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Original article

Identification of profilin 1 as the primary target for the anti-cancer activities of Furowanin A in colorectal cancer

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ABSTRACT

Background: Furowanin A (Fur A) is a flavonoid compound isolated from medicinal plant *Millettia pachycarpa* Benth. This study aims to explore the effect of Fur A on Colorectal cancer (CRC) and its molecular mechanisms.

Methods: Cell proliferative capacity of CRC cells was assessed by CCK-8 assay. Cell apoptosis and cell cycle distribution were detected by flow cytometry. Cell migration and invasion were detected by wound healing and Transwell assay, respectively. EMT markers, apoptosis and profilin 1(Pfn1) expression were detected by immunohistochemistry (IHC). The protein expression levels were examined by western blotting. i-TRAQ analyses were conducted to identify the differentially expressed genes in CRC cells. CRC xenograft model was also used to validate the *in vivo* anti-cancer activity of Fur A.

Results: Fur A exhibited anti-prolifertive, blocked cell cycle progression and promoted apoptotic cell death in CRC cells. Fur A suppressed the migration, invasion and epithelial-to-mesenchymal transition (EMT) *in vitro*, and tumor growth and pulmonary metastasis *in vivo*, without causing obvious toxicity. iTRAQ analysis identified Pfn1 as a gene up-regulated by Fur A. In xenograft tumor tissue, the expression of Pfn1 was also elevated by Fur A treatment. In clinical CRC samples, high expression of Pfn1 was correlated with lower stage and longer survival. Knockdown of Pfn1 significantly dampened the pro-apoptotic and anti-metastatic activities of Fur A in CRC cells. Ectopic Pfn1 expression augmented the anti-neoplastic activities of Fur A.

Conclusion: Fur A exhibited anti-cancer activities *in vitro* and *in vivo* in CRC by up-regulating Pfn1.

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies worldwide, and accounts for 600,000 deaths annually [1]. Recent statistics indicate a marked increase in its incidence rate in China, making it the most prevalent cancer among the Chinese after lung and gastric cancer [2]. At present, the primary treatment for CRC is surgical resection [3]. However, despite advances in its therapeutic regimen, such as systemic chemotherapy, radiotherapy, immunotherapy and targeted therapy, the clinical outcomes and prognosis for CRC patients undergoing surgery remain dismal [4] due to distant metastasis and the aggressive nature of colon tumors [5]. Therefore, it is necessary to identify and develop novel chemotherapeutics for the effective targeting and clearing of CRC cells.

Cellular activities like motility, division and endocytosis are dependent on the dynamic remodeling of the actin cytoskeleton [6,7]. The neoplastic transformation of cells is accompanied by alteration of the dynamic equilibrium between monomeric and filamentous actin *via* the concerted actions of different actin-binding proteins (ABPs) [8]. Profilin1 (Pfn1), a widely expressed ABP, is essential for normal cell motility, proliferation and differentiation [9]. Pfn1 is down-regulated in different epithelial cell-derived tumors, such as those originating in the breast, liver, pancreas and bladder [10–12]. Pfn1 is known to interact with actin, polyproline ligands and phosphoinositide [13]. A recent study showed a correlation between Pfn1 up-regulation and decreased CRC tumor growth [14], thereby indicating its potential role in CRC progression.

Millettia pachycarpa Benth (Houguojixueteng in Chinese), a herb widely distributed in the Southwest part of China, has been widely used as a folk medicine to clear parasitic intestinal worms [15]. Studies have shown the anti-inflammatory effects of *M. pachycarpa* Benth extract *in vitro* [15], and a number of flavonoid and isoflavonoid compounds have been identified as its active

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ingredients. Furowanin A (Fur A), an isoflavonoid extracted from the leaves of *M. pachycarpa* Benth, induces apoptosis in leukemia HL-60 cells [16], but its potential anti-neoplastic effects against solid tumors are unknown. The aim of this study was to determine the therapeutic effects of Fur A in CRC, and its role in Pfn1 regulation.

Materials and methods

Patients and clinical samples

A total of 47 CRC patients who underwent surgical tumor resection at the Affiliated Hospital of Qingdao University between Jun 2011 and Feb 2013 were recruited for the study. CRC and adjacent non-cancer tissues were excised, flash frozen in liquid nitrogen, and stored at −80 °C. Tumor diagnosis and staging was verified by two independent senior oncologists blinded to the clinico-pathological features of the patients. This study was approved by the ethics committee of the hospital, and all patients provided written informed consent.

Immunohistochemistry (IHC)

The *in situ* expression level of Pfn1 and cleaved caspase-3 in the CRC and non-CRC tissue samples was determined by IHC. Tissue sections were probed with specific polyclonal antibodies against Pfn1 and cleaved caspase-3 (Cell Signaling, Danvers, MA, USA), and the frequency of positively stained cells and the intensity of immunostaining were quantified. According to the percentage of positively stained cells, the sections were graded as 0 (≤ 5%), 1 (6%–25%), 2 (26%–50%), 3 (51%–75%) and 4 (> 76%). Similarly, the samples were graded in terms of staining intensity as 0 for negative staining, 1 for weakly positive, 2 for moderately positive and 3 for strongly positive staining. The total immuno-reactive score of each specimen (0–12) was calculated by multiplying the respective scores of the percentage of positive cells and the staining intensities. Based on the total score, the samples were classified into the low-expression (0–3) and high-expression (4–12) groups.

Cell lines and culture

The human colon cancer cell lines HCT116 and LOVO were provided by the Centre for Cell Resources of Shanghai Institute for Life Sciences, Chinese Academy of Sciences (Shanghai, China). Both lines were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 25 mg/ml amphotericin B, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C under 5% CO_2.

Cell counting Kit-8 (CCK-8) assay

Following treatment with indicated conditions, the viability of CRC cells was tested by the CCK-8 assay. Briefly, the cells were seeded in 96-well plates at the density of 5×10^3 cells/well. After culturing the cells for indicated time, 10 μl CCK-8 solution was added per well and the cells were incubated for another 2 h. The absorbance at 450 nm was measured using a microplate reader (BioTek, Winooski, VT, USA).

Vector construction and transfection of CRC cells

Pfn1 overexpression vector was constructed as previously described using the pEGFP-N1 vector (Wuhan Miaoling Bioscience & Technology Co. Ltd, Wuhan, China) [17]. The human Pfn1 shRNA was provided by Santa Cruz (Santa Cruz, CA, USA), and a non-silencing scrambled shRNA control was synthesized as previously described [18,19]. The overexpression/shRNA constructs and the respective empty vectors were transfected into CRC cells using Lipofectamine 3000 reagent (Invitrogen, Grand Island, NY, USA). Pfn1 overexpression/knockdown was measured by Western blotting 48 h after transfection.

Apoptosis assays

CRC cells were stained with the TUNEL dye (Beyotime, Wuxi, China) as per the manufacturer's instructions. At least 300 cells in five random fields (100 × magnification) per sample were counted to calculate the percentage of TUNEL positive apoptotic cells. In addition, CRC cells were also double stained using Annexin V-FITC/PI for 15 min as previously described [20]. The stained cells were observed by flow cytometry, and the percentage of apoptotic cells was calculated.

Cell cycle analysis

CRC cells were seeded in 6-well plates at the density of 1×10^5/well, cultured for 48 h, washed and fixed with ice-cold 70% ethanol at 4 °C for 2 h. The cells were stained with PI as per standard protocols and analyzed by FACScan.

Wound healing assay

LOVO and HCT116 cells were seeded into 6-well plates. When cells were grown to approximately 90% confluence, a wound was created by using the 20 μl pipette tip on the cell monolayer. And the cell monolayer was clearly distinguished into different areas by horizontal linear markings. The scratched wound was observed after 24 h in three fields under the microscope at 100 × magnification. Effect of Fur A on cellular migration capabilities was evaluated through calculating the distance between the edges of wound. Experiments were carried out at least in triplicate.

Transwell assays

Cells invasion was measured by using transwell inserts (8 μM pore size, CORNING, Corning, NY, USA). Transwell inserts were coated with 100 μl Matrigel for 30 min at 37° C before the experiment. CRC cells were plated in Transwell inserts with serum-free RPMI-1640 medium, while RPMI-1640 supplemented with 20% fetal bovine serum was added to the bottom chamber of 24-well plates and incubated 24 h at 37° C. Then carried out transwell inserts and fixed in 4% paraformaldehyde for 30 min and strained with 0.1% crystal violet for 50 min. Cells were imaged in 3 random fields of each chamber under an inverted fluorescence microscope (Nikon, Japan), each group was analyzed at least in triplicate.

iTRAQ reagent labeling and LC–MS/MS analysis

iTRAQ-based proteomics analysis was quantitatively analyzed by Shanghai OE Biotech (Shanghai, China) as described previously [21]. In briefly, following Fur A treatment, cells were dissolved in lysis buffer and labelled with iTRAQ labelling reagents. The samples were subjected to LC analysis and tandem mass spectrometry analysis. Protein identification and relative iTRAQ quantification service were provided by Oebiotech (Shanghai, China). The cutoff value for the differentially expressed proteins was adjusted to $p < 0.05$ and the fold change was set to > 1.5 or < 0.5.

Western blotting

Total proteins were extracted from the cells and tissues using RIPA sample buffer (Beyotime, China) supplemented with protease

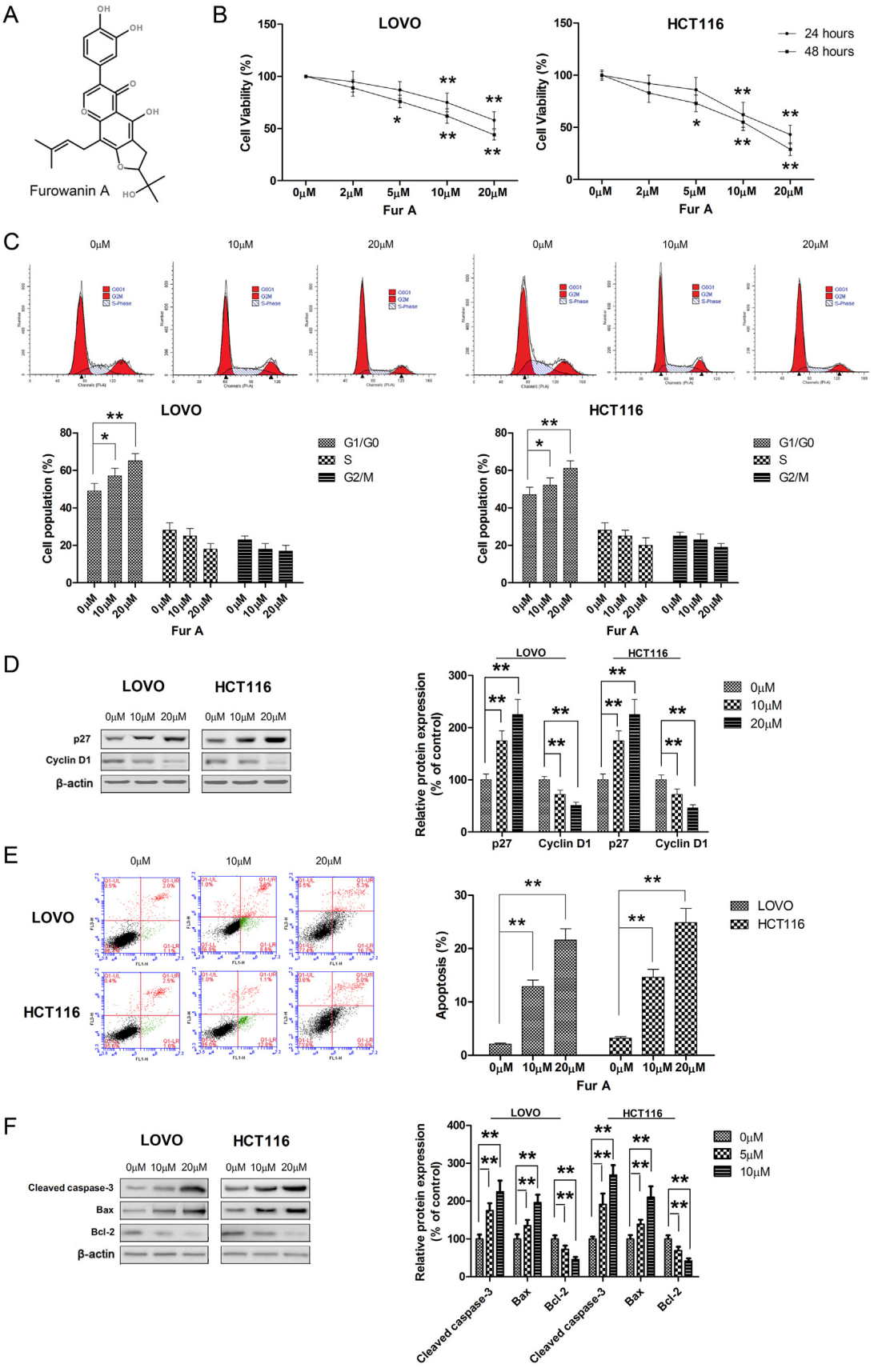

Fig. 1. Fur A suppresses proliferation, and causes cell cycle arrest and apoptosis in CRC cells. A. Chemical structure of Fur A. Fur A suppresses cell growth (B), blocks cell cycle progression at the G0/G1 stage (C), upregulates p27 and downregulates cyclin D1 (D), increases the percentage of apoptotic cells (E), and activates caspase-3, upregulates Bax and downregulates Bcl-2 (F) in CRC cells in a dose- and time-dependent manner. * p <0.05, ** p <0.01.

inhibitors (Complete, EDTA-free; Roche, USA) and PMSF (Beyotime, China). Equal amount of proteins per sample were separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). After blocking the membrane with 5% non-fat milk, they were incubated overnight with the specific primary antibodies at 4 °C. Goat anti-mouse IgG-HRP (1:1000; Abcam, USA) was used as the secondary antibody. The positive bands were visualized by an enhanced chemiluminescent (ECL) detection reagent (Thermo Fisher, USA). The band intensities were analyzed and protein expression was quantified using Gel Doc 2000 (BioRad, USA).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cultured CRC cells using TRIzol Reagent (Life Technology, Carlsbad, CA, USA), and cDNA was synthesized using a reverse transcription kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The primers specific for Pfn1 and GAPDH (internal control) were synthesised by Sangon (Shanghai, China) according to previously published sequences [22]. The PCR reaction was conducted using SYBR GREEN master-mix (Takara, Shanghai, China), and the relative expression of Pfn1 was calculated by the $2^{-\Delta\Delta}$Ct method.

In vivo *tumor modeling*

All animal experiments were approved by the Medical Ethics Committee of the Affiliated Hospital of Qingdao University. HCT116 cells (1×10^7 cells/ml) were subcutaneously injected into 8-week-old male athymic BALB/c nu/nu mice on their left flanks. The body weight of the mice and tumor volumes were measured every three days. Tumors were harvested after 27 days, and processed for protein extraction, or frozen and cut into 4 μm cryostat sections for hematoxylin-eosin (HE) and TUNEL staining as previously described [23].

Establishment of pulmonary metastasis model

HCT116 cells (1×10^6 cells) were injected into the mice *via* their tail veins. The mice were sacrificed 12 weeks after inoculation, and the macroscopic metastatic lesions on the lung surface were counted to assess pulmonary metastasis. In addition, the lungs were fixed and consecutive sections were stained with HE.

Statistical analysis

Statistical analysis was conducted using SPSS software 16.0 (SPSS Inc., Chicago, IL, USA). Spearman's correlation test was used to analyze the association between Pfn1 expression and clinicopathological characteristics of the patients. The survival data was analyzed using the log-rank test and Kaplan-Meier method. One-way ANOVA followed by Dunnett's *t*-test was used for multiple comparisons. *p* Values less than 0.05 were considered statistically significant.

Results

Fur A suppresses proliferation and promotes apoptosis of CRC cells

The chemical structure of Fur A is shown in Fig. 1A. To determine the effect of Fur A on CRC cell proliferation, the HCT116 and LOVO cell lines were challenged with 2, 5, 10 and 20 μM Fur A for 24 and 48 h. In both lines, 10 and 20 μM Fur A caused a significant decrease in the number of viable cells after 24 h (Fig. 1B), which was sustained by the 20 μM dose after 48 h. In contrast, 5 and 10 μM Fur A resulted in a significant loss of viability only after 48 h (Fig. 1B). In the subsequent experiments therefore, LOVO and HCT116 cells were incubated with Fur A for 24 h at the indicated dosage. In addition, Fur A treatment significantly increased the cell population at the G0/G1 phase (Fig. 1C), and markedly elevated p27 and repressed Cyclin D1 expression (Fig. 1D) levels, indicating that Fur A blocked cell-cycle progression by inducing a G1/G0 phase arrest. Finally, the higher doses of Fur A induced apoptosis in LOVO and HCT116 cells (Fig. 1E), and increased the levels of the pro-apoptotic cleaved caspase-3 and the Bax/Bcl-2 ratio (Fig. 1F).

Fur A suppresses CRC cell migration and invasion

The *in vitro* migration and invasion capacity of the CRC cells was determined by the wound healing (Fig. 2A) and Transwell (Fig. 2B) assays, which respectively showed that Fur A significantly inhibited the migration and invasion of CRC cells in a dose-dependent manner. The extra-cellular matrix proteases MMP-2, MMP-9 and their inhibitor TIMP-3 are key molecules driving the migratory and invasive behavior of tumor cells. We found that Fur A markedly down-regulated MMP-2 and MMP-9 and up-regulated TIMP-3 in both LOVO and HCT116 cells (Fig. 2C). Since epithelial to mesenchymal transition (EMT) of tumor cells is the initial step in metastasis [24], we also analyzed the expression patterns of the epithelial (E-cadherin) and mesenchymal (N-cadherin, Vimentin) markers in CRC cells after Fur A treatment. Fur A increased the expression of E-cadherin in the CRC cells in a dose-dependent manner (Fig. 2D), and significantly decreased N-cadherin and Vimentin protein levels compared to that in the control cells (Fig. 2E). Taken together, Fur A effectively suppressed the migration and invasive capability of CRC cells.

Fur A suppressed tumor growth and pulmonary metastasis in a xenograft model

We also analyzed the effects of Fur A in a CRC xenograft murine model. As shown in Fig. 3A and B, 20 and 40 mg/kg Fur A significantly inhibited tumor growth in terms of both volume and weight. Furthermore, Fur A treatment resulted in a significant increase in the percentage of TUNEL positive apoptotic cells (Fig. 3C), and a decrease in the *in situ* expression of the proliferation marker Ki-67 and anti-apoptotic Bcl-2 (Fig. 3D) in the xenograft tumor tissue, in a dose-dependent manner. In contrast, the higher dose of Fur A significantly increased the expression of cleaved caspase-3 and Bax proteins in the tumor tissue (Fig. 3D). The anti-metastatic effect of Fur A was evaluated using a pulmonary metastasis model. As shown in Fig. 3E, the number of macroscopic metastatic nodules on the lung surface was significantly decreased by Fur A in a dose-dependent manner. Furthermore, the metastatic nodules of the Fur A-treated mice showed higher *in situ* expression of E-cadherin and lower expression of N-cadherin (Fig. 3F).

The general toxicity of Fur A was also assessed by monitoring the body weight of the mice, in addition to histological change in the major organs, and hematological and serum biochemical indicators. As shown in Supplementary Fig. 1A, both vehicle and Fur A-treated mice showed an increase in body weight over time. In addition, no discernible anomalies were seen in the tissues of both mice groups (Supplementary Fig. 1B). Finally, Fur A treatment did not result in any significant changes in the plasma indices of liver metabolism (ALT, AST and Urea nitrogen) (Supplementary Table 1), or in the hematological parameters (Supplementary Table 2) compared to the mice treated with vehicle. Taken together, Fur A suppressed CRC tumor growth and pulmonary metastasis *in vivo* without any systemic adverse effects.

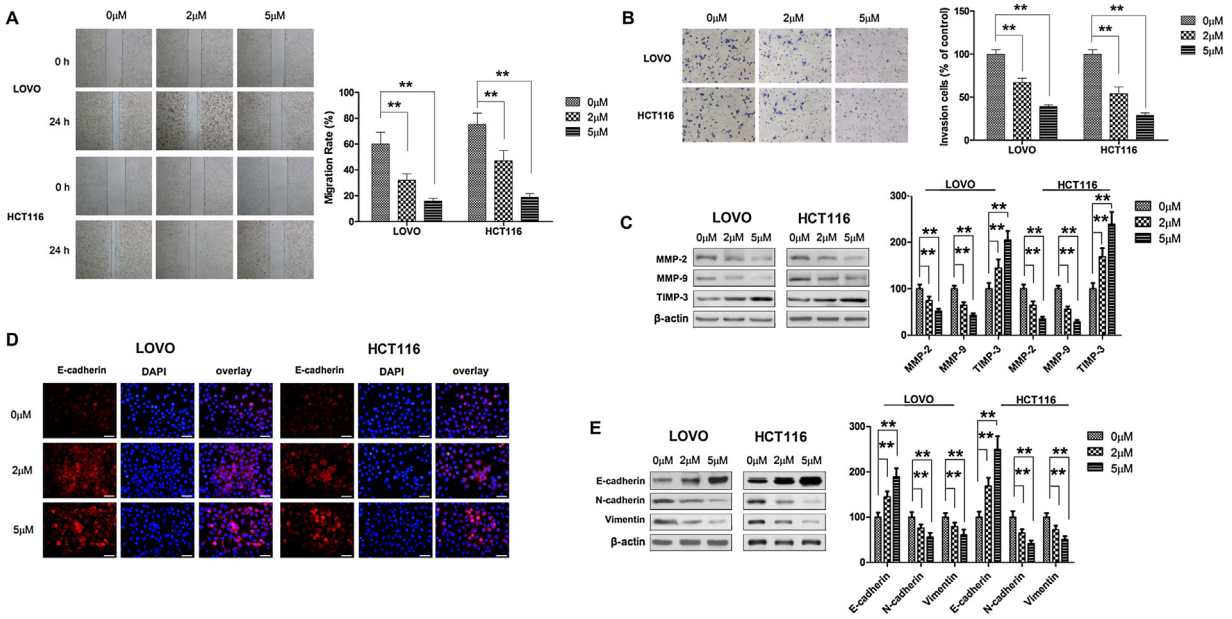

Fig. 2. Fur A suppresses cell migration, invasion and EMT of CRC cells. Fur A suppresses *in vitro* wound healing (A) and invasiveness (B) of CRC cells, downregulates MMP-2 and MMP-9 and upregulates TIMP3 (C), elevates the *in situ* expression of E-cadherin (D), and downregulates N-cadherin and vimentin (E) in CRC cells. ** $p < 0.01$.

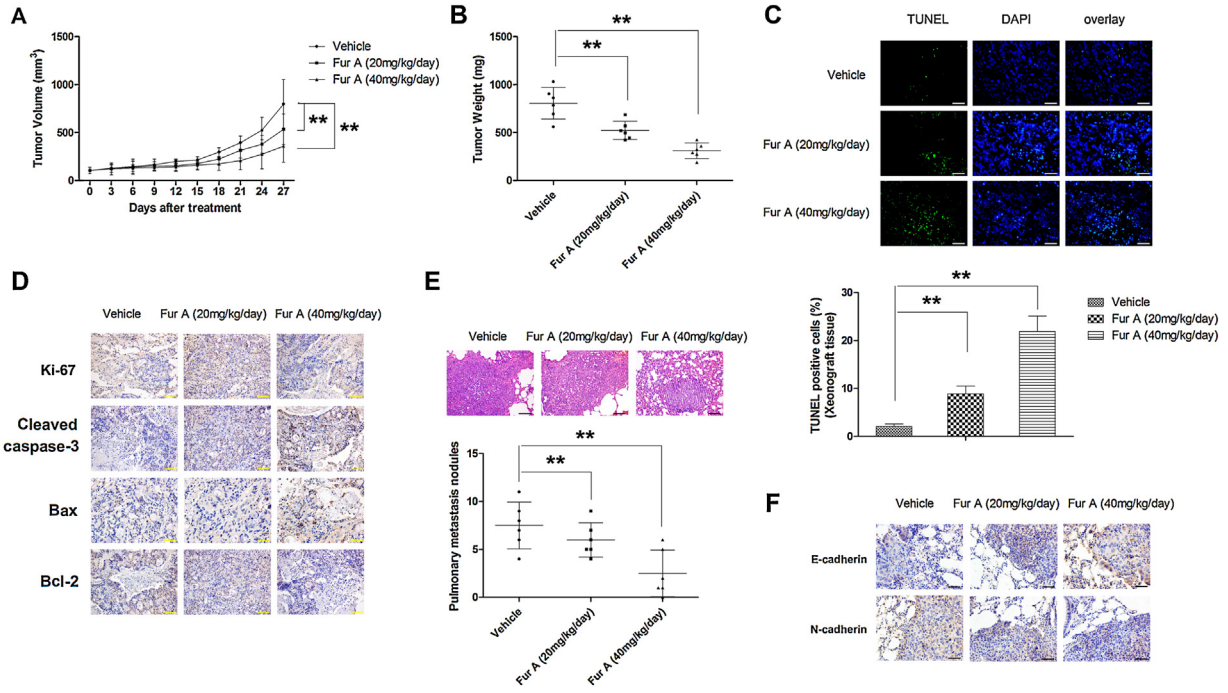

Fig. 3. Fur A suppresses CRC tumor growth and metastasis *in vivo*. Fur A inhibits increase in tumor volume(A) and weight (B), and increases apoptosis (C), increases *in situ* expression of cleaved caspase-3 and Bax, and decreases that of Ki-67 and Bcl-2 (D) in the primary tumor xenografts. Fur A decreases the number of pulmonary metastatic nodules (E), and increases the expression of E-cadherin and decreases that of N-cadherin in the metastatic nodules (F). ** $p < 0.01$.

Fur A upregulates Pfn1 in the CRC cells

To identify the potential target of Fur A, we conducted the iTRAQ proteomic analysis on HCT116 cells incubated with 20 μM Fur A for 24 h. The heat map of the expression levels of different proteins is shown in Fig. 4A, which indicates that 32 proteins were up-regulated and 84 were down-regulated. Furthermore, the differentially expressed genes (DEGs) were then functionally annotated and classified into the biological process, cell component and molecular function groups. Since Pfn1 was up-regulated by more than 5 folds, we subsequently focused on this protein. To verify the results of the iTRAQ assay, the expression of Pfn1 mRNA and protein levels in the CRC cells was first examined. As shown in Fig. 4B and C, Pfn1 mRNA and protein was markedly elevated in both LOVO and HCT116 cells following Fur A treatment. Furthermore, Pfn1 was also up-regulated in the primary tumor xenograft (Fig. 4D and E), as well as in the metastatic nodules (Fig. 4F) in mice treated with Fur A. Taken together, Fur A likely exerted its anti-proliferative and anti-metastatic effects on CRC cells by up-regulating Pfn1.

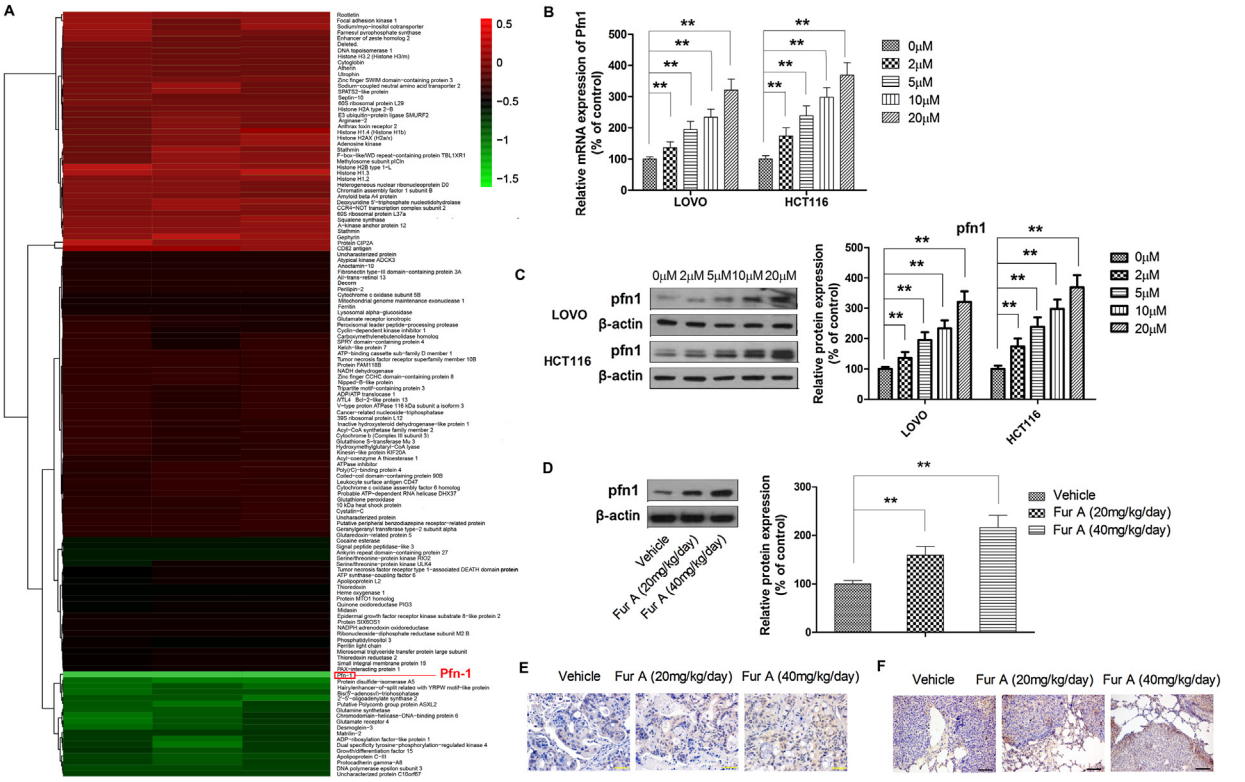

Fig. 4. Fur A upregulates Pfn1 in CRC cells *in vitro* and *in vivo*. A. Differentially expressed proteins identified by iTraq in HCT116 cells treated with 20μM Fur A for 24 h. Fur A upregulates Pfn1 mRNA (B) and protein (C) levels in CRC cells in a dose-dependent manner, and increases *in situ* Pfn1 mRNA (D) and protein (E) in tumor xenografts, and in pulmonary metastatic noules (F). $^{*}p < 0.05$, ** $p < 0.01$.

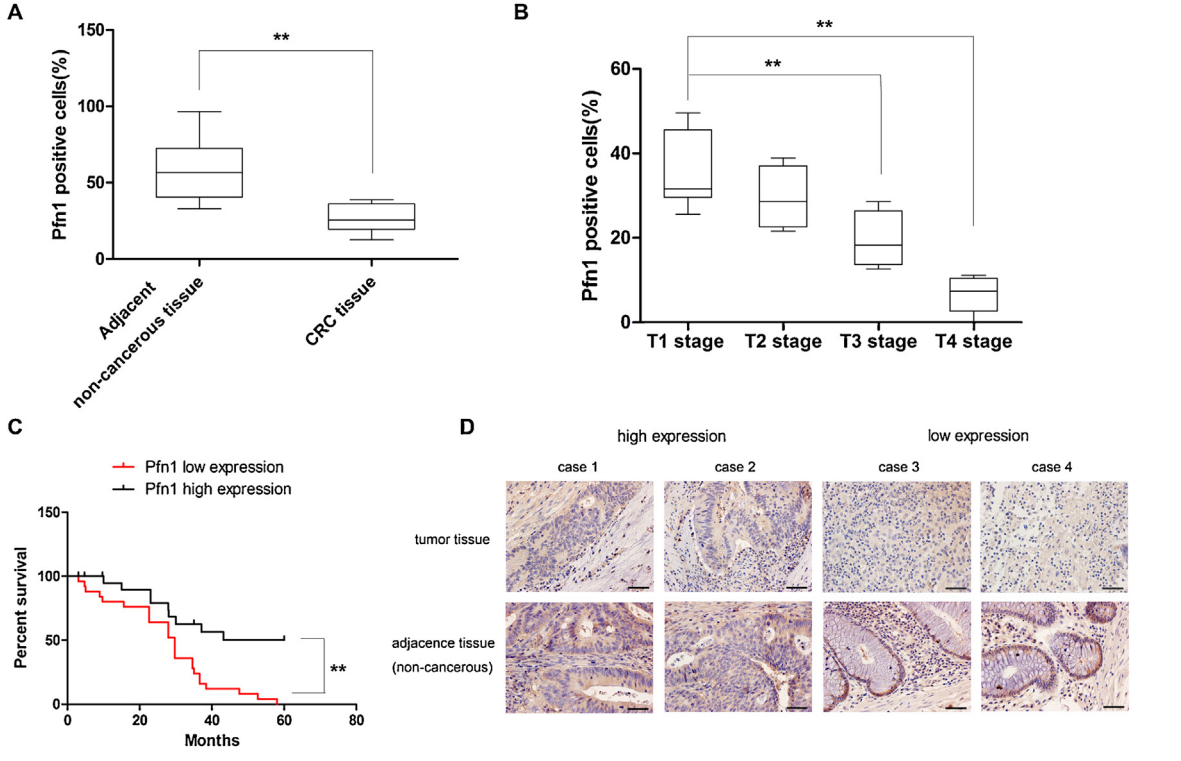

Fig. 5. Pfn1 expression is aberrantly lower in CRC tissues and correlates with poor prognosis of patients. Pfn1 expression is markedly lower in CRC tissues relative to the adjacent non-cancerous tissue (A), and in the advanced disease stage (B). C. High expression of Pfn2 is associated with improved survival. D. Representative data of high and low expression of Pfn1. ** $p < 0.01$.

Pfn1 expression is aberrantly lower in CRC tissues and correlates with poor survival

Compared to the adjacent non-cancerous tissues, the percentage of Pfn1 positive cells was significant lower in the CRC tissues ($p < 0.01$, Fig. 5A). In addition, patients with advanced disease and higher tumor-node-metastasis (TNM) stage had relatively lower percentage of Pfn1 positive cells compared to the patients with milder disease progression (Fig. 5B). To further determine the association between the clinico-pathological features of the patients and Pfn1 expression levels, the patients were divided into Pfn1 high- and low-expression groups. As listed in Table 1, Pfn1 expression level was significantly associated with TNM stage and lymph node metastasis, while no significant correlation was seen with age, gender and tumor size. Finally, high Pfn1 expression correlated with significantly longer survival duration among the patients (Fig. 5C and D). Taken together, our findings indicated that Pfn1 acts as a tumor suppressor in CRC.

Fur A exerts its anti-neoplastic effects via *Pfn1 upregulation*

Based on our prior findings, we further explored the role of Pfn1 in the anti-neoplastic effects of Fur A by manipulating Pfn1 expression in the CRC cells (Supplementary Fig. 2A and 2B). Pfn1 knockdown markedly abrogated the pro-apoptotic effects of Fur A in both LOVO and HCT116 cells (Fig. 6A), and also rendered the CRC cells more resistant to the pro-apoptotic effects of chrysophanol, as demonstrated by attenuated activation of caspase-3, increase in Bax and decrease in Bcl-2 levels (Fig. 6B). In contrast, Pfn1 overexpression effectively enhanced the effect of Fur A on cell apoptosis (Fig. 6C and D). Furthermore, Pfn1 knockdown markedly attenuated the suppressive effects of Fur A on CRC cell migration (Fig. 7A) and invasion (Fig. 7B), reversed Fur A-induced inhibition of MMP-2 and MMP-9 and activation of TIMP-3 (Fig. 7C), and restored EMT of CRC cells treated with Fur A (Fig. 7D). In contrast, the inhibitory effect of Fur A on CRC cells invasive capability was effectively enhanced by Pfn1 overexpression. (Fig. 7E-H). Fur A exerts its anti-neoplastic effects in CRC at least partly by up-regulating Pfn1.

Discussion

Fur A is an active ingredient of traditional Chinese medicine *Millettia pachycarpa* Benth [15], and has shown potent anti-neoplastic effects in the leukemia cell line HL-60 [16]. However, little is known about its potential therapeutic role against solid tumors, and the underlying molecular mechanisms. In the present study, we found that Fur A suppressed proliferation, caused cell cycle arrest and promoted apoptosis in CRC cells, in addition to inhibiting *in vitro* EMT and invasion, and *in vivo* tumor growth and pulmonary metastasis. Furthermore, our findings indicate Pfn1 as the primary molecular target of Fur A in the CRC cells.

Proteomics offer a platform for global expression analyses, and have been widely used to elucidate complex biological networks [25]. The iTRAQ-based proteomics approach has helped uncover the underlying mechanisms of anti-cancer chemotherapeutic agents [26,27]. In the present study, iTRAQ revealed Pfn1 as the strongest molecular target of Fur A in the CRC cells, which was validated by standard expression assays in Fur A-treated CRC cells. Pfn1, a widely expressed ABP, is involved in many cellular activities, such as cell proliferation and motility. The tumor suppressive role of Pnf1 has been extensively reported. Wang et al. found that the aberrantly down-regulated of Pnf1 was strongly associated with aggravation of HCC and poor prognosis [28]. In current study, we found the expression of Pfn1 was also markedly increased in the primary and metastatic tumor xenografts in response to Fur A treatment. In addition, ectopic expression of Pfn1 further augmented the pro-apoptotic and anti-metastatic effects of Fur A while Pfn1 knockdown diminished these effects of Fur A in the CRC cells. Taken together, there was a significant association between the anti-cancer effects of Fur A and increased Pfn1 expression, indicating that Pfn1 mediated the therapeutic effects of Fur A in CRC cells.

Apoptosis, or programmed cell death I, is one of the most common pathways targeted by the chemotherapeutic agents in cancer cells [29]. Previous studies have shown a pro-apoptotic role of Pfn1 in various cancers. For example, Pfn1 sensitized breast cancer cells to apoptosis induced by Doxorubicin, Vinblastine and Paclitaxel by suppressing NF-κB and upregulating p53 [30]. In pancreatic cancer cells, Pfn1 functioned as a sensitizing factor to irradiation-induced apoptosis and suppressed tumorigenesis [31,32]. Staurosporine-induced apoptosis in human neuroblastoma and breast cancer cells was also facilitated by Pfn1 overexpression [33], which has also been reported to promote apoptosis in breast cancer cells by stabilizing the integrin β1-actin complex [34]. In line with these findings, Fur A exerted its pro-apoptotic effects in CRC cells at least partly by up-regulating Pfn1. Similarly, ectopic expression also promoted apoptosis in CRC cells, thus further supporting its role in the apoptotic signaling pathway.

The role of Pfn1 in cancer cell motility and metastasis is however ambiguous. Ding et al showed that Pfn1 enhanced metastasis of breast cancer cells [35], and another study found that Pfn1 knockdown impaired adhesion and motility of T24 M cells, indicating its pro-metastatic role in bladder cancer [18]. In gastric cancer cells also, Pfn1 knockdown suppressed migration and invasion with concomitant suppression of MMP-2 and MMP-9 [36]. However, ectopic Pfn1 expression in MDA-MB-231 mammary carcinoma cells suppressed their micro-metastasis in nude mice [37], and restoration of Pfn1 levels in hepatocellular carcinoma cells also suppressed their motility and metastasis [22]. In line with their studies, we found that the anti-metastatic effects of Fur A against CRC were associated with Pfn1 upregulation. Nevertheless, further studies are needed to fully investigate the role of Pfn1 in cancer cell metastasis and the underlying mechanisms.

Aberrant down-regulation of Pfn1 has been documented in various human malignancies, including cancers of the breast, pancreas, bladder and liver [10–12,22]. In this study also, Pfn1 expression was significantly lower in the CRC tissues compared to the adjacent non-cancerous tissues. In addition, lower Pfn1 expression was associated with advanced disease stage, higher

Table 1
Correlation between Pfn1 expression and the clinico-pathological features of patients.

Parameters	Pfn1 expression		P value
	High(22)	Low(25)	
Age			0.5624
≤60	12	16	
>60	10	9	
Gender			0.5583
Male	13	17	
Female	9	8	
Tumor size			0.1237
≤5cm	17	13	
>5cm	5	12	
TNM stage			0.0190*
TI/II	16	9	
TIII/IV	6	16	
Lymph node metastasis			0.0174*
N0	17	10	
N1/2	5	15	

* $p < 0.05$.

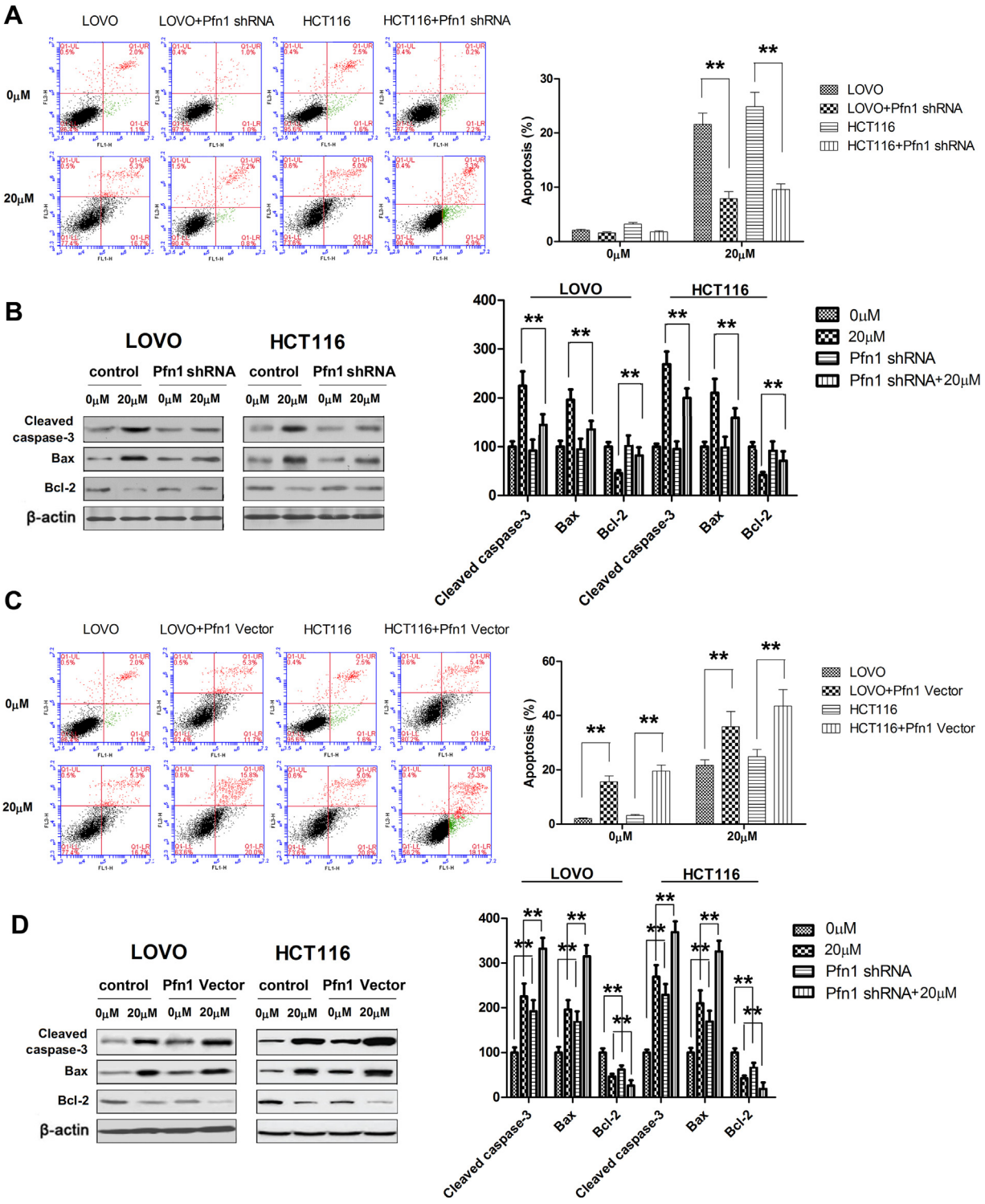

Fig. 6. Fur A exerts its pro-apoptotic effects by upregulating Pfn1. Knockdown of Pfn1 diminishes the pro-apoptotic effects of Fur A (A) and attenuates Fur A-induced activation of caspase-3, increase in Bax expression and decrease in Bcl-2 expression (B) in CRC cells. Ectopic Pfn1 expression augments the pro-apoptotic effects of Fur A (C) and augments Fur A-induced activation of casaspe-3, increase in Bax and decrease in Bcl-2 (D) in CRC cells. ** p <0.01.

TNM stage and lymph mode metastasis in CRC patients. In contrast, higher Pfn1 expression predicted longer overall survival of CRC patients. These findings collectively indicate that Pfn1 functions as a tumor suppressor in CRC.

In summary, Fur A suppresses CRC growth and metastasis by upregulating Pfn1. Our findings provide a novel insight into the therapeutic potential of Fur in CRC, and present Pfn1 as a prognostic biomarker of CRC.

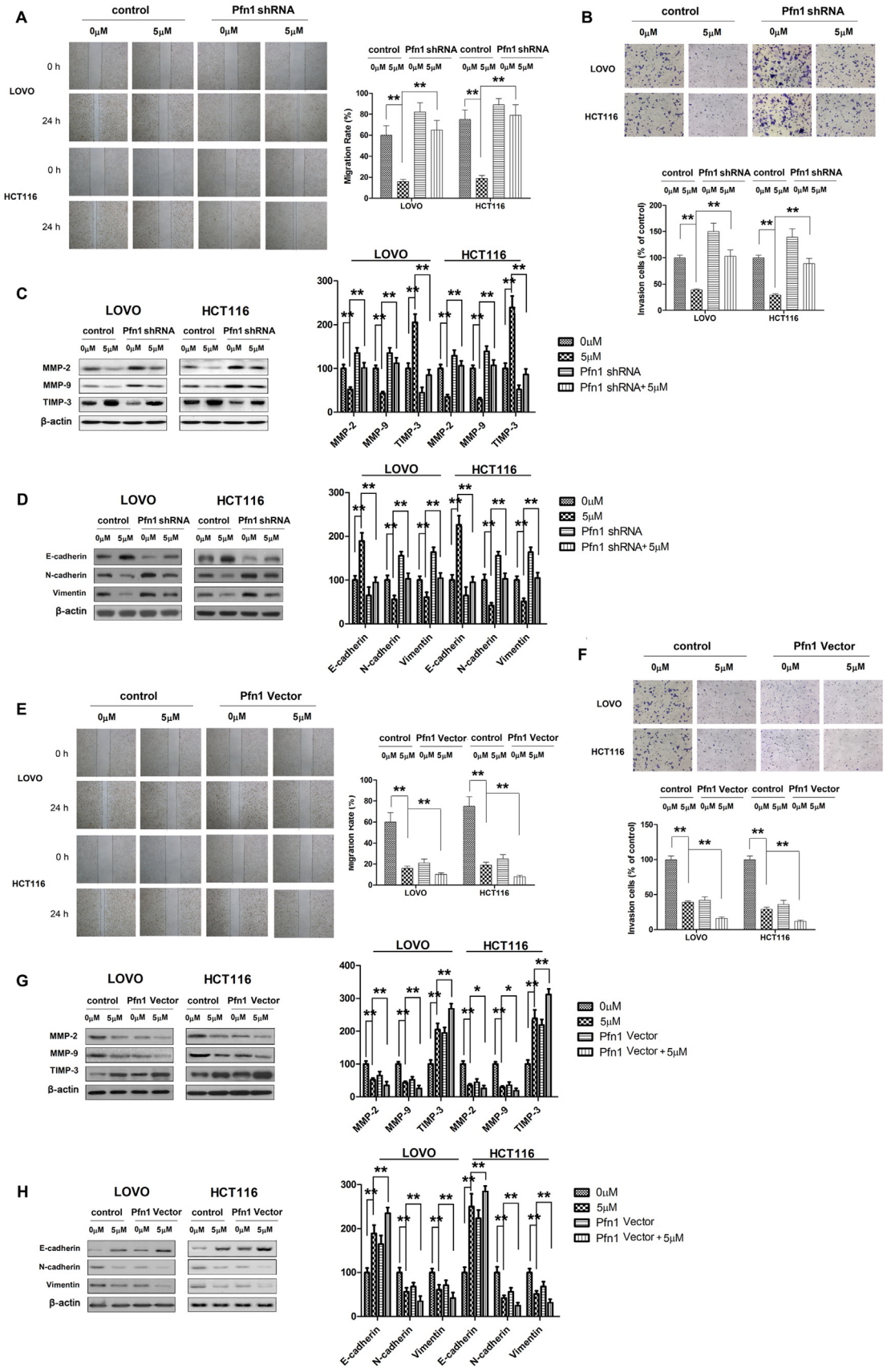

Fig. 7. Fur A-induced Pfn1 upregulation mediates its anti-metastatic effects in CRC cells. Pfn1 knockdown attenuates *in vitro* migration (A), invasion (B), decrease in MMP-2 and MMP-9, and increase in Bcl-2 expression (C), increase in E-cadherin, and decrease in N-cadherin and vimentin expression (D) induced by Fur A in CRC cells. Ectopic Pfn1 expression augments *in vitro* migration (E), invasion (F), decrease in MMP-2 and MMP-9, and increase in Bcl-2 expression (G), increase in E-cadherin, and decrease in N-cadherin and vimentin expression (H) induced by Fur A in CRC cells. $^{**}p < 0.01$.

Conflict of interest

None.

Author contribution

Jing Lv designed the research and provided the funds. Jinxia Zhao performed the experiments, analyzed the data and wrote the paper. Junhua Xu performed the experiments and analyzed the data.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pharep.2019.05.007.

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