



# miRNA-125b Signaling Ameliorates Liver Injury Against Obstructive Jaundice-Induced Excessive Fibrosis in Experimental Rats

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**Purpose:** Multiple pathways are involved in inducing liver fibrosis, which can damage the integrity of liver. Among them, miR-125b has been found to exert an activating action on hepatic stellate cells. Endoplasmic reticulum stress and autophagy lead to liver disorders. Here, we evaluated the therapeutic influence of miR-125b on the endoplasmic reticulum function in injured livers submitted to bile duct ligation.

**Materials and Methods:** For inducing injury, bile duct ligation was done on miR-125b transgenic rats (miR-125b-Tg) in wild type rats. The rat T-6 cells received transfection of miR-125b mimic and Tunicamycin. Protein expressions were observed by western blot analysis.

**Results:** Compared to wild type rats, liver-injured rats showed significant impairment of liver function as assessed by the total bilirubin levels. The miR-125b-Tg rats showed decrease in activity of aspartate transaminase and alanine transaminase. Liver tissues of miR-125b-Tg rats showed weaker fibrotic matrix formation. Upregulation of miR-125b decreased the bile duct ligation-mediated hepatic disturbances for the expressions of endoplasmic reticulum kinase, inositol-requiring kinase 1alpha, sXBP1, CHOP, LC3, p62, ULK, and caspase-3/-8/-9. T-6 cells transfected with miR-125b mimic and treated with Tunicamycin caused decrease in levels of cleaved caspase-3, sXBP1, CHOP, and LC3. The miR-125b signaling showed protective effect on the liver tissues subjected to injury and fibrosis histopathology.

**Conclusion:** This study demonstrates a novel insight into the miR125b-mediated stabilization of endoplasmic reticulum integrity, which slows the progression of injury-induced hepatic deterioration.

**Key Words:** miR-125b, liver injury, bile duct ligation, endoplasmic reticulum

## INTRODUCTION

Long-term damage to liver by cholestasis and hepatitis can stimulate the production of fibrosis, which is harmful and associat-

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ed with deregulation of multiple molecular and cellular events.<sup>1</sup> Hepatic stellate cells have been discovered to undergo a phenomenon that leads to the deposition of extracellular matrix on a damaged lever by differentiating into myofibroblastic cells.<sup>2</sup>

Unfolded protein response is a damaging reaction that results from the irregular folding of proteins in endoplasmic reticulum, and this response is responsible for the survival and apoptosis of cells.<sup>3</sup> Three proteins known as inositol-requiring enzyme 1 (IRE1), activating transcription factor 6, and protein kinase R-like endoplasmic reticulum kinase (PERK), which are found at the site of endoplasmic reticulum membrane, have been identified to initiate unfolded protein response in stressed cells.<sup>4</sup> In liver cells, the stress in endoplasmic reticulum is found to induce fibrogenic reactions in hepatic stellate cells by regu-

lating autophagy.<sup>5</sup> Long-term exposure of endoplasmic reticulum to stress increases apoptosis which causes cell death, while blockade of IRE1 maintains the process of autophagy and its inactiveness leads to decreased fibrogenic activity in the hepatic stellate cells.<sup>5-7</sup> These three proteins have also been found to regulate CHOP (CCAAT/enhancer-binding protein homologous protein), which is a main regulator of endoplasmic reticulum stress that leads to apoptosis.<sup>8,9</sup> However, the mechanisms involved in endoplasmic reticulum stress-mediated hepatic fibrosis remains unclear.

Recent studies have shown that miR-125b is implicated in various hepatic abnormalities, such drug-induced hepatic failure.<sup>10</sup> Also, miR-125b has been found to be upregulated in hepatic stellate cells.<sup>11</sup> miR-125b has been reported to regulate the number of pathways that control the hepatic fibrosis reactions; for example, miR-125b inhibited the levels of TGF- $\beta$ .<sup>12</sup> In addition, miR-125b has been found to regulate the function of DNA methyl transferase.<sup>13</sup> As it has been reported that miR-125b inhibits CCl<sub>4</sub>-induced hepatic fibrosis,<sup>12</sup> we postulated that the unfolded protein response cascade in injured hepatic tissue may be associated with its protective effect. Here, we used miR-125b transgenic rats (miR-125b-Tg) to affirm the correlation between unfolded protein response pathway, hepatic fibrosis in the endoplasmic reticulum, and miR-125b signaling in the liver subjected to obstructive jaundice.

## MATERIALS AND METHODS

### Animals and construction of miR-125b transgenic rats

For the study, we selected transgenic rats showing over-expression of miR-125b for the same breeding of rats, which was done under pathogen-free conditions as described earlier.<sup>14</sup> Prior to this, all of the protocols for animal studies were approved by the Institutional Ethical Review Board of Shandong Provincial Qianfoshan Hospital, Shandong University China (Number A0145LI). For this study, we used genetically modified Gunn rat, which is a mutant strain of Wistar rats. The rats were probed with PCR and miR-125b primers (Sense: 5'-UCCCUGAGACCU AACUUGUGA-3', Anti-sense: 5'-ACAAGUUAGGGUCUCAGG GAUU-3'). Wild type rats were devoid of constructs. The rats showed over-expression of miR-125b, and were housed under specific pathogen-free conditions at 25°C while maintaining 55% humidity with 12 hours of light-and-dark cycle. The rats were provided with sterile food and water ad libitum.

### Experimental protocol

Each group consisted of six animals. The rats were subjected to ligation of bile duct for which they were submitted to Ketamine and Xylazine anesthesia via intramuscular route. Ligation was done by operating the rats for midline laparotomy, and hepatic hilum was identified to confirm the common bile duct. The process of duct ligation was done under a microscope; bile

duct was ligated two times, and this group of rats was named bile duct ligation (BDL) group while rats subjected to sham surgery were named sham group. The rats were euthanized 7 days after the operation, and their liver tissues were removed, frozen, and processed to isolate the total proteins and RNA.<sup>15</sup> The tissues were stored at -80°C for further use.

### RNAi transfection and culture of T6 cells

For the study, we utilized T6 stellate cells that were initially maintained in Waymouth MB 752/1 media (Gibco, Gaithersburg, MD, USA), and the media was supplemented with fetal bovine serum (FBS) 10% and penicillin 100 IU/mL and streptomycin (ThermoFisher, Waltham, MA, USA). The cells were transfected with simian virus-40, as described in a previous study.<sup>16</sup> The T6 cells incubated in MEM media (ThermoFisher) were added with 10% FBS, penicillin, streptomycin, and glutamax-1 under humid conditions with 5% CO<sub>2</sub>. T6 cells were then plated in culture plates at a density of 6×10<sup>5</sup> cells, and then incubated in Tunicamycin for 6 hours (0.6 mg/mL, dissolved in DMSO) to produce endoplasmic reticulum stress. The culture medium was removed, and cells were rinsed with phosphate-buffered saline. Consistent with the present study, T6 cells were transfected with miR-125b mimic or miR-control for 1 day (24 hours) using Lipofectamine reagent (Invitrogen, California, CA, USA) following the supplied instructions. Each experiment was repeated three times.

### Masson's trichrome staining

The isolated liver tissues were fixed using formalin (10%), and then embedded in paraffin. The tissue blocks were sliced using a microtome for obtaining 5- $\mu$ m sections. The sections were deparaffinized and were hydrated before being subjected to Masson's trichrome staining. The staining was done using Masson's trichrome staining kit (Abcam, Cambridge, MA, USA) according to the supplied instructions. The sections were evaluated for the intensity of staining using animage J developer's analysis software. Each experiment was repeated three times.

### Western blot analysis

For western blot analysis, about 10 mg of liver tissue was measured and mixed with 500- $\mu$ L lysis buffer. The resultant was homogenized and centrifuged at 10000 rpm for 10 minutes, followed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (10-15%). The isolated proteins were transferred to polyvinylidene fluoride membrane, and the blots were then exposed to primary antibodies against Caspase-3/-9/-8, Phospho-ULK1, sXBP1, CHOP, IRE1, LC3 (Cell Signal Technology, Beverly, CA, USA), COL4A1 (GeneTex, Irvine, CA, USA), SQSTM1/P62, and GAPDH (Abcam). The immuno-blots were washed with mixture of tris-buffered saline in tween-20, and were incubated with horseradish peroxidase (HRP) coupled with ant-rabbit IGG antibodies (1:2000), HRP-anti-mouse IGG antibodies (1:1000) and HRP-ant-goat IGG (1:1000) were stored

at room temperature for 1 hour. The blots were developed using enhanced chemiluminescence, followed by exposure to film and densitometry analysis.

### Statistical analysis

Data are presented as mean±standard error. One-way ANOVA was performed to establish the level of significance. *p*-values<0.05 were considered significant.

## RESULTS

### Over-expression of miR-125b decreases fibrosis and hepatic damage in injured liver

To study the change in endoplasmic reticulum stress in the early

stage of liver damage, we submitted the miR-125b-Tg and wild type rats to 1 week of bile duct ligation procedure. The results of biochemical analysis (Table 1) suggested that bile duct ligation caused a significant increase in the levels of alanine transaminase (ALT), bilirubin, and aspartate transaminase (AST) in wild type rats (*p*<0.001). Nevertheless, the procedure of bile duct ligation resulted in weaker levels of AST and ALT in miR-125b-Tg rats compared to wild type rats.

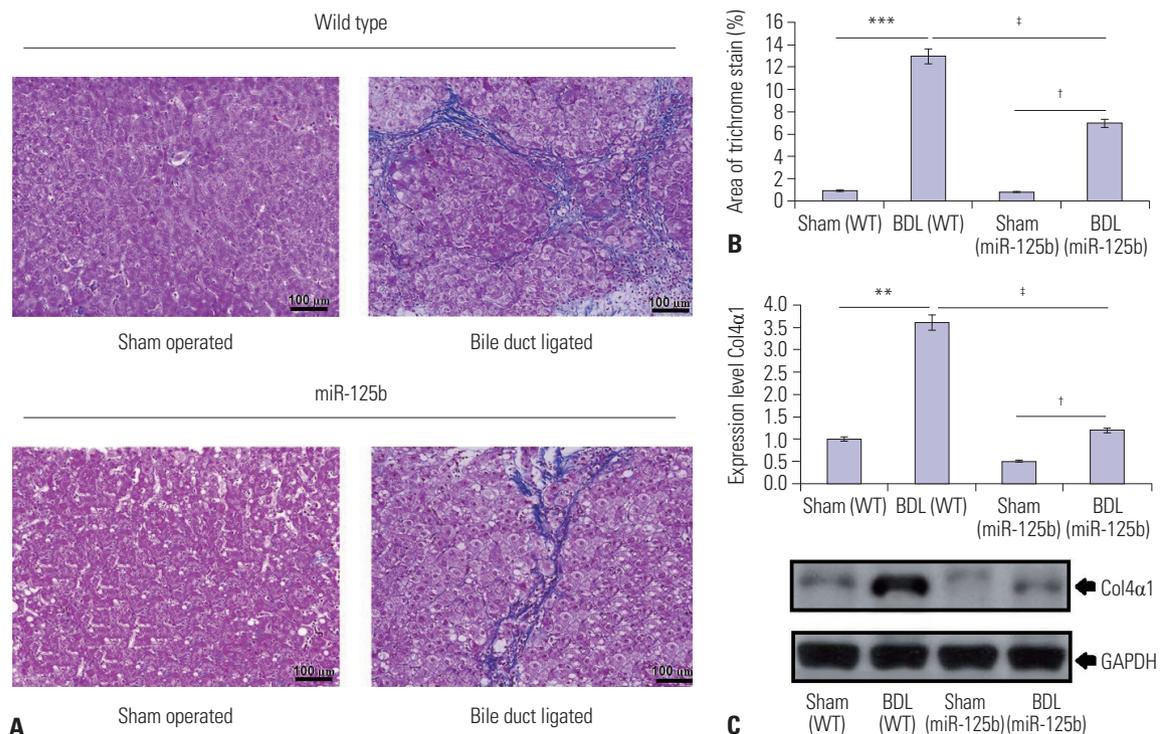
The outcomes of Masson's trichrome staining suggested a high accumulation of collagenous matrix around the portal region of liver tissues recovered from BDL wild type rats, while sham-operated rats did not show the same results. The outcomes of histopathology were distinctly mitigated in the bile duct-ligated miR-129b rats (Fig. 1A and B); parallel to this, the bile duct ligation-mediated increase in collagen levels decreased signifi-

**Table 1.** Comparison of Liver Function Changes in the Four Groups

Group (n=6)	Bilirubin (mg/dL)	Aspartate aminotransferase (IU/L)	Alanine aminotransferase (IU/L)
Wild type sham-operated	<0.5	61.22±4.11	21.11±1.22
Wild type bile duct-ligated	12.89±1.01*	788.22±44.14*	637.22±24.56*
miR-125b sham-operated	<0.5	61.22±1.55	15.77±1.44
miR-125b bile duct-ligated	6.98±0.55**†	378.54±34.15**†	354.54±38.59**†

Data are expressed as mean±standard error.

\**p*<0.05 compared to sham wild type, †*p*<0.05 compared to wild type bile duct-ligated, ‡*p*<0.05 compared to miR-125b sham-operated.



**Fig. 1.** Over-expression of miR-125b transgenic rats decreased fibrosis and hepatic damage in injured liver. (A) Masson's trichrome staining demonstrated the presence of moderate fibrosis in wild type rats, as seen by blue staining showing mild fibrosis in miR-125b transgenic rats. (B) Intensity of Masson's trichrome staining in tissue sections analyzed by image analysis software. (C) Quantitative analysis showed that the expression of Col4α1 in liver tissues of bile duct-ligated rats was significantly higher compared to sham group wild type rats. It was also found that over-expression of miR-125b caused a decrease in Col4α1 protein levels in the transgenic rats subjected to bile duct ligation compared to wild type rats. The results are presented as mean±standard error (n=6). \*\**p*<0.01 compared to sham wild type group of rats, \*\*\**p*<0.001 compared to sham wild type group of rats, †*p*<0.05 compared to sham+miR-125b, ‡*p*<0.01 compared to BDL wild type rats. BDL, bile duct ligation.

cantly in miR-129b-Tg rats ( $p < 0.001$ ) (Fig. 1C).

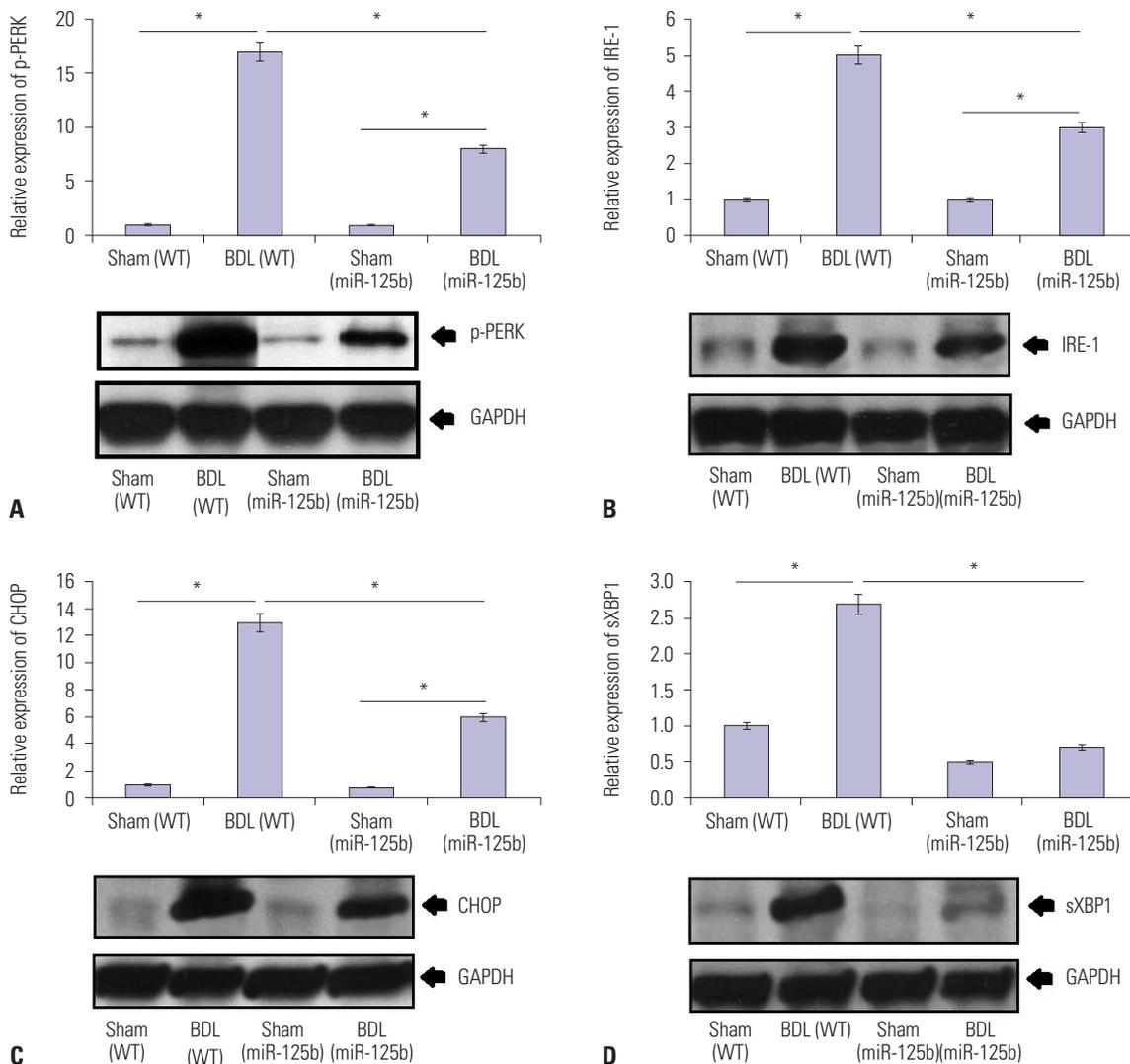
**Over-expression of miR-125b inhibits the bile duct ligation-mediated aggravation of endoplasmic reticulum stress and unfolded protein response-associated protein expression**

This study evaluated whether miR-125b could alter the endoplasmic reticulum stress in BDL-injured liver tissues. It was observed that the rats of wild type group showed significantly high levels of phosphorylated-PERK (p-PERK) and IRE1 after bile duct ligation injury (Fig. 2A and B). The miR-125b-Tg rats showed a significant decrease in bile duct ligation-mediated increase of p-PERK and IRE1, suggesting an active action for miR-125b signaling in the endoplasmic reticulum. Ahead, it was investigated whether bile duct ligation or miR-125b altered the levels of CHOP or sXBP1, as both of them are downstream ef-

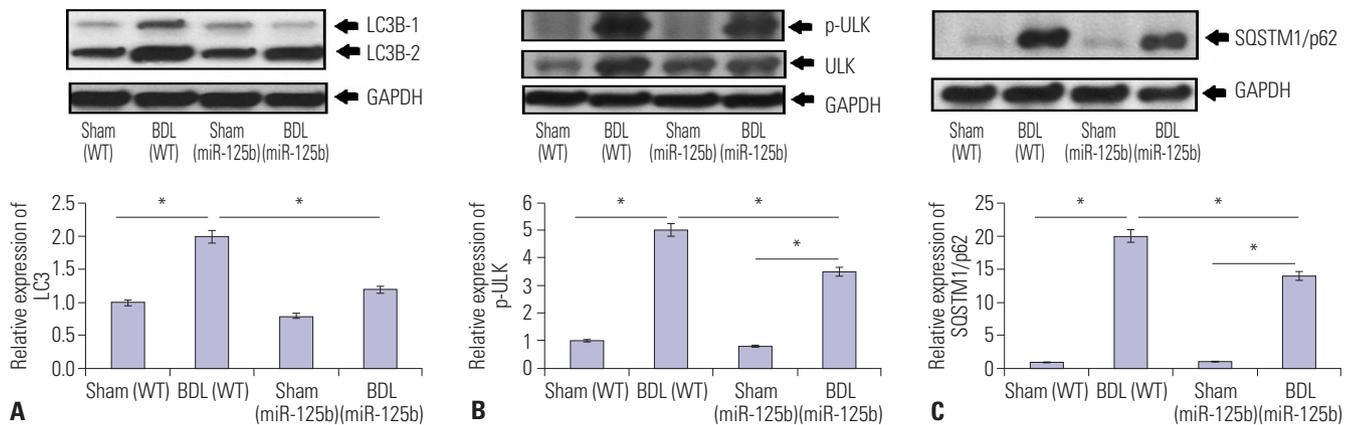
factors of endoplasmic reticulum stress that regulate the activation of hepatic stellate cells.<sup>17</sup> In the bile duct-ligated wild type rats, the levels of both sXBP1 and CHOP were significantly increased compared to sham-operated rats (Fig. 2C and D). However, miR-125b-Tg rats demonstrated a moderate effect on the bile duct ligation-mediated exasperation of both sXBP1 and CHOP (Fig. 2C and D).

**miR-125b mitigates autophagy in bile duct injury of liver in rats**

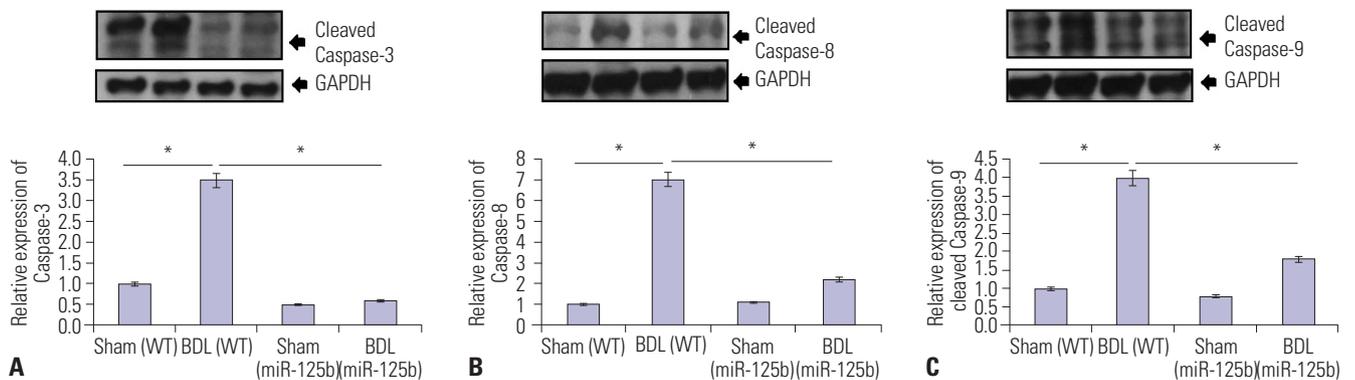
The current study evaluated whether miR-125b signaling altered the autophagy in the bile duct ligation-injured liver tissues. We found that the bile duct ligation-induced wild type rats showed high levels of LC3, p-ULK (Ser317), and SQSTM1/p62 (Fig. 3). The bile duct ligation miR-125b-Tg rats showed decreased levels of LC3, p-ULK (Ser317), and SQSTM1/p62 (Fig.



**Fig. 2.** Over-expression of miR-125b blocked the bile duct ligation-induced aggravation of endoplasmic reticulum stress. Western blot analysis and quantitative analysis of proteins such as (A) p-PERK, (B) IRE1, (C) CHOP, and (D) sXBP1 in wild type and miR-125b transgenic rats subjected to bile duct ligation. The results are presented as mean±standard error (n=6). \* $p < 0.05$  compared to respective groups. p-PERK, phosphorylated-protein kinase R-like endoplasmic reticulum kinase; IRE1, inositol-requiring enzyme 1.



**Fig. 3.** miR-125b increased autophagy in bile duct-ligated livers. Western blot analysis and quantitative analysis of (A) LC3B2, (B) p-ULK, and (C) SQSTM1/p62 expressions in wild type and miR-125b transgenic rat livers after bile duct ligation. The results are presented as mean  $\pm$  standard error (n=6), \* $p < 0.05$  compared to respective groups.



**Fig. 4.** miR-125b down-regulated the expression of caspase proteins in damaged livers. Western blot analysis showing expression of caspase proteins including (A) cleaved-Caspase-3, (B) Caspase-8, and (C) Caspase-9 in wild type and miR-125b transgenic rat livers after bile duct ligation. The results are presented as mean  $\pm$  standard error (n=6), \* $p < 0.05$  compared to respective groups.

3), suggesting miR-125b signaling in autophagy. The miR-125b down-regulated the expression of caspase proteins in damaged livers in addition to endoplasmic reticulum stress, and we also found a significant increase in the levels of caspase -3/-8/-9 in bile duct ligation-damaged liver tissues in wild type rats. Specifically, we observed that miR-125b over-expression caused a significant decrease in the bile duct ligation-induced cleavage of caspase-3/-8/-9 (Fig. 4).

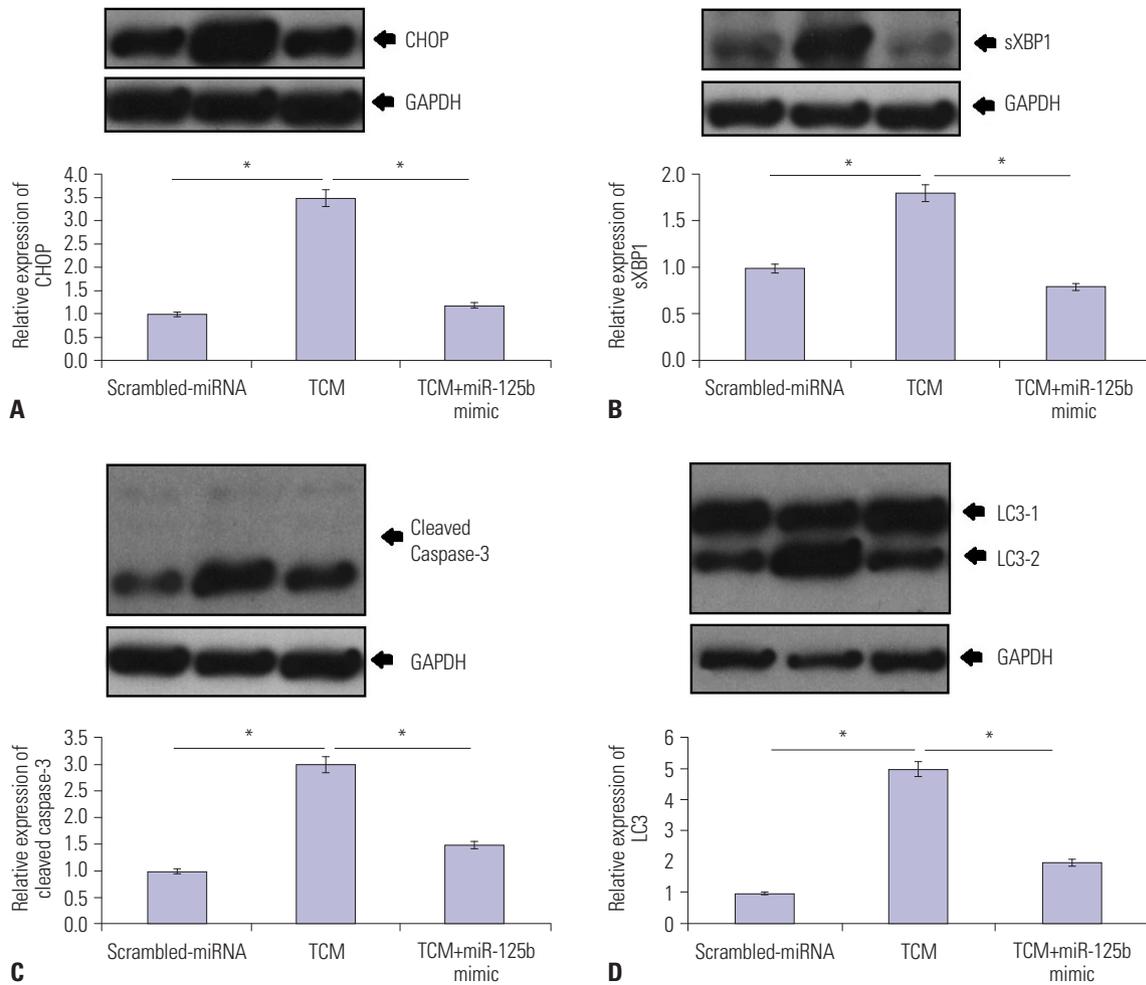
#### Over-expression of miR-125b decreases the expressions of CHOP and sXBP1 in T6 cells

We studied whether miR-125b regulated the functioning of endoplasmic reticulum in the T6 hepatic stellate cells. We treated the T6 cells to Tunicamycin into induce stress in endoplasmic reticulum, and found a significant increase in the expression levels of sXBP1, CHOP, cleaved caspase-3, and LC3 in the T6 cell cultures. The levels of sXBP1, CHOP, cleaved caspase-3, and LC3 were significantly decreased in the miR-125b mimic-transfected T6 cells (Fig. 5).

## DISCUSSION

The deposition of extracellular fibrotic matrix is a remarkable feature of hepatic stellate cell differentiation in the development of liver fibrosis.<sup>2</sup> In addition to this, the hepatic stellate cells derived from immortalized hepatic rat stellate cell line show activated phenotype.<sup>18</sup> The T6 cells are found to express some specific cytoskeletal proteins that are responsible for activating the hepatic stellate cells.

miR-125b has been found to be upregulated in hepatic stellate cells. Recent studies have confirmed the role of miR-125b in various hepatic abnormalities, such as paracetamol-induced hepatic toxicity.<sup>19</sup> miR-125b has also been found to promote apoptosis in hepatocellular carcinoma cells.<sup>20</sup> A recent study confirmed over-expression of miR-125b in fibrotic liver of mice as well as in Humans subjects suffering from liver fibrosis.<sup>21,22</sup> The present work provides evidence that miR-125b signaling may exert a protective action on the microenvironment of hepatic cells against the endoplasmic reticulum stress, which weakens the apoptosis activity, autophagic activity, and formation of fibrotic tissues, thereby attenuating the liver function. Such findings shed a new light on the mechanisms that are



**Fig. 5.** Over-expression of miR-125b suppressed the expressions of CHOP and sXBP1 in T6 cells. Western blot analysis of (A) CHOP, (B) sXBP1, (C) Caspase-3, and (D) LC3B2 in T6 cells from immortalized rat HSCs. The results are presented as mean±standard error (n=6), \**p*<0.05 compared to respective groups. HSCs, human stem cells.

involved in miR-125b-mediated reduction of hepatic fibrosis.

Liver cirrhosis and fibrosis are the results of chronic liver injury attributed to the apoptosis of liver cells, infiltration of inflammatory cells, and activation of stellate cells.<sup>23</sup> The presence of bile acid in the liver tissue is found to be responsible for over-production of unfolded proteins in the endoplasmic reticulum.<sup>24</sup> The unfolded protein response acts as an adaptive mechanism responsible for maintaining homeostasis against any injury.<sup>22</sup> Increased unfolded protein response has been found to produce adverse actions on the structural and functional integrity of liver cells in the presence of toxic agents. If the endoplasmic reticulum stress is high, the molecule CHOP can degenerate the functioning of cell and decrease its survival.<sup>22</sup> Unfolded protein response has been found to become an important cascade involved in the activation of hepatic stellate cells and secretion of collagen.<sup>23</sup> Stress activator, such as Brefeldin, leads to increase in the deposition of collagen along with expression of Smad-3 in hepatic stellate cells.<sup>24</sup> The results of our study were in agreement to the previous findings which confirmed that miR-125b-mediated suppression in fibrosis was

associated with the maintenance of endoplasmic reticulum stress.<sup>25,26</sup>

Previous studies have reported that CHOP is associated with endoplasmic reticulum stress-mediated apoptosis.<sup>27</sup> With regards to liver tissues, CHOP was reported to be associated with proapoptotic pathways participating in liver damage and fibrosis.<sup>27</sup> Lower levels of CHOP showed protective action in hepatocytes against the hepatic damage and fibrosis induced by alcohol, cholestasis, and steato hepatitis.<sup>28</sup> The present findings showed that increased expression of miR-125b caused down regulation of cleaved caspase-3 signaling and CHOP levels, which prevented liver fibrosis in the injured liver.

X-Box Binding Protein 1, also known as XBP-1, is identified as the important downstream target of IRE1.<sup>9</sup> XBP-1 is identified as a potential fibrogenic agent generated in hepatic stellate cells in both animal and human models. In addition, the abnormal upregulation of XBP-1 leads to production of collagen in hepatic stellate cells.<sup>29</sup> TANGO-1 is a protein which found to be involved in the biosynthesis of collagen. Unfolded protein response signaling is arbitrated XBP-1, which leads to over-

expression of TANGO-1 against TGF $\beta$  stress. Also, XBP-1-arbitrated unfolded protein response leads to the activation of hepatic stellate cells, which is functionally linked to autophagy.<sup>29</sup> The findings of our study suggested that over-expression of miR-125b caused the suppression of XBP-1, IRE1, CHOP, and PERK in injured liver tissues and hepatic stellate cells, which protected the liver from fibrosis and activation of stellate cells.

Autophagy is a process of programmed destruction and recycling of cells.<sup>30</sup> Previous studies have suggested that autophagy is an important regulatory pathway that contributes to liver fibrosis and is directly linked with the activation of hepatic stellate cells.<sup>24,29</sup> Reports have also shown increased autophagy in animal models with hepatic injury by inducing carbon tetra chloride or bile duct ligation,<sup>31</sup> whereas inhibiting the autophagic function decreased fibrogenesis and activation of hepatic stellate cells.<sup>32</sup> In addition, p62 has been confirmed to regulate liver inflammation and fibrosis negatively.<sup>33</sup> The outcomes of our study showed that over-expression of miR-125b led to a significant inhibition of autophagy, and attenuated liver injury as well as fibrosis mediated by bile duct ligation. Therefore, our findings have demonstrated a novel potential of miR-125b by modulating the endoplasmic reticulum stress as well as the autophagy in hepatic fibrosis.

In conclusion, as reported, the inhibition of fibrogenic character in hepatic stellate cells is important for halting the development of fibrosis.<sup>34</sup> The findings of this study demonstrate the profitability of controlling miR-125b signaling as an advanced strategy to control the miRs of endoplasmic reticulum homeostasis for preventing injury-mediated liver disorders.

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## AUTHOR CONTRIBUTIONS

**Conceptualization:** Xingyuan Zhang. **Data curation:** Xingyuan Zhang. **Formal analysis:** Xingyuan Zhang. **Investigation:** Changxi Zhang. **Methodology:** Xingyuan Zhang. **Project administration:** Jie Li. **Resources:** Jie Li. **Software:** Changxi Zhang. **Supervision:** Jie Li. **Validation:** Jie Li. **Visualization:** Fang Zhang. **Writing—original draft:** Xingyuan Zhang. **Writing—review & editing:** Jie Li. **Approval of final manuscript:** all authors.

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