Skp2-mediated degradation of p27 regulates cell cycle progression in compressed human bladder smooth muscle cells

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Abstract  Bladder outlet obstruction (BOO) results in smooth muscle cell hyperplasia, decreased bladder wall compliance, and lower and upper urinary tract pathology. Mechanical stimulus on detrusor tissue is critical to BOO disease progression. Our previous studies confirm that mechanical stimulus triggers human bladder smooth muscle cell (HBSCM) proliferation. To better understand the signal transduction mechanisms for this process we detected cell cycle machinery of HBSCM (Bose® Biodynamic, Minnetonka, MN, USA). HBSCMs cultured in scaffolds were subjected to four different pressures (0 cmH2O, 100 cmH2O, 200 cmH2O, and 300 cmH2O) for 24 hours, which were controlled by a BOSE BioDynamic bioreactor. Then we used flow cytometry to examine cell cycle distribution, polymerase chain reaction, and immunoblotting to quantify Skp2, p27, and p21 expression in each group. Additionally, Skp2 was silenced in HBSCMs using small interfering RNA to validate the role of Skp2 in mediating pressure-induced cell cycle progression. Compared with the 0 cmH2O control, HBSCMs in the 200 cmH2O and 300 cmH2O groups exhibited high-level expression of Skp2 gene and low-level expression of p27 protein. However, p21, another downstream signal of Skp2, showed no significant change between groups. In addition, Skp2 silencing abolished increases in cell proliferation induced by pressure. To the best of our knowledge, this is the first report on the functional importance of Skp2 in cyclic hydrodynamic pressure stimulated HBSCM proliferation. The signal transduction mechanism for this process involves p27 as well as p21 signaling pathway.

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Introduction

Increased growth of bladder smooth muscle cells (SMCs) is a hallmark of bladder outlet obstruction (BOO) progress [1–4]. The detrusor muscle hypertrophy results in a deleterious change in bladder wall compliance [5], which in turn causes substantial renal injury [3]. It has been established that chronic infravesical obstruction results in abnormally increased intravesical pressure, which in turn results in increased mechanical stimulus on bladder SMCs. It is the increased mechanical stimuli that lead to bladder SMC proliferation [4]. However, the pathophysiologic mechanisms remains poorly understood. To elucidate the molecular mechanisms of this pathophysiologic process, it is necessary to establish a mechanic-cell proliferation model in vitro. Many studies showed that hydrostatic pressure less than physical stress (commonly 40 cmH2O) could induce proliferation of bladder SMCs [6–8]. However, in our previous experiment, hydrostatic pressure lower than 40 cmH2O had no effect on cell cycle distribution of human bladder smooth muscle cells (HBSMCs), which is the most accurate and readily quantifiable parameter of cell proliferation [9]. Next, we demonstrated that the cyclic hydrodynamic pressure of more than 100 cmH2O could trigger proliferation of HBSMCs [10]. The possible reasons might be: (1) theoretically native bladder tissues are subjected to hydrodynamic pressure other than hydrostatic pressure during filling and voiding cycles, or (2) the same pressure leads to weaker stimuli on cells in vitro than in vivo [10].

With this mechanic-cell proliferation module, we carried out a series of studies on cell signaling and found that the P38/MAPK, p38/MAPK, and PI3K/AKT, which initiated proliferation of SMCs [7,11,12]. Although these signal transduction pathways differ from each other, they finally converge at the level of the cell cycle machinery. Cell cycle progression depends on the regulation of the cyclin proteins and their associated cyclin-dependent kinases (CDKs) [13]. Cyclin/CDK complexes regulate proliferation by phosphorylating and, thus, inactivating the transcriptional repressor retinoblastoma protein and related proteins, which allows the expression of genes that promote cell cycle progression and proliferation [14]. A primary means of regulating CDKs and thus, cellular proliferation, is via cyclin-dependent kinase inhibitors (CDKIs), which associate with and inactivate cyclin-CDK complexes to prevent cell cycle progression and inhibit proliferation [15]. However, there are a series of CDKIs in SMCs, including p16, p21, and p27 [16,17], and just which CDKI may be responsible for SMC proliferation is controversial.

As previously reported, some growth factors such as angiotensin II and platelet-derived growth factor (PDGF)-BB stimulate vascular SMC proliferation by inhibiting p21 expression [18,19]. However, some antiproliferative agents such as crocetin, JY0691, and trichostatin A inhibit cell cycle progression of vascular SMC by increasing the level of p27 but not p21 [16,20,21]. In addition, cell-specific differences also exist. For example, p21 is actively involved in airway SMC proliferation [22]. The current study, therefore, aimed to elucidate the molecular mechanism of bladder SMC cycle progression induced by cyclic hydrodynamic pressure. We specifically quantified cell cycle distribution, p27 and its counterpart p21 expression, and degradation after exposure of HBSMCs to cyclic hydrodynamic pressure. We also investigated the potential regulatory mechanism of CDKIs and tested mechanoregulation of Skp2, which is the substrate recognition subunit and limiting component of a Skp1–CUL1–F-box ubiquitin ligase complex that promotes proliferation primarily by targeting CDKIs for ubiquitination and, consequently, proteasomal degradation [23,24]. Here, ubiquitination is the process whereby ubiquitin, a small regulatory protein, is being attached to proteins and labeling them for destruction.

Materials and methods

Cell culture

HBSMCs (Sciencell, Carlsbad, CA, USA; Cat.No.4310) were cultured in Dulbecco’s modification of Eagle’s medium (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% CO2. Experiments were performed on cells between passages three and seven.

Cyclic hydrodynamic pressure

According to the mechanic-cell proliferation module we previously established [10], HBSMCs were seeded into polyvinyl alcohol-based scaffolds (Bose ® Biodynamic, Minnetonka, MN, USA) contained by Petri dishes (1 x 105 cells/piece). After low medium starve or small interfering RNA (siRNA) transfection, HBSMCs in scaffolds were transplanted into the computer-controlled bioreactor (BOSE, BioDynamic). The HBSMCs were then subjected to cyclic hydrodynamic pressure simulating bladder cycle (2 hours/cycle, increasing from 0 cmH2O to 100 cmH2O at the first 1.75 hours, then up to 100 cmH2O, 200 cmH2O, or 300 cmH2O rapidly and maintaining for 0.25 hours, and finally back to 0 cmH2O) for up to 24 hours. HBSMCs in the control group were maintained under static conditions (0 cmH2O). Real-time readouts from the control software were plotted versus time.

Cell cycle analysis by flow cytometry

After HBSMCs were seeded into scaffolds for 24 hours, the medium was replaced by DMEM containing no serum to make them quiescent for 48 hours. Scaffolds with HBSMCs were transplanted into the bioreactor and were subjected to cyclic hydrodynamic pressure as described previously. HBSMCs in the control group were maintained under static conditions. Cells were then harvested and fixed in 70% ethanol in cold phosphate buffered saline (PBS) overnight at 4°C. After centrifugation, the pellet was washed with cold PBS and resuspended in 500 µL PBS containing 100 µg/mL RNase A and 50 µg/mL propidium iodide for 1 hour in the
dark. The cell cycle distribution was then determined by flow cytometry using an EPICS ELITE ESP flow cytometer (Beckman Coulter, Miami, FL, USA). In addition, cell proliferation index, a parameter cell reflecting proliferation rate, was calculated as: proliferation index (%) = (S + G2/M)/(G0/G1+S + G2/M) × 100%.

RNA isolation and real-time PCR

Total RNA was extracted with an RNAiso Plus reagent (TaKaRa, Dalian, China) based on the manufacturer’s instructions. RNA was eluted in 20 µL of nuclease-free water and stored at −70°C prior to use. Complementary DNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Richmond, CA, USA). With a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as an internal control, the messenger RNA (mRNA) expressions of p27, p21, and Skp2 were analyzed by a real-time PCR using the SYBR Premix EX Taq premix reagent (TaKaRa) and Bio-Rad iQ5 machine. The following primers were used: (1) human Skp2: sense, 5'-GGCGTTTGGAGTGGTAGA-3'; antisense, 5'-TGCAACTTGGCTCTTATTCT3'-3'; (2) human p27: sense, 5'-TAATTGGGGCTCCGGCTAACT-3' and antisense, 5'-TTGCAAGGCTGCTTCTTATTCT3'-3'; (3) human p21: sense, 5'- AGCAGAGGAAGACCATGTGGAC-3' and antisense, 5'-GGCGTTTGGAGTGGTAGA-3'; and (4) human GAPDH: sense, 5'-GCTTCGCTCTCTGCTCCTC-3' and antisense, 5'-CGCCCAATACGACCAAAT-3'. PCR cycling conditions were 94°C for 3 minutes and 40 cycles of (94°C for 5 seconds, 54°C for 30 seconds, and 72°C for 20 seconds). Polymerase chain reaction (PCR) product quality was monitored by post-PCR melt curve analysis. Data analysis was performed using comparative computed tomography method in Bio-Rad iQ5 software. Averages of three experiments, each performed in triplicate with standard errors, are presented.

Immunoblotting

Cells were lysed in RIPA buffer (Beyotime, Shanghai, China), and 30 µg of proteins were separated by electrophoresis on SDS-polyacrylamide gels and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). Membranes were incubated with specific antibodies. The antibodies used for immunoblotting were GAPDH, p27, p21 and Skp2 (Cell Signaling Technology, Beverly, MA, USA).

siRNA transfection

siRNAs were transfected with Lip2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Cells were transfected twice at an interval of 24 hours with Skp2 siRNAs. The following siRNA sequences were used: Skp2 sense, 5'-AAGGGAGUGAACAAGACAUUG-3'; antisense, 5'-CAAAGUCUUUGUCACUCUCU-3'. Scrambled siRNAs were purchased from Invitrogen.

Statistical analysis

Each experiment was performed at least in triplicate. Means and standard deviation (SD) were calculated. Results between two groups were compared by one-way analysis of variance (ANOVA). A p value <0.05 was considered significant. Data in the figures are expressed as the mean, the error bars showing SD.

Results

Effect of cyclic hydrodynamic pressure on p27 and p21

Our previous research demonstrated that cyclic hydrodynamic pressure more than 100 cmH2O can induce proliferation of HBSCMs. To begin to understand the mechanism by which proliferative signals regulated by mechanical stimulus are coupled to the central cell cycle machinery, we first chose 100 cmH2O, 200 cmH2O, and 300 cmH2O pressures as the pressure group, and static (no pressure) as the control group to examine the effect of mechanical stimulus in HBMC on the expression of two CDKIs, p27 and p21, which have been implicated in the regulation of smooth muscle proliferation [17,19,20].

After the cells were exposed to the cyclic hydrodynamic pressure for 24 hours and no pressure group was under static conditions, the mRNA and protein of p27 and p21 were detected by real-time PCR and Western blots. We found that although the level of p21 mRNA and protein in the cells was unaffected by cyclic hydrodynamic pressure, the level of p27 protein fell dramatically when pressure was increased (Table 1, Figs. 1 and 2). The decrease in p27 correlated with an increase in cell proliferation index, a parameter reflecting cell proliferation rate, was calculated as: proliferation index (%) = (S + G2/M)/(G0/G1+S + G2/M) × 100%.

Effect of cyclic hydrodynamic pressure on Skp2

As reported previously, the degradation of CDKIs such as p27 and p21 is mediated by the upregulation of expression of Skp2, a component of a ubiquitin ligase complex that can target p27 or p21 for ubiquitination, and, consequently, proteasome-mediated degradation [23,24]. Therefore, we also examined the effect of cyclic hydrodynamic pressure

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| Table 1 Relative messenger RNA level of Skp2, p27, and p21 between control and cyclic hydrodynamic pressure groups. |
|----------------|----------------|----------------|----------------|
| Control        | 100 cmH2O      | 200 cmH2O      | 300 cmH2O      |
| Skp2           | 1.00           | 1.46 ± 0.11    | 4.43 ± 0.46    | 2.13 ± 0.27*   |
| p27            | 1.00           | 1.25 ± 0.18    | 1.14 ± 0.07    | 1.03 ± 0.14    |
| p21            | 1.00           | 1.29 ± 0.22    | 1.25 ± 0.38    | 1.64 ± 0.53    |

*p < 0.05 based on one-way analysis of variance.

| Three independent assays were done, and each value represents the mean ± standard deviation. |
on Skp2, and we found that Skp2 mRNA and protein were both increased in the 200 cmH₂O and 300 cmH₂O pressure treatment groups as compared with the 100 cmH₂O pressure group \((p < 0.05)\). The increase in Skp2 correlated with an increase in cell proliferation index (Fig. 1) [10]. These findings suggested that the proliferative response that occurred in response to cyclic hydrodynamic pressure was mediated in part by Skp2.

### Skp2-mediated degradation of p27 rather than p21 in compressed HBSMC

We then wanted to confirm whether Skp2 was important in the regulation of CDKIs in compressed HBSMC. We chose the 200 cmH₂O group and the control group as the pressure and no pressure group respectively, and siRNA was used to inhibit Skp2 expression in HBSMC. Compared with the scrambled siRNA control, the expression of Skp2 was remarkably suppressed by target siRNA transfection in both pressure and no pressure groups (Fig. 3). Inhibition of Skp2 expression increased the level of p27 protein; however, the level of p21 protein in the cells was unaffected (Fig. 3). Our results identified that Skp2 mediated degradation of p27 rather than p21 in compressed HBSMC.

### Lack of Skp2 suppressed HBSMC proliferation induced by pressure

To determine whether the lack of Skp2 expression in bladder is an important factor in limiting HBSMC proliferation, the siRNA was also used to inhibit Skp2 expression in HBSMCs, which were further subjected to cell cycle detection. We found that Skp2 siRNA significantly inhibited cell proliferation induced by cyclic hydrodynamic pressure. In the pressure groups, cell proliferation index decreased from 46.90 ± 6.68% to 36.70 ± 3.05% \((p < 0.05)\), whereas in the no pressure groups, cell proliferation index decreased from 29.37 ± 2.32% to 25.53 ± 0.91% \((p = 0.67;\) Fig. 4, Table 2). Taken together, our results strongly suggest that increased cyclic hydrodynamic pressure on HBSMCs promotes proliferation, at least in part, by stimulating Skp2/p27 but not Skp2/p21 signaling pathway.

### Discussion

Bladder compliance is adversely affected by chronically increased intravesical pressure sustained from BOO and the resulting proliferation of bladder SMCs [3,4,27]. Our earlier

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**Figure 1.** Mechanoregulation of Skp2, p27, and p21 at gene level. Samples were prepared from pooled cells of one culture chamber. Three or more independent assays were done and a representative result was chosen. Real-time polymerase chain reaction was used to quantify Skp2, p27, p21, and control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA).

**Figure 2.** Mechanoregulation of Skp2, p27, and p21 at protein level. Samples were prepared from pooled cells of one culture chamber. Three or more independent assays were done and a representative result was chosen. Immunoblotting was used to quantify Skp2, p27, p21, and control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). siRNA = small interfering RNA.

**Figure 3.** Small interfering RNA (siRNA) transfection inhibited pressure-induced Skp2 expression. Immunoblotting was used for Skp2, p27, and p21 in HBSMCs harvested from pressure and no pressure chamber. Three or more independent assays were done and a representative result was chosen. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.
findings demonstrated that the cyclic hydrodynamic pressure of over 100 cmH₂O could induce proliferation of HBSCMs by our validated model [10], but how do HBSCMs convert diverse cyclic hydrodynamic pressure into biochemical signals? It is worthwhile to note that numerous studies illustrate that several signal pathways such as Rho family GTPases, STAT3, ERK1/2 MAPKs, p38/MAPK, PI3K/Akt, and PI3K/SGK1 initiated proliferation of SMCs [7,11,12]. However, the more signaling pathways that are found, the more difficult it becomes to choose a reliable way as the target to inhibit HBSMC proliferation and to treat BOO. Fortunately, it was demonstrated that cell cycle progression finally depended on the negative regulation of CDKIs, although the intermediate signal varied [15,18,19]. Therefore, we hypothesized that cyclic hydrodynamic pressure might modulate the expression or degradation of CDKIs. This hypothesis was confirmed in the current in vitro study, and we found that cyclic hydrodynamic pressure stimulated degradation of CDKI p27 but not p21. In addition, the decrease in p27 correlates with an increase in HBSMC proliferation (Fig. 2) [10]. Taken together, these findings clearly indicate that p27 rather than its counterpart p21 is the mediator of mechanical signaling events in HBSMC.

However, our findings are in direct conflict with growth factor stimulation tests in vascular systems, because vascular SMC proliferation was mediated by down-regulation of p21, which was stimulated by mitogens such as angiotensin II and PDGF-BB [18,19]. Two types of explanation are proposed: (1) cell-specific difference and/or (2) stimulus-specific difference. To determine which is predominant, studies must fix one of them and then research the other. One study reported that, when they fixed the stimulus as sustained tension, no difference was found between several cell types such as bladder SMC, vascular SMC, and fibroblast, and sustained tension also stimulates cell proliferation by regulation of p27 [28]. Therefore, we can reasonably conclude that mechanical stimulus-mediated p27 degradation promotes cell proliferation. On the contrary, chemical stimulus such as angiotensin II and PDGF-BB improves cell cycle progression through p21 but not p27.

The following questions focus on the molecular mechanism of p27 degradation. Previously, Skp2 was reported as a node at which proliferative signals from mitogens were integrated in the regulation of CDKIs. In tumor cells and vascular SMCs, it is the substrate recognition subunit and limiting component of a SKP1–CUL1–F-box ubiquitin ligase complex that promotes proliferation primarily by targeting CDKIs for ubiquitination and, consequently, proteasomal degradation [23,24]. Therefore, we speculated that cyclic hydrodynamic pressure may modulate the expression or the activity of Skp2, which in turn results in p27 degradation and bladder SMC proliferation. The pressure and siRNA experiments should support this hypothesis. Cyclic hydrodynamic pressure obviously promotes both Skp2 gene expression and its protein synthesis in HBSMC, and the increase in Skp2 correlated with a decrease in p27 (Fig. 2). In addition, Skp2 “knockdown” by target siRNA transfection decreased degradation of p27 (Fig. 3). The upregulated

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<th>Cell proliferation indices between groups.</th>
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<td>Scrambled siRNA control</td>
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<tr>
<td>No pressure</td>
<td>29.37 ± 2.32</td>
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<td>Pressure groupa</td>
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Data are presented as %. siRNA = small interfering RNA. a The pressure is 200 cmH₂O cyclic hydrodynamic pressure.
proliferation of HBSMCs induced by 200 cmH2O cyclic hydrodynamic pressure was repressed by Skp2 "knockdown" (Fig. 4). To our knowledge this is the first report of the functional importance of Skp2 in engineered detrusor muscle. Our findings strongly implicate Skp2 as a promising target for therapy in chronic BOO cases. Additional investigation, therefore, is necessary to elucidate the relationship between mechanical stimuli and Skp2 expression in HBSMCs.

In conclusion, the current study indicates the functional importance of Skp2 in cyclic hydrodynamic pressure stimulating HBSMC proliferation. The signal transduction mechanism for cell cycle progression involves p27 but not p21 signaling pathway. Better understanding of cell cycle and proliferative signaling pathways may provide important novel targets to treat lower urinary tract disorders. Skp2/p27 is a molecular target that may have future significance for treating patients with altered detrusor dynamics.

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