International Journal of Biochemistry and Cell Biology 114 (2019) 105572

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Knockdown of IRE1α suppresses metastatic potential of colon cancer cells through inhibiting FN1-Src/FAK-GTPases signaling

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ARTICLE INFO

Keywords:
IRE1α
Migration
Invasion
Fibronectin
Colon cancer

ABSTRACT

The inositol-requiring enzyme 1α (IRE1α) is an endoplasmic reticulum (ER)-resident transmembrane protein and senses cellular unfolded/misfolded proteins. Upon activation, IRE1α removes a 26-bp nucleotide from the mRNA encoding X-box binding protein (XBP) 1 to generate a spliced active form of this transcription factor (XBP1s). Though IRE1α is implicated in development of cancer, the role and underlying mechanism remain unclear. Here, we demonstrate that IRE1α regulates colon cancer cell metastasis through regulating the expression of fibronectin-1 (FN1). We found that knockdown of IRE1α inhibited colon cancer cell migration and invasion *in vitro* and metastasis *in vivo*. Knockdown of IRE1α decreased the formation of XBP1s and attenuated the expression of FN1, leading to inhibition of phosphorylation of Src and FAK and inactivation the downstream effector GTPases including RhoA, Rac1 and CDC42. Addition of exogenous FN1 reversed Src/FAK phosphorylation and cell migration inhibited by IRE1α knockdown. We found that XBP1s bound *FN1* promoter and acted as a transcription factor to initiate *FN1* expression. Our results suggest that IRE1α modulates metastatic potential of colon cancer cells through regulating the expression of FN1.

1. Introduction

In eukaryotic cells, the endoplasmic reticulum (ER) is the main subcellular compartment involved in protein folding and maturation. Perturbations can cause the accumulation of unfolded or misfolded proteins in the lumen of the ER, resulting in ER stress [Kozutsumi et al., 1988; Hetz et al., 2013]. Cellular adaptation to ER stress is called unfolded protein response (UPR) [Cox and Walter, 1996; Chevet et al., 2015]. The UPR is mainly transduced by three ER-resident sensor proteins, inositol-requiring enzyme 1 alpha (IRE1α), protein kinase R-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) [Cox et al., 1993; Mori et al., 1993; Shi et al., 1998; Haze et al., 1999]. IRE1α, the most evolutionally conserved one among them, is a type I ER-resident transmembrane protein and possesses both endoribonuclease (RNase) and kinase activities [Tirasophon et al., 1998]. The activated IRE1α excises a 26-nucleotide of the mRNA that encodes the transcription factor X-box binding protein 1 (XBP1), causing a frame-shift and producing an active form XBP1s [Hetz et al., 2013; Calfon et al., 2002]. XBP1s controls the expression of genes involved in protein folding, secretion and ER-associated degradation (ERAD) [Lee et al., 2003; Acosta-Alvear et al., 2007].

Cancers usually arise and progress in a stressful microenvironment, which leads to perturbation of ER functions [Schroder and Kaufman, 2005; Moenner et al., 2007; Wang and Kaufman, 2016]. Sustained and high-level activation of UPR has been found in many forms of cancer [Fernandez et al., 2000; Song et al., 2001; Chen et al., 2002; Shuda et al., 2003; Carrasco et al., 2007; Gardner and Walter, 2011]. As part of the UPR, IRE1α-XBP1 signaling was shown to be involved in tumor development [Carrasco et al., 2007; Romero-Ramirez et al., 2004; Denoyelle et al., 2006; Auf et al., 2010; Dejeans et al., 2012; Chen et al., 2014; Jabouille et al., 2015]. Activation of IRE1α contributes to angiogenesis and tumor growth in glioblastoma [Auf et al., 2010; Drogat et al., 2007]. In triple-negative breast cancers, loss of XBP1 reduces tumor growth and metastasis due to impaired angiogenesis,

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https://doi.org/10.1016/j.biocel.2019.105572
Received 16 April 2019; Received in revised form 2 July 2019; Accepted 16 July 2019
Available online 18 July 2019
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independently of cell proliferation or apoptosis [Chen et al., 2014]. Reports are indicating that XBP1 is over-expressed in hepatocellular carcinoma [Shuda et al., 2003]. Sarcoma cells deficient for XBP1 have a large reduction in its ability to form solid tumors in nude mice [Spiotto et al., 2010]. A recent study demonstrated that high level of IRE1α in colon cancer was important in the acquisition of a poor prognostic phenotype and overexpression of IRE1α promoted the growth of colon cancer cells [Jin et al., 2016].

IRE1α-XBP1 was found to play a role in migration and invasion of cancer cells. It was found that XBP1 promoted tumor invasion *via* up-regulating the expression of MMP9 in esophageal squamous cell carcinoma and triggering the epithelial-mesenchymal transition (EMT) through inducing snail in breast cancer cells and hepatocellular carcinoma cells [Li et al., 2015; Xia et al., 2016; Wu et al., 2018]. XBP1 is expressed at high levels in metastatic and poorly differentiated colon cancer tissue samples, and loss of XBP1 was shown to severely inhibit tumor metastasis *in vitro* and *in vivo* [Mhaidat et al., 2015]. Consistent with this, a recent report suggested that activation of the IRE1α-XBP1 pathway induces cell invasion in colorectal carcinoma by regulating EMT [Jin et al., 2016]. Another report showed that IRE1α controls actin cytoskeleton dynamics and affects cell migration upstream of filamin A, and the regulation is independent of its canonical role as a UPR mediator [Urra et al., 2018]. These results suggest that IRE1α-XBP1 signaling acts as a positive regulator of migration and invasion of cancer cells. However, several studies showed that IRE1α might have an opposite effect in other types of cancer. For example, antagonism of IRE1α leads to extensive invasiveness in glioblastoma [Auf et al., 2010]. Besides, loss of functional IRE1α signaling mostly resulted in the up-regulation of genes that encode extracellular matrix (ECM) proteins in a glioma model [Dejeans et al., 2012]. These results suggest that IRE1α regulates cell migration and invasion in a cancer type-specific manner.

Colon cancer is one of the most common cancers and a serious threat to human health [Ferlay et al., 2015; Siegel et al., 2019]. Metastasis is the most lethal feature of colon cancers [Wanebo et al., 2012]. Therefore, the study on migration and invasion of colon cancer cells is important for exploring better diagnostic methods and improving treatment. Though IRE1α is implicated in the regulation of cancer cell migration and invasion, the underlying mechanism remains elusive. Here, we demonstrate that IRE1α acts as a positive regulator of metastasis of colon cancer cells *via* FN1-Src/FAK-GTPases signaling. We found that inhibition of IRE1α impairs the metastatic potential of colon cancer cells both *in vitro* and *in vivo*. Knockdown of IRE1α attenuated the expression of extracellular matrix protein Fibronectin-1 (FN1) *via* XBP1s, leading to inhibition of phosphorylation of Src and FAK and inactivation of Rho GTPases. Our results reveal a new mechanism underlying the regulation of colon cancer cell migration and invasion through the IRE1α-XBP1 pathway.

2. Materials and methods

2.1. Reagents

The fetal bovine serum (FBS) was from Gibco, ThermoFisher. Antibiotics were obtained from Life Technologies. The IRE1α RNase inhibitor 4μ8C was a product of Selleck Chemicals. Fibronectin human plasma was purchased from Sigma. All other reagents used were of the highest grade available.

2.2. Cells

Human colon cancer HCT116 cells were maintained in McCoy's 5A medium. Human colon cancer SW480 cells were maintained in Leibovitz's L-15 medium. All the medium was supplemented with 10% FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin.

2.3. Small interference RNA (siRNA) and short hairpin RNA (shRNA)

siRNA oligos were purchased from Gene Pharma (Shanghai). Cell transfection was conducted using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instruction. The sense sequences of the siRNA oligos are as follows.

si*IRE1a*-1: 5'GGAGAGAAGCAGCAGACUU3′;
si*IRE1a*-2: 5'GCGUAAAUUCAGGACCUAU3′;
si*XBP1*: 5'GGAACAGCAAGUGGUAGAUTT3′;
Control: 5'UUCUCCGAACGUGUCACGUTT3′.

The lentivirus expressing shRNAs were purchased from Genechem. Infection and stable cell selection were performed according to the technical manual. The targeted sequences are as follows:

sh*IRE1a*: 5'GCGTAAATTCAGGACCTAT3′.
shControl: 5'TTCTCCGAACGTGTCACGT3′.

siIRE1α-1 targets exon 6 of *IRE1a* and siIRE1α-2 targets exon 20 of *IRE1a*. shIRE1α targets exon 20 of *IRE1a*. siXBP1 targets exon 2 of *XBP1*. The cells stably expressing shRNA were infected with aforementioned virus and selected with puromycin.

2.4. Construction of vectors

The *FN1* promoter sequence from -3000 upstream to 100 downstream of human *FN1* gene transcription starting site was cloned by genomic PCR using human genomic DNA as a template. The *FN1* promoter (-3000~+100) luciferase reporter plasmid (FN1(-3000~+100)-Luc) was constructed into pGL3.0 vector (Promega). The FN1(-500~+100)-Luc reporter plasmid was constructed as described above. A mutated FN1-Luc plasmid derived from FN1(-500~+100)-Luc was constructed by means of site mutagenesis.

2.5. Western blot analysis

The cells were lysed in RIPA buffer containing protease inhibitor cocktail (Meilunbio) and phosphatase inhibitor cocktail (Bimake). Protein concentration was determined using Pierce™ BCA Protein Assay Kit (ThermoFisher). Identical amounts of proteins were subjected to SDS–PAGE and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% fat-free milk for 1 h at room temperature and incubated with primary antibody at 4 °C overnight, followed by incubation with secondary antibody at room temperature for 1 h. Antibodies against IRE1α, FAK, p-FAK(Tyr397), Src, p-Src(Tyr416), RAC1, CDC42 and RhoA were purchased from Cell Signaling Technology. Antibody against XBP1s was from BioLegend. SANI1(13,099) and TWIST(15,393) antibodies were from Proteintech and Santa Cruz, respectively. β-actin antibody was obtained from Sigma-Aldrich. The GAPDH antibody was a product of MultiSciences Lianke Biotech. All protein bands were detected using the LumiGLO® Reagent and Peroxide kit (Cell Signaling Technology).

2.6. Transwell assay

*M*igration assay was performed in a 24-well Transwell plate (Costar, 6.5 mm diameter, 8 μm pore size). Cells (1×10^5 per well) suspended in serum-free medium were seeded into the upper inserts and 500 u L medium containing 10% FBS was added to the bottom chamber. After certain hours of migration, cells on the upper face of the porous membrane were removed with a sterile cotton swab. Cells migrated to another side of the membrane were fixed in 4% paraformaldehyde and stained with Richard-Allan scientific Three-Step Stain Set. Inserts were digitally photographed on the lower side and migrated cells were counted on at least five different fields. For cell invasion assay, the experimental procedure was similar as described above, except that the transwell inserts were pre-coated with growth factor reduced Matrigel.

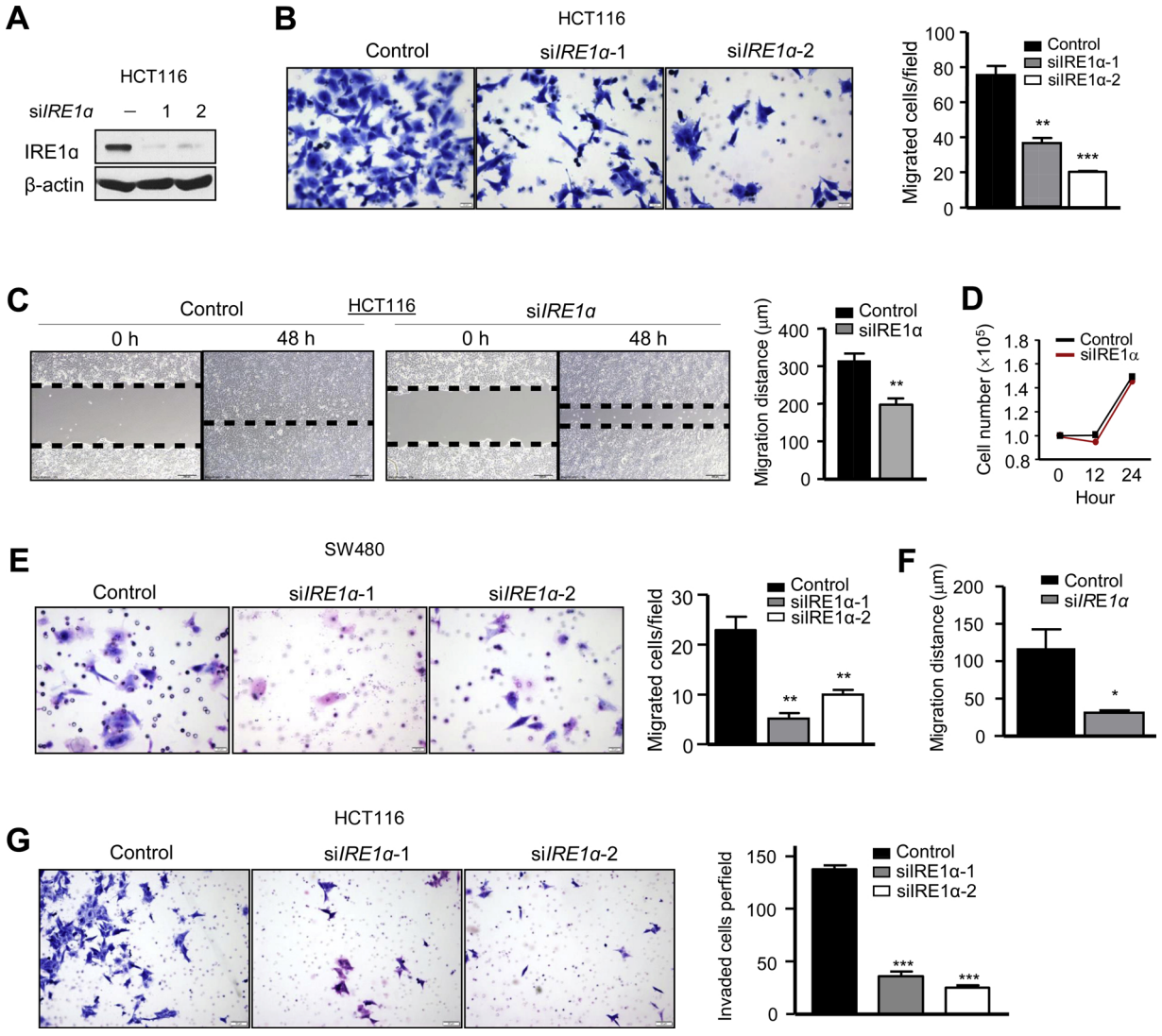

Fig. 1. Knockdown of IRE1α inhibits migration of colon cancer cells.
(A) HCT116 cells were transfected with control or IRE1α siRNA oligos. After 48 h, the cells were harvested for western blot. (B) HCT116 cells were transfected with control siRNA or IRE1α siRNA oligos. After 48 h, the transfected cells were seeded in a Transwell and allowed to migrate for 12 h. The migrated cells were determined as described in Methods. The right panel shows the quantification of migrated cells. (C) Wound-healing assay with HCT116 cells. The left panel shows the migration of the cells and the right panel shows the distance of migration of the cells. (D) Knockdown of IRE1α had little effect on proliferation of HCT116 cells. Cell proliferation was determined by counting the cells in a hemocytometer. (E) SW480 cells were transfected as above. After 48 h, the cells were seeded in a Transwell and allowed to migrate for 48 h. (F) Wound healing assay with SW480 cells. (G) HCT116 cells were transfected with siIRE1α oligos and cell invasion was determined as described in Methods. The cells were allowed to invade for 28 h. ns, not significant; *, $P < 0.05$; **, $P < 0.01$.

2.7. Wound healing assays

Monolayer (100% confluence) of the cells was wounded with a 20–200 μL pipette tip. The cells were washed twice with PBS and allowed migration for certain hours. The images of the cells were acquired under a microscope. The migrated distance of the cells was calculated using ImageJ software.

Migrated distance = [scratch width (start time)—scratch width (end time)]/2

2.8. In vivo metastasis assay

Five-week-old BALB/c nude mice (male) were anaesthetised by intraperitoneal injection of pentobarbital sodium (50 mg/kg). A laparotomy was conducted, followed by a slow injection of HCT116 cells (2×10^6) expressing control or IRE1α shRNA into the spleen. Ten minutes after injection, the spleen was removed. After 4 weeks, the mice were sacrificed and the livers were collected and the number of metastatic lesions was counted. The tissues were formalin-fixed and paraffin-embedded, followed by hematoxylin and eosin (H&E) stain. The mice were purchased from the Shanghai Experimental Animal Center (Shanghai) and maintained under a specific pathogen-free condition, kept in laboratory cages at 23 ± 3 °C with a humidity of 35 ± 5% under a 12 h dark/light cycle, and were maintained on a regular chow diet. All animals were used in accordance with the guidelines of the Institutional Animal Care and Use Committee of Shanghai Institute for Nutrition and Health, Chinese Academy of Sciences.

2.9. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted in TRIzol® (Invitrogen) and quantified in NanoDrop 2000 (Thermo Fisher Scientific). Five μg of total RNA was

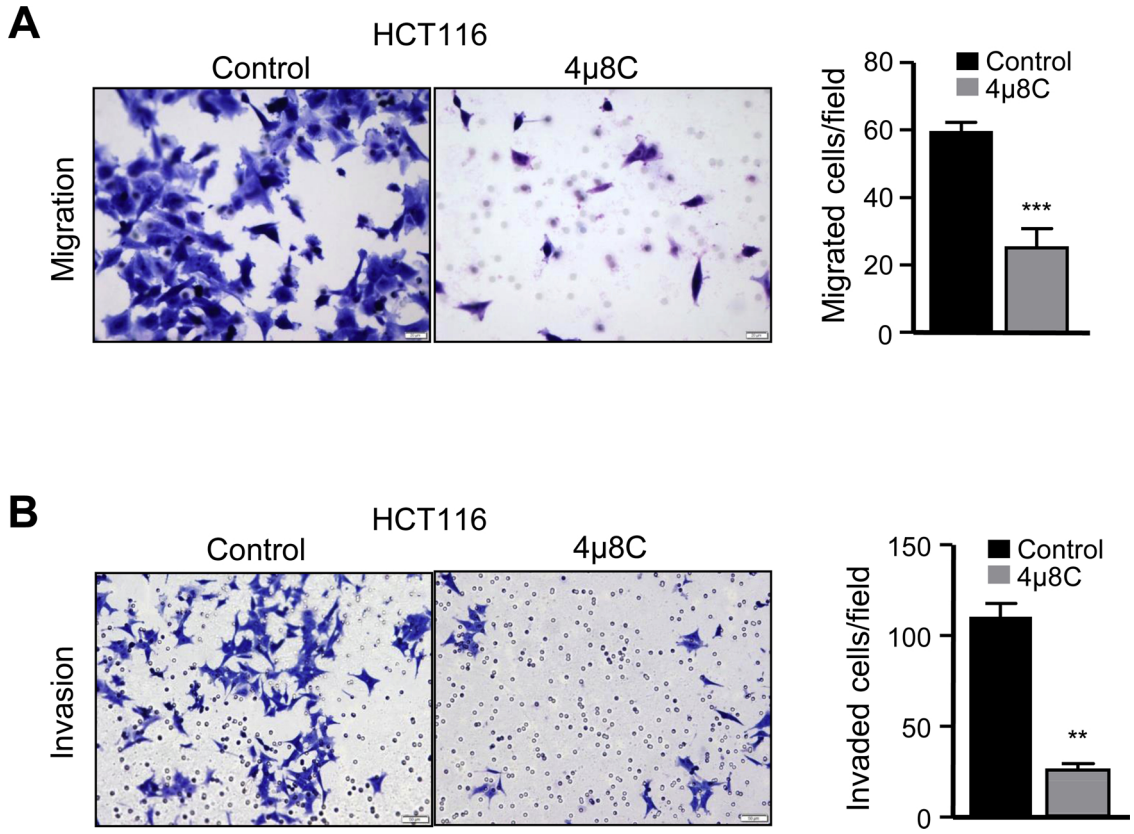

Fig. 2. 4μ8C inhibits colon cancer cell migration and invasion.
(A) 4μ8C inhibits migration of HCT116 cells. 4μ8C-treated cells were allowed to migrate for 12 h. The migrated cells were counted under a microscope. The left panel shows the image of migrated cells and the right panel is the quantification of migrated cells. (B) 4μ8C inhibits invasion of HCT116 cells. 4μ8C-treated cells were allowed to invade for 28 h. 4μ8C (20 μM). ns, not significant; **, $P < 0.01$; ***, $P < 0.001$.

reverse-transcribed using the M-MLV reverse transcriptase (Promega). qRT-PCR was performed on the Applied Biosystems Step Two Real-Time PCR System (Applied Biosystems). The SYBR® Green Realtime PCR Master Mix (Toyobo) was employed to detect the expression level of target genes. β-actin was used as internal control. The primers are as following:

FN1: 5'AGTCAAAGCAAGCCCGGTTG3′(F), 5'CAGTTAAAACCTCGGCTTCCTC3′(R);

E-cadherin: 5'TTCTTCGGAGGAGAGCGGTG3′(F), 5'CAAGTCAAAGTCCTGGTCCTC3′(R);

N-cadherin: 5'TCAGGCGTCTGTAGAGGCTT3′(F),5'ATGCACATCCTTCGATAAGACTG3′(R);

Vimentin: 5'AGTCCACTGAGTACCGGAGAC3′(F),5'CATTTCACGCATCTGGCGTTC3′(R);

TWIST1: 5'CTCCATCCTCCAGACCGAGA3′(F), 5'TCTACCAGGTCCTCCAGAGC3′(R);

SNAI1: 5'TCGGAAGCCTAACTACAGCGA3′(F), 5'AGATGAGCATTGGCAGCGAG3′(R);

IRE1a: 5'GTTTGTGTCAACGCTGGATGG3′(F), 5'TTCCACATGTGTTGGGACCTG3′(R);

MMP1: 5'CACGCCAGATTTGCCAAGAG3′(F), 5'GTCCCGATGATCTCCCCTGA3′(R);

MMP2: 5'TGGATGATGCCTTTGCTCGT3′(F), 5'CCAGGAGTCCGTCCTTACCG3′(R);

MMP3:5'TGGGCCAGGGATTAATGGAG3′(F), 5'GGCCAATTTCATGAGCAGCA3′(R);

MMP7: 5'TGAGCTACAGTGGGAACAGG3′(F), 5'TCATCGAAGTGAGCATCTCC3′(R);

MMP9: 5'GGGACGCAGACATCGTCATC3′(F), 5'GGGACCACAACTCGTCATCG3′(R);

MMP13: 5'CCTTCCCAGTGGTGGTGATG3′(F), 5'CGGAGCCTCTCAGTCATGGA3′(R);

β-actin: 5′GATCATTGCTCCTCCTGAGC3′(F), 5′ACTCCTGCTTGCTGATCCAC3′(R).

2.10. Dual luciferase reporter assay

Cells grown in 24 well plates were transfected with FN1 promoter luciferase reporter plasmid and Renilla luciferase reporter plasmid that is used as an internal control. After 24 h, the luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) as per the manufacturer's instruction.

2.11. GTPases pull-down assay

The recombinant protein GST-fused Rho-binding domain of the effector Rhotekin (GST-RBD) [Pertz et al., 2006] or GST-fused p21-binding domain of PAK1 (GST-PBD) [Price et al., 1998] were expressed in *E. coli* BL21 and purified by Glutathione-Sepharose 4B beads (GE Healthcare) according to the manufacturer's instruction. The cell lysate was incubated with Glutathione-Sepharose 4B beads coated with GST-PBD (PAK1) or GST-RBD (Rhotekin) at 4 °C for 2 h. The beads were washed three times with cold lysis buffer and the proteins were eluted and subjected to SDS-PAGE. Rac1-GTP and Cdc42-GTP bound to GST-PBD and RhoA-GTP bound to GST-RBD were detected by western blot.

2.12. Chromatin immunoprecipitation

The cells were fixed in 1% formaldehyde at 37 °C for 10 min. The glycine solution was added to stop the cross-linking. After that, cells

were washed and lysed in SDS lysis buffer containing protease inhibitors. Then chromatin was sheared to lengths between 200 and 1000 base-pairs by ultrasonic lysis. The chromatin fragments were immunoprecipitated with anti-XBP1s antibody or IgG by incubation at 4 °C for 2 h, followed by incubation with Protein A/G Magnetic Beads (MedChem Express) overnight. After washing, the complexes were eluted with the elution buffer. Purified immunoprecipitated DNA and input DNA were used as templates for qRT-PCR. The primers that were used are as follows.

Primer 1:

5'AGTTTGATGACCGCAAAGGAAAC3'(F), 5'AAGAGATGCTGATGGCCCGC3'(R);

Primer 2:

5'ATCCCTTCCCCCATCCCC3'(F), 5'ACGCTCCCCCTTCCCTTTTC3'(R);

Control:

5'CGTGCTTGTTAGCAGCAGGA3'(F), 5'ACACTTGCTAGTGGTCATCCA3'(R).

2.13. Statistical analysis

Data presented as the mean ± sem are from at least three independent experiments unless where indicated. Statistical analysis was performed using the unpaired two-tailed Student's *t*-test with GraphPad Prism 5.0.

3. Results

3.1. Knockdown of IRE1α inhibits migration of colon cancer cells

To define the role of IRE1α in the migration of colon cancer cells, we inhibited the expression of IRE1α by small interfering RNA (siRNA) and performed transwell and wound-healing assay. Colon cancer cell HCT116 were examined. Western-blot analysis showed that IRE1α was knocked down efficiently (Fig. 1A). Transwell assay revealed that depletion of IRE1α resulted in a dramatic decrease of migration of

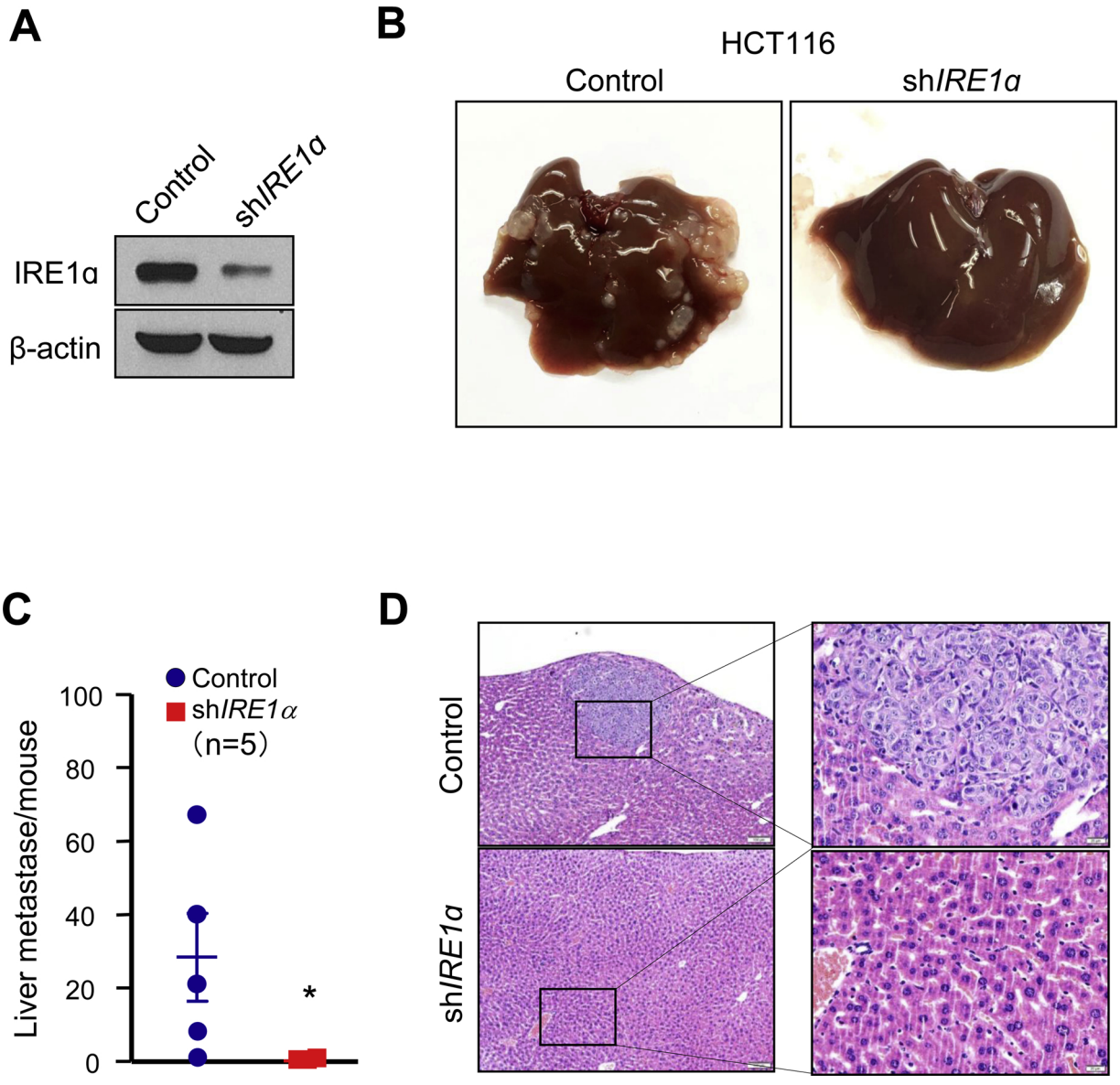

Fig. 3. Knockdown of IRE1 inhibits metastasis of HCT116 cells *in vivo*.
(A) HCT116 cells stably expressing shIRE1α were selected as described in Methods. The knockdown efficiency of IRE1α was determined by western blot. (B) Representative pictures of liver metastatic lesions. HCT116 cells (2×10^6) expressing control or shIRE1α were implanted into spleens of 5-week old nude mice (n = 5). After 4 weeks, the mice were sacrificed and liver lesion was photographed. (C) The number of metastatic lesions in each liver was counted (n = 5). (D) H&E stain of liver tissues of mice. *, $P < 0.05$.

HCT116 cells (Fig. 1B). Wound-healing assay results also show that knockdown of IRE1α suppressed migration of the cells (Fig. 1C). We determined proliferation of the cells under the experimental conditions and found that knockdown of IRE1α had little effect (Fig. 1D), suggesting that the reduced migration is not mainly due to the inhibition of cell proliferation. We examined another colon cancer cell SW480 and similar results were obtained (Fig. 1E and F). Besides, knockdown of IRE1α also inhibited invasion of HCT116 cells (Fig. 1G). These results suggest that IRE1α plays a positive role in the migration and invasion of colon cancer cells.

3.2. 4μ8C inhibits colon cancer cell migration

We then defined whether the RNase activity is required for IRE1α to modulate cell migration and invasion. We applied 4μ8C in our work. 4μ8C is a specific inhibitor of IRE1α RNase activity and it inhibits the formation of XBP1s [Cross et al., 2012; Kemp et al., 2013]. The results show that treatment of the cells with 4μ8C significantly inhibited the migration of HCT116 cells (Fig. 2A). 4μ8C also blocked the invasion of these cells (Fig. 2B). The results suggest that the RNase activity is required for IRE1α to regulate the migration of colon cancer cells.

3.3. Knockdown of IRE1α suppresses metastasis of colon cancer cells in vivo

Next, we determined the effect of knockdown of IRE1α on metastasis of colon cancer cells *in vivo*. IRE1α was knocked down by lentivirus-mediated shRNAs interference. HCT116 cells that stably expressed control or shIRE1α were selected. Western blot analysis showed the knockdown efficacy of IRE1α (Fig. 3A). The selected cells were implanted into the spleens of nude mice and liver metastases of the cells were determined. The results show that IRE1α knockdown inhibited significantly liver metastases of the cells (Fig. 3B and C). H&E stain of mice liver was shown (Fig. 3D). These results indicate that IRE1α-deficiency suppressed metastasis of colon cancer cells *in vivo*.

3.4. Knockdown of IRE1α suppresses FN1-Src/FAK-GTPases signaling

To identify the mediators of IRE1α-XBP1 signaling in the regulation of metastasis of colon cancer cells, we examined the activity of three Rho-family GTPases, Ras homolog family member A (RhoA), Rac family small GTPase 1 (Rac1) and cell division cycle 42 (CDC42). These three GTPases are key regulators of actin cytoskeleton dynamics and cell motility [Tapon and Hall, 1997; Ridley, 2001]. The activities of these GTPases were determined by using GTPases pull-down assay as described in Methods. The results show that GTP-coupled RhoA, Rac1 and CDC42 were decreased markedly in IRE1α knockdown cells compared with control cells (Fig. 4A). The protein tyrosine kinases Src and focal adhesion kinase (FAK) function as a central signaling scaffold that is critical for the activation of downstream GTPases signaling pathways [Cary et al., 1996; Hildebrand et al., 1996; Hsia et al., 2003; Zhai et al., 2003; Lim et al., 2008]. The activity of FAK depends on the phosphorylation of tyrosine residue 397, which induces an interaction with Src that stabilizes the active conformation of Src, leading to increased catalytic activity [Mitra et al., 2005]. We found that there was a remarkable decrease of phosphorylation of Src and FAK in IRE1α knockdown cells (Fig. 4B).

We did qRT-PCR to determine the influence of knockdown of IRE1α on the expression of EMT related genes including *E-cadherin*, *N-cadherin*, *Vimentin*, *Fibronectin 1*, *TWIST* and *SNAI1*. We found that knockdown of IRE1α caused a decrease in the transcription of *Vimentin*,

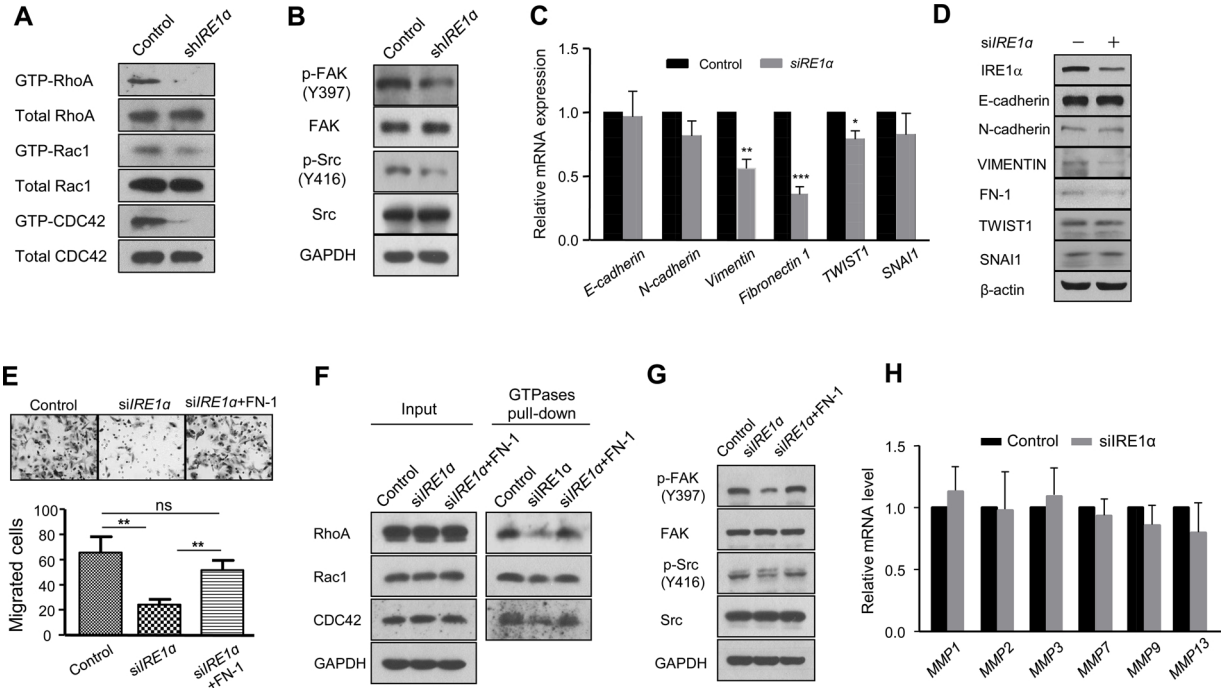

Fig. 4. Knockdown of IRE1α inhibits Src/FAK/GTPase signaling *via* FN1.
(A) HCT116 cells expressing control or shIRE1α were used in the test. The determination of activation of RhoA, Rac1 and CDC42 was performed as described in Methods. (B) Knockdown of IRE1α inhibited p-FAK and p-Src in HCT116 cells. (C) qRT-PCR was performed to determine the expression of the genes indicated. HCT116 cells stably expressing control or shIRE1α were examined. (D) Knockdown of IRE1α decreased the protein level of FN-1. (E) HCT116 cells were transfected as indicated. After 48 h, the cells were seeded on plates pre-coated with coating buffer in the absence or presence of FN1 (5 μg/mL) and allowed to migrate for 12 h. (F) HCT116 cells were transfected with siIRE1α oligos for 48 h. The cells were then seeded on plates that were pre-coated with coating buffer in the absence or presence of FN1 (5 μg/mL). After 4 h, the cells were collected and RhoA-GTP, Rac1-GTP and CDC42-GTP levels were determined by a GTPases pull-down assay followed by western blot. (G) HCT116 cells were treated as described above. FAK and Src phosphorylation was determined. (H) Knockdown of IRE1α had little effect on expression of MMP1, 2, 3, 7, 9 and 13. HCT116 cells were transfected with siIRE1α oligos. After 48 h, cells were harvested for qRT-PCR. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Fibronectin 1 and TWIST1 in HCT116 cells (Fig. 4C). We also did westernblot and found that knockdown of IRE1α decreased the protein levels of fibronectin 1 (FN1) and vimentin, with little effect on the levels of other proteins including E-cadherin, N-cadherin, TWIST and SNAI1 (Fig. 4D). FN1, a major ECM constituent [Topalovski and Brekken, 2016], binds to membrane-spanning receptor proteins called integrins, which leads to phosphorylation of FAK and Src [Lipfert et al., 1992; Vuori, 1998; Giancotti and Ruoslahti, 1999], resulting in activation of Rho GTPases and cell migration [Bourdoulous et al., 1998; Mukai et al., 2002]. Based on these results, we proposed that FN1 was involved in the regulation of cell migration by IRE1α. To verify this, we added exogenous FN1 in cell migration assay. As expected, the inhibition of cell migration by IRE1α-knockdown was reversed by addition of FN1 (Fig. 4E). Furthermore, the activation of small GTPases RhoA, Rac1 and CDC42 and their upstream regulators Src and FAK was also reversed by addition of FN1 (Fig. 4F and G). Together, these results suggest that IRE1α regulates colon cancer cells migration *via* FN1-Src/FAK-GTPases signaling.

The matrix metalloproteinase (MMPs) play an important role in the metastasis of cancer cells. We also determine the effect of IRE1α-knockdown on expression of the genes encoding *MMP1*, *MMP2*, *MMP3*, *MMP7*, *MMP9* and *MMP13*. Our results show that knockdown of IRE1α had little effect on the transcript levels of these genes (Fig. 4H).

3.5. IRE1α regulates FN1 expression through XBP1

Since IRE1α regulates FN1 expression at a transcriptional level (Fig. 4A) and the transcription factor XBP1s is a well-known product of IRE1α, we presumed that XBP1s was involved in the regulation of FN1 expression by IRE1α. To study the transcription of *FN1* gene, we constructed a *FN1* promoter (−3000˜+100)-driven luciferase reporter plasmid (Fig. 5A). We found that the knockdown of IRE1α suppressed luciferase activities of the reporter (Fig. 5B_1). Inhibition of IRE1α RNase activity by 4μ8C also resulted in a decrease of luciferase activities (Fig. 5B_2). Moreover, knockdown of XBP1 decreased the luciferase activities as well (Fig. 5B_3). These results suggest that XBP1s regulates the transcription of *FN1*. To narrow down the range of possible binding site for XBP1s, we constructed a luciferase reporter plasmid driven by a shorter *FN1* promoter (−500˜+100) (Fig. 5C). 4μ8C or XBP1-knockdown also resulted in a similar reduction of FN1(−500˜+100) reporter activities (Fig. 5D and E). These results suggest that XBP1s may bind the region (−500˜+100) of *FN1* promoter. Analysis of this region revealed a putative XBP1s binding site ACGTCA that is located from −181 to −176. On the basis of FN1(−500˜+100) luciferase reporter plasmid, we constructed a mutated *FN1* reporter (ACGTCA→ACTTCA) (Fig. 5F). We found that knockdown of XBP1 inhibited luciferase activity of the wild-type reporter but had little effect on luciferase activity of the

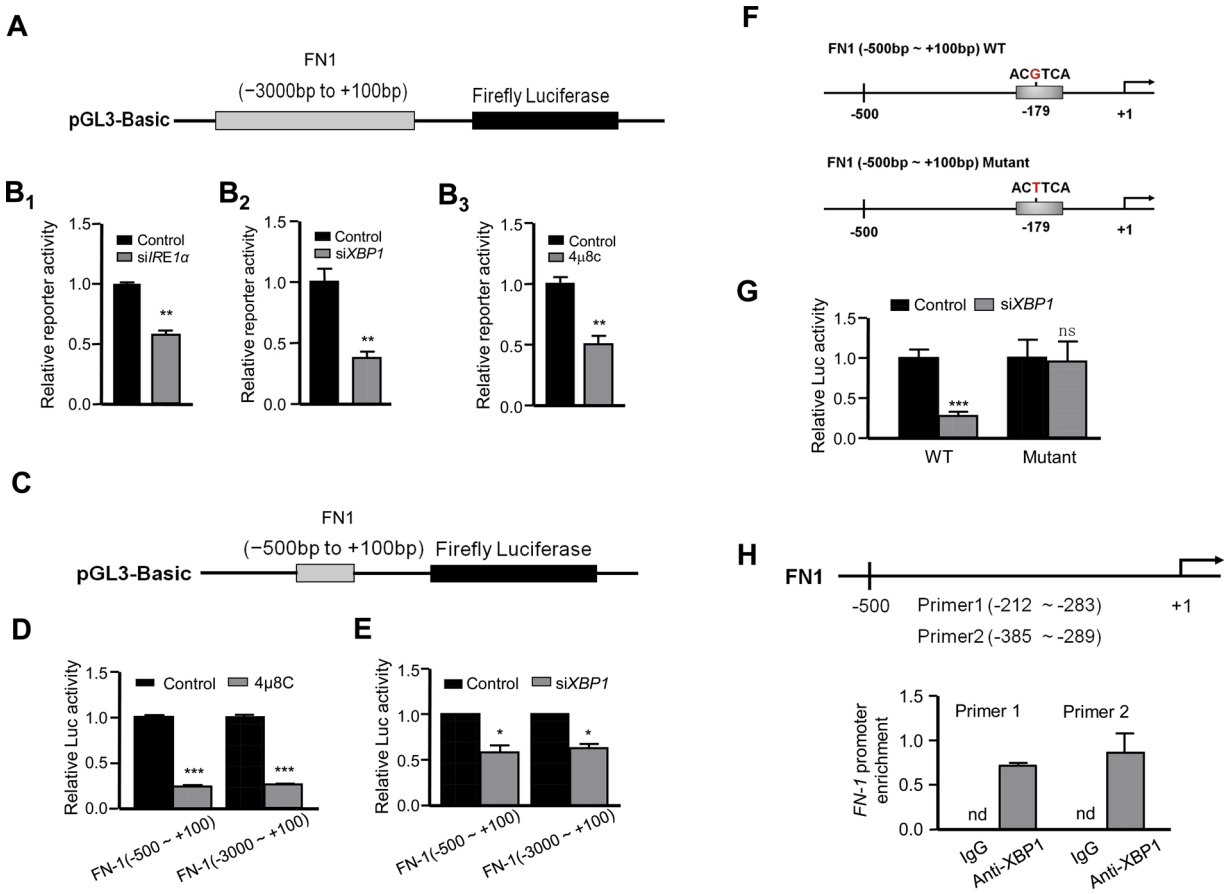

Fig. 5. XBP1s acts as a transcription factor of FN1.
(A) The diagram of the FN1(-3000˜+100) luciferase reporter plasmid. (B) HCT116 cells expressing FN1(-3000˜+100)-Luc reporter were transfected with siIRE1α (B_1) or siXBP1 (B_2) oligos as indicated. After 48 h, the cells were harvested for determining luciferase activity. The cells expressing this reporter were also treated with 4μ8C (20 μM) (B_3) for 24 h, followed by luciferase activity assay. (C) The schematic diagram of the FN1(-500˜+100)-Luc reporter plasmid. (D) HCT116 cells were transfected with FN1(-500˜+100)-Luc or FN1(-3000˜+100)-Luc reporter plasmid and the transfected cells were treated with 4μ8C (24 μM) for 24 h. (E) HCT116 cells expressing luciferase reporter were transfected with siXBP1 oligos. After 48 h, the cells were harvested. (F) The schematic diagrams of the wild-type *FN1* promoter with putative XBP1 binding site ACGTCA and mutated *FN1* promoter (ACTTCA). The wild-type and mutated sites were indicated in grey box. (G) HCT116 cells expressing the wild-type or mutated FN1 reporter were transfected with siXBP1 oligos. After 48 h, the cells were harvested for luciferase activity assay. (H) XBP1s binds the *FN1* promoter. HCT116 cells were used in the experiment. The upper panel indicated the design of primers used for ChIP. The lower panel shows the enrichment of *FN1* promoter. nd, not detected. ns, not significant; *, $P < 0.05$; **, $P < 0.01$.

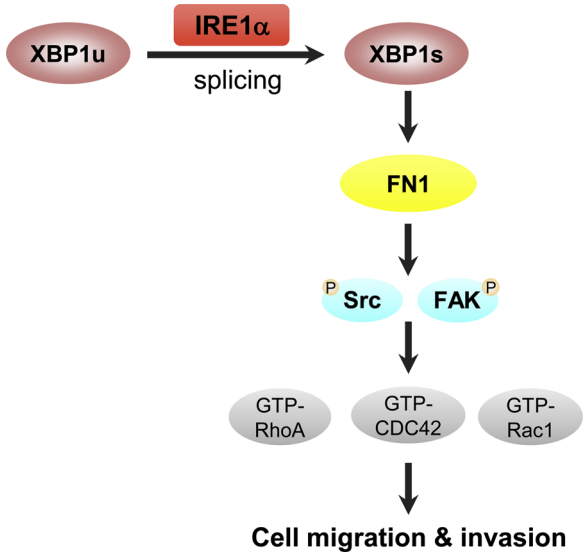

Fig. 6. Proposed working model for IRE1α.
IRE1α splices XBP1 mRNA to form activated transcription factor XBP1s that induces the expression of FN1. FN1 then activates downstream Src/FAK-GTPases signalling, promoting cell migration and invasion.

mutated one (Fig. 5G). Thus, $^{-181}$ACGTCA^{-176} might be the binding site for XBP1s.

To verify that XBP1s could bind the *FN1* promoter, we performed chromatin immunoprecipitation (ChIP) assay. Two pairs of PCR primer were designed for the experiment. The results indicate that immunoprecipitation by XBP1s antibody substantially enriched *FN1* promoter, but not by the IgG control (Fig. 5H). Taken together, these results suggest that XBP1s binds *FN1* promoter and regulates its transcription (Fig. 6).

4. Discussion

Here, we have demonstrated that loss of IRE1α suppresses migration and invasion of colon cancer cells. Inhibition of IRE1α suppresses the expression of FN1 by the transcription factor XBP1s, which in turn results in inhibition of Src/FAK phosphorylation and Rho GTPases (RhoA, Rac1 and CDC42) activation, leading to inhibition of cell migration and invasion.

ER stress is a common feature of cancers. Cancer cells adapt to ER stress through activating UPR. The UPR is involved in the acquisition of a few malignant characteristics that allow tumor growth. Recent studies indicate UPR may affect all the cardinal hallmarks of cancer, including angiogenesis, metastasis, genome stability, inflammation, and drug resistance (Urra et al., 2016; Hetz and Papa, 2018). XBP1s, the product of IRE1α, functions as a transcription factor during ER stress by regulating UPR to maintain ER homeostasis. Besides, XBP1s may have other functions. For instance, XBP1s induces phospholipid biosynthesis and ER expansion [Sriburi et al., 2004] and modulates endostatin-induced autophagy in through BECN1 transcriptional activation [Margariti et al., 2013]. XBP1s also plays a role in tumor progression. It was found in breast cancer cells that it stimulated *SNAI1* transcription to promote the metastasis of breast cancer cells [Li et al., 2015]. We demonstrate in this manuscript that XBP1s controls the transcription of *FN1*. Our results indicate that XBP1s binds the promoter region of *FN1* and initiates the transcription of *FN1*. We have discovered in our work a new target of IRE1α-XBP1 signaling that is involved in the regulation of cell migration. In addition to FN1, our results show that knockdown of IER1α also inhibits the expression of vimentin (Fig. 4C). So, it is possible that IRE1α may also regulate cancer cell metastasis through vimentin and this needs investigation in future.

Tumor cells are prone to ER stress, because of dysregulated protein synthesis and the tumor microenvironments such as nutrient deficiency or lack of oxygen. And this leads to elevated activation of UPR in tumors, compared with normal tissues. IRE1α activation has been found in cancers [Wang and Kaufman, 2014; Oakes and Papa, 2015] and it is believed that the activation of IRE1α contributes to oncogenic processes including metastasis [Ma and Hendershot, 2004; Chevet et al., 2015]. In summary, our studies suggest that IRE1α regulates the metastasis of colon cancer cells through the FN1-Src/FAK-GTPases axis. The findings may help understand the mechanism underlying the regulation of migration and invasion of colon cancer cells by IRE1α.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Acknowledgements

This work was supported by the Ministry of Science and Technology of China (2017YFD0400206), National Natural Science Foundation of China (31670785, 31525016) Shanghai Ministry of Science and Technology (16JC1406100) and Chinese Academy of Sciences (KFZD-SW-213).

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