Cyclic Hydrodynamic Pressure Induced Proliferation of Bladder Smooth Muscle Cells via Integrin α5 and 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 α5 siRNA was applied to validate the role of integrin α5 in cell proliferation. Here, we showed that cyclic hydrodynamic pressure promoted proliferation of HBSMCs. The cyclic hydrodynamic pressure also increased expression of integrin α5 and phosphorylation of FAK, the key mediator of integrin α5 signaling, but not that of integrin α1, α3, α4, αv, β1 and β3. Moreover, inhibition of integrin α5 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 α5-FAK signaling pathway controls the proliferation of HBSMCs in response to cyclic hydrodynamic pressure.

Key words

Hydrodynamic pressure • Proliferation • Human bladder smooth muscle cells

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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 Cheresh 2002).

Activation of specific integrins has been documented in different cells upon exposure to various mechanical stimuli. For example, cyclic stretch up-regulated αvβ3 in mice and rat vascular smooth muscle cells (VSMCs) (Chen *et al.* 1999, Cheng *et al.* 2007), while cyclic strain induced an association of α1β1 in mice VSMCs (Pyle *et al.* 2008). Wilson showed that cyclic strain induced an association of both β3 and αvβ5 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 α5 Antibody(Cell Signalling Technologies, CST, USA), Lipofectamine2000 reagent and scrambled siRAN (Invitrogen, USA).

Cell culture

HBSMCs were cultured in DMEM supplemented with 10 % fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 95 % air/5 % CO_2. Cells between generations 3 and 7 were studied.

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 h/cycle, increasing from 0 cm H_2O to 10 cm H_2O at first 1.75 h, then up to 100 cm H_2O, 200 cm H_2O, or 300 cm H_2O rapidly and maintaining for 0.25 h, lowering to 0 cm H_2O at last) for up to 24 h. HBSMCs in the control groups were maintained under static conditions.

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;
α1, forward: 5′-GGTTCCTACTTTGGCAGTATT-3′,
reverse: 5′-AACCTTGTCTGATTGAGAGCA-3′;
α3, forward: 5′-AAGGGACCTTCAGGTGCA-3′,
reverse: 5′-TGTAGCCGGTGATTTACCAT-3′;
α4, forward: 5′-GCTTCTCAGATCTGCTCGTG-3′,
reverse: 5′-GTCACTTCCAACGAGGTTTG-3′;
α5, forward: 5′-TGCAGTGTGAGGCTGTGTACA-3′,
reverse: 5′-GTGGCCACCTGACGCTCT-3′;
αV, forward: 5′-AATCTTCCAATTGAGGATATCAC-3′,
reverse: 5′-AAAACAGCCAGTAGCAACAAT-3′;
β1, forward: 5′-GAAGGGTTGCCCTCCAGA-3′,
reverse: 5′-GCTTGAGCTTCTCTGCTGTT-3′;
β3, forward: 5′-CCGTGACGAGATTGAGTCA-3′,
reverse: 5′-AGGATGGACTTTCCACTAGAA-3′.

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.

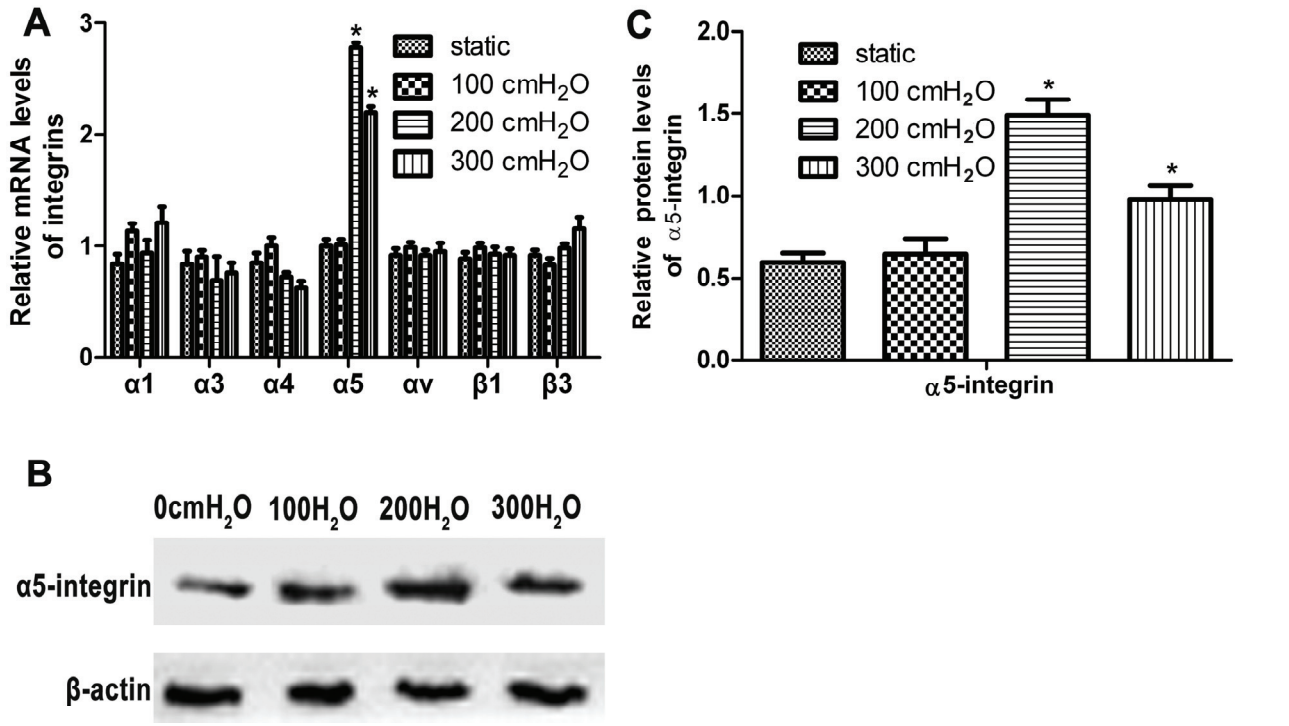

Fig. 1. Expression levels of integrin subunits under different cyclic hydrodynamic pressure groups. **A:** relative mRNA levels of integrin α1, α3, α4, α5, αv, β1 and β3 subunits about HBSMCs cultured in vitro under different cyclic hydrodynamic pressure. **B:** representative protein bands of integrin α5 subunit and control β-actin. **C:** statistical graph of integrin α5. * $p<0.05$ vs. static groups

Cell transfection

A siRNA specific for integrin α5 (UGGCUCAGACAUUCGAUCC) 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 α5 expression. The control groups were transfected by scrambled siRNA.

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 x 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 *et al.* 1998). Membranes were incubated with specific antibodies. The antibodies used for western blotting were β-actin, integrin α5, 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 μg/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 ± standard deviation (M ± SD). Statistical analysis was performed using Students t test, and $p<0.05$ was considered statistically significant.

Results

Cyclic hydrodynamic pressure increased integrin α5 expression level of HBSMCs cultured in vitro

To investigate which integrin subunit in HBSMCs was changed under hydrodynamic pressure, the

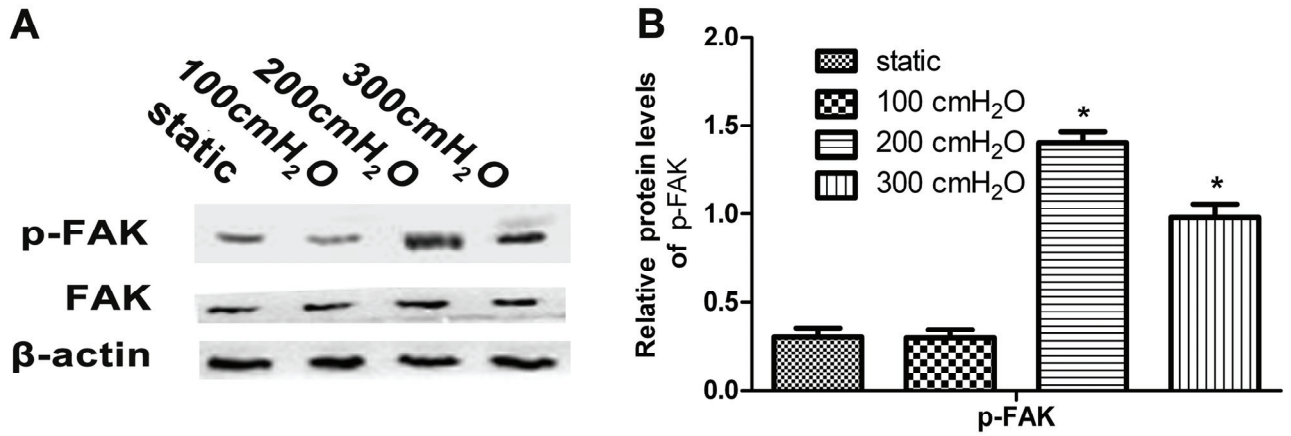

Fig. 2. Expression levels of p-FAK under different cyclic hydrodynamic pressure groups (static, 100 cm H_2O, 200 cm H_2O, 300 cm H_2O). **A:** representative protein bands of FAK and phosphorylated FAK protein. Control (Con), α5 integrin siRNA (α5-inte siRNA). **B:** the data revealed levels of p-FAK under different cyclic hydrodynamic pressure groups. What the data shows are mean values (± SD) of a representative experiment performed in triplicate. * $p<0.05$ vs. static groups

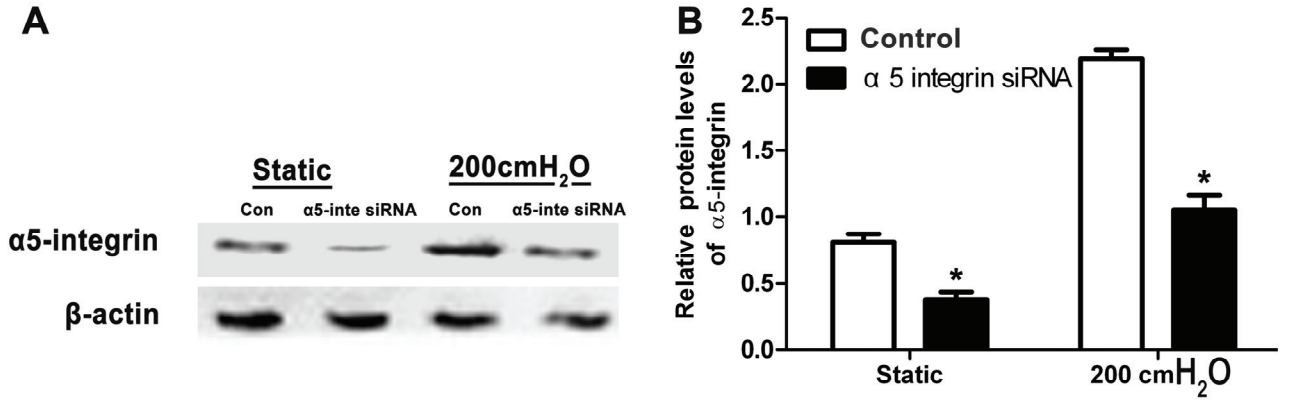

Fig. 3. Expression of integrin α5 was greatly suppressed after using integrin α5 siRNA for static groups or 200 cm H_2O groups. **A:** representative protein bands of integrin α5 protein. Control (Con), α5 integrin siRNA (α5-inte siRNA). **B:** after cells were transfected with siRNA specified for integrin α5 or with scrambled sequences, the data revealed that the expression of integrin α5 was greatly suppressed static groups or 200 cm H_2O groups. What the data shows are mean values (± SD) of a representative experiment performed in triplicate. * $p<0.05$ vs. control groups

genetic expression of integrin subunits α1, α3, α4, α5, αv, β1 and β3 were measured by RT-PCR. The data showed that only integrin α5 expression increased in groups of 200 and 300 cm H_2O groups, whereas integrin subunits α1, α3, α4, αv, β1 and β3 were not greatly affected by cyclic hydrodynamic pressure. Compared to 0 cm H_2O group, the HBSMCs in 200 and 300 cm H_2O groups had higher integrin α5 expression ($p<0.05$) in (Fig. 1A). At protein level, integrin α5 expression significantly increased in both 200 and 300 cm H_2O groups with comparison to static control group ($p<0.05$), but no significant difference was found between 100 cm H_2O group and static control group (Fig. 1B, 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 α5, the p-FAK level significantly increased in both 200 and 300 cm H_2O groups ($p<0.05$), but there was no difference with 100 cm H_2O 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 α5 and FAK signal pathway in proliferation of HBSMCs promoted by hydrodynamic pressure, the HBSMCs were treated with integrin α5 siRNA. 200 cm H_2O was chosen as magnitude of pressure for this study. Compared to the scrambled siRNA control, the expression of integrin α5 was remarkably suppressed by target siRNA transfection in both pressure and static groups (Fig. 3), and cell

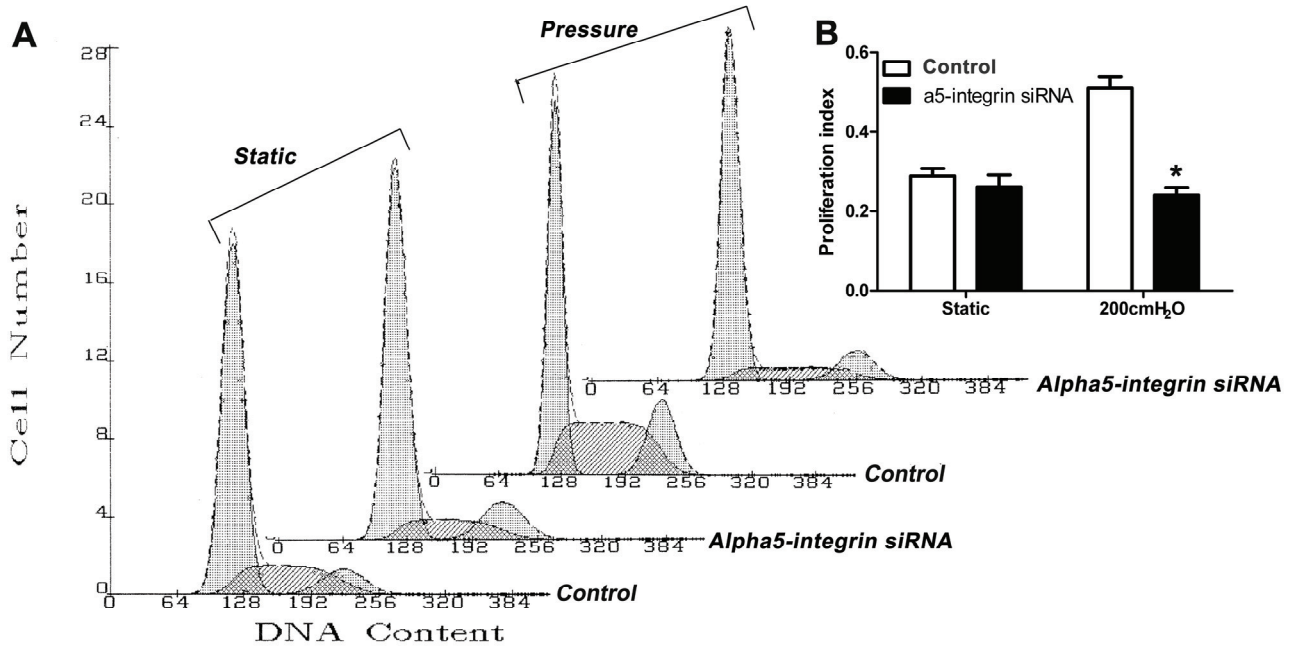

Fig. 4. Cell proliferation induced by specific cyclic hydrodynamic pressure was suppressed as expression of integrin α5 was down-regulated with siRNA specific for integrin α5. **A:** representative flow cytometric profiles demonstrated cell cycle distribution. 3 independent assays were done and representative result was chosen. **B:** cell proliferation index (%) of each group. Data were expressed as mean ± SD. * $p<0.05$ vs. control groups

proliferation activity induced by cyclic hydrodynamic pressure was inhibited. In pressure groups, cell proliferation index decreased from 50.93±5.14 % to 23.99±3.46 % ($p<0.05$); while in static groups, cell proliferation index decreased from 28.81±3.38 % to 26.02±5.33 % ($p>0.05$) (Fig. 4).

After mRNA of integrin α5 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 cm H_2O groups, but p-FAK/FAK ratio slightly dropped ($p>0.05$) in static groups (Fig. 5). Under cyclic hydrodynamic pressure, the level of integrin α5, 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 α5-FAK signaling transduction pathway. FAK was responsible for downstream signaling transduction of integrin α5.

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 H_2O to 10 cm H_2O at first 1.75 h, followed by increase to 100 cm H_2O, 200 cm H_2O, or 300 cm H_2O rapidly and maintained for 0.25 h, then decreased to 0 cm H_2O). 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 H_2O) *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 αv and β1 regulated dynamic compression-induced proteoglycan synthesis (Chai *et al.* 2010). In VSMCs, Liu *et al.* (2008) found that cyclic stretch

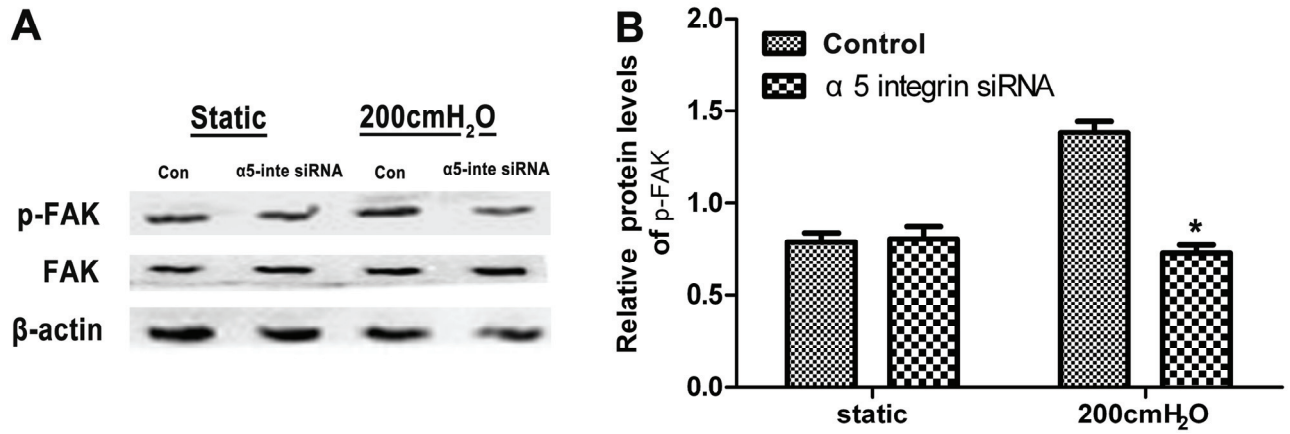

Fig. 5. Inhibition of integrin α5 resulted in declined level of p-FAK. **A:** representative protein bands of p-FAK protein. Control (Con), α5 integrin siRNA (α5-inte siRNA). **B:** statistical graph of p-FAK/FAK ratio. What the data shows are mean values (± SD) of a representative experiment performed in triplicate. * $p<0.05$ vs. control groups

regulated alignment of VSMCs *via* integrin β1, and Chen *et al.* (1999) also reported that integrin αVβ3 can serve as mechanosensors of shear stress to transduce mechanical stimuli into chemical signals *via* their association with Shc.

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 α5 was obviously increased in groups of 200 or 300 cm H_2O groups, but integrin subunits α1, α3, α4, αv, β1 and β3 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 α5 with siRNA, and the level of p-FAK has parallel relationship with integrin α5 under cyclic hydrodynamic pressure. After silencing integrin α5 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 α5-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 α5 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 α5-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.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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