product was digested with *Eco*R I and *Sma* I (New England Biolabs, USA), and the product was ligated into pEGFP-N1 vector, resulting in a recombinant expression vector pEGFP-N1-Fyn, Fyn gene was expressed as a GFP-Fyn fusion protein in cell.

Cell culture and transfection: The CHO cells (Chinese Hamster Ovary) were prepared in our lab and cultured in F12K medium (Gibco, USA) containing 10% fetal bovine serum (FBS) including penicillin (50 U/ml) and streptomycin (50 U/ml). Transient transfection was carried out using X-tremeGENE HP (Roche, USA) according to the manufacturer's instructions.

Quantitative real-time PCR and western blot: Cells were collected after 24h of transfection for quantitative real-time PCR and 48h for western blotting. Fyn fragment, F: 5'-CACAGACCCCACCC CTCA-3'; R: 5'-CCGTCCGTGCTTCATAGT-3'; β-actin, F: 5'-TCCCTGGAGAAGAGCTACGA-3'; R: 5'-AGCA CTGTGTTGGCGTACAG-3'. Quantitative real-time PCR amplification reactions were carried out on a Bio-Rad CFX96 by SYBR Premix Ex TaqTM II (Takara, Japan) chemistry detection under amplification conditions. The mixtures were incubated for initial denaturation at 95°C for 10 min, followed by 38 cycles consisting of 95°C for 15s and 60°C for 1min.

The cellular protein was extracted in the lysis buffer (solarbio, china) with 1 mM PMSF (Phenylmethane-sulfonyl fluoride) Protein concentrations were measured and equal amounts were loaded in each condition. The extracted protein was electrophoresed in 10% SDS-PAGE gels and transferred to polyvinylidence fluoride (PVDF) membranes (Millipore, USA). After blocking with 5% nonfat milk in Tris-buffered saline/0.1% Tween 20 (TBST) at RT (room temperature) for 30mins, the membranes were incubated with primary antibodies (Mouse monoclonal anti-GAPDH antibody and mouse monoclonal anti-Fyn, Santa Cruz, USA) overnight at 4°C and the membranes were hybridized with a 1:2000 dilution of goat-anti-mouse HRP-labeled antibody for 2h at RT. The targeted proteins were detected using the ECL substrate kit (Thermo, USA) according to the manufacturer's instruction.

Immunohistochemistry: Cells were fixed with 4% Paraformaldehyde (PFA) in PBS for 1h at RT, and fixed cells were incubated with primary antibody (mouse anti-tubulin or mouse anti-vinculin) overnight at 4°C, then the cells were incubated in secondary antibody Alexa Fluor 568-conjugated goat anti-mouse (1:300; invitrogen) for 2 h at room temperature. Cells were stained for F-actin with TRITC-conjugated phalloidin (1:1000; millipore). All of cells were counterstained with DAPI (1:1000; millipore). Fluorescent image analysis was done on fluorescent microscope and stained cells were analyzed on a Zeiss microscope (Axio Observer Z1, Carl Zeiss).

Statistical analysis: Data are expressed as mean ± standard error of the mean (SEM). The data were evaluated for significant differences using a Student's *t*-test. $P<0.05$ (*), $P<0.01$ (**) and $P<0.0001$ (***) was considered significant, very significant and extremely significant, respectively.

RESULTS

Identification and over-expression of the recombinant vector: Using recombinant clone, the Fyn gene was inserted into vector pEGFP-N1 and formed pEGFP-N1-Fyn. We identified the over-expression of GFP-Fyn in CHO cells using quantitative RT-PCR and western blot. The normal transcription of Fyn gene in CHO cells transfected with pEGFP-N1-null (GFP-Control) only maintained at extremely low level (Fig. 1A & 1B), while the transcription of Fyn mRNA in the cells transfected with pEGFP-N1-Fyn increased significantly 24 h post-transfection ($P<0.0001$). The Fyn protein was only found in cells transfected with pEGFP-N1-Fyn group (Fig. 1C), indicating that Fyn was successfully expressed in vitro.

Fyn induces dramatic morphological changes in CHO cells: Filopodia, a slender cytoplasmic projection, is composed of microfilament and extend beyond the lamellipodia under the condition of movement (Mattila and Lappalainen, 2008). Following pEGFP-N1-Fyn treatment, an association between Fyn and filopodia in the cell processes was observed. Over-expression of Fyn-GFP induced dramatic morphological changes (Fig. 1A). The formation of filopodia and lamellipodia at the cell periphery were characterized, and the mean number and length analysis of cells filopodia were measured and calculated. As shown in Figure 1D/E, the number and mean length of filopodia in cells transfected with pEGFP-N1-Fyn increased dramatically compared to those in control cells ($P<0.0001$). The results showed that distribution of Fyn in the cells not only overlay with actin, but also with the formation of filopodia and lamellipodia. In addition, the distribution of filopodia is always located at the edge of lamellipodia/cell, and the mean length is up to 10 microns as long as a cell diameter.

Fyn leads to formation of filopodia and lamellipodia via F-actin reorganization: Stress fiber, a structure of actin filament, has been found in eukaryotic cell and it is closely related to morphological changes and movement, indicating that when cell moves, the stress fibers will disappear and transform into fragmentation in order to adapt and stabilize the changes. Consequently, the changes of stress fibers were observed. As can be seen in Figure 2, the F-actin did not organize into typical bundles of stress fibers in cytoplasma of cells transfected with pEGFP-N1-Fyn, but appeared to be highly concentrated in the filopodia and cell periphery. It was shown that stress fiber number declined as well as total length whereas the average length remained unchanged (Fig. 2B/C, $P<0.0001$), therefore, the significant difference of

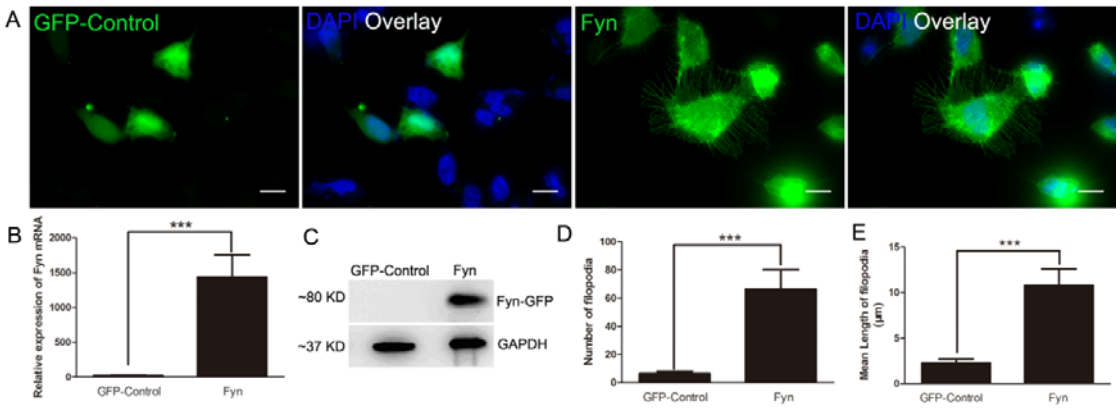

Fig. 1: Over-expression of Fyn-GFP in CHO cells induced dramatic morphological changes. Although GFP protein expressed in both control group and pEGFP-N1-Fyn group, formation of filopodia and lamellipodia were found in the pEGFP-N1-Fyn group. An increased filopodia/lamellipodia was observed and analysed between the two groups. (A) Left: the GFP-Control transfected cells. Right: the Fyn transfected cells. Scale bar, 10 μm. (B) The transcription of Fyn in CHO cells transfected with pEGFP-N1-Fyn was higher than the control group. (C) Western blot analysis of Fyn in CHO cells. (D/E) The number of filopodia transfected with pEGFP-N1-Fyn was analyzed, and the length of filopodia was similar to the analysis (P<0.0001). For quantification, Number/length was counted using the Image J.

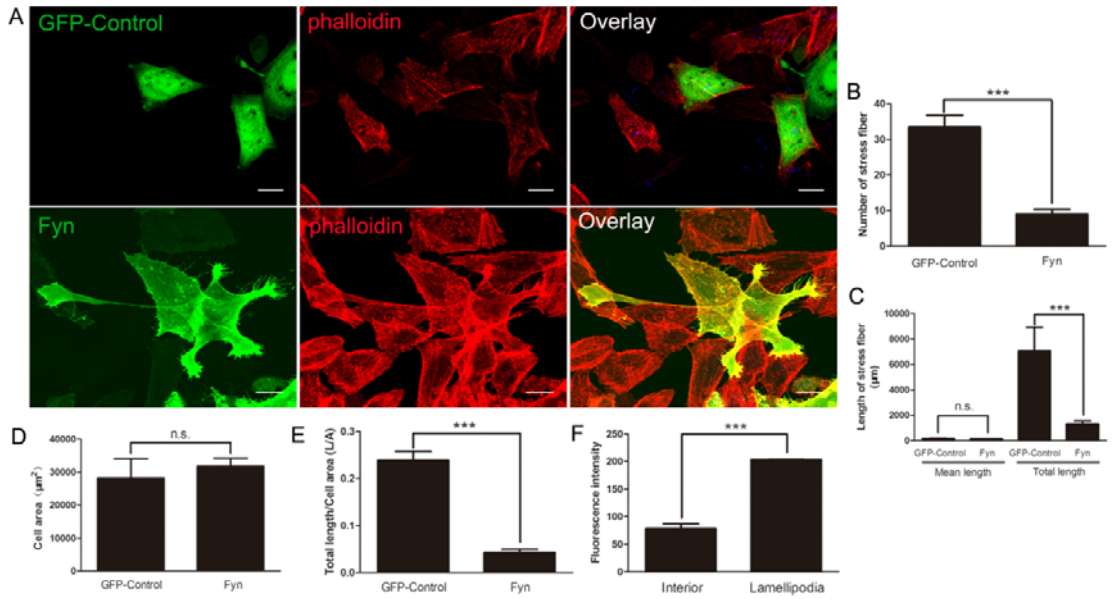

Fig. 2: The CHO cells transfected with pEGFP-N1-Fyn induced the depolymerization of stress fiber. (A) Upper panel: the GFP-Control transfected cells. Lower panel: the Fyn transfected cells. The cells were stained with TRITC-phalloidin and DAPI. Scale bar, 10 μm. (B) The number of stress fiber was counted between the two groups (P<0.0001). (C) The mean length of stress fiber did not change in the cells with pEGFP-N1-Fyn, therefore, total length in the pEGFP-N1-Fyn group significantly decreased (P<0.0001). (D) The result showed that the area of cells was unaffected by Fyn; (E) The ratio between total length of stress fibers and area of cells (L/A), significantly reduced (P<0.0001). For quantification, length/area was counted using the Image J. (F) The analysis of fluorescence intensity in Fyn transfected cells. The distribution of Fyn-GFP on lamellipodia was significantly higher than cell interior (P<0.0001).

L/A means the state of stress fibers has changed from linear bundles to random fragment (Fig. 2D/E, P<0.0001). The results suggested that Fyn-induced formation of filopodia and lamellipodia depending on actin filament rearrangement which involved in the activity of microfilaments. Interestingly, we noticed that: in Fyn transfected cells, a high concentration of Fyn instead of low concentration of Fyn could arrest depolymerization of stress fiber showing that high concentration Fyn could induce formation of filopodia and lamellipodia (Fig. 2F, P<0.0001).

Vinculin and tubulin might be absent to Fyn-induced morphological changes: In the present study, the CHO cells were double stained with the anti-tubulin/vinculin mAb and DAPI to investigate whether Fyn was associated or stabilized with tubulin/vinculin in the process of outgrowth. In figure 3, Fyn could cause the shifting of vinculin accompanied by protrusions partially, but it was not necessary. Moreover, the distribution of tubulin was not always co-localized with GFP-Fyn (Fig. 3B), the distribution of tubulin was not altered in the process of formation of lamellipodia/filopodia, suggesting that Fyn-induced filopodia/lamellipodia is mainly actin-based, while vinculin/tubulin is absent to the filopodia/ lamellipodia predominantly. The previous study proved that Fyn does not associate or stabilize microtubules (MTs) in COS-7 cells (Zamora-Leon and Shafit-Zagardo,

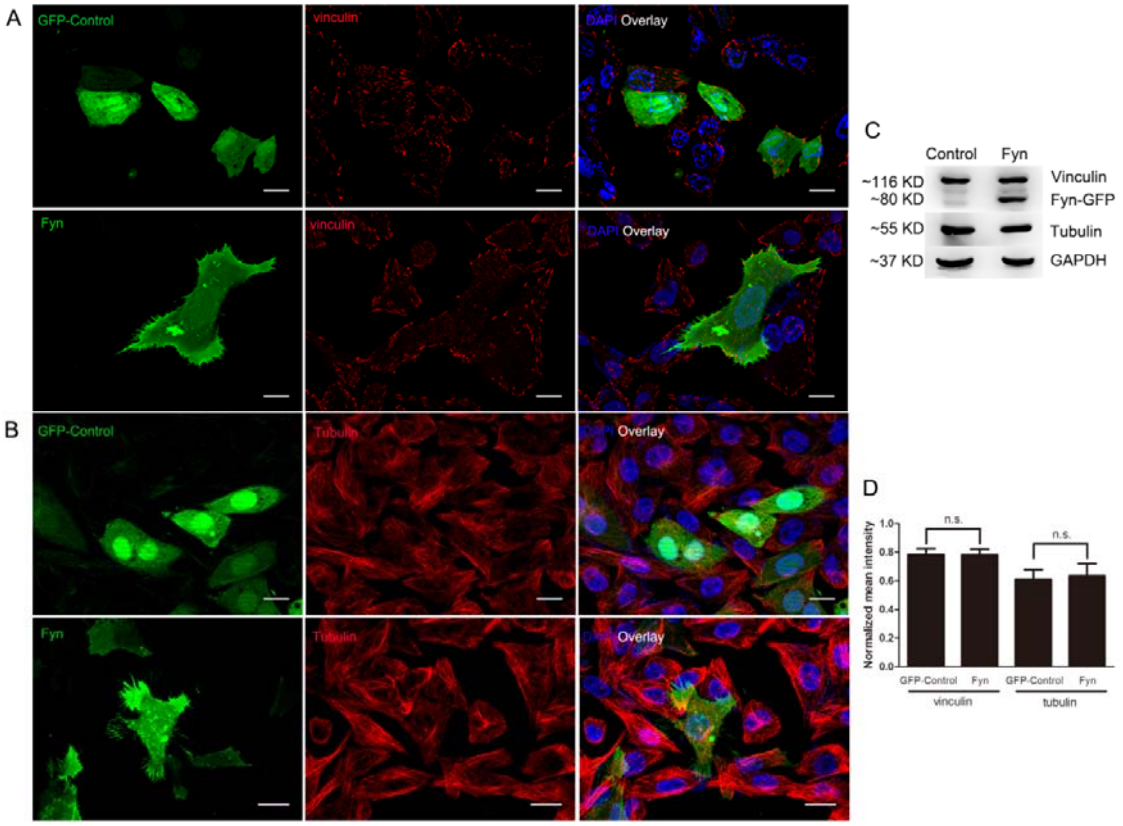

Fig. 3: The effect of Fyn on vinculin/tubulin. (A) Fyn did promote shifting of vinculin in the process of formation of filopodia/lamellipodia. (B) Fyn did not induce remarkable change of tubulin in CHO cells. (C) Fyn did not induce changes of tubulin/vinculin protein. The protein expression levels of both tubulin and vinculin were no difference between the two groups. (D) Densitometric analysis of vinculin and tubulin protein in CHO cells transfected with GFP-Control or Fyn. Results were from three independent experiments; statistically significant differences are indicated (unpaired t test, n.s., not significant). Scale bar, 10 μm.

2005), our results showed similar characteristics. There was not a statistically significant difference between the expression of vinculin/tubulin between the GFP-Control group and the Fyn group (Fig. 3C/D), indicating that tubulin is an unnecessary factor within the process of filopodia/lamellipodia, in which pseudopodia were stimulated by F-actin polymerization and stabilized at focal adhesion sites. Our results showed that these distributions of vinculin always pitch matures membrane protrusion in the process of cell motility and morphogenetic movement and this affection is limited and during the process of actin polymerization, the role of tubulin seems to maintain cell shape.

DISCUSSION

Using biochemical and molecular approaches, we demonstrated that mouse Fyn interact with cytoskeleton in vitro, moreover, the relationship between Fyn and vinculin/tubulin was detected and the changes of stress fibers were observed primarily. Since microtubule depolymerization in fibroblasts had been shown to induce stress fibers formation, Fyn did not interact with acetylated tubulin or enhance MT (microtubule) stabilization (Zamora-Leon and Shafit-Zagardo, 2005). Vinculin is thought to anchor F-actin to the membrane and regulate the links between F-actin and the cadherin and integrin families of cell-adhesion molecules. Our previous research proved that SFKs played a part in neuronal migration and movement (Zhao and Frotscher, 2010). Cofilin, an actin-depolymerizing protein, promotes the disassembly of F-actin leading to the process (Frotscher *et al*., 2009). In this study, we have provided evidence that Fyn had positive effects on the formation of filopodia and lamellipodia, and Fyn instructed the actin filament toward depolymerizing and acted as a joint cue to the active cells via F-actin rearrangement in the process of filopodia/ lamellipodia when the cell got a signal to change morphology.

At the normal physiological state, the morphology of a CHO cell deliver signal that it has a smooth edge around the cell surface, where very few filopodia or lamellipodia are found. Here we have shown that over-expression of Fyn in CHO cells resulted in filopodia and lamellipodia formation via F-actin rearrangement, and vinculin and tubulin were unchanged at the level of protein expression when the stress fiber disappeared. Thus, our experiments have further revealed the mechanism of Fyn-mediated morphological changes depended on cytoskeleton factor. Over-expression of Fyn further changed the state of stress fiber, and the most important point is the change of stress fibers from lineation to dispersion in CHO cells (Fig. 2). In order to verify our idea, number, length and area of actin filament have been measured. Moreover, the analysis of fluorescence intensity in Fyn transfected cells was detected. The results proved that Fyn produced strong

effect on the formation of pseudopodium, and the cells had longer and more filopodia than that in control group. We used (Image J for measuring the mean length, total length of stress fibers (summation of stress fibers in the cell), cell area, and the ratio between total length of stress fibers and cell area (L/A), thus we got some results to check F-actin rearrangement. Interestingly, the typical bundles of parallel stress fibers stimulated had a tendency to disappear, and the disappeared filaments were drifted to random fragment or arranged fragment for maintaining filopodia and lamellipodia. And the distribution of Fyn-GFP on lamellipodia was higher than cell interior, and a high concentration of Fyn instead of low concentration of Fyn could cause the result (Fig. 2F). We first performed an analysis and found the stress fiber changes during the filopodia/lamellipodia formation.

Vinculin acted as structural components of the maturing adhesion, which led to a local increase in actin polymerization and protrusion activity. Although the shifting vinculin reached on the verge of lamellipodia or filopodia, the Fyn transfected cells labeled for vinculin revealed that it was co-located in filopodia/lamellipodia partially (Fig. 3A), and the figure 3C also demonstrated that vinculin had no significant difference at the protein level. In accordance with the level of vinculin, tubulin seemed not involved in process of filopodia/lamellipodia. As a cytoskeleton protein associated with junctions, focal adhesion and binding sites, vinculin binds to specific domains with many cytoskeleton on proteins, plays a critical role in the control of cellular morphology, protrusion and cell motility. The recruitment of vinculin to lamellipodia might be one supplementary mechanism to which actin depolymerization and membrane protrusion are coupled to mediate focal adhesion. Zamora-Leon has suggested that Fyn-transfected cells did not associate with acetylated tubulin but has the capacity to induce process outgrowth (Zamora-Leon and Shafit-Zagardo, 2005), and MAP-2C (microtubule associated protein 2C) did further increased the interaction with actin cytoskeleton. On the basis of our observation that tubulin did not participate in the formation of lamellipodia/filopodia (Fig. 3), where tubulin was shown to be disassociated with lamellipodia/ filopodia because it was not the composition components of filopodia. One reason for these discrepancies might be, in the CHO cells, due to the phosphorylation state of downstream factors including arp2/3, Talin, FAK (Focal Adhesion Kinase) LKB1, Filamin, Rho, Rac, and Cdc42 (Yamada *et al*., 2010; Case and Waterman, 2011).

Conclusion: Based on the present study, we have shown that over-expression of Fyn elevated the formation of filopodia and lamellipodia via F-actin rearrangement and shifting of vinculin might participate in this process. However, whether other factors participated in Fyn-induced cell motility and migration were open for further study.

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