BIOCELL
2017 41(2-3): 45-53

ISSN 1667-5746
ELECTRONIC

# Membrane fluidity regulates high shear stress-induced FAK activation at different subcellular compartments

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**Key words:** FAK activity, shear stress, FRET biosensor

**Abstract:** Focal adhesion kinase (FAK) plays a vital role in mediating the adaptability of tumor cells under mechanical stimuli. Previous studies revealed that FAK can locate to different cell compartments, and its regulation is highly dependent on its subcellular localization. However, the local FAK activities and its regulation mechanism in different cell compartments of tumor cells in response to fluid shear stress are still unclear. In this study, 5 dyn/cm$^2$ and 20 dyn/cm$^2$ of shear stress was applied to HeLa cells for 30 min. The activities of FAK targeting different subcellular compartments (lipids rafts, non-rafts, focal adhesions and cytoplasm) were investigated with fluorescence resonance energy transfer (FRET) technology. Results showed that the activity of FAK in response to high shear stress at focal adhesion sites was lower than that of other three areas, while no difference among four areas was observed in response to low shear stress. Furthermore, high shear stress-induced distinct FAK activation at different compartments was inhibited by decreasing membrane fluidity, but Src inhibition prevented high shear stress-induced FAK activation only in the cytoplasm. This study revealed the spatiotemporal characteristics of FAK under the different magnitude of shear stress, which provides a deeper understanding of mechanotransduction in tumor cells.

## Introduction

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase consisting of a FERM domain (protein 4.1, ezrin, radixin and moesin homology), a kinase domain, and a FAT domain (targeting focal adhesion (Seong *et al.*, 2011a). Phosphorylation of FAK at Tyr397 leads its binding to Src kinase, and then regulates many signaling molecules such as PI3K, Grb7 and Rho GTPases (Zhao *et al.,* 2009). Lee et al. ( Lee *et al.,* 2015) found FAK overexpressed in a variety of cancer cells, and this was directly related to poor clinical treatment. It was also found that FAK plays a vital role in tumor cells mechanotransductions, which is associated with tumor progression (Lachowski *et al.*, 2017). For example, high-stiffness matrix promotes the internal tension via activating FAK and P130Cas, and further mediates focal adhesion dynamics (Levental *et al.*, 2009; Puig *et al.*, 2015; Pang *et al.*, 2017). And low-stiffness matrix promotes the growth of cancer stem cells by inhibiting FAK activity and expression (Tan *et al.*, 2017). In addition, low shear stress-induced cytoskeletal rearrangement in breast cancer cells, which is fundamental for cell migration, also depends on FAK/Src and ROCK/p-MLC signaling pathways (Xiong *et al.*, 2017). The migration direction of tumor cells is influenced by shear flow pattern. Evidence showed that breast carcinoma cells and hepatocellular carcinoma cells on a 2D substrate migrate downstream (Xiong *et al.*, 2017; Yu *et al.*, 2017). In contrast, breast carcinoma cells in a 3D matrix migrate in interstitial upstream direction, which is regulated by the polarization of FAK (Polacheck *et al.*, 2011; Polacheck *et al.*, 2014).

The activity of FAK is directly related to its subcellular localization (Seong *et al.*, 2011a). It not only exists in the cytoplasm, but also targets focal adhesion through its carboxyl-terminal FAT region (Lee *et al.*, 2015). It has been found that FAK could not be activated by fibronectin in high-hardness matrix when it is unable to target focal adhesion (Seong *et al.*, 2013). In addition, FAK can also be recruited to cell membrane through binding to cytoplasmic of integrin (Staubach *et al.*, 2011; Lee *et al.*, 2015), and its membrane targeting promotes PI3K/Akt activation and cancer cell adhesion (Baillat *et al.*, 2008). This membrane activity of FAK is affected by membrane lipid rafts. For instance, disruption of the lipid caveolin protein prevents integrin-mediated

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FAK phosphorylation (Seong *et al.*, 2011b). A fluorescence resonance energy transfer (FRET) study found that the degree of FAK activation in lipid rafts is higher than in non-rafts domain during cell adhesion and platelet-derived growth factor (PDGF) stimulation (Guan, 2004; Seong *et al.*, 2011a; Seong *et al.*, 2011b). The fluid shear stress can also regulate the localization and activity of FAK in endothelial cells, thereby regulating different processes (Li *et al.*, 2002; Andersen *et al.*, 2017; Lee *et al.*, 2017). For example, lipid rafts targeted FAK has a higher activity at the upstream of the endothelial cells in response to 65 dyn/cm$^2$ of shear stress) (Liu *et al.*, 2014). Shear stress induces FAK accumulation and promotes new focal adhesions in the direction of the flow (Li *et al.*, 2002). Therefore, FAK at different subcellular domains shows distinct sensitivity to mechanical force. Therefore, the characteristics of FAK activity in specific subcellular compartments of tumor cells under different magnitude of shear stress need to be elucidated.

Tumor cells are regulated by different magnitude of shear stress from interstitial fluid and blood flow. In spite of preferring to adhere and survive in a low shear stress environment (Wirtz *et al.*, 2011), tumor cells still retain adhesion ability after 50 dyn/cm$^2$ of shear stress application (Jabbar *et al.*, 2006). Massive studies have reported that low shear stress can promote adhesion of tumor cells in a FAK dependent manner (Thamilselvan *et al.*, 2007; Xiong *et al.*, 2017), whereas FAK activity in response to high shear stress is rarely reported. In this paper, the classical parallel plate flow chamber system was used to apply 5 dyn/cm$^2$ (low shear stress, occur in vein) and 20 dyn/cm$^2$ (high shear stress, occur in artery) of shear stress respectively to the HeLa cells (Kroll *et al.*, 1996), and the FRET technology was used to observe the spatiotemporal pattern and possible mechanism of FAK activity at the lipid rafts, non-rafts, focal adhesion and the cytoplasm in response to different magnitudes of shear stress. The results show that FAK at different subcellular compartments can sense the different magnitude of shear stress, which is likely mediated by membrane fluidity and partially by Src.

## Material and Methods

*Cell culture and transfection*

HeLa cells were cultured in DMEM medium (GIBCO, USA) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Hyclone, USA). The cells were maintained at 37°C with 5% $CO_2$. The transfections were performed using Lipofectamine LTX reagent (Invitrogen, USA).

*Plasmids*

The cytosolic FAK biosensor (Cyto-FAK) consists of SH2 domain of c-Src, a linker sequence, a substrate peptide, and a pair of ECFP/Ypet fluorescent protein (Seong *et al.*, 2013). The focal adhesion-targeting FAK biosensor (FAT-FAK) was developed by fusion of FAT domain to the C-terminus of cytosolic FAK biosensor (Seong *et al.*, 2013). The lipid rafts-targeting FAK biosensor (Lyn-FAK) was constructed by fusing a raft-targeting motif from Lyn kinase to the N-terminus of cytosolic FAK biosensor. The non-rafts FAK biosensor (Kras-FAK) was generated by adding a non-raft-targeting sequence to the C-terminus of cytosolic FAK biosensor.

*Flow system*

HeLa cells transfected with various plasmids were starved in 0.5% FBS medium overnight before shear stress loading. Shear stress was applied by the classical parallel plate flow system as previously described (Shao *et al.*, 2017). The magnitude of shear stress was calculated as: $\tau=6\mu Q/(bh^2)$, where μ=fluid viscosity of solution, Q=flow rate, h=channel height, b=channel width.

*Image acquisition and analysis*

Images were collected by an inverted microscope (Nikon Eclipse Ti-FI Epi-fl/1, Nikon, Tokyo, Japan) equipped with a cooled charge-coupled device camera (Photometrics, Tucson, AZ), a 440DF20 excitation filter, a 455DRLP dichroic mirror and two emission filters controlled by a filter charger (480DF30 for CFP and 535DF25 for YFP). Time-lapse fluorescence images were acquired using MetaMorph 7.0 software (Universal Imaging, Downingtown, PA) and quantified by MetaFluor 7.0 software (Universal Imaging, Downingtown, PA). The FRET ratio data were analyzed by Excel (Microsoft, Redmond, WA).

*Statistical analysis*

The FRET ratio values were normalized by their basal levels to unit before stimulation in the same cell. Student's t-Test was employed to evaluate the statistical difference among groups. A significant difference was assumed by the *P*-value was <0.05.

## Results

*Fluid shear stress affected FAK activation at different compartments of HeLa cells*

To detect FAK activity at the rafts lipids, non-rafts, focal adhesions and cytoplasm in response to shear stress, Kras-FAK, Lyn-FAK, FAT-FAK and Cyto-FAK FRET biosensors were employed. As shown in Fig. 1, the FRET ratio, which represents FAK activity, increases about 10% under 5 dyn/cm$^2$ of shear stress within 30 min, and there was no significant difference at the four compartments ($p>0.05$). While upon 20 dyn/cm$^2$ of shear stress application, the FRET ratio of Kras-FAK, Lyn-FAK and Cyto-FAK increased about 17%, but the FRET ratio of FAT-FAK increases only about 7%. Therefore, FAK activity at the cytoplasm, lipids rafts and non-rafts were higher than focal adhesion upon 20 dyn/cm$^2$ of shear stress application. These results suggest that low shear stress induces similar FAK activity at different subcellular compartments, while high shear stress induces lower FAK activity at focal adhesion.

*Membrane fluidity regulated shear stress-induced FAK activation in HeLa cells*

To further investigate whether cell membrane fluidity is associated with the shear stress-induced FAK activation at different areas, HeLa cells were pre-treated with 45 mmol/L benzyl alcohol (BA) for 15 min to increase membrane fluidity, or pre-treated with 0.1 mmol/L cholesterol (Cho) for 3 h to

reduce membrane fluidity. As shown in Fig. 2, the FRET ratio of four biosensors in HeLa cells, which were pretreated with Cho, showed no difference from that of control group upon 5 dyn/cm$^2$ of shear stress application. However, 5 dyn/cm$^2$ of shear stress-induced Lyn-FAK and Cyto-FAK activation were inhibited after increasing membrane fluidity by BA. When 20 dyn/cm$^2$ of shear stress was applied, the FRET ratio of Cyto-FAK, Kras-FAK, Lyn-FAK was found lower than that of control group ($p$<0.05), whereas the FAT-FAK showed no significant difference ($p$>0.05) after decreasing the fluidity of cell membrane (Fig. 2). Furthermore, the FRET ratio of four biosensors showed no significant difference under 20 dyn/cm$^2$ of shear stress ($p$>0.05) (Fig. 2). These results suggest that the difference of FAK activity induced by high shear stress at different subcellular compartments was prevented by inhibiting membrane fluidity.

In addition, increasing membrane fluidity inhibited the FRET ratio of all four biosensor upon 20 dyn/cm$^2$ of shear stress application (Fig. 3). The down-regulated FRET ratio of FAT-FAK was still lower than that of Kras-FAK and Cyto-FAK ($p$<0.05), but showed no difference with Lyn-FAK($p$>0.05). These results indicate that increasing cell membrane fluidity can inhibit high shear stress-induced FAK activation.

*Src regulates shear stress-induced FAK activation in the cytoplasm of HeLa cells*

To investigate the role of Src in the shear stress-induced FAK activation, HeLa cells were pre-treated with 50 μmol/L PP1 (Src inhibitor) for 30 min. The FRET ratio of the four biosensors showed no significant difference from control groups upon 5 dyn/cm$^2$ of shear stress application ($p$>0.05) (Fig. 4). Pretreating with PP1 did not inhibit the FAK activation under 5 dyn/cm$^2$ of shear stress-induced FAK activation at four areas. Under 20 dyn/cm$^2$ of shear stress, the FRET ratio of Cyto-FAK was found lower than that of control group ($p$<0.05), whereas the other three biosensors showws no significant difference ($p$>0.05) (Fig. 4). These results suggest that high shear stress-induced FAK activation in the cytoplasm is regulated by Src.

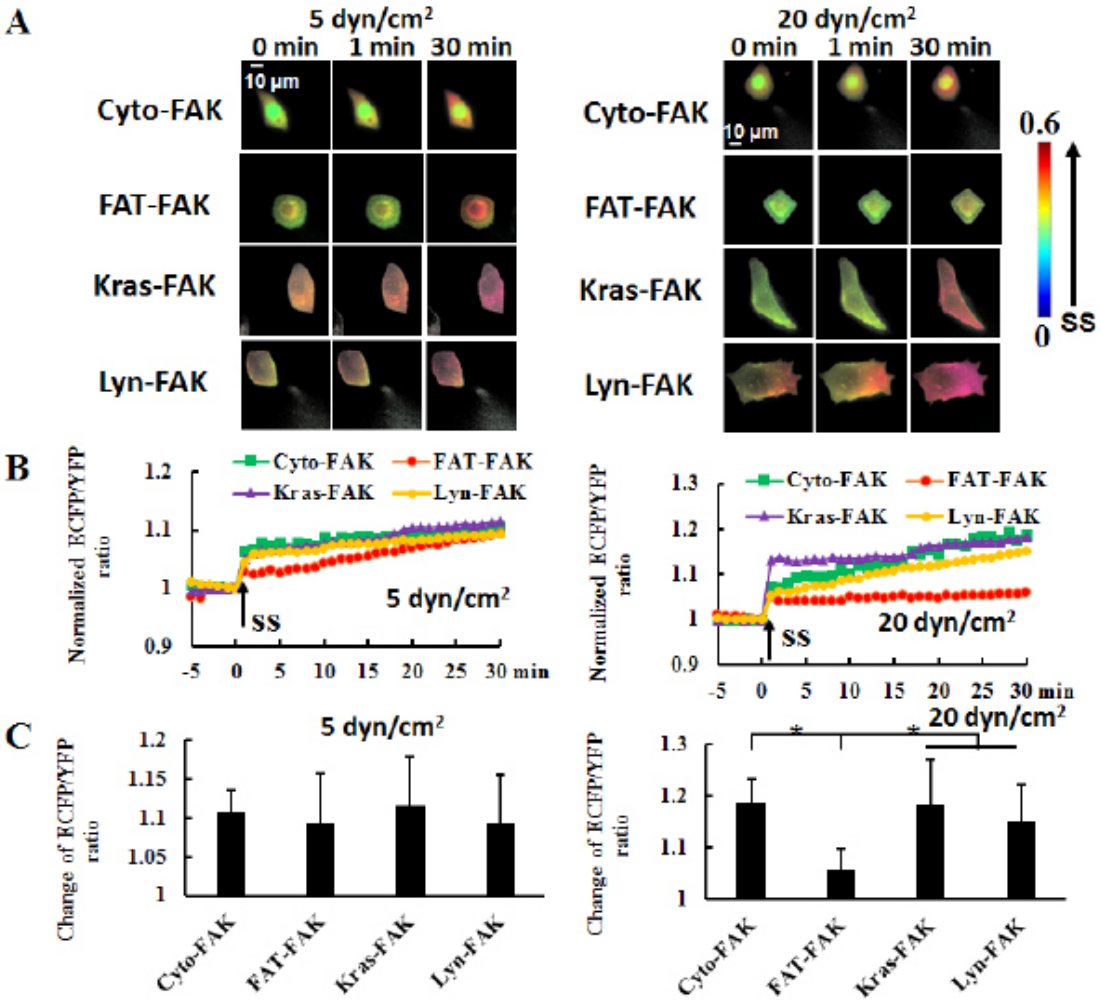


**FIGURE 1.** Distinct FAK activity at different subcellular compartments of HeLa cells upon 5 dyn/cm$^2$ and 20 dyn/cm$^2$ of shear stress application. The representative FRET ratio images (A) and averaged time courses (B) of the Cyto-FAK, FAT-FAK, Kras-FAK, Lyn-FAK FRET biosensors to represent different subcellular FAK activation upon 5 dyn/cm$^2$ and 20 dyn/cm$^2$ of shear stress application. (C) Averaged values of ECFP/YFP emission ratios of the Cyto-FAK, FAT-FAK, Kras-FAK, Lyn-FAK FRET biosensor upon 30 min of 5 dyn/cm$^2$ and 20 dyn/cm$^2$ of shear stress application. * represents a statistically significant $P$<0.05.

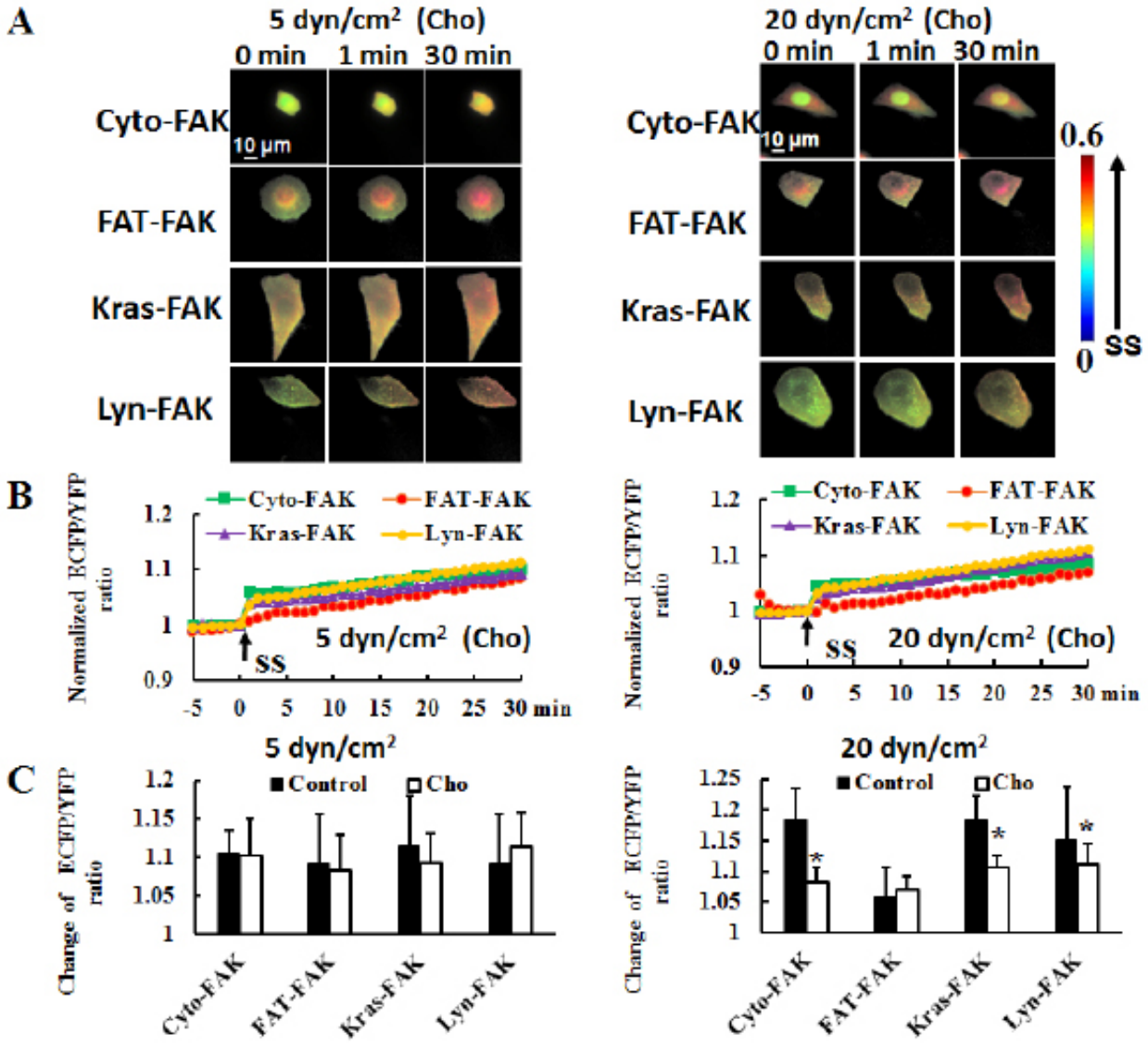


**FIGURE 2**: High shear stress-mediated FAK activities at different subcellular compartments of HeLa cells pre-treated with Cho. The representative FRET ratio images (A) and averaged time courses (B) of the Cyto-FAK, FAT-FAK, Kras-FAK, Lyn-FAK FRET biosensors pre-treated with BA to represent different subcellular FAK activation upon 5 dyn/cm$^2$ and 20 dyn/cm$^2$ of shear stress application. (C) Averaged values of ECFP/YFP emission ratios of the Cyto-FAK, FAT-FAK, Kras-FAK, Lyn-FAK FRET biosensor pre-treated with Cho upon 30 min of 5 dyn/cm$^2$ and 20 dyn/cm$^2$ of shear stress application. * represents a statistically significant $P<0.05$.

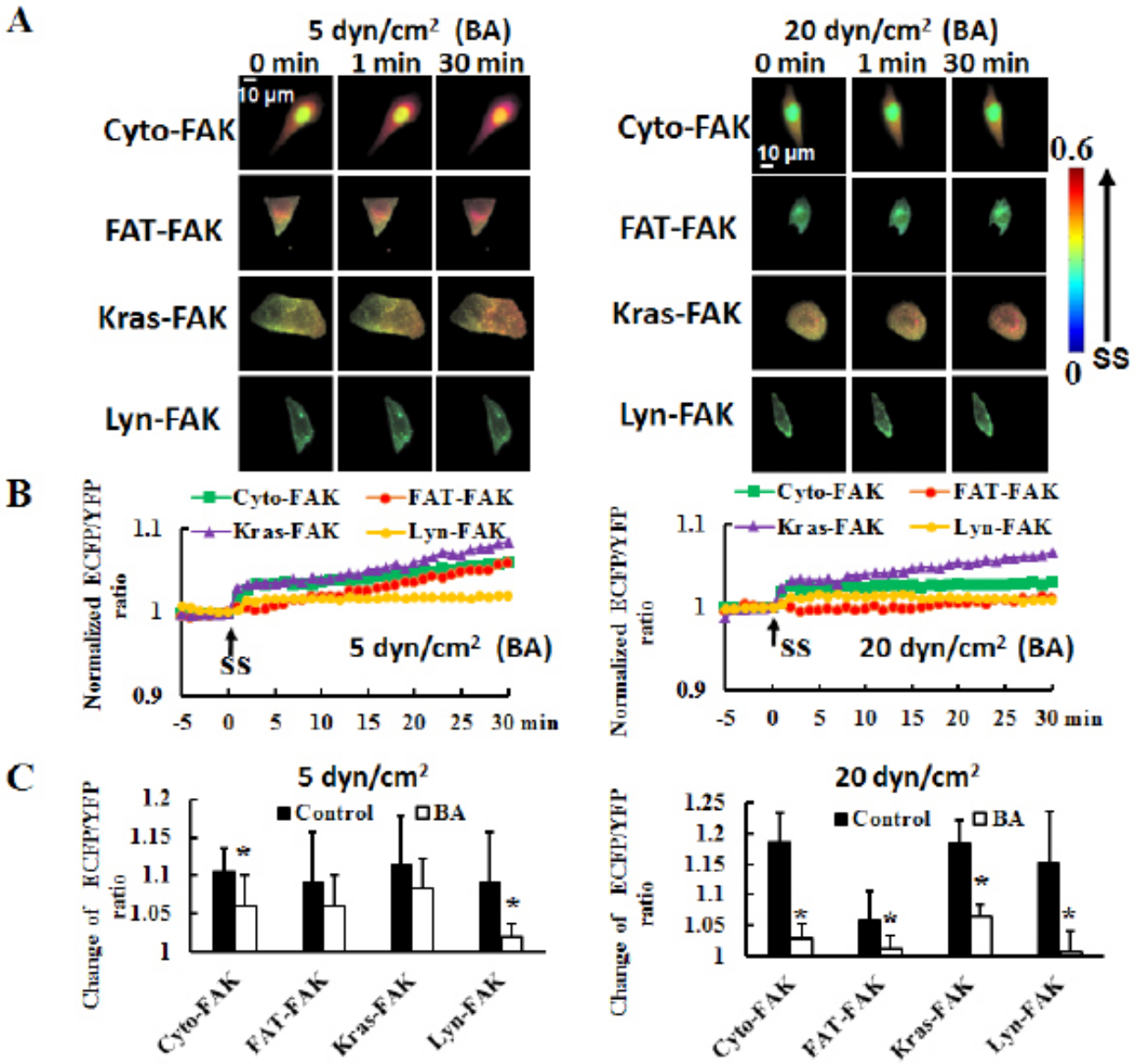


**FIGURE 3**: High shear stress-mediated FAK activities at different subcellular compartments of HeLa cells pre-treated with BA. The representative FRET ratio images (A) and averaged time courses (B) of the Cyto-FAK, FAT-FAK, Kras-FAK, Lyn-FAK FRET biosensors pre-treated with BA to represent different subcellular FAK activation upon 5 dyn/cm$^2$ and 20 dyn/cm$^2$ of shear stress application. (C) Averaged values of ECFP/YFP emission ratios of the Cyto-FAK, FAT-FAK, Kras-FAK, Lyn-FAK FRET biosensor pre-treated with BA upon 30 min of 5 dyn/cm$^2$ and 20 dyn/cm$^2$ of shear stress application. * represents a statistically significant $P<0.05$.

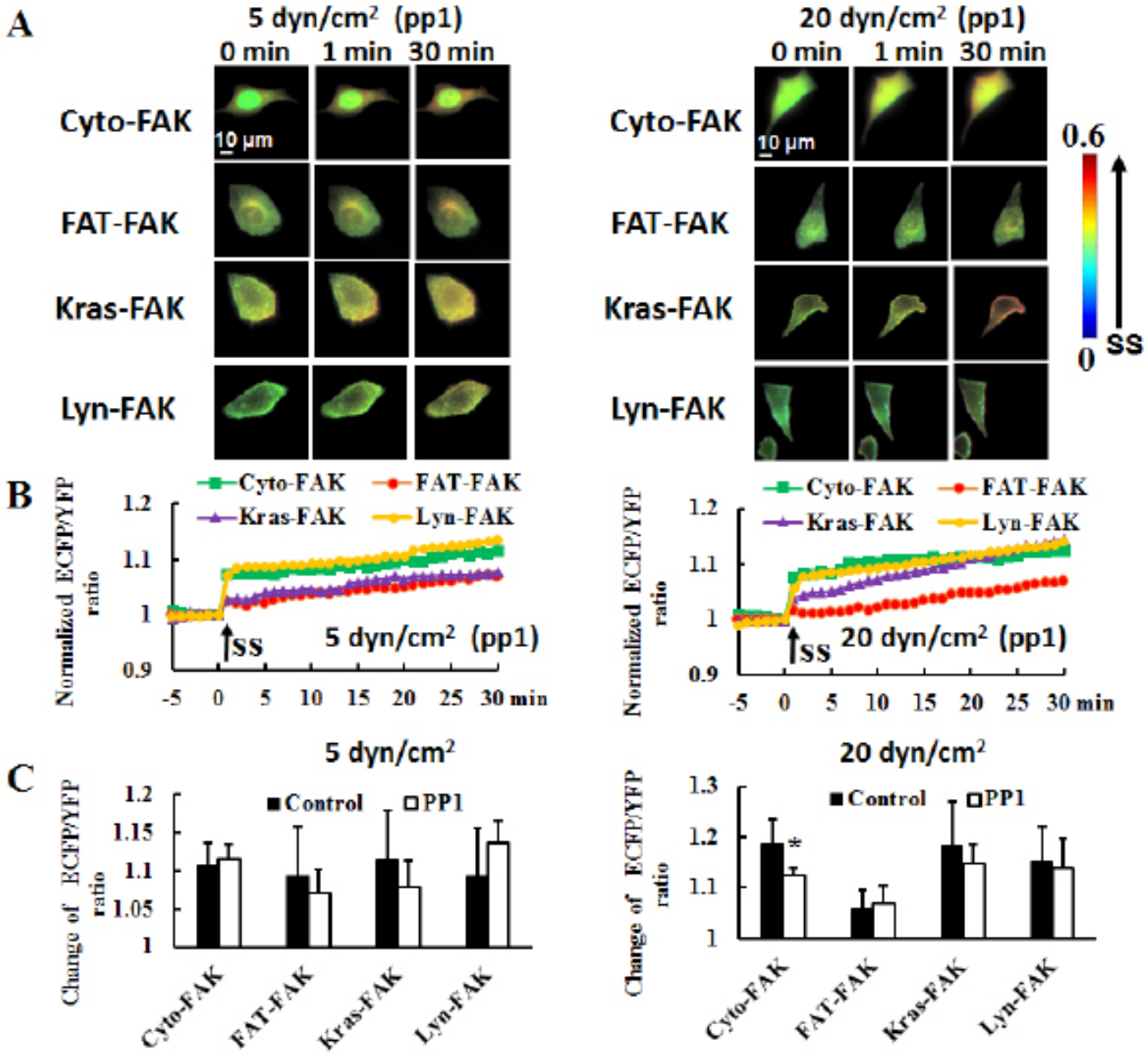


**FIGURE 4**: High shear stress-mediated FAK activities at different subcellular compartments of HeLa cells pre-treated with PP1. The representative FRET ratio images (A) and averaged time courses (B) of the Cyto-FAK, FAT-FAK, Kras-FAK, Lyn-FAK FRET biosensors pre-treated with PP1 to represent different subcellular FAK activation upon 5 dyn/cm$^2$ and 20 dyn/cm$^2$ of shear stress application. (C) Averaged values of ECFP/YFP emission ratios of the Cyto-FAK, FAT-FAK, Kras-FAK, Lyn-FAK FRET biosensor pre-treated with PP1 upon 30 min of 5 dyn/cm$^2$ and 20 dyn/cm$^2$ of shear stress application. * represents a statistically significant $P<0.05$.

## Discussion

Massive studies have reported that FAK activity increasing is critical for cancer cell mechanotransduction (Wang *et al.*, 2011; Seong *et al.*, 2013; Xiong *et al.*, 2017). For example, shear stress activates signal pathways such as ERK and JNK in osteosarcoma cells by elevating FAK activity (Wang *et al.*, 2011); low shear stress phosphorylates FAK and Src through caveolin-1 to promote the dynamics of focal adhesion and promote the movement of breast cancer cells adhesion (Xiong *et al.*, 2017). This study shows that shear stress-induced FAK activation in tumor cells is dependent on the cellular localization and the magnitude of shear stress. It is further found that membrane fluidity and Src are involved in the regulation of asymmetric FAK activation at different cellular compartments.

Shear stress can alter the physical properties of tumor cell membrane, and sequentially activates membrane-bound molecules, such as integrin and Rho GTPases (Butler *et al.*, 2001; Butler *et al.*, 2002; Zhang *et al.*, 2011; Chakraborty *et al.*, 2012; Liu *et al.*, 2014). It has been found that shear stress increases HeLa cells membrane fluidity in a magnitude dependent manner, which is proposed as a potential mechanism for tumor cells deformation and survival

under high shear stress (Das *et al.*, 2011; Wirtz *et al.*, 2011; Chakraborty *et al.*, 2012). To reduce the influence of different cells lines, HeLa cells were used here for further exploring the role of membrane fluidity in shear stress-induced FAK activation. In the present study, low shear stress induced similar FAK activation at different cellular compartments, whereas high shear stress decreases the activation of FAK at focal adhesion but not cytoplasm, lipid rafts and non-rafts. A possible mechanism for distinct FAK activity at different cellular compartments is the shear stress-induced membrane fluidity alternation. As the alternation of membrane fluidity can regulate the mobility of membrane receptors (Butler *et al.*, 2001), high shear stress-induced membrane fluidity alternation might induce the relocation of integrins from focal adhesion and then down-regulate integrin-mediated FAK activation at focal adhesion (Ahlen *et al.*, 2004). Recent studies also found decreasing focal adhesion and its components (Talin, Vinculin) upon shear stress application to tumor cells (Das *et al.*, 2011; Wirtz *et al.*, 2011; Yu *et al.*, 2017). This might inhibit binding of Talin to integrin, which is critical for integrin activation, and subsequently inhibit FAK activation at focal adhesion (Janostiak *et al.*, 2014). High shear stress-induced distinct FAK activities among different cellular compartments disappear after inhibiting membrane fluidity. In addition, increasing membrane fluidity further down-regulates high shear stress-induced FAK activation at focal adhesion. These results further provide the evidence that high shear stress down-regulate FAK activity at focal adhesion through increasing membrane fluidity. This might subsequently weakens the adhesion of tumor cells and promote tumor cells migration, because transforming growth factor (TGF)-$\beta$1 decreases tumor cells adhesion strength through inhibiting FAK activity (Zheng and Lu, 2009).

In addition, the activities of FAK in the cytoplasm, lipid rafts and non-rafts, which are also regulated by membrane fluidity, are still high in response to high shear stress. A membrane fluidity-independent mechanisms may exist in maintaining FAK activities at these three areas in response to high shear stress. FAK can interact with EGFR through its FERM-domain (Tomar and Schlaepfer, 2010). The unit of integrin or EGFR was found to promote tumor cell metastases through activating FAK/AKT pathway (Leng *et al.*, 2016). Evidence showed that low shear stress-induced EGFR activation is mainly dependent on EGF, whereas high shear stress can directly activate EGFR in a EGF-independent manner (Das *et al.*, 2011). It is speculated that high shear stress might activate EGFR, which maintains FAK activity in the lipid rafts, non-rafts and cytoplasm domain. Whereas the activity of FAK at focal adhesion is attenuated by shear stress-induced focal adhesion disassembly.

Phosphorylation of FAK at Tyr397 leads to its binding and activating Src, which can further phosphorylate and activate FAK (Hamadi *et al.*, 2005). The results show that Src is involved in the shear stress-induced FAK activation in the cytoplasm, but not membrane lipid rafts, non-rafts or focal adhesion. It may be that shear stress firstly activates membrane-bound FAK, which subsequently leads to Src activation and cytoplasmic FAK activation. Similarly, recent studies have also shown that shear stress induced both membrane lipid rafts and non-rafts of FAK activation are not affected by Src activity (Wan *et al.*, 2017).

This study demonstrates that high shear stress differentially activates FAK at specific compartments of tumor cells via membrane fluidity. It is speculated that high shear stress might increase membrane fluidity, which promotes the mobility of membrane receptors and decreases FAK activity at focal adhesion. The activity of FAK in the cytoplasm, but not in other three areas, was regulated by Src. These results imply that membrane fluidity transmits extracellular signal to FAK with different regulation mechanism. Uneven FAK activity may induce different signal transduction, and then promotes tumor cell deformation and survival under high shear stress. This research provides a novel insight for understanding the mechanism of malignant tumor cells adapting to mechanical stimuli, which may be helpful in suppressing tumor metastasis.

**Acknowledgements:** This work was supported by National Natural Science Foundation of China(11532004, 31670867, 31670961); Natural Science Foundation Project of CQ (CSTCJCYJBX0003,CSTCJCYJAX0286); The Program for Innovation Teams in University of Chongqing (CXTDX201601032) and program of Chongqing University of Science & Technology(CK2017ZKYB017, YKJCX1720201); Cooperative Project of Academician Workstation of Chongqing University of Science & Technology. We appreciate Yingxiao Wang's lab in University of California, San Diego for the kindly gifted plasmids.

**Conflict of interest:** The authors declare no conflicts of interest.

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