Endoplasmic reticulum oxidase 1α is critical for collagen secretion from and membrane type 1-matrix metalloproteinase levels in hepatic stellate cells

Received for publication, February 24, 2017, and in revised form, July 21, 2017 Published, Papers in Press, August 3, 2017, DOI 10.1074/jbc.M117.783126

Mizuki Fujii[‡§], Akihiro Yoneda[§1], Norio Takei[§], Kaori Sakai-Sawada[§], Marina Kosaka[§¶], Kenjiro Minomi[§¶], Atsuro Yokoyama[‡], and Yasuaki Tamura[§]

From the [‡]Department of Oral Functional Prosthodontics, Division of Oral Functional Science, Graduate School of Dental Medicine, Hokkaido University, Nishi-7, Kita-13, Kita-ku, Sapporo 060-8486, Japan, the [§]Department of Molecular Therapeutics, Center for Food and Medical Innovation, Institute for the Promotion of Business-Regional Collaboration, Hokkaido University, Nishi-11, Kita-21, Kita-ku, Sapporo 001-0021, Japan, and the [¶]Research and Development Department, Nucleic Acid Medicine Business Division, Nitto Denko Corporation, Nishi-11, Kita-21, Kita-ku, Sapporo 001-0021, Japan

Edited by Amanda J. Fosang

Upon liver injury, excessive deposition of collagen from activated hepatic stellate cells (HSCs) is a leading cause of liver fibrosis. An understanding of the mechanism by which collagen biosynthesis is regulated in HSCs will provide important clues for practical anti-fibrotic therapy. Endoplasmic reticulum oxidase 1α (ERO1α) functions as an oxidative enzyme of protein disulfide isomerase, which forms intramolecular disulfide bonds of membrane and secreted proteins. However, the role of ERO1α in HSCs remains unclear. Here, we show that ERO1α is expressed and mainly localized in the endoplasmic reticulum in human HSCs. When HSCs were transfected with ERO1α siRNA or an ERO1α shRNA-expressing plasmid, expression of ERO1α was completely silenced. Silencing of ERO1α expression in HSCs markedly suppressed their proliferation but did not induce apoptosis, which was accompanied by impaired secretion of collagen type 1. Silencing of ERO1α expression induced impaired disulfide bond formation and inhibited autophagy via activation of the Akt/mammalian target of rapamycin signaling pathway, resulting in intracellular accumulation of collagen type 1 in HSCs. Furthermore, silencing of ERO1α expression also promoted proteasome-dependent degradation of membrane type 1-matrix metalloproteinase (MT1-MMP), which stimulates cell proliferation through cleavage of secreted collagens. The inhibition of HSC proliferation was reversed by treatment with MT1-MMP–cleaved collagen type 1. The results suggest that ERO1α plays a crucial role in HSC proliferation via posttranslational modification of collagen and MT1-MMP and, therefore, may be a suitable therapeutic target for managing liver fibrosis.

Hepatic stellate cells (HSCs)[2] are located in the space of Disse between the basolateral surfaces of hepatocytes and the antiluminal sides of sinusoidal endothelial cells (1, 2). In the liver, under physiological conditions, HSCs mainly accumulate vitamin A as retinyl ester in lipid droplets in the cytoplasm. Upon injury to or inflammation of the liver, HSCs lose vitamin A and transdifferentiate to myofibroblastic cells, so-called activated HSCs, which produce and secrete excessive extracellular matrices, including collagens, suggesting that activated HSCs are a major contributor to liver fibrosis and attractive target cells for anti-fibrotic therapy (3–5). Indeed, some studies have demonstrated that resolution of collagens in the liver of cirrhotic animal models is triggered by *in vivo* delivery of siRNA against the collagen-specific chaperone heat shock protein 47 (HSP47) or collagen type 1α1 to activated HSCs (6, 7).

During liver fibrosis, there is dramatic proliferation of activated HSCs, accompanied by excessive secretion and deposition of collagens in liver tissue. Our previous study and other studies also revealed that activated HSCs proliferate through the interaction of integrins with collagen molecules secreted by the HSCs (8–10). Collagens secreted by activated HSCs are cleaved by membrane type 1-matrix metalloproteinase (MT1-MMP) on the cell surface, leading to exposure of the RGD motif on the surfaces of collagen molecules. Integrin αvβ1 on activated HSCs binds to the RGD motif in collagen molecules, resulting in promotion of proliferation and survival of activated HSCs. Therefore, the collagen/MT1-MMP/integrin signaling axis is indispensable for the proliferation and survival of activated HSCs; however, the molecular mechanism by which the collagen/MT1-MMP/integrin signaling axis in HSCs is regulated remains elusive.

Intramolecular disulfide bond formation of the membrane and secreted proteins mainly relies on the protein disulfide isomerase (PDI) machinery, which accepts electrons from client cysteine thiols (11). Endoplasmic reticulum oxidase 1 (ERO1) has been identified as a major client of the PDI machin-

This work was supported in part by grants from the Center of Innovation Program from the Ministry and Education, Culture, Sports, Science and Technology of Japan (MEXT) and Japan Science and Technology Agency (JST). This work was supported in part by Nitto Denko Corporation (Osaka, Japan). The authors declare that they have no conflicts of interest with the contents of this article.

[1] To whom correspondence should be addressed. Tel.: 81-11-706-9464; Fax: 81-11-706-9463; E-mail: ayoneda@fmi.hokudai.ac.jp.

[2] The abbreviations used are: HSC, hepatic stellate cell; MT1-MMP, membrane type 1-matrix metalloproteinase; PDI, protein disulfide isomerase; ER, endoplasmic reticulum; FAK, focal adhesion kinase; mTOR, mammalian target of rapamycin; AMPK, AMP-activated protein kinase.

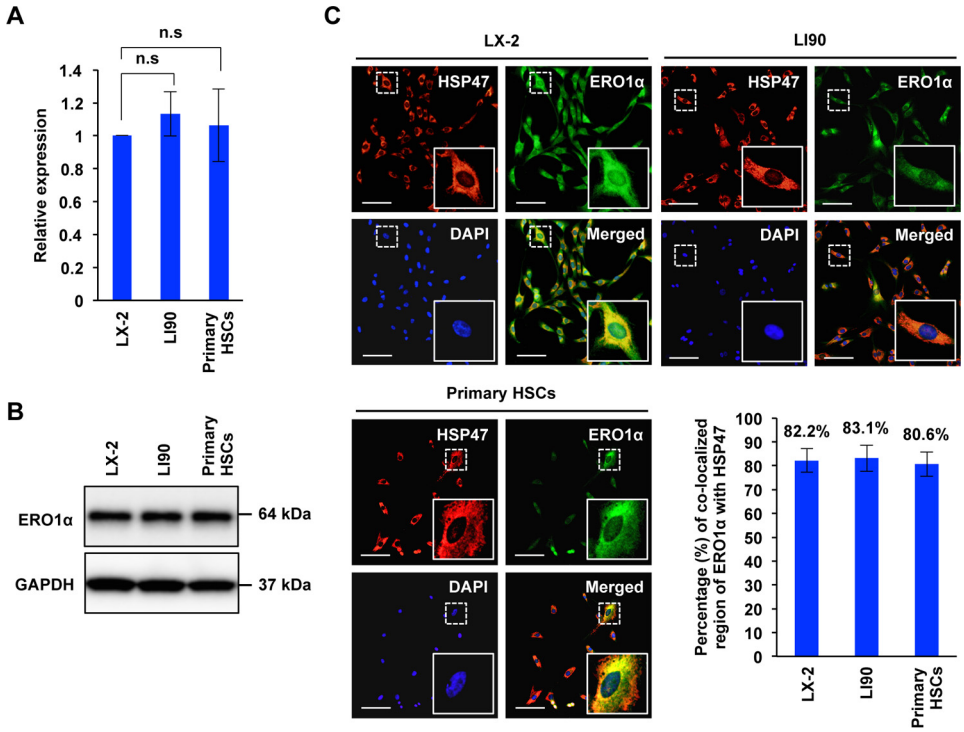

Figure 1. Expression and localization of ERO1α in human hepatic stellate cells. *A*, expression of ERO1α in the human hepatic stellate cell lines LX-2 and LI90 and primary HSCs was examined by quantitative RT-PCR. *n.s.*, not significant. *B*, immunoblots of ERO1α protein in LX-2 cells, LI90 cells, and primary HSCs. *C*, immunofluorescent staining of ERO1α and HSP47 in LX-2 cells, LI90 cells, and primary HSCs. HSP47 served as an endoplasmic reticulum marker. The degree of co-localization of ERO1 with HSP47 in cells was determined by NIH ImageJ. *Scale bars* = 100 μm.

ery (12, 13). ERO1 accepts electrons from reduced PDI and hands them over to oxygen molecules, catalyzing oxygen-mediated disulfide bond formation (14, 15). In mammals, two types of ERO1 (ERO1α and ERO1β) have been identified, and loss-of-function mutations in mammalian ERO1 genes result in rather subtle phenotypes (16, 17). Mice lacking ERO1α, which is ubiquitously expressed in several tissues and cells, have an abnormal cardiac response to adrenergic stimulation, and mice lacking ERO1β, which is specifically expressed in pancreatic β cells, develop mild nonprogressive pancreatic β cell dysfunction with glucose intolerance (18). The combined loss of function of ERO1α and ERO1β has been shown to compromise the extracellular matrix in mice and interfere with the intracellular maturation of procollagen (19). Collagen type 1 consists of two molecules of collagen type 1α1 and one molecule of collagen type 1α2 and also has some disulfide bonds between these molecules, leading to the formation of a triple helix structure (20). It has been reported that the disulfide bond in collagen molecules is formed by PDI (21, 22), suggesting that ERO1α plays a crucial role in the disulfide bond formation of collagen molecules, which is involved in the maintenance of HSC proliferation. However, the functional significance of ERO1α in HSCs remains unclear. Here we investigated the expression of ERO1α in human HSCs and examined the effect of ERO1α silencing on the proliferation of HSCs via the collagen/MT1-MMP/integrin signaling axis to explore the functional significance of ERO1α in HSCs.

Results

Silencing of ERO1α inhibits proliferation of HSCs

We first confirmed the expression of ERO1α in human hepatic stellate cell lines (LX-2 and LI90) and primary human HSCs. Quantitative RT-PCR and Western blotting showed that ERO1α is expressed in LX-2 cells, LI90 cells, and primary HSCs (Fig. 1, *A* and *B*). ERO1α was co-localized with HSP47, which is a collagen-specific chaperone and a marker of the endoplasmic reticulum (ER), in LX-2 cells (82.2%), LI90 cells (83.1%), and primary HSCs (80.6%) (Fig. 1*C*), indicating that ERO1α is expressed and mainly localizes in the ER in HSCs.

To clarify the significance of ERO1α in HSCs, we investigated the proliferation of HSCs by silencing ERO1α expression. When LX-2 cells were transfected with three batches of ERO1α siRNA, the expression of ERO1α mRNA and protein was completely silenced (Fig. 2*A*). Because the knockdown efficiency of ERO1α expression in LX-2 cells transfected with ERO1α-A siRNA was the highest among the three batches of ERO1α siRNAs, and transfection with ERO1α-A siRNA inhibited the expression of ERO1α protein in LX-2 cells, LI90, cells and primary HSCs (Fig. 2*B*), we used ERO1α-A siRNA for the cell proliferation assay. Silencing of ERO1α expression induced impaired proliferation of LX-2 cells, LI90 cells, and primary HSCs but did not induce cell death during a period of 5 days after transfection with ERO1α-A siRNA (Fig. 2, *C* and *D*).

Based on the results of the ERO1α siRNA–silencing experiment, we generated HSCs stably expressing ERO1α shRNA by transfection of two cell lines (LX-2 and LI90) with ERO1α shRNA–expressing plasmids to establish HSCs constitutively silencing ERO1α expression (Fig. 3*A*). Proliferation of the cell lines (LX-2 and LI90) stably expressing ERO1α shRNA was markedly suppressed compared with that of the cell lines stably expressing control shRNA (Fig. 3*B*). It has been reported that HSCs proliferate through activation of the FAK/MAPK signaling pathway and Akt/IκB signaling pathway (23–25). Thus, we

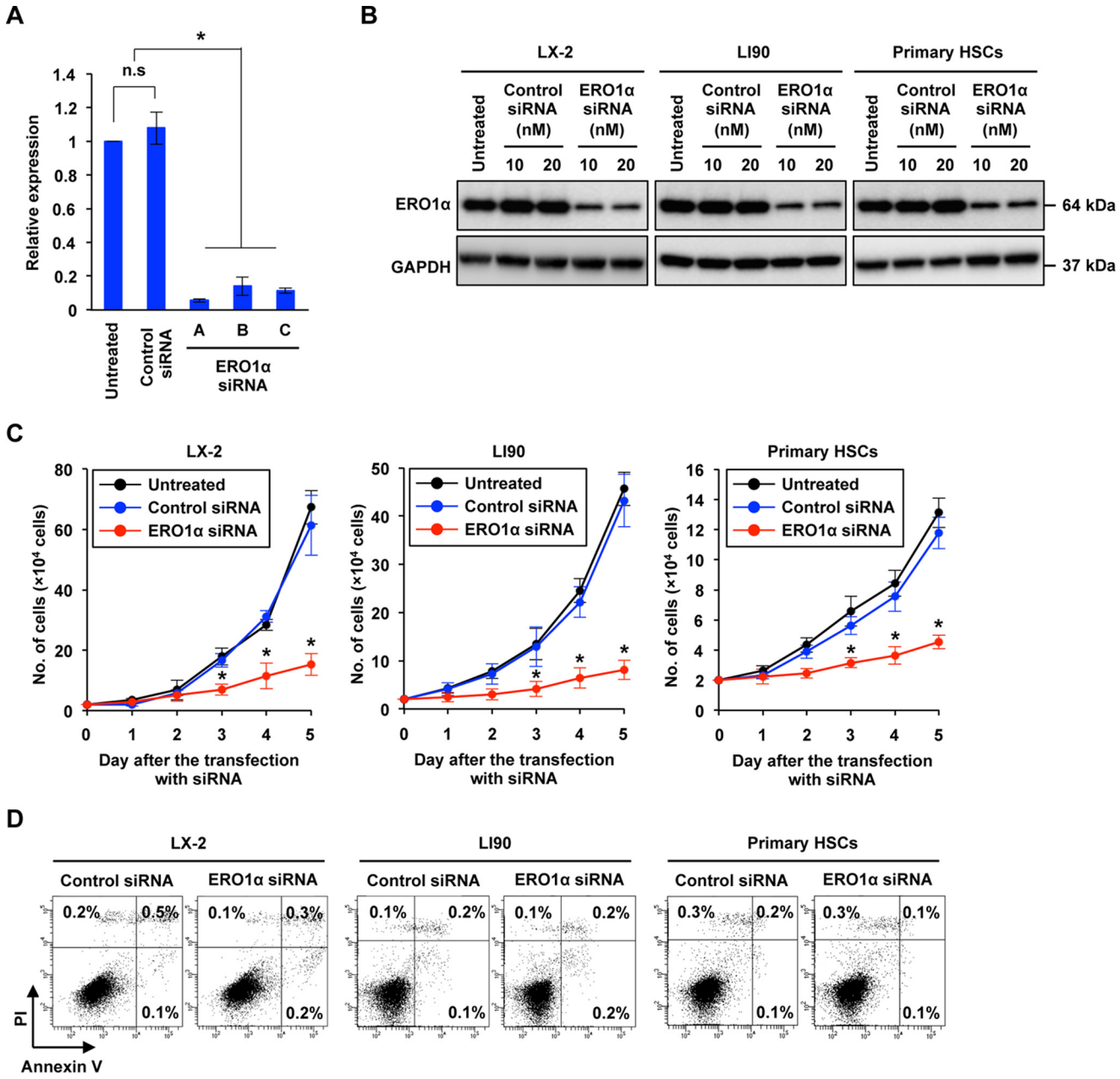

Figure 2. Silencing of ERO1α in human hepatic stellate cells suppresses their proliferation. *A*, expression of ERO1α mRNA in the HSC line LX-2 transfected with three batches of ERO1α siRNA at a concentration of 10 nM was examined by quantitative RT-PCR. *, $p < 0.05$; *n.s.*, not significant. *B*, immunoblots of ERO1α protein in LX-2 cells, LI90 cells, and primary HSCs transfected with control siRNA (10 nM and 20 nM) or ERO1α siRNA (10 nM and 20 nM). *C*, proliferation of LX-2 cells, LI90 cells, and primary HSCs after transfection with control siRNA (10 nM) or ERO1α siRNA (10 nM). Cell proliferation for 5 days is shown. *, $p < 0.05$. *D*, percentages of apoptotic LX-2 cells, LI90 cells, and primary HSCs transfected with control siRNA (10 nM) or ERO1α siRNA (10 nM) were determined by flow cytometric analysis for annexin V and propidium iodide (*PI*).

examined the phosphorylation of FAK and activation of the Raf-1/MEK/MAPK signaling pathway in two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA. As shown in Fig. 3*C*, the phosphorylation of FAK and activation of Raf-1/MEK/MAPK in both LX-2 cells and LI90 cells were markedly inhibited by silencing of ERO1α expression. Intriguingly, phosphorylation of Akt at Ser-473 and Thr-308 and phosphorylation of IκB at Ser-32/36 were clearly detected in two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA. Activation of the Akt/IκB signaling pathway in HSCs sustains their survival apart from the induction of cell proliferation (10, 23, 24), suggesting that activation of the Akt/IκB signaling pathway is responsible for the survival of HSCs stably expressing ERO1α shRNA regardless of impaired cell proliferation via inactivation of the FAK/Raf-1/MEK/MAPK signaling pathway and that ERO1α plays a critical role in the proliferation of HSCs.

ERO1α regulates the secretion of collagen type 1 from HSCs

We examined the secretion of collagen type 1 from HSCs stably expressing ERO1α shRNA. ELISA and immunoblotting of collagen type 1 showed that the amount of collagen type 1 secreted from the two hepatic stellate cell lines (LX-2 and LI90) stably expressing ERO1α shRNA was significantly smaller than that secreted from the two hepatic stellate cell lines (LX-2 and

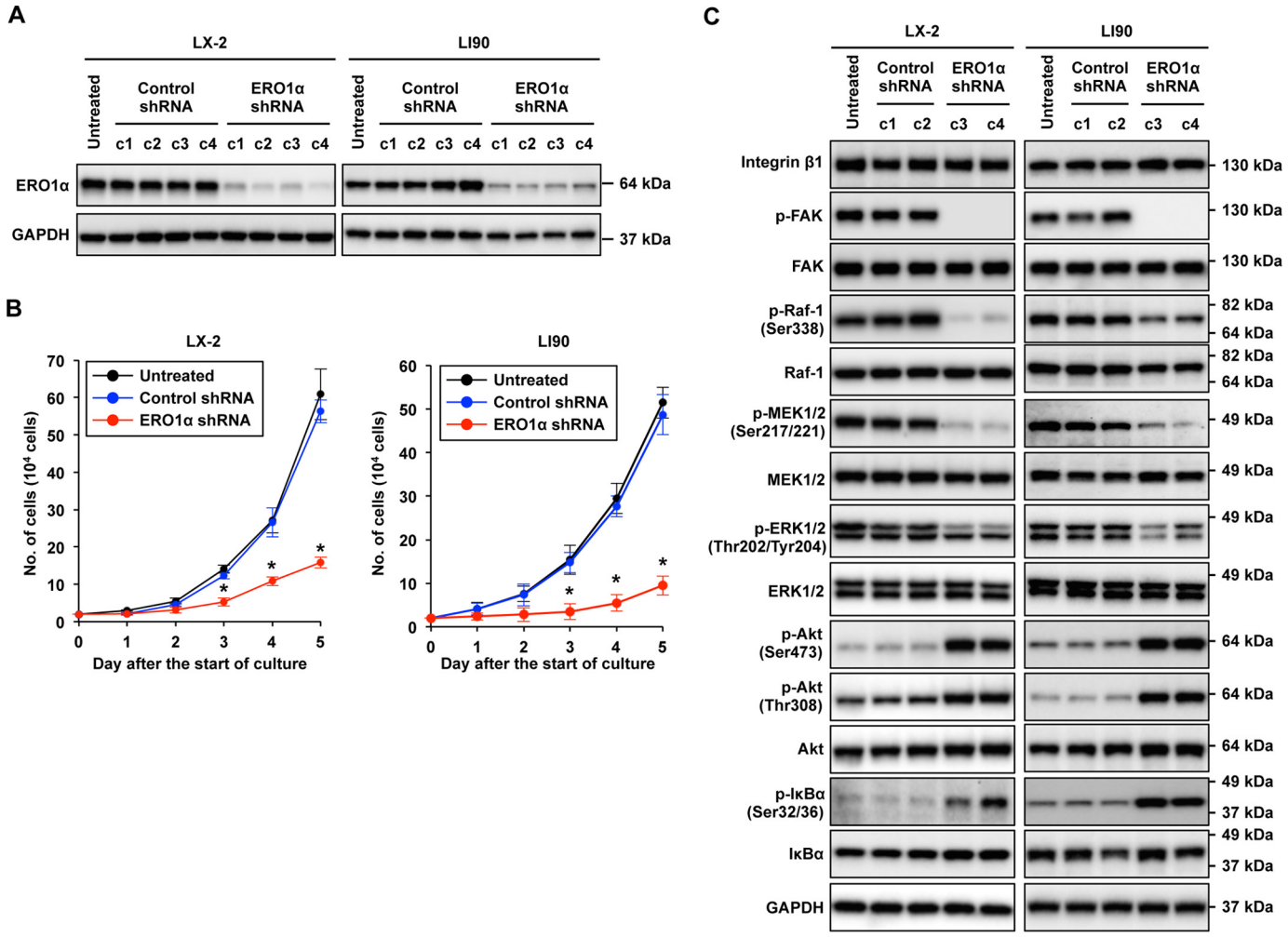

Figure 3. Silencing of ERO1α in human hepatic stellate cells suppresses growth signaling pathways. *A*, immunoblots of ERO1α protein in two human hepatic stellate cell lines (LX-2 and LI90) stably expressing control shRNA (clone 1 (*c1*), c2, c3, and c4) or ERO1α shRNA (c1, c2, c3, and c4). *B*, proliferation of two cell lines (LX-2 and LI90) stably expressing control shRNA or ERO1α shRNA. Cell proliferation for 5 days is shown. *, $p < 0.05$. *C*, immunoblots of the integrin β1/FAK/Raf/MEK/ERK and Akt/IκB signaling pathways in two cell lines (LX-2 and LI90) stably expressing control shRNA or ERO1α shRNA.

LI90) or from two hepatic stellate cell lines (LX-2 and LI90) stably expressing control shRNA (Fig. 4*A*). On the other hand, the expression levels of collagen 1α1 and 1α2 mRNAs in the two hepatic stellate cell lines (LX-2 and LI90) stably expressing ERO1α shRNA were slightly increased compared with those in the counterparts (Fig. 4*B*). Immunoblotting and immunofluorescence revealed that collagen type 1 had accumulated excessively in the two hepatic stellate cell lines (LX-2 and LI90) stably expressing ERO1α shRNA compared with the amount in two cell lines (LX-2 and LI90) stably expressing control shRNA (Fig. 4, *C* and *D*). Furthermore, immunoblotting under non-reducing conditions showed that collagen type 1 formed a triple helix in LX-2 cells and the LX-2 stably expressing control shRNA, but only a single chain of collagen type 1 was detected in LX-2 cells stably expressing ERO1α shRNA (Fig. 4*E*). These results indicated that silencing of ERO1α expression in HSCs causes impaired disulfide bond formation of collagen type 1, resulting in inhibition of collagen type 1 secretion.

We then investigated whether the impaired proliferation of HSCs by silencing of ERO1α expression is rescued by addition of collagen type 1. Surprisingly, proliferation of two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA progressed only modestly with addition of collagen type 1, and the rate of proliferation was significantly lower than the rates of proliferation of two cell lines (LX-2 and LI90) stably expressing control shRNA (Fig. 5, *A* and *B*). These results suggest that an additional obstacle other than impaired secretion of collagen type 1 is involved in the inhibited proliferation of HSCs by silencing of ERO1α expression.

Silencing of ERO1α inhibits autophagy in HSCs via activation of Akt

Some studies have demonstrated that unfolded or immature procollagens that have accumulated in the ER in fibroblasts and HSCs are mainly degraded by autophagy (26–28). Thus, we examined autophagy in two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA. Immunofluorescence and immunoblotting of LC3 showed that there was no aggregation of LC3 or an increased level of LC3II in the two cell lines with or without ERO1α expression (Fig. 6, *A* and *B*). Formation of autophagosomes was also not observed in LX-2 cells stably expressing control shRNA or ERO1α shRNA (Fig. 6*C*). Intriguingly, phosphorylation of adenosine monophosphate–activated protein kinase α1 (AMPKα1), an inducer of autophagy, was detected in

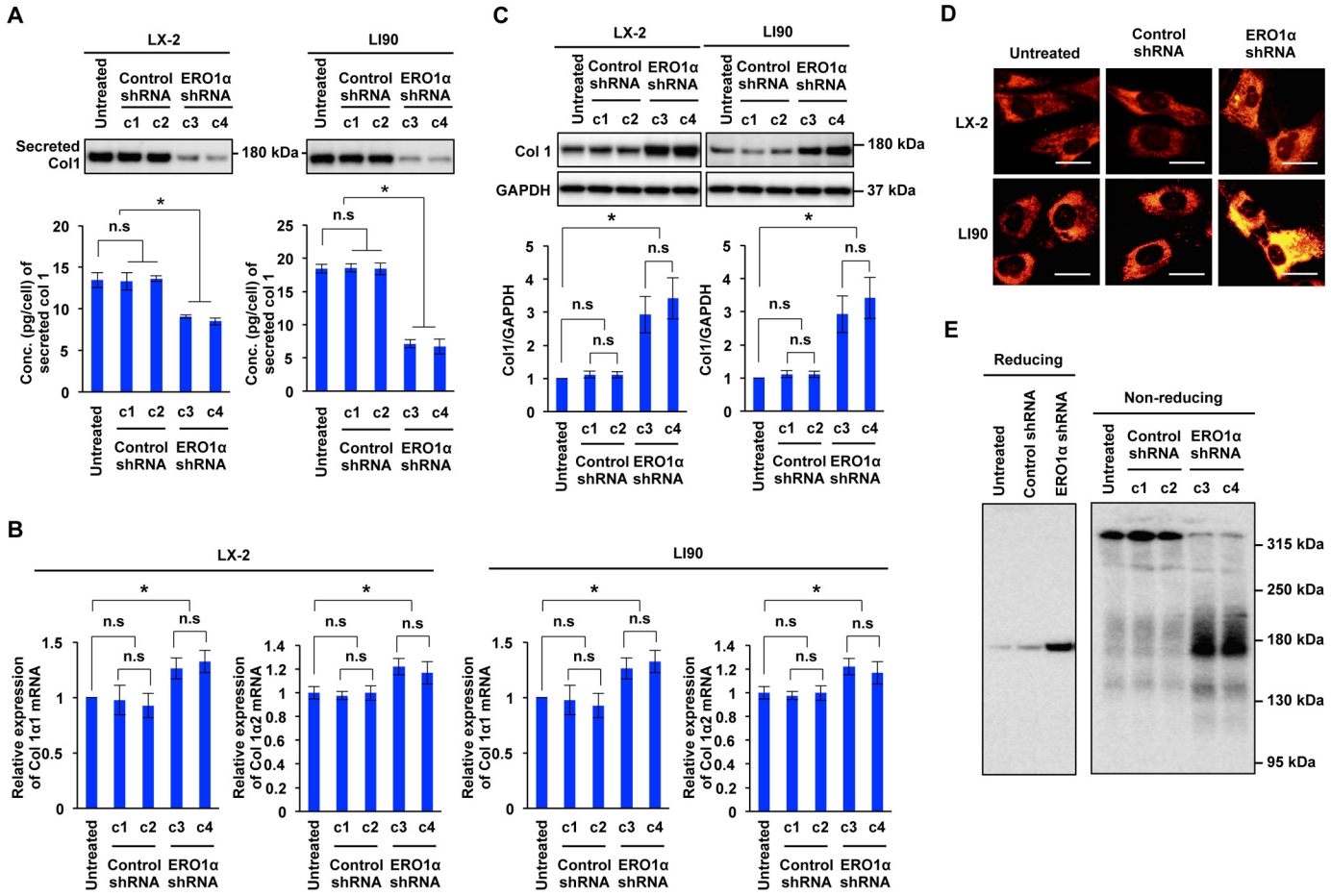

Figure 4. ERO1α regulates secretion of collagen type 1 from human hepatic stellate cells. *A*, secretion of collagen type 1 from two cell lines (LX-2 and LI90) stably expressing control shRNA (clone 1 (*c1*) and c2) or ERO1α shRNA (c3 and c4). *, $p < 0.05$; *n.s.*, not significant. *B*, expression of collagen 1α1 and 1α2 mRNAs in two cell lines (LX-2 and LI90) stably expressing control shRNA or ERO1α shRNA. *, $p < 0.05$. *C*, immunoblots (*top panel*) of intracellular collagen type 1 in two cell lines (LX-2 and LI90) stably expressing control shRNA or ERO1α shRNA. Quantification (*bottom panel*) of intracellular pro-collagen type 1 was determined by National Institutes of Health ImageJ. *D*, localization of intracellular collagen type 1 in two cell lines (LX-2 and LI90) stably expressing control shRNA or ERO1α shRNA. *Scale bars* = 20 μm. *E*, immunoblots of collagen type 1 LX2 cells stably expressing control shRNA or ERO1α shRNA under non-reducing and reducing conditions.

two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA but not in two cell lines (LX-2 and LI90) stably expressing control shRNA (Fig. 6*B*). On the other hand, phosphorylation of ULK1 at Thr-555, a target site of AMPKα1, was inhibited in two cell lines (LX-2 and LI90) by silencing ERO1α expression (Fig. 6*D*). Because silencing of ERO1α expression in HSCs stimulated phosphorylation of Akt (Figs. 3*C* and Fig. 6*B*), we further examined the phosphorylation status of mammalian target of rapamycin (mTOR), an inhibitor of ULK1. As expected, phosphorylation of mTOR at Ser-2448, a target site of Akt, was observed in two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA (Fig. 6*B*). Treatment with an Akt inhibitor at a concentration of 5 or 10 μM induced phosphorylation of ULK1 at Thr-555 and the formation of autophagosomes in LX-2 cells stably expressing ERO1α shRNA (Fig. 6, *D* and *E*). Treatment with the Akt inhibitor partially inhibited the expression of collagen 1α1 mRNA and collagen 1α2 mRNA in two cell lines (LX-2 and LI90) stably expressing control shRNA and ERO1α shRNA compared with that in the counterparts (Fig. 6*F*). However, although there was no significant difference in the expression levels of collagen 1α1 mRNA and collagen 1α2 mRNA between cells stably expressing control shRNA with the Akt inhibitor and cells stably expressing ERO1α shRNA with the Akt inhibitor, immunoblotting revealed that excessive accumulation of intracellular collagen type 1 in cells (LX-2 and LI90) stably expressing ERO1α shRNA was markedly ameliorated by treatment with the Akt inhibitor (Fig. 6*G*). These results indicate that silencing of ERO1α inhibits autophagy through activation of the Akt signaling pathway, causing excessive accumulation of collagens in HSCs.

MT1-MMP is regulated by ERO1α in HSCs

It has been shown that the proliferation of HSCs progresses through the modification of collagens with MMP2 and MT1-MMP, which are expressed by HSCs themselves (8, 10). We examined the expression of MMP2 and MT1-MMP in HSCs stably expressing control shRNA or ERO1α shRNA. The expression levels of MT1-MMP mRNA, but not those of MMP2 mRNA, in two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA were slightly higher than those in two cell lines (LX-2 and LI90) expressing control shRNA (Fig. 7*A*). On the other hand, the levels of MT1-MMP protein in two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA were significantly lower than those in the counterparts, reflecting the

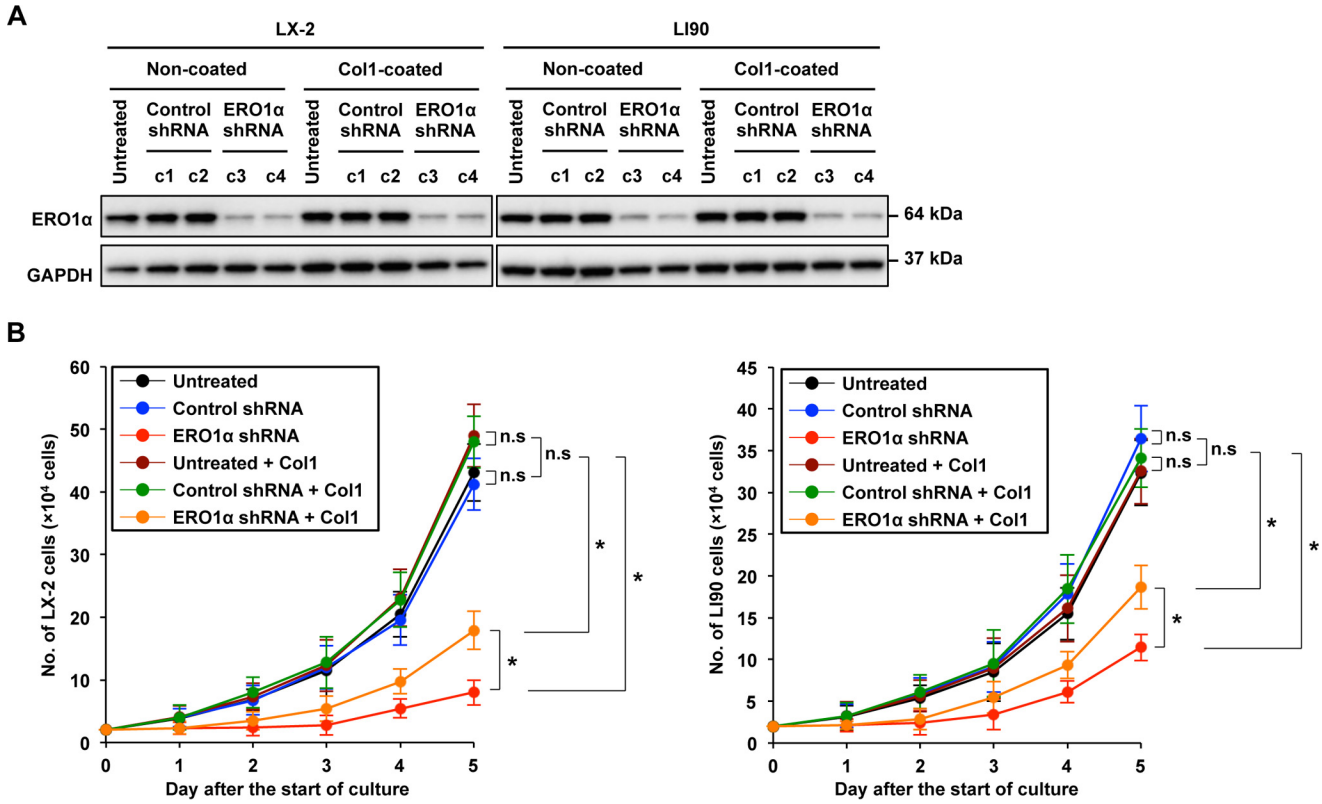

Figure 5. Treatment with collagens partially recovers the impaired proliferation of ERO1α-silenced hepatic stellate cells. *A*, immunoblots of ERO1α in two cell lines (LX-2 and LI90) stably expressing control shRNA or ERO1α shRNA. *c1*, clone 1. *B*, proliferation of two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA during *in vitro* culture on native collagen type 1. Cell proliferation for 5 days is shown. *, $p < 0.05$; *n.s.*, not significant.

reduced level of active MMP2 to which pro-MMP2 is transformed by the MT1-MMP–mediated activation process in two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA (Fig. 7*B*) (29, 30).

Intracellular proteins and misfolded proteins are degraded by the ubiquitin-proteasome pathway and by autophagy (31, 32). In this study, phosphorylation of Akt, but not of AMPKα1, was detected, and aggregation of LC3 was not observed in two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA (Figs. 3*C* and Fig. 6), suggesting that the ubiquitin–proteasome pathway is responsible for the decrease in the level of MT1-MMP protein induced by silencing of ERO1α expression. Thus, we examined whether recovery of MT1-MMP proteins in HSCs stably expressing ERO1α shRNA is induced by treatment with a proteasome inhibitor, MG132. As shown in Fig. 7*C*, the expression of MT1-MMP protein in two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA was completely rescued by treatment with 5 μM MG132. On the other hand, flow cytometric analysis showed that MT1-MMP was not localized in the cellular membrane but in the cytoplasm in LX-2 cells stably expressing ERO1α shRNA, regardless of recovery of MT1-MMP protein expression by treatment with MG132 (Fig. 7*D*). MT1-MMP protein has disulfide bond formation in the hemopexin domain (33), suggesting that unfolded MT1-MMP protein that accumulates in HSCs by silencing of ERO1α expression is degraded through the ubiquitin–proteasome pathway.

MT1-MMP–cleaved collagens promote proliferation of ERO1α-silenced HSCs

As described above, secretion of collagens was impaired and degradation of MT1-MMP was enhanced in HSCs by silencing of ERO1α expression. The proliferation of HSCs is enhanced through the interaction of integrin β1 with collagens (10). Because the expression of integrin β1 in HSCs was sustained despite the silencing of ERO1α expression (Fig. 3*A*), we examined whether addition of MT1-MMP–cleaved collagens promotes the proliferation of HSCs stably expressing ERO1α shRNA. A cell proliferation assay showed that the proliferation of two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA was significantly enhanced by addition of MT1-MMP–cleaved collagen type 1, as was also observed in two cell lines (LX-2 and LI90) stably expressing control shRNA (Fig. 8, *A* and *B*).

Discussion

Excessive deposition of extracellular matrix containing collagens in response to injury or inflammation is a leading cause of liver fibrosis, which mainly depends on activation of HSCs, which synthesize collagen and promote their own proliferation via interaction with collagen. Thus, an understanding of the mechanism by which HSCs regulate the biosynthesis of collagen will provide a meaningful clue for practical anti-fibrotic therapy of organs. In this study, we demonstrate for the first time that ERO1α, an oxidative enzyme of PDI that forms intramolecular disulfide bonds of membrane and secreted proteins

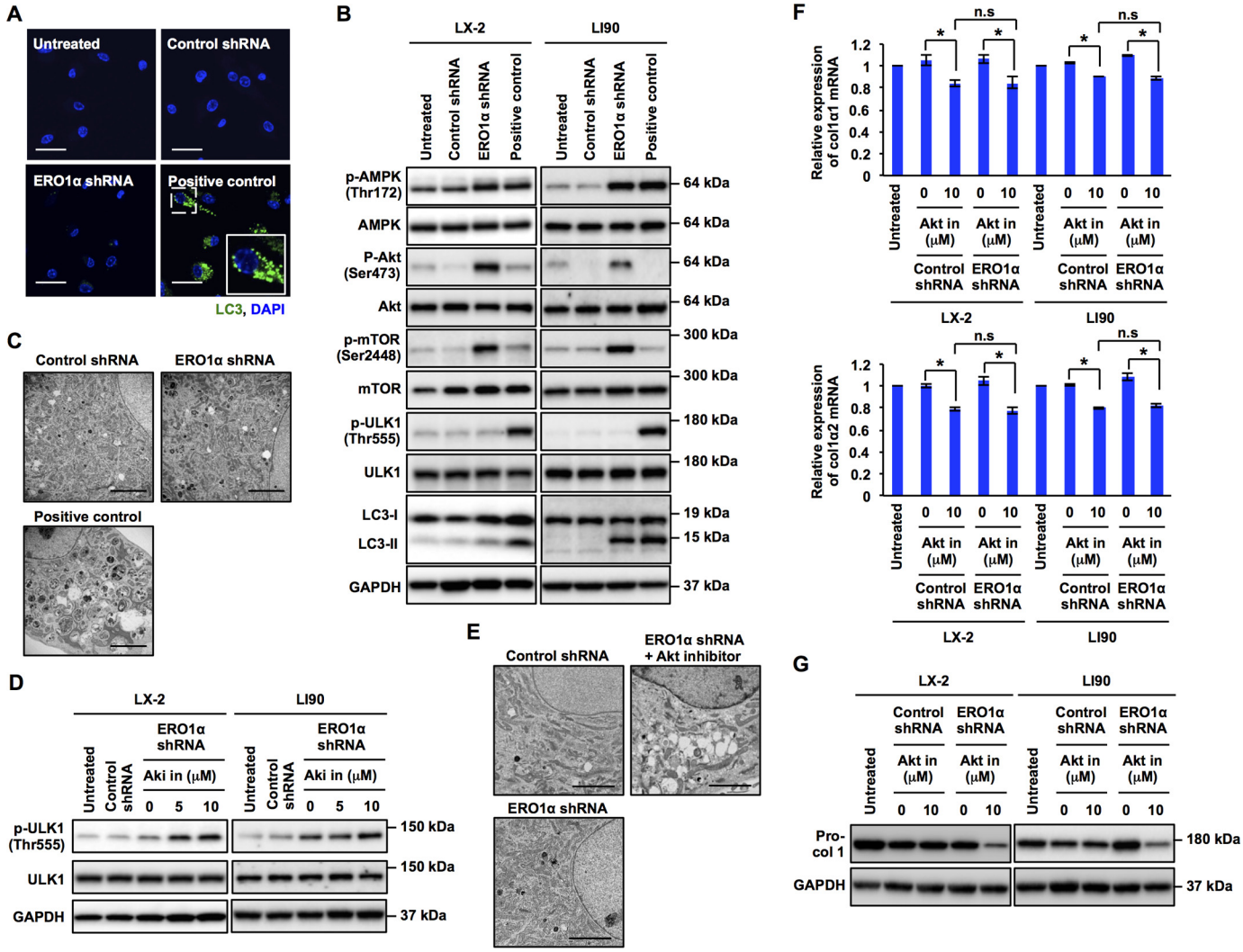

Figure 6. Silencing of ERO1α in human hepatic stellate cells inhibits autophagy through activation of Akt. *A*, immunofluorescence of LC3B in LX-2 cells stably expressing control shRNA or ERO1α shRNA. LX2 cells cultured in serum-starved medium were used as a positive control. *Scale bars* = 50 μm. *B*, immunoblots of phospho-AMPKα1, AMPKα1, phospho-Akt, Akt, phospho-mTOR, mTOR, phospho-ULK1, ULK1, and LC3B in two cell lines (LX-2 and LI90) stably expressing control shRNA or ERO1α shRNA. *C*, formation of autophagosomes in LX-2 cells stably expressing control shRNA or ERO1α shRNA. *Scale bars* = 2 μm. *D*, immunoblots of ULK1 in two cell lines (LX-2 and LI90) stably expressing control shRNA or ERO1α shRNA after treatment with an Akt inhibitor (5 and 10 μM). *E*, formation of autophagosomes in LX-2 cells stably expressing control shRNA or ERO1α shRNA after treatment with an Akt inhibitor (10 μM). *Scale bars* = 2 μm. *F*, expression of collagen type 1α1 and collagen type 1α2 mRNAs in two cell lines (LX-2 and LI90) stably expressing control shRNA or ERO1α shRNA after treatment with an Akt inhibitor (10 μM). *, $p < 0.05$; *n.s.*, not significant. *G*, immunoblots of collagen type 1 protein in two cell lines (LX-2 and LI90) stably expressing control shRNA or ERO1α shRNA after treatment with an Akt inhibitor (10 μM).

(14, 15), is expressed in human HSCs and that silencing of ERO1α expression markedly inhibits the proliferation of HSCs, accompanied by inactivation of the integrin/FAK/Raf/MEK/ERK signaling pathway. Silencing of ERO1α expression in HSCs resulted in excessive intracellular accumulation and impaired secretion of collagen type 1. Western blot analysis under non-reducing conditions showed that silencing of ERO1α expression causes accumulation of single chains of collagen type 1 in HSCs, indicating inhibition of disulfide bond formation between collagen molecules. The proliferation of HSCs is mainly dependent on the interaction of integrins with collagens secreted from HSCs (8, 10, 26, 34). Collagen type 1 consists of two molecules of collagen type 1α1 and one molecule of collagen type 1α2 and also has some disulfide bonds between these molecules, leading to the formation of a triple helix structure (20). It has been reported that the combined loss of function of ERO1α and ERO1β interferes with the intracellular maturation of pro-collagen (19). Therefore, ERO1α may play a critical role in the proliferation of HSCs through the proper structural formation and secretion of collagens.

It has been reported that unfolded or immature procollagens that have accumulated in fibroblasts and HSCs are degraded by an autophagy-dependent degradation system to prevent them from exposure to prolonged ER stress, which leads to apoptosis (26–28). Activation of autophagy, which consists of the formation of autophagosomes and activation of the AMPK/ULK1 signaling pathway, is indispensable for the maintenance of cell survival under serum starvation or low-glucose conditions (35–37). Moreover, inhibition of autophagy evokes an accumulation of misfolded or aggregated proteins in the ER (38–40). This study shows excessive accumulation of unfolded collagen type 1 in human HSCs by silencing ERO1α expression. Aggregation of

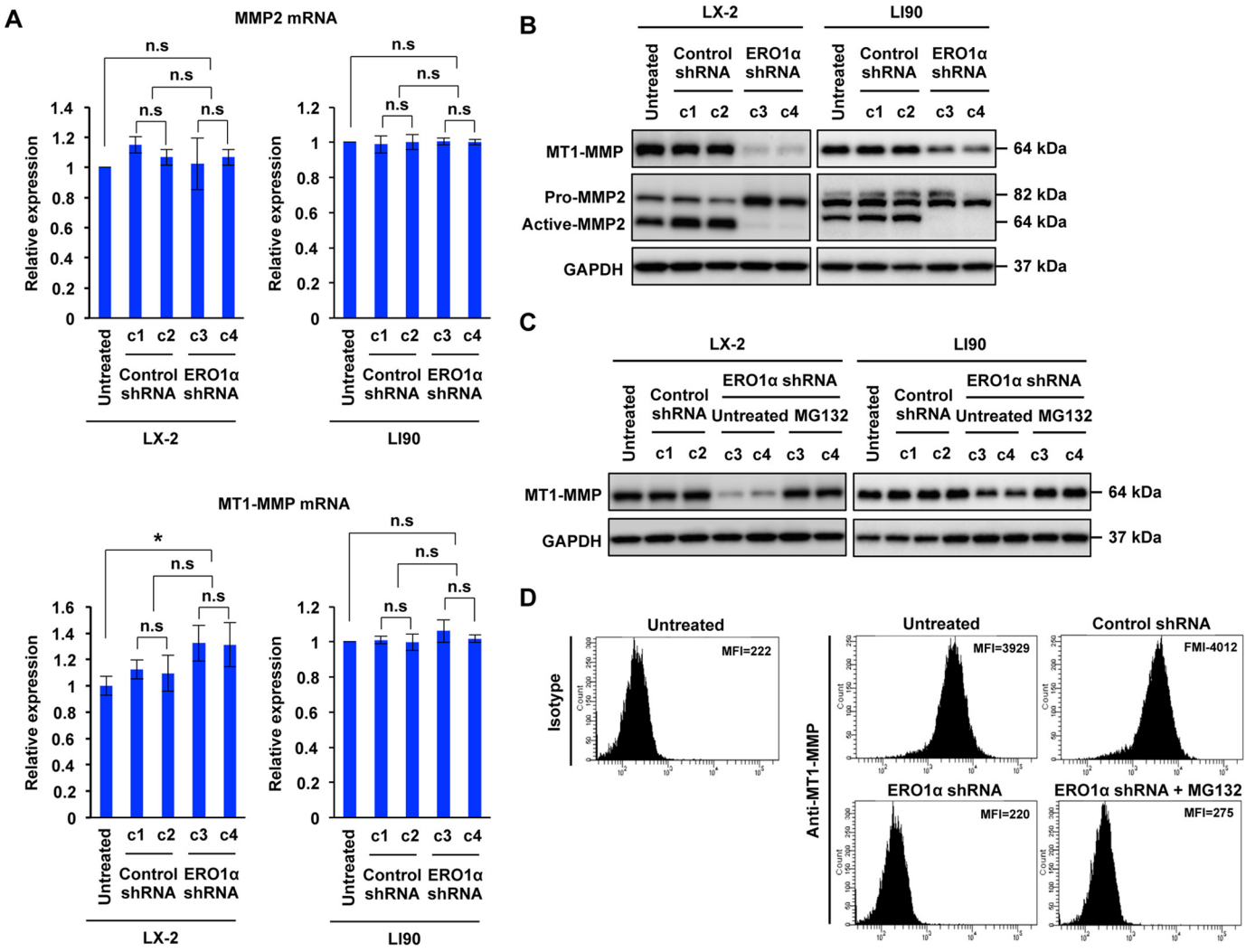

Figure 7. ERO1α regulates the transport of membrane type 1-matrix metalloproteinase to the cellular membrane. *A*, expression of MMP2 and MT1-MMP mRNA in two cell lines (LX-2 and LI90) stably expressing control shRNA (clone 1 (*c1*) and c2) or ERO1α shRNA (c3 and c4). *, $p < 0.05$; *n.s.*, not significant. *B*, immunoblots of MT1-MMP and MMP2 proteins in two cell lines (LX-2 and LI90) stably expressing control shRNA or ERO1α shRNA. *C*, immunoblots of MT1-MMP protein in two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA after treatment with the proteasome inhibitor MG132 (10 μM). *D*, flow cytometric analysis of MT1-MMP protein in LX-2 cells stably expressing ERO1α shRNA after treatment with or without MG132 (10 μM). *MFI*, mean fluorescent intensity.

LC3, formation of autophagosomes, and phosphorylation of ULK1 were not triggered, although phosphorylation of AMPK was induced, by silencing of ERO1α expression in human HSCs. Intriguingly, silencing of ERO1α expression in human HSCs induced activation of the Akt/mTOR signaling pathway (Fig. 3*B*), which suppresses autophagy through inhibition of phosphorylation of ULK1 (41, 42). We found that treatment of ERO1α-silenced human HSCs with an Akt inhibitor ameliorated excessive accumulation of collagen type 1 and induced activation of autophagy, accompanied by formation of autophagosomes and phosphorylation of ULK1. Although the molecular mechanism by which activation of Akt is stimulated by silencing of ERO1α expression remains unclear, the results imply that silencing of ERO1α expression triggers inhibition of autophagy via activation of Akt, which is responsible for excessive accumulation of collagens in HSCs.

It has been shown that proliferation of HSCs progresses through modification of collagens with MMP2 and MT1-MMP, which are expressed by HSCs themselves (8, 10). MT1-MMP has disulfide bond formation in the hemopexin domain in its molecule (33), suggesting that expression and synthesis of MT1-MMP are involved in the impaired proliferation of human HSCs by silencing ERO1α expression. In this study, silencing of ERO1α expression resulted in progressive degradation of MT1-MMP protein through the proteasome pathway, although no reduction of MT1-MMP mRNA expression was detected. Flow cytometric analysis also revealed that localization of MT1-MMP on the surface of human HSCs was inhibited by silencing ERO1α expression. These results suggest that ERO1α may regulate the trafficking of MT1-MMP to the cell surface through proper structural formation of MT1-MMP protein. Collagens secreted by activated HSCs are cleaved by MT1-MMP on the cell surface, leading to exposure of the RGD motif to the surfaces of collagen molecules (10). Integrin αvβ1 on activated HSCs binds to the RGD motif in collagen molecules, resulting in promotion of proliferation and survival of activated HSCs. This study shows that the proliferation of ERO1α-silenced human HSCs is completely recovered by

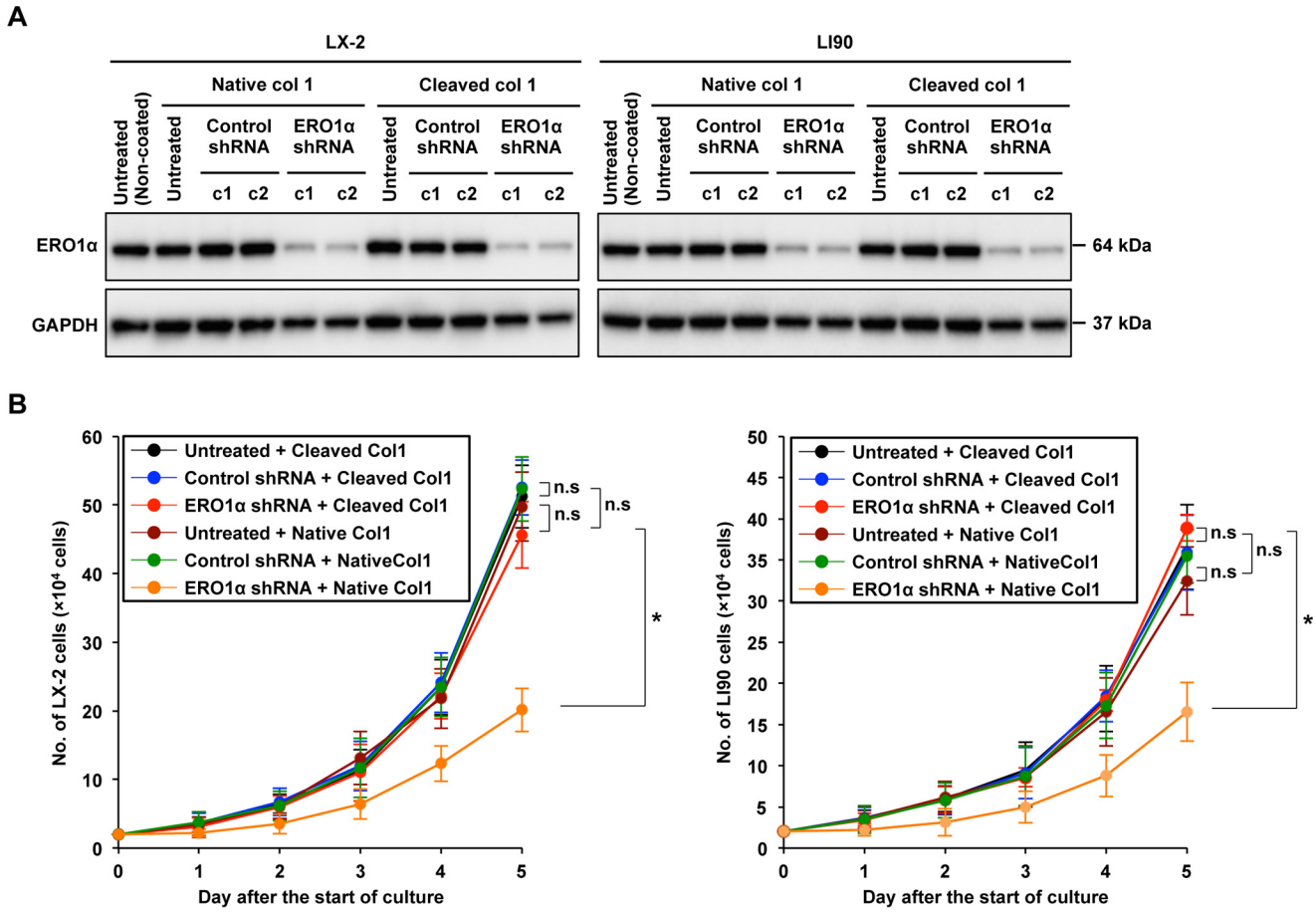

Figure 8. Treatment with MT1-MMP–cleaved collagen type 1 restores impaired proliferation of ERO1α-silenced human hepatic stellate cells. *A*, immunoblots of ERO1α protein in two cell lines (LX-2 and LI90) stably expressing control shRNA (clone 1 (*c1*) and c2) or ERO1α shRNA (c3 and c4) after treatment with native or MT1-MMP–cleaved collagen type 1. *B*, proliferation of two cell lines (LX-2 and LI90) stably expressing ERO1α shRNA after treatment with native or MT1-MMP–cleaved collagen type 1. Cell proliferation for 5 days is shown. *, $p < 0.05$; *n.s.*, not significant.

treatment with MT1-MMP–cleaved collagen type 1. Treatment with MT1-MMP–cleaved collagen type 1 also triggered activation of the FAK/Raf/MEK/ERK signaling pathway in ERO1α-silenced human HSCs. These results imply that ERO1α plays a critical role in collagen secretion and MT1-MMP biosynthesis in human HSCs.

Numerous studies on the resolution of organ fibrosis and cirrhosis have been carried out. Excessive secretion of collagens from activated stellate cells and myofibroblasts is a leading cause of organ fibrosis and cirrhosis, suggesting that elimination of these causative agents is necessary for anti-fibrotic therapy (43–45). In fact, inhibition of collagen secretion from stellate cells has been demonstrated to partially induce the resolution of organ fibrosis (6, 46–49). Suppression of proliferation and reduction of the number of activated stellate cells are also critical for fibrosis regression. For example, suppression of HSC proliferation through p53 activation has been reported to contribute to the reversion of fibrosis (50). It has also been shown that inhibition of expression of NFκB and decreased expression of the tissue inhibitor of metalloproteinase 1 reduce the number of activated HSCs, resulting in regression of liver fibrosis (51). However, targeting only one component in a complex signaling pathway may not be sufficiently potent, and targeting more than one molecule is considered to be a meaningful clue for anti-fibrotic therapy. In this context, this study showed that silencing of ERO1α expression results in impaired secretion of collagens, activation of AMPK, and progressive degradation of MT1-MMP protein, causing suppression of the proliferation of human HSCs. Therefore, targeted disruption of ERO1α in stellate cells may be one of the therapeutic modalities for tissue fibrosis.

In conclusion, this study demonstrates that ERO1α is expressed and localizes in the ER of human HSCs and that silencing of ERO1α expression inhibits the proliferation of HSCs through impaired secretion of collagen type 1, inhibition of autophagy, and progressive degradation of MT1-MMP protein. Treatment with MT1-MMP–cleaved collagen type 1 restored the inhibited proliferation of HSCs by silencing of ERO1α expression through activation of the collagen/MT1-MMP/integrin β1/focal adhesion kinase signaling axis. These results suggest that ERO1α plays a crucial role in the proliferation of HSCs through regulation of collagen secretion and biosynthesis of MT1-MMP protein.

Experimental procedures

Culture of hepatic stellate cells

The human hepatic stellate cell lines LX-2 and LI90 were purchased from Millipore (Billerica, MA) and from the JCRB

Cell Bank (National Institutes of Biomedical Innovation, Health, and Nutrition, Osaka, Japan), respectively. Primary human HSCs were obtained from ScienCell Research Laboratories (Carlsbad, CA). LX-2 cells, LI90 cells, and primary human HSCs were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Gibco) and 100 μM ascorbic acid (Sigma).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde (Wako Pure Chemicals, Tokyo, Japan). After washing with PBS (Life Technologies), the cells were permeabilized with PBS supplemented with or without 0.5% Triton X-100 for 3 min at room temperature and treated with 5% goat serum in PBS for 60 min at room temperature. The cells were then incubated with primary antibodies against ERO1α (Millipore), HSP47 (Abcam, Cambridge, UK), collagen type 1 (Abcam), and MT1-MMP (Abcam). After washing with PBS, the cells were incubated with the following secondary antibodies: Alexa 488–conjugated goat anti-rabbit IgG (Invitrogen) and Alexa 555–conjugated goat anti-mouse IgG (Invitrogen). Finally, the cells were counterstained with DAPI and analyzed with an immunofluorescence microscope. The degree of co-localization of ERO1α with HSP47 in the cells was determined by National Institutes of Health ImageJ.

Transfection of small interfering RNA

Three batches of siRNAs against human ERO1α (ERO1α-A siRNA, 5′-ACCAGACAAGAAAUAGUAUCAUUat-3′ (sense) and 5′-AAUGAUACUAUUUCUUGUCUGGUat-3′ (antisense); ERO1α-B siRNA, 5′-GGUAUAACAUGUUGAAAUGUCACat-3′ (sense) and 5′-GUGACAUUUCAACAUGUUAUACCat-3′ (antisense); ERO1α-C siRNA, 5′-AGCUGAAUAUGUAGAUUUGCUUCtt-3′ (sense) and 5′-GAAGCAAAUCUACAUAUUCAGCUtt-3′ (antisense)) and control siRNA were purchased from OriGene (Rockville, MD). LX-2 cells were transfected with ERO1α siRNA or control siRNA at concentrations of 10 nM and 20 nM using Lipofectamine RNAiMAX (Life Technologies). 5 h after transfection with siRNA, the treated cells were further cultured in DMEM supplemented with 10% FBS and 100 μM ascorbic acid.

Cell proliferation assay

LX-2 cells, LI90 cells, and primary HSCs were plated at 2×10^4 cells/well in a 6-well plate and cultured for 24 h. After transfection with control siRNA or ERO1α-A siRNA at a concentration of 10 nM, the treated cells were cultured for 120 h. Proliferation of LX-2 cells treated with control siRNA or ERO1α-A siRNA was determined by trypan blue staining. Cell proliferation assays were carried out 24, 48, 72, 96, and 120 h after transfection with siRNA.

Western blot analysis

Cultured cells were washed with ice-cold PBS and lysed with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, and 1% Nonidet P-40) supplemented with a protease inhibitor (Roche Diagnostics). For immunoblotting of secreted collagen, the culture medium at 96 h after *in vitro* culture of the cells was collected and concentrated by a Vivaspin column (Vivaspin 500-3K, GE Healthcare). After heating cell lysates for 5 min at 100 °C in SDS sample buffer supplemented with 5% 2-mercaptoethanol (Sigma-Aldrich), proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). The membranes were incubated with 5% skim milk in PBS containing 0.05% Tween 20 (PBS-T) for 60 min at room temperature and then incubated with primary antibodies against ERO1α (Millipore), integrin β1 (Abcam), phospho-FAK (Cell Signaling Technology), total FAK (Cell Signaling Technology), phospho-Raf-1 (Cell Signaling Technology), total Raf-1 (Cell Signaling Technology), phospho-ERK (Cell Signaling Technology), total ERK (Cell Signaling Technology), phospho-MAPK (Cell Signaling Technology), total MAPK (Cell Signaling Technology), phospho-PI3K (Cell Signaling Technology), total PI3K (Cell Signaling Technology), phospho-Akt (Ser-473) (Cell Signaling Technology), phospho-Akt (Thr-308) (Cell Signaling Technology), total Akt (Cell Signaling Technology), phospho-AMPKα1 (Cell Signaling Technology), total AMPKα (Cell Signaling Technology), LC3B (Cell Signaling Technology), collagen type 1 (Abcam), MT1-MMP (Abcam), MMP2 (Cell Signaling Technology), and GAPDH (Abcam). After washing with PBS-T, the membranes were reacted for 60 min with the following secondary antibodies: HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology) and HRP-conjugated goat anti-mouse IgG (Cell Signaling Technology). Proteins were detected by a chemiluminescent method using ECL (GE Healthcare).

Quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from cultured cells using RNeasy Mini kits (Qiagen, Valencia, CA) according to the instructions of the manufacturer. cDNAs were synthesized from total RNA (1 μg) using a high-capacity RNA-to-cDNA kit (Applied Biosystems) according to the protocol of the manufacturer. Quantitative PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) and the following primers: collagen type1α1 (forward, 5′-GTGCTAAAGGTGCCAATGGT-3′; reverse, 5′-ACCAGGTTCACCGCTGTTAC-3′), collagen type 1α2 (forward, 5′-CCTGGTAATCCTGGAGCAAA-3′; reverse, 5′-CAGATCCAGCTTCCCCATTA-3′), human ERO1α (forward, 5′-TGCTTCTGCCAGGTTAGTGG-3′; reverse, 5′-TCCACTGCTCCAAGTCGTTC-3′), human MMP2 (forward, 5′-ATGACAGCTGCACCACTGAG-3′; reverse, 5′-ATTTGTTGCCCAGGAAAGTG-3′), MT1-MMP (forward, 5′-GCAGAAGTTTTACGGCTTGC-3′; reverse, 5′-TAGCGCTTCCTTCGAACATT-3′), and GAPDH (forward, 5′-GAGTCAACGGATTTGGTCGT-3′; reverse, 5′-TTGATTTTGGAGGGATCTCG-3′).

Establishment of HSCs stably expressing ERO1α short hairpin RNA

LX-2 cells and LI90 cells were transfected with plasmids containing a control shRNA sequence (OriGene) or ERO1α shRNA sequence (5′-AGAGCATTCTACAGACTTATATCTGGCCT-3′). 48 h after transfection with the plasmid, the treated cells were cultured in DMEM supplemented with 10% FBS and 2 μg/ml puromycin (Invitrogen) for 2 weeks to select cell clones stably expressing control shRNA or ERO1α shRNA.

To confirm silencing of ERO1α expression in the selected cell clones, the expression of ERO1α mRNA and protein was determined by quantitative RT-PCR and Western blotting.

Enzyme-linked immunosorbent assay

Cells plated at 2×10^4 cells/well in 6-well plates were cultured for 96 h. All samples were stored at −80 °C until use. The amount of mature collagen type I in supernatants was determined using a human collagen type 1 ELISA kit (ACEL). Absorbance was determined at 450 nm. The amount of secreted collagen type I was determined by normalizing the amount of collagen type 1 in supernatants with cell number.

Transmission electron microscopy

LX-2 cells and LX-2 cells stably expressing control shRNA or ERO1α shRNA cultured with or without Akt inhibitor (Akt inhibitor IV, Millipore) were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). After washing with 0.1 M cacodylate buffer (pH 7.3), the cells were post-fixed in 1% OsO_4 and 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer. Samples were stained with uranyl acetate for 2 h at room temperature and then washed, dehydrated, and embedded in Epon 812. Ultrathin sections were cut with a diamond knife and stained with lead citrate. Samples were examined with an electron microscope (JEM-3200FS, JEOL) at an acceleration voltage of 300 kV.

Flow cytometric analysis

LX-2 cells stably expressing control shRNA or ERO1α shRNA were cultured in DMEM supplemented with 10% FBS, and 100 μM ascorbic acid, and with or without 5 μM proteasome inhibitor MG132 for 12 h and further cultured in DMEM supplemented with 10% FBS for 24 h. The treated cells were incubated with isotype control rabbit IgG (Abcam) and a primary antibody against MT1-MMP at 4 °C for 30 min. After washing with PBS, the cells were incubated with Alexa 488 – conjugated goat anti-rabbit IgG as a secondary antibody. Fluorescence of the treated cells was determined by flow cytometry (FACS Canto, BD Biosciences).

Preparation of MT1-MMP– cleaved collagen type 1

MT1-MMP– cleaved collagen type 1 was prepared by a method reported previously (10, 52). In brief, recombinant human MT1-MMP (100 μg/ml, R&D Systems) was activated by recombinant human active trypsin3/PRSS3 (100 ng/ml, R&D Systems) at 37 °C for 60 min in activation buffer (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 5 mM $ZnCl_2$, and 0.05% Brij-35 (pH 7.5)), and the activation was terminated with 4(2-aminoethyl-benzensulfonyl fluoride hydrochloride) (1 mM, R&D Systems) at 25 °C for 15 min. Rat tail collagen type 1 (6 μg, Sigma-Aldrich) was cleaved by active MMP14 for 20 h in assay buffer (50 mM Tris, 3 mM $CaCl_2$, and 1 mM $ZnCl_2$). Digestion was terminated by addition of 20 mM EDTA. The fragments were dialyzed against PBS using a dialysis kit (GE Healthcare).

Statistical analysis

All experiments were carried out independently three times. Results are shown as means ± S.D. Comparisons between two groups were performed using Student's t test, with $p < 0.05$ considered to be statistically significant.

Author contributions—M. F. and A. Yoneda designed all experiments and performed most of them. A. Yoneda wrote the manuscript. K. S. S. and M. K. performed Western blotting and the cell growth assay. A. Yoneda, N. T., K. M., A. Yokoyama, and Y. T. prepared the manuscript.

References

1. Wake, K. (1980) Perisinusoidal stellate cells (fat-storing cells, interstitial cells, lipocytes), their related structure in and around the liver sinusoids, and vitamin A-storing cells in extrahepatic organs. *Int. Rev. Cytol.* **66,** 303–353
2. Friedman, S. L. (2008) Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol. Rev.* **88,** 125–172
3. Friedman, S. L. (2008) Mechanisms of hepatic fibrogenesis. *Gastroenterology* **134,** 1655–1669
4. Karsdal, M. A., Manon-Jensen, T., Genovese, F., Kristensen, J. H., Nielsen, M. J., Sand, J. M., Hansen, N. U., Bay-Jensen, A. C., Bager, C. L., Krag, A., Blanchard, A., Krarup, H., Leeming, D. J., and Schuppan, D. (2015) Novel insights into the function and dynamics of extracellular matrix in liver fibrosis. *Am. J. Physiol. Gastrointest. Liver. Physiol.* **308,** G807-G830
5. Mehal, W. Z., and Schuppan, D. (2015) Antifibrotic therapies in the liver. *Semin. Liver. Dis.* **35,** 184–198
6. Sato, Y., Murase, K., Kato, J., Kobune, M., Sato, T., Kawano, Y., Takimoto, R., Takada, K., Miyanishi, K., Matsunaga, T., Takayama, T., and Niitsu, Y. (2008) Resolution of liver cirrhosis using vitamin A-coupled liposomes to deliver siRNA against a collagen-specific chaperone. *Nat. Biotechnol.* **26,** 431–442
7. Jiménez Calvente, C., Sehgal, A., Popov, Y., Kim, Y. O., Zevallos, V., Sahin, U., Diken, M., and Schuppan, D. (2015) Specific hepatic delivery of procollagen α1(I) small interfering RNA in lipid-like nanoparticles resolves liver fibrosis. *Hepatology* **62,** 1285–1297
8. Zhou, X., Jamil, A., Nash, A., Chan, J., Trim, N., Iredale, J. P., and Benyon, R. C. (2006) Impaired proteolysis of collagen I inhibits proliferation of hepatic stellate cells: implications for regulation of liver fibrosis. *J. Biol. Chem.* **281,** 39757–39765
9. Henderson, N. C., Arnold, T. D., Katamura, Y., Giacomini, M. M., Rodriguez, J. D., McCarty, J. H., Pellicoro, A., Raschperger, E., Betsholtz, C., Ruminski, P. G., Griggs, D. W., Prinsen, M. J., Maher, J. J., Iredale, J. P., Lacy-Hulbert, A., *et al.* (2013) Targeting of αv integrin identifies a core molecular pathway that regulates fibrosis in several organs. *Nat. Med.* **19,** 1617–1624
10. Birukawa, N. K., Murase, K., Sato, Y., Kosaka, A., Yoneda, A., Nishita, H., Fujita, R., Nishimura, M., Ninomiya, T., Kajiwara, K., Miyazaki, M., Nakashima, Y., Ota, S., Murakami, Y., Tanaka, Y., *et al.* (2014) Activated hepatic stellate cells are dependent on self-collagen, cleaved by membrane type 1 matrix metalloproteinase for their growth. *J. Biol. Chem.* **289,** 20209–20221
11. Hudson, D. A., Gannon, S. A., and Thorpe, C. (2015) Oxidative protein folding: from thiol-disulfide exchange reactions to the redox poise of the endoplasmic reticulum. *Free. Radic. Biol. Med.* **80,** 171–182
12. Frand, A. R., and Kaiser, C. A. (1998) The ERO1 gene of yeast is required for oxidation of protein dithiols in the endoplasmic reticulum. *Mol. Cell* **1,** 161–170
13. Pollard, M. G., Travers, K. J., and Weissman, J. S. (1998) Ero1p: a novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum. *Mol. Cell* **1,** 171–182
14. Tsai, B., and Rapoport, T. A. (2002) Unfolded cholera toxin is transferred to the ER membrane and released from protein disulfide isomerase upon oxidation by Ero1. *J. Cell Biol.* **159,** 207–216
15. Araki, K., Iemura, S., Kamiya, Y., Ron, D., Kato, K., Natsume, T., and Nagata, K. (2013) Ero1-α and PDIs constitute a hierarchical electron transfer network of endoplasmic reticulum oxidoreductases. *J. Cell Biol.* **202,** 861–874

16. Cabibbo, A., Pagani, M., Fabbri, M., Rocchi, M., Farmery, M. R., Bulleid, N. J., and Sitia, R. (2000) ERO1-L, a human protein that favors disulfide bond formation in the endoplasmic reticulum. *J. Biol. Chem.* **275,** 4827–4833
17. Pagani, M., Fabbri, M., Benedetti, C., Fassio, A., Pilati, S., Bulleid, N. J., Cabibbo, A., and Sitia, R. (2000) Endoplasmic reticulum oxidoreductin 1-lβ (ERO1-Lβ), a human gene induced in the course of the unfolded protein response. *J. Biol. Chem.* **275,** 23685–23692
18. Zito, E., Chin, K. T., Blais, J., Harding, H. P., and Ron, D. (2010) ERO1b, a pancreas-specific disulfide oxidase promotes insulin biogenesis and glucose homeostasis. *J. Cell Biol.* **188,** 821–832
19. Zito, E., Hansen, H. G., Yeo, G. S., Fujii, J., and Ron, D. (2012) Endoplasmic reticulum thiol oxidase deficiency leads to ascorbic acid depletion and noncanonical scurvy in mice. *Mol. Cell* **48,** 39–51
20. Hulmes, D. J. (2002) Building collagen molecules, fibrils, and suprafibrillar structures. *J. Struct. Biol.* **137,** 2–10
21. Wilson, R., Lees, J. F., and Bulleid, N. J. (1998) Protein disulfide isomerase acts as a molecular chaperone during the assembly of procollagen. *J. Biol. Chem.* **273,** 9637–9643
22. Bottomley, M. J., Batten, M. R., Lumb, R. A., and Bulleid, N. J. (2001) Quality control in the endoplasmic reticulum: PDI mediates the ER retention of unassembled procollagen C-propeptides. *Curr. Biol.* **11,** 1114–1118
23. Carloni, V., Defranco, R. M., Caligiuri, A., Gentilini, A., Sciammetta, S. C., Baldi, E., Lottini, B., Gentilini, P., and Pinzani, M. (2002) Cell adhesion regulates platelet-derived growth factor-induced MAP kinase and PI-3 kinase activation in stellate cells. *Hepatology* **36,** 582–591
24. Reif, S., Lang, A., Lindquist, J. N., Yata, Y., Gabele, E., Scanga, A., Brenner, D. A., and Rippe, R. A. (2003) The role of focal adhesion kinase-phosphatidylinositol 3-kinase-akt signaling in hepatic stellate cell proliferation and type I collagen expression. *J. Biol. Chem.* **278,** 8083–8090
25. Gao, R., Ball, D. K., Perbal, B., and Brigstock, D. R. (2004) Connective tissue growth factor induces c-fos gene activation and cell proliferation through p44/42 MAP kinase in primary rat hepatic stellate cells. *J. Hepatol.* **40,** 431–438
26. Kawasaki, K., Ushioda, R., Ito, S., Ikeda, K., Masago, Y., and Nagata, K. (2015) Deletion of the collagen-specific molecular chaperone Hsp47 causes endoplasmic reticulum stress-mediated apoptosis of hepatic stellate cells. *J. Biol. Chem.* **290,** 3639–3646
27. Ishida, Y., Yamamoto, A., Kitamura, A., Lamandé, S. R., Yoshimori, T., Bateman, J. F., Kubota, H., and Nagata, K. (2009) Autophagic elimination of misfolded procollagen aggregates in the endoplasmic reticulum as a means of cell protection. *Mol. Biol. Cell* **20,** 2744–2754
28. Ishida, Y., and Nagata, K. (2009) Autophagy eliminates a specific species of misfolded procollagen and plays a protective role in cell survival against ER stress. *Autophagy* **5,** 1217–1219
29. Kinoshita, T., Sato, H., Okada, A., Ohuchi, E., Imai, K., Okada, Y., and Seiki, M. (1998) TIMP-2 promotes activation of progelatinase A by membrane-type 1 matrix metalloproteinase immobilized on agarose beads. *J. Biol. Chem.* **273,** 16098–16103
30. Zucker, S., Drews, M., Conner, C., Foda, H. D., DeClerck, Y. A., Langley, K. E., Bahou, W. F., Docherty, A. J., and Cao, J. (1998) Tissue inhibitor of metalloproteinase-2 (TIMP-2) binds to the catalytic domain of the cell surface receptor, membrane type 1-matrix metalloproteinase 1 (MT1-MMP). *J. Biol. Chem.* **273,** 1216–1222
31. Nedelsky, N. B., Todd, P. K., and Taylor, J. P. (2008) Autophagy and the ubiquitin-proteasome system: collaborators in neuroprotection. *Biochim. Biophys. Acta* **1782,** 691–699
32. Lilienbaum, A. (2013) Relationship between the proteasomal system and autophagy. *Int. J. Biochem. Mol. Biol.* **4,** 1–26
33. Nagase, H., Visse, R., and Murphy, G. (2006) Structure and function of matrix metalloproteinase and TIMPs. *Cardiovasc. Res.* **69,** 562–573
34. Zhou, X., Murphy, F. R., Gehdu, N., Zhang, J., Iredale, J. P., and Benyon, R. C. (2004) Engagement of αvβ3 integrin regulates proliferation and apoptosis of hepatic stellate cells. *J. Biol. Chem.* **279,** 23996–24006
35. Mizushima, N., and Komatsu, M. (2011) Autophagy: renovation of cells and tissues. *Cell* **147,** 728–741
36. Shen, H. M., and Mizushima, N. (2014) At the end of the autophagic road: an emerging understanding of lysosomal functions in autophagy. *Trends Biochem. Sci.* **39,** 61–71
37. Egan, D. F., Shackelford, D. B., Mihaylova, M. M., Gelino, S., Kohnz, R. A., Mair, W., Vasquez, D. S., Joshi, A., Gwinn, D. M., Taylor, R., Asara, J. M., Fitzpatrick, J., Dillin, A., Viollet, B., Kundu, M., *et al.* (2011) Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science* **331,** 456–461
38. Schuck, S., Gallagher, C. M., and Walter, P. (2014) ER-phagy mediates selective degradation of endoplasmic reticulum independently of the core autophagy machinery. *J. Cell Sci.* **127,** 4078–4088
39. Khaminets, A., Heinrich, T., Mari, M., Grumati, P., Huebner, A. K., Akutsu, M., Liebmann, L., Stolz, A., Nietzsche, S., Koch, N., Mauthe, M., Katona, I., Qualmann, B., Weis, J., Reggiori, F., *et al.* (2015) Regulation of endoplasmic reticulum turnover by selective autophagy. *Nature* **522,** 354–358
40. Mochida, K., Oikawa, Y., Kimura, Y., Kirisako, H., Hirano, H., Ohsumi, Y., and Nakatogawa, H. (2015) Receptor-mediated selective autophagy degrades the endoplasmic reticulum and the nucleus. *Nature* **522,** 359–362
41. Kim, J., Kundu, M., Viollet, B., and Guan, K. L. (2011) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat. Cell Biol.* **13,** 132–141
42. Alers, S., Löffler, A. S., Wesselborg, S., and Stork, B. (2012) Role of AMPK-mTOR-Ulk1/2 in the regulation of autophagy: cross talk, shortcuts, and feedbacks. *Mol. Cell Biol.* **32,** 2–11
43. Hardie, D. G. (2003) Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status. *Endocrinology* **144,** 5179–5183
44. Long, Y. C., and Zierath, J. R. (2006) AMP-activated protein kinase signaling in metabolic regulation. *J. Clin. Invest.* **116,** 1776–1783
45. Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M. F., Goodyear, L. J., and Moller, D. E. (2001) Role of AMP-activated protein kinase in mechanism of metformin action. *J. Clin. Invest.* **108,** 1167–1174
46. Hardie, D. G. (2007) AMP-activated protein kinase as a drug target. *Annu. Rev. Pharmacol. Toxicol.* **47,** 185–210
47. Viollet, B., Foretz, M., Guigas, B., Horman, S., Dentin, R., Bertrand, L., Hue, L., and Andreelli, F. (2006) Activation of AMP-activated protein kinase in the liver: a new strategy for the management of metabolic hepatic disorders. *J. Physiol.* **574,** 41–53
48. Caligiuri, A., Bertolani, C., Guerra, C. T., Aleffi, S., Galastri, S., Trappoliere, M., Vizzutti, F., Gelmini, S., Laffi, G., Pinzani, M., and Marra, F. (2008) Adenosine monophosphate-activated protein kinase modulates the activated phenotype of hepatic stellate cells. *Hepatology* **47,** 668–676
49. Adachi, M., and Brenner, D. A. (2008) High molecular weight adiponectin inhibits proliferation of hepatic stellate cells via activation of adenosine monophosphate-activated protein kinase. *Hepatology* **47,** 677–685
50. Handy, J. A., Fu, P. P., Kumar, P., Mells, J. E., Sharma, S., Saxena, N. K., and Anania, F. A. (2011) Adiponectin inhibits leptin signalling via multiple mechanisms to exert protective effects against hepatic fibrosis. *Biochem. J.* **440,** 385–395
51. Kumar, P., Smith, T., Rahman, K., Thorn, N. E., and Anania, F. A. (2014) Adiponectin agonist ADP355 attenuates CCl4-induced liver fibrosis in mice. *PLoS ONE* **9,** e110405
52. Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M., and Okada, Y. (1997) Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. *J. Biol. Chem.* **272,** 2446–2451

Endoplasmic reticulum oxidase 1α is critical for collagen secretion from and membrane type 1-matrix metalloproteinase levels in hepatic stellate cells

Mizuki Fujii, Akihiro Yoneda, Norio Takei, Kaori Sakai-Sawada, Marina Kosaka, Kenjiro Minomi, Atsuro Yokoyama and Yasuaki Tamura

J. Biol. Chem. 2017, 292:15649-15660.
doi: 10.1074/jbc.M117.783126 originally published online August 3, 2017