Endoplasmic reticulum stress enhances fibrosis through IRE1α-mediated degradation of miR-150 and XBP-1 splicing

Femke Heindryckx, François Binet, Markella Ponticos, Krista Rombouts, Joey Lau, Johan Kreuger & Pär Gerwins

Abstract

ER stress results in activation of the unfolded protein response and has been implicated in the development of fibrotic diseases. In this study, we show that inhibition of the ER stress-induced IRE1α signaling pathway, using the inhibitor 4p8C, blocks TGFβ-induced activation of myofibroblasts in vitro, reduces liver and skin fibrosis in vivo, and reverts the fibrotic phenotype of activated myofibroblasts isolated from patients with systemic sclerosis. By using IRE1α−/− fibroblasts and expression of IRE1α-mutant proteins lacking endoribonuclease activity, we confirmed that IRE1α plays an important role during myofibroblast activation. IRE1α was shown to cleave miR-150 and thereby to release the suppressive effect that miR-150 exerted on αSMA expression through c-Myb. Inhibition of IRE1α was also demonstrated to block ER expansion through an XBP-1-dependent pathway. Taken together, our results suggest that ER stress could be an important and conserved mechanism in the pathogenesis of fibrosis and that components of the ER stress pathway may be therapeutically relevant for treating patients with fibrotic diseases.

Keywords: endoplasmic reticulum stress; fibrosis; liver cirrhosis; myofibroblast; scleroderma

Subject Categories: Digestive System; Pharmacology & Drug Discovery; Skin

Introduction

Fibrosis is characterized by an excessive accumulation of fibrous connective tissue that typically occurs in response to chronic inflammation caused by toxic substances, infections, mechanical injury, or as a result of autoimmune reactions (Darby & Hewitson, 2007; Kramann et al., 2013; Kendall & Feghali-Bostwick, 2014). Progressive accumulation of connective tissue ultimately leads to disruption of tissue architecture and organ dysfunction that can be fatal as seen in end-stage liver disease, kidney disease, pulmonary fibrosis, and in heart failure. Chronic autoimmune diseases such as scleroderma, ulcerative colitis, Crohn’s disease, and rheumatoid arthritis are additional examples of diseases where fibrosis is a prominent pathological feature (Gilbane et al., 2013; Li & Kuehmerle, 2014; Pattanaik et al., 2015). Despite that fibrosis can affect nearly every tissue in the body and is a major cause of morbidity and mortality in a variety of diseases, there are few treatments that specifically target the pathogenesis of fibrosis.

Although the etiology and causative mechanisms may vary widely, fibrosis always involves excessive production and deposition of collagen and other extracellular matrix proteins by activated myofibroblasts. Myofibroblasts express alpha-smooth muscle actin (αSMA) and are formed and activated as part of a repair mechanism triggered by tissue damage (Kendall & Feghali-Bostwick, 2014). Several cell types have been shown to serve as progenitors for myofibroblasts: residential fibroblasts, epithelial cells (through epithelial-to-mesenchymal transition) as well as blood-borne mesenchymal progenitor cells called fibrocytes. In the liver, activated myofibroblasts derived from hepatic stellate cells (HSCs) are the principal fibroblastic cell type responsible for matrix deposition.

The formation and function of myofibroblasts is promoted by a number of secreted soluble factors such as cytokines, growth factors, and matrix-related factors that act in synergy in the development of fibrosis (Kramann et al., 2013). TGFβ is considered a key factor that coordinates the interplay between parenchymal cells, inflammatory cells, and myofibroblasts (Leask & Abraham, 2004). Accordingly, overexpression of TGFβ in transgenic mice results in multi-organ fibrosis (Sime et al., 1997; Kanzler et al., 1999; Rahmutula et al., 2013). Homeostatic regulation of protein folding in the endoplasmic reticulum (ER) is under the control of three evolutionary conserved
pathways: IRE1α-XBP-1, PERK-elf2α, and ATF6 (Schroder & Kaufman, 2005). These pathways, also referred to as the unfolded protein response (UPR), are activated in response to ER stress, which occurs when cells have to cope with an increased burden in the protein folding machinery. Importantly, the UPR makes life/death decisions for the cell and the final outcome of ER stress is either recovery and survival or apoptosis depending on the severity and duration of ER stress (Sovolyova et al., 2014). All three pathways of the UPR form a coordinated reaction to the accumulation of unfolded proteins and several studies have demonstrated that there is cross talk between the different pathways (Yamamoto et al., 2004; Arai et al., 2006; Adachi et al., 2008). ER stress and activation of the UPR can be caused by mechanical injury, inflammation, genetic mutations, infections, and oxidative stress, and has been implicated in diseases that result in fibrotic remodeling of internal organs, such as chronic liver diseases (Galligan et al., 2012; Shin et al., 2013; Ji, 2014), pulmonary fibrosis (Baek et al., 2012; Tanjore et al., 2012, 2013), kidney fibrosis (Chiang et al., 2011), cardiovascular disease (Spitler & Webb, 2014), and inflammatory bowel disease (Bogaert et al., 2011; Cao et al., 2013).

IRE1α is a kinase located in the ER membrane that transmits information across the ER lipid bilayer (Tirasophon et al., 2000). Increased ER protein load and presence of unfolded proteins leads to the dissociation of the ER chaperone GRP78/BiP from IRE1α. The free IRE1α then dimerize and autophosphorylate, which leads to activation of the IRE1α ribonuclease domain in the cytosol. Regulated IRE1α-dependent decay of mRNA (RIDD) reduces both protein translation and import of proteins into the ER (Hollien & Weissman, 2006). In addition, IRE1α has the ability to splice XBP-1 mRNA, which is required for the translation of active XBP-1. After transport into the nucleus, XBP-1 binds to UPR promoter elements to initiate expression of genes that enhance the ability of the ER to cope with unfolded proteins, including molecular chaperones like BiP and transcription factors such as CHOP. ER stress is in addition associated with enlargement of the ER volume, which has been interpreted as an adaptive mechanism to increase protein folding capacity (Sriburi et al., 2004).

Besides degrading mRNA (Binet et al., 2013), it was recently shown that IRE1α also has the ability to degrade microRNAs (miRs) (Upton et al., 2012). miRs are short noncoding RNA oligonucleotides consisting of 17–25 nucleotides that generally act to inhibit gene expression by binding to complementary sequences in the 3′-untranslated region of target mRNAs, either to repress mRNA translation or to induce mRNA cleavage. A number of cellular functions such as proliferation, differentiation, and apoptosis are regulated by miRs, and aberrant miR expression is observed in a variety of human diseases including fibrosis (Bowen et al., 2013).

Inhibitors that specifically target individual components of the UPR have recently been developed. The selective inhibitor 4a8C that stably binds to lysine 907 in the IRE1α endoribonuclease domain has been shown to inhibit both RIDD activity and XBP-1 splicing (Cross et al., 2012). High levels of 4a8C cause no measurable toxicity in cells and concentrations ranging from 80 to 128 μM of 4a8C completely block XBP-1 splicing without affecting IRE1α kinase activity (Cross et al., 2012). The inhibitor 4a8C thus represents both a potential drug and an important tool to delineate the functions of IRE1α in vivo as IRE1α-knockout mice die during embryonic development.

In this report, we show that inhibition of IRE1α prevents activation of myofibroblasts and reduces fibrosis in animal models of liver and skin fibrosis. Our finding that pharmacological inhibition of IRE1α could revert the profibrotic phenotype of activated myofibroblasts isolated from patients with scleroderma indicates that ER stress inhibitors should be taken into consideration when developing new strategies for the treatment of fibrotic diseases.

Results

The RNase activity of IRE1α is required for activation of myofibroblasts

Enhanced secretion of extracellular matrix proteins as well as increased αSMA expression are typical features of fibroblast to myofibroblast conversion (Baum & Duffy, 2011). Treatment of human fetal lung fibroblasts (HFL1) with TGFβ resulted in formation of myofibroblasts as judged by the increased expression of αSMA (Fig 1A) and collagen I (Fig 1B). Since increased collagen I production may increase the protein folding demand and activate the UPR, we measured the expression of several ER stress pathway components and found increased mRNA expression of CHOP and BiP (Fig 1C) as well as increased protein levels of CHOP (Fig 1D) and increased splicing of XBP-1 (Fig 1C and E). To investigate whether this activation of ER stress, and specifically the activation of IRE1α, is involved in myofibroblast activation, we inhibited IRE1α function using the inhibitor 4a8C, which blocked TGFβ-induced activation of fibroblasts as measured by expression of mRNA and protein levels of αSMA (Fig 1A and F) and collagen I (Fig 1B and G). Further, TGFβ-induced expression of αSMA mRNA was reduced in cells overexpressing two different mutant forms of IRE1α that lack RNase activity (Fig 1H) or when complete loss-of-function experiments were performed using IRE1α−/− MEF cells (Fig 1I).

miR-150 exhibits anti-fibrotic effects and is directly cleaved by IRE1α

IRE1α has recently been shown to directly cleave miRs (Upton et al., 2012) and it was therefore hypothesized that 4a8C could modulate fibrosis by preventing cleavage of miRs involved in activation of myofibroblasts. Decreased levels of miR-29 (Khalil et al., 2015), miR-148 (Van Keuren-Jensen et al., 2015), and miR-150 (Honda et al., 2013; Zheng et al., 2013) have been implicated in fibrotic tissue remodeling and were therefore selected for analysis. The combined treatment of HFL1 fibroblasts with TGFβ and 4a8C led to a significant increase in miR-150 (Fig 2A), while the levels of miR-29 and miR-148 were unaffected (Fig EV1). These results suggested that an IRE1α-dependent signaling pathway could be responsible for regulating the levels of miR-150. This was further supported by the finding that overexpression of RNase inactive IRE1α mutants in HFL1 fibroblasts resulted in increased levels of miR-150 in response to TGFβ (Fig 2B). Further, miR-150 levels were also higher in TGFβ-treated IRE1α−/− MEF cells, as compared to WT cells (Fig 2C). To further investigate whether IRE1α-mediated cleavage of miR-150 contributes to TGFβ-induced myofibroblast activation, IRE1α−/−...
Figure 1. The RNase activity of IRE1α is required for activation of myofibroblasts.

A αSMA mRNA levels in TGFβ-treated HFL1 fibroblasts and the effect of the IRE1α inhibitor 4μ8C. *P = 0.001.

B Collagen 1α2 mRNA levels in TGFβ-treated HFL1 fibroblasts ± the IRE1α inhibitor 4μ8C. *P = 0.01.

C mRNA levels of ER stress-related genes in TGFβ-treated HFL1 fibroblasts. *P CHOP = 0.045, *P Bip = 0.023, *P spliced XBP-1 P = 0.001, compared to corresponding control.

D Western blot of CHOP protein levels in TGFβ-treated HFL1 cells ± the IRE1α inhibitor 4μ8C.

E Agarose gel showing the presence of spliced and unspliced XBP-1 mRNA in TGFβ-treated HFL1 fibroblasts ± the IRE1α inhibitor 4μ8C. *P = 0.049.

F Immunohistochemical staining of αSMA in TGFβ-treated HFL1 fibroblasts ± the IRE1α inhibitor 4μ8C. Scale bars = 20 µm.

G Quantification of secreted soluble collagen proteins produced by TGFβ-treated HFL1 cells ± the IRE1α inhibitor 4μ8C. *P = 0.025.

H αSMA mRNA expression in TGFβ-treated IRE1α−/− or WT MEF cells. *P = 0.025.

I αSMA mRNA expression in TGFβ-treated IRE1α−/− or WT MEF cells. *P = 0.025.

Data information: Statistical significance was evaluated using Student's t-test. Significant differences are indicated with * and exact P-values are given. Error bars indicate s.e.m. n = 3 in (A–C) and (G–I).

Source data are available online for this figure.
MEF cells were transfected with an anti-miR-150-5p miRNA inhibitor, which was shown to suppress the levels of miR-150 (Fig 2D) and to increase αSMA mRNA in response to TGFβ (Fig 2E). Based on this, it was hypothesized that miR-150 is directly involved in the negative regulation of αSMA expression.

The transcription factor c-Myb represents the top target gene of miR-150 with 3 conserved miR-150 binding sites in the human mRNA according to TargetScan (Lewis et al., 2003). Several studies have shown that miR-150 suppresses c-Myb expression (Lin et al., 2008; Feng et al., 2014; Yang et al., 2015) and c-Myb in turn is known to increase αSMA expression (Kitada et al., 1997; Buck et al., 2000; Zheng et al., 2013). We therefore investigated whether miR-150 could regulate c-Myb and αSMA expression in HFL1 fibroblasts. Transfection of HFL-1 fibroblasts with a miR-150 expression plasmid (Fig 2F) resulted in reduced c-Myb and αSMA protein expression (Fig 2G and H) supporting an anti-fibrotic function of miR-150. To further establish the link between c-Myb and αSMA, HFL1 fibroblasts were transfected with siRNA targeting c-Myb (Fig 2I). In line with previous studies (Kitada et al., 1997; Buck et al., 2000; Zheng et al., 2013), silencing of c-Myb reduced TGFβ-induced αSMA expression (Fig 2J).

Sequence analysis of pre-miR-150 showed the presence of several putative IRE1α cleavage sites, including one positioned 10 bp from the 5’-terminus (Fig 2K). The possibility that IRE1α may directly cleave miR-150 was investigated using an in vitro cleavage assay, with 32P-labeled pre-miR-150 as a substrate and a synthetic stem-loop RNA corresponding to the XBP-1 cleavage site as a positive control. It was found that recombinant IRE1α cleaved pre-miR-150, which generated a small cleavage product of approximately 10 bp (Fig 2L). Cleavage of pre-miR-150 and the XBP-1 stem-loop control was almost completely inhibited by addition of 4μB, which confirmed that 4μB is an inhibitor of IRE1α RIDD activity (Fig 2L). Next, HFL1 cells were pre-treated with TGFβ and 4μB, and transcription inhibited using actinomycin D for 4 and 24 h to see whether the stability of miR-150 was affected. Indeed, 4μB was shown to slow down miR-150 degradation in TGFβ-treated HFL1-cells (Fig 2M). Thus, degradation of miR-150 can be directly mediated by IRE1α, and loss of IRE1α Rnase activity results in increased levels of miR-150, which results in c-Myb suppression and reduced TGFβ-induced αSMA gene expression.

**ER expansion in myofibroblasts is coupled to XBP-1 splicing**

Activation of UPR pathways, in particular splicing of XBP-1, has been linked to ER expansion and increased secretory capacity (Sriburi et al., 2004; Bonmiyasamy et al., 2009). Therefore, we hypothesized that ER stress, through splicing of XBP-1 by IRE1α, mediates ER expansion and is at least partially required for TGFβ-induced activation of myofibroblasts and secretion of extracellular matrix. TGFβ-stimulation of HFL1 fibroblasts resulted in an enlargement of the ER (Fig 3A and B) in agreement with previous studies on myofibroblasts (Baum & Duffy, 2011). Treatment of cells with 4μB inhibited TGFβ-induced ER expansion suggesting a central role of IRE1α (Fig 3A and B). To specifically investigate the role of XBP-1, we treated HFL1 fibroblasts with siRNA targeting XBP-1, which caused an 85% reduction in XBP-1 gene expression (Fig 3C) and inhibited TGFβ-induced ER expansion (Fig 3A and B), as well as collagen secretion (Fig 3D). These observations suggest that the IRE1α-XBP-1 axis contributes to myofibroblast activation by expanding the ER in order to cope with increased protein folding demands.

**Pharmacological inhibition of IRE1α attenuates liver fibrosis**

The UPR is activated in several liver diseases (Malhi & Kaufman, 2011). To investigate whether inhibition of IRE1α could reduce development of liver cirrhosis, we used a mouse model in which hepatic fibrosis was induced by alcohol and CCl4 (Geerts et al., 2008). Mice developed typical bridging liver fibrosis with an increased META VIR score reflecting F3-F4 cirrhosis (Bedossa & Poynard, 1996) (Fig 4A and B). Treatment with 4μB reduced fibrosis (Fig 4A and B) and the number of αSMA-positive

---

**Figure 2. miR-150 exhibits anti-fibrotic effects and is directly cleaved by IRE1α.**

A miR-150 levels in TGFβ-treated HFL1 fibroblasts and the effect of the IRE1α inhibitor 4μB. *P = 0.046 compared to control.
B miR-150 levels in HFL1 fibroblasts overexpressing two RNase-dead mutants (K999A and K907A) of IRE1α. *P = 0.0043 (K999A), P = 0.043 (K907A), compared to control.
C miR-150 levels in TGFβ-treated WT (IRE1α+/+) and IRE1α–/– MEF cells. *P = 0.018. Results are expressed as fold change compared to the Ctrl.
D miR-150 levels in IRE1α–/– MEF cells that were either untransfected (UT) or transfected with miRNA inhibitor control (siCtrl) or with a miR-150-5p miRNA inhibitor. *P = 0.034 compared to UT.
E αSMA mRNA expression in TGFβ-treated IRE1α+/– MEF cells that were either untransfected (UT) or transfected with miRNA inhibitor control (siCtrl) or with a miR-150-5p miRNA inhibitor. *P = 0.043 compared to UT.
F αSMA mRNA expression in TGFβ-treated IRE1α+/– MEF cells that were either untransfected (UT) or transfected with miRNA inhibitor control (siCtrl) or with a miR-150-5p miRNA inhibitor. *P = 0.043 compared to UT.
G Western blot of c-Myb in TGFβ-treated HFL1 fibroblasts that were either untransfected (UT), transfected with a control plasmid (pCtrl) or a miR-150 expression plasmid (miR-150). *P = 0.038.
H Western blot of αSMA in the same experimental setup as in G. *P = 0.0405 in UT Ctrl versus UT TGFβ, and P = 0.0284 in pCtrl Ctrl versus pCtrl TGFβ.
I c-Myb mRNA levels in untransfected HFL1 cells (UT) and in cells transfected with a control siRNA (siCtrl) or an siRNA targeting c-Myb (sicMyb). *P = 0.00001 compared to UT.
J αSMA mRNA expression in transfected HFL1 cells (UT) and in cells transfected with a control siRNA (siCtrl) or with an siRNA targeting c-Myb (sicMyb). *P = 0.0042 in UT Ctrl versus UT TGFβ.
K Putative IRE1α cleavage sites in miR-150 based on sequence analysis are indicated with arrows.
L In vitro cleavage assay with recombinant IRE1α and miR-150 (left) or XBP-1 (right).
M miR-150 expression in HFL1 cells that were pre-treated with TGFβ in the absence of presence of 4μB and then treated with actinomycin D (ActD) for 4 and 24 h. *P = 0.027 compared to TGFβ.

Data information: Statistical significance was evaluated using Student’s t-test. Significant differences are indicated with * and exact P-values given. Error bars indicate ± s.e.m. n = 3 in (A–E, G–J, M) and n = 4 in (F).

Source data are available online for this figure.
Figure 2.

A. miR-150 expression in response to TGF-β and 4μ8C.

B. miR-150 expression in IRE1α KO cells.

C. miR-150 expression in response to TGF-β in the presence of simiR-150.

D. Control and siCtrl expression in response to simiR-150.

E. αSMA mRNA expression in response to TGF-β.

F. cMyb protein expression in response to miR-150.

G. cMyb protein expression in response to TGF-β.

H. αSMA and βActin protein expression in response to TGF-β and miR-150.

I. cMyb mRNA expression in response to siCtrl and sicMYB.

J. dsMα mRNA expression in response to siCtrl and sicMYB.

K. XBP-1 and IRE1α reporter assays.

L. Microarray analysis of miR-150 expression.

M. Relative expression of miR-150 in response to TGF-β and 4μ8C.

© 2016 The Authors

EMBO Molecular Medicine Vol 8 No 7 2016 733
myofibroblasts in pericentral areas (Fig 4C). Fibrotic livers typically have an increased size and weight and treatment with 4μ8C decreased the liver-to-body weight ratio to further support the hypothesis that inhibition of the UPR reduces disease progression (Fig 4D). To verify that 4μ8C affected activation of the UPR, markers of the UPR pathway were measured. The mRNA levels of BiP

![Image](image_url)

**Figure 3.** ER expansion in myofibroblasts is coupled to XBP-1 splicing.

A Analysis of ER size in HFL1 fibroblasts treated with TGFβ in the absence (Ctrl) or presence of the IRE1α inhibitor 4μ8C, or in HFL1 cells transfected with a siRNA control siRNA (siCtrl) or with siRNA targeting XBP-1 (siXBP-1). The ER was visualized with ER-Tracker Red and nuclei stained with Hoechst (left, scale bars = 20 μm) or by electron microscopy (right, scale bars = 200 nm) where red lines mark the ER.

B Quantification of ER size relative to the nucleus from the experimental setup in (A). *P = 0.0001 in Ctrl, and *P = 0.0223 in siCtrl.

C XBP-1 mRNA expression in untransfected HFL1 cells (UT) and in cells transfected with control siRNA (siCtrl) or siRNA targeting XBP-1 (siXBP-1). *P = 0.0001.

D Collagen protein levels in TGFβ-treated HFL1 fibroblasts transfected as described in (C). *P = 0.005.

Data information: Statistical significance was evaluated using Student’s t-test. Significant differences are indicated with * and exact P-values given. Error bars indicate s.e.m. n = 3 in (B, C) and n = 4 in (D).
Endoplasmic reticulum stress enhances fibrosis

Inhibition of IRE1α reduces skin fibrosis

In order to investigate whether interference with ER stress at the level of IRE1α affects fibrosis in other models, and hence could be of more general applicability, we used a mouse model of skin fibrosis where TGF-β is continuously infused through subcutaneously implanted mini-osmotic pumps. TGF-β caused a fibrotic response in the hypodermis with an increased amount of deposited collagen, which was significantly decreased in 4µ8C-treated mice (Fig 5A–C). Selective analysis of the hypodermis by laser capture microdissection (Fig 5D) confirmed that treatment with 4µ8C reduced αSMA mRNA expression in HSCs isolated from human liver (Fig 4L). These results suggest that inhibition of IRE1α RNAse activity can attenuate liver fibrosis by hindering HSCs to become activated myofibroblasts.

Further supports that 4µ8C inhibits fibrosis by preventing activation of myofibroblasts.

The fibrotic phenotype of myofibroblasts isolated from patients with scleroderma can be reverted by inhibition of IRE1α

Scleroderma, or progressive systemic sclerosis (SSc), is a connective tissue disease characterized by severe multi-organ fibrosis, which results from the excessive synthesis of extracellular matrix by myofibroblasts (Gilbane et al., 2013). To explore the possibility that IRE1α could be a potential therapeutic target for treatment of SSc, fibroblasts isolated from SSc patients were treated with 4µ8C. These experiments showed that pharmacological inhibition of IRE1α significantly decreased mRNA levels of CTGF (Fig 6A) and collagen 1 (Fig 6B) and soluble collagen protein expression (Fig 6C) in fibroblasts from both skin (dSSc) and lung (LSSc). Fibroblasts from SSc patients also showed increased levels of BiP, which were reduced after treatment with 4µ8C (Fig 6D). To further study the general applicability of treatment with 4µ8C, primary lung fibroblasts from patients with inflammatory lung diseases, such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and asthma, were exposed to 4µ8C. CF, COPD, and asthma are diseases that cause chronic inflammation of the airways, involving myofibroblast activation and ECM deposition. Primary fibroblasts from asthma patients, but not fibroblasts isolated from CF or COPD patients, expressed significantly increased levels of αSMA and BiP compared to controls, which were decreased by treatment with 4µ8C (Fig 6E and F). These results suggest that inhibition of IRE1α not only can prevent formation and activation of myofibroblasts, but also can revert the fibrotic phenotype of myofibroblasts in cells isolated from patients with fibrotic disease.

Discussion

There is increasing evidence that ER stress and activation of the UPR is a feature of fibrosis in different organs caused by different etiologies. On a cellular level, the myofibroblast is considered to have a central role in development of fibrosis, but little is known...
Figure 4.
about how ER stress influences the activation of fibroblasts to myofibroblasts. It has been shown that treatment of fibroblasts with TGFβ induces ER stress and formation of myofibroblasts, which could be inhibited with a chemical chaperone that inhibits ER stress by improving the ER protein folding capacity (Baek et al., 2012). In this report, we further define a direct role of
the UPR in myofibroblast differentiation as we provide evidence that inhibition of IRE1α prevents TGFβ-induced formation of myofibroblasts.

The recent finding that IRE1α is capable of degrading miRs, in addition to its well-known ability to degrade mRNA, provides a new possible connection between IRE1α and miRs that regulate the activation of myofibroblasts (Upton et al., 2012). IRE1α, miRs, and components of the RISC machinery are found on the cytosolic side of the rough ER membrane (Daniels et al., 2009; Li et al., 2013; Stalder et al., 2013), which would allow a direct interaction between IRE1α and miRs. Sequence analysis of miR-150 revealed several putative IRE1α cleavage sites (TGTC-) (Upton et al., 2012) and our data demonstrate that IRE1α directly cleaves miR-150, providing a mechanism for the increased levels of miR-150 that were observed when IRE1α was inhibited in TGFβ-treated fibroblasts. Cleavage of pre-miR-150 by IRE1α probably destabilizes pre-miR-150 to prevent further processing by Dicer, as described for another ribonuclease, MPCI (Suzuki et al., 2011). Previous studies on isolated HSCs have similarly shown that TGFβ reduces miR-150 levels and that overexpression of miR-150 inhibits TGFβ-induced sSMA and collagen I and IV expression (Buck et al., 2000; Zheng et al., 2013). These results in combination with reports that miR-150 is decreased in fibrotic conditions and correlates with disease severity (Honda et al., 2013) indicate an anti-fibrotic function of miR-150.

One of the top target genes of miR-150 is the transcription factor c-Myb (Lin et al., 2008; Feng et al., 2014; Yang et al., 2015). c-Myb is known to bind the sSMA promoter and overexpression of c-Myb in HSCs increases sSMA expression, while antisense c-Myb inhibits sSMA expression (Buck et al., 2000). In addition, miR-150 has been shown to regulate collagen I expression by regulating the transcription factor SP1 (Zheng et al., 2013). These findings would suggest a pathway where fibrosis-inducing agents like TGFβ induce ER stress with activation of IRE1α. Activated IRE1α then degrades miR-150, removing the inhibitory effect of miR-150 on expression of transcription factors like c-Myb and SP1. Increased levels of these transcription factors are essential for the formation of myofibroblasts as they induce expression of sSMA and collagen I. Under normal conditions, miR-150 would continuously suppress expression of profibrotic transcription factors and prevent formation of myofibroblasts.

A similar mechanism whereby activated IRE1α inactivates miRs that normally repress protein translation has been demonstrated for other biological responses. For example, it has previously been shown that increased caspase-2 expression and induction of apoptosis can be a result of IRE1α activation leading to destruction of miRs that under normal conditions repress caspase-2 expression (Upton et al., 2012). Suppression of these miRs increased caspase-2 without any additional stimuli. Further, another study showed that the thioredoxin-interacting protein (TXNIP) is increased as a result of activated IRE1α destroying miR-17 that otherwise mediates degradation of TXNIP mRNA (Lerner et al., 2012).

In a parallel pathway, we observed an IRE1α- and XBP-1-dependent increase in ER size when fibroblasts were stimulated with TGFβ. An enlarged ER would prepared the myofibroblast for increased production and folding of extracellular matrix proteins during wound healing and fibrosis. It has been suggested that splicing and hence activation of XBP-1 in response to ER stress promotes an increase in ER volume through increased phospholipid biosynthesis in the ER membrane (Sriburi et al., 2004). A functional role of XBP-1 in membrane biogenesis was originally described for differentiation of B lymphocytes into antibody-secreting plasma B cells (Reimold et al., 2001) and later also for ER expansion in pancreatic β-cells (Lee et al., 2005) and in hepatocytes (Lee et al., 2008).

In conclusion, our study indicates that activation of the UPR is a conserved mechanism in fibrotic pathologies in various organs and that targeting the IRE1α RNase activity with the inhibitor 4μ8C could be a potential treatment strategy for patients with progressive fibrotic diseases. We suggest that there are two mechanisms by which IRE1α contributes to myofibroblast activation (Fig 7). Firstly, the RIDD activity of IRE1α is responsible for degrading the anti-fibrotic miR-150 to allow for increased c-Myb activity leading to increased sSMA expression, thereby resulting in increased formation of myofibroblasts. Secondly, IRE1α mediates splicing of XBP-1, leading to ER expansion that contributes to the enhanced secretory capacity needed for the secretion of extracellular matrix proteins.
Figure 6. Inhibition of IRE1α reverts the fibrotic phenotype of myofibroblasts isolated from scleroderma patients.
A CTGF mRNA expression levels in skin (dSSc) and lung (LSSc) fibroblasts isolated from scleroderma patients and treated with the IRE1α inhibitor 4μ8C. *P = 0.018 for dSSc (n = 6) and P = 0.003 for LSSc (n = 3).
B Collagen 1α2 mRNA expression levels in the same samples as in (A). *P = 0.0030 for dSSc and P = 0.0036 for LSSc.
C Quantification of collagen protein levels in the same samples as in (A). *P = 0.010.
D Immunohistochemical staining to detect BiP protein in fibroblasts isolated from scleroderma patients treated or not with 4μ8C. Scale bars = 20 μm.
E mRNA levels of αSMA in primary lung fibroblasts isolated from patients with either cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), or asthma.
*P = 0.00001 for asthma versus healthy controls, and P = 0.00003 for ctrl versus 4μ8C in asthma patients.
F Quantification of mRNA levels for BiP in same samples as in (E). *P = 0.0023 between asthma and healthy controls and P = 0.0031 between 4μ8C-treated and control treatment in fibroblasts from asthma patients.

Data information: Statistical significance was evaluated using Student’s t-test. Significant differences are indicated with * and exact P-values given. Error bars indicate s.e.m. n = 3 in (E, F).
Published online: May 25, 2016
EMBO Molecular Medicine

Endoplasmic reticulum stress enhances fibrosis  Femke Heindryckx et al

Mouse embryonic fibroblasts (MEFs) either WT or deficient for IRE1α (kind gift from R.J. Kaufman, Sanford-Burnham Institute) were grown in DMEM media supplemented with 10% fetal bovine serum. Primary lung fibroblasts from patients with asthma (194912, Lonza), COPD (195277, Lonza), and CF (194843, Lonza) and healthy controls (CC-2512; Lonza) were grown in FGM-2 medium (CC3131 and CC4134, Lonza).

For in vitro experiments, cells were detached using trypsin–EDTA (Gibco), resuspended in growth medium, and plated at a density of 5 × 10^4 cells/cm². Cells were allowed to attach and left undisturbed for 8 h before being starved for 16 h. After that time, fresh starvation medium containing indicated growth factors or substances was added. Cells were exposed for 48 h to 5 ng/ml TGFβ and/or 100 μM 4μ8C, unless stated otherwise. Previous studies have shown that concentrations up to 128 μM of 4μ8C cause no measurable toxicity and that a concentration of 100 μM leads to a complete inhibition of XBP-1 splicing (Cross et al., 2012). Actinomycin D (A1410, Sigma) was added to the cells at a concentration of 5 μg/ml.

**miR cleavage assay**

Pre-miR-150 was synthesized by Thermo Fisher Scientific miRIDIAN using the published sequence in the miRBase (www.mirbase.org). The dephosphorylated pre-miRNA was end-labeled with 32P-ATP and incubated with 2.5 μg of recombinant IRE1α for 6 h at 37°C in a reaction buffer (20 mM Hepes, 50 mM NaCl, 1 mM DTT, 10 mM ATP). Cleaved products were resolved using a 15% denaturing polyacrylamide gel. The dried gel was exposed to an autoradiographic film overnight. The XBP-1 mini-stem loop was synthesized by Integrated DNA Technologies and processed similarly to pre-miR-150. The pre-miR-150 and XBP-1 mini-stem-loop sequences are provided in Table EV1.

**Materials and Methods**

**Cell culture and reagents**

Human fetal lung fibroblast (HFL1, ECACC) was routinely cultured in MEM supplemented with nonessential amino acids, 25 mM HEPES (Sigma), 10% FBS (Gibco), 2 mM glutamax (Gibco), and 1 mM sodium pyruvate (Gibco). Cells were cultured at 37°C with 5% CO2. For starvation, stimulation and migration growth medium was used without FBS was used.

Primary cells were isolated from skin and lung, using the tissue explant outgrowth method. Shortly, fresh tissue was obtained post-mortem from adult mice, rinsed in PBS, cut in 5-mm² pieces, and subsequently added to culture plates with medium (DMEM, 10% FCS, penicillin, streptomycin, and fungizone) to allow outgrowth of fibroblasts. Mouse hepatic stellate cells were isolated using a pronase/collagenase digestion, followed by Nycodenz gradient centrifugation (Maschmeyer et al., 2011). Human hepatic stellate cells were isolated as previously described (Rombouts et al, 2012). Fibroblasts were explanted and cultured from skin or lung tissue taken from patients with scleroderma or from normal control subjects (kind gift of Prof Chris Denton, Royal Free Hospital UK). The patients fulfilled the American College of Rheumatology criteria. All samples were obtained after informed consent and under institutional local research ethics committee approval (Royal Free and Medical School Local Research Ethics Committee (Ponticos et al., 2015). Each of the primary cell lines in this study is derived from a different individual or mouse.

![Diagram of ER stress and XBP1 splicing](image)

**Figure 7.** Model for the role of IRE1α in myofibroblast activation.

In the absence of ER stress, miR-150 inhibits c-Myb expression to prevent c-Myb-induced αSMA expression. ER stress, for example, as a result of fibrotic stimuli, activates IRE1α that directly cleaves and inactivates miR-150 resulting in increased c-Myb expression and as a consequence increased expression of αSMA and activation of myofibroblasts. In addition, IRE1α-mediated splicing of XBP-1 mRNA leads to ER expansion and enhanced secretory capacity of extracellular matrix proteins. Inhibition of the endoribonuclease activity of IRE1α by 4μ8C will lead to increased miR-150 levels followed by reduced c-Myb and αSMA levels. Treatment with 4μ8C will also reduce XBP-1 splicing, ER expansion, and secretion of matrix proteins.

**Quantitative RT–PCR of mRNA or miRNA and XBP-1 splicing assay**

RNA was isolated from tissue or cell culture using the Qiagen miNeasy Mini kit according to the manufacturer’s instructions. Briefly, 500 ng of miRNA/mRNA was reverse-transcribed using miScript II RT Kit, and amplifications performed using primers summarized in Table EV2. Normalization was done using ActB/RPS18/TBP for mRNA and SNORD68/SNORD95 for miRNA. The spliced form of XBP-1 was detected by incubation of the semi-quantitative PCR product with PstI enzyme for 3 h at 37°C. Cleaved products were resolved using a 15% denaturing polyacrylamide gel. The dried gel was exposed to an autoradiographic film overnight. The XBP-1 mini-stem loop was synthesized by Integrated DNA Technologies and processed similarly to pre-miR-150. The pre-miR-150 and XBP-1 mini-stem-loop sequences are provided in Table EV1.

**Transfections and nucleofections**

HFL1 cells were transiently transfected with 300 ng of IRE1α WT, K599A or K907A, and miR-150 expression plasmid (in courtesy of D. Bartel, Addgene #26310) using Lipofectamine LTX and the PLUS reagent for 24 h. Nucleofection of siXBP-1 (sc-38627, Santa Cruz), si-c-Myb (4392420, Life Technologies), si-miR-150 (219300, Qiagen), or siCtrl (100 pmol) was done using Amamax Nucleofector program S-05 in Ingenio electroporation solution (Mirus Bio LLC). Cells were starved overnight and subsequently treated with 5 ng/ml TGFβ and/or 4μ8C (100 μM) for 48 h.
Animal experiments

Eight-week-old, male C57Bl/6 mice were obtained from Scanbur (Denmark). Mice were housed in standard conditions with normal dark–light cycle. Animals were acclimatized to their environment for 1 week and given *ad libitum* access to water and food throughout the experiments. Mice were randomly divided into different treatment groups prior to starting the experiment. During experiments, mice were weighted twice per week and their health was evaluated using the Karolinska Institute health monitoring template, whereby a total score of >0.4 or a single score of >0.3 was considered a humane endpoint for euthanasia. Sample size was determined from previous experience (Geerts et al., 2008; Van de Veire et al., 2010; Van Steenkiste et al., 2011).

Skin fibrosis model

Mini-osmotic pumps (Alzet 1003D, 1 µl/h) containing TGFβ (200 ng in 0.1% BSA in saline) or controls (0.1% BSA in saline) were implanted subcutaneous in 32 mice under isoflurane (Forane) anesthesia. Postoperative analgesia (Temgesic) was provided daily for 3 days. Mice were subcutaneously injected every day near the implantation site with 10°C in sodium citrate (Denmark). Mice were housed in standard conditions with normal dark–light cycle. Animals were acclimatized to their environment for 1 week and given *ad libitum* access to water and food throughout the experiments. Mice were randomly divided into different treatment groups prior to starting the experiment. During experiments, mice were weighted twice per week and their health was evaluated using the Karolinska Institute health monitoring template, whereby a total score of >0.4 or a single score of >0.3 was considered a humane endpoint for euthanasia. Sample size was determined from previous experience (Geerts et al., 2008; Van de Veire et al., 2010; Van Steenkiste et al., 2011).

Liver cirrhosis model

Sixteen mice were injected twice per week with CCl₄ (Sigma) (1:1 dissolved in olive oil; 1 mg/kg) and 5% alcohol was added to drinking water. Similarly, 16 control mice received physiological saline solution (1 ml/kg) and no alcohol was added. Mice were injected twice per week with 10 µg/g 4µBC in saline or 0.08% DMSO in saline (control). Skin biopsies were collected after 3 days using 4-mm biopsy punches.

Stainings and immunohistochemistry

Tissue samples were fixed in 4% paraformaldehyde for 24 h and subsequently embedded in paraffin. 5-µm slides were cut and dried overnight. Sections were de-paraffinized and rehydrated prior to staining. Collagen was stained using the picrosirus red staining with an incubation time of 30 min, followed by 10-min washing in distilled water. For the immunohistochemical staining of αSMA, an antigen retrieval was done at 95°C in sodium citrate buffer, endogenous peroxidase activity was blocked using 3% H₂O₂, and endogenous mouse IgG was blocked using a mouse-on-mouse staining kit (ab127055, Abcam) prior to a 1-h incubation with the primary antibody (A2547, Sigma) followed by a HRP-coupled secondary antibody using DAB as substrate. Slides were washed in TBS-T (0.1% Triton X-100) 3 × 10 min after primary and secondary antibody incubations. Slides were counterstained with Mayer’s hematoxylin. For the immunofluorescent stainings of skin and liver tissue, antigen retrieval was done at 95°C in sodium citrate buffer and endogenous mouse IgG was blocked using a rodent blocking buffer (ab127055, Abcam) following the manufacturer’s protocol. Samples were incubated overnight at 4°C with primary antibodies against αSMA (A2547, Sigma), BiP (ab21685, Abcam), and/or CHOP (sc575, Santa Cruz). A 20-min incubation was used for the secondary antibody (Alexa Fluor 488 and Alexa Fluor 555) and cell nuclei were stained with Hoechst for 5 min.

Stainings and immunohistochemistry

Tissue samples were fixed in 4% paraformaldehyde for 24 h and subsequently embedded in paraffin. 5-µm slides were cut and dried overnight. Sections were de-paraffinized and rehydrated prior to staining. Collagen was stained using the picrosirus red staining with an incubation time of 30 min, followed by 10-min washing in distilled water. For the immunohistochemical staining of αSMA, an antigen retrieval was done at 95°C in sodium citrate buffer, endogenous peroxidase activity was blocked using 3% H₂O₂, and endogenous mouse IgG was blocked using a mouse-on-mouse staining kit (ab127055, Abcam) prior to a 1-h incubation with the primary antibody (A2547, Sigma) followed by a HRP-coupled secondary antibody using DAB as substrate. Slides were washed in TBS-T (0.1% Triton X-100) 3 × 10 min after primary and secondary antibody incubations. Slides were counterstained with Mayer’s hematoxylin. For the immunofluorescent stainings of skin and liver tissue, antigen retrieval was done at 95°C in sodium citrate buffer and endogenous mouse IgG was blocked using a rodent blocking buffer (ab127055, Abcam) following the manufacturer’s protocol. Samples were incubated overnight at 4°C with primary antibodies against αSMA (A2547, Sigma), BiP (ab21685, Abcam), and/or CHOP (sc575, Santa Cruz). A 20-min incubation was used for the secondary antibody (Alexa Fluor 488 and Alexa Fluor 555) and cell nuclei were stained with Hoechst for 5 min.

For staining of cells in culture, cells were fixed for 10 min in 4% paraformaldehyde, washed with PBS, and blocked for 30 min using 1% BSA in PBS + 0.1% Tween, followed by a 1-h incubation with antibodies against αSMA (clone 1A4, Sigma) or BiP (ab21685). A 20-min incubation was used for the secondary antibody (Alexa Fluor 488) and cell nuclei were stained with Hoechst staining for 5 min. Images were acquired using a Nikon eclipse 90i microscope equipped with a DS-Qi1Mc camera and Nikon Plan Apo objectives. NIS-Elements AR 3.2 software was used to save and export the images. The different channels (RGB) of immunofluorescent images were merged using ImageJ software. Quantifications were performed blindly with ImageJ software by conversion to binary images after color de-convolution to separate DAB staining, as previously described (Ruijrok & Johnston, 2001).

Immunoblotting

Samples were lysed and protein concentrations were determined using the BCA reagent. Equal amounts of proteins were analyzed by standard SDS–PAGE technique. Membranes were incubated with the respective antibodies (see Table EV2) and analyzed using the Li-Cor Odyssey imaging system.

Laser microdissection

Mouse skin samples were embedded in OCT and cut into 8-µm sections and mounted on frame slides (POL-Membrane 0.9 µm, Leica Microsystems, Wetzlar, Germany). Sections were fixed in acetone, washed with RNase-free water, then stained with RNase-free hematoxylin (Arcturus® Histogene® Staining Solution, Applied Biosystems, Foster City, CA, USA) for 1 min, dehydrated, and dried. The hypodermis layer was laser microdissected using a Leica LMD6000 B microscope. Quantification of mRNA or miRNA expression in the microdissected layers was performed as previously described.

Quantification of ER expansion

For ER staining, cells were washed once with HBSS and incubated for 30 min with 1 µM of ER-Tracker Red (Invitrogen). After 5-min fixation with 3.7% formaldehyde at 37°C, cells were counterstained with Hoechst and visualized using a Zeiss LSM 700 confocal microscope. Quantification of ER morphology was done blindly using ImageJ software. For the electron microscopy images, cells were fixed in 2% paraformaldehyde with 0.25% glutaraldehyde in PBS, postfixed in 1% OsO₄, and embedded in Epon (Ladd Research Industries, Burlington, VT, USA). The material was studied at 100 kV in a Jeol 1230 electron microscope (Jeol, Akishima, Tokyo, Japan). Electron micrographs were taken with a Gatan multiscan...
The paper explained

Problem
Fibrosis is characterized by an excessive accumulation of fibrous connective tissue that typically occurs in response to chronic inflammation. Despite that fibrosis can affect nearly every tissue in the body and is a major cause of morbidity and mortality in a variety of diseases, there are few treatments that specifically target the pathogenesis of fibrosis.

Results
We provide evidence that the ER stress sensor IRE1α mediates fibrosis by directly cleaving and inactivating miR-150. Under normal conditions, miR-150 represses c-Myb expression and thereby c-Myb-induced αSMA expression in fibroblasts which hinders formation of activated myofibroblasts. In addition, we find that IRE1α-mediated splicing of XBP-1 is required for expansion of the ER in activated myofibroblasts, which could be a mechanism that supports the enhanced secretion of extracellular matrix proteins. Inhibition of IRE1α, using the inhibitor 4μ8C, was found to inhibit TGFβ-induced activation of myofibroblasts in vitro, reduced liver and skin fibrosis in vivo, and reverted the profibrotic phenotype of activated myofibroblasts isolated from patients with systemic sclerosis.

Impact
Our results indicate that ER stress could be an important and conserved mechanism in the pathogenesis of fibrosis and that targeting components of the ER stress pathway might be therapeutically relevant for treating patients with fibrotic diseases.

camera model 791 with Gatan digital micrograph software version 3.6.4 (Gatan, Pleasanton, CA, USA).

Statistics
Data are presented as mean ± s.e.m. An unpaired, two-tailed Student’s t-test was used to compare different groups; a P < 0.05 was considered statistically different.

Study approval
The ethical committee for animal studies approved all methods involving animals (C45/13 and C95/14).

Expanded View for this article is available online.

Acknowledgements
We thank Dr Paul O’Callaghan for valuable input during preparation of the manuscript. FH is funded by the Wenner-Gren Foundation and Cancerfonden. PG is supported by Barnacancerfonden, Cancerfonden, and Lions Cancer Research Fund in Uppsala. JK is supported by Cancerfonden. FB is supported by Fonds de recherche du Québec-Santé.

Author contributions
FH was involved in the study design, animal experiments, and in vitro work. FB contributed to the study design and the in vitro experiments. MP was involved in the experiments with fibroblasts from scleroderma patients. KR provided hepatic stellate cells from patients. JL assisted with the laser capture microdissection. JK and PG were involved with the study design and project management. All authors contributed to writing of the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

References
Endoplasmic reticulum stress enhances fibrosis

EMBO Molecular Medicine


License: This is an open access article under the terms of the Creative Commons Attribution 4.0 License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.