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Mechanical regulation of cancer cell apoptosis and autophagy: Roles of bone morphogenetic protein receptor, Smad1/5, and p38 MAPK

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ABSTRACT

Mechanical forces induced by interstitial fluid flow in and surrounding tissues and by blood/lymphatic flow in vessels may modulate cancer cell invasion and metastasis and anticancer drug delivery. Our previous study demonstrated that laminar flow-induced shear stress induces G_2/M arrest in tumor cells. However, whether shear stress modulates final cell fate remains unclear. In this study, we investigated the role of flow-induced shear stress in modulating the survival of four human tumor cell lines, i.e., Hep3B hepatocarcinoma cells, MG63 osteosarcoma cells, SCC25 oral squamous carcinoma cells, and A549 carcinomic alveolar basal epithelial cells. Laminar shear stress (LSS) ranging from 0.5 to 12 dyn/cm^2 induced death of these four tumor cell lines. In contrast to LSS at 0.5 dyn/cm^2, oscillatory shear stress (OSS) at 0.5 ± 4 dyn/cm^2 cannot induce cancer cell death. Both LSS and OSS had no effect on human normal hepatocyte, lung epithelial, and endothelial cells. Application of LSS to these four cell lines increased the percentage of cells stained positively for annexin V–FITC, with up-regulations of cleaved caspase-8, -9, and -3, and PARP. In addition, LSS also induced Hep3B cell autophagy, as detected by acidic vesicular organelle formation, LC3B transformation, and p62/SQSTM1 degradation. By transfecting with small interfering RNA, we found that the shear-induced apoptosis and autophagy are mediated by bone morphogenetic protein receptor type (BMPR)-IB, BMPR-specific Smad1 and Smad5, and p38 mitogen-activated protein kinase in Hep3B cells. Our findings provide insights into the molecular mechanisms by which shear stress induces apoptosis and autophagy in tumor cells.

1. Introduction

Mechanical microenvironment of cells and tissues has been shown to play important roles in modulating cellular responses and tissue functions [1]. There is increasing evidence that mechanical forces exert significant effects on the growth and development of solid tumors. Compressive stress on tumor cells can suppress tumor cell proliferation, induce apoptosis, and stimulate their migration [2,3]. Extracellular matrix stiffness can regulate tumor malignancy through integrin-dependent mechanotransduction [4]. In the body, fluid shear stress may be generated by interstitial flow, blood or lymphatic flow, or some other type of fluid flow. Cancer cells may experience shear stress induced by interstitial flow in tumor tissues and by blood or lymphatic flow during their metastasis. The role of flow-induced mechanical forces in modulating cancer pathophysiology has just emerged [5]. Our previous study demonstrated that laminar shear stress at 12 dyn/cm^2 induces G_2/M arrest in tumor cells [6]. Although flow environment has been shown to modulate cancer cell cycle distribution, invasion, and metastasis [7,8], whether flow-induced shear stress can mediate the survival and death of cancer cells remains unclear.

Apoptosis and autophagy are both forms of programmed cell death. Apoptosis, which is the first known programmed cell death mechanism, has been extensively studied, and its contribution to the pathogenesis of disease has been well documented [9]. Apoptotic cell death manifests as condensation and shrinkage of the cell, membrane blebbing, DNA fragmentation, and caspase activation [10,11]. Autophagy is now known to play a role in the processes of many diseases, including tumorigenesis [12]. At the cellular level, autophagy is defined as a pro-survival function that facilitates cellular response to stress by clearing damaged proteins and organelles, and provides energy to the cells during starvation [13]. The process of autophagy is mediated by autophagy-related genes (Atgs). In this process, an isolation membrane is initially formed and transformed into an autophagosome, which is

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accompanied by microtubule-associated protein light chain 3 (LC3) protein conversion of LC3-I to LC3-II and p62/sequestosome 1 (SQSTM1) protein degradation [14]. Subsequently, autophagosomes fuse with the lysosome and degrade the contents of the cells [14]. Recent studies have indicated that autophagy may be a mechanism of caspase- and apoptosis-independent cell death [15,16]. The molecular connection between autophagy and apoptosis has become a burgeoning area poorly understood. Apoptosis and autophagy may exert synergetic effects on cells [17]. On the other hand, autophagy may only be triggered in the cells when apoptosis is suppressed [18]. Numerous studies have proposed chemical methods to lead cell apoptosis and autophagy for anti-cancer therapies. However, the detailed mechanisms of cell death progression in tumor cells and the interaction between these two types of programmed cell death modulated by mechanical stimulation remain unclear.

Bone morphogenetic proteins (BMPs), which are multifunctional cytokines that belong to transforming growth factor (TGF)-β superfamily, play critical roles in cell differentiation, proliferation, migration, and apoptosis [19]. BMPs transduce signals by forming heteromeric complex of cognate type I (i.e., BMPRIA or BMPRIB) and type II serine/threonine kinase receptors [20], and function through BMP receptor-regulated Smads (R-Smads, i.e., Smad1/5/8). It has been reported that BMP signaling plays important roles in mediating tumorigenesis [21]. However, the functional significance in regulating mechanical responses of tumor cells has not been reported. In addition, mitogen-activated protein kinases (MAPKs), including extracellular signal-related kinase (ERK), Jun NH2 terminal kinase (JNK), and p38 MAPK, have been shown to modulate apoptosis in response to many types of cellular stress [22]. However, the shear stress-dependent activation of MAPKs in cancer cell and its role in the regulation of cancer cell survival and death are unclear. It has been reported that BMP/Smad signaling mediates MAPK activation in cancer cell metastasis and growth [23,24]. Thus, it is important to elucidate the role of BMP/Smad and MAPK signaling pathways and their interactions in modulating apoptosis and autophagy of cancer cells in response to shear stress.

In this study, we investigated the regulatory role and its underlying mechanism of shear stress in modulating cell death of four cancer cell lines, including Hep3B hepatocarcinoma cells, MG63 osteosarcoma cells, SCC25 oral squamous carcinoma cells, and A549 carcinomic alveolar basal epithelial cells. We found that laminar shear stress (LSS) induces the progress of cellular apoptosis and autophagy in these cells through the BMPRIB/Smad1/5/p38 MAPK signaling cascade, which is accompanied by the increased expression of cleaved caspases and LC3B-II formation. Our findings demonstrate a crucial role of mechanical stimuli in tumor pathobiology and generate a new insight into the mechanism of shear-regulation in tumor cell death.

2. Materials and methods

2.1. Materials

The following antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA): polyclonal antibodies against cleaved caspase-3, caspase-9, poly-(ADP-ribose) polymerase (PARP), p62/SQSTM1, phospho-Smad1/5, and p38 MAPK and monoclonal antibodies against cleaved caspase-8. The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): monoclonal antibodies against phospho-ERK and phospho-JNK and polyclonal antibodies against Smad1/5/8. A polyclonal antibody against LC3B was obtained from Sigma (St Louis, MO, USA). The phospho-p38 MAPK antibody was purchased from BD Biosciences Pharmingen (San Diego, CA, USA). The anti-GAPDH mouse monoclonal antibody was obtained from Millipore (Temecula, CA, USA). SB203580 (a specific p38 MAPK inhibitor) was from Merck (Frankfurter, Darmstadt, Germany). z-DEVD-FMK (a specific caspase-3 inhibitor), 3-methyladenine (3-MA, a specific autophagy inhibitor by blocking the autophagosome formation via inhibition in type III phosphatidylinositol 3-kinase), and all other reagent grade chemicals were purchased from Sigma.

2.2. Cell culture

The human cancer cell lines SCC25 (oral squamous carcinoma cells), MG63 (osteosarcoma cells), A549 (carcinomic alveolar basal epithelial cells), and Hep3B (hepatocarcinoma cells), normal lung cell line NL20, and normal hepatocyte Chang liver cell line (CHL) were purchased from ATCC (Manassas, VA, USA). Hep3B, MG63, and CHL cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (P/S; Gibco). 0.1 mM MEM NEAA was added to the Hep3B culture medium. SCC25 and A549 cells were cultured in RPMI-1640 medium (RPMI; Gibco) supplemented with 10% FBS and 1% P/S. NL20 cells were cultured in F12 medium (Gibco) supplemented with 10% FBS and 1% P/S. Human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cords by means of the collagenase perfusion technique [25], and grown in Petri dishes in medium 199 (M199, Gibco) supplemented with 20% FBS and 1% penicillin/streptomycin (P/S, Gibco). Before the flow experiments, cells ($1–2 \times 10^5$ cells/cm^2) were trypsinized and seeded onto glass slides (75×38 mm; Corning) pre-coated with type I collagen (30 μg/mL). Cells were refreshed with media containing 2% FBS and then incubated for 24 h before experiments. All cells were grown at 37 °C in a 5% CO_2 humidified atmosphere.

2.3. Flow apparatus

Slides with cultured cells were mounted in a parallel-plate flow chamber, which was characterized and described in detail elsewhere [26]. The chamber was connected to a perfusion loop system and maintained under a constant temperature-controlled enclosure at pH 7.4 by continuous gassing with a mixture of 5% CO_2 in air. LSS generated on the cells by flow was calculated using the formula $\tau = 6\mu Q/wh^2$, where h is the channel height, w is the channel width, Q is the flow rate, and μ is the dynamic viscosity of the perfusate. The magnitudes of LSS used in this study are 0.5, 6, and 12 dyn/cm^2. For some experiments, cells were pre-treated with 3-MA (4 mM), z-DEVD-FMK (50 μM), and SB203580 (10 μM) for 1 h, and then subjected to shear flow in the presence of the same reagent. The static control cells were incubated in new culture medium, whereas the experimental cells were placed under flow conditions. The cultured cells were subjected to oscillatory fluid flow in a parallel plate flow chamber as previously described [27]. The oscillatory flow was composed of a low level of mean flow (shear stress = 0.5 dyn/cm^2), which was supplied by a hydrostatic flow system to provide basal nutrients and oxygen and the superimposition of a sinusoidal oscillation, using a piston pump with a frequency of 1 Hz and peak-to-peak amplitude of ± 4 dyn/cm^2.

2.4. Cell viability assay

Cell viability was determined using trypan blue exclusion method. The stimulated and control cells were collected by trypsinization, and the cell pellets were resuspended in 1 mL serum-free complete medium. An equal volume of 0.4% trypan blue and cell suspension was mixed and incubated for 3 min at room temperature. A drop of the trypan blue/cell mixture was analyzed using a hemacytometer observed under a binocular microscope. Unstained (viable) and stained (nonviable) cells were separately counted, and the percentages of dead cells were calculated.

2.5. Measurement of the formation of acidic vesicular organelles (AVOs) and annexin-V/propidium iodide (PI) binding assay

To quantify the formation of AVOs, cells were washed twice in phosphate buffered saline (PBS), harvested, and stained with 1 μg/mL acridine orange for 15 min, and then analyzed by flow cytometry to detect emission in the red wavelength (650 nm). In the apoptosis detection assay, a FITC Annexin-V Apoptosis Detection Kit (Invitrogen, San Diego, CA) was used according to the manufacturer's instructions. Briefly, cells were trypsinized and collected in PBS by centrifugation, resuspended in 1× binding buffer, and FITC–annexin-V and PI were then added. After incubation at room temperature for 15 min in the dark, the cells were analyzed by flow cytometry. Control cells stained with annexin-V or PI alone were used to compensate for the flow cytometric analysis.

2.6. Western blot analysis

Cells were lysed with a buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and a protease inhibitor cocktail (Roche, Basal, Switzerland). Total cell lysates (50 μg of protein) were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane (Immobilon P, 0.45-μm pore size). The membrane was then incubated with the designated antibodies. Immunodetection was performed using the Western-Light chemiluminescent detection system (Applied Biosystems, Foster City, CA, USA).

2.7. Small interfering RNA transfection assay

Control, Atg5, Smad1, Smad5, BMPRIA, BMPRIB, and BMPRII-specific small interfering RNAs (siRNA) were purchased from Invitrogen Technology. p38 MAPK-specific siRNA was purchased from Cell Signaling Technology. For siRNA delivery, cells with a 70–80% confluence were transfected with an appropriate concentration of siRNA using the Lipofectamine RNAiMAX Reagent (Invitrogen), according to the manufacturer's instructions.

2.8. Statistical analysis

Results are expressed as mean ± SEM, with $n = 3$ per group for all comparisons. Statistical analysis was performed using one-way analyses of variance (ANOVA), followed by the Scheffe's test. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. LSS, but not oscillatory shear stress (OSS), induces cancer cell death

To determine whether shear stress could induce cancer cell death, four cancer cell lines, i.e., SCC25, MG63, A549, and Hep3B, were cultured on collagen-coated glass slides and exposed to LSS at 0.5, 6, or 12 dyn/cm^2 at different time points. LSS significantly increased the percentage of dead cells during the shearing time period, and the decrease in cell viability was in a shearing time- and dose-dependent manner (Fig. 1A). To determine which type of shear stress could induce cancer cell death, we applied OSS at 0.5 ± 4 dyn/cm^2 and LSS at 0.5 dyn/cm^2 to these four cancer cell lines for the designated times. The results showed that while LSS induces death of these four cancer cell lines, there was no significant increase in the percentage of dead cells when these four cancer cell lines were exposed to OSS, as compared with static control cells (Fig. 1B and C). To validate whether shear stress could induce non-cancerous cell death, Chang liver (CHL) cells were exposed to LSS at 0.5 or 12 dyn/cm^2 for 12 and 24 h. In addition, NL20 normal lung cells were exposed to 12 dyn/cm^2 for 48 h. The percentage of death of sheared-CHL (Fig. 1D) and NL20 cells (Fig. 1E) was significantly lower than that of sheared-Hep3B and A549 cells at the same time points, respectively. As controls, there was no significant difference in cell death between static and sheared HUVECs (Fig. 1E).

3.2. LSS induces autophagy and apoptosis in cancer cells

AVO formation occurs during autophagy process, and it can be detected and measured by vital staining with acridine orange. Acridine orange moves freely across biological membranes and accumulates in acidic compartments, where it is observed as a bright red fluorescence [28]. Fig. 2A demonstrates that LSS caused increase of AVO formation in the four cancer cells along with shearing force and treatment time. The representative images for the AVO quantification of Hep3B cells were shown in Fig. 2B. The autophagy level was significantly higher in sheared cells in all four cancer cell lines for various durations, as compared with the static control cells. In addition, the changes in LC3B and p62/SQSTM1 patterns were investigated. Fig. 2C shows that cells subjected to LSS increased the LC3B-II levels and decreased the p62/SQSTM1 levels in a dose- and time-dependent manner. However, the shear-induced LC3B-II expression in the CHL cells (Fig. 2D) and NL20 cells (Fig. 2E) was significantly lower than that of the Hep3B cells and A549 cells, respectively. LSS did not induce LC3B-II expression in HUVECs either (Fig. 2E). To further investigate the mechanism of LSS-induced apoptosis, we detected apoptotic cells (annexin-V$^+$/PI$^-$) by flow cytometry. As shown in Fig. 2F, LSS increased the amount of apoptotic cells in all four cancer cell lines. We next examined the expression of various apoptosis-related proteins in Hep3B cells. LSS enhanced the cleavage of caspase-8, caspase-9, caspase-3, and PARP (Fig. 2G). These increases in the FITC–annexin V/PI staining and caspase cleavage caused by LSS occurred in a shearing time- and dose-dependent manner. The shear-induced expressions of cleaved forms of caspase-8, caspase-9, caspase-3, and PARP between CHL and Hep3B cells were also compared. Fig. 2H shows that the expression of these apoptosis-related proteins in sheared CHL cells was significantly lower than that of sheared Hep3B cells. In addition, the expression of these proteins in sheared NL20 cells was also lower than that of sheared A549 cells (Fig. 2I). No significant difference was found for the expression of these apoptosis-related proteins between static and sheared HUVECs (Fig. 2I).

3.3. Interaction between shear stress-induced autophagy and apoptosis in cancer cells

Given our findings that LSS induced autophagy and apoptosis in cancer cells, we further investigated the interaction between these two cellular processes. Hep3B cells were pre-treated with a specific caspase-3 inhibitor z-DEVD-FMK (50 μM), an autophagy inhibitor 3-MA (4 mM), or co-treated with both reagents for 1 h and then exposed to LSS at 0.5 dyn/cm^2 for an additional 24 h. The shear-induced death of Hep3B cells was partially but significantly decreased with 3-MA and z-DEVD-FMK treatments (Fig. 3A). Co-treatment with both inhibitors exerted additive effects to decrease the percentage of shear-induced cell death. In Hep3B cells, cleaved PARP was partially but significantly attenuated with 3-MA and z-DEVD-FMK treatments (Fig. 3B). The shear-induced LC3B-II protein expression was significantly attenuated only with 3-MA, but not z-DEVD-FMK treatment (Fig. 3B). It appears that the shear-induced autophagy occurred earlier than apoptosis. To further confirm this result, Hep3B cells were first transfected with Atg5-specific siRNA (20 nM) and then exposed to LSS for 24 h. The percentage of apoptosis (i.e., annexin V-positive and PI-negative cells) in Atg5-specific siRNA-transfected cells was attenuated after exposing to shear stress, as compared with the control siRNA-transfected cells (Fig. 3C). Shear-inductions of cleaved PARP, caspase-3, and LC3B-II protein expression were partially but significantly decreased in Atg5-specific siRNA-transfected cells, as compared with control siRNA-transfected cells (Fig. 3D).

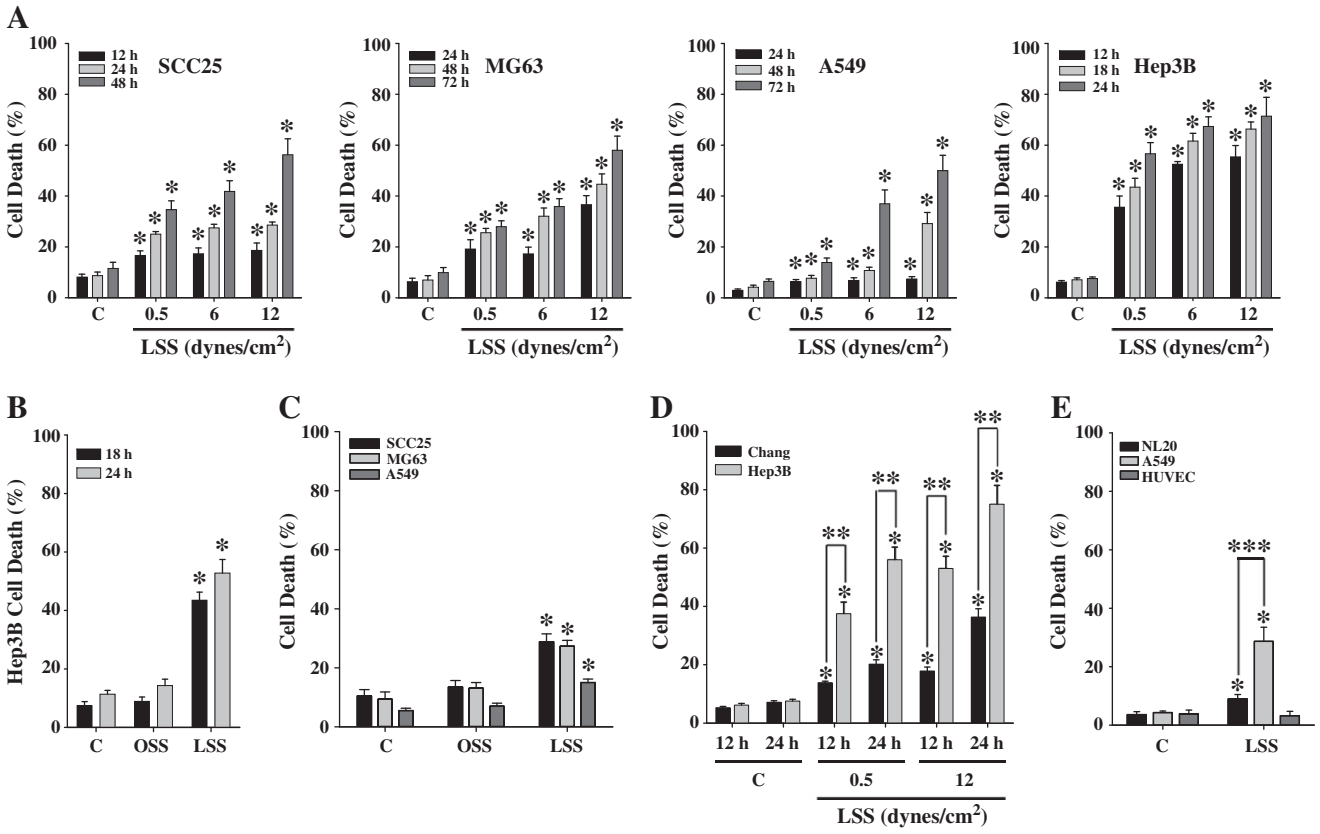

Fig. 1. LSS, but not OSS, induces cancer cell death. (A) Human tumor cell lines SCC25, MG63, A549, and Hep3B were maintained under static conditions (C) or subjected to LSS at 0.5, 6, or 12 dyn/cm^2 for various durations. (B) Hep3B cells were maintained under static conditions or subjected to LSS or OSS for 18 or 24 h. (C) SCC25, MG63, and A549 cells were kept under static conditions or subjected to LSS or OSS for 24 h, 48 h, and 72 h, respectively. (D) CHL and Hep3B cells were maintained under static conditions or subjected to LSS at 0.5 or 12 dyn/cm^2 for 12 or 24 h. (E) A549, NL20, and HUVECs were maintained under static conditions or subjected to LSS at 12 dyn/cm^2 for 48 h. HUVECs served as negative controls. The percentage of cell death was calculated using trypan blue staining method. The results are shown as mean ± SEM from three independent experiments. *, $P < 0.05$ sheared cells vs. static cells at the same point; **, $P < 0.05$ Hep3B cells vs. CHL cells under the same condition; ***, $P < 0.05$ A549 cells vs. NL20 cells under the same condition.

3.4. Shear stress-induced autophagy and apoptosis in cancer cells are mediated by BMP/Smad signaling pathway

Application of LSS at 0.5 dyn/cm^2 to Hep3B cells induced a rapid increase (within 10 min) in Smad1/5 phosphorylation, which reached a maximal level within 1 h and then declined but remained elevated after 24 h of shearing (Fig. 4A). Hep3B cells were transfected with control and Smad1/5-specific siRNAs (20 nM each) and then exposed to LSS at 0.5 dyn/cm^2 for 24 h. The shear-induced expressions of cleaved PARP and LC3B-II were partially but significantly attenuated in Smad1/5-specific siRNA-transfected cells (Fig. 4B). These data suggest that Smad1/5 is involved in the shear-induced apoptosis and autophagy pathways. To clarify whether Smad knockdown-mediated attenuation of PARP cleavage was mediated through the extrinsic or intrinsic apoptosis pathway, the expression levels of cleaved caspase-8 and caspase-9 in Hep3B cells were examined. The expression of cleaved caspase-9 was partially but significantly attenuated in Smad1/5-specific siRNA-transfected cells, but there was no difference in that of cleaved caspase-8 expression (Fig. 4B). To determine which BMP receptor subtypes are responsible for the shear-mediated activation of Smad1/5, Hep3B cells were transfected with BMPRIA-, BMPRIB-, or BMPRII-specific siRNAs (40 nM), which reduced the expression of corresponding proteins by ~70% in comparison to that by control siRNA (Fig. 4C), and then exposed to LSS for 1 h. The shear-induced Smad1/5 phosphorylation was abolished by BMPRIB- and BMPRII-specific siRNAs, but not BMPRIA-specific siRNA (Fig. 4D). Subsequently, Hep3B cells were transfected with BMPRIB- or BMPRII-specific siRNA (40 nM) and then maintained under static conditions or exposed to LSS for 24 h. After shear stress, Hep3B cells transfected with BMPRIB- or BMPRII-specific siRNA resulted in significant inhibitions in the shear-induced cleavage of caspase-9 and PARP, as well as LC3B-II in comparison to control siRNA-transfected group (Fig. 4E). These results indicate that shear-induced apoptosis and autophagy pathways are regulated by BMPRIB and Smad1/5 in cancer cells.

3.5. p38 MAPK, but not ERK and JNK, mediates shear-induced autophagy and apoptosis in cancer cells

We further investigated whether LSS could induce cancer cell apoptosis and autophagy through the activation of MAPK signaling pathway. A time course experiment ranging from 10 min to 24 h was carried out to measure ERK, JNK, and p38 MAPK phosphorylation levels by Western blotting. Fig. 5A shows that the levels of phosphorylated p38 MAPK (p-p38) increased 10 min after the onset of LSS treatment in Hep3B cells. However, there was no significant difference in the levels of phosphorylated ERK (p-ERK) or phosphorylated JNK (p-JNK) in these cells in comparison to static control cells. To determine whether BMP-specific receptors and Smad1/5 were involved in p38 MAPK activation, Hep3B cells were transfected with BMPRIB- or BMPRII-specific siRNA (40 nM) or Smad1/5-specific siRNA (20 nM) and then maintained under static conditions or exposed to LSS for 1 h. The shear-induced p38 MAPK phosphorylation was abolished by BMPRIB-, BMPRII- (Fig. 5B), and Smad1/5-specific siRNAs (Fig. 5C). To confirm the role of p38 MAPK in modulating the LSS-induced apoptosis or autophagy in Hep3B cells, the expression levels of cleaved caspase-9, caspase-3, and PARP, and LC3B-II were assayed by Western blotting. Hep3B cells

were transfected with p38 MAPK-specific siRNA (20 nM) or pre-treated with a specific p38 MAPK inhibitor SB203580 (10 μM) for 1 h and then maintained under static conditions or exposed to LSS for 24 h. The LSS-induced cleavages of caspase-9, caspase-3, and PARP, and LC3B-II expressions were abolished by p38 MAPK specific-siRNA or SB203580 treatment in comparison to static control cells (Fig. 5D). To validate whether the BMPRIB/Smad1/5/p38 MAPK signaling cascade is involved in shear-induced cancer cell death, Hep3B cells were transfected with BMPRIB-, Smad1/5-, and p38 MAPK-specific siRNA and then exposed to LSS for 24 h. The percentage of death of Hep3B cells was decreased in the sheared cells transfected with BMPRIB-, Smad1/5-, and p38 MAPK-specific siRNAs, as compared with control siRNA-transfected

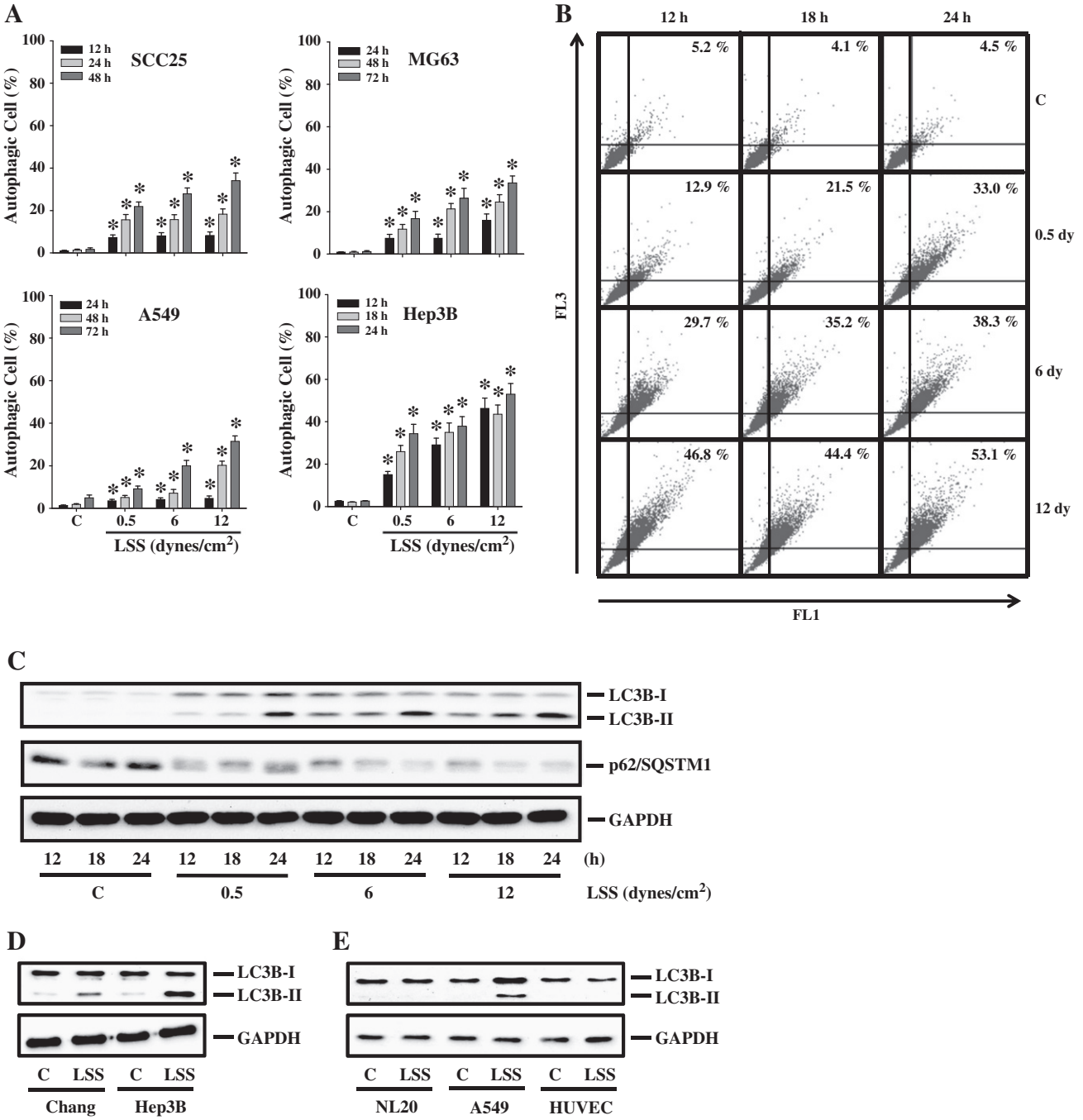

Fig. 2. LSS induces autophagy and apoptosis in cancer cells. (A and F) SCC25, MG63, A549, and Hep3B were maintained under static conditions (C) as controls or subjected to LSS at 0.5, 6, or 12 dyn/cm^2 for various durations. The percentages of autophagic and apoptotic cells were determined. (B) Hep3B cells were maintained under static conditions as controls or subjected to LSS at 0.5, 6, or 12 dyn/cm^2 for various durations. The AVO formation was then analyzed with a flow cytometer. The percentage indicates the sample proportion in the upper part (including the upper left and upper right quadrants) in the figure. (C and G) Hep3B cells were maintained as controls or subjected to LSS at 0.5, 6, or 12 dyn/cm^2 for 12, 18, or 24 h. The expressions of the LC3B, p62/SQSTM1, cleaved caspase-8 (c-Casp8), cleaved caspase-9 (c-Casp9), cleaved caspase-3 (c-Casp3), and cleaved PARP (c-PARP) proteins were examined. (D and H) Chang and Hep3B cells were maintained under static conditions as controls or subjected to a LSS at 0.5 dyn/cm^2 for 24 h. The expressions of the LC3B, c-Casp8, c-Casp9, c-Casp3, and c-PARP protein were examined. (E and I) A549, NL20, and HUVECs were maintained under static conditions as controls or subjected to LSS at 12 dyn/cm^2 for 48 h. The expressions of LC3B, c-Casp8, c-Casp9, c-Casp3, and c-PARP proteins were examined. HUVECs were used as negative controls. The results in A and F depict the mean ± SEM of three independent experiments. The results in C, D, E, G, H, and I depict three experiments with similar results. *, $P < 0.05$ sheared cells vs. static cells at the same point.

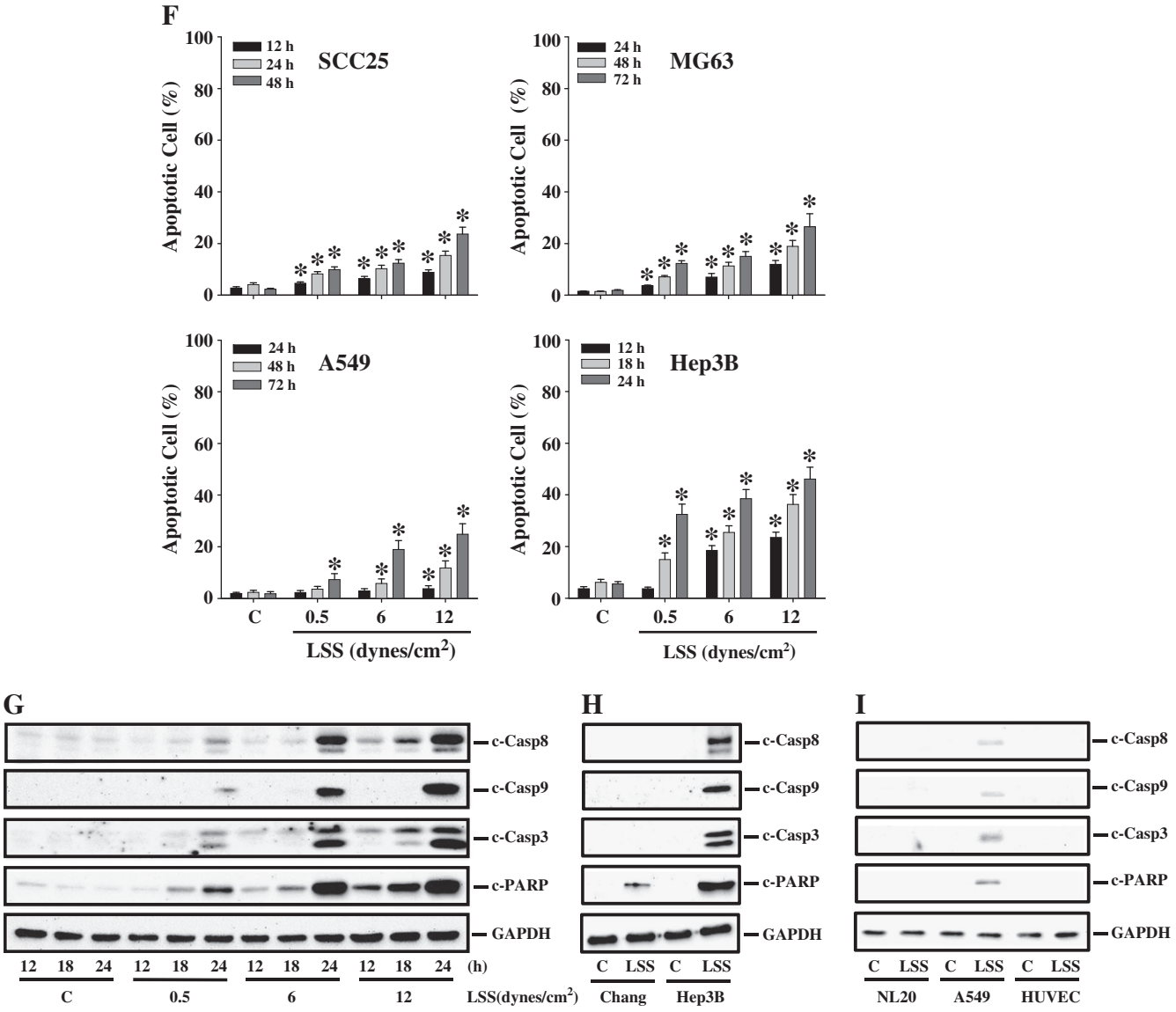

Fig. 2 (*continued*).

cells (Fig. 5E). Taken together, these results indicate that LSS induces cancer cell apoptosis and autophagy through the activation of the BMPRIB/Smad1/5/p38 MAPK signaling pathway.

4. Discussion

In this study, we investigated the role of shear stress in tumor cell apoptosis and autophagy and its underlying mechanism. We have characterized the mechanisms by which shear stress regulates apoptosis and autophagy in tumor cells through BMP receptors, BMPR-specific Smads, and p38 MAPK (Fig. 6). Our study has generated the following findings. (i) Shear stress induced cancer cell death through apoptosis and autophagy in several types of tumor cells. This shear-induced cell death occurred in response to LSS, but not OSS, and was not found in normal cells. (ii) In our experimental model, LSS-induced cell autophagy was essential, at least in part, for the cell apoptosis. This result indicates that in addition to being an independent pathway to modulate LSS-induced cell death, autophagy may play synergistic roles as an upstream mechanism to modulate cancer cell apoptosis in response to shear. (iii) Our study demonstrates that the BMPR/Smad1/5/p38 MAPK cascade modulates the expression of cleaved caspases and LC3B-II proteins, thereby inducing apoptosis and autophagy in cancer cells in response to mechanical forces. Thus, our findings provide insight into the molecular mechanisms by which flow-induced shear force regulates cancer cell fate.

In the body, fluid shear stress may be generated by interstitial flow, blood or lymphatic flow, or some other types of fluid flow. Cancer cells may experience shear stress induced by interstitial flow in tumor tissues and by blood or lymphatic flow during their metastasis. It remains challenged to measure interstitial flow-induced shear stress in vivo and that interstitial flow is generally not considered oscillatory in nature. Recent studies indicated that interstitial flow-induced shear stress ranges from 0.09 to 0.68 dyn/cm^2 in brain tumor [7] and from 8 to 30 dyn/cm^2 for bone cells [29]. In addition, experimental measurements using different methods have shown that in humans the magnitude of shear stress ranges from 1 to 6 dyn/cm^2 in the venous system and from 10 to 70 dyn/cm^2 in the arteries [30]. Thus, in our study we used unidirectional/laminar pattern of flow with different magnitudes of shear stress (i.e., 0.5, 6, and 12 dyn/cm^2) to investigate the effects of shear stress on cancer cell apoptosis and autophagy. Although interstitial flow is not considered oscillatory in nature, it is probable that cancer cells may still be able to encounter disturbed pattern of flow during their

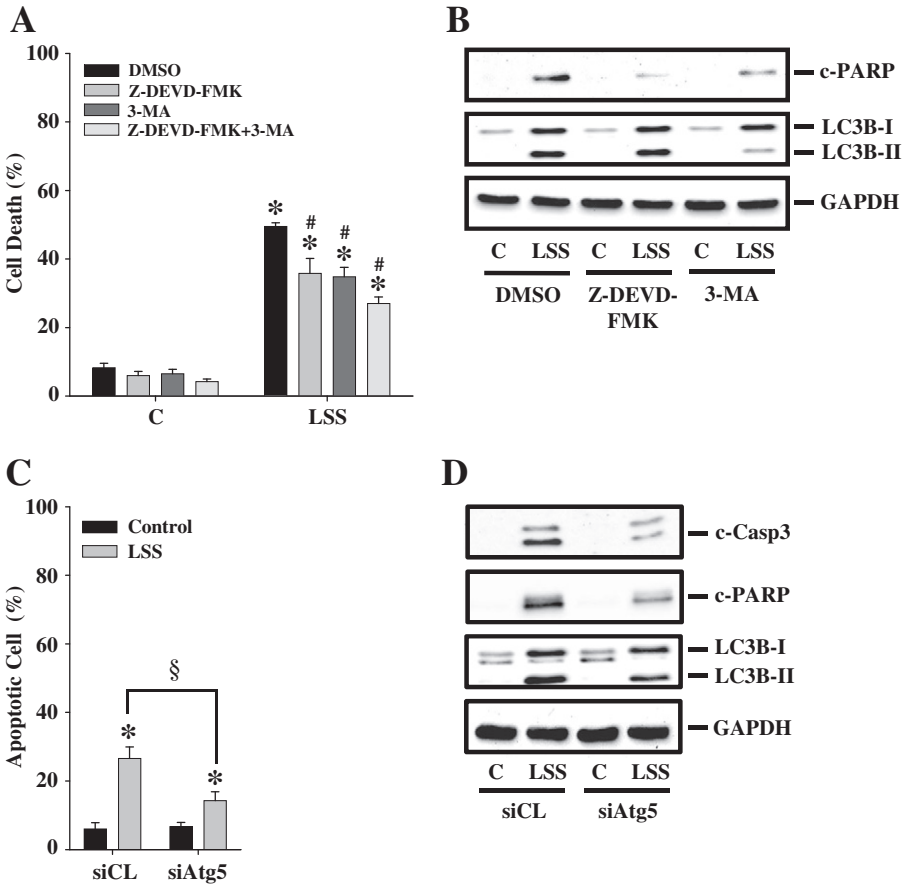

Fig. 3. Shear stress induces autophagic cell death earlier than apoptotic cell death. (A and B) Hep3B cells were pre-treated with DMSO, z-DEVD-FMK, 3-MA, or both for 1 h, and then kept as controls (C) or exposed to LSS at 0.5 dyn/cm^2 for 24 h. The percentage of cell death was calculated and the expressions of the cleaved PARP and LC3B proteins were examined. (C and D) Hep3B cells were transfected with control (siCL) or Atg5-specific siRNA (siAtg5) for 48 h, and maintained as controls or exposed to LSS at 0.5 dyn/cm^2 for 24 h. The percentage of apoptotic cells and the expressions of the cleaved caspase-3 (c-Casp3), cleaved PARP (c-PARP), and LC3B proteins were determined. The results in A and C depict the mean ± SEM of three independent experiments. The results in B and D depict three experiments with similar results. *, $P < 0.05$ sheared cells vs. static cells (A and C); #, $P < 0.05$ reagent-treated cells vs. untreated cells (A); §, $P < 0.05$ siAtg5-transfected cells vs. siCL-transfected cells.

metastasis through vascular trees, where the local flow is often oscillatory [30]. Millions of cells are shed from a tumor daily; however, hematogenous metastasis is believed to be inefficient [31]. This inefficiency is assumed to be a result of the destruction of cells in the bloodstream by shear stress and the immune system [31]. It is known that cancer cell metastasis occurs mainly in certain tissues/organs (e.g., liver) [32], but not in other places (e.g., heart and large arterial wall) [33], where the local flow patterns in these areas are quite different. Our results on the differential roles of laminar vs. oscillatory flows in modulating cancer cell apoptosis and autophagy suggest that this regional specificity of cancer cell metastasis may be attributable, at least in part, to the different patterns of flow and associated shear stress in these different regions.

Caspases have been shown to play important roles in the modulation of apoptotic signaling pathways in cells and hence their survival and death. There are two major apoptotic pathways for caspase activation: the extrinsic and intrinsic pathways. The extrinsic pathway is initiated by death receptor (e.g., tumor necrosis factor receptor and Fas) activation and induces the cleavage of procaspase-8, which is the initiator caspase of the extrinsic pathway. The intrinsic pathway is induced by cellular stresses and releases apoptotic mitochondrial molecules, leading to the cleavage and activation of procaspase-9. Although the cascades involved in the extrinsic and intrinsic pathways are different, both signaling pathways ultimately merge at caspase-3 [34]. On the other hand, autophagy enables a cell to survive under various stress conditions, including nutrient deprivation and growth factor depletion, by self-digesting its proteins and organelles to maintain macromolecule synthesis and ATP production. However, autophagy has recently been proposed to induce cell death. Atgs play a crucial role in autophagy. An autophagosome is formed by the conjugation of Atg12 to Atg5 and the lipid conjugation of LC3 (one of the mammalian homologues of Atg8) to transform into the autophagic vesicle-associated form. p62/SQSTM1 protein is down-regulated during autophagy [14]. In this study, we found that shear stress induces increases in the formations of cleaved caspase-8, caspase-9, and caspase-3 and transformation of LC3B-I to LC3B-II, as well as a down-regulation of p62/SQSTM1. The shear-induced changes in these protein expressions contribute to shear-induced apoptosis and autophagy in cancer cells.

Although the cellular characteristics and physiological process are different for apoptosis and autophagy, there is a cross-talk between these two processes [35]. Autophagy and apoptosis may be linked to each other and occur simultaneously or sequentially in a cell type-, death stimulation-, or environment-dependent manner. Three different types of interactions may occur: apoptosis and autophagy partner, leading to cell death; autophagy antagonizes apoptotic cell death by promoting cell survival; and autophagy, although not leading to cell death by itself, enables the apoptotic cell death [35]. Our study showed that blocking shear-induced autophagy by a specific inhibitor (3-MA) or Atg5-specific siRNA can inhibit, at least in part, the shear-induced apoptosis in cancer cells. The percentage of shear-induced apoptotic cells was attenuated by approximately 50% when cells were pre-treated with 3-MA for 1 h and then exposed to LSS at 0.5 dyn/cm^2 for 24 h, as

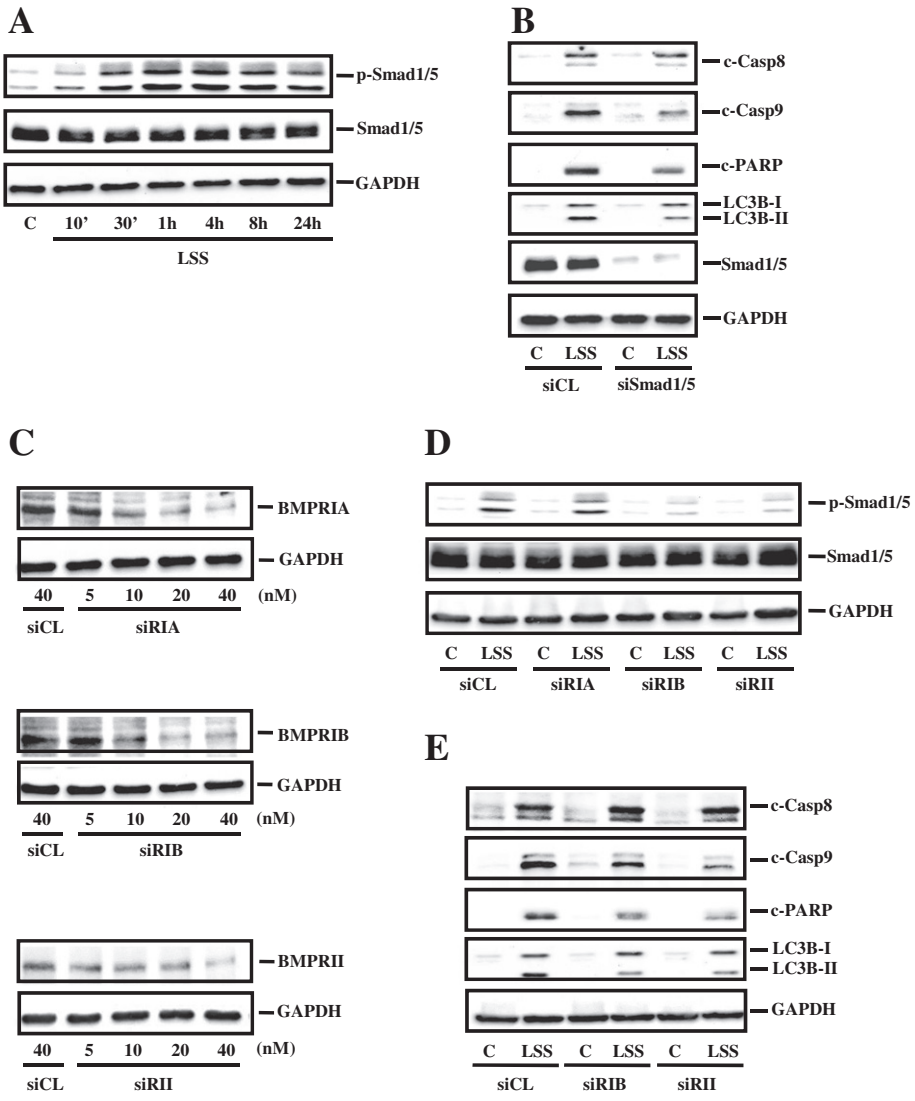

Fig. 4. Shear-induced autophagy and apoptosis in Hep3B cells are mediated by Smad1/5 and BMP receptors. (A) Hep3B cells were kept as controls (C) or subjected to LSS at 0.5 dyn/cm^2 for the indicated times, and their levels of phospho-Smad1/5 were examined. (B) Hep3B cells were transfected with control (siCL) or Smad1/5-specific siRNA (siSmad1/5) (20 nM each) for 48 h before exposure to flow and then maintained under static conditions or subjected to LSS at 0.5 dyn/cm^2 for 24 h. The expressions of cleaved caspase-8 (c-Casp8), caspase-9 (c-Casp9), PARP (c-PARP), and LC3B proteins were examined. (C) Hep3B cells were transfected with specific siRNAs for BMPRs at the indicated concentrations. (D) Hep3B cells were transfected with control (siCL) or specific siRNAs of BMPRIA (siRIA), BMPRIB (siRIB), and BMPRII (siRII) for 48 h and then maintained in static conditions or subjected to LSS at 0.5 dyn/cm^2 for 1 h. (E) Hep3B cells were transfected with indicated siRNAs for 48 h before exposure to flow and then maintained under static conditions or subjected to LSS at 0.5 dyn/cm^2 for 24 h. The expressions of c-Casp8, c-Casp9, c-PARP, and LC3B proteins were examined. The results are representative of three experiments with similar results.

compared with the sheared cells without 3-MA pre-treatment. However, our results demonstrated that treating Hep3B cells with a specific caspase-3 inhibitor (i.e., z-DEVD-FMK) does not inhibit shear-induced autophagy. Thus, shear-induced autophagy may be essential, at least in part, for apoptotic cell death. Our result also indicates that in addition to being an upstream mechanism to modulate cancer cell apoptosis in response to shear, autophagy may also act as an independent mechanism to modulate LSS-induced cell death.

TGF-β induces apoptosis in many types of cells and activates autophagy in hepatocellular carcinoma cells [36]. However, the detailed mechanism underlying BMP-regulated apoptosis and autophagy remains unclear. In this study, we demonstrated that shear-induced apoptosis and autophagy are mediated by BMP receptors and BMPR-specific Smad1/5. Our previous study demonstrated that shear stress induces G_2/M arrest in tumor cells through a signaling pathway involving integrins, BMPRIA, and Smad1/5, and this pathway is independent of BMPs [6]. It has been shown that BMPRs may play a role as intermediate regulators of mechanosensors, such as integrins, to transduce signals downstream, thus leading to apoptosis and autophagy in cancer cells.

MAPKs mediate intracellular signaling and are associated with a variety of cellular activities, including cell proliferation, survival, and death [37]. The ERK signaling pathway plays a role in tumorigenesis. In lung and colorectal cancers, a mutation in epidermal growth factor receptor activates the ERK signaling cascade [38,39]. In many human cancers, JNKs can exert dual functions, either oncogenic or tumor suppressive [40,41], and p38 MAPK plays a role as tumor suppressor in human cancer cell lines [42,43]. In hepatocarcinoma cells, JNK1 appears to be oncogenic, and p38 MAPK activity is lower than that in non-tumorigenic tissues [43]. Some reports have indicated that JNK and p38 MAPK mediate autophagy in cancer cells [35,44], but their effects in response to shear stress are unknown. In our study, we demonstrated that LSS induces apoptosis and autophagy in cancer cells, and these responses are regulated by p38 MAPK, but not ERK and JNK.

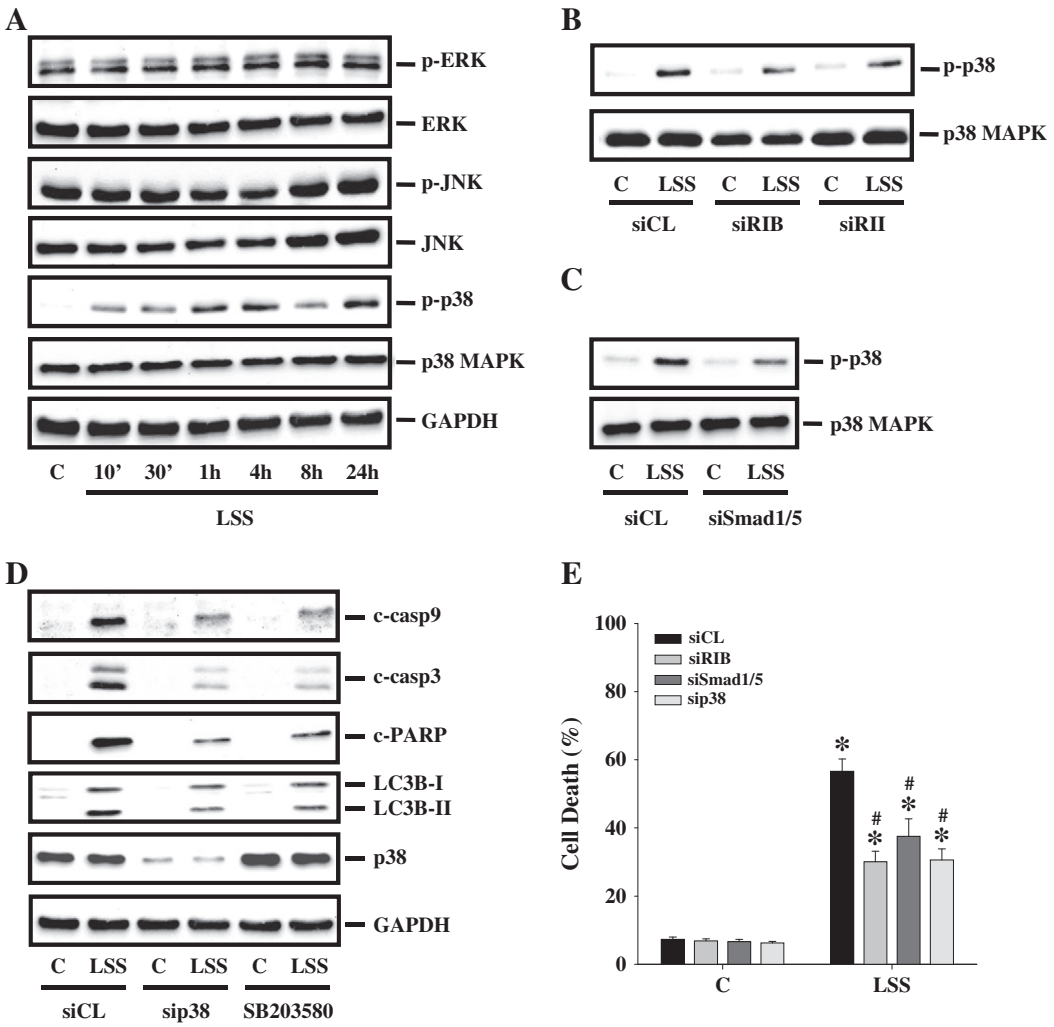

Fig. 5. Shear-induced autophagy and apoptosis in Hep3B cells are mediated by p38 MAPK. (A) Hep3B cells were maintained under static conditions (C) or subjected to LSS at 0.5 dyn/cm^2 for the indicated times, and the levels of phospho-ERK (p-ERK), phospho-JNK (p-JNK), and phospho-p38 MAPK (p-p38) were examined. (B and C) Hep3B cells were transfected with the designated siRNAs for 48 h and then maintained as controls or subjected to LSS at 0.5 dyn/cm^2 for 1 h. (D) Hep3B cells were transfected with control (siCL) or p38 MAPK-specific siRNA (sip38) for 48 h. A sample of siCL-transfected cells was pre-treated with SB203580 for 1 h. All cells were maintained under static conditions or subjected to LSS for 24 h. The expressions of the cleaved caspase-9 (c-Casp9), caspase-3 (c-Casp3), PARP (c-PARP), and LC3B proteins were examined. (E) Hep3B cells were transfected with the designated siRNAs for 48 h and then maintained as controls or subjected to LSS at 0.5 dyn/cm^2 for 24 h. The percentage of cell death was calculated. The results are representative of three independent experiments with similar results. *, $P < 0.05$ sheared cells vs. static cells; #, $P < 0.05$ cells transfected with BMPRIB-, Smad1/5-, or p38 MAPK-specific siRNA vs. control siRNA.

There have been several reports using 3-D models to study interstitial flow [45–47]. These 3-D systems represent a significant advance over a 2-D system for studying interstitial flow. However, to better define and control the levels of shear stress imposed on cancer cells, in this study we used a 2-D culture system with a parallel-plate flow chamber to create the flow. This homogeneous culture and well-controlled levels of shear stress allowed us to collect more cells experiencing the same levels of force for analysis. Development of a more sophisticated 3-D culture system for studying the effect of shear stress on cancer cells warrants further investigation.

In summary, this study demonstrated that shear stress induces autophagy and apoptosis in cancer cells. These shear-induced responses are mediated by BMPRs, BMPR-specific Smads, and p38 MAPK, leading to changes in the expression of cleaved caspase-9 and caspase-3 proteins and transformation of LC3B-II protein. Our data on shear-modulation in cancer cell autophagy and apoptosis suggest that mechanical microenvironment of cancer cells may play important roles in cancer biology and pathobiology, and should be taken into account in tumor therapy and management.

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Contributors

S.-C.L. designed the study, performed the experiments, and wrote the manuscript. S.-F.C. designed the study and participated in the discussion. P.-L.L. performed the experiments. S.-Y.W. designed the study and wrote the manuscript. M.D.-T.C. and J.-Y.C. participated in the discussion and provided a financial support. J.-J.C. designed the study, wrote the manuscript, and provided a financial support.

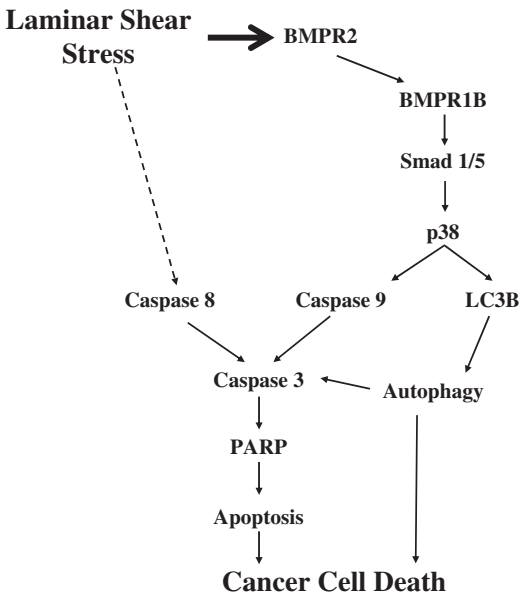

Fig. 6. Schematic representation of the signaling pathways regulating autophagy and apoptosis in cancer cells in response to shear stress. Solid arrow indicates the results obtained from this study. Dash arrow shows the signaling required for further investigation.

Conflict of interest

The authors declare that they have no competing interests.

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