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Original Article

BAG3 regulates ECM accumulation in renal proximal tubular cells induced by TGF-β1

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Abstract: Previously we have demonstrated that Bcl-2-associated athanogene 3 (BAG3) is increased in renal fibrosis using a rat unilateral ureteral obstruction model. The current study investigated the role of BAG3 in renal fibrosis using transforming growth factor (TGF)-*β*1-treated human proximal tubular epithelial (HK-2) cells. An upregulation of BAG3 in vitro models was observed, which correlated with the increased synthesis of extracellular matrix (ECM) proteins and expression of tissue-type plasminogen activator inhibitor (PAI)-1. Blockade of BAG3 induction by shorting hairpin RNA suppressed the expression of ECM proteins but had no effect on PAI-1 expression induced by TGF-*β*1. Forced overexpression of BAG3 selectively increased collagens. TGF-β1-induced BAG3 expression in HK-2 cells was attenuated by ERK1/2 and JNK MAPK inhibitors. In addition, forced BAG3 overexpression blocked attenuation of collagens expression by ERK1/2 and JNK inhibitors. These data suggest that ERK1/2 and JNK signaling events are involved in modulating the expression of BAG3, which would ultimately contribute to renal fibrosis by enhancing the synthesis and deposition of ECM proteins.

Keywords: BAG3, ECM, MAPK, tubular epithelial cell

Introduction

In recent years, accumulating studies have focused on exploring the pathogenesis of renal fibrosis, as there is a strong correlation between the extent of tubulointerstial fibrosis and loss of renal functions in end stage renal disease (ESRD) [1, 2]. Renal tubulointerstitial fibrosis is characterized by an excess deposition of ECM, which is partly due to unbalanced synthesis and degradation of ECM proteins. Major components of ECM proteins include fibronectin (FN) and collagens, especially type I and type IV, and their excessive synthesis and deposition are observed in the process of renal fibrosis and experimental animal models [3]. PAI-1 is a major inhibitor of plasminogen activators and elevated PAI-1 level is identified to reduce plasmin generation and further decrease plasmin-dependent ECM degradation [4]. Both degradation of the existing matrix and deposition of the newly synthesized ECM are responsible for ECM reforming [5].

TGF-*β*1 has been described as the core cytokine leading to the synthesis of ECM and is well known to trigger fibrosis in kidneys [1, 6, 7]. All three MAPK family proteins, including p38 MAPK, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), have been shown to correlate with ECM accumulation induced by TGF-*β*1. In addition, MAPK signaling contributes to TGF-*β*1 induced transition of tubular epithelial cells into myofibroblasts [8].

BAG3 was identified as an interacting partner of Bcl-2 by yeast two hybrid assay [9]. Expression of BAG3 is induced by many stressful stimuli, such as high temperature and heavy metal exposure [10-12], though its expression is limited to the striated muscle cells [13-15]. BAG3 is involved in multiple biological functions, such as cell survival, cell adhesion, and invasion [16-19]. Suppression of BAG3 has been identified to involve in apoptosis of kidney cancer cells, which is regulated by the inhibition of JNK sig-

nal pathway [20]. Recently, we have demonstrated that BAG3 is involved in epithelial-mesenchymal transition (EMT) of HK2 cells induced by fibroblast growth factor-2 (FGF2), and its expression is augmented in tubular epithelium in unilateral urinary obstruction (UUO) rat models [21]. These previous results urged us to investigate the potential role of BAG3 on ECM accumulation of renal epithelial cells stimulated by TGF-*β*1. In the current study, we examined the role of BAG3 in ECMs accumulation induced by TGF-β1 in HK2 cells. Induction of BAG3 by TGF-β1 in vitro models was observed, which correlated with the increased synthesis of ECM proteins and expression of tissue-type PAI-1. In addition, suppression of BAG3 reduced the expression of ECM proteins but had no effect on PAI-1 expression. We also demonstrated that TGF-β1-induced BAG3 expression in HK2 cells was attenuated by ERK1/2 and JNK MAPK inhibitors, and forced overexpression of BAG3 partly blocked the suppressive effects of ERK1/2 and JNK inhibitors. These findings suggested the involvement of ERK1/2 and JNK signaling events in regulating the expression of BAG3, which would ultimately contribute to renal fibrosis by enhancing the synthesis and deposition of ECM proteins.

Materials and methods

Reagents and antibodies

PD98059, SB203580 and SP600125 were purchased from Calbiochem (La Jolla, CA). TGF-β1 was purchased from PeproTech. The following antibodies were used in the current study: a rabbit antibody against BAG3 (Abcam), a rabbit antibody against Col I (Abcam), a rabbit antibody against Col IV (Abcam), a rabbit antibody against FN (Abcam), a rabbit antibody against PAI-1 (Abcam) and a mouse antibody against GAPDH (Sigma-Aldrich).

Cell culture

Human kidney 2 (HK2) cells were cultured in DMEM/F12 (Sigma-Aldrich, Saint Louis, MO) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Saint Louis, MO).

RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR)

RNA isolation and real-time RT-PCR were performed as previously reported [20]. For BAG3, the forward primer was 5'-CATCCAGGAGTGCTGAAAGTG-3' and the reverse primer was 5'-TCTGAACCTTCCTGACACCG-3'. For Col I, the forward primer was 5'-ACGTGATCTGTGACGAGACC-3' and the reverse primer was 5'-AGGCTGTCCAGGGATGCCATC-3'. For Col IV, the forward primer was 5'-GTACATCTCTGCCAGGACCAAG-3' and the reverse primer was 5'-CTGCAACACCATCTCTGCCAG-3'. For FN, the forward primer was 5'-CTACTCTGTGGGGATGCAGTG-3' and the reverse primer was 5'-AAGGCACCATTGGAATTTCCTC-3'. For PAI-1, the forward primer was 5'-GCTCCAGCTGACAACAGGAG-3' and the reverse primer was 5'-GTGACCGTGCTCCGGAACAG-3'.

Western blot analysis

Cells were lysed in protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO) and lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton-X100). Cell extract protein amounts were quantified using the BSA protein assay kit. Equal amounts of protein (25 μg) were separated by 12% SDS-PAGE and transferred to PVDF membrane (Millipore Corporation, Billerica, MA).

Dot blot analysis

Supernatant of the cultured cell was collected and normalized by cell number. Samples (300 μl/well) for dot blot analysis were loaded onto the dot blot apparatus (BioRad) using a multichannel pipette (Matrix) and allowed to adsorb to the protan nitrocellulose membrane (Whatman) under vacuum.

Transduction of HK2 cells using lentiviral vectors

Lentiviral plasmids containing BAG3 shRNA and LUC shRNA target sequences and a GFP expression cassette were produced by GeneChem Corporation (Shanghai, China). The target sequences against BAG3 (shBAG3) were as followed: shBAG3#1 5'-AATTCAAGTGATCCGCAAA-3'; shBAG3#2 5'-ATCTCCATTCCGGTGATAC-3'; shBAG3#3 5'-AATTACCCATCACATAAAT-3'; shBAG3#4 5'-TGGACACATCCCAATTCAA-3' and shBAG3#5 5'-GGAGGATTCTAAACCTGTTT-3'; Target HK2 cells were incubated with vector supernatants for 12 h. Transduction efficiency was determined by the measurement of GFP+ cells by a fluorescence microscopy. Transduced cells were cultured for 2 days

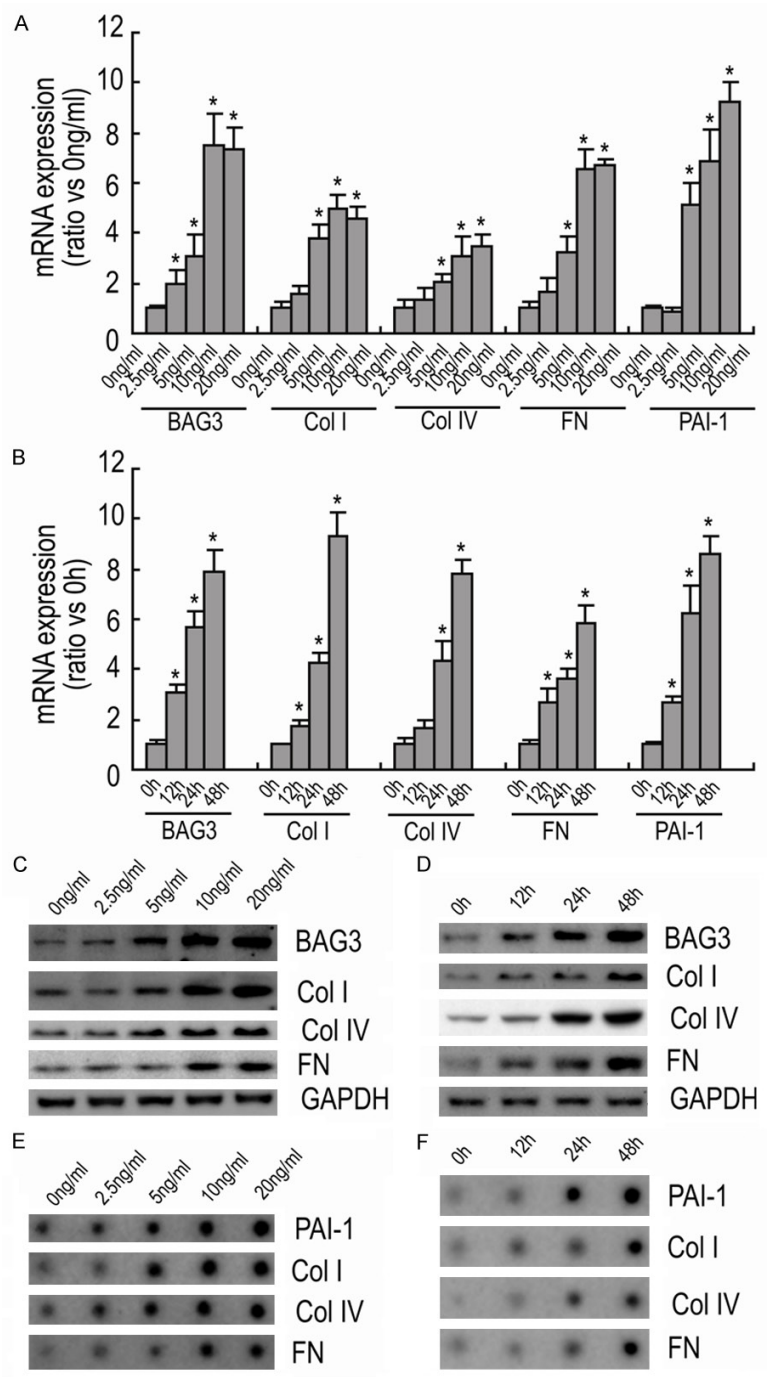

Figure 1. Induction of BAG3, Col I, Col IV, FN, and PAI-1 by TGF-β1 in HK2 cells. A. HK2 cells were treated with indicated dose of TGF-β1 for 24 h, mRNA levels of BAG3, Col I, Col IV, FN, and PAI-1 were measured by real time RT-PCR. B. HK2 cells were treated with 10 ng/ml TGF-β1 for indicated time, mRNA levels of BAG3, Col I, Col IV, FN, and PAI-1 were measured by real time RT-PCR. C. HK2 cells were treated with indicated dose of TGF-β1 for 24 h, western blot analysis was performed using the indicated antibodies. D. HK2 cells were treated with 10 ng/ml TGF-β1 for indicated time, western blot analysis was performed using the indicated antibodies. E. HK2 cells were treated with indicated dose of TGF-β1 for 24 h, culture supernatants were collected and dot blot was performed using the indicated antibodies. F. HK2 cells were treated with 10 ng/ml TGF-β1 for indicated time. After that, supernatant was collected and dot blot was performed using the indicated antibodies. *, $P<0.01$.

before proteins were extracted and analyzed by western blot.

Viable cell count

Cells were stained with trypan blue, viable (negative staining) and dead (positive staining) cell numbers were counted, respectively.

Statistical analysis

Statistical analysis between groups was performed with one-way ANOVA. All data were acquired from at least 3 individual experiments. Values are expressed as the mean ± SD. The statistical significance was defined as $p<0.05$.

Result

TGF-b1 augments BAG3 expression in HK2 cells

HK2 cells were treated with increasing concentrations of TGF-β1 for 24 h. RT-PCR analyses demonstrated dose-dependent increases of BAG3, and maximal increases were observed when 10 ng/ml TGF-β1 was included in the culture medium (**Figure 1A**). HK2 cells were then treated with 10 ng/ml of TGF-β1 for different time and time-dependent increases of BAG3 mRNA were observed as assessed by RT-PCR (**Figure 1B**). RT-PCR analyses demonstrated that TGF-β1 resulted in dose- and time-dependent increases of collagen type I (Col I), collagen type IV (Col IV), fibronectin (FN) and plasminogen inhibitor 1 (PAI-1) (**Figure 1A**, **1B**). Consistent with mRNA expression, western blot analyses revealed TGF-β1 induced increases of BAG3, Col I, Col IV and FN proteins in HK2 cells treated with

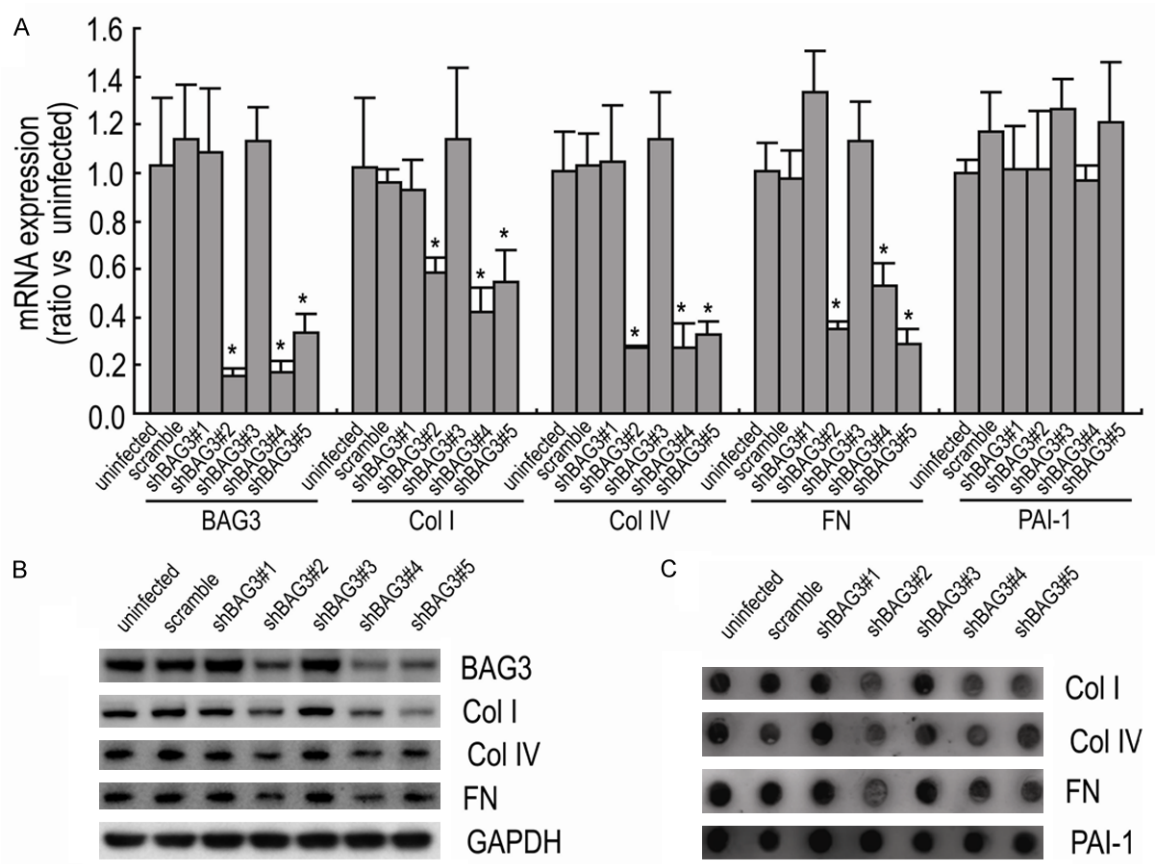

Figure 2. Attenuation of TGF-β1 mediated accumulation of ECM proteins by BAG3 knockdown. A. HK2 cells were infected with shBAG3 or scramble lentiviral vectors for 12 h and cultured for additional 48 h. Cells were then treated with 10 ng/ml of TGF-β1 for additional 24 h. BAG3 and ECM proteins mRNA expression was analyzed using real-time RT-PCR. B. HK2 cells were transduced with shBAG3 or scramble lentiviral vectors for 12 h and cultured for additional 48 h. Cells were then treated with 10 ng/ml of TGF-β1 for additional 24 h and Western blot was performed using the indicated antibodies. C. HK2 cells were infected with shBAG3 or scramble lentiviral vectors for 12 h and cultured for additional 48 h. Cells were then treated with 10 ng/ml of TGF-β1 for additional 24 h. Culture supernatants were collected and dot blot was performed using the indicated antibodies.

TGF-β1 in dose- (**Figure 1C**) and time-dependent (**Figure 1D**) manner. Dot blot analyses also confirmed the dose- (**Figure 1E**) and time-dependent (**Figure 1F**) increases in PAI-1, Col I, Col IV and FN in culture supernatants of HK2 cells cultured in the presence of TGF-β1. The concomitant increases in BAG3 and ECM protein expression after TGF-β1 treatment suggests that BAG3 might be implicated in profibrogenic effect of TGF-β1 and thus could play a role in renal interstitial fibrosis.

Knockdown of BAG3 expression attenuates expression of ECM proteins induced by TGF-β1

To investigate whether the induction of BAG3 expression was possibly implicated in deposition of ECM proteins, HK2 cells were infected with lentivirus containing shRNAs against BAG3 (shBAG3) before addition of TGF-β1 in the culture media. Three shBAG3 (#2, #4 and #5) resulted in a significant reduction in the mRNA expression of BAG3 induced by TGF-β1 (**Figure 2A**). RT-PCR demonstrated that knockdown of BAG3 expression resulted in reduction in TGF-β1-induced mRNA expression of Col I, Col IV and FN, but not PAI-1 in HK2 cells (**Figure 2A**). Consistent with changes in mRNA levels, western blot demonstrated that shBAG3#2, shBAG3#4 and shBAG3#5 resulted in significant reduced protein expression of BAG3, Col I, Col IV and FN in HK2 cells treated with TGF-β1 (**Figure 2B**). Also, similar reduction of protein expression of Col I, Col IV and FN was observed by dot blot in supernatants of HK2 cells treated with TGF-β1 (**Figure 2C**). Consistent with mRNA expression, knockdown of BAG3 had no obvious effect on PAI-1 expression in supernatants

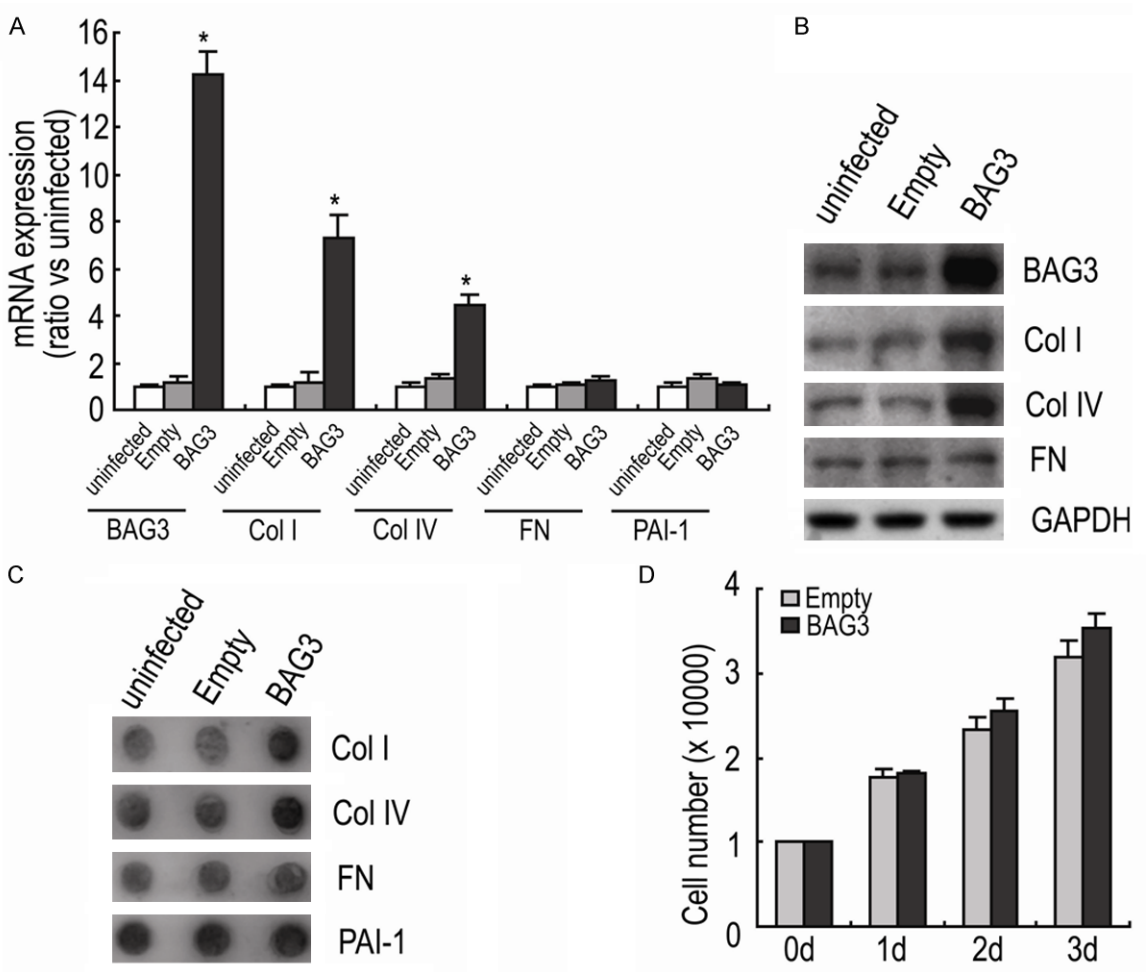

Figure 3. Selective induction of collagens generation by overexpression of BAG3. A. HK2 cells were infected with lentiviral vectors containing empty or BAG3 construct for 12 h and cultured for additional 48 h. mRNA levels of BAG3 and ECM proteins were analyzed using real-time RT-PCR. B. HK2 cells were infected with lentiviral vectors containing empty or BAG3 construct for 12 h and cultured for additional 48 h, Western blot was performed using the indicated antibodies. C. HK2 cells were infected with lentiviral vectors containing empty or BAG3 construct for 12 h and cultured for additional 48 h. Culture supernatants were collected and dot blot was performed using the indicated antibodies. D. HK2 cells were infected with lentiviral vectors containing empty or BAG3 construct, viable cells were counted daily.

of HK2 cells treated with TGF-β1 (**Figure 2C**). These data support that BAG3 specifically modulates de novo synthesis of ECM proteins induced by TGF-β1 in HK2 cells.

Overexpression of BAG3 selectively increases collagens

To further confirm the role of BAG3 in ECM protein components in HK2 cells, HK2 cells were infected with lentivirus containing empty or BAG3 construct. RT-PCR demonstrated that overexpression of BAG3 resulted in increased mRNA expression of Col I and Col IV, but not FN and PAI-1 in HK2 cells (**Figure 3A**). Consistent with mRNA expression, augment of protein expression of Col I and Col IV was observed (**Figure 3B**). BAG3 upregulation had no obvious influence on FN or PAI-1 expression (**Figure 3B**, **C**). Cell count demonstrated that BAG3 overexpression exhibited no obvious influence on proliferation of cultured HK2 cells (**Figure 3D**). These results indicate that BAG3 *per se* may selectively affect collagen generation.

JNK and ERK1/2 signaling pathways are involved in induction of BAG3 expression by TGF-β1

To investigate whether MAPK pathways are involved in TGF-β1-induced BAG3 expression, HK2 cells were individually pretreated with the

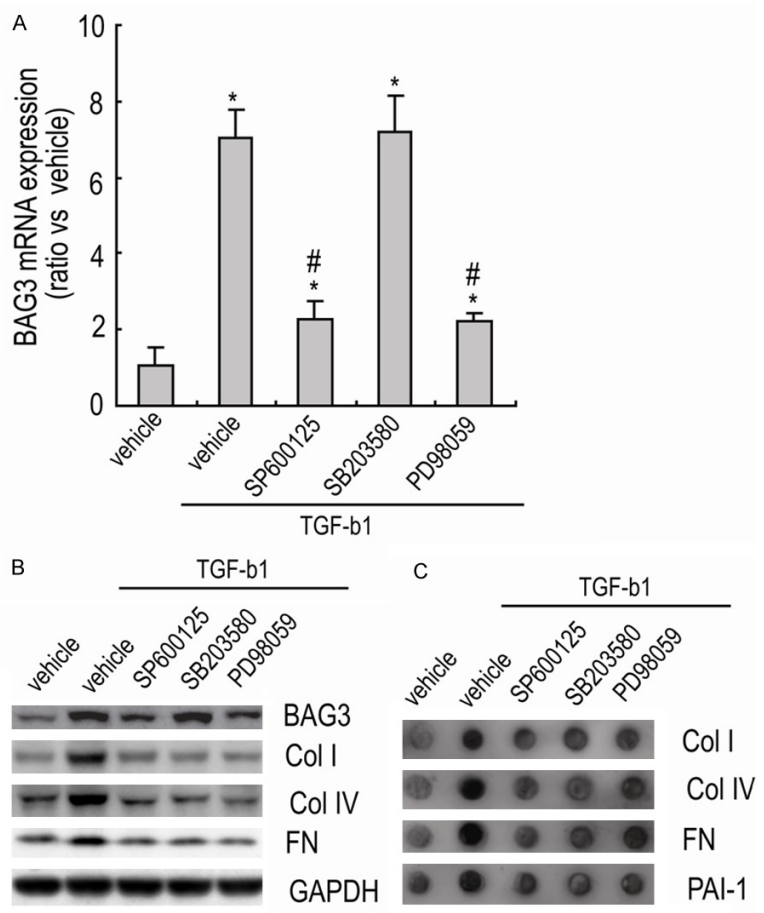

Figure 4. Implication of JNK and ERK1/2 signaling pathways in induction of BAG3 expression by TGF-β1. A. HK2 cells were individually pretreated with the JNK inhibitor SP600125, p38 inhibitor SB203580, or the ERK1/2 inhibitor PD98059 for 60 min before treatment with TGF-β1. mRNA levels of BAG3 was measured by real time RT-PCR. B. HK2 cells were individually pretreated with the JNK inhibitor SP600125, p38 inhibitor SB203580, or the ERK1/2 inhibitor PD98059 for 60 min before treatment with TGF-β1. Western blot was performed using the indicated antibodies. C. HK2 cells were individually pretreated with the JNK inhibitor SP600125, p38 inhibitor SB203580, or the ERK1/2 inhibitor PD98059 for 60 min before treatment with TGF-β1. Culture supernatants were collected and dot blot was performed using the indicated antibodies.

JNK inhibitor SP600125, p38 inhibitor SB203580, or the ERK1/2 inhibitor PD98059 for 60 min before treatment with TGF-β1. RT-PCR analysis revealed that pretreatment with SP600125 and PD98059 attenuated the TGF-β1-induced BAG3 mRNA expression (**Figure 4A**). SB203580, the inhibitor of p38 MAPK demonstrated no obvious influenced on TGF-β1-induced BAG3 mRNA expression (**Figure 4A**). Western blot analyses confirmed that treatment with SP600125 and PD98059 suppressed TGF-β1-induced BAG3 expression, while SB203580 exhibited no effect (**Figure 4B**). Western blot (**Figure 4B**) and dot blot (**Figure 4C**) analyses demonstrated that inhibition of MAPK pathways suppressed increases in ECM proteins and PAI-1 induced by TGF-β1. These data indicate that JNK and ERK1/2 pathways might be responsible for induction of BAG3 by TGF-β1 in HK2 cells.

BAG3 overexpression partly blocks the suppressive effects of ERK1/2 and JNK inhibition on ECM accumulation in HK2 cells

To further investigate whether BAG3 could rescue the effect mediated by inhibition of JNK and ERK1/2 pathways, HK2 cells infected with lentivirus containing empty or BAG3 constructed were individually pretreated with SP600125 or PD98059 for 60 min before treatment with TGF-β1. SP600125 and PD98059 significantly suppressed induction of Col I and Col IV by TGF-β1 in empty vector-infected cells, while their suppressive effects were attenuated in BAG3-infected cells (**Figure 5A**). BAG3 overexpression attenuated SP600125-mediated inhibition of FN, whiled showed no obvious influence on PD98059-mediated inhibition of FN (**Figure 5A**). SP600125 and PD98059 inhibited PAI-1 mRNA levels, and overexpression of BAG3 had no effect on their suppressive effects (**Figure 5A**). Consistent with mRNA expression, SP600125 and PD98059 suppressed Col I, Col IV, FN and PAI-1 protein expression in empty vector-infected cells (**Figure 5B**, **5C**). In BAG3-infected cells, inhibitory effects of SP600125 and PD98059 on Col I and Col IV were attenuated (**Figure 5B**, **5C**). BAG3 attenuated the suppressive effect of SP600125 on FN expression, while showed no obvious influence on the suppressive effect of PD98059 (**Figure 5B**, **5C**). PAI-1 demonstrated

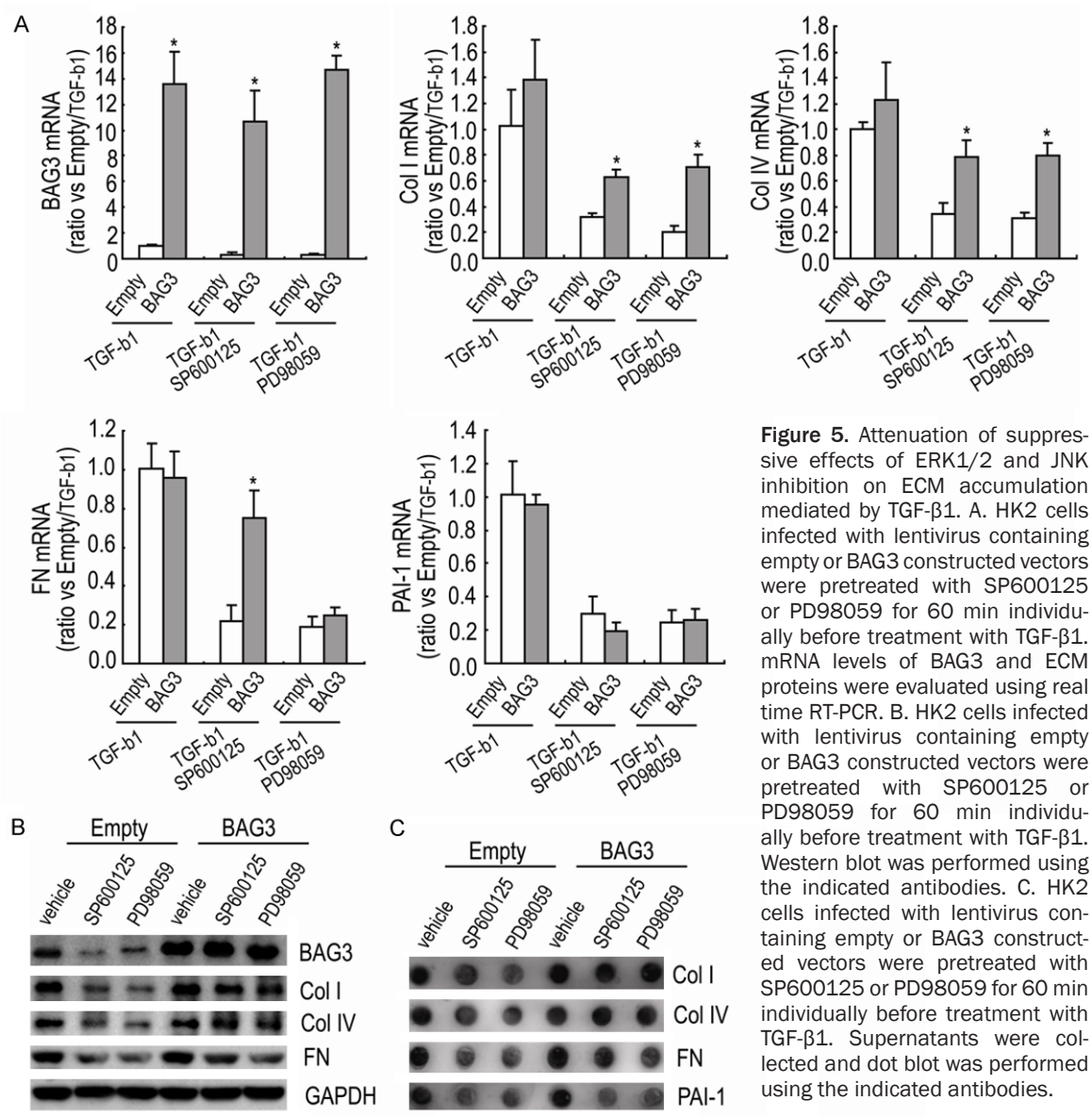

Figure 5. Attenuation of suppressive effects of ERK1/2 and JNK inhibition on ECM accumulation mediated by TGF-β1. A. HK2 cells infected with lentivirus containing empty or BAG3 constructed vectors were pretreated with SP600125 or PD98059 for 60 min individually before treatment with TGF-β1. mRNA levels of BAG3 and ECM proteins were evaluated using real time RT-PCR. B. HK2 cells infected with lentivirus containing empty or BAG3 constructed vectors were pretreated with SP600125 or PD98059 for 60 min individually before treatment with TGF-β1. Western blot was performed using the indicated antibodies. C. HK2 cells infected with lentivirus containing empty or BAG3 constructed vectors were pretreated with SP600125 or PD98059 for 60 min individually before treatment with TGF-β1. Supernatants were collected and dot blot was performed using the indicated antibodies.

similar expression levels in empty vector- and BAG3-infected cells (**Figure 5B**, **5C**). These data indicate that ERK1/2 and JNK signaling pathways are involved in ECM accumulation induced by TGF-β1, at least in part, via induction of BAG3.

Discussion

Interstitial fibrosis is associated with extensive accumulation of ECM constituents in the cortical interstitium and is also directly correlated to progression of renal disease [22]. Myofibroblasts are considered to be the terminally differentiated cells that are responsible for the synthesis and accumulation of interstitial ECM components such as collagens and fibronectin in various pathologies, including cancer and renal fibrosis [22, 23]. Epithelial to mesenchymal transition (EMT) program play a role, though a small part, in the formation of myofibroblast cells [24]. BAG3 has been demonstrated on its role in EMT of thyroid cancer cells [25]. Recently, we also demonstrated the increased expression of BAG3 during the EMT process in UUO rat models and HK2 cells induced by FGF2 [21]. However, the role of BAG3 in accumulation of ECM is still unknown. In the present study, we demonstrated that TGF-β1 induced the increased expression of BAG3 in HK2 cells. The upregulation of BAG3 is correlated with the increased synthesis of ECM

proteins and expression of PAI-1. The equilibrium of ECM components relies on both the synthesis and the degradation of ECM proteins [5]. The current study demonstrated that blockade of BAG3 by shRNA inhibited the expression of ECM proteins but showed no effect on the expression of PAI-1. Similarly, PAI-1 level was not affected by overexpression of BAG3 in HK2 cell. These results indicated that in HK2 cells BAG3 specifically regulated de novo synthesis of ECM proteins induced by TGF-β1 without affecting its degradation. The present study also identified a new role of BAG3, other than EMT, on implicating the ECM accumulation in renal fibrosis.

In HK2 cells BAG3 upregulation induced by TGF-β1, as well as forced overexpression of BAG3, resulted in the increased level of collagen. However, distinct effect of TGF-β1-induced BAG3 and overexpressed BAG3 on FN was demonstrated. BAG3 induction was implicated in FN expression induced by TGF-β1, as knockdown of BAG3 suppressed TGF-β1 mediated FN expression. On the other hand, forced BAG3 overexpression *per se* demonstrated no obvious influence on FN expression. BAG3 has a modular structure containing multiple protein interacting motifs. In addition, it has been reported that BAG3 is phosphorylated at Ser187, phosphorylated and non-phosphorylated BAG3 appear to act opposite in EMT and invasion of thyroid cancer cells [25, 26]. TGF-β1 exposure lead to complicated crosstalk among the signaling pathways and downstream proteins [27], which may alter phosphorylation status of BAG3 or its interacting partners. Thus the altered interacting partners, as well as different phosphorylation status of endogenous TGF-β1 induced BAG3 and overexpression BAG3 may explain the different function. Nevertheless, further investigations are needed to elucidate the mechanism responsible for the distinction between these two formations.

MAPKs are intricately implicated in the pathobiology of various kidney diseases, including renal fibrosis. It is well known that TGF-β1 and MAPKs signaling pathways cross-talk considerably in the synthesis and degradation of ECM by fibroblast-like cells in the kidney. In addition, MAPK signaling is considered to contribute to transition of tubular epithelial cells into myofibroblasts induced by TGF-β1 [8, 28-31]. The present study demonstrated MAPK signaling pathway also participated in ECM accumulation induced by TGF-β1, and this process is, at least in parted, regulated by BAG3. In the current study, overexpression of BAG3 rescued the inhibition of FN mRNA expression induced by JNK inhibitor SP600125, indicating that BAG3 might be involved in FN expression mediated by JNK signaling pathway. On the other hand, overexpression of BAG3 had no influence on FN mRNA levels inhibited by ERK1/2 inhibitor PD98059. These data indicated that BAG3 may not ascribe to FN expression mediated by ERK1/2. Alternatively, ERK1/2-mediated modulation is critical for its role in FN expression, predicting the possibility for BAG3 as a down stream target protein of ERK signaling pathway.

In summary, the present study, for the first time, demonstrated that BAG3 was implicated in TGF-β1 induced ECM accumulation in HK2 cells, and MAPK signaling contributed to the regulation of this process. The current study described the role of BAG3 in the pathogenesis of tubulointerstitial fibrosis, and this may give us a new idea on developing therapeutic method in renal disease.

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Disclosure of conflict of interest

None.

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