Fluid Shear Stress Induces Cell Cycle Arrest in Human Urinary Bladder Transitional Cell Carcinoma Through Bone Morphogenetic Protein Receptor-Smad1/5 Pathway

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(Received 23 January 2018; accepted 13 April 2018; published online 17 April 2018)

Associate Editor Partha Roy oversaw the review of this article.

Abstract—Introduction—Mechanical force generated from the interstitial fluid flow inside and surrounding tissue has been known to play a significant role in cancer pathophysiology. In this study, we aimed to investigate the role of laminar shear stress (LSS) in modulating the cell cycle of human bladder transitional carcinoma (BFTC-905) cells which are frequently stimulated by not only the interstitial fluid flow, but also the urine flow transported from kidney to bladder in the urinary tract.

Methods—The BFTC-905 cells were subjected to 0–12 dynes cm^{-2} LSS for 1, 4, 8, or 12 h, respectively, followed by cellular and molecular assays for investigations of cell cycle regulation protein expressions, cell growth rates, and the potential mechanism.

Results—The results showed that the LSS with ≥ 8 dynes cm^{-2} for ≥ 8 h significantly increased protein expressions of cyclin B1, Wee1, p21, and p-CDK1^(Tyr15) ($p < 0.05$ for each), but conversely decreased protein expressions of cyclin A2, D1, E1, and CDK-1, -2, -4, and -6 ($p < 0.05$ for each) in the BFTC-905 cells, indicating that a G₂/M cell cycle arrest was obtained after shearing stimulation. Furthermore, our data demonstrated that the LSS-induced G₂/M arrest and the corresponding changes in cell cycle regulatory protein expressions were modulated by bone morphogenetic protein (BMP) receptor-Smad1/5 signaling pathway.

Conclusions—Our findings provided evidences for the effect of mechanical microenvironment on urothelial cancer pathobiology and generated insights into mechanism of LSS-regulated bladder tumor cell cycle.

Keywords—Laminar shear stress, Urothelial carcinoma, Bladder cancer, Cell cycle, BMP, Smad.

INTRODUCTION

Clinically, urothelial carcinoma (UC) remains one of the most fatal diseases in urological system, while that bladder cancer; the lower urinary tract carcinoma, is the most frequently diagnosed UC and accounts for a number of deaths in the past years.¹³ According to the statistics of World Health Organization, bladder cancer ranks as the ninth most frequently-diagnosed cancer with a higher incidence in men, and is the 13th leading death of cancer worldwide.² Moreover, UC occurring in upper urinary tract such as renal pelvis and/or ureter have also gained increasing attention since statistical analyses show that both of their morbidity and mortality rates are elevating over the last decade,²⁷ indicating that UC is currently one of the prevalent cancer worldwide and thus an efficacious strategy for UC therapy is urgently required.

Patients with UC can be classified into two histopathological stages: the major one is confined to the mucosa and does not damage the basement membrane (noninvasive UC, stage Ta), whereas the minority is presented as an invasive cancer that it may breach the basement membrane (stages T1–T4) and cause unpredictable metastasis subsequently. Although more than two-thirds of newly diagnosed bladder carcinoma are superficial/non-muscle-invasive, they are usually at high risk of recurrence after transurethral resection of tumor tissue and/or prophylactic intravesical therapy.^{1,19} In addition, even though the technologies of aggressive treatment including chemotherapy and radical cystectomy have been greatly advanced in the last decades, the invasive bladder cancer remains incurable and that the mean survival time following cancer recurrence in patients

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with invasive UC is merely 5.6 months.²⁰ These circumstances indicate that identifying the key factor responsible for the development and/or progression of urological malignancy, rather than persistently changing the anticancer drugs for trying, is truly needed to successfully cure UC.

Cell proliferation, performing in either a limited (one cell cycle) or an extensive degree (≥ 12 cell cycles), is the central phenotypic expression in all types of malignant neoplasia, and has long been known to play a crucial role in initiation, promotion, and progression of cancer.⁶ In general, cancer cell proliferation often disturbs the growth of neighbor normal cells in the tissue and may enhance the opportunity of neoplastic migration and/or invasion, leading to a dysfunctional organ and cancer metastasis accordingly.¹⁴ Whereas in terms of UC progression, the UC proliferation is highly growth rate-limited and thus is usually considered as the main determinant for cancer development.⁸ Therefore, it is applicable to control the progress of urothelial carcinogenesis through inhibition of cell proliferation.

Among various physiological factors, interstitial, blood, and/or lymphatic fluid flows inside and around the tissue (i.e., cellular microenvironment) have been known to play a critical role in cellular responses and functions²² since they could generate shear stress and tethering force to cell surface and/or cell–matrix connections as reported in the previous studies.^{25,28} Such interstitial fluid flow-induced mechanical stresses may be able to modulate prostate cancer cell growth through Tafazzin (TAZ)-mediated signaling pathway,¹⁷ as well as enhance expression levels of adhesion molecules in breast carcinoma triggered by downstream caveolin-1 (Cav-1)-mediated FAK/Src and ROCK/p-MLC pathways.²⁹ In addition, a study further revealed that epithelial tumor cell (A431 and A549) proliferation can be influenced by intratumoral pressure where both mitogen-activated protein kinases and nuclear antigen Ki-67 are involved in this mechanical modulation.¹⁰ Overall these research efforts demonstrated that the mechanical forces may be exploited as a potential tool to alter tumor cell proliferation and subsequently stop/retard cancer progression.

It is foreseeable that UCs which grow in the urinary tract are highly influenced by fluid-induced mechanical forces because they are frequently sheared by not only the interstitial and blood/lymphatic fluid flows, but also the urine flow drained from kidney to bladder. Therefore, in this study, we aimed to investigate the regulatory effects of fluid shear stress on cell cycle of bladder UC and the underlying mechanism. We first examined the cell cycle fluctuation of bladder cancer cells under impact of laminar shear stress (LSS) with

various intensities and/or time frame through imaging cytometric and protein expression analyses, followed by analyzing the expressions of Smad proteins; the known cell proliferation- and cell cycle-regulated molecules⁸ to identify the potential mechanism.

MATERIALS AND METHODS

Cell Culture

The human bladder female transitional carcinoma cells (BFTC-905 cells) purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan R.O.C.) were normally maintained using RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 1.5 g L^{-1} sodium bicarbonate, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin in a 37°C incubator with 5% CO_2 . The BFTC-905 cells in the exponential growth phase were transferred to sterilized glass slides 24 h prior to the experiment.

LSS Setup

To provide LSS on the cell surface, a laminar shearing system was established in which the BFTC-905 cells were seeded on the glass slides in the parallel plate flow chamber and sheared by growth medium flow as illustrated in the previous report.¹⁸ The intensity of applied LSS was determined based on its physiological level in UCs. However, it remains challenged to directly measure the total flow-induced shear stresses on UCs *in vivo* because of their slowness and heterogeneity.²⁵ Previous study indicated that the urine flow-induced shear stress ranges from 0.2 to 2 dynes cm^{-2} at normal flow rate (10 mL day^{-1}) and from 2 to 20 dynes cm^{-2} at high flow rate (100 mL day^{-1}) in inner medullary collecting ducts.⁴ In addition, it is known that the interstitial fluid-induced shear stress in tumors is nearly as high as the blood pressure because tumor vessel leakage is so extreme that implicates there is unhindered fluid communication between blood and interstitial spaces in tumors.^{12,23,24} Based on the experimental measurements using different methods, the magnitude of blood flow-induced shear stress in the venous system is ranged from 1 to 6 dynes cm^{-2} , whereas that in arteries is from 10 to 70 dynes cm^{-2} .⁵ Taken all together, in this study we used unidirectional/laminar pattern of flow with intensities of 2, 4, 8, and 12 dynes cm^{-2} to simulate different levels of LSS (τ) possibly encountered by the transitional UCs, and those can be obtained by setting appropriate medium flow rate (Q) according to the Navier–Stokes equation:

$$\tau = \frac{6\mu Q}{bh^2}, \quad (1)$$

where μ is the viscosity of the flowing solution (i.e., cell culture medium) with and without dorsomorphin (~ 0.01 dynes-s cm^{-2}); b and h represent the width and height, respectively, of the space above the cell monolayer. Cells in the shearing experiments were continuously stimulated by LSS and dorsomorphin (if it was used) simultaneously throughout the time course. In this study, dorsomorphin (Sigma, St. Louis, MO) with concentration of $1 \mu\text{M}$ was exploited to abolish the Smad1/5 signaling pathway according to the previous result.³⁰

Cell Cycle Assay

The cell cycle analysis was carried out by using a NC-3000TM cell cycle assay (Chemometec Inc., Allend, Denmark) in association with imaging cytometric measurement as reported previously.²⁶ Briefly, 24 h prior to the experiment, BFTC-905 cells were seeded onto glass slides with 1×10^6 cells per each. After sheared by LSS with designated intensity ($2\text{--}12$ dynes cm^{-2}) and treatment time ($1\text{--}12$ h), cells in each group were washed twice with PBS and fixed in 1-mL pre-chilled 70% ethanol, followed by a two-step sample preparation according to the manufacturer's instruction. For the static group, 1×10^6 BFTC-905 cells were normally cultured on the sterilized glass slide for 24 h, followed by the two-step sample preparation as described above. The cell cycle distribution of each group reflected by the DNA content up was detected using the NucleoCounter[®] NC-3000 system (Chemometec Inc.) and then quantitatively analyzed by using the NucleoView NC-3000TM software (Chemometec Inc.).

Effect of LSS on Cell Growth

To assess the effect of LSS on cell growth, a total of 1×10^7 BFTC-905 cells in the exponential growth phase were aliquoted onto ten sterilized glass slides at 24 h prior to the experiments. Cells on eight of ten slides were separately sheared with 2, 4, 8, or 12 dynes cm^{-2} LSS for 1 or 12 h that one set of LSS intensity was applied to two slides for each treatment time, whereas the cells on the rest two slides were statically cultured for additional 1 or 12 h as the controls. After LSS treatment, the cells for each group were transferred to six culture flasks with 5×10^4 cells per each flask. The kinetic growth curve of each group was established by counting the cell number every 24 h for 6 days using a hemocytometer with trypan blue exclusion method, whereas the specific growth rate (μ) was calculated by the formula:

$$\mu \times (t_2 - t_1) = \ln\left(\frac{C_{t_2}}{C_{t_1}}\right), \quad (2)$$

where C_{t_1} and C_{t_2} represent the cell concentrations at time points t_1 and t_2 , respectively.

Western Blot

In this study, monoclonal antibodies (mAbs) against cyclin-dependent protein kinases (CDK)-1, -2, -4, -6, phospho-CDK1 at Tyr15 (p-CDK1^(Tyr15)), CDK regulatory subunit cyclin A2, B1, D1, and E1, mitosis inhibitor protein kinase (Wee1), cyclin-dependent kinase inhibitor 1 at CIP1 (p21), receptor-regulated Smad-2/3, p-Smad1/5, p-Smad3, common-mediator Smad4, AMP-activated protein kinase alpha (AMPK α), p-AMPK at Thr172 (p-AMPK α ^(Thr172)) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were all purchased from Cell Signaling Technology (Danvers, MA). The expressions of aforementioned proteins in the BFTC-905 cells under various treatments were detected using Western blot as described in elsewhere.¹⁸ Briefly, 30 μg of whole protein extract of each sample was resolved by sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and transferred to 0.45- μm polyvinylidene difluoride membranes afterward. The membrane was then treated by blocking buffer (5% w/v skim dry milk in PBS-Tween-20 (0.05% v/v; PBS-T)) for 90 min at room temperature. The primary antibodies of aforementioned proteins were first diluted 1:1000 in blocking buffer, followed by incubation with membranes at 4 °C for 12 h. Membranes were then washed three times with PBS-T and incubated with HRP-conjugated goat anti-mouse/rabbit IgG secondary antibodies for 90 min at room temperature. After washed with PBS-T, the membranes were treated with chemiluminescent substrates and the expression level of each molecular band was quantitatively analyzed by using the ChemiDoc-IT 810 imaging system (UVP, Upland, CA). In this study, GAPDH was employed as the reference gene in all Western blot analyses.

Statistical Analysis

All data were obtained from triplicate experiments and presented as mean $\pm$ standard error (SE). Statistical analyses were conducted by using MedCalc software (MedCalc Software, Ostend, Belgium) in which comparisons for one condition between two groups were performed using one-way analyses of variance (ANOVA), followed by Dunnett's *post-hoc* test at a significance level of $p < 0.05$ throughout the study.

RESULTS AND DISCUSSION

LSS Induced Change of Cell Cycle Distribution in the BFTC-905 Cells

Figure 1(I; A–T) shows the imaging cytometric results for cell cycle distribution of BFTC-905 cells after treated with various LSS intensities (0 – 12 dynes cm^{-2}) for 0 – 12 h in the laminar shearing system, in which the groups without LSS (Fig. 1(I); A, F, K, and P) were served as the static controls. The cell cycle percentage of each experimental setting was further quantified and plotted in Fig. 1(II). Our data showed that the percentage of cell number in each cell cycle stage (i.e., G_0/G_1 , S, and G_2/M) remained constant when the cells were treated with ≤ 2 dynes cm^{-2} LSS for up to 12 h (Fig. 1(II); A and B), whereas the ratio of cell amount in G_0/G_1 significantly decreased but that in S and G_2/M phases increased when the cells were treated with ≥ 8 dynes cm^{-2} LSS for ≥ 8 h ($p < 0.05$; Fig. 1(II); D and E). Moreover, as comparing the percentages of the cells with ≥ 8 dynes cm^{-2} LSS (Fig. 1(II); D and E) in different cell cycle stages between 8 th and 12 th h, we found that the cell percentages in both G_1/G_0 and S phases decreased but that in G_2/M increased, showing that the cell cycle was only progressed from S to G_2 phase without mitosis in the duration of this time period. These results implied that the LSS with intensity of ≥ 8 dynes cm^{-2} may cause a G_2/M cell cycle arrest in human bladder transitional carcinoma after treatment for ≥ 8 h.

LSS Altered Cell Cycle Regulation Protein Expressions in the BFTC-905 Cells

To further confirm the LSS-induced cell cycle arrest obtained from the imaging cytometric analysis (Fig. 1), the protein expressions of cell cycle regulatory proteins (cyclin and CDK), Wee1, and p21 in the cells with various LSS treatments (0 – 12 dynes cm^{-2}) for 12 h were examined by Western blot and the results were exhibited in Fig. 2a. After quantitative analysis of each blotting, our data showed that all protein expressions in the cells with ≤ 4 dynes cm^{-2} LSS were maintained in a similar level within 12 -h treatment (Figs. 2b–2e; $p = \text{NS}$). For the cells with ≥ 8 dynes cm^{-2} LSS, however, the expression levels of cyclin B1, p-CDK1^(Tyr15), Wee1, and p21 were significantly increased (Figs. 2d and 2e, $p < 0.05$ for each) while those of cyclin A2, D1, E1, and CDK-1, -2, -4, and -6 were markedly decreased after treatment for 12 h (Figs. 2b–2d, $p < 0.05$ for each).

Based on the analyses of cell cycle regulation protein expressions, our data showed that the levels of cell distribution in G_1 phase (cyclin D1/CDK-4/-6), G_1/S

transformation (cyclin E1/CDK2), S/ G_2 transformation (cyclin A2/CDK2), G_2 phase (cyclin A2/CDK1), and G_2/M transformation (cyclin B1/CDK1) were all markedly declined after sheared with ≥ 8 dynes cm^{-2} LSS for ≥ 8 h. Moreover, we found that the decrease of CDK-1 expression was accompanied by an increase of its tyrosine 15 phosphorylation (p-CDK1^(Tyr15)) (Fig. 2d), indicating that the binding capability of cyclin B1 and CDK1 was reduced. Such circumstances may be interpreted by the Wee1 expression as described in the following. Wee1 is one of the negative regulators for cell cycle by phosphorylating the amino acid fragment Tyr15 and Thr14 on CDK1 and consequently inactivates the formation of CDK1–cyclin B1 complex.⁷ In this study, the results showed that the expression level of Wee1 in the BFTC-905 cells with 8 or 12 dynes cm^{-2} LSS was significantly enhanced by approximately 2 folds (Fig. 2e, $p < 0.05$ for each) whereas the expression level of p-CDK1^(Tyr15) was indeed correspondingly increased as shown in Fig. 2d. Therefore, here we surmise that because Wee1-mediated CDK1 phosphorylation at Tyr15 hindered the combination of CDK1 and cyclin B1 and such hindrance was performed in a LSS intensity-dependent manner, more cyclin B1 was accumulated in the cells with higher LSS magnitude as exhibited in Fig. 2d. On the other hand, p21, a tumor suppressor protein, has been known to play a crucial role in cell cycle regulation since it could mediate cyclin B1 accumulation in nuclei near the G_2/M boundary and cause a transient block in late G_2 phase accordingly.⁹ As plotted in Fig. 2e, the expression level of p21 was certainly elevated along with increase of LSS intensity that an enhancement of 2.3 - ($p < 0.05$) or 2.5 - ($p < 0.05$) fold was obtained after the cells were sheared with 8 or 12 dynes cm^{-2} LSS for 12 h, respectively. Combined with the previous result showing that tumor cell cycle arrest at G_2/M is associated with an increase of cyclin B1,¹⁶ our data clearly demonstrated that the impact of ≥ 8 dynes cm^{-2} LSS for ≥ 8 h may cause a G_2/M arrest in the BFTC-905 cells and that is consistent with the imaging cytometric analyses as shown in Fig. 1.

LSS Affected Cell Growth in the BFTC-905 Cells

To investigate how the LSS-induced cell cycle arrest influenced the cell proliferation, the growth of BFTC-905 cells after sheared with LSS was subsequently monitored for continuous six days. As plotted in Fig. 3, our data showed that all the shearing cells with 2 – 12 dynes cm^{-2} LSS for 1 h exhibited a resembling growth rate of ~ 0.681 day⁻¹ (Fig. 3(I)) and that is similar to the value obtained from the static culture (0.685 day⁻¹; $p = \text{NS}$). However, it could be observed that the cell growth kinetics was markedly retarded

FIGURE 1. Analyses of BFTC-905 cell cycle under different treatments. (I) Scatter plots and histograms of the cells sheared with 0 (static), 2, 4, 8, or 12 dynes cm⁻² LSS for 1, 4, 8, or 12 h, respectively, as indicated in the figure. The M1, M2, and M3 represented in the histogram A were used to demarcate cells in cell cycle phase of G₀/G₁, S, and G₂/M, respectively, based on the NucleoCounter[®] NC-3000[™] cell cycle assay instruction and such analytical approach was applied to all the other groups (B–T). (II) Quantitative analyses of cell number percentage in G₀/G₁, S, and G₂/M phase for the cells treated with 0 (A; static setting), 2 (B), 4 (C), 8 (D), or 12 (E) dynes cm⁻² LSS for 0, 1, 4, 8, and 12 h. Values are mean ± SE (*n* = 3). Symbols of *, †, and ‡ represent *p* < 0.05 compared with the percentage values of G₀/G₁, S, and G₂/M measured at *t* = 0, respectively.

FIGURE 2. Effect of LSS on expressions of cell cycle regulatory proteins in the BFTC-905 cells. (a) Western blots of GAPDH and cell cycle regulatory proteins in the BFTC-905 cells which were treated by 0, 2, 4, 8, or 12 dynes cm⁻² LSS for 12 h as indicated on the top of the blot images. The protein expression obtained from the group without LSS (i.e., LSS intensity = 0 dynes cm⁻²) was employed as the blank control. (b–e): Quantitative analyses of protein expression levels of cyclin D1, CDK4, and CDK6 (b); cyclin A2, cyclin E1, and CDK2 (c); cyclin B1, CDK1, and p-CDK1^(Tyr15) (d); and Wee1 and p21 (e) displayed above. Each bar represents the relative protein expression level after normalization to the GAPDH. Values are mean $\pm$ SE (*n* = 3). **p* < 0.05 compared to the value obtained from the group without LSS.

when the cells were sheared with 8 ($\mu = 0.651 \text{ day}^{-1}$) or 12 ($\mu = 0.642 \text{ day}^{-1}$) dynes cm⁻² LSS for 12 h, resulting in a 15% (*p* < 0.05) and 20% (*p* < 0.05) decrease of cell number, respectively, at the 6th day compared to the group without impact of LSS (Fig. 3(II)). These results showed that the LSS with intensity of ≥ 8 dynes cm⁻² for ≥ 8 h was able to decelerate cell proliferation in the BFTC-905 cells and we reason that it was attributed to LSS-induced G₂/M arrest as illustrated above.

LSS Induced Phosphorylation of Smad1/5 in the BFTC-905 Cells

According to the previous study efforts, mechanoreceptors bone morphogenetic protein (BMP)³¹ and/or transforming growth factor beta (TGF- β)¹¹ play the key role in mediating LSS stimulation signaling to cells through BMP-Smad1/5 pathway and TGF- β /activin-Smad2/3 pathway, respectively. In addition, it has been known that reg-

ulation of AMPK activity may provide significant effects on cell cycle progression.²¹ To identify how the LSS induced cell cycle arrest in the BFTC-905 cells, the expression levels of AMPK and Smad proteins in the cells with 0–12 dynes cm⁻² LSS for 12 h were comprehensively examined. As shown in Fig. 4, it can be seen that the expressions of Smad-2, -3, p-Smad3, AMPK α , and p-AMPK α ^(Thr172) were maintained in a constant level (Fig. 4a) throughout the range of LSS intensity applied, whereas the levels of Smad4 and p-Smad1/5 were markedly enhanced along with LSS intensity used, indicating that the LSS-induced activation of Smad4 and Smad1/5 phosphorylation were shearing dose-dependent within the range of 0–12 dynes cm⁻² LSS. Based on the quantitative analyses of blot intensities (Fig. 4c), the expression levels of Smad4 and p-Smad1/5 in the cells with 12 dynes cm⁻² LSS were significantly increased by 64% (*p* < 0.05) and 88% (*p* < 0.05), respectively, compared to the group without LSS. These outcomes suggest that the LSS stimulation to BFTC-905 cells were mediated

FIGURE 3. Growth kinetic curves of the BFTC-905 cells after different LSS treatments. After sheared with 2, 4, 8, or 12 dynes cm^{-2} LSS for 1 (I) or 12 (II) h, the cells of all settings were statically incubated at 37 °C and the cellular growth curve of each group was established through measurement of cell number every 24 h for 6 days. The group without LSS (LSS intensity = 0 dynes cm^{-2}) was employed as the control. Values are mean $\pm$ SE ($n = 3$). * $p < 0.05$ compared to the cell number obtained from the control group.

through BMP receptor (BMPR)-Smad1/5 pathway instead of TGF- β /activin-Smad2/3 pathway, nor induced by AMPK activity in the cells.

To further verify the effectiveness of BMP-Smad1/5 pathway on LSS-treated UC, expression levels of Smad4 and p-Smad1/5 proteins in the 12-h shearing BFTC-905 cells in the presence and absence of dorsomorphin, an inhibitor for BMP-Smad1/5 pathway, were examined. As shown in Fig. 4b, the 1- μM dorsomorphin was able to reduce the level of Smad4 and p-Smad1/5 in the cells even when the intensity of LSS was ≥ 8 dynes cm^{-2} . In comparison to the shearing group without dorsomorphin, the expression level of p-Smad1/5 in the cells simultaneously treated with 12 dynes cm^{-2} LSS and 1- μM dorsomorphin was significantly reduced by about 2-fold (Fig. 4d; $p < 0.05$), while that of Smad4 was slightly decreased by 15% ($p = \text{NS}$) as shown in Fig. 4e. These results clearly demonstrated the correlation between BMP-Smad1/5 pathway and LSS stimulation. Furthermore, on the other hand, one may concern that dorsomorphin, also known as Compound C, is also an inhibitor of AMPK and thus question that whether AMPK pathway played a role in LSS signaling delivery. Here we exclude such hypothesis because, although treatment with dorsomorphin may inhibit both p-Smad1/5 and pAMPK, the levels of AMPK α and p-AMPK $\alpha^{\text{(Thr172)}}$ did not change with increase of LSS in 2–12 dynes

cm^{-2} as illustrated in Figs. 4a and 4c ($p = \text{NS}$ for each), suggesting that the effects of LSS were mediated by Smad1/5 but not AMPK in the BFTC-905 cells.

In addition to determining the role of BMP receptor in LSS-induced mechanotransduction, our data implied that 1- μM dorsomorphin was able to reduce the effect of LSS on BFTC-905 cells by down-regulating the level of Smad1/5 phosphorylation and hindered the BMP-Smad1/5 signaling delivery consequently. In fact, BMP signaling through BMPRII has been known to be able to suppress tumorigenesis at gastric epithelial junction zones.³ Moreover, it has been demonstrated that mice with (1) inactivated BMPRII, (2) overexpressed BMP antagonist, and/or (3) Smad4-deficient T cells may develop epithelial tumor in the intestinal tract.¹⁵ Combined with the detrimental effect of LSS on cell proliferation as illustrated in Figs. 1, 2, and 3, our experimental results suggest that the BMP-specific Smad1/5 signaling pathway may play a tumor-suppressive role in human UC and such anticancer efficacy can be induced by ≥ 8 dynes cm^{-2} LSS for ≥ 8 -h treatment.

LSS Induced G₂/M Arrest in the BFTC-905 Cells Through BMP-Smad1/5 Pathway

We subsequently examined how dorsomorphin affected the cell cycle of BFTC-905 cells when they were simultaneously treated with 0–12 dynes cm^{-2} LSS for 12 h. As shown in Fig. 5a, the results showed that the expression levels of CDK-1 (Fig. 5b), CDK-2 (Fig. 5e), and cyclin E1 (Fig. 5f) in the cells with ≥ 8 dynes cm^{-2} LSS and 1- μM dorsomorphin were markedly increased, whereas those of p-CDK1^(Tyr15) (Fig. 5c), cyclin B1 (Fig. 5d), and p21 (Fig. 5g) were significantly reduced compared to the LSS-treated cells without dorsomorphin ($p < 0.05$ for each).

Considering the correlation between p21 and cell cycle regulatory proteins, as well as the effect of dorsomorphin on phosphorylation of Smad1/5 proteins (Fig. 4), the above outcome was reasoned that because dorsomorphin inhibited BMP-Smad1/5 signaling pathway, the LSS stimulation (≥ 8 dynes cm^{-2}) to the cells was blocked and led to a deactivation of p21 transcription as shown in Fig. 5g. Thereafter, the binding of cyclin B1 and CDK1 can be normally processed and thus the cyclin B1 accumulation was no longer taken place, leading to a reduced level of cyclin B1 compared to the shearing group without dorsomorphin as shown in Fig. 5d. In comparison to the results of cell cycle protein expressions obtained from the LSS-treated cells without dorsomorphin (Fig. 2), our data clearly showed that the application of 1- μM dorsomorphin successfully abolished the LSS-induced G₂/M cell cycle arrest and was able to facilitate the cell

FIGURE 4. Analyses of Smad and AMPK protein expressions in the BFTC-905 cells under various treatments. (a and b): Western blots of Smad2, Smad3, Smad4, p-Smad1/5, p-Smad3, AMPK α , p-AMPK α ^(Thr172), and GAPDH proteins in the BFTC-905 cells which were statically cultured (LSS intensity = 0 dynes cm⁻²) or treated with 2, 4, 8, or 12 dynes cm⁻² LSS in the absence (a) and/or presence (b) of 1- μ M dorsomorphin for 12 h. The condition of each lane is indicated on the top of the blot images. (c) Quantitative analyses of the expression levels of the seven proteins shown in Fig. 4a. Each bar represents the relative protein expression level after normalization to the GAPDH. Values are mean $\pm$ SE ($n = 3$). * $p < 0.05$ compared to the value obtained from the group without LSS. (d and e): Quantitative analyses of the expression levels of p-Smad1/5 (d) and Smad4 (e) in the cells without and with dorsomorphin shown in Figs. 4a and 4b, respectively. Each bar represents the relative protein expression level after normalization to the GAPDH and the blank control (i.e., the one with neither LSS nor a dorsomorphin). Values are mean $\pm$ SE ($n = 3$). * $p < 0.05$. † $p < 0.05$ compared to the value gained from the group with neither LSS nor a dorsomorphin. ‡ $p < 0.05$ compared to the value gained from the group statically cultured with 1- μ M dorsomorphin for 12 h.

FIGURE 5. Effect of dorsomorphin on expressions of cell cycle regulatory proteins in the LSS-treated BFTC-905 cells. (a) Western blots of GAPDH and cell cycle regulatory proteins in the BFTC-905 cells which were treated by 0, 2, 4, 8, or 12 dynes cm⁻² LSS in the presence or absence of 1- μ M dorsomorphin for 12 h as indicated on the top of the blot images. The expression of the protein obtained from the group with neither LSS (LSS intensity = 0 dynes cm⁻²) nor a dorsomorphin was employed as the blank control. (b-g): Quantitative analyses of protein expression magnitudes of CDK1 (b), p-CDK1^(Tyr15) (c), cyclin B1 (d), CDK2 (e), cyclin E1 (f), and p21 (g) displayed above. Each bar represents the relative protein expression level after normalization to the GAPDH and the blank control (i.e., the one with neither LSS nor a dorsomorphin). Values are mean $\pm$ SE ($n = 3$). * $p < 0.05$. † $p < 0.05$ compared to value obtained from the blank control.

cycle process from G₁ to S phase in the shearing cells based on the enhanced protein expressions of CDK2 and cyclin E1 as plotted in Figs. 5e and 5f, respec-

tively. However, more experiments are certainly needed to precisely evaluate the cell cycle regulatory capability of BMP-Smad1/5 pathway in response to

FIGURE 6. Schematic diagram of the signaling pathway regarding the cell cycle regulation in human bladder transitional carcinoma/BFTC-905 cells after stimulated by LSS. ↑ and ↓ denote up- and down-regulation of the protein induced by LSS. ⊥ represents an inhibitory effect on cyclin–CDK interaction. The block with black background indicates that the molecule is not involved in the mechanism of LSS-induced cell cycle arrest.

LSS stimulation because AMPK pathway may be concurrently inactivated after use of dorsomorphin and thereby affected cell cycle progression accordingly. Nonetheless, these results have prompted a clinical interest since the LSS administration with ≥ 8 dynes cm^{-2} for ≥ 8 h may be helpful to decelerate cell proliferation of UC and the potential mechanism was hypothetically mediated through BMP-specific Smad1/5 pathway.

In summary, we have successfully demonstrated that LSS with ≥ 8 dynes cm^{-2} for ≥ 8 h is able to induce a G_2/M cell cycle arrest in human bladder transitional carcinoma (BFTC-905 cells). Such shearing-induced cellular response is likely mediated through BMP-specific Smad1/5 signaling pathway in association with Wee1 interaction, leading to increased protein expressions of p-CDK1^(Tyr15) and p21 but decreased levels in cyclin D1, E1 A2 and CDK-1, -2, -4, -6, resulting in a G_2/M transition arrest in the BFTC-905 cells as illustrated in Fig. 6. Since mechanical microenvironment plays a significant role in cancer cell pathobiology whereas it is greatly affected by fluid flow-induced shear stress, our findings suggest that the response of UC to ≥ 8 dynes cm^{-2} LSS induced from the combination of interstitial, blood/lymphatic and/or urine flow should be taken into account in cancer therapy and disease management.

ACKNOWLEDGMENTS

This work was financially supported by Ministry of Science and Technology, Taiwan R.O.C. (MOST 106-2221-E-008-060; Y.-H. Lee and MOST 106-2314-B-281-001-MY3; Y.-C. Cheng).

CONFLICT OF INTEREST

Yu-Hsiang Lee, Chia-Wei Lai, and Yu-Che Cheng declare no conflict of interest.

COMPLIANCE WITH ETHICAL STANDARDS

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

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