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Novel involvement of leukotriene B_4 receptor 2 through ERK activation by PP2A down-regulation in leukotriene B_4-induced keratin phosphorylation and reorganization of pancreatic cancer cells

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ABSTRACT

Perinuclear reorganization via phosphorylation of specific serine residues in keratin is involved in the deformability of metastatic cancer cells. The level of leukotriene B_4 is high in pancreatic cancers. However, the roles of LTB_4 and its cognate receptors in keratin reorganization of pancreatic cancers are not known. LTB_4 dose-dependently induced phosphorylation and reorganization of Keratin 8 (K8) and these processes were reversed by LY255283 (BLT2 antagonist). BLT2 agonists such as Comp A and 15(S)-HETE also induced phosphorylation of serine 431 in K8. Moreover, Comp A-induced K8 phosphorylation and reorganization were blocked by LY255283. Gene silencing of BLT2 suppressed Comp A-induced K8 phosphorylation and reorganization in PANC-1 cells. Over-expression of BLT2 promoted K8 phosphorylation. Comp A promoted the migration of PANC-1 cells in a dose-dependent manner, and LY255283 blocked Comp A-induced migration, respectively. PD98059 (ERK inhibitor) suppressed Comp A-induced phosphorylation of serine 431 and reorganization of K8. Gene silencing of BLT2 suppressed the expression of pERK, and over-expression of BLT2 increased the expression of pERK even without Comp A. Comp A induced the expression of active ERK (pERK) and BLT2. These inductions were blocked by PD98059. Comp A decreased PP2A expression and hindered the binding of PP2A to the K8, leading to the activation of ERK. PD98059 suppressed the Comp A-induced migration of PANC-1 cells and BLT2 over-expression-induced migration of PANC-1 cells. Overall, these results suggest that BLT2 is involved in LTB_4-induced phosphorylation and reorganization through ERK activation by PP2A downregulation, leading to increased migration of PANC-1 cells.

1. Introduction

Recently, novel approaches have been tested to characterize the properties of cells such as cell elasticity and mechanical properties [1,2]. The clinical importance of viscoelasticity or cell stiffness was shown by several researchers including Cross et al. [3,4]. The stiffness of live metastatic cancer cells taken from the body (pleural) fluids of patients with suspected lung, breast and pancreas cancer was more than 70% softer than that of normal cells [3]. The importance of cell elasticity or viscoelasticity in several metastatic cancer cell lines and other diseases has also been reported [5]. For example, SPC-induced molecular reorganization of keratin in PANC-1 human epithelial pancreatic cancer cells and the resulting changes in the mechanical deformability of cells have been examined as possible pathways that facilitate migration and increase the metastatic competence of pancreatic tumor cells [6–9].

Leukotriene B4 (LTB_4) is a downstream metabolite of 5-lipoxygenase (5-LO) [10]. LTB_4 stimulates growth and proliferation of pancreatic cancer cells by activating phosphatidylinositol 3-kinase/ERK [10]. It has been found that LTB_4 levels are significantly higher in pancreatic carcinoma than in tumor-free tissues [11]. Overexpression of its cognate receptors such as BLT1 or BLT2 has been observed in several cancers including pancreatic cancers [12–14]. Furthermore, BLT2 levels were markedly increased in invasive human cancer tissues compared to non-neoplastic tissues [12,14,15]. More specifically, BLT2 is expressed in PanIN, IPMN, and pancreatic cancers [16]. Most studies on LTB_4 receptors have focused on the high-affinity receptor, BLT1. In contrast, very little is known about the physiological function of BLT2. Recently, BLT2 signaling was shown to be important in oncogenic Ras-induced cancer progression in Rat-2 fibroblast cells [17]. BLT2 is also involved in the regulation of VEGF-induced angiogenesis [18].

Because keratin reorganization is involved in the deformability of pancreatic cancer cells that lead to efficient migration, we were

Abbreviations: LTB_4, Leukotriene B_4; K8, Keratin-8; BLT2, Leukotriene B_4 receptor 2; Comp A, Compound A; PP2A, Protein phosphatase 2A; SPC, Sphingosylphosphorylcholine; PanIN, Pancreatic intraepithelial neoplasia; IPMN, Intraductal papillary mucinous neoplasm

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interested in finding endogenous materials that induce keratin phosphorylation and reorganization [6]. Previous studies have shown that several chemicals, proteins and shear stress induce keratin reorganization in several cell types including the pancreatic cancer cell line PANC-1 and the gastric cancer cell line AGS [6,19–23]. However, endogenous mediators that evoke keratin reorganization are not well known. Therefore, we examined whether LTB_4 and its cognate receptors are involved in keratin phosphorylation and reorganization in PANC-1 cells.

In this report, we found that LTB_4 induced keratin-8 (K8) phosphorylation and reorganization in PANC-1 cells. BLT2 receptor was involved in LTB_4-induced in K8 phosphorylation and reorganization of PANC-1 cells via ERK activation by PP2A downregulation, leading to increased migration of PANC-1 cells.

2. Materials and methods

2.1. Material

The phosphospecific antibody detecting K8 Ser 431 was purchased from Abcam (Cambridge, UK). Peroxidase-labeled secondary antibodies were acquired from Santa Cruz Biotechnology (1:5000, Santa Cruz, CA, USA). Alexa Fluor 594 goat anti-mouse antibody was obtained from Molecular Probes, Inc. (1:500, Eugene, OR, USA).

Leukotriene B_4, LY255283, 15(S)-HETE, PD98059, SP600125 and SB203580 were purchased from Cayman Chemical (Ann Arbor, MI). Compound A was a gift from Dr. Sanghee Kim. It was supplied as a crystalline solid and then dissolved in dimethylsulfoxide (DMSO) or ethanol and stored at −20 °C. All of these chemicals were freshly prepared at the time of each experiment. BLT2 plasmid (pBLT2) was kindly provided by Dr. Shimizu (University of Tokyo).

2.2. Cell culture

Human pancreatic cancer cell lines, namely PANC-1, AsPC1, BxPC1, HPAC, Capan1, Capan2, and MiaPaCa2 were purchased from the American Type Culture Collection (Manassas, VA). Stocks were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified atmosphere of 5% carbon dioxide and 95% air at 37 °C and were passaged every 3 days. Cells were washed twice in serum-free DMEM and subsequently incubated in serum-free DMEM for 15 hours before the respective experiments.

2.3. Western Blot

After incubation, the cells were collected and washed twice with cold PBS. The cells were lysed in lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1% Deoxycholic acid (DOC), 0.1% SDS, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT] and centrifuged to yield whole-cell lysates. Protein concentration was measured by the Bradford method. Aliquots of the lysates (10 μg of protein) were separated on 10% SDS-polyacrylamide gels and then transferred onto polyvinylidene fluoride (PVDF) or nitrocellulose (NC) membranes (Invitrogen, Carlsbad, CA) with glycine transfer buffer [192 mM glycine, 25 mM Tris–HCl (pH 8.8), 10% MeOH (v/v)]. After blocking the nonspecific sites with 5% non-fat dry milk, the membrane was then incubated with specific primary antibody in 3% bovine serum albumin (BSA) at 4 °C overnight. The following primary antibodies were used: anti-cytokeratin 8 (1:1000, Abcam, Cambridge, MA); anti-ß-actin (1:1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-BLT2 (1:200, Cayman Chemical, Ann Arbor, MI), anti-PP2A (1:1000, BD Transduction Laboratories, Franklin Lakes, NJ), 5-lipoxygenase (1:1000, Abcam, Cambridge, MA), 12-lipoxygenase (1:1000, Abcam, Cambridge, MA), phospho-ERK (1:1000, Cell Signaling, Beverly, MA), ERK (1:1000, Cell Signaling, Beverly, MA). The membrane was further incubated for 60 min with HRP-labeled anti-rabbit or anti-mouse IgG secondary antibody (1:5000, Santa Cruz, CA) at room temperature. The protein-antibody complexes were detected by HRP–conjugated secondary antibodies followed by enhanced chemiluminescence Western blotting detection reagents (Amersham, Arlington Heights, IL).

2.4. Confocal microscopy

Cells were seeded onto coverslips and treated with chemicals. 24 h later, cells were fixed with 100% methanol for 30 min at room temperature and permeabilized with 0.1% Triton X-100 for 10 min followed by several washes with PBS. After blocking with 3% BSA in PBS at room temperature for 1 h, anti-cytokeratin 8 (1:250, Abcam, Cambridge, UK) primary antibody was incubated with coverslips overnight at 4 °C. Excess antibody was removed with PBS and then species-specific secondary antibody conjugated to Alexa Fluor 594 goat anti-mouse antibody obtained from Molecular Probes, Inc. (1:500, Eugene, OR, USA) was reacted with the coverslips for 1 h at room temperature. Followed by four washes in PBS, the slides were mounted with mounting solution and visualized using a Zeiss Axiophot confocal microscope (400×).

2.5. Transfection with small interference RNA (siRNA) or plasmid containing BLT2

PANC-1 cells were seeded into 6-well microplates in DMEM supplemented with 10% FBS without antibiotics. BLT2 siRNA (sense CCACGCAGUCAACCUUCUGTT, antisense CAGAAGGUUGACUGCGUGG TA) was purchased from Samchully Pharm. For siRNA or BLT2 transfection experiments, the cells were plated on 6-well plates until they reached 70% confluence and were then transfected with BLT2 siRNA (100 pmol) or plasmid containing BLT2 using Lipofectamine™ 2000 reagent according to the manufacturer's instructions. 48 hours after transfection, PANC-1 cells were grown in complete culture medium. After this, the cells were starved for 15 hours and treated with specific chemicals. Proteins were extracted from the cells and analyzed by Western blot.

2.6. Immunoprecipitation

The cell extracts (typically 600 μg) were prepared with protein A beads before being incubated with the appropriate antibody for 2 hours, followed by addition of more protein A beads for an additional hour. The beads were washed with lysis buffer before being boiled in the SDS sample buffer and then subjected to SDS PAGE and Western blotting. The phosphospecific antibody detecting K8 Ser431 (1:1000, Abcam, Cambridge, MA) was used for immunoprecipitation.

2.7. Migration

Migration of PANC-1 cells through 8-μm size-limited pores was assessed in response to LTB_4 according to Park's report [24]. PANC-1 cells (5×10^4 cells per well) were treated with the indicated concentrations of LTB_4, Compound A, or 15(S)-HETE for 1 hour. PANC-1 cells plated in the upper chamber were allowed to migrate for 5 hours to establish the temporal kinetics of migration. The transwell membranes were then fixed and stained with Diff-Quik® staining kit (Kobe, Japan). Membranes were removed from transwells, and cells on the undersurface of the millipore membrane were counted under a light microscope (average of 5 semi-random non-overlapping fields at 200x magnification). All treatments were performed in triplicate wells.

2.8. Flow cytometry analysis

For surface staining of BLT2, a total of 5×105 cells were harvested and blocked with 10% bovine serum albumin in FACS buffer (PBS with

25 mM HEPES, 1 mM EDTA, 1% FBS) for 30 min at 4 °C. Then, cells were incubated with the primary antibody against BLT2 (1:100 dilution) (Cayman Chemical Company Ann Arbor, MI) for 1 h, and washed three times with FACS buffer. Cells incubated with anti-rabbit-Alexa 488 (1:500, Molecular Probes) for 30 min, and washed three times with FACS buffer. BLT2 receptor expression was analyzed with a FACS Calibur flow cytometer.

2.9. Statistical analysis

The data are expressed as mean ± s.e.m. of at least three independent experiments performed in triplicate. A p value < 0.05 was considered significant.

3. Results

3.1. Effects of LTB_4 on K8 organization and phosphorylation

The effect of LTB_4 on K8 phosphorylation was examined using a specific monoclonal antibody for serine 431 (Ser431) of K8 in several pancreatic cancer cell lines, namely PANC-1, HPAC, BXPC3, ASPC1, Capan2, and MiaPaCa2 (Fig. S1). After this, the effects of LTB_4 on the phosphorylation of serine residues in K8 were examined with site-specific phosphoserine K8 antibodies, anti-Ser23, anti-Ser73, and anti- Ser431, in PANC-1 cells which is well-characterized cells suited to examine keratin phosphorylation and reorganization [6–8]. We found that phosphorylation of Ser431 of K8 was remarkable in LTB_4-treated PANC-1 cells (Fig. S2). Thus, subsequent studies were performed in PANC-1 cells with antibody specific for Ser431 of K8. LTB_4 dose-dependently induced phosphorylation of keratin-8 in PANC-1 and ASPC-1 cells (Fig. 1A). Maximum phosphorylation of K8-serine 431 (Ser431) was achieved at 50 nM LTB_4. An increased induction of phosphorylation of K8 was first detected after 30 min and reached a maximum after 1-hour of incubation with LTB_4 (Fig. 1B).

PANC-1 and ASPC-1 cells exhibited the typical pan-cytoplasmic pattern of keratin filaments (Figs. 1C, S3). LTB_4 induced reorganization of keratin filaments to a perinuclear, ring-like structure (Figs. 1C, S3). This effect was rapid, reaching a maximum after 1 hour (Figs. 1D, S4).

3.2. Effects of LY255283, Compound A, and 15(S)-HETE on LTB_4-induced K8 phosphorylation and reorganization

The involvement of BLT2 receptor in LTB_4-induced K8 phosphorylation and perinuclear reorganization was confirmed with the BLT2 receptor antagonist LY255283 or BLT2 agonists such as 15(S)-HETE and Compound A (Comp A, Fig. S5). LY255283, a well-known BLT2 antagonist, suppressed the LTB_4-induced phosphorylation and reorganization of K8 (Figs. 2A, B, S6). However, U75302, a well-known BLT1 antagonist, did not prevent LTB4-induced K8 events (Fig. S7). We also tested whether the BLT2 agonists, Comp A or 15(S)-HETE induced phosphorylation of K8. The results showed that Comp A or 15(S)-HETE induced K8 phosphorylation and reorganization (Fig. 2C, D). Comp A-induced phosphorylation and reorganization of K8 were also suppressed by LY255283 (Figs. 2E, F, S8).

3.3. Effects of BLT2 gene silencing and overexpression on Comp A-induced K8 phosphorylation and reorganization

To examine the involvement of BLT2, we mainly used Comp A in the following experiments instead of LTB_4 since Comp A is specific for BLT2. The involvement of BLT2 receptor in Comp A-induced K8 phosphorylation and perinuclear reorganization was confirmed with BLT2 receptor gene silencing (siBLT2) and overexpression (pBLT2). Gene silencing of BLT2 suppressed the Comp A-induced phosphorylation and reorganization of K8 (Fig. 3A, B). Gene silencing of BLT2 also suppressed the LTB_4-induced phosphorylation of K8 (Fig. S9). Gene silencing of BLT2 was confirmed by Western blot (Fig. 3A). Transfection of the BLT2 gene (pBLT2) induced phosphorylation and reorganization of K8 in PANC-1 cells (Fig. 3C, D). Overexpression of BLT2 was also confirmed by Western blot (Fig. 3C).

3.4. Effect of LY255283 on Comp A-induced migration of PANC-1 cells

The expected final output of Comp A-induced reorganization of the keratin network in PANC-1 cells was increased migratory properties [6]. Therefore, the involvement of BLT2 in the Comp A-induced migration of PANC-1 cells was examined using LY255283. At first, we confirmed whether a BLT2 agonist, Comp A, induced the migration of PANC-1 cells. PANC-1 cells were treated for 1 hour with various amounts Comp A ranging from 10 nM to 100 nM. Comp A induced the migration of PANC-1 cells in a dose-dependent manner. At 50 nM, the increase in migration reached a maximum (Fig. 4A). LY255283, a BLT2 antagonist, suppressed the Comp A-induced migration of PANC-1 cells (Fig. 4B). LTB_4 also induced the migration of PANC-1 cells, but LY255283 suppressed it (Figs. S10, S11). These results suggest that BLT2 is involved in LTB_4- or Comp A-induced migration.

3.5. Effect of ERK inhibitor on Comp A-induced K8 phosphorylation and reorganization

MAP kinase is believed to be involved in K8 phosphorylation and reorganization in PANC-1 cells [6], but a detailed study using kinase inhibitors has not been done. Therefore, the effects of several kinase inhibitors on Comp A-induced phosphorylation of K8 were examined in PANC-1 cells. PD98059 (MEK1 inhibitor) suppressed the Comp A-induced phosphorylation and reorganization of K8 (Figs. 5A, B, S12, S13). PD98059 also suppressed the LTB4-induced phosphorylation of K8 (Fig. S14). Next, the relationship between BLT2 and ERK was examined. Comp A induced phosphorylation of ERK in wild-type PANC-1 cells, but the phosphorylation was repressed in BLT2-silenced PANC-1 cells (Fig. 5C). Overexpression of BLT2 induced ERK activation (Fig. 5D). Comp A also induced BLT expression and this induction was suppressed by gene silencing of BLT2 (Fig. 5C, D). Comp A-induced migration of PANC-1 cells was suppressed by PD98059, and overexpression of BLT2 increased the migration of PANC-1 cells; this migration was also inhibited by PD98059 (Fig. 5E). Comp A also affected the expression of PP2A and the inverse relationship between pERK and PP2A was observed with Comp A treatment (Fig. 5F). PP2A and pERK were coimmunoprecipitated with phosphoserine antibody specific for Ser431 of K8. Compound A treatment decreased PP2A binding but increased pERK binding to K8 (Fig. 5G). Comp A induced BLT2 expression and this expression was suppressed by PD98059 (Fig. 5H). These findings were also confirmed by flow cytometry (Fig. S15).

4. Discussion

Metastatic cancer cells are reported to have unique mechanical characteristics including soft stiffness and less elasticity [3]. Keratins are one of the main intermediate filaments that control the mechanical characteristics of cells [25]. Precise characterization of the dynamic functions of keratin in vivo has been hampered by the fact that very few physiological regulators are known to specifically trigger keratin reorganization and phosphorylation. This study focused on the effects of LTB_4 on keratin reorganization in pancreatic cancer cells including PANC-1 cells and on the underlying mechanisms in the migratory properties of PANC-1 cells. The effects of inflammatory mediators such as LTB_4 on keratin phosphorylation and reorganization have not been investigated, although inflammation is regarded as one of the hallmarks in cancer [26]. Such findings along with the fact that LTB_4 levels and BLT2 expression are high in these cancers evoked our interest in the role and the related mechanisms of LTB_4

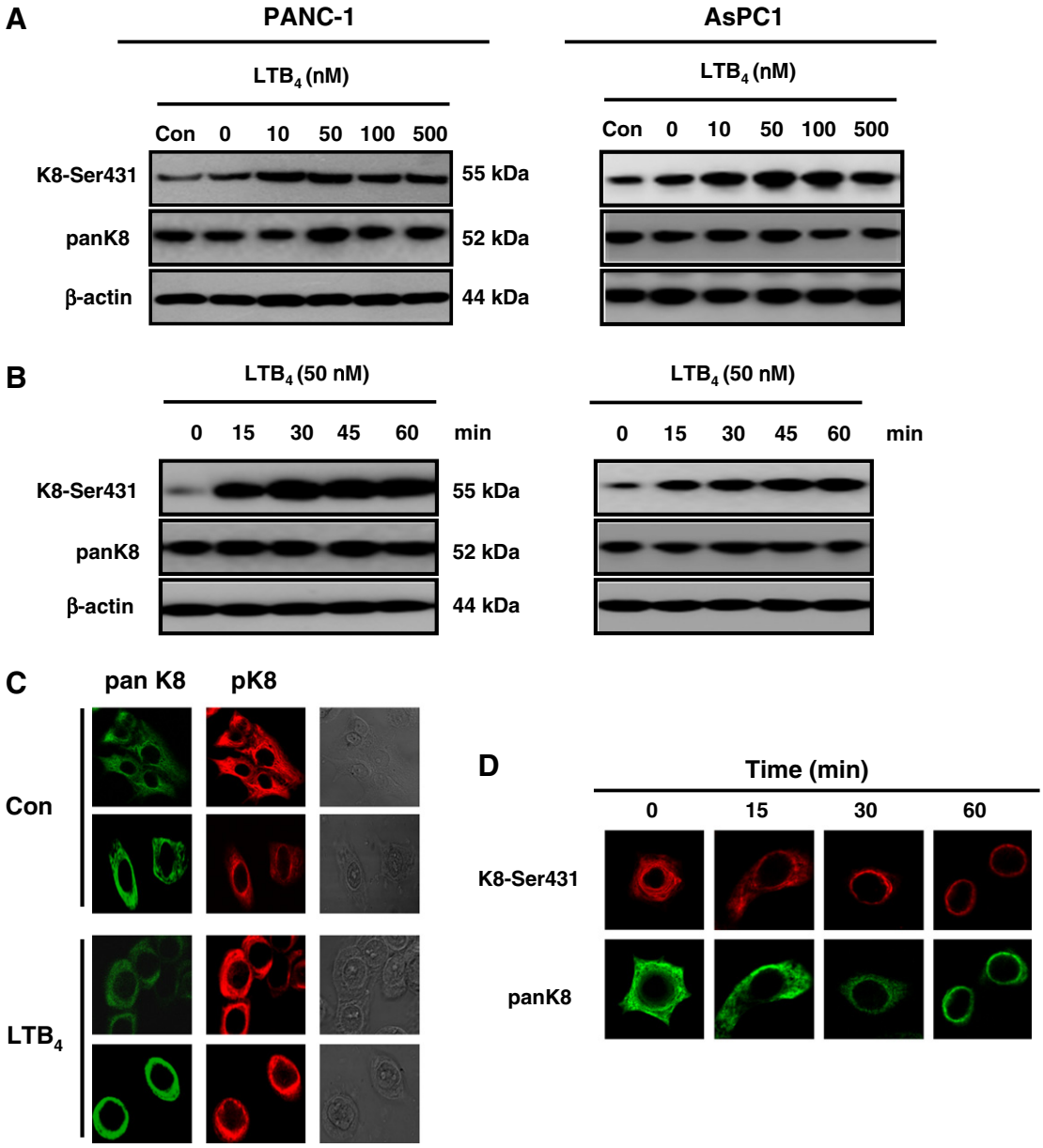

Fig. 1. LTB_4 induces phosphorylation and reorganization of keratin 8 in PANC-1 and AsPC1 cells. (A) Dose dependency of LTB_4-induced serine 431 (Ser431) phosphorylation of K8 in PANC-1 and AsPC1 cells (B) Time dependent phosphorylation of Ser431 of K8 in PANC-1 and AsPC1 cells. (C) Confocal microscopic examination of phosphorylation of Ser431 in K8 of PANC-1 cells. (D) Confocal microscopic examination of time-dependent phosphorylation of Ser431 in K8 of PANC-1 cells. In (B), (C), and (D), cells were treated with 50 nM LTB_4 for the times indicated. In (A) and (B), phosphorylated Ser431 was analyzed by Western blotting with a monoclonal antibody specific for phospho-Ser431 of K8. Blots were stripped and reprobed with β-actin antibody. In (C) and (D), immunostaining was performed using K8-Ser431 (red) or panK8 (green). The results shown are representative of three independent experiments with similar results.

in migration of pancreatic cancer cells [16]. Therefore, we examined LTB_4-induced keratin phosphorylation and reorganization, which are related to deformability and migration of cancer cells [6,7,27].

Using fluorescence microscopy, we demonstrated that LTB_4 rapidly induced a redistribution of intact keratin filaments from the cell periphery to the perinuclear region (Fig. 1C, D). K8 is one of the intermediate filaments consisting of a conserved central coiled-coil α-helical rod domain that is flanked by N- (head) and C-terminal (tail) domains [23]. Three phosphorylation sites have been reported for K8, namely K8pSer23, K8pSer73, K8pSer431 [28]. Phosphorylation of K8 is regulated by various protein kinases such as PKC and mitogen-activated protein kinase (MAPK) [29–32]. The effects of LTB_4 on phosphorylation of Ser431 of K8 were examined in several pancreatic cancer cell lines, including PANC-1, HPAC, BxPC3, AsPC3, Capan2, and MiaPaCa2 cells (Fig. S1). LTB_4-induced K8 phosphorylation in MiaPaCa2, BxPC3, and Capan2 is less significant than other pancreatic cancer cell lines. It is not known why pancreatic cancer cell lines respond differently. We could not find any correlation between K8 phosphorylation and the characteristics of pancreatic cancer cell lines, such as grade of differentiation and origin [33]. The phosphorylated status of K8 in MiaPaCa2 and BxPC3 without LTB_4 treatment was higher than those of other pancreatic cancer cell lines (Fig S1). Thus, MiaPaCa2 and BxPC3 cells did not respond well with LTB_4 treatment. Pancreatic cancer cell lines contain Ras mutation leading to Raf/MEK/MAPK activation, and MAPK is known to phosphorylate serine 431 of K8 [8,34–36]. Therefore, these different responses in different pancreatic ductal adenocarcinoma cell lines might be contributed by different intracellular environments

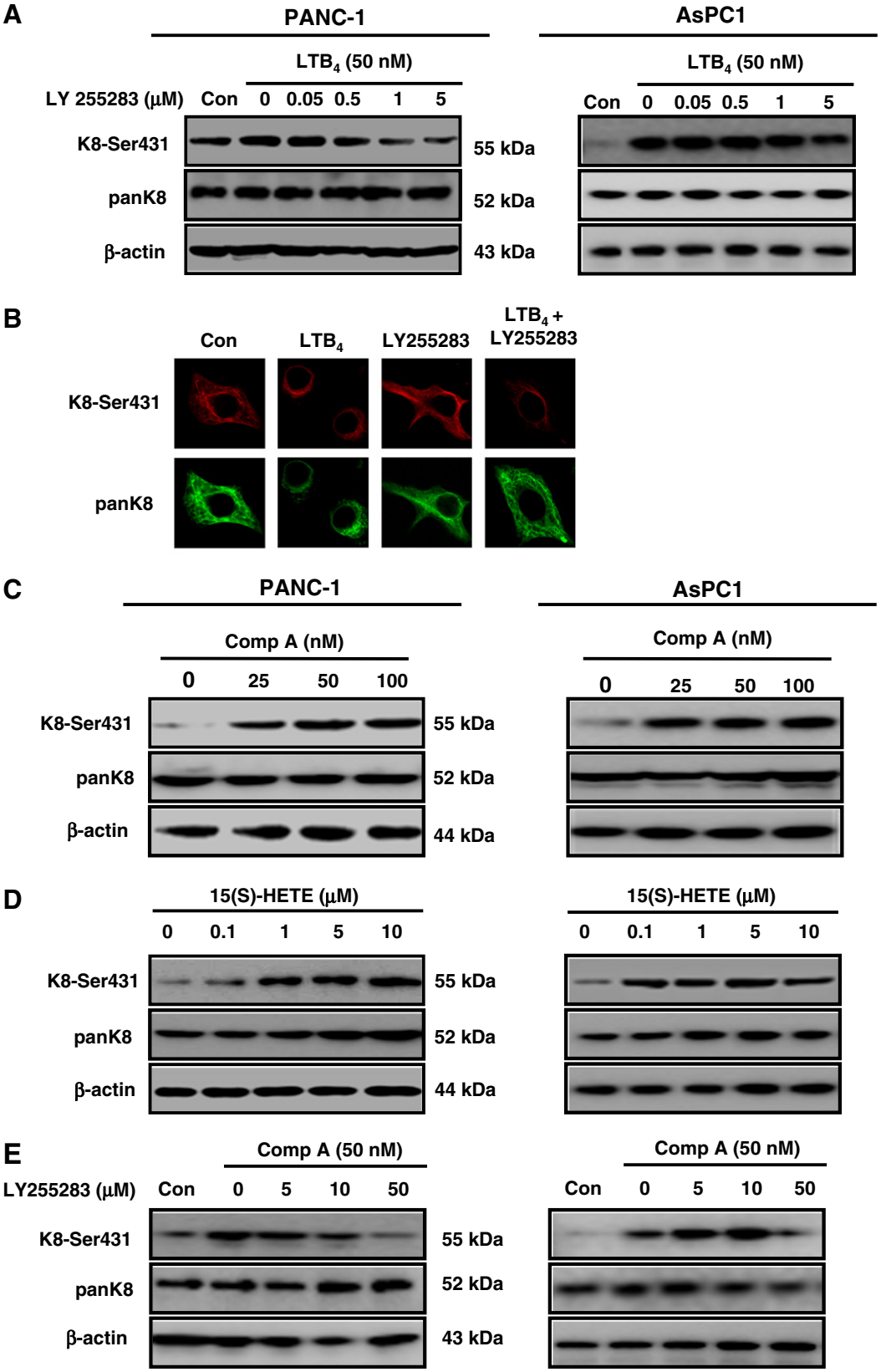

Fig. 2. BLT2 is involved in LTB_4-induced K8 phosphorylation and reorganization of PANC-1 and AsPC1 cells. (A) Effect of LY255283 on LTB_4-induced K8 phosphorylation. PANC-1 or AsPC1 cells were treated with varying amounts of LY255283 and LTB_4 (50 nM). (B) Confocal microscopic examination of the effect of LY255283 on LTB_4-induced K8 phosphorylation of PANC-1 cells. (C) Dose dependency of Compound A (Comp A)-induced serine 431 (Ser431) phosphorylation of K8 in PANC-1 or AsPC1 cells. (D) Dose dependency of 15(S)-HETE-induced serine 431 (Ser431) phosphorylation of K8 in PANC-1 or AsPC1 cells. (E) Effect of LY255283 on Comp A-induced K8 phosphorylation. PANC-1 or AsPC1 cells were treated with varying amounts of LY255283 and Comp A (50 nM). (F) Confocal microscopic examination of the effect of LY255283 on Comp A-induced K8 phosphorylation. In (A), (C), (D) and (E), phosphorylated Ser431 was analyzed by Western blotting with a monoclonal antibody specific for phospho-Ser431 K8. Blots were stripped and reprobed with β-actin antibody. In (B) and (F), immunostaining was performed using K8-Ser431 (red) or panK8 (green).

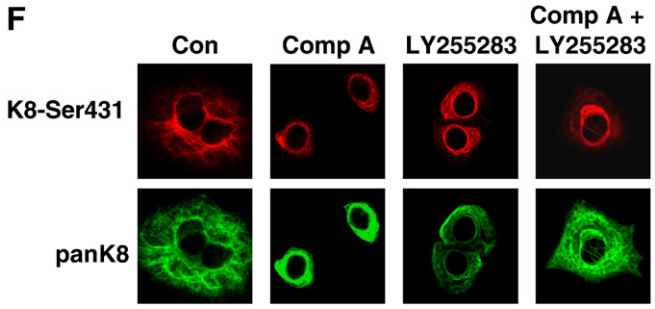

Fig. 2 (*continued*).

affecting the MAPK signaling pathway and expression pattern of keratin [16]. However, these notions may require further research.

The exact phosphoserine residues of K8 affected by LTB_4 were determined by site-specific phosphoserine K8 antibodies, anti-Ser23, anti-Ser73, and anti-Ser431. Phosphorylation of Ser431 of K8 was remarkable in LTB_4-treated PANC-1 cells (Fig. S2). Since SPC-induced phosphorylation in Ser431 of K8 is already well-defined, we focused on the effects of LTB_4 on phosphorylation of Ser431 in K8 [6] (Fig. 1A, B). Several inducers, such as EGF, phosphatase inhibitor and sheared stress, can induce phosphorylation and reorganization of keratin in many types of cells [22,23,31]. In particular, SPC-induced keratin reorganization via phosphorylation is related to the viscoelasticity and migration of tumor cells [6,7]. Moreover, LTB_4 is among the compounds that induce keratin phosphorylation and reorganization (Fig. 1).

LTB_4 is a product of 5-LO and acts via BLT1 and BLT2 receptors. BLT1 and BLT2 are expressed in pancreatic cancers [12,16]. LTB_4-induced phosphorylation and reorganization of K8 were inhibited by LY255283, but U755302, a BLT1 antagonist, did not suppress the LTB_4-induced phosphorylation and reorganization of K8 (Figs. 2, S7). The involvement of BLT2 was confirmed by Comp A or 15(S)-HETE, specific BLT2 agonists, and the increased phosphorylation of K8 by Comp A was also suppressed by LY255283 (Fig. 2). Different concentrations of LY255283 were used in Fig. 2A and E. Comp A is known as a higher affinity agonist than LTB_4 [37]. These amounts used were not cytotoxic to PANC-1 or AsPC1 cells in our experimental conditions (Fig. S 16). Thus, higher concentrations of LY255283 were used to suppressed the Comp A-induced BLT2 activation than the LTB4-induced BLT2 activation. These relationships between Comp A and LY255283 were also observed in our previous paper [38]. The suppressive effects of LY255283 were confirmed by gene silencing of BLT2.

Overexpression of BLT2 led to increased phosphorylation of K8. Taken together, these observations suggest that BLT2 is involved in LTB_4-induced phosphorylation and reorganization of K8 in PANC-1 cells.

Migration of PANC-1 cells through size-limited pores was increased by Comp A or LTB_4 via keratin reorganization (Figs. 4A, S10). Comp A- or LTB_4-induced migration of PANC-1 cells was suppressed by LY255283 (Figs. 4B, S11). These results suggest that BLT2 was involved in Comp A- or LTB_4-induced migration and the fact that BLT2-mediated reorganization and phosphorylation of keratin in PANC-1 cells led to increased deformability may in part explain the BLT2-mediated migration of PANC-1 cells induced by Comp A or LTB_4 (Figs. 4, S10, S11). BLT2

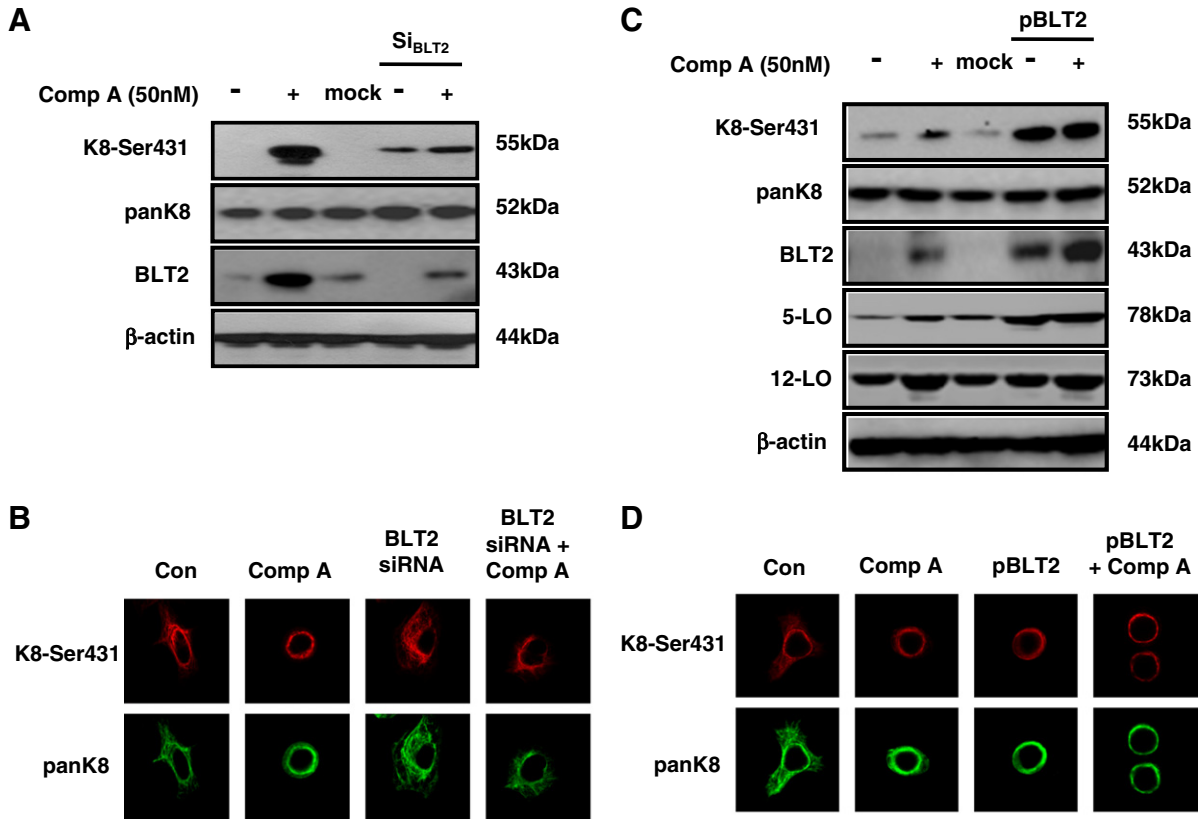

Fig. 3. Effects of BLT2 on phosphorylation of Ser431 in K8 in PANC-1 cells. (A) Effects of BLT2 gene silencing on phosphorylation of Ser431 in K8. PANC-1 cells were transfected with the indicated amounts of BLT2 siRNAs or control siRNA (mock) and stimulated with or without Comp A, respectively (50 nM). (B) Confocal microscopic examination of the effects of BLT2 gene silencing on Comp A-induced reorganization of K8. (C) Effects of BLT2 overexpression on phosphorylation of Ser431 in K8 in Comp A-treated PANC-1 cells. PANC-1 cells were treated with or without Comp A (50 nM) for 1 hour after transfection with the plasmid containing BLT2 (pBLT2) and control (mock). (D) Confocal microscopic examination of the effects of BLT2 overexpression on Comp A-induced reorganization of K8. In (B) and (D), immunostaining was performed using K8-Ser431 (red) or panK8 (green). In (C), 5-LO, 12-LO, BLT2, and panK8 was analyzed by Western blotting. Blots were stripped and reprobed with β-actin antibody.

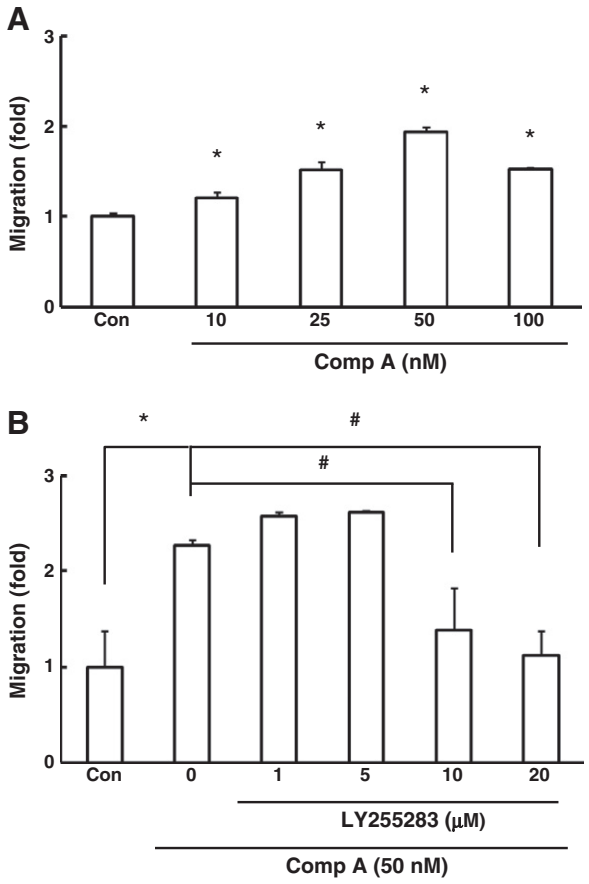

Fig. 4. BLT2 is involved in Comp A-induced migration of PANC-1 cells. (A) Migration of PANC-1 cells through 8-μm size-limited pores in response to Comp A. (B) Effect of LY255283 on Comp A-induced migration of PANC-1 cells. In (A), and (B), PANC-1 cells (5×10^4 cells per well) were treated with Comp A (50 nM) and varying concentrations of LY255283 as indicated for 1 hour. A representative of three independent experiments + s.e.m., each performed in triplicate, is shown. $^*p<0.05$ compared to control, $^{**}p<0.05$ compared to Comp A (50 nM).

expression is specific for pancreatic cancers because the expression of BLT2 is low in normal pancreas [16]. BLT2 is also highly expressed in bladder cancer, causing survival and metastasis of cancer cells [39]. However, the role of BLT2 and its underlying mechanism in the metastasis of pancreatic cancer cells are not clear. Phosphorylation and reorganization of K8 mediated by BLT2 might contribute to the metastatic properties of pancreatic cancers by enhancing deformability. Enhanced deformability allows PANC-1 cells to squeeze through size-limited spaces [6,7].

Nothing is known about how BLT2 promotes phosphorylation and reorganization of K8. Since phosphorylation and reorganization of keratin occurs via PKC and MAP kinases, and LTB_4 stimulates growth of pancreatic cancer cells via MAPK pathways [8,9], BLT2 might induce keratin phosphorylation and reorganization via PKC and MAPK pathways [6,10]. Another possibility is activation of ROS by BLT2 [14,17]. Our data showed that BLT2 induced ERK activation in PANC-1 cells, leading to keratin reorganization and increased migration of PANC-1 cells (Fig. 5). A relationship between BLT2 and ERK was also observed in mouse keratinocytes [37]. BLT2 signaling has been reported to regulate the expression of cyclooxygenase-2 through the RAS/RAF/ERK pathway [40].

BLT2 overexpression enhanced phosphorylation of Ser431 in K8 and migration in the absence of LTB_4 (Figs. 3C, 5E). There is no report on how BLT2 can overexpress itself without comp A treatment and induce synthesis of BLT2 ligands such as LTB_4 or 12(S)-HETE in PANC-1 cells. However, overexpression of BLT2 itself exerts matrix metalloproteinase-1 expression in HaCaT cells [41]. Furthermore, BLT2 overexpression itself is shown to induce the production of LTB_4 or 12(S)-HETE, ligands of BLT2 via 5-lipoxygenase and 12-lipoxygenase in Rat2 cells [42]. Therefore, it is plausible that BLT2 overexpression itself might enhance the phosphorylation via increasing the synthesis of BLT2 ligands. We confirmed the expression of 5-lipoxygenase and 12-lipoxygenase, which were involved in the synthesis of BLT2 ligands such as LTB_4 or 12(S)-HETE, respectively (Fig. 3C).

Reduced expression of PP2A is observed via Comp A treatment (Fig. 5F). Changes of PP2A expression might be the new mechanism for ERK activation and phosphorylation of K8. PP2A is reported to be involved in the phosphorylation of Ser 431 in K8 after hypoosmotic stress conditions [43]. PP2A can directly dephosphorylate pERK [44]. A detailed mechanism of PP2A's action is not clear and the related studies are currently underway.

We also examined the expression of PP2A in pancreatic cancer cells. We found that the expression of PP2A was reduced in MiaPaCa2 and BxPC3 cell lines (Fig. S17). These results might explain the increases of K8 phosphorylation in these two cell lines with no treatment (Fig. S1). These results also suggest that different expression levels of PP2A in pancreatic cancer cells contribute the factors that explain the response of keratin phosphorylation in different pancreatic cancer cell lines.

Fig. 5H shows that the Comp A-induced BLT2 expression was suppressed by PD98059. The finding that PD98059 suppressed the expression of BLT2 suggests the involvement of the ERK pathway in the expression of BLT2. To our knowledge, there is no report about the details on the interaction of transcription factors and the promoter region of BLT2 and cloning of the promoter region of BLT2. Therefore, we estimated the binding site for transcription factors in the upstream sequences of BLT2 using the UCSG genome browser. Upstream regions of BLT2 have many possible binding sequences for transcription factors such as FosB, Fra-1, HSF1, PPAR-γ1, γ2, c-fos, c-jun, and c-Myc, which are known to be the substrates of ERK [45]. As a result, Comp A induces basal BLT2 activation leading to increased ERK activity and ERK phosphorylates transcription factors resulting in binding to the promoter region of BLT2 and enhances the transcription of BLT2. PD98059 inhibited ERK activation via blocking the binding of transcription factors to the promoter region of BLT2, resulting in suppression of BLT2 expression.

All together, LTB_4 induced K8 phosphorylation and reorganization via BLT2/ERK activation through decreased expression of PP2A in PANC-1 cells (Fig. 5I). This suggests that BLT2 plays an active role in the deformability of metastatic cancer cells by inducing K8 reorganization, and BLT2 could be a new target to modulate phosphorylation and reorganization of K8, which may alter the viscoelasticity and deformability of cells.

Conflict of interest statement

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2012.09.004.

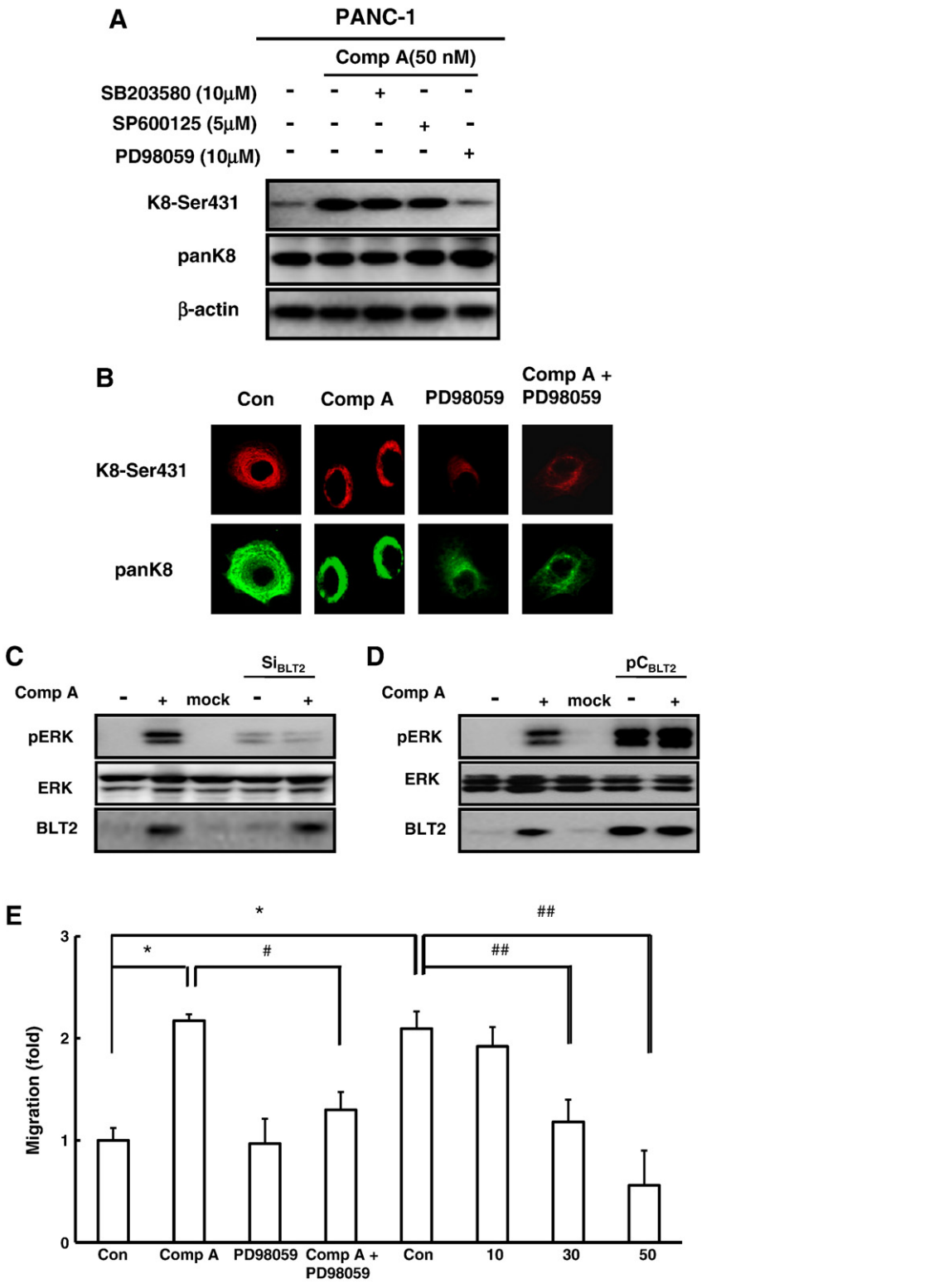

Fig. 5. ERK activation is dependent on BLT2. (A) Effect of several kinase inhibitors on Comp A-induced keratin phosphorylation in PANC-1 cells. PANC-1 cells were treated with or without 50 nM Comp A for 1 hour in the presence of PD98059 (MEK inhibitor, 10 μM), SP600125 (JNK inhibitor, 5 μM), and SB203580 (p38 kinase, 10 μM). (B) Confocal microscopic examination of the effects of PD98059 on Comp A-induced K8 phosphorylation in PANC-1 cells. (C) Effect of BLT2 gene silencing on Comp A-induced activation of ERK in PANC-1 cells. PANC-1 cells were treated with or without 50 nM Comp A for 1 hour after transfection with BLT2 siRNA (50 pmol) and control siRNA (mock). (D) Effect of BLT2 overexpression on Comp A-induced ERK activation in PANC-1 cells. PANC-1cells were treated with or without 50 nM Comp A for 1 hour after transfection with the plasmid containing BLT2 (pBLT2) and control (mock). (E) Effect of ERK inhibitor, PD98059 at 10 μM on the migration of PANC1$_{WT}$. PANC1$_{BLT2}$ cells (5×10^4 cells per well) were used in migration assay. $^{*}p<0.05$ compared with control, $^{**}p<0.05$ compared with LTB_4. (F) Effect of Comp A on PP2A expression in PANC-1 cells. PANC-1 cells were treated with or without 50 nM Comp A. Immunostaining was performed using PP2A antibody. (G) Binding of PP2A with pSer431 of K8. Immunoprecipitation with the 431 phosphoserine-specific keratin 8 antibody (IP: K8-Ser431) was performed on the extracts of PANC-1 cells with or without 50 nM Comp A for 1 hour. The resulting immunocomplexes were immunoblotted with PP2A, pERK, 431 phosphoserine-specific keratin 8 antibodies. IgG was used as negative control. (H–I) Effect of PD98059 on Comp A-induced ERK activation and BLT2 expression in PANC-1 cells. PANC-1 cells were treated with or without 50 nM Comp A for 1 hour in the presence of PD98059 (MEK inhibitor, 10 μM). In (H), pERK, ERK, and BLT2 were analyzed by Western blot. (I) Signaling pathway of BLT2-involved keratin 8 phosphorylation.

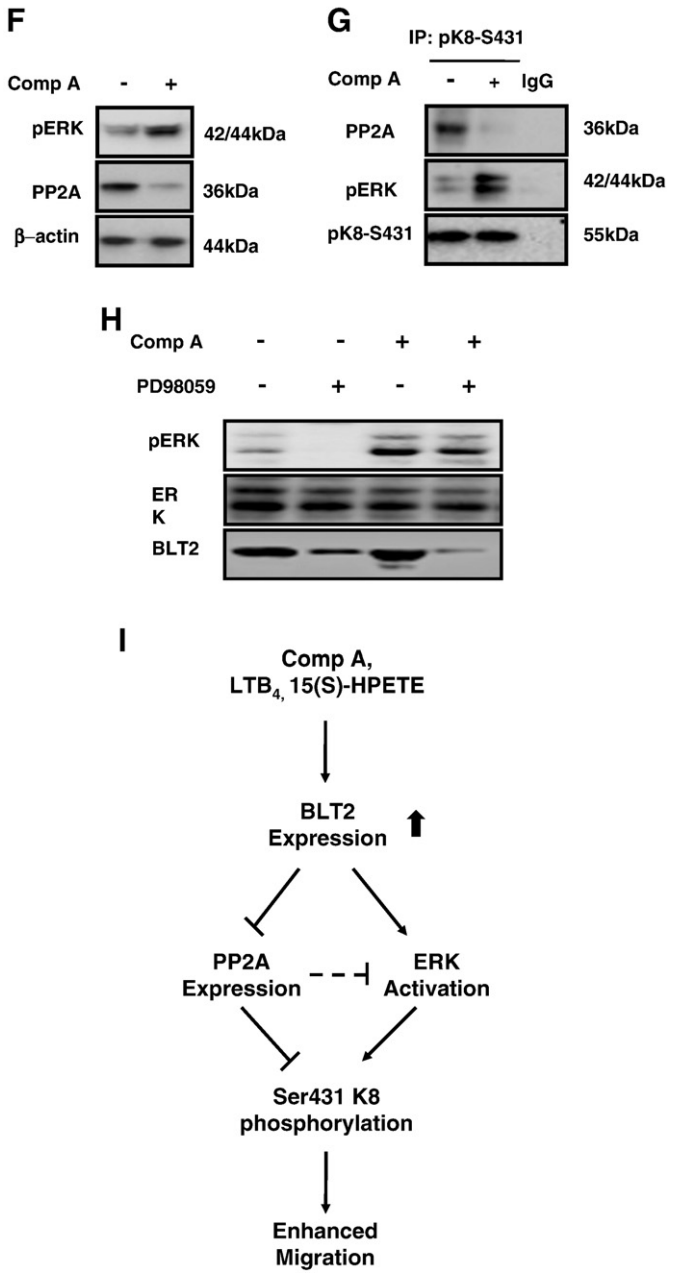

Fig. 5 (*continued*).

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