Fluid shear stress regulates HepG2 cell migration though time-dependent integrin signaling cascade

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ABSTRACT
Hepatocellular carcinoma (HCC) is a subtype of malignant liver cancer with poor prognosis and limited treatment options. It is noteworthy that mechanical forces in tumor microenvironment play a pivotal role in mediating the behaviors and functions of tumor cells. As an instrumental type of mechanical forces in vivo, fluid shear stress (FSS) has been reported having potent physiologic and pathologic effects on cancer progression. However, the time-dependent mechanochemical transduction in HCC induced by FSS remains unclear. In this study, hepatocellular carcinoma HepG2 cells were exposed to 1.4 dyn/cm² FSS for transient duration (15s and 30s), short duration (5 min, 15 min and 30 min) and long duration (1h, 2h and 4h), respectively. The expression and translocation of Integrins induced FAK-Rho GTPases signaling events were examined. Our results showed that FSS endowed HepG2 cells with higher migration ability via reorganizing cellular F-actin and disrupting intercellular tight junctions. We further demonstrated that FSS regulated the expression and translocation of Integrins and their downstream signaling cascade in time-dependent patterns. The FSS downregulated focal adhesion components (Paxillin, Vinculin and Talin) while upregulated the expression of Rho GTPases (Cdc42, Rac1 and RhoA) in long durations. These results indicated that FSS enhanced tumor cell migration through Integrins-FAK-Rho GTPases signaling pathway in time-dependent manners. Our in vitro findings shed new light on the role of FSS acting in physiologic and pathological processes during tumor progression, which has emerged as a promising clinical strategy for liver carcinoma.

KEYWORDS
adhesion; fluid shear stress; hepatocellular carcinoma; integrins; migration; tumor microenvironment

Introduction
Hepatocellular carcinoma (HCC, also known as malignant hepatoma) is a common subtype of liver cancer that occurs predominantly in patients with dormant chronic liver diseases and cirrhosis. The past decades have witnessed a surge in morbidity of HCC, which ranks only second to lung cancer in term of mortality nationwide. One of the major contributing causes of high mortality rate is that tumor cells are prone to be invasive and metastatic, which is initiated by tumor cell migration. Thus dampening tumor cell migration and metastasis may pave the way for tumor treatment. With the booming of cancer research, biologists have found that tumor microenvironment plays an essential role in tumor growth, invasion and metastasis. There are increasing evidences revealing that multiple mechanical forces have been involved in tumor microenvironment. Dynamic stresses presenting in all living tissues, which are caused by osmotic and hydrostatic pressure gradients among blood vessels, interstitium, and the lymphatics, can propel small fluid flows (called interstitial flows) through the extracellular matrix. Interstitial flow can give rise to fluid shear stress (FSS), which is identified as a crucial biophysical factor in cancer initiation and progression. FSS plays an important role in modulating cell behavior and coupling biophysical and biochemical signals. However, the time-dependent mechanochemical transduction in HCC induced by FSS remains poorly understood.

Present studies support that the parameter of FSS is under 0.1 dyn/cm² in normal physiologic tissue environment. It is elevated significantly in tumor microenvironment due to tumor-associated angiogenesis and lymphangiogenesis, as well as changes in the tumor stroma. FSS on tumor cell surface ranges from 0.1 to 2.0 dyn/cm². So far, no consensus has yet been reached on the effect of FSS on migration capacity in hepatocellular carcinoma cells.

As the main receptors that connect cytoskeleton with extracellular matrix (ECM), Integrins respond rapidly to
the shear stress less than 1 min, hence it is mechanosensitive. They integrate mechanical and chemical signals to regulate the activities of cytoplasmic kinases, growth factor receptors, and ion channels and control intracellular actin cytoskeleton organization. Shyy et al reviewed the role of Integrins that are involved in cell attachment and migration. Focal adhesion kinase (FAK) is located at focal adhesions and contributes to the regulation of cell motility and adhesion. At the molecular level, the dynamic rearrangement of focal adhesions may be related to spatial and temporal responses of some associated proteins, including Paxillin, Talin and Vinculin. Phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B, PKB), which serves as significant downstream molecules, can be activated by shear stress in 15 seconds. In this regard, PI3K/Akt is of great value in modulating migration and adhesion. The SH2 domains phosphorylation of the p85 subunit binding to FAK at Y397 may activate the p110 catalytic subunit in PI3K-AKT signal pathway. Then activated AKT is recruited to the plasma membrane through direct contact of its pleckstrin homology domain with phosphatidylinositol triphosphate, and is phosphorylated at Thr308 and Ser473. Rho family is composed of Cdc42, Rac, and RhoA, which is correlated with cell adhesion and migration in the down-stream signaling of Integrins-FAK. RhoA facilitates cell contractility, focal adhesions, and actin stress fiber formation; Cdc42 regulates filopodia formation; and Rac functions at membrane ruffling and lamellipodia formation respectively. However, the time-dependent mechano-chemical transduction and functional manner of Integrins in cancer cells that are exposed to the FSS remain unclear. To work out this question, we exert 1.4 dyn/cm² FSS with different durations as shown in Table 1), 5 min, 15 min, 30 min (defined as short duration as shown in Table 1), 1h, 2h, 4h (defined as long duration as shown in Table 1).

**Materials and methods**

**Cells and cell culture**

HepG2 cells were obtained from the Institute of biochemistry and cell biology (Shanghai, China). HepG2 cells were cultured in DMEM high glucose culture media (Gibco BRL) with 10% fetal bovine serum (FBS, Gibco BRL), 100 U/ml penicillin and streptomycin (Beyotime Institute of Biotechnology), and grown at 37 °C in 95% air and 5% CO₂.

**Loading condition of FSS**

Until 90% confluence on the glass slides, the HepG2 cells were exposed to a stable uniform 0.14 Pa (1.4 dyn/cm²) FSS. A peristaltic pump (Masterflex model7518–10, Cole-Parmer Instrument Company) was used to drive a stable FSS in the system. The flow experiments were performed by using the parallel plate flow chamber with modifications, so that multiple slides could be loaded simultaneously. The system was kept constantly by gassing with 5% CO₂, 95% air and the temperature was maintained in a 37°C water bath. The HepG2 cells were exposed to FSS for 15s, 30s (defined as transient duration as shown in Table 1), 5 min, 15 min, 30 min (defined as short duration as shown in Table 1), 1h, 2h, 4h (defined as long duration as shown in Table 1).

**Cells migration–scratch wound healing assay**

The HepG2 cells were cultured on the slides until 90% confluence, and then cells were starved without serum overnight in the CO₂ incubator. A cross wound region was created by scraping cells off the surface with a plastic scraper. The slides after scraping were rinsed with PBS buffer. After exposure to 1.4 dyn/cm² FSS for 1h, 2h and 4h, the images were acquired and analyzed, respectively.

**Western-blot analysis**

HepG2 cells with almost 90% confluence were exposed to 1.4 dyn/cm² FSS with different durations. The cells were washed twice with PBS on the ice, and then harvested in 120 μL RIPA buffer, containing 1% Phenylmethanesulfonyl fluoride (PMSF) and

| Identification codes | Exposure duration to shear stress (min) | Fluid shear stress (dyn/cm²) |
|----------------------|----------------------------------------|----------------------------|
| Control group        | —                                      | —                          |
| Transient duration   | 15s                                    | 1.4                        |
| group                | 30s                                    | 1.4                        |
| Short duration group | 5 min                                  | 1.4                        |
|                      | 15 min                                 | 1.4                        |
|                      | 30 min                                 | 1.4                        |
| Long duration group  | 1h                                     | 1.4                        |
|                      | 2h                                     | 1.4                        |
|                      | 4h                                     | 1.4                        |
Complete™ protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis). After putting cell suspension on the ice for 30 min, the lysed cells were centrifuged at 12,000 rpm at 4°C for 10 min, and the supernatant was collected. Total protein content of the lysates was determined using a bicinchoninic acid (BCA) Protein Assay kit (Pierce, Rockford). Proteins in the lysates were subjected to SDS-PAGE electrophoresis and transferred to PVDF membranes. Membranes were blocked in 3% BSA or 5% non-fat milk TBST buffer at 37°C for at least 1 h, and then incubated with the diluted primary antibody in 5% BSA TBST buffer (with dilution 1:200) at 4°C overnight. After incubation, membranes were washed at room temperature with TBST buffer and incubated with the appropriate peroxidase-conjugated secondary antibody (in 5% skim milk TBST buffer, 1:3000) for 1 h. Membranes were then rinsed with TBST buffer. Target proteins were detected using enhanced chemiluminescence (ECL). Images of bands were determined using Molecular Image ChemiDoc™ XRS+ with Image Lab™ Software (Bio-Rad Laboratories, Inc.) The tests were performed 3 times and quantification was done and analyzed by Image J 1.44p software (National Institutes of Health, USA). The endogenous control β-actin was used to guarantee the uniformity of equal loaded protein among all groups. The detailed information of primary antibodies was shown in Table 2.

### Immunofluorescence staining

HepG2 cells with almost 90% confluence were treated with FSS (1.4 dyn/cm²) after different loading duration (30s, 30 min, 4h) respectively. At each time point, the cells were washed twice with cold PBS, and fixed with 4% paraformaldehyde for 8 min at 4°C, permeabilized for 5 min with 1% (v/v) Triton X-100. Next cells were pre-incubated with 1% BSA in PBS for 15 min to decrease non-specific antibody binding and washed for 30s to 1 min with PBS. Then Cells were incubated with Talin/Vinculin, RhoA/Rac1 primary antibodies (with 1:100, Santa Cruz Biotechnology) overnight at 4°C, respectively. After primary antibody incubation, the slides were incubated with appropriate peroxidase-conjugated secondary antibody (in TBST, 1:5000) for 1 h or in BODIPY FL Phalloidin solution for 90 min (with 1:100, Invitrogen™, Table 2. The detailed information of antibodies used in this study.

| Category       | Antibody       | Isotype | Blocking Conditions 1-hr room temperature | Primary Ab incubation | Manufacturer   | Cat. No |
|----------------|----------------|---------|-----------------------------------------|-----------------------|----------------|---------|
| Integrins      | α2 (C-9)       | mouse mAb | 5% nonfat dry milk in PBS                | Overnight at 4°C,    | Santa Cruz, Inc. | sc-74466 |
|                | α5 (A-11)      | mouse mAb | 5% nonfat dry milk in PBS                | One:200 dilution     |                | sc-166665 |
|                | αV (H-2)       | mouse mAb | 5% nonfat dry milk in PBS                | Overnight at 4°C,    |                | sc-376156 |
|                | β1 (A-4)       | mouse mAb | 5% nonfat dry milk in PBS                | One:200 dilution     |                | sc-374429 |
|                | β3 (B-7)       | mouse mAb | 5% nonfat dry milk in PBS                | One:200 dilution     |                | sc-46655  |
| FA components  | Talin (8D4)    | mouse mAb | 5% nonfat dry milk in PBS                | Overnight at 4°C,    | Santa Cruz, Inc. | sc-59881 |
|                | Paxillin (D-9) | mouse mAb | 5% nonfat dry milk in PBS                | One:200 dilution     |                | sc-365174 |
|                | Vinculin (G-11)| mouse mAb | 5% nonfat dry milk in PBS                | One:200 dilution     |                | sc-55465  |
| (p)FAK         | FAK (A-17)     | rabbit pAb | 5% nonfat dry milk in PBS                | Overnight at 4°C,    | Santa Cruz, Inc. | sc-557   |
|                | pFAK (2D11)    | mouse mAb | 3% BSA in PBS                           | One:200 dilution     |                | sc-81493  |
| PI3K/AKT       | PI3K C2β (16L9)| mouse mAb | 5% nonfat dry milk in PBS                | Overnight at 4°C,    | Santa Cruz, Inc. | sc-100407|
|                | AKT1 (B-1)     | mouse mAb | 5% nonfat dry milk in PBS                | One:200 dilution     |                | sc-5298  |
|                | pAKT1 (Thr 308)| rabbit pAb | 3% BSA in PBS                           | One:200 dilution     |                | sc-135650|
| Rho GTPases    | Rac1 (C-11)    | rabbit pAb | 5% nonfat dry milk in PBS                | Overnight at 4°C,    | Santa Cruz, Inc. | sc-95    |
|                | pRac1 (Ser 71) | rabbit pAb | 3% BSA in PBS                           | One:200 dilution     |                | sc-12924-R|
|                | RhoA (26C4)    | mouse mAb | 5% nonfat dry milk in PBS                | Overnight at 4°C,    |                | sc-418   |
|                | Cdc42          | rabbit mAb | 5% nonfat dry milk in PBS                | One:100 dilution     | Abcam®, Inc.   | ab64533  |
USA). DAPI (4′,6′-diamidino-2′-phenylindole dihydrochloride, Roche Diagnostics GmbH, Germany) (with 1:800) was subsequently added for nuclei staining for 30 min. Each step was followed by washing with PBS 3 times. Using a laser scanning confocal microscope (Leica TCS SP5), cell morphologies and intercellular gaps that indicated endothelial permeability were examined.

The secondary FTIC-conjugated goat anti-mouse immunoglobulin (IgG), PE-conjugated goat anti-rabbit IgG were purchased from Biosynthesis biotechnology Co., LTD. The BODIPY FL Phallacidin (Invitrogen) was used for F-actin staining.

**TEM analysis**

For transmission electron microscope (TEM) observation, samples were treated with FSS after 1h, 2h, 4h, respectively, and harvested gently by a plastic cell scraper (Corning) to maintain original and intact intercellular junctions. The samples were centrifuged at 1,500 rpm for 10 min, fixed with 0.5% glutaraldehyde, and stored at 4°C for 10 min. Subsequently, the samples were centrifuged again at 12,000 rpm for 10 min; the supernatant was discarded and 3% glutaraldehyde was added slowly. All samples were fixed by 1% OsO4, dehydrated by different concentration of acetone and embedded by epoxy resin (Epon812), then these samples were cut into slices and doubled dyed with uranyl acetate and lead citrate. The micro-structure of intercellular junctions was observed under TEM (H-600IV).

**Statistical analysis**

Data obtained from this study was manifested as mean ± SD. Data represented for at least 3 independent experiments. To reveal differences among the groups, one-way ANOVA followed by Tukey’s test was used. *P* values of < 0.05 were regarded as significance.

**Results**

**FSS promotes the migration ability of HepG2 cells**

The FSS-induced HepG2 cells migration was examined by a scratch wound healing assay. As shown in Fig. 1, HepG2 cells were able to migrate under static condition without the stimulation of FSS. However, the cells have showed strikingly increased migration ability, which were exposed to FSS after 2h and 4h (Fig. 1A). With the stimulation of FSS, the gaps between the leading edges of the cells are narrower, associated with a faster migration speed (Fig. 1B). In conclusion, FSS results in faster rate of HepG2 cell

![Figure 1](image-url)

Figure 1. Effects of FSS on HepG2 cell migration and F-actin reorganization. (A) Images of scratch wound migration under static occasion and FSS exertion (1.4 dyn/cm²), respectively; Black dotted lines indicate the location of wound edges at the beginning of the experiment. (B) The statistical image of migration distance at different time. *P* < 0.05 is regard as significant. (C) The impact on FSS-induced cytoskeleton and F-actin rearrangement in HepG2 cells. The actin-based cytoskeletal structure is shown in green. The white arrows show the junctions and gaps among cells, and the red arrows indicate cytoskeleton emerged filamentary structure. Scale bar in images = 10 μm.
migration for wound healing. In addition, the direction of HepG2 cell migration is related to exposed FSS direction. The red arrow indicates that cells in upstream region, whose migration direction is parallel with FSS direction, have a faster speed than those in downstream area, whose migration direction is vertical to FSS direction (Fig. 1A, down panel).

The adhesion-associated actin cytoskeleton remodels instantly in response to dynamics alteration, adhesion reinforcement or strengthening is the widely-studied cases.\textsuperscript{14} Interestingly, we found that the elongation and alignment of the flow direction in HepG2 cells induced by FSS (Fig. 1C) and the F-actin stress fibers were remodeled in accordance with flow direction. The Fig. 1C showed clearly visible F-actin stress fibers with distinct orientation. One hour later, stress fiber had a rearrangement. Additionally, the formation and alignment of thick stress fiber were observed clearly after 4h. The results indicated that cytoskeletal arrangement and its translocation occurred drastically as FSS exerted on HepG2 cells. Furthermore, it can be found that HepG2 cells had a time-dependent alteration in cell morphology from polygon-shape to spindle-shape and become uniformly oriented to flow. Disrupted intercellular junction and enlarged gaps could be observed after 2h mechanical loading (Fig. 1C).

**FSS induces the morphological changes of cell-cell junction in HepG2 cells**

For intravasation and extravasation, cells are subjected to dramatic elastic deformations to transmigrate through endothelial cell-cell junctions.\textsuperscript{15,16} In this regard, malignant tumor cells must undermine intercellular junctions and cell-extracellular matrix to disassociate from primary carcinoma for metastasis. We found that the expanded distance (red arrows indicated in Fig. 2A) is positive correlated with longer mechanical loading. The expression level of ZO proteins and Claudins decreased after persistent FSS exertion (Fig. 2B). In particular, the expression level of ZO-1 only had a transient increase after exposed to FSS at 15s. Subsequently its expression decreased until 4h. Compared to ZO-1, the expression level of

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**Figure 2.** TEM images show the effect of FSS(1.4 dyn/cm\textsuperscript{2}) exerted on intercellular junctions. (A) The altered morphology of intercellular junctions after FSS loading. The red arrows in Fig. 2A show the cell-cell junctions and gaps in HepG2 cells. Scale bar in images = 5μm. (B) Representative western blot results of tight junction proteins and quantification of those proteins expression level are showed in Fig. 2B. The intrinsic control β-actin is used to guarantee the uniformity of equal loaded protein among all groups. Values represent the Mean ± S.D from 3 independent experiments. *, P<0.05 is regard as significant.
Claudin-5 decreased sharply from the beginning to the end. As a result, it demonstrated that FSS may disrupt the intercellular junctions in HepG2 cells.

**FSS induces time-dependent expression pattern of Integrins**

To investigate whether Integrin signaling is involved in the process of FSS induced HepG2 cell migration, we further detected the expression of Integrin subunits $\alpha_2$, $\alpha_5$, $\alpha_v$, $\beta_1$ and $\beta_3$, respectively.

The cells with exposure to FSS had expression level changes of different Integrin subunits $\alpha$ (Fig. 3A) and $\beta$ (Fig. 3B) according to their different functions responded to FSS. The expression of Integrin $\alpha_2$ increased at 15s, 30s immediately, and then reduced in short duration and 2 experimental time nodes in long duration (1h and 2h). Nonetheless, the expression of Integrin $\alpha_2$ reached the peak value at 4h. Similar to Integrin $\alpha_2$ subunit, the expression of Integrin $\alpha_5$ increased at 15s and 2h remarkably. In contrast, the expression of Integrin $\alpha_v$ subunit decreased during the whole time after FSS exposure. This evidence indicates that FSS can activate $\alpha_2$ and $\alpha_5$ subunit which results in increased ligand affinity and downstream signaling molecules recruitment during transient and long duration. Meanwhile our results also suggest that $\alpha_v$ subunit does not function significantly in mechanical signaling transduction process (Fig. 3A). The expression of $\beta_1$ subunit increased sharply at 15s, 5min and 1h respectively. In the case of $\beta_3$, it increased at 30s and 30min, associated with significant decrease in long duration. It suggests that as a transporter of mechanical signal, $\beta_1$ subunit plays an indispensable role in different time duration when cells are exposed to FSS (Fig. 3B).

In conclusion, these results illustrate that FSS is responsible for modulating the expression of Integrin $\alpha$.

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**Figure 3.** FSS regulates the expression of integrin $\alpha$ and $\beta$ subunits in HepG2 cells. (A) The expression level of integrin $\alpha_2$, $\alpha_5$, $\alpha_v$ in HepG2 cells treated with FSS and quantification of each integrin $\alpha$ subunits expression level by image analysis of western blot bands. *, $P<0.05$ denotes significantly statistical difference compared with other groups. (B) The expression level of integrin $\beta_1$ and $\beta_3$ in HepG2 cells treated with FSS and quantification of each integrin $\beta$ subunits expression level via image analysis of western blot bands. $\beta$-actin in each group is used as intrinsic controls, and relative expression of integrins is calculated. Values represent the Mean ± S.D from 3 independent experiments. **, $P<0.05$ is regard as significant.
and β subunits and their time-dependent expression manner that may contribute to cancer cell migration and invasion.

**FSS induces change of focal adhesion kinase (FAK) expression and phosphorylation level**

We further explored the effects of FSS on the expression and phosphorylation state of FAK by Western blots. We found that the total expression of FAK reduced after mechanical stimulation (Fig. 4A). Meanwhile, the ratio of pFAK/ tFAK obviously downregulated as same as the total expression of FAK. It implicates that FSS alters the focal adhesion through down-regulating the phosphorylation level of FAK, since the decreased FAK phosphorylation state is capable of degrading focal adhesions (FAs) arrangement. To validate this hypothesis, we investigated the expression and translocation of representative FA components including Paxillin, Vinculin and Talin (Fig. 4B and C).

The expression level of Paxillin reduced at 15s transiently but significantly increased from 30s to 15min, which had a subsequent recession during 30min to 4h. There was always lower expression of Paxillin compared with control group (P < 0.05, Figure 4B). We observed that the expression of Vinculin was continuously reduced in contrast to control group (P < 0.05, Fig. 4B). Whereas, the expression of Talin downregulated from 15s but upregulated at 2h and then decreased again. Given the results of Western blot, it demonstrated that FA complexes were disassembled in response to FSS, which enhanced cell motility and disrupted adhesion.

To further explore whether FSS affect the translocation of FA components, the double-labeled immunofluorescence analysis was performed (Fig. 4C). There are multiple spot-shaped structures dispersed around the cell membrane in static condition (As the white arrow indicates in Fig. 4C). The expression level of Talin shows a distinct downregulation when exposed to FSS (Fig. 4B) with a decreasing number of spots. Similarly, the Vinculin expression is similar to Talin (Fig. 4C). In addition, spotted structures in cells disappeared when exposed to FSS for 4h (Fig. 4C), which suggests that FSS may downregulate the expression of Talin and Vinculin to disassemble FAs for strengthening cell motility eventually.

**FSS down-regulates the phosphorylation level of AKT**

As downstream molecules of FAK, the phosphatidylinositol 3-kinase (PI3K)/AKT (protein kinase B, PKB) have been found involved in regulating cell physiologic process such as cell proliferation, differentiation, and apoptosis. Herein, we suspected that the PI3K-(p)AKT signaling contributed to migration and adhesion of HepG2 cells exposed to FSS. The results demonstrated that the expression of PI3K was inconsistent with FAK in response to FSS (Fig. 5A). FSS-induced PI3K was upregulated promptly at initial 15s, and then an obvious decrease was observed at 4h. Analogously, the expression of AKT had a significant increase in FSS initiation (15s, 15min and 1h). It is noteworthy that AKT shows a low expression level at other time points (Fig. 5B). The statistical analysis shows a significant difference of pAKT/tAKT at 30s, 5 min in comparison to other groups during FSS duration (P < 0.05, right panel in Fig. 5B).

**Time-dependent expression and translocation of Rho-GTPases during FSS loading**

The temporal expression changes of Rho family members were also detected in our research (Fig. 6A). The expression level of RhoA did not show an increase until 2h, meanwhile Cdc42 increased at 30min. Intriguingly, Rac1 was activated initially at 15s, 30s. With longer duration of FSS stimulation, Rho family members manifested a significant increase at 4h. The difference of Rho-GTPases time-dependent expression indicates that various mechanisms of FSS-mediated cell migration have been involved in HepG2 cells.

To investigate the relationship between the distribution of Rho family molecules and FSS loading, RhoA and Rac1 were detected by double-labeled immunofluorescence. As a result, Rac1 and RhoA was distributed around the cytoplasm under static condition. When exposed to FSS, these proteins began to translocate to the plasma membrane with higher expression level (Fig. 6B). The result was consistent with the western blot analysis of RhoA and Rac1 (Fig. 6A).

**Discussion**

Recently hepatocellular carcinoma (HCC) has already become the second leading cause of cancer-related mortality.\(^{17}\) It is widely acknowledged that in early stage there are several potentially curative treatments including local ablation, resection and liver transplantation. However, a myriad of HCC patients have been diagnosed in advanced stage with a 5-year survival rate of < 5%. Thus promising therapeutic applications need to be urgently provided for HCC treatment.

The intricate microenvironment is composed of several cells, cytokines, growth factors and some proteins.\(^{18}\) Numerous findings have elucidated that multiple biochemical signals may contribute to HCC...
Figure 4. FSS regulates the expression and translocation of FAs through down-regulating the ratio of pFAK/tFAK. (A) The impact of FSS exerted on expression of tFAK and pFAK in hepatoma cells and quantification of phosphorylated FAK in total FAK expression by image analysis of Western blot bands. (B) The expression level of Vinculin, Talin and Paxillin in hepatoma cells treated with FSS and quantification of each protein expression level by image analysis of western blot bands. The expression of β-actin in each group was used as intrinsic controls and relative expressions of FAs proteins were calculated. Values represent the Mean ± S.D from 3 independent experiments. (C) FSS exerted influence on protein expression of Talin (green), Vinculin (red) detected by immunofluorescence analysis. Scale bar = 15 μm. *P < 0.05 is regard as significant.
progression. In addition, prevalent studies have provided evidences for mechanical factors involved in the tumor microenvironment, which is thought to promote tumor cell invasion and metastasis. However, the role of FSS in cancer progression remains unclear due to the difficulty of velocity measuring for interstitial flow. The velocity of interstitial flow ranges from 0.1 to 1.0 mm/s in normal tissues. It is elevated significantly in tumors that results from tumor-associated angiogenesis and lymphangiogenesis, as well as the alterations in tumor stroma. Elevated interstitial flow in the tumor microenvironment is likely due to the increased tumor interstitial fluid pressure (IFP). No consensus has yet been reached on accurate velocity of interstitial flows with the difficulties in measuring interstitial flows velocity in neo-plastic tissues. Recent studies have reported that tumor cell are exposed to the value of 0.1–2.0 dyn/cm² FSS. Cell migration plays a pivotal role in tumor metastasis as well as in many other physiologic procedures including embryogenesis, immune response, and wound healing and homeostasis maintenance. Inhibition of cancer cell migration and subsequent metastasis is served as an effective clinical strategy for cancer treatment. Herein we exert 1.4 dyn/cm² FSS on HepG2 cell for different time durations, which shows that HepG2 cells acquire an enhanced migration ability (as shown in Fig. 1)

The Integrins recruitment is found crucially involved in a variety of cell physiologic activities, such as migration and adhesion. The extracellular domain of Integrins can interact with specific extracellular matrix ligands including Fibronectin, Vitronectin, and Collagen. As a downstream signaling factor of Integrins, FAK is recruited to the Integrins intracellular domain. Studies have demonstrated that cells derived from FAK−/− mouse embryos exhibit reduced migration ability. Conversely, cells with overexpression of FAK show increased motility compared with wild-type cells. Associated with small GTP protein, Integrin-mediated FSS functions in regulating expression profile of genes which are related to cell junctions. The Integrins are activated and aggregated on the cell membrane within 1 minute and can last to 6 hours after FSS stimulation. Nonetheless, time-dependent expression patterns of Integrin-FAK-Rho GTPases signaling axis in HepG2 cells migration and adhesion still remains poorly understood. In this study, we found that FSS was able to promote the migration ability of HepG2 cells via wound healing assay. Different Integrin subunits is capable of sensing FSS and modulating the biologic behaviors of cell migration according to different time duration (as shown in Fig. 3). Furthermore, the disruption of intercellular junctions and cell-matrix adhesions was also observed in our research. In comparison to HepG2 cells in static condition, the elevated migration distance between 2 cells is
identified in a time-dependent manner during FSS loading. Cancer cell migration is regulated by actin dynamics accompanied with the polymerization and depolymerization of actin filaments. The longer FSS exerted on, the clearer visible F-actin stress fibers were observed in HepG2 cells with a defined mechanical direction (As shown in Fig. 2). Ishida et al.\textsuperscript{29} have demonstrated that the phosphorylation level of FAK increased at 30min

**Figure 6.** The expression and translocation of small G-protein in HepG2 cell exposed to FSS. (A) The expression level of small Rho GTPases in HepG2 cells stimulated by FSS and quantification of each protein expression level via image analysis of Western blot bands. The expression of β-actin in each group was used as intrinsic controls, and relative expressions of Rho GTPases were calculated. Values represent the Mean ± S.D from 3 independent experiments. (B) The immunofluorescence analysis of FSS influencing Rac1 (green) and RhoA (red) expression. Scale bar=15μm. *$P < 0.05$ is regard as significant.
and lasted to 2h in endothelial cells induced by FSS. We found that compared with static condition, FAK expression reduced at 15s treated with FSS but augmented significantly at 4h, which illustrated that FAK responded to FSS in HepG2 cell is delayed compared with endothelial cells. It can be hypothesized that the phenomenon results from different microenvironments that generate diverse intensity of FSS exerted on 2 types of cells. Similarly, the activity of PI3K is induced by FSS at 15s with an obvious recession to resting level after 1 min exposure to 5 dyn/cm² FSS.³⁰ Herein the activation of PI3K increased at 15s and can be sustained to 4h, which supports the longer activation of PI3K in HepG2 cells. As a crucial downstream molecule responded to PI3K, Akt is activated at 30min and sustained to 6h in endothelial cells exposed to 15 dyn/cm² FSS.³¹ In terms of HepG2 cells, we corroborate that the activation of Akt can be stimulated by 1.4 dyn/cm² FSS at 15s and maintain to 1h.

Cytoskeletal proteins including Talin, Vinculin have a much broader functionality in mediated ligands binding of cell adhesions.³² Talin is an actin-crosslinking protein that binds to the cytoplasmic domains of Integrin β1, β2 and β3 in vitro.³³ Multiple researches have highlighted the importance of Talin in inducing the formation and degradation of focal adhesion.³⁴ Served as a binding partner of Talin, more and more evidences indicate that Vinculin is more than a simple linker protein but also involved in cell motility. Cells lacking of Vinculin are highly motile due to fewer FAs, which illustrates the central role of Vinculin in cell motility modulation. Conversely, cells with overexpression of Vinculin are prone to be less motile with enhanced FAs.³⁵ Paxillin is an adaptor protein with important functions in matrix adhesion assembly, and binds to Talin simultaneously when it is incorporated into αvβ3-containing focal complexes.³⁶ Our results showed that the expression level of FAs protein significantly downregulated with the increased expression of FAK (as shown in Fig. 4).

Current findings suggest that Ras superfamily is involved in a variety of cell physiologic activities. Particularly, the GTPases RhoA, Rac1, and Cdc42 are known to regulate cell shape through exerting mainly impacts on cytoskeleton. Moreover, these molecules can also influence cell polarity, microtubule dynamics, and transcription factor activity.³⁷ The phosphorylation of FAK regulates the activation of small GTPases, which results in controlling the directional cell migration. It is demonstrated that Cdc42 is enhanced promptly by FSS in transient time (initiated at 1 min, peaked at 5 min, and then reduced at 15 min). Meanwhile RhoA is subjected to longer activation (initiated at 1 min, reach to highest level at 2 h), which is identified by their translocations from cytosol to membrane. Interestingly, FSS-induced Rac1 is upregulated at 30s and maintained to 4h. Similar result was supported by Tzima et al.³⁸ that the activations of Rac1 and Cdc42 were more significant at the downstream side of the flow direction, and lasted for at least 120 min.

Tumor progression is composed of tumor cell migration, invasion and metastasis, which induces poor prognosis and survival rate. There are a myriad of complex signaling events related to mechanical-chemical microenvironment participating in cancer progression. Our results revealed that Integrins-FA(s)-FAK-PI3K(AKT)-Rho GTPases signaling pathway was involved in regulating adhesion and migration in HepG2 cell. Several major proteins in this pathway display different time-dependent manners, suggesting diverse contributions of signal molecules to HepG2 cell migration at different durations. In tumor microenvironment, cancer cell is influenced by chemical and mechanical factors, especially, it is indicated that FSS can enhance the invasive ability of tumor cells. Herein, we discussed new insights into FSS-regulated expression and translocation of Integrins and their downstream signaling cascade in a time-dependent manner, which implicates novel approaches to a promising clinical strategy for liver carcinoma.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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