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ERAP2 is a novel target involved in autophagy and activation of pancreatic stellate cells via UPR signaling pathway

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ABSTRACT

Background/objectives: Pancreatic ductal adenocarcinoma (PDAC) is characterized by excessive desmoplasia and autophagy-dependent tumorigenic growth. Pancreatic stellate cells (PSCs) as a predominant stromal cell type play a critical role in PDAC biology. We have previously reported that autophagy facilitates PSC activation, however, the mechanism remains unknown. We investigated the mechanism of autophagy in PSC activation.
Methods: We compared gene expression profiles between patient-derived PSCs from pancreatic cancer and chronic pancreatitis using a microarray. The stromal expression of target gene in specimen of PDAC patients (n = 63) was analyzed. The effect of target gene on autophagy and activation of PSCs was investigated by small interfering RNAs transfection, and the relationship between autophagy and ER stress was investigated. We analyzed the growth and fibrosis of xenografted tumor by orthotopic models.
Results: In analysis of gene expression microarray, endoplasmic reticulum aminopeptidase 2 (ERAP2) upregulated in cancer-associated PSCs was identified as the target gene. High stromal ERAP2 expression is associated with a poor prognosis of PDAC patients. Knockdown of ERAP2 inhibited unfolded protein response mediated autophagy, and led to inactivation of PSCs, thereby attenuating tumor-stromal interactions by inhibiting production of IL-6 and fibronectin. In vivo, the promoting effect of PSCs on xenografted tumor growth and fibrosis was inhibited by ERAP2 knockdown.
Conclusions: Our findings demonstrate a novel mechanism of PSCs activation regulated by autophagy. ERAP2 as a promising therapeutic target may provide a novel strategy for the treatment of PDAC.
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1. Introduction

Pancreatic cancer is an aggressive malignant tumor with a 5-year survival rate of 9% [1]. It is projected to become the second leading cause of cancer-related death by 2030 [2]. Surgery is the only potentially curative option and chemotherapy can extend overall survival [3,4]. Because the current treatments of pancreatic cancer are insufficient to improve the survival rate significantly, it is urgent to find a viable strategy to improve therapeutic effectiveness.

Pancreatic ductal adenocarcinoma (PDAC) is characterized by excessive desmoplasia, in which pancreatic stellate cells (PSCs) as the predominant stromal cell type play a major role through tumor-stromal interactions [5]. When exposed to various stimuli in the tumor microenvironment, PSCs transdifferentiate from a quiescent to activated state represented by losing vitamin A-containing lipid droplets in the cytoplasm, undergoing morphological and functional changes, becoming “myofibroblast-like” cells, and producing large amounts of extracellular matrix (ECM) components, cytokines, and chemokines to facilitate pancreatic cancer cell (PCC) aggressiveness and therapeutic resistance [6].

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We have previously shown that macroautophagy (hereafter referred to as autophagy) is associated with PSC activation [7]. Autophagy is a dynamic process involved in energy and nutrient homeostasis [8], and a broad spectrum of human diseases [9,10]. The roles of autophagy in cancer are contradictory in different cancer contexts [11,12]. In pancreatic cancer, production and secretion of activated PSCs is critical for cancer cell metabolism and aggressiveness, which is also regulated by autophagy [7,13]. Therefore, stroma-targeting therapy is a potentially promising strategy for pancreatic cancer [14,15]. However, the mechanism of PSC activation remains to be further elucidated [16]. Therefore, autophagy may fully or partially explain the mechanism of PSC activation.

Endoplasmic reticulum (ER) is an organelle where native folding and initial post-translational modifications occur [17]. Under conditions such as hypoxia, nutrient deprivation, and infection, the folding capacity of ER is exceeded, which is referred to as ER stress, resulting in accumulation of unfolded proteins in the ER. Correspondingly, the unfolded protein response (UPR) activates to restore ER protein-folding homeostasis [18]. Autophagy is initiated at the ER membrane and induced via the UPR pathway to relieve the burden of accumulating aberrant proteins during ER stress [19–21]. Furthermore, ER stress is implicated in activation of hepatic stellate cells (HSCs) in liver fibrosis [22]. Therefore, we hypothesized that ER activity is the crosstalk between autophagy and activation of PSCs.

Here, we applied a gene expression microarray and demonstrated that ER-resident target endoplasmic reticulum aminopeptidase 2 (ERAP2) is highly expressed in activated PSCs. We found that ERAP2 plays an important role in autophagy of PSCs, which regulates activation of PSCs through ER-derived autophagy. This process is mediated by ER stress and consequent UPR signaling pathways. Therefore, ERAP2 as a promising therapeutic target may provide a novel strategy for the treatment of PDAC.

2. Materials and methods

2.1. Patient specimens

Pancreatic cancer specimens were obtained from 63 patients who underwent a pancreatoduodenectomy for PDAC at our institution from 2005 to 2014. Normal pancreatic tissue specimens were obtained from subjects with bile duct or duodenal papilla cancers. For a detailed description, please refer to the Supplementary Materials and Methods section.

2.2. Cells and culture conditions

PSCs were established from fresh surgical specimens of patients with pancreatic cancer or chronic pancreatitis using the outgrowth method, as described in our previous reports [7,14]. PSCs up to passage 8 were used for experiments. HPaSteCs isolated from human normal pancreas were purchased from ScienCell Research Laboratories (#3830; Carlsbad, CA) and maintained in Stellate Cell Medium (#5301; ScienCell Research Laboratories). PCC lines SUIT-2 (Health Science Research Resources Bank, Osaka, Japan) and Panc-1 (Riken BioResource Center, Tsukuba, Japan) were used in this study.

Activation of PSCs was induced by treatment with TGF-β1 (#100-21; PeproTech, Rocky Hill, NJ). Tunicamycin was purchased from EMD Millipore (#654380; Billerica, MA) for ER stress induction.

2.3. Immunofluorescence staining

Immunofluorescence staining was performed as described in Supplementary Materials and Methods section. Images were acquired under the fluorescence microscope.

2.4. Lipid droplet accumulation assay

PSCs (4×10^4 cells) were seeded in a 35-mm glass-bottom dish and incubated for 24 h, followed by staining with 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503, 1 μg/ml, #D-3922; Life Technologies, Carlsbad, CA) and 1 μg/ml 4′,6-diamidino-2-phenylindole at room temperature for 1 h. BODIPY-positive puncta were calculated per cell in a total of twenty cells using ImageJ software (National Institutes of Health, Bethesda, MD).

2.5. Quantitative reverse transcription-polymerase chain reaction

Quantitative reverse transcription polymerase chain reaction was performed as described previously [7]. Primers for α-SMA, GAPDH (Takara Bio, Shiga, Japan), and ERAP2 (Integrated DNA Technologies, Coralville, IA) were used, the sequences were listed in Supplementary Table S1.

2.6. Western blot analysis

Western blot analysis was performed as described in the Supplementary Materials and Methods section. Antibodies used for protein detection are shown in Supplementary Table S2.

2.7. Enzyme-linked immunosorbent assay

After transfection of PSCs with siERAP2 for 72 h, the medium was replaced with serum-free DMEM, followed by incubation for 48 h. Concentrations of IL-6 in PSC culture supernatants were determined by a Human IL-6 Quantikine ELISA Kit (#D6050; R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The experiment was performed in triplicate and repeated twice.

2.8. In vivo experiments

Female 4-week-old BALB/c-nu/nu mice were purchased from Clea Japan (Tokyo, Japan). For orthotopic models, SUIT-2 cells (5×10^5) or SUIT-2 cells (5×10^5) and shERAP2- or shControl-transfected imPSCs (5×10^5) were suspended in 50 μl DMEM and implanted into the pancreatic tail of mice. For a detailed description, please refer to the Supplementary Materials and Methods section.

Cell viability assay, migration and invasion assays, and small interfering RNA and small hairpin RNA transfection procedures are described in the Supplementary Materials and Methods section.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA). Results are presented as the mean ± standard deviation. Differences between two groups were statistically analyzed using the Student's *t*-test. Comparison of ERAP2 expression in the database, and analysis of xenografted tumor were performed using the Wilcoxon test. Survival analysis was performed by Kaplan–Meier method, and curves were analyzed using the log-rank test.

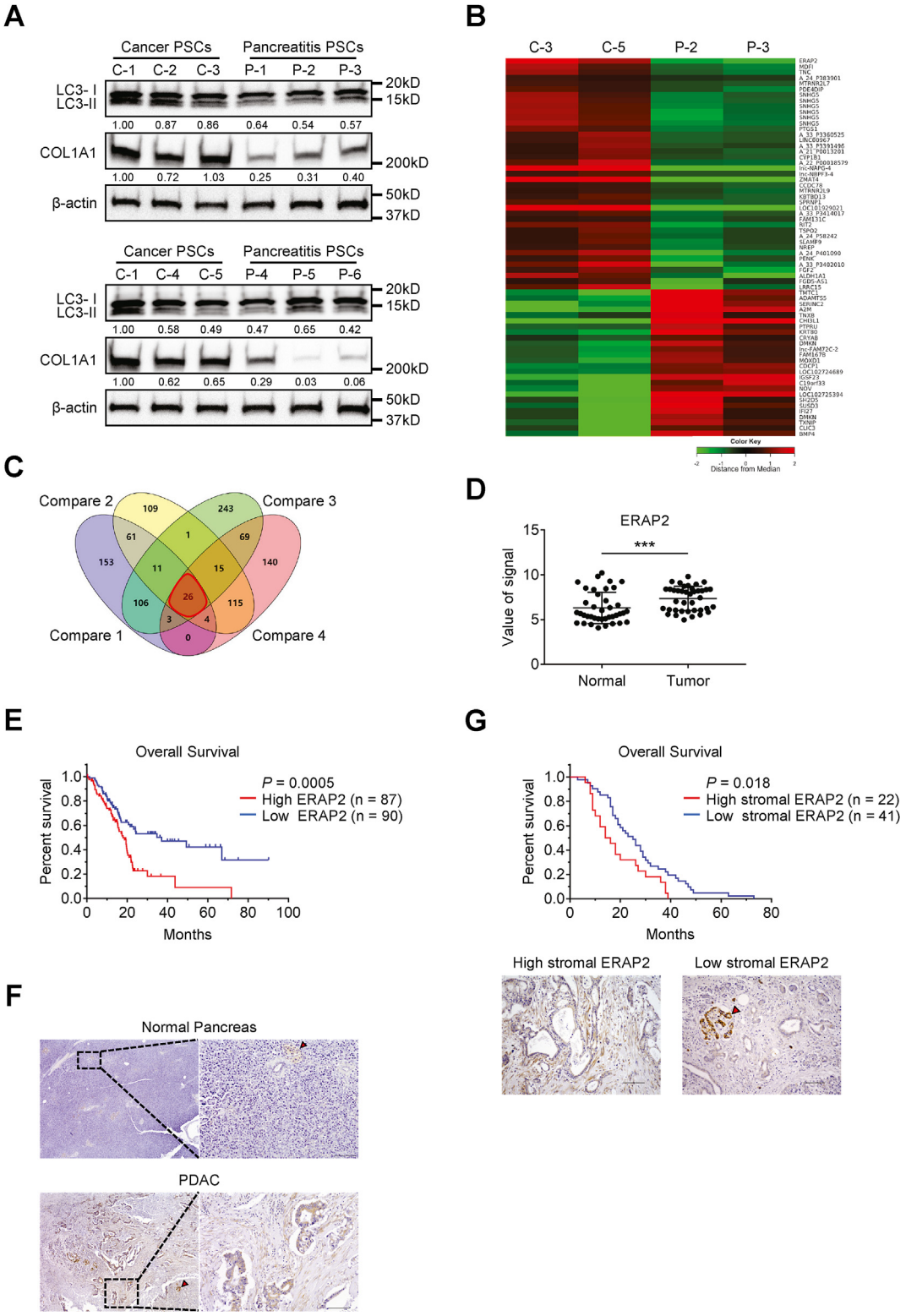

Fig. 1. ERAP2 is highly expressed in cancer-associated PSCs and associated with poor prognosis of PDAC patients. (A) Levels of LC3 and COL1A1 in human primary cultured PSCs. Values indicate densitometric ratios normalized to β-actin. (B) Heatmap of gene microarray analysis between cancer and pancreatitis-derived PSCs (GEO accession number: GSE172168). (C) Venn diagram of the microarray analysis. (D) Relative mRNA levels of ERAP2 in normal pancreatic and PDAC tissues ($n = 39$) from the GEO database (Accession number: GSE15471). (E) Kaplan–Meier analysis of overall survival based on ERAP2 expression in PDAC ($n = 177$) using the TCGA database. ERAP2 mRNA expression exponential>0 was defined as high expression. (F) Representative images of immunohistochemical staining for ERAP2. ERAP2 expression was not detected in normal pancreatic tissue (top), and variously expressed both in pancreatic cancer tissue and adjacent stromal tissue (bottom). Original magnification: 40× (left), 200× (right). (G) Top, Kaplan–Meier analysis of overall survival based on stromal ERAP2 expression in PDAC ($n = 63$). Bottom, representative images of immunohistochemical staining of stromal ERAP2 in PDAC. Islet cells are positive for ERAP2 (arrowhead). Original magnification: 200× . Scale bar = 100 μm. *P*-value was determined by Wilcoxon test. ***$P < 0.001$.

3. Results

3.1. Gene expression profiles of cancer-associated pancreatic stellate cells

We compared pancreatic stellate cells (PSCs) derived from pancreatic cancer and chronic pancreatitis patients. Autophagy and collagen levels were higher in cancer-associated PSCs than in those derived from chronic pancreatitis, suggesting a higher level of activation in cancer-associated PSCs (Fig. 1A). Considering the characteristics of these PSCs, two primary cultures of PSCs were selected as representative of cancer-associated PSCs (C-3 and C-5)

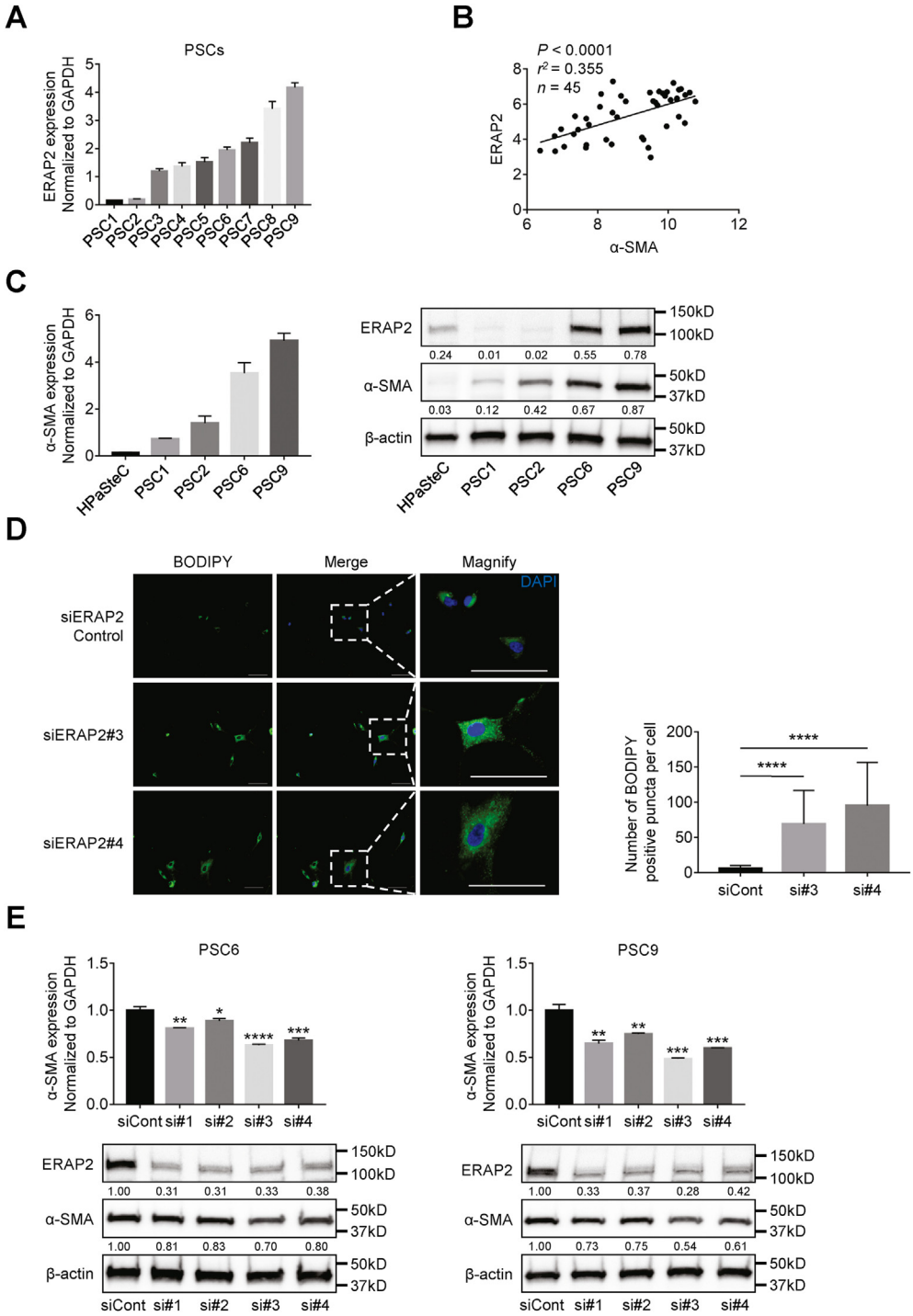

Fig. 2. Activation of PSCs is inhibited by ERAP2 knockdown. (A) Levels of ERAP2 mRNA in PSCs. (B) Linear relationship between ERAP2 and α-SMA mRNA levels in non-tumor tissues of PDAC ($n = 45$) from the GEO database (Accession number: GSE28735). ERAP2 is correlated with α-SMA expression. (C) Levels of α-SMA mRNA (left) and α-SMA and ERAP2 proteins (right) in normal PSCs (HPaSteCs) and human primary cultured PSCs. Values indicate densitometric ratios normalized to β-actin. (D) Left, representative images of neutral lipid staining with BODIPY in PSCs. Right, quantification of BODIPY-positive puncta per cell ($n = 20$ cells). Scale bar = 100 μm. (E) Effect of siRNA-mediated ERAP2 knockdown on the levels of α-SMA mRNA (top) and protein (bottom) in PSCs. ERAP2 knockdown inhibited α-SMA expression in PSCs. Values indicate densitometric ratios normalized to β-actin. Data are mean ± S.D. *P*-values were determined by unpaired two-tailed *t*-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

and their pancreatitis-derived counterparts (P-2 and P-3). To determine the potential targets involved in autophagy and activation of PSCs, gene expression profiles of these PSC groups were compared using a gene expression microarray (Fig. 1B). With a Z-score of >2.0 and ratio of >1.5, 26 hits were upregulated in cancer-associated PSCs compared with their counterparts (Fig. 1C). Among these genes, endoplasmic reticulum aminopeptidase 2 (ERAP2), which resides in the endoplasmic reticulum (ER) membrane, was ranked as the top-scoring gene in microarray analysis (Fig. 1B). Previous reports have demonstrated that the ER in activated PSCs undergoes a morphological change to become more prominent and increases in numbers [23,24]. Thus, we hypothesized that ERAP2 is associated with PSC activation through ER functions.

To test our hypothesis, clinical relevance of ERAP2 in pancreatic cancer was analyzed using the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geoprofiles. ID: 78910644). The ERAP2 mRNA level in pancreatic ductal adenocarcinoma (PDAC) was significantly higher than that in normal pancreatic tissue (Fig. 1D). The Cancer Genome Atlas dataset analysis revealed that high ERAP2 mRNA expression was associated with a poor prognosis of PDAC patients (Fig. 1E). Immunohistochemical staining showed that ERAP2 protein was expressed in both fibroblasts and pancreatic carcinoma cells, but not detected in chronic pancreatitis and normal pancreatic tissue (Fig. 1F and Supplementary Fig. S1A). Then, we focused on the stromal ERAP2 expression and found that its expression was related to poor prognosis of patients with PDAC (Fig. 1G).

3.2. ERAP2 affects pancreatic stellate cell activation through autophagy

ERAP2 mRNA was variously expressed in primary cultured human PSCs (Fig. 2A). GEO profiles (ID: 200028735) showed that α-smooth muscle actin (α-SMA), a marker of PSC activation, was positively correlated with ERAP2 expression in non-tumor tissues adjacent to PDAC tumor tissue ($r^2 = 0.355$, $P < 0.0001$) (Fig. 2B). This correlation was further confirmed in PSCs (Fig. 2C). These data indicated that ERAP2 expression was associated with PSC activation. We previously reported that inhibition of autophagy induces a quiescent state of PSCs [7]. Therefore, we investigated whether inhibition of PSC autophagy also resulted from ERAP2 knockdown, followed by conversion of PSCs into an inactivated state. After effective knockdown of ERAP2 in PSCs (Supplementary Fig. S2A), lipid droplet re-accumulation (Fig. 2D) and decreased mRNA and protein levels of α-SMA were observed (Fig. 2E), indicating conversion of PSCs into a quiescent state after ERAP2 knockdown.

Because the ER has been recently reported to be essential for autophagy initiation [19,25,26], we next investigated whether ERAP2 was related to autophagy in PSCs. After ERAP2 knockdown in PSCs, a decreased level of LC3-II and increased level of p62 were

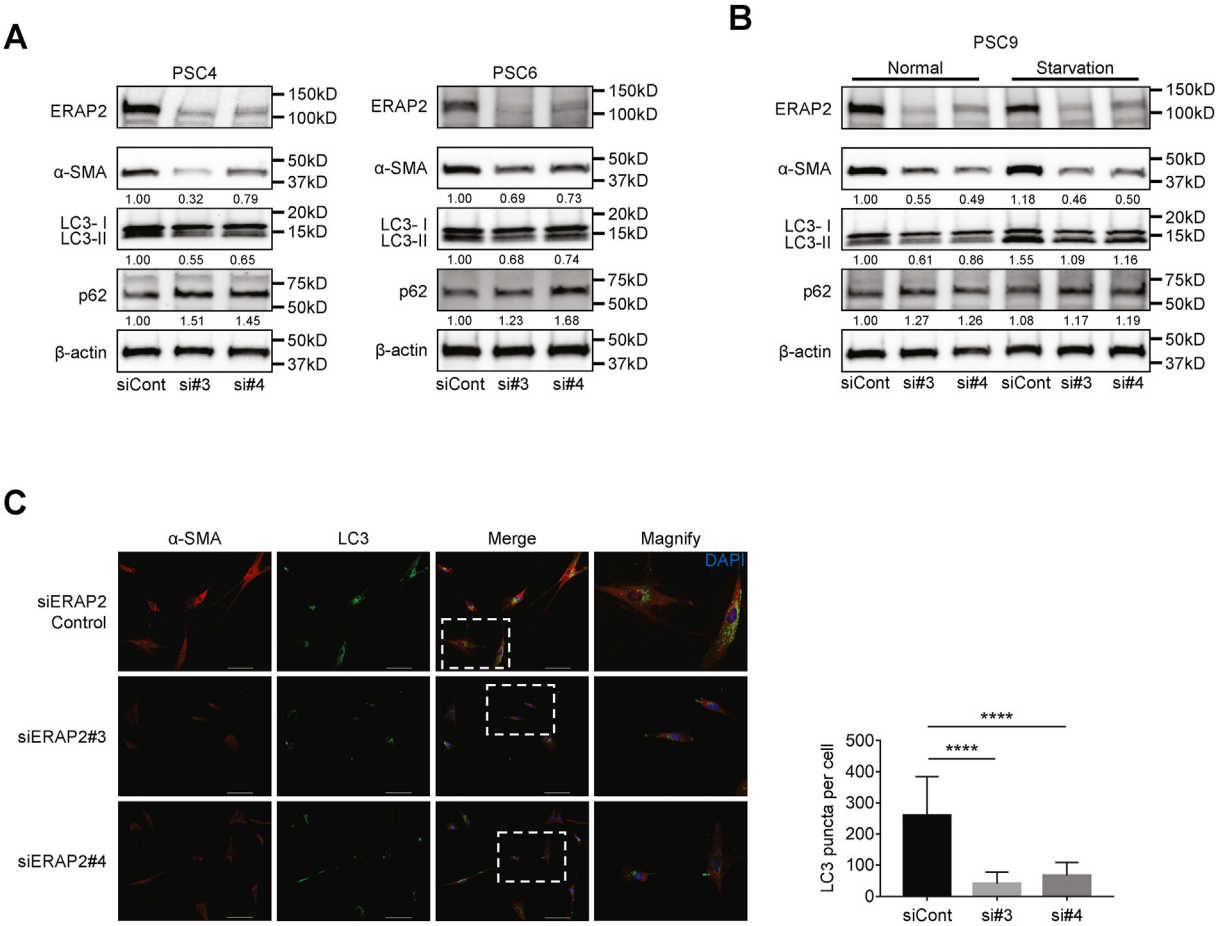

Fig. 3. ERAP2 affects the autophagy activity of PSCs. (A) Effect of siRNA-mediated ERAP2 knockdown on the levels of α-SMA, LC3 and p62 proteins in PSCs. Values indicate densitometric ratios normalized to β-actin. (B) Comparison of the effect of ERAP2 knockdown on α-SMA, LC3, and p62 protein levels in PSCs. Under normal and amino acid starvation conditions, autophagy activity was suppressed by ERAP2 knockdown. Values indicate densitometric ratios normalized to β-actin. (C) Representative images of immunofluorescence staining for α-SMA and LC3 in PSCs (left) and quantification of LC3 puncta per cell in 20 cells (right). Scale bar = 100 μm. Data are mean ± S.D. *P*-values were determined by unpaired two-tailed *t*-test. ****$P < 0.0001$.

found (Fig. 3A). We next evaluated autophagy activity under autophagy induction conditions. Under starvation conditions, LC3-II expression and degradation of p62 were inhibited in ERAP2 knockdown PSCs (Fig. 3B). Moreover, inhibition of autophagic flux was also confirmed after ERAP2 knockdown (Supplementary Fig. S3A). Immunofluorescence staining also confirmed the inhibition of α-SMA and LC3-II expression after ERAP2 knockdown (Fig. 3C). Thus, we concluded that ERAP2 functions in PSC activation through autophagy.

3.3. ERAP2 affects tumor-stromal interactions

Migratory and invasive potentials of PCCs, which were indirectly cocultured with PSCs, were enhanced by tumor-stromal interactions, as reported previously [27,28]. ERAP2 knockdown weakened the capacity of PSCs to promote migration and invasion of PCCs (Fig. 4A and B), while the proliferative capacity of PSCs was unaffected by ERAP2 knockdown (Supplementary Fig. S4A). Expression of fibronectin, a major component of the extracellular

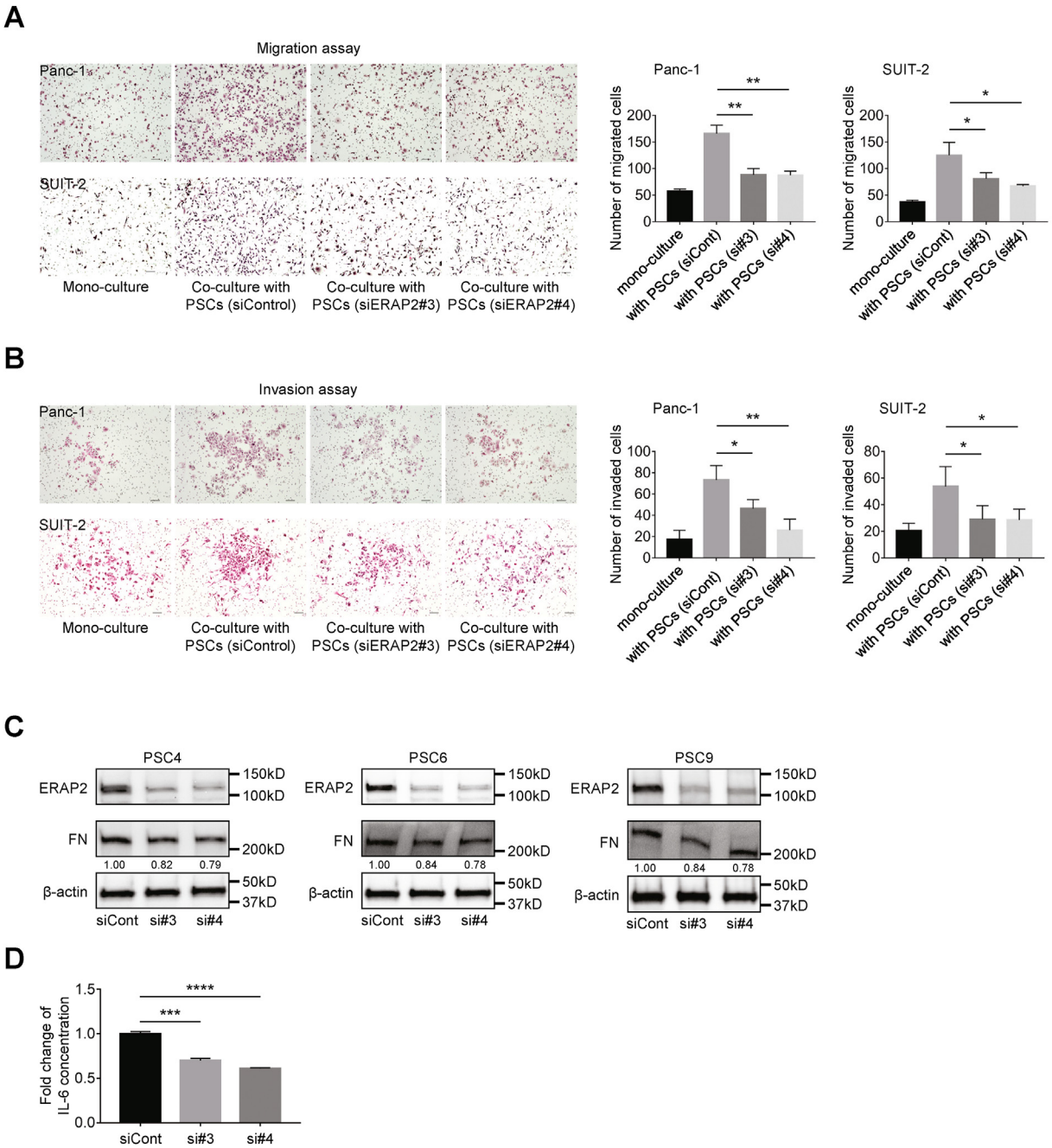

Fig. 4. ERAP2 affects tumor-stromal interactions by regulating fibronectin production and IL-6 secretion from PSCs. Representative images of migrated (A) and invaded (B) pancreatic cancer cells and quantification. Original magnification: 100× , scale bar = 100 μm. (C) Effect of siRNA-mediated ERAP2 knockdown on the level of fibronectin (FN) protein in PSCs. Values indicate densitometric ratios normalized to β-actin. (D) ELISA-based quantification of IL-6 secreted by PSCs after ERAP2 knockdown. (E), (F) Representative images of migrated (left) and invaded (right) pancreatic cancer cells treated with or without 10 ng/ml recombinant human IL-6 (E) and 10 μg/ml recombinant human fibronectin (rhFN) (F). Original magnification: 100× , scale bar = 100 μm. Data are the mean ± S.D. *P*-values were determined by unpaired two-tailed *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

matrix (ECM), was suppressed by ERAP2 knockdown in PSCs (Fig. 4C). Proinflammatory cytokine interleukin-6 (IL-6) secreted from PSCs plays an important role in promoting tumor growth and progression [16,29]. Thus, we measured the IL-6 concentration in the culture supernatant of 48 h-cultured PSCs and found a decrease in IL-6 secretion after ERAP2 knockdown (Fig. 4D). Because fibronectin and IL-6 promoted migration and invasion of PCCs (Fig. 4E and F), we concluded that ERAP2 knockdown inhibited ECM production and IL-6 secretion from PSCs. In addition, we also found that PSC activation induced by PCC supernatant or TGF-β1 was inhibited with ERAP2 knockdown (Supplementary Figs. S5A and B). These findings suggested that ERAP2 knockdown in PSCs attenuated tumor-stromal interactions.

3.4. Effect of the unfolded protein response on autophagy and activation of PSCs

We investigated the relationship between ER stress and PSC activation, and found that autophagy was induced during PSC activation in response to TGF-β1 treatment (Fig. 5A). Moreover, ER stress and UPR activation were observed with TGF-β1 treatment (Fig. 5B). Because autophagy is also induced by ER stress and the consequent UPR pathway [21,30], it was reasonable to consider that ER stress might account for PSC activation through autophagy induction. To test our hypothesis, tunicamycin was used to pharmacologically induce ER stress in PSCs at a concentration that did not affect cell viability. Similar to PSCs treated with TGF-β1, activation of normal PSCs (HPaSteCs) (Supplementary Fig. S6A), was also induced by ER stress (Fig. 5C). In addition, exposure of PSCs to tunicamycin increased both LC3-II expression and p62 degradation, indicating induction of autophagy by ER stress. Moreover, under conditions of tunicamycin treatment, LC3-II expression and p62 degradation were inhibited with ERAP2 knockdown, suggesting ER stress-mediated autophagy was suppressed (Fig. 5D). These results further supported our conclusion that ERAP2 plays an important role in autophagy. Knockdown of ERAP2 decreased the levels of Calnexin, IRE1α, and PERK in PSCs. These results suggested that the corresponding UPR pathways were inactivated under both starvation and ER stress conditions, particularly the PERK-mediated UPR pathway, because it significantly suppressed the level of PERK phosphorylation (Fig. 5D). These findings revealed that ER stress and the consequent UPR pathway were the crosstalk between autophagy and PSC activation, through which ERAP2 had an important function.

3.5. ERAP2 knockdown in PSCs retards xenografted tumor growth

To further confirm our conclusion obtained from *in vitro* experiments, the effect of ERAP2 knockdown in PSCs on orthotopic models was investigated. Immortalized PSC (imPSC) lines were

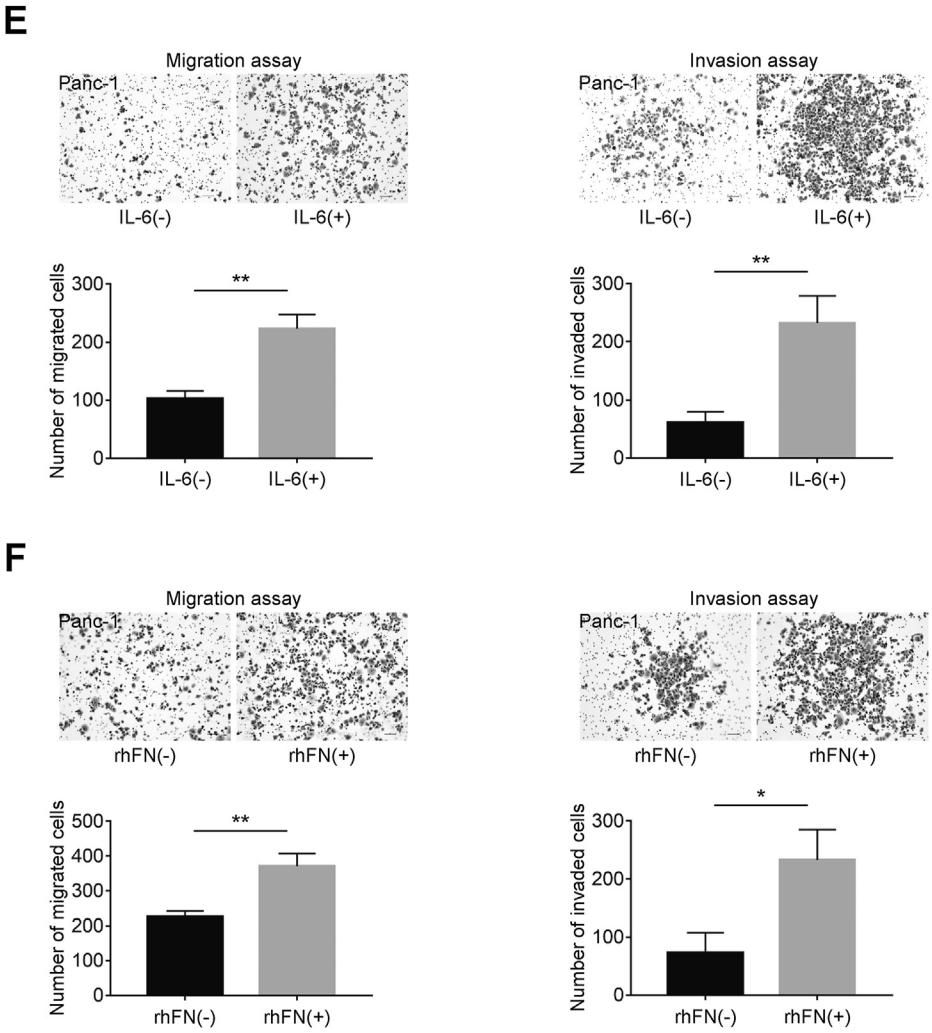

Fig. 4. (*continued*).

established without affecting ERAP2 expression as reported previously [7,31], followed by shRNA transfection for stable knockdown of ERAP2 (Supplementary Fig. S7A). Consistent with siERAP2 transfection of PSCs, autophagy and activation levels were decreased in shERAP2-transfected imPSCs (Fig. 6A), while cell viability was unaffected (Fig. 6B). We next orthotopically implanted shERAP2- and shControl-transfected imPSCs and SUIT-2 cells into immunocompromized mice. The promoting effect of imPSCs on tumor growth was significantly attenuated by ERAP2 knockdown (Fig. 6C). In addition, gemcitabine treatment further inhibited tumor growth (Fig. 6D). We then evaluated histopathological changes in the xenografted tumors. Consistent with our previous *in vitro* finding, the α-SMA-positive area was increased significantly by imPSC coimplantation, which was ablated by ERAP2 knockdown (Fig. 6E). Through Sirius red staining, we found that the fibrotic level of xenografted tumors increased by imPSC coimplantation ($P < 0.001$) was reduced by ERAP2 knockdown ($P < 0.01$) (Fig. 6E). In addition, we investigated the PCC proliferative capacity by proliferating cell nuclear antigen (PCNA) staining. The number of PCNA-positive PCCs was significantly higher in xenografted tumors with imPSC coimplantation compared with those implanted with PCCs alone ($P < 0.001$), and knockdown of ERAP2 abolished the capacity of imPSCs to promote PCCs proliferation ($P < 0.0001$) (Fig. 6E). Collectively, ERAP2 knockdown in PSCs inhibited the growth and fibrotic level of xenografted tumors *in vivo*.

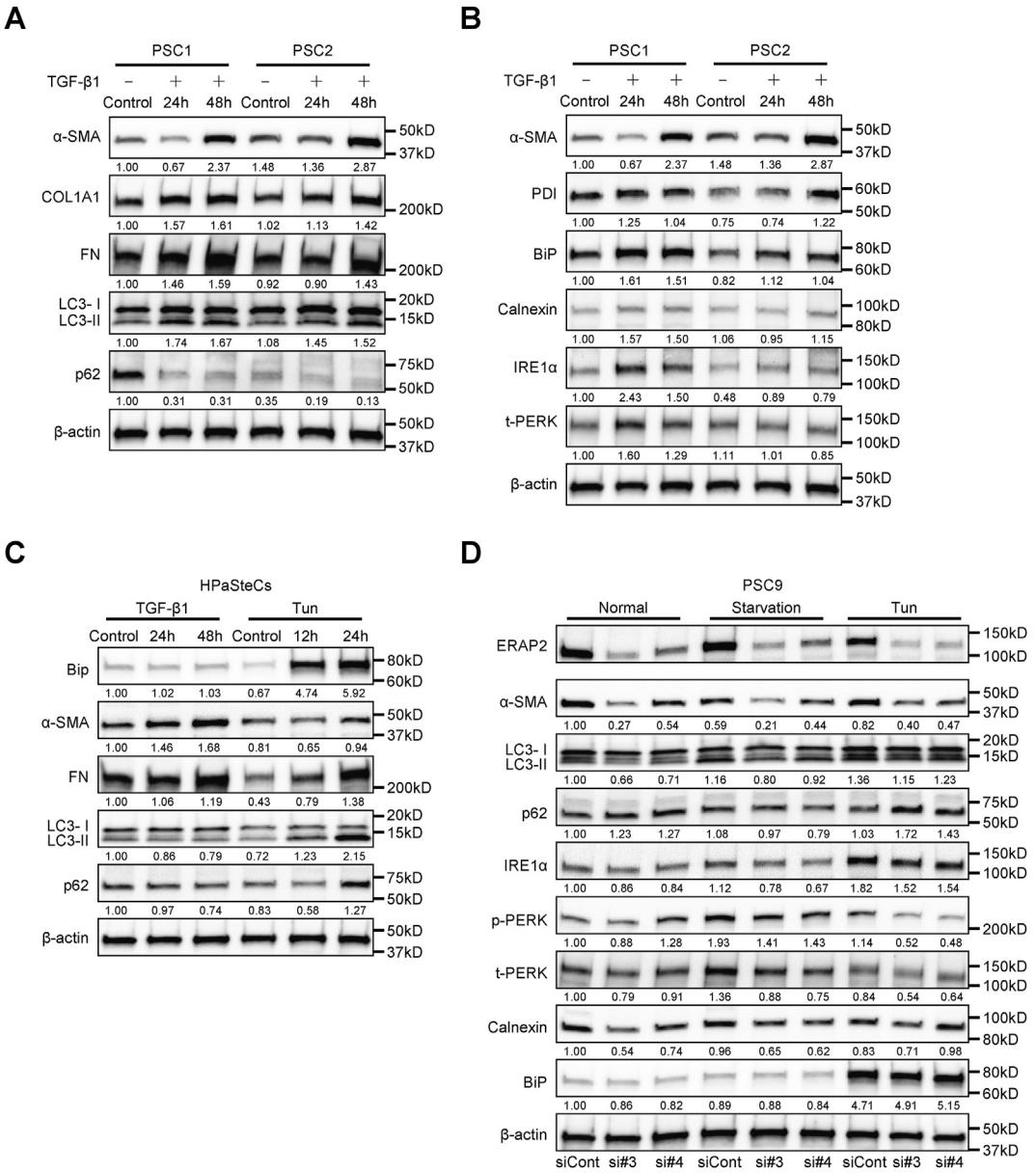

Fig. 5. ERAP2 functions in ER-derived autophagy via ER stress and the consequent UPR pathway. (A) Levels of fibrosis and autophagy markers in PSCs treated with 5 ng/ml TGF-β1 for the indicated times. TGF-β1 induced autophagy and PSC activation. (B) Levels of ER stress and UPR markers in PSCs treated with 5 ng/ml TGF-β1 for the indicated times. ER stress and consequent UPR occurred in response to TGF-β1 treatment. (C) Levels of ER stress, fibrosis and autophagy markers in normal PSCs treated with 5 ng/ml TGF-β1 or 2.5 μg/ml tunicamycin for the indicated times. Autophagy and activation of normal PSCs were induced by ER stress. (D) Western blot analysis showing levels of IRE1α, phosphorylation of PERK (Thr 981) and PERK in PSCs were inhibited by ERAP2 knockdown under autophagy induction conditions (by starvation or ER stress). Autophagy induced by ER stress was also inhibited by ERAP2 knockdown. Values indicate densitometric ratios normalized to β-actin.

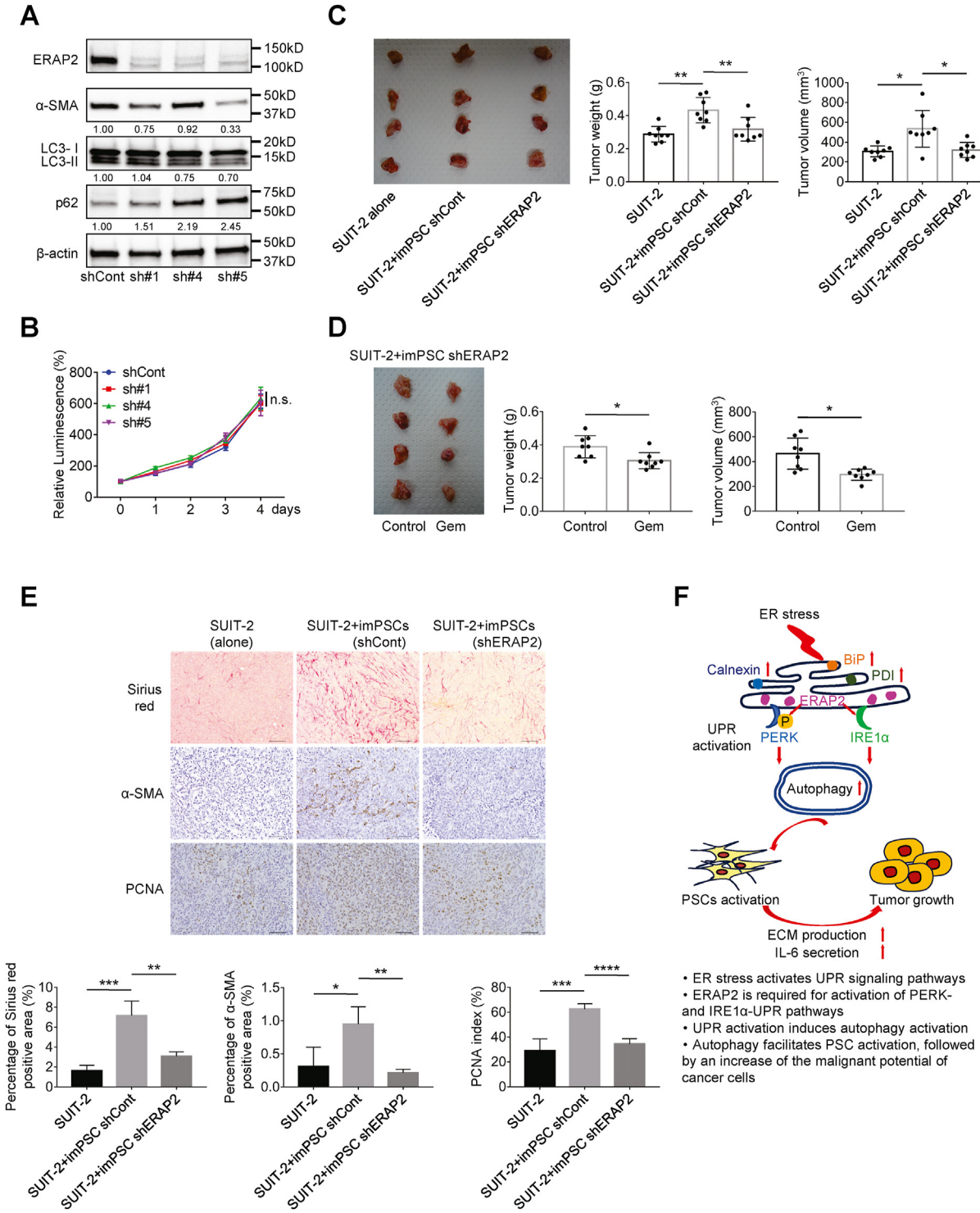

Fig. 6. ERAP2 knockdown in PSCs retards xenografted tumor growth and fibrosis. (A) Effect of shRNA-mediated ERAP2 knockdown on the levels of α-SMA, LC3, and p62 proteins in immortalized PSCs. Values indicate densitometric ratios normalized to β-actin. (B) Proliferation rate of immortalized PSCs. Cell viability was unaffected by ERAP2 knockdown with shERAP2. (C) Left, image of an excised xenograft tumors. Right, tumor weight and volume analyses of total xenografted tumors. (D) Left, image of an excised xenograft tumors with or without gemcitabine administration. Right, tumor weight and volume analyses of total xenografted tumors. Experiments were performed twice and a representative image of one experiment is shown ($n = 4$ per group) in (C, D). Dot indicates an individual mouse. (E) Top, representative images of immunohistochemical staining of xenografted tumor sections stained with Sirius red, and anti-α-SMA and -PCNA antibodies. Original magnification: 200× , scale bar = 100 μm. Bottom, quantification of Sirius red-stained and α-SMA-positive areas, and the percentage of PCNA-positive cancer cells. (F) Schematic diagram showing that autophagy activation induced by ER stress and consequent IRE1α- and PERK-UPR pathways facilitates PSC activation, thereby promoting PCC progression and aggressiveness. Data are the mean ± S.D. *P*-values were determined by Wilcoxon test and in (C, D), and unpaired two-tailed *t*-test in (B, E). Not significant: n.s. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

4. Discussion

Our previous study revealed that PSC activation is associated with autophagy [7]. However, there was little known about the underlying mechanism. In this study, we showed that ERAP2, which belongs to the zinc metallopeptidases of the oxytocinase M1 subfamily for peptide trimming in the endoplasmic reticulum [32], plays important roles in autophagy of PSCs. Mechanistically, ERAP2 regulates activation of PSCs through ER-derived autophagy. After knockdown of ERAP2, autophagy and activation of PSCs were inhibited, and then tumor-stromal interactions were attenuated by the reduction of ECM components and IL-6 produced by PSCs. We revealed a new mechanism of autophagy activation modulated by ERAP2. Therefore, ERAP2 is a potential therapeutic target for treatment of pancreatic cancer.

ERAP2 is originally described as a peptide-trimming enzyme located in the ER, but it has not been studied extensively [33,34]. Recent reports indicate that ERAP2 is a pan-cancer type gene [35,36]. In agreement with previous studies, we demonstrated that ERAP2 was expressed in pancreatic carcinoma cells. Moreover, upregulation of ERAP2 was identified in activated PSCs. We confirmed that tumor-stromal interactions were attenuated by ERAP2 knockdown in PSCs. Consistent with our previous study showing that autophagy regulates IL-6 secretion from PSCs [7], we demonstrated that IL-6 secretion was affected by ERAP2 expression, which possibly or partially explained the mechanism of IL-6 secretion regulated by autophagy.

Maiers reported that ER stress and its consequent UPR are activated during hepatic stellate cell (HSC) activation [37]. In contrast, Koo showed that ER stress within HSCs leads to activation, thereby promoting liver fibrosis [22]. However, the interplay of ER stress and PSC activation had never been explored. In this study, during PSC activation induced by TGF-β1 treatment, ER stress and its consequent UPR were activated, and unexpectedly, autophagy was also induced. To ascertain their causality, tunicamycin was used to induce ER stress in PSCs. As a result, autophagy in response to ER stress was also inhibited by ERAP2 knockdown. We also revealed that ER stress in human normal PSCs (HPaSteCs) isolated from the pancreas led to autophagy induction and PSC activation. Therefore, we confirmed that ERAP2 regulates PSC activation through ER-derived autophagy. We also found that this process is mediated by IRE1α- and PERK-UPR signaling pathways. It should be noted that a direct effect of UPR on PSC activation cannot be excluded in this process. In addition, expression of calnexin, an ER chaperone for quality control of protein folding [38], was suppressed after ERAP2 knockdown, accounting for a previous study indicating that ERAP2 colocalizes to a large extent with calnexin [33].

In vivo, ERAP2 is involved in antigen-processing for immune surveillance and there are no analogues in rodents [33]. Therefore, the effect of ERAP2 per se on tumorigenesis was precise in orthotopic models of immunocompromized mice. Knockdown of ERAP2 weakened the capacity of PSCs to support xenografted tumor growth. Because the proliferative capacity of imPSCs was unaffected by ERAP2 knockdown, we considered that this retarded tumor growth resulted from attenuation of tumor-stromal interactions. In addition, the reduction of α-SMA-positive area was not due to the decrease in the number of PSCs. In orthotopic models, liver metastasis was not found, therefore, we did not analyze the effect of ERAP2 knockdown on tumor metastasis. Studies of stroma-targeting therapeutic approaches for pancreatic cancer are controversial. Rhim reported that stromal ablation promotes rather than limits pancreatic cancer progression [39]. In stark contrast to stromal ablation, Sherman determined that stromal remodeling by targeting PSCs to convert into the quiescent state hinders tumor-stromal interactions and tumor growth [40]. In agreement with the latter view, we revealed that PSC remodeling by targeting ERAP2 inhibited tumor growth and fibrosis.

Pancreatic ductal adenocarcinoma is characterized by desmoplasia and autophagy-dependent tumorigenic growth [41–43]. Our study uncovered the benefit of targeting ERAP2 because it inhibited autophagy of PSCs, and consequent PSC activation. In this study, we did not use a specific inhibitor of ERAP2 because it is unavailable. Recently, several phosphonic and phosphinic compounds were reported to exhibit potent and selective inhibition of ERAP2 [44]. Therefore, we consider ERAP2 targeting as a potentially promising strategy for treatment of pancreatic cancer.

Ethics approval

Informed consent was obtained from all patients prior to collection of surgical specimens. The study was approved by the Ethics Committee of Kyushu University (IRB: 28–189; Fukuoka, Japan), and conducted under strict compliance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government and Helsinki Declaration.

Declaration of competing interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pan.2021.09.012.

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