

Original article

Title: Cyclic hydrodynamic pressure induced proliferation of bladder smooth muscle cells via integrin $\alpha 5$ and FAK

Running title: Pressure induces HBSMCs proliferation via integrin $\alpha 5$ -FAK

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Summary

According to previous studies, integrins play an important role in the mechanotransduction. The aim of this study was to examine the role of integrin subunits and its down-stream signaling molecules in the cyclic hydrodynamic pressure-induced proliferation of human bladder smooth muscle cells (HBSMCs) cultured in scaffolds. The HBSMCs cultured in scaffolds were subjected to four different levels of cyclic hydrodynamic pressure for 24 hours, which were controlled by a BOSE BioDynamic bioreactor. Flow cytometry was used to examine cell cycle distribution. Real-time RT-PCR and western blotting were used to examine the expression levels of integrin subunits and their downstream signaling molecules. Integrin $\alpha 5$ siRNA was applied to validate the role of integrin $\alpha 5$ in cell proliferation. Here, we showed that cyclic hydrodynamic pressure promoted proliferation of HBSMCs. The cyclic hydrodynamic pressure also increased expression of integrin $\alpha 5$ and phosphorylation of FAK, the key mediator of integrin $\alpha 5$ signaling, but not that of integrin $\alpha 1$, $\alpha 3$, $\alpha 4$, αv , $\beta 1$ and $\beta 3$. Moreover, inhibition of integrin $\alpha 5$ decreased the level of p-FAK and abolished proliferation of HBSMCs stimulated by cyclic hydrodynamic pressure. Taken together, we demonstrate for the first time that the integrin $\alpha 5$ -FAK signaling pathway controls the proliferation of HBSMCs in response to cyclic hydrodynamic pressure.

Key words: hydrodynamic pressure; proliferation; human bladder smooth muscle cells

Introduction

Mechanical stimuli are the major regulatory factor of normal tissue morphology and function, and an important determinant factor for cell or tissue fate under physiological and pathological conditions (Kessler et al. 2001; Zhou et al. 2005). Particularly, smooth muscle (SM)-rich tissues, such as the bladder smooth muscle, are constantly exposed to mechanical stimuli arising from fluctuations in cyclical hydrodynamic pressure, strain and periodic stretching. Breakdown of force equilibrium within these tissues occur often as a result of obstructive diseases or pressure overload triggered by pathophysiological changes, such as hypertrophy, hyperplasia, and various degrees of fibrosis (Chang et al. 2009; Koitabashi et al. 2011; Zhang et al. 2004). In response to mechanical stimuli, smooth muscle cells have altered expression of cell surface molecules, such as integrins, which act locally to orchestrate wide molecular and cellular changes.

Integrins are heterodimers of associated subunits of α and β , which include 18 α and 8 β subunits that can assemble into 24 different receptors with different binding properties and different tissue distribution (Barczyk et al. 2010; Hynes 2002). A lot of data showed that the mechanical signals transduced by integrin-mediated signaling pathways, which modulate various cellular functions, including growth and proliferation, cytoskeletal organization and motility, ion channel function, gene and protein expression, and extracellular matrix production and autocrine secretion (Engers and Gabbert 2000; Gerthoffer and Gunst 2001; Hood and Cheresch 2002).

Activation of specific integrins has been documented in different cells upon exposure

to various mechanical stimuli. For example, cyclic stretch up-regulated $\alpha\text{v}\beta\text{3}$ in mice and rat vascular smooth muscle cells (VSMCs) (Chen et al. 1999; Cheng et al. 2007), while cyclic strain induced an association of $\alpha\text{1}\beta\text{1}$ in mice VSMCs (Pyle et al. 2008). Wilson showed that cyclic strain induced an association of both β3 and $\alpha\text{v}\beta\text{5}$ integrins in rat VSMCs (Wilson et al. 1995). The mechanisms of mechanotransduction involved in integrin signal pathway in the other cell types, such as VSMC and osteocyte, have been fully illustrated (Davis 2010; Jacobs et al. 2010; Shi and Tarbell 2011). However, the role of integrins in proliferation of human bladder smooth muscle cells (HBSMCs) is not well defined. In addition, most studies used bladder smooth muscle cells isolated from animals such as rat and rabbit to investigate cellular response to mechanical stimuli (Deng et al. 2007; Stover and Nagatomi 2007). It is necessary to validate these studies using human bladder smooth muscle cells.

In this study, we treated HBSMCs with cyclic hydrodynamic pressure, which is different from mechanical stretch and hydrostatic pressure. By analyzing the expression of integrins upon cyclic hydrodynamic pressure exposure, we identified integrin α5 as the key molecules that mediated the signaling pathway induced by cyclic hydrodynamic pressure in HBSMCs.

Materials and methods

Materials

HBSMCs (ScienCell, USA, Cat.No.4310), polyvinyl alcohol based scaffolds (BOSE, BioDynamic, USA), the computer-controlled bioreactor (BOSE, BioDynamic, USA), EPICS ELITE ESP flow cytometer (Beckman Coulter, FL), RNAiso Plus reagent

(TaKaRa, Dalian, China), iScript cDNA Synthesis Kit (Bio-Rad, USA), SYBR Premix EX Taq premix reagent (TaKaRa, Dalian, China) and Bio-Rad iQ5 machine, RIPA buffer (Beyotime, China), PVDF membranes (Millipore, USA), the GAPDH antibody, Phospho-FAK (Tyr397) antibody, FAK antibody and integrin $\alpha 5$ Antibody (Cell Signalling Technologies, CST, USA), Lipofectamine2000 reagent and scrambled siRNA (Invitrogen, USA).

Cell culture

HBSMCs were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) at 37 °C in a humidified atmosphere of 95% air/5% CO₂. Experiments were performed on cells between generations 3 and 7.

Cyclic hydrodynamic pressure

HBSMCs were seeded onto polyvinyl alcohol based scaffolds (BOSE, BioDynamic, USA) contained in Petri dishes (1×10^5 cells/piece). After low medium starving or siRNA transfecting, HBSMCs in scaffolds were transplanted into the computer-controlled bioreactor. The HBSMCs were then subjected to cyclic hydrodynamic pressure simulating bladder cycle (2 hour/cycle, increasing from 0 cm H₂O to 10 cm H₂O at first 1.75 h, then up to 100 cm H₂O, 200 cm H₂O, or 300 cm H₂O rapidly and maintaining for 0.25 h, lowering to 0 cm H₂O at last) for up to 24 h. HBSMCs in the control groups were maintained under static conditions.

Cell transfection

A siRNA specific for integrin $\alpha 5$ (UGGCUCAGACAUCGAUCC) siRNA was

transfected by using Lipfectamine™ 2000 reagent according to the manufacturer's protocol. For each transfection, Real-Time PCR was used to assess integrin $\alpha 5$ expression. The control groups were transfected by scrambled siRNA.

Semiquantitative reverse transcription-PCR and real-time PCR

Total RNAs were isolated from HBSMCs using RNAiso Plus reagent according to the manufacturer's instructions. Total RNAs were reversed transcribed using iScript cDNA Synthesis Kit and amplified by PCR using primers for each integrin as follows;

$\alpha 1$: forward: 5'-GGTTCCTACTTTGGCAGTATT-3', reverse: 5'-AACCTTGTCTGATTGAGAGCA-

$\alpha 3$, forward: 5'- AAGGGACCTTCAGGTGCA-3', reverse: 5'- TGTAGCCGGTGATTTACC AT-3'

$\alpha 4$, forward: 5'- GCTTCTCAGATCTGCTCGTG-3', reverse: 5'- GTCACTTCCAACGAGGTTTG-

$\alpha 5$, forward: 5'- TGCAGTGTGAGGCTGTGTACA-3', reverse: 5'- GTGGCCACCTGACGCTCT-3';

αV , forward: 5'- AATCTTCCAATTGAGGATATCAC-3', reverse: 5'- AAAACAGCCAGTAGCAACAA

$\beta 1$, forward: 5'- GAAGGGTTGCCCTCCAGA -3', reverse: 5'- GCTTGAGCTTCTCTGCTGTT-3

$\beta 3$, forward: 5'- CCGTGACGAGATTGAGTCA -3' reverse: 5'- AGGATGGACTTTCCACTAGAA

Real time PCR was assayed with Bio-Rad iQ5 machine. Normalization of samples was achieved by measurement of the endogenous reference gene, GAPDH. All reactions were run in triplicate, and the mean value was used to calculate the ratio of target gene/GAPDH expression in each sample.

Western blotting analysis

HBSMCs were collected, washed twice in PBS, and lysed in RIPA buffer for 30 min at 4°C, and then centrifuged at 15,000 \times g for 30 min at 4°C. The resultant supernatant (30 mg protein/lane) was subjected to SDS-PAGE on 7.5% polyacrylamide gels, and

the separated proteins were transferred electrophoretically onto a PVDF membrane. Western blotting analysis was performed as described previously (Tanaka S, Nemoto K, Yamamura E, Ichikawa A. Intracellular localization of the 74- and 53-kDa forms of L-histidine decarboxylase in a rat basophilic/mast cell line, RBL-2H3. *J Biol Chem* 1998, 273:8177–82). Membranes were incubated with specific antibodies. The antibodies used for western blotting were β -actin, integrin α 5, p-FAK (Thr397) and FAK. The membranes were stained using an ECL kit according to the manufacturer's instructions. Protein concentrations were determined by Bradford Ultra reagent using bovine serum albumin for the standards.

Cell cycle analysis by flow cytometry

Flow cytometry was used to assess cell growth and to determine the percentage of cells in various phases of the cell cycle. After HBSMCs were treated, they were re-suspended in 1 ml of Propidium Iodide (PI) staining solution (20 ug/ml PI/ 10 Units/ml RNase One-Promega in 1X PBS) at a density of 10,000 cells/ml for 20 min in the dark at room temperature. Then the samples were examined using an EPICS ELITE ESP flow cytometer and data was analyzed using FCS Express V2 (De Novo Software, Thornhill, Ontario, Canada). A minimum of 10,000 cells were collected in list mode files. PI histograms were used to analyze the cell cycle.

Statistical analysis

All results were expressed as means \pm standard deviation (M \pm SD). Statistical analysis was performed using Student's t test, and $p < 0.05$ was considered statistically significant.

Results

Cyclic hydrodynamic pressure increased integrin $\alpha 5$ expression level of HBSMCs cultured in vitro

To investigate which integrin subunit in HBSMCs was changed under hydrodynamic pressure, the genetic expression of integrin subunits $\alpha 1$, $\alpha 3$, $\alpha 4$, $\alpha 5$, αv , $\beta 1$ and $\beta 3$ were measured by RT-PCR. The data showed that only integrin $\alpha 5$ expression increased in groups of 200 and 300 H₂O cm groups, whereas integrin subunits $\alpha 1$, $\alpha 3$, $\alpha 4$, αv , $\beta 1$ and $\beta 3$ were not greatly affected by cyclic hydrodynamic pressure. Compared to 0 H₂O cm group, the HBSMCs in 200 and 300 H₂O cm groups had higher integrin $\alpha 5$ expression ($p < 0.05$) in (**Fig. 1A**). At protein level, integrin $\alpha 5$ expression significantly increased in both 200 and 300 H₂O cm groups with comparison to static control group ($p < 0.05$), but no significant difference was found between 100 H₂O cm group and static control group (**Fig. 1 B, C**).

Cyclic hydrodynamic pressure increased p-FAK (Tyr397) level of HBSMCs cultured in vitro

To identify the downstream signal molecular of integrin, the p-FAK and FAK was assessed using RT-PCR. Similarly as expression of integrin $\alpha 5$, the p-FAK level significantly increased in both 200 and 300 H₂O cm groups ($p < 0.05$), but there was no difference with 100 H₂O cm group compared to static control group (**Fig. 2**).

Integrin modulate the hydrodynamic pressure induced-proliferation of HBSMCs through the p-FAK/FAK ratio

To clarify the role of integrin $\alpha 5$ and FAK signal pathway in proliferation of HBSMCs

promoted by hydrodynamic pressure, the HBSMCs were treated with integrin $\alpha 5$ siRNA. 200 cm H₂O was chosen as magnitude of pressure for this study. Compared to the scrambled siRNA control, the expression of integrin $\alpha 5$ was remarkably suppressed by target siRNA transfection in both pressure and static groups (**Fig. 3**), and cell proliferation activity induced by cyclic hydrodynamic pressure was inhibited. In pressure groups, cell proliferation index decreased from (50.93 \pm 5.14)% to (23.99 \pm 3.46)% ($p < 0.05$); while in static groups, cell proliferation index decreased from (28.81 \pm 3.38)% to (26.02 \pm 5.33)% ($p > 0.05$) (**Fig. 4**).

After mRNA of integrin $\alpha 5$ was inhibited by targeted siRNA, p-FAK level was greatly abolished (Fig. 5), and the proliferation was also suppressed (**Fig. 4**). Compared to the scrambled siRNA control, p-FAK /FAK ratio greatly decreased ($p < 0.05$) in 200 H₂O cm groups, but p-FAK/FAK ratio slightly dropped ($p > 0.05$) in static groups (**Fig. 5**). Under cyclic hydrodynamic pressure, the level of integrin $\alpha 5$, p-FAK /FAK ratio and proliferation of HBSMCs had parallel relationship with each other. That is, cyclic hydrodynamic pressure promoted proliferation of HBSMCs via integrin $\alpha 5$ -FAK signaling transduction pathway. FAK was responsible for downstream signaling transduction of integrin $\alpha 5$.

Discussion

In relatively fixed period of time, normal urinary bladder experiences the cycle of emptying-filling. In this process, bladder smooth muscle cells undertake cyclic hydrodynamic pressure. This present study simulated the cyclic bladder physiological pressure based on the modified BOSE BioDynamic system, which applied cyclic

hydrodynamic pressure simulating bladder cycle to HBSMCs cultured on scaffold. (2 h /cycle: increased from 0 cm H₂O to 10 cm H₂O at first 1.75 h, followed by increase to 100 cm H₂O, 200 cm H₂O, or 300 cm H₂O rapidly and maintained for 0.25 h, then decreased to 0 cm H₂O). These parameters used in our experiment based on our previous studies (Chen et al. 2012; Wu et al. 2012). Pressures applied to HBSMCs in this study were higher than physiological bladder pressure (<40 cm H₂O) in vivo. The possible explanations are that, the pressure in native bladder is dynamic not continual hydrostatic pressure during filling and voiding, and the same pressure induces weaker biological effects on cells in vitro compare to that in vivo. As the bladder detrusor experience cyclic dynamic pressure in vivo, cyclic hydrodynamic pressure is optimal to physiological condition of HBSMCs in vitro.

Integrins are a family of cell-surface receptors, which transduce mechanical signals (Chowdhury et al. 2006; Friedland et al. 2009; Shemesh et al. 2005). Integrins have numerous subunits, and different subunits have different functions. In bovine articular chondrocytes integrins α_v and β_1 regulated dynamic compression-induced proteoglycan synthesis (Chai et al. 2010). In VSMCs, Liu et al. found that cyclic stretch regulated alignment of VSMCs via integrin β_1 (Liu et al. 2008), and Chen et al. also reported that integrin $\alpha_V\beta_3$ can serve as mechanosensors of shear stress to transduce mechanical stimuli into chemical signals via their association with Shc (Chen et al. 1999).

However, up to now, it is still a mystery to know which integrin subunit is involved in mechanotransduction in the HBSMCs. Although the previous studies showed that

integrins mediate key responses to mechanical stimuli in the bladder (Upadhyay et al. 2003), it is still necessary to explore whether mechanical stimuli induce extensive or limited changes in gene expression of integrin subunits in HBSMCs. To address this question, we have examined the level of expression of integrin subunits in HBSMCs subjected to cyclic hydrodynamic pressure. The results showed that the expression of integrin $\alpha 5$ was obviously increased in groups of 200 or 300 H₂O cm groups, but integrin subunits $\alpha 1$, $\alpha 3$, $\alpha 4$, αv , $\beta 1$ and $\beta 3$ were not affected under cyclic hydrodynamic pressure (**Fig. 1A**).

Integrins in the smooth muscle cells exhibited various biological effects, such as inducing cell growth (Upadhyay et al. 2003), proliferation (Aitken et al. 2006), etc. And our previous studies indicated that cyclic hydrodynamic pressure stimulated proliferation of HBSMCs cultured in scaffolds (Chen et al. 2012; Wu et al. 2012). These findings led us to hypothesize that integrin controls HBSMCs proliferation under hydrodynamic pressure. In order to verify this hypothesis, we found that proliferation activity of HBSMCs was greatly suppressed by down-regulating integrin $\alpha 5$ with siRNA, and the level of p-FAK has parallel relationship with integrin $\alpha 5$ under cyclic hydrodynamic pressure. After silencing integrin $\alpha 5$ with siRNA, the ratio of p-FAK/FAK obviously declined, but not greatly changed in static groups (**Fig. 5**). We concluded that cyclic hydrodynamic pressure stimulated proliferation of HBSMCs cultured in scaffolds in vitro via integrin $\alpha 5$ -FAK signaling pathway. FAK is a cytoplasmic tyrosine kinase identified as a key mediator of intracellular signaling by integrins (Guan 2010; Hanks and Polte 1997; Miranti and Brugge 2002). FAK has

been shown to facilitate the generation of integrin-stimulated signals to downstream targets such as the ERK2 and JNK/mitogen-activated protein kinase cascades (Schlaepfer and Hunter 1998), to play a role in the regulation of cell cycle progression (Zhao et al. 2001). In this study, only one tyrosine kinase phosphorylation site (Thy397) was preliminarily examined, other tyrosine kinase phosphorylation site of FAK and its down-stream signaling molecules still need to be further explored.

The periodical hydrodynamic pressure is one of the main mechanical stimuli to HBSMCs during filling and voiding cycles in vivo. Appropriate mechanical stimuli are needed for growth and development of the urinary bladder (Haberstroh et al. 1999). In contrast, the abnormal mechanical environment created by various voiding dysfunctions, for example, the paraplegia or bladder outlet obstruction (BOO), can cause cellular alterations that can jeopardize the integrity of the bladder (Korossis et al. 2006; Stover and Nagatomi 2007). In constructing tissue engineered urinary bladder, amplification of the seeded cell is a key step. Maybe the periodical hydrodynamic pressure is to make up for the ex vivo expansion of the seeded cells. Meanwhile, the mechanism of single cyclic hydrodynamic pressure promoting HBSMCs proliferation may offer a new insight into some urinary bladder diseases caused by excessive mechanical forces, such as BOO studies, because previous studies about high pressures induced detrusors proliferation in BOO studies is not a single mechanical stimulus, but a complicated mechanical strains that mainly contain hydrostatic pressure and stretch force (Chang et al. 2009; Kim et al. 2005; Zhang et al. 2004). Down-regulation of integrin $\alpha 5$ could prevent the bladder hypertrophy or

hyperplasia process, which was often seen under pathological conditions such as spinal cord injury and BOO.

In conclusion, we provide the first report of relationship among cyclic hydrodynamic pressure, integrins, FAK, and proliferation on HBSMCs cultured in scaffolds. The findings of this study demonstrate that integrin $\alpha 5$ -FAK is a key signaling pathway in the proliferation of human bladder smooth muscle cells in response to cyclic hydrodynamic pressure, which may be used for construction of functional bladder tissue engineering and also offer a new insight into some urinary bladder diseases.

Acknowledgement

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alpha-v-integrin, and alpha-actin genes through cytoskeletonally based mechanotransduction mechanisms in bladder smooth muscle cells. *J Appl Physiol* **98**: 2344-2354, 2005.

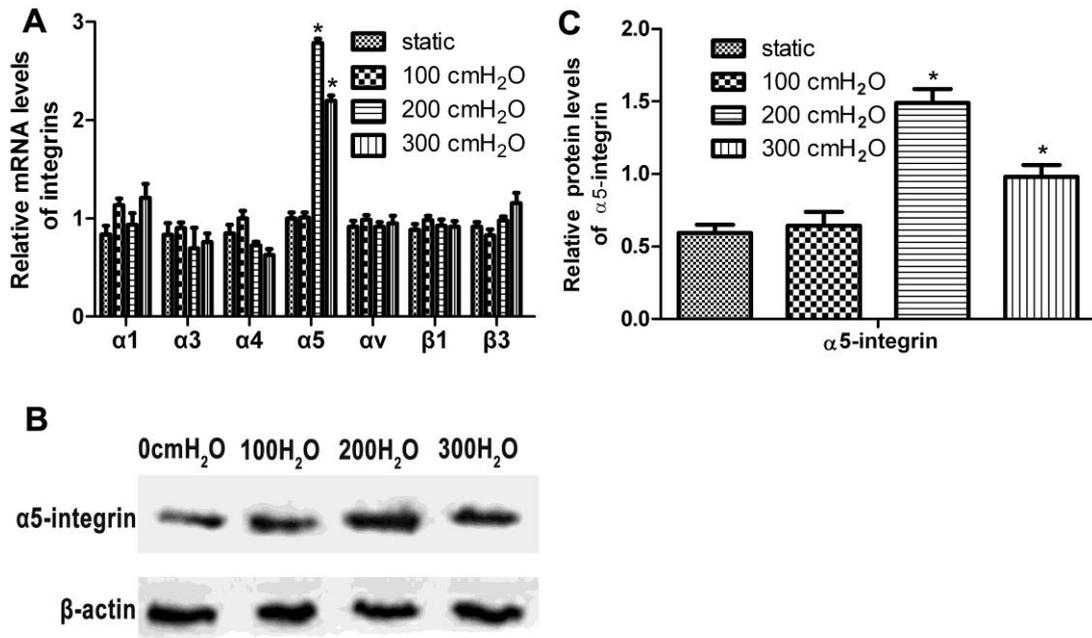


Fig. 1. Expression levels of integrin subunits under different cyclic hydrodynamic pressure groups. A: relative mRNA levels of integrin $\alpha 1$, $\alpha 3$, $\alpha 4$, $\alpha 5$, αv , $\beta 1$ and $\beta 3$ subunits about HBSMCs cultured in vitro under different cyclic hydrodynamic pressure. **B:** representative protein bands of integrin $\alpha 5$ subunit and control β -actin. **C:** statistical graph of integrin $\alpha 5$. * $p < 0.05$ vs static groups.

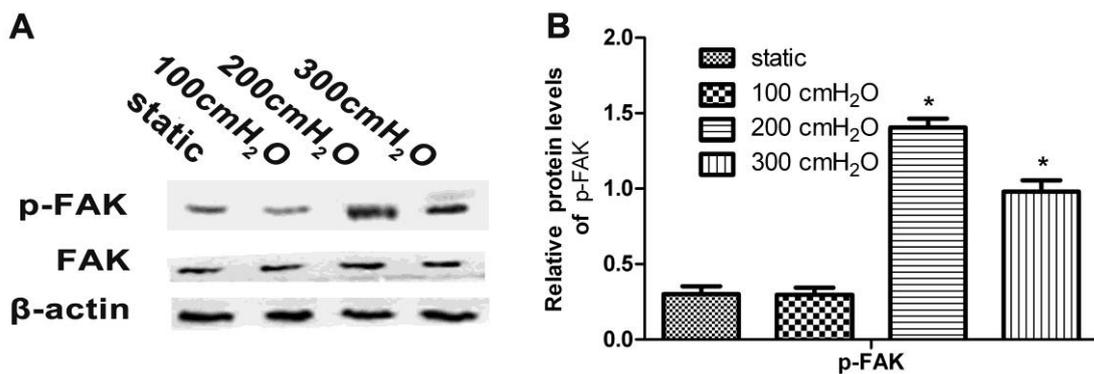


Fig. 2. Expression levels of p-FAK under different cyclic hydrodynamic pressure groups (static, 100 cm H₂O, 200 cm H₂O, 300 cm H₂O). A: representative protein bands of FAK and phosphorylated FAK protein. Control (Con), $\alpha 5$ integrin siRNA ($\alpha 5$ -inte siRNA). **B:** the data revealed levels of p-FAK under different cyclic

hydrodynamic pressure groups. What the data shows are mean values (\pm SD) of a representative experiment performed in triplicate. * $p < 0.05$ vs static groups.

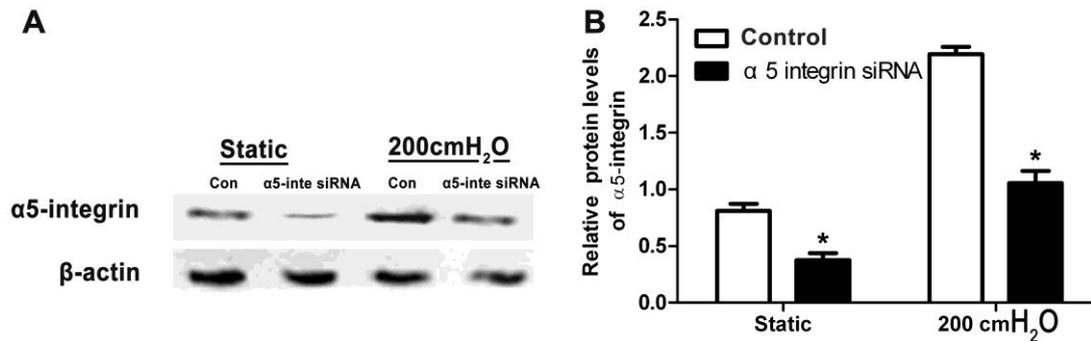


Fig. 3. Expression of integrin $\alpha 5$ was greatly suppressed after using integrin $\alpha 5$ siRNA for static groups or 200 cm H₂O groups. **A:** representative protein bands of integrin $\alpha 5$ protein. Control (Con), $\alpha 5$ integrin siRNA ($\alpha 5$ -inte siRNA). **B:** after cells were transfected with siRNA specified for integrin $\alpha 5$ or with scrambled sequences, the data revealed that the expression of integrin $\alpha 5$ was greatly suppressed static groups or 200 cm H₂O groups. What the data shows are mean values (\pm SD) of a representative experiment performed in triplicate. * $p < 0.05$ vs control groups.

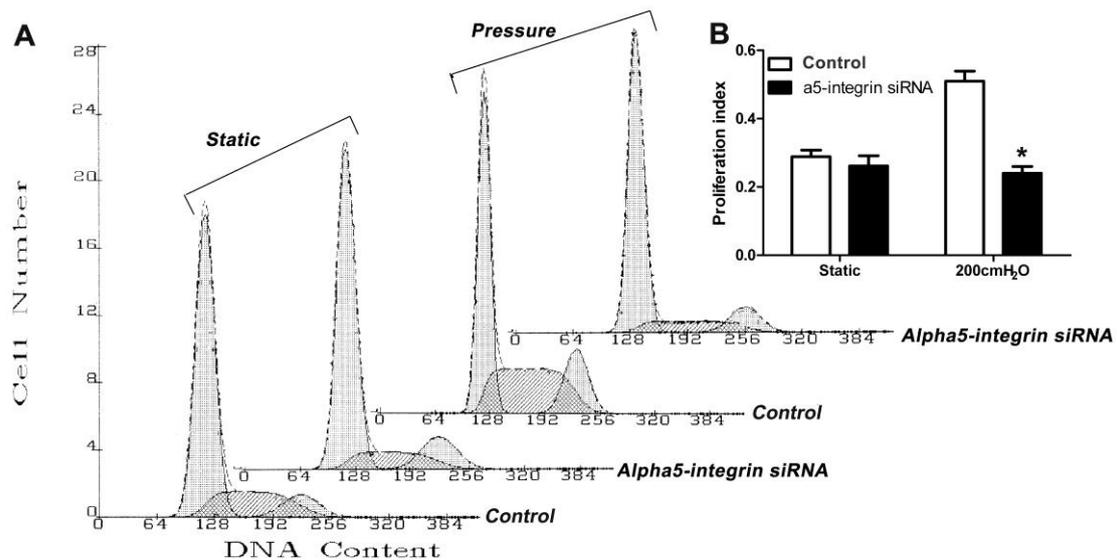


Fig. 4. Cell proliferation induced by specific cyclic hydrodynamic pressure was suppressed as expression of integrin $\alpha 5$ was down-regulated with siRNA specific

for integrin $\alpha 5$. **A:** representative flow cytometric profiles demonstrated cell cycle distribution. 3 independent assays were done and representative result was chosen. **B:** cell proliferation index (%) of each group. Data were expressed as mean \pm SD. * $p < 0.05$ vs control groups.

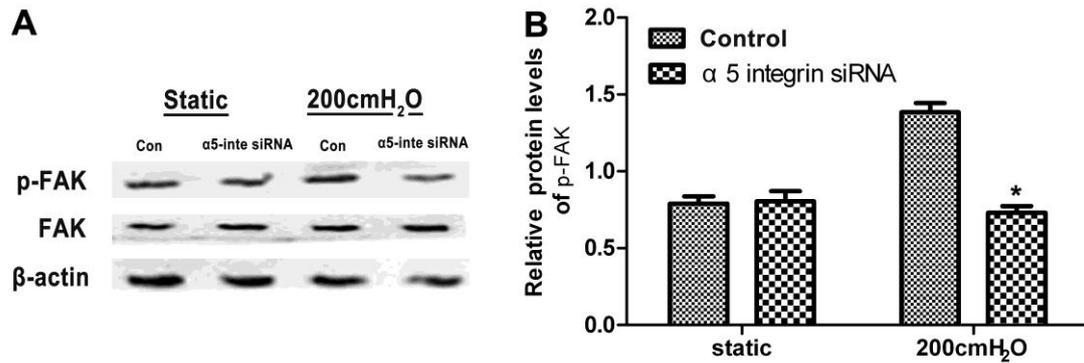


Fig. 5. Inhibition of integrin $\alpha 5$ resulted in declined level of p-FAK. **A:** representative protein bands of p-FAK protein. Control (Con), $\alpha 5$ integrin siRNA ($\alpha 5$ -inte siRNA). **B:** statistical graph of p-FAK/FAK ratio. What the data shows are mean values (\pm SD) of a representative experiment performed in triplicate. * $p < 0.05$ vs control groups.