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Original Articles

Scattered DUSP28 is a novel biomarker responsible for aggravating malignancy *via* the autocrine and paracrine signaling in metastatic pancreatic cancer

Jungwhoi Lee[a,b,**], Jungsul Lee[c], Jae-Hoon Kim[a,b,*]

[a] Department of Applied Life Science, SARI, Jeju National University, Jeju-do, 63243, Republic of Korea
[b] Subtropical/tropical Organism Gene Bank, Jeju National University, Jeju-do, 63243, Republic of Korea
[c] Department of Bio and Brain Engineering, KAIST, Daejeon, 34141, Republic of Korea

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ABSTRACT

Pancreatic cancer remains one of the most dangerous cancers with a grave prognosis. We have reported that dual specificity phosphatise 28 (DUSP28) could be secreted in pancreatic cancer cells. However, its biological function is poorly understood. Here, we distinguish the function of scattered DUSP28 in human pancreatic cancer. DUSP28 was specifically secreted to cultured medium in metastatic pancreatic cancer cells. Treatment with recombinant DUSP28 significantly increased the migration, invasion, and viability of metastatic pancreatic cancer cells through the activation of CREB, AKT, and ERK1/2 signaling pathways. In addition, administration of recombinant DUSP28 elicited pro-angiogenic effects in human umbilical vein endothelial cells. Injection of recombinant DUSP28 also produced tumor growth *in vivo*. Of interest, DUSP28 formed an autocrine loop with integrin α1 (ITGα1) by transcriptional regulation and recombinant DUSP28 acted as an oncogenic reagent through the interaction with ITGα1. Notably, scattered DUSP28 could be detected in whole blood samples of pancreatic cancer patients by accessible immunoassay. These results provide the basis for DUSP28 as a promising therapeutic target and a biomarker for metastatic pancreatic cancer.

1. Introduction

Pancreatic ductal adenocarcinoma, also known as pancreatic cancer, is one of the most aggressive diseases in the world. The five year survival rate is below 7% and median survival is approximately 6 months. While relatively low in prevalence, pancreatic cancer is the fourth leading cause of cancer-related death [1–3]. More seriously, the prognosis of pancreatic cancer patients remains unchanged, in spite of significant improvements in overall survival rates of other cancers [4]. Effective therapy for pancreatic cancers is urgently required.

One of the major features of pancreatic cancer is the high resistance to conventional cancer therapies including chemotherapy and radiation therapy. Another serious feature of pancreatic cancers is its early distant metastasis and local abnormal progression, which contributes to the relative rarity of surgery [5,6]. The more fundamental reason for the extremely poor prognosis may be that there are very few patients undergoing surgery because of lack of diagnosis [7]. Thus, early diagnosis is of vital significance.

To date, the diagnosis of pancreatic cancer after the onset of symptoms includes the use of computed tomography (CT) scan, endoscopic ultrasound (EUS), and endoscopic cholangiopancreatography (ERCP). Although these techniques have been proven effective, their ability to diagnose early-stage pancreatic cancer are still disappointing [8]. The elucidation of genetic abnormalities such as *KRAS, P16^{INK4A}*, *p53*, and *SMAD* has also been developed for pancreatic cancer diagnosis [9,10]. In addition, human serum or plasma markers have been continuously developed due to their easier accessibility for diagnostic testing than tissue samples. In the last two decades, potential biomarkers examined for pancreatic cancer diagnosis include CA19-9, DUPAN-2, CAM17.1, TPS, SPan-1, TAT1, POA, YKL-40, TUM2-PK, and various MMPs. CA19-9 is believed to the superior marker, still, it is not the perfect with variable or sometimes poor sensitivity (41–86%) and specificity (33–100%) [9,10]. These unsatisfactory results prompt us to exploit alternative biomarkers of pancreatic cancer.

* Corresponding author. Faculty of Biotechnology, College of Applied Life Science, Jeju National University, 102 Jejudaehak-ro, Jeju-si, Jeju-do, 63243, Republic of Korea.
** Corresponding author. Faculty of Biotechnology, College of Applied Life Science, Jeju National University, 102 Jejudaehak-ro, Jeju-si, Jeju-do, 63243, Republic of Korea.
E-mail addresses: sdjd1108@kaist.ac.kr (J. Lee), kimjh@jejunu.ac.kr (J.-H. Kim).

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Dual specificity phosphatases (DUSPs) are a heterogeneous group of protein phosphatases that regulate mitogen-activated protein kinases (MAPKs). DUSPs have been implicated as critical modulators of intracellular signaling pathways involved in various diseases including malignant cancers [11,12]. So far, 25 DUSP genes have been listed in Human Genome Organization databases and can be subdivided into three groups by subcellular expression. Class I DUSPs including DUSP1, 2, 4, and 5 are localized to the nucleus, while Class II DUSPs including DUSP6, 7, and 16 are found in the cytoplasm. Class III DUSPs including DUSP8, 9, and 10 can be localized in the nucleus or the cytoplasm [13–15]. Of particular interest, DUSP28 is a member of Class III, but unlike other members, dephosphorylating activity is abnormally low due to the substrate accommodation hindrance [16]. We have previously shown that DUSP28 could be detected as a scattered form *in vitro*, especially human metastatic pancreatic cancer cells [17]. However, its biological effects on autocrine signaling and paracrine signaling in the environments and blood vessels of cancer cells are still ambiguous.

Considering the pleiotropic expression features of DUSP28, the biological features evoked by scattered DUSP28 should be examined with respect to not only mode of autocrine and paracrine actions but also potential as a diagnostic biomarker in pancreatic cancer. In the present study, we verified the biological meanings of scattered DUSP28 in human metastatic pancreatic cancer *in vitro* and *in vivo* models. Our reports are the first to suggest that scattered DUSP28 aggravates the malignancy of human metastatic pancreatic cancer through the autocrine and paracrine signaling. Therefore, it might be a promising therapeutic target and a biomarker for metastatic pancreatic cancer.

2. Material and methods

2.1. Cell culture and reagents

Capan-1, Panc-1, SNU-213, and SNU-410 cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). CFPAC-1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown as described previously [16]. Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC, Manassa, VA, USA). HUVECs were grown in endothelial growth medium (EGM-2) Bulletkit medium (Lonza Biologics, Hopkinton, MA) at 37 °C in a humidified atmosphere containing 5% CO_2. All experiments were performed using HUVECs within 3–6 passages. H6c7 cells was obtained from Kerafast (Boston, MA, USA) and grown as described previously [17]. The recombinant DUSP28 protein was kindly provided by Dr. D.G. Jeong (Korea research institute of bioscience & biotechnology, KRIBB) (16). Polyclonal antibody for DUSP28 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and monoclonal antibody for DUSP28 was purchased from Calbiochem (La Jolla, CA). Antibodies for cAMP response element binding protein (CREB), phospho-CREB (Ser133), protein kinase B (AKT), phospho-AKT (Ser473), extracellular signal-regulated kinase (ERK), phospho-ERK (Thr202/204), proliferating cell nuclear antigen (PCNA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. Phospho-MAPK array

To demonstrate the intracellular signaling by recombinant DUSP28 treatment, a phospho-MAPK array kit (R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions.

2.3. Network analysis and enrichment analysis of chemical targets

Target protein network was constructed with direct target proteins and their interaction partners according to the protein-protein interaction data from The Human Protein Reference Database (http://www.hprd.org/) [18,19].

2.4. Transfection with small interfering RNA (siRNA)

Transfection of siRNAs was performed using Effectene reagent (Qiagen, Hilden, Germany) as reported previously [20]. Oligonucleotides specific for DUSP28 (sc-94445 and 1120164) and ITGα1 (sc-94445 and 1075668) were obtained from Santa Cruz Biotechnology and Bioneer (Daejeon, Korea) respectively. Scrambled control (sc-37007) was purchased from Santa Cruz Biotechnology. The efficacy of siRNA transfection was confirmed by Western blot analysis of corresponding proteins.

2.5. DUSP28 overexpression plasmid constructs

Plasmids expressing FLAG-tagged DUSP28 were constructed to overexpress DUSP28 in human pancreatic cancer cells, as described previously [16]. The target sequences for DUSP28 were: *Nhe*I, 5′-GCTA GCTAGC CACC ATG GGA CCG GCA GAA GCT GG-3′ and *Xho*I, 5′-GCCG CTCGAG TTA CTTGTCATCGTCGTCCTTGTAGTC AGC CTC AGG GCC CAA CCC TA-3'. Point-directed mutation PCR was used for the C103S-DUSP28 mutant construct, as described previously [21]. The primers were: 5′- GCCTGCCTAGTCTACAGCAAGAACGGCCGCAGC-3′, 5′- GCT GCGGCCGTTCTTGCTGTAGACTAGGCAGGC-3'.

2.6. Measurement of cell viability

To evaluate cell viability with si-DUSP28 transfection or exogenous treatment with conditioned medium following si-DUSP28 transfection, WST-1 reagent (Nalgene, Rochester, NY, USA) was used as described previously [22]. After a 10-min incubation at room temperature, absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Richmond, CA, USA).

2.7. Trans-well migration assay

Migration assay was performed using a 24-well Trans-well apparatus (Corning, Corning, NY, USA), as described previously [23]. Cells were applied to the upper chamber containing serum-free RPMI and the cells that migrated to the underside of the filter in 6 h were stained. The eluted dye was measured at 560 nm in an ELISA reader (Bio-Rad).

2.8. Cell invasion assay

Cell invasion assay was performed using growth factor-reduced Matrigel (BD Biosciences, San Diego, CA, USA) coated on 24-Transwell permeable supports as described previously [22]. Cells were layered in the upper chamber containing RPMI without serum for 18 h, and cells that invaded to the back side of the filter after 24 h were stained. The eluted dye was measured at 560 nm in an ELISA reader.

2.9. Tube formation

Tube formation assay was performed as previously described with some modifications [24]. In brief, 250 μL growth factor-reduced matrigel (BD Biosciences, San Jose, CA, USA) was used to coat 24 well plate (SPL Life Sciences, Seoul, Korea) and allowed to polymerize at 37 °C for 30 min. HUVECs (3×10^4 cells/well) were suspended in 500 μL serum-free medium containing different dosages of recombinant DUSP28. After incubation for 16 h at 37 °C, photographs of four representative fields per well were taken using phase contrast microscopy. Endothelial tubes were quantified by counting the number of junctions defined as the origin of two or more branch protrusions.

2.10. Western blot analysis

To evaluate the effect of recombinant DUSP28 treatment in a time dependent manner on Capan-1 cells, Western blot analysis was performed as described previously [25].

2.11. RNA preparation and reverse transcript polymerase chain reaction (RT-PCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and converted to cDNA using a RNA PCR kit (Takara Bio Inc., Shiga, Japan) as described previously [16]. RT-PCR analysis was performed using a Takara RT-PCR kit according to the manufacturers' protocol. Primers for human DUSP28 were obtained from Bioneer (Daejeon, Korea) and GAPDH gene primers were designed using Primer Express 3.0 (Applied Biosystems, Franklin Lakes, NJ, USA). Primer sequences for RT-PCR were as follows: GAPDH (forward primer, 5′-TCACTGGCATGGCCTTCCGTG-3′; reverse primer, 5′-GCCATGAGGTCCACCACCCTG-3′), DUSP28 (forward primer, 5′-GCGGGATCCATGGGACCGGCAGAAGCTGGGCG-3′; reverse primer, 5′-CCCGCTCGAGTCAAGCCTCAGGGCCCAACCCTAA-3′), and ITGα1 (forward 5′-GTGCTTATTGGTTCTCCGTTAGT-3′; reverse 5′-CACAAGCCAGAAATCCTCCAT-3′).

2.12. Enzyme-linked immunosorbent assay (ELISA)

To measure scattered DUSP28 in whole blood of pancreatic cancer patients, blood samples (ten from pancreatic cancer patients and sixteen from healthy individuals) were purchased from Innovative Research (Novi, MI, USA). Ninety six well plates (SPL) were coated with anti-DUSP28 polyclonal antibody (Santa Cruz Biotechnology) at 1 μg/mL. Bound scattered DUSP28 was detected using anti-DUSP28 monoclonal antibody (Abcam, Cambridge, UK) as described previously [26]. The absorbance was measured at 450 nm by using a microplate reader (Bio-Rad).

2.13. Immunoprecipitation (IP)

To detect scattered DUSP28 in whole blood of pancreatic cancer patients, the aforementioned blood samples were incubated with anti-polyclonal DUSP28 antibody, followed by addition of Protein G agarose (Amersham Bioscience, Little Chalfont, UK). Bound proteins were subjected to SDS-PAGE and Western blotting using a monoclonal DUSP28 antibody.

2.14. Xenograft tumor model

BALB/c nude mice were obtained from Orient (Seongnam, Korea) at 6–8 weeks of age. Capan-1 (1×10^7) cells were injected subcutaneously into left side of the flank as described previously [27]. Once the tumors achieved a size of approximately 60 mm^3, mice were randomized to two experimental groups to receive phosphate buffered saline (PBS) or 10 mg/kg of recombinant DUSP28. Tumor volume (V) was calculated as 0.523 LW^2 (L = length, W = width). Body weight was recorded regularly. Animal care and experiments were carried out in accordance with guidelines approved by the animal bioethics committee of Jeju National University (2016-0049). Animals were anesthetized by chloroform, perfused with PBS, and fixed with formalin (Sigma-Aldrich). Specimens were excised, immersed in formalin and transferred to 30% sucrose (Sigma-Aldrich) solution. Immunohistochemistry was performed using the VECTASTAIN® ABC-kit (Vector Laboratories, Inc., Burlingame, CA, USA) as manufacturer's recommendation.

2.15. GSE data-set analysis

GSE57495 data-set was obtained from the Gene Expression Omnibus (GEO) public microarray data-base. We independently integrated data set using the absolute normalization method SCAN.UPC [28]. To test prognostic value of a gene, samples were divided into two groups using median gene expression level as threshold. Log-rank test was then performed using Graph Prism version 5.

2.16. Statistical analyses

All data are presented as mean ± standard deviation. Levels of significance for comparisons between two independent samples were determined by Student's t-test. Groups were compared by one-way ANOVA with Tukey's *post hoc* test applied to significant main effects (SPSS 12.0K for Windows; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of DUSP28 treatment in human pancreatic cancer cells

To confirm the release of sDUSP28 in metastatic pancreatic cancer cells, we additionally used CFPAC-1 human metastatic pancreatic cancer cells and SNU-213 cells, which almost negligibly express DUSP28, for sandwich-ELISA and immune-precipitation. Capan-1 and CFPAC-1 cells are representative metastatic pancreatic cancer cells isolated from metastatic site of liver as previously reported [29–31]. sDUSP28 were clearly detected in cultured supernatants of Capan-1 and CFPAC-1 cells, but not SNU-213 cells (Fig. 1A and B). Next, we prepared recombinant DUSP28 (rDUSP28) to evaluate the effects of the sDUSP28 in features of human metastatic pancreatic cancer (Supplementary Fig. 1). As shown in Fig. 1C, treatment of rDUSP28 showed a significant and dose-dependent effect on migration of Capan-1 and CFPAC-1 cells. Treatment with maximal concentration of rDUSP28 (25 μg/mL) for 6 h increased Capan-1 and CFPAC-1 cell migration by approximately 1.34- and 1.33-fold compared to control cells; in contrast, denatured rDUSP28 (25 μg/mL) had no effect on migration of Capan-1 and CFPAC-1 cell. Exogenous treatment with rDUSP28 also significantly induced the invasion of Capan-1 and CFPAC-1 cells in a dose-dependent manner; in contrast, denatured rDUSP28 had no effect on invasion of Capan-1 and CFPAC-1 cell (Fig. 1D). In addition, the viability of Capan-1 and CFPAC-1 cells was significantly increased in a dose-dependent manner by rDUSP28 treatment; however, denatured rDUSP28 had no effect on viability of Capan-1 and CFPAC-1 cell (Fig. 1E). These results indicated that DUSP28 is scattered and functionally activates the malignancy in human metastatic pancreatic cancer cells.

3.2. Effect of DUSP28 treatment in the intracellular MAPK signaling pathway in human pancreatic cancer cells

We examined the signal transduction pathways using phospho-MAPK array to understand the mechanism by which DUSP28 aggravated the malignancy of human metastatic pancreatic cancer cells. Exogenous recombinant DUSP28 (rDUSP28) treatment induced a significant increase in phosphorylation of various MAPK molecules including AKT2, CREB, ERK1, ERK2, heat shock protein (HSP)27, and target of rapamycin (TOR) in Capan-1 cells compared to control cells (Fig. 2A). To further analyze the detailed mechanism of rDUSP28 treatment in Capan-1 cells, the levels of phosphorylated CREB, AKT, and ERK1/2 were investigated at different rDUSP28 treatment times. Exogenous rDUSP28 treatment increased phosphorylation of CREB, AKT, and ERK1/2 in a time-dependent manner (Fig. 2B). Similar result was obtained from CFPAC-1 cells (Supplementary Fig. 2). *In silico* protein networks of molecules regulated by exogenous treatment of rDUSP28 were constructed with direct target proteins and their interaction partners through the protein-protein interaction data from The Human Protein Reference Database [18]. According to the protein-protein interaction (PPI) network, AKT2, CREB, ERK1, ERK2, HSP27, and TOR regulated by rDUSP28 treatment markedly established the

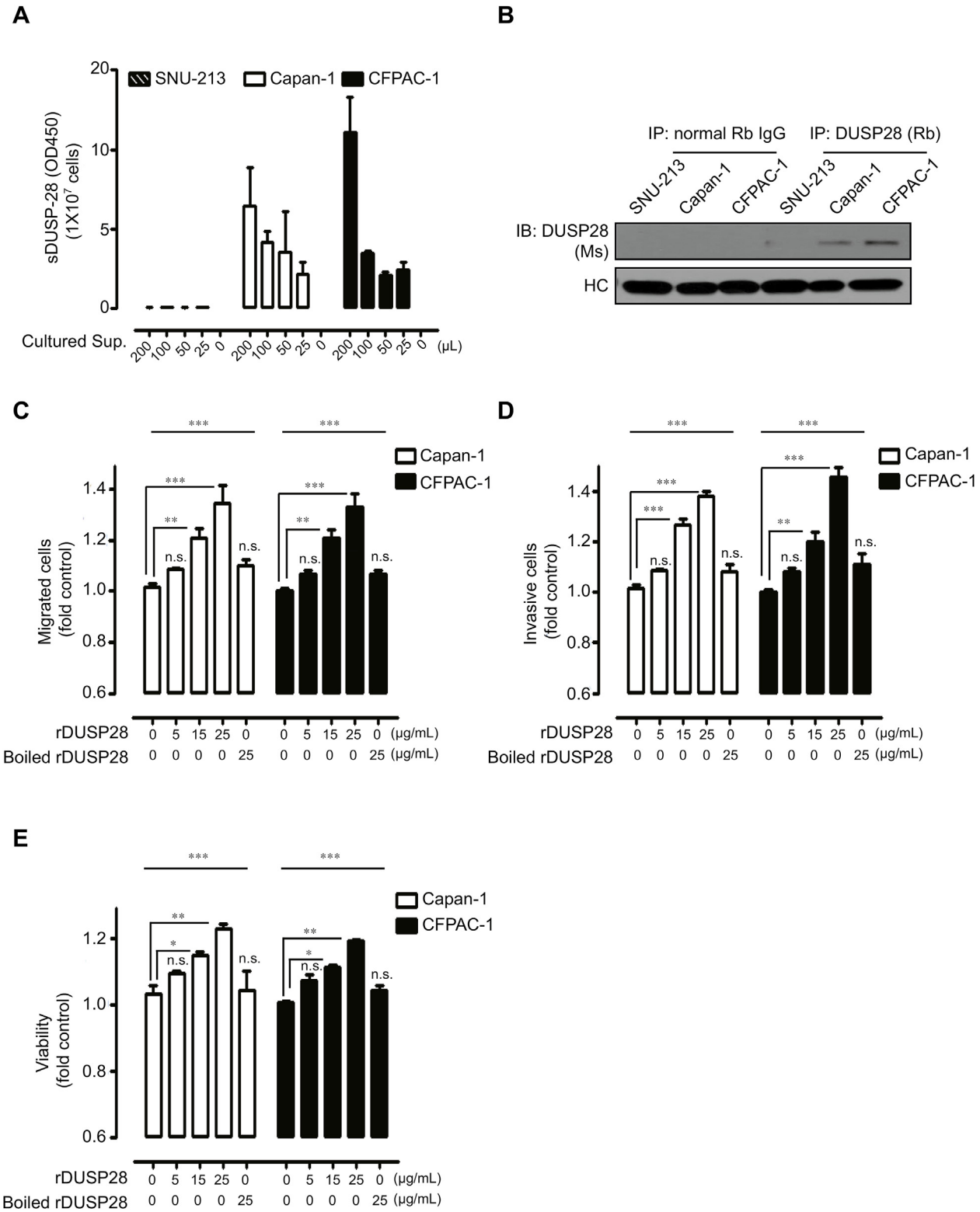

Fig. 1. Sensitivity of human metastatic pancreatic cancer cells to recombinant DUSP28 (rDUSP28). (A) sDUSP28 was measured by a sandwich ELISA in SNU-213, Capan-1, and CFPAC-1 cells cultured supernatants. **(B)** sDUSP28 was precipitated using polyclonal DUSP28 and normal rabbit IgG antibodies in SNU-213, Capan-1, and CFPAC-1 cells cultured supernatants. Bound DUSP28 was subjected to Western blot analysis using monoclonal antibody specific for DUSP28. Heavy chain (HC) of antibodies was used as a control. **(C)** Capan-1 and CFPAC-1 cells were incubated with various concentrations of rDUSP28 or denatured rDUSP28 for 6 h. Migration was evaluated using the Transwell migration assay ($n = 3$; Tukey's post-hoc test was applied to detect significant group effects as determined by analysis of variance, $P < 0.0001$; asterisks indicate a significant difference vs. 0% inhibition, $^{***}P < 0.001$, $^{**}P < 0.01$, n.s., non-significant). **(D)** Capan-1 and CFPAC-1 cells were incubated with various concentrations or denatured sDUSP28 for 24 h. Invasion was evaluated using the Transwell invasion assay ($n = 3$; Tukey's post-hoc test was applied to detect significant group effects as determined by analysis of variance, $P < 0.0001$; asterisks indicate a significant difference vs. 0% inhibition, $^{***}P < 0.001$, $^{**}P < 0.01$, n.s., non-significant). **(E)** Capan-1 and CFPAC-1 cells were incubated with various concentrations or denatured sDUSP28 for 72 h under serum-free cultured condition. Viability was measured by the WST-1 assay ($n = 3$; Tukey's *post hoc* test was applied to significant group effects in ANOVA, $p < 0.0001$; asterisks indicate a significant difference compared with 0% inhibition, $^{*}P < 0.05$, $^{**}P < 0.01$, n.s., non-significant).

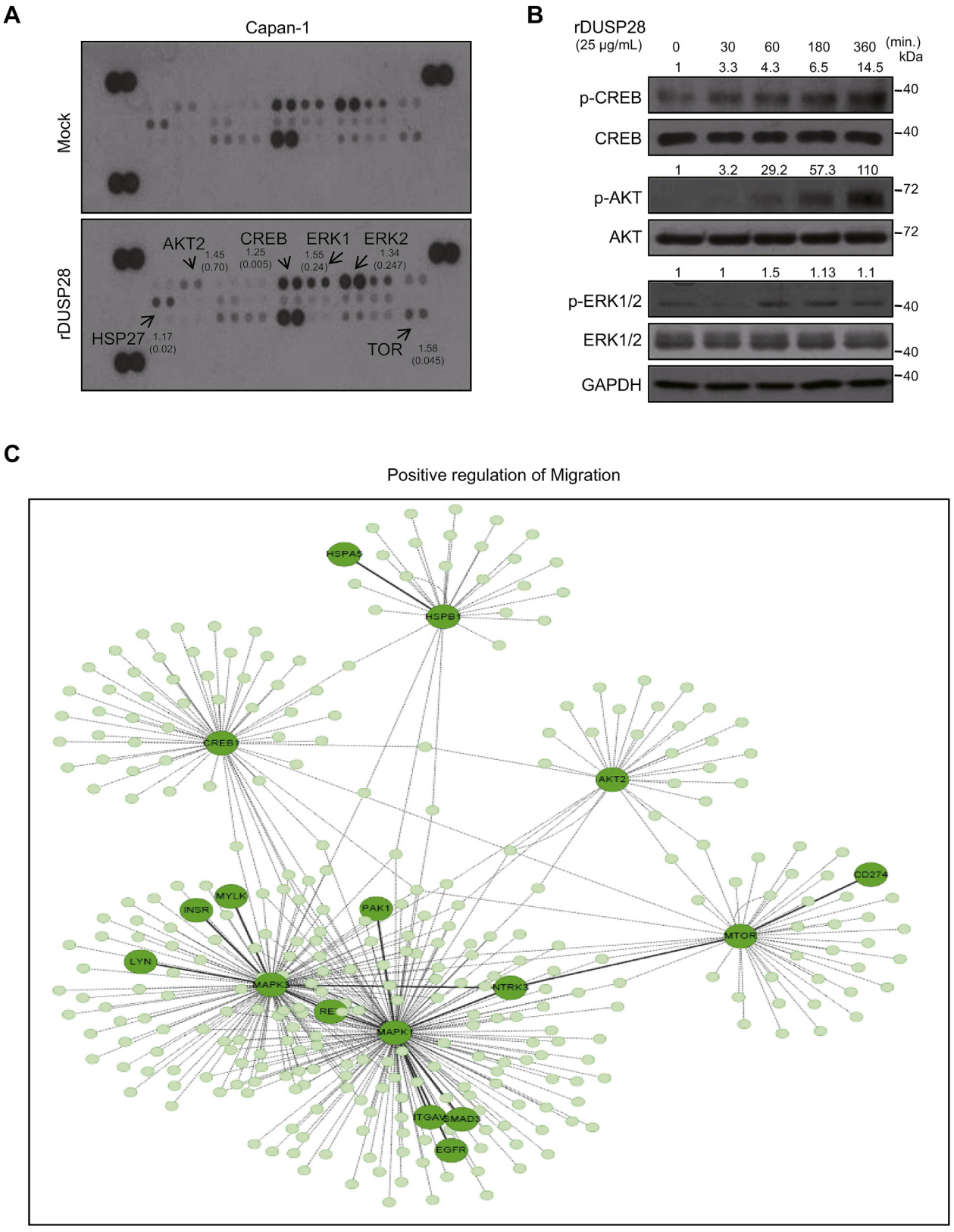

Fig. 2. Regulation of Mitogen-activated protein kinase (MAPK) signaling by recombinant DUSP28 (rDUSP28). (A) Capan-1 cells were incubated with rDUSP28 (25 μg/mL) for 24 h. Phospho-MAPK array was used to determine differences in rDUSP28 treated, or control human pancreatic cancer cells. Expression changes of various molecules are indicated by the arrow. Relative pixel intensities for phospho-AKT2, phospho-CREB, phospho-ERK1, phospho-ERK2, phospho-HSP27, and phospho-TOR were measured by densitometry analysis using ImageJ analysis software and compared to positive control spots. **(B)** Capan-1 cells were incubated with rDUSP28 (25 μg/mL) for various times, and the cell lysates were subjected to Western blot analysis using antibodies specific for phospho-CREB, total CREB, phospho-AKT, total AKT, phospho-ERK1/2, total ERK1, and GAPDH. Relative pixel intensities for phospho-CREB, phospho-AKT, phospho-ERK1/2 were measured using phospho-CREB/total-CREB, phospho-AKT/total-AKT, and phospho-ERK1/2/total-ERK1/2 by densitometry analysis using ImageJ analysis software. **(C).** Protein-protein interaction (PPI) networks of AKT2, CREB, ERK1, ERK2, HSP27, and TOR in pancreatic cancer were constructed using The Human Protein Reference Database (http://www.hprd.org/).

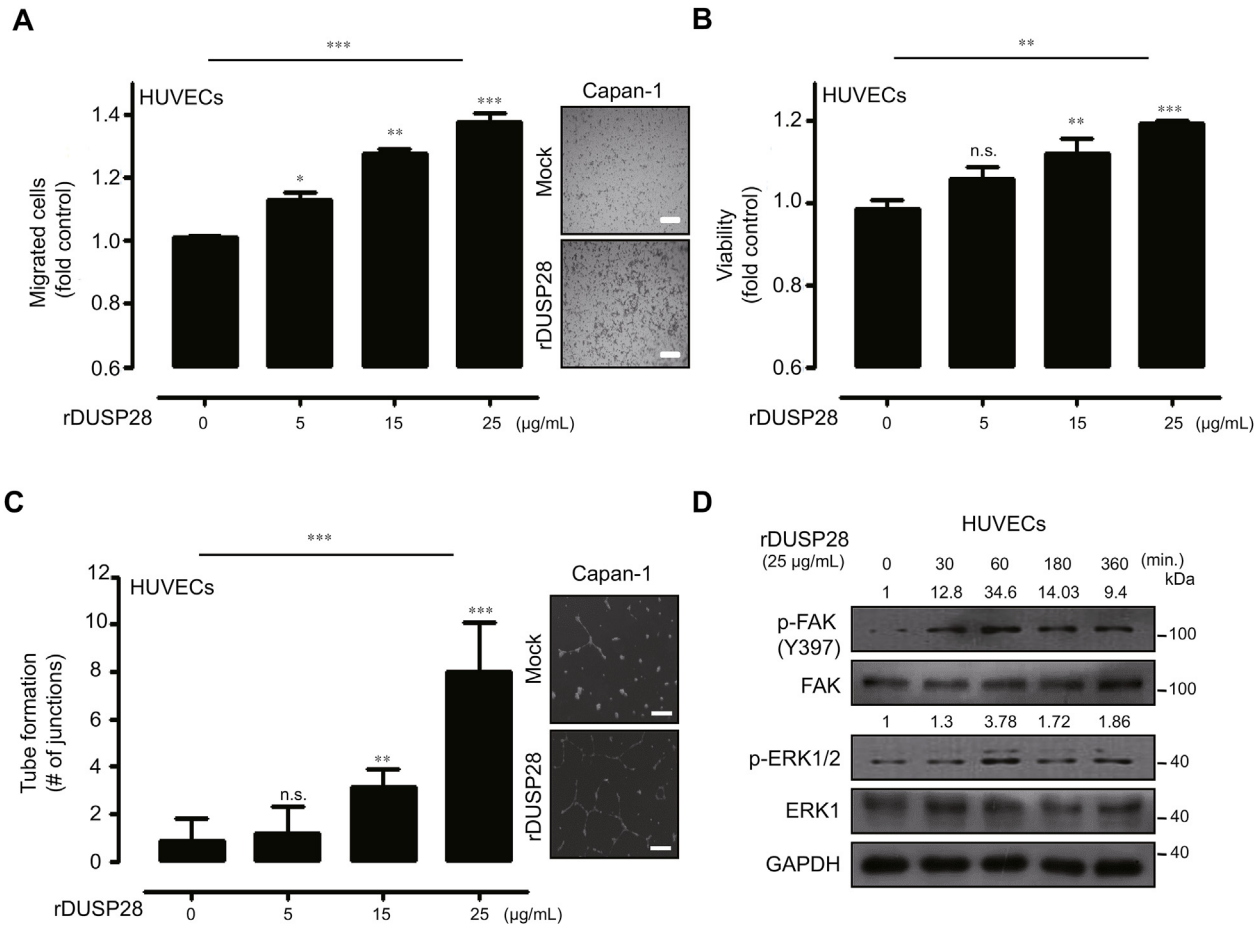

Fig. 3. Pro-angiogenic effects of recombinant DUSP28 (rDUSP28). (A) HUVECs were incubated with varying concentrations (0, 5, 15, and 25 μg/mL) of rDUSP28. Migration was evaluated using the Transwell migration assay ($n = 3$; Tukey's post-hoc test was applied to detect significant group effects as determined by analysis of variance, $P < 0.0001$; asterisks indicate a significant difference vs. 0% inhibition, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, n.s., non-significant, scale bar = 50 μm). **(B)** HUVECs were incubated with varying doses of rDUSP28 for 72 h. Viability was measured by the WST-1 assay ($n = 3$; Tukey's *post hoc* test was applied to significant group effects in ANOVA, $P < 0.0001$; asterisks indicate a significant difference compared with 0% inhibition, $^{**}P < 0.01$, $^{***}P < 0.001$, n.s., non-significant). **(C)** Capillary like structure (CLS) formation of HUVECs assayed after 16 h of incubation of cells in the varying concentrations of rDUSP28 (data represent the percentage ± SD and are representative of three individual experiments, $^{**}P < 0.01$, $^{***}P < 0.001$, n.s., non-significant). **(D)** HUVECs were incubated at 25 μg/mL of rDUSP28 in a time-dependent manner, and cell lysates were subjected to Western blot using antibodies for phospho-FAK and phospho-ERK1/2. GAPDH was used as loading control. Relative pixel intensities for phospho-FAK and phospho-ERK1/2 were measured using phospho-FAK/total-FAK and phospho-ERK1/2/total-ERK1/2 by densitometry analysis using ImageJ analysis software.

oncogenic signaling pathway including positive regulation of migration (Fig. 2C and Supplementary table) and negative regulation of apoptosis (Supplementary Fig. 3). These results clearly indicated that rDUSP28 treatment regulates the functional MAPK signaling pathway in human metastatic pancreatic cancer cells.

3.3. Effects of recombinant treatment in HUVECs

We next tested whether rDUSP28 can induce pro-angiogenic effects in HUVECs due to its external-cellular action potential. As shown in Fig. 3A, rDUSP28 treatment significantly induced a HUVECs migration as compared to the control cells in a dose-dependent manner. Treatment with rDUSP28 also significantly increased on the viability of HUVECs as the treated dosages increased (15 μg/mL: 1.12-fold; 25 μg/mL: 1.19-fold) (Fig. 3B). Since endothelial cell morphogenesis is critically required for angiogenesis [32], we next examined the effect of rDUSP28 on tube formation in HUVECs using a capillary-like tubular structures formed on matrigel. Quantitative evaluation of tube formation by counting the junctions of branches revealed that exposure to rDUSP28 significantly increased the number of junctions of the tubular structure compared to control treatment in a dose-dependent manner (15 μg/mL: 3.16-fold; 25 μg/mL: 8-fold) (Fig. 3C). To understand the functional mechanism of the pro-angiogenic effect of rDUSP28 in HUVECs, we examined the time course of rDUSP28-induced signal transduction. We have previously reported that FAK and ERK1/2 signaling pathway was critical for pro-angiogenic effect [33]. Therefore, alteration of phosphorylated FAK and ERK1/2 were investigated following exogenous rDUSP28 administration. Treatment with rDUSP28 (25 μg/mL) time-dependently increased the levels of phosphorylated FAK (Y397) and ERK1/2 (Fig. 3D). These results show that rDUSP28 treatment induces pro-angiogenic effects through the activation of the FAK and ERK1/2 signaling pathways.

3.4. In vivo effects of recombinant DUSP28

To validate the effect of rDUSP28 *in vivo*, we prepared xenograft models using Capan-1 cells. Tumor-laden mice were injected intraperitoneally with PBS or rDUSP28 (10 mg/kg) when the tumors reached an average size of approximately 60 mm^3. PBS-treated Capan-1 xenograft tumors grew to an average size of 157.23 ± 11.33 mm^3 by 22 days after transplantation, while rDUSP28-treated Capan-1 xenograft tumors grew to an average size of 295.33 ± 108.79 mm^3 after the same time (Fig. 4A). There was no significant weight loss in either the control and rDUSP28-treated xenograft models (Fig. 4B). As expected,

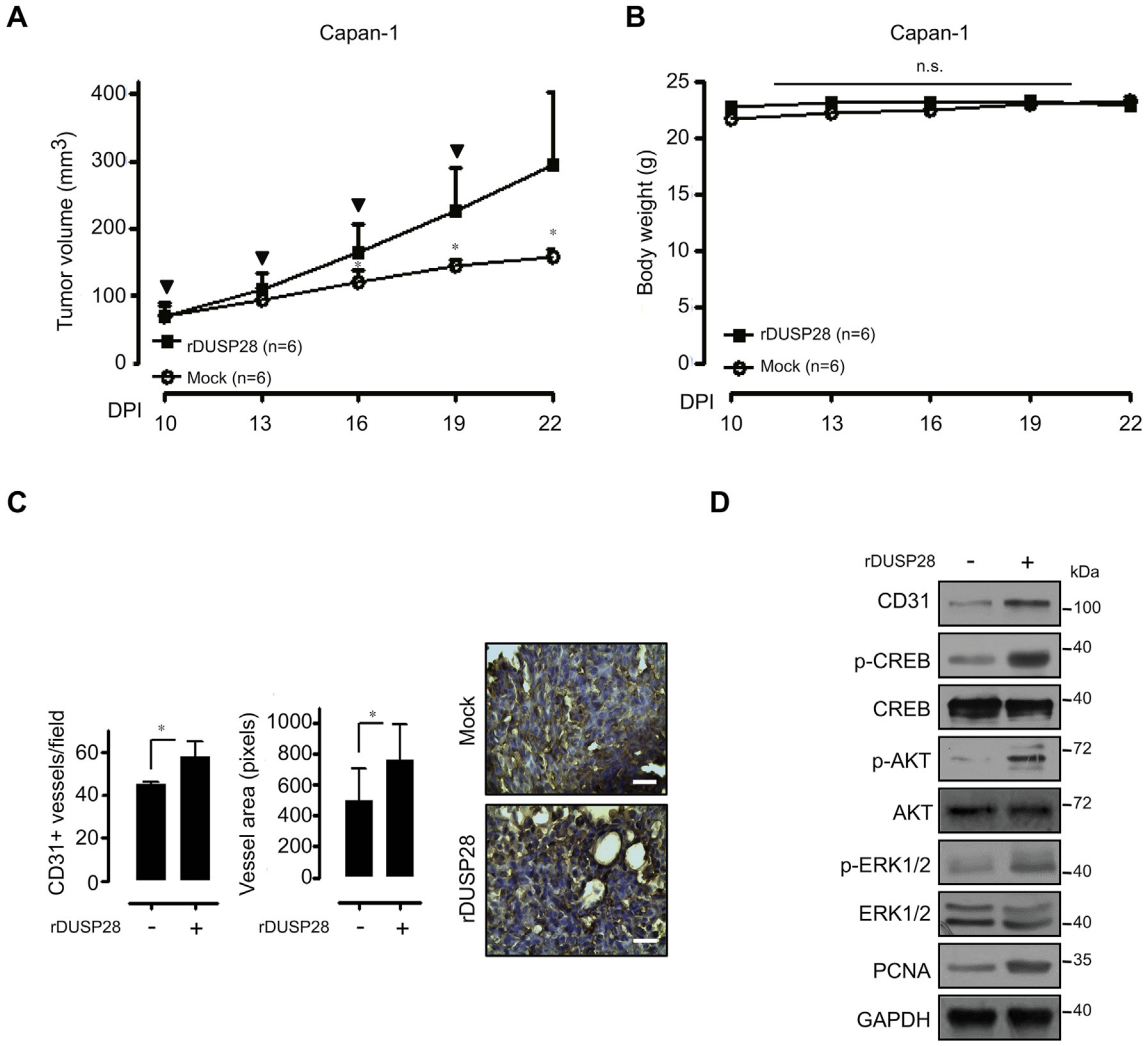

Fig. 4. *In vivo* effects of recombinant DUSP28 (rDUSP28). (A) Effects of rDUSP28 (30 mg/kg) injection in Capan-1 xenograft models (Mock group: $n = 6$ and rDUSP28 injection group: $n = 6$) were measured for 22 days using the formula: $V = 0.523\ LW^2$ (L = length, W = width). Bold arrows indicate the time of rDUSP28 injection (Tukey's *post hoc* test was applied to significant group effects in ANOVA, $P < 0.0001$; asterisks indicate a significant difference between the control group and the sDUSP28-injected group, $^*P < 0.05$, DPI indicates the day post injection). **(B)** The body weight in each group was measured regularly. **(C)** Immunohistochemistry was performed for the number of CD31 positive vessels and vessel areas in control or rDUSP28 treated xenograft tumors. CD31 (brown) was counterstained with hematoxylin (blue) (Magnification: X200, scale bar = 200 μm). **(D)** Western blot analysis of control or rDUSP28 treated tumor lysate was probed with anti CD31, phospho-CREB, phospho-AKT, phospho-ERK1/2, and PCNA antibodies. GAPDH was used for loading control. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the vessel areas and the number of CD31-positive endothelial cells also were significantly increased in rDUSP28-treated tumors compared to the control group (Fig. 4C). To ascertain the growth stimulating effect of rDUSP28 at the molecular level, we determined the levels of CD31, phospho-CREB, phospho-AKT, phospho-ERK1/2, and proliferating cell nuclear antigen (PCNA) in control and rDUSP28-treated xenograft models. Treatment with 10 mg/kg of rDUSP28 upregulated the levels of phospho-CREB, phospho-AKT, phospho-ERK1/2, CD31, and PCNA compared to the PBS-treated group (Fig. 4D). These results strongly suggest that rDUSP28 stimulates tumor growth through the activation of the MAPK signaling pathway and angiogenesis *in vivo*.

In addition, we used conditioned medium (CM) containing sDUSP28 to investigate the effect of sDUSP28 blockade in human metastatic pancreatic cancer cells. We firstly checked the efficiency of si-DUSP28 transfection in Capan-1 cells using RT-PCR and Western blot. Transient transfection of si-DUSP28 strongly reduced the expression of *DUSP28* mRNA in Capan-1 cells (Supplementary Fig. 4A, Top) and sDUSP28 in cultured medium (Supplementary Fig. 4A, Bottom). Different volumes of si-DUSP28-transfected CM significantly reduced a migration of Capan-1 and CFPAC-1 cells compared to scrambled siRNA-transfected CM (Supplementary Fig. 4B) Treatment of 10% si-DUSP28-transfected CM (to total volume of cultured medium) also weakly but statistically significantly inhibited the viability of Capan-1 and CFPAC-1 cells (Supplementary Fig. 4C). In addition, we examined the effect of si-DUSP28-transfected CM on migration and tube formation of HUVECs using the Transwell migration assay and formation of capillary-like tubular structure formed on matrigel, respectively. Treatment with different volumes of si-DUSP28- transfected CM significantly reduced migration and tube-formation by HUVECs compared to the scrambled siRNA transfected CM treated HUVECs (Supplementary Figs. 4D and 4E). Treatments with different volumes of si-DUSP28-transfected CM also showed weakly, though statistically significant decreases in the viability of HUVECs as the treated dosages increased (Supplementary Fig. 4F). These results clearly indicated that blockade of sDUSP28 effectively induces anti-cancer effects in human metastatic pancreatic cancer cells and anti-angiogenesis effects in HUVECs.

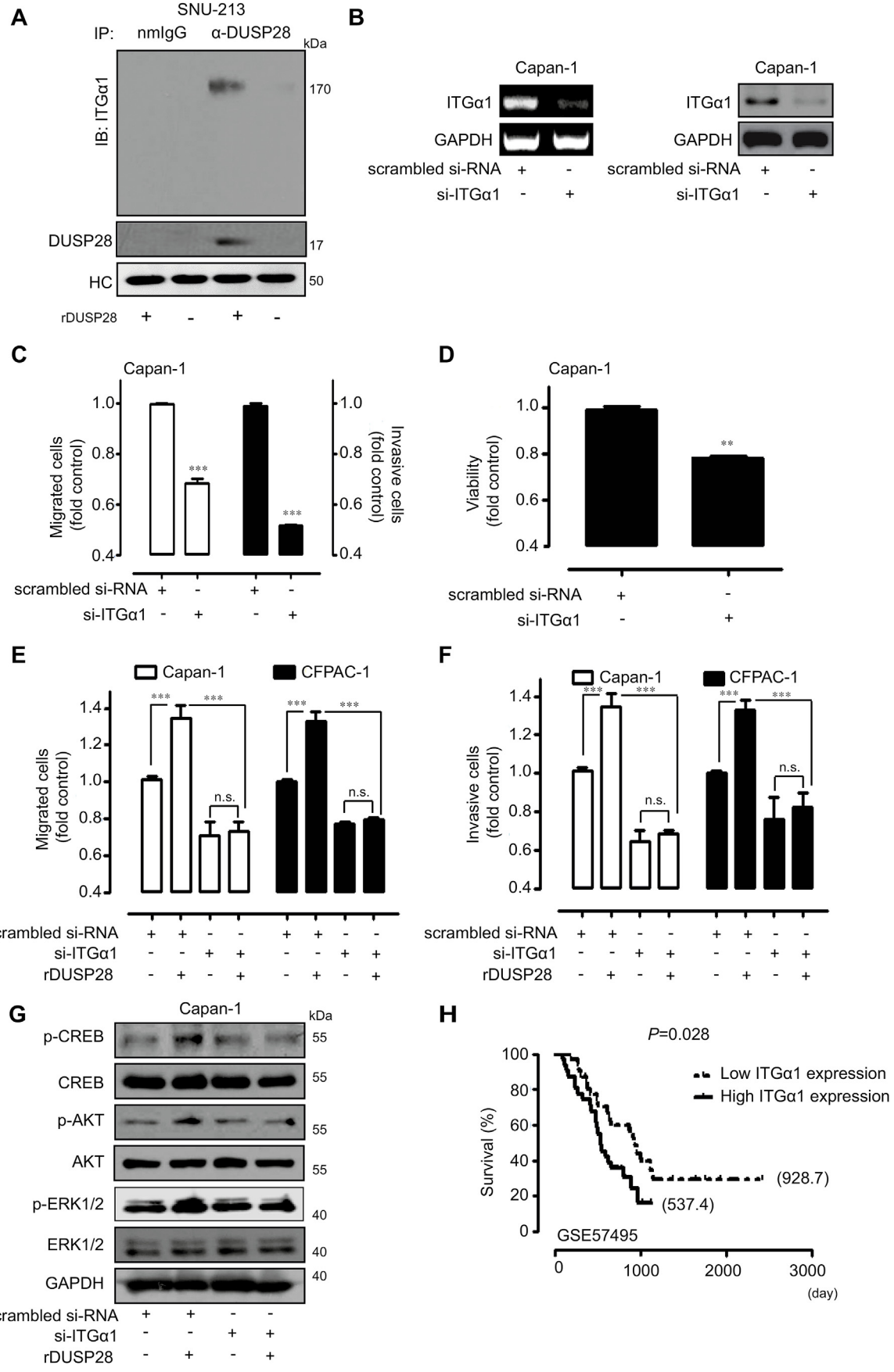

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Fig. 5. Molecular interaction partner with recombinant DUSP28 (rDUSP28). (A) ITGα1 and exogenously treated rDUSP28 were precipitated using polyclonal DUSP28 and normal rabbit IgG antibodies in SNU-213 cells. Bound ITGα1 and rDUSP28 were subjected to Western blot analysis using monoclonal antibody specific for ITGα1 and DUSP28. Heavy chain (HC) of antibodies was used as a control. **(B)** Capan-1 cells were transfected with scrambled or ITGα1-specific siRNA. After 72 h of transfection, *ITGα1* mRNA levels were analyzed by reverse-transcription polymerase chain reaction (RT-PCR, Left) and ITGα1 proteins were subjected to Western blot analysis, Right. GAPDH was used as a control. **(C)** Capan-1 cells were transfected with scrambled or ITGα1-siRNAs for 48 h. Migrated or invasive cells was evaluated using the Transwell-assay for an additional 6 for migration or 24 h for invasion, respectively ($n = 3$; Tukey's *post-hoc* test was used to detect significant difference in ANOVA, $p < 0.0001$; asterisks indicate a significant difference compared with 0% inhibition, $^{***}P < 0.001$). **(D)** Capan-1 cells were transfected with scrambled or ITGα1-siRNAs for 72 h. Viability was measured by the WST-1 assay (*P* value was calculated using the Student's *t*-test). **(E)** Capan-1 and CFPAC-1 cells were transfected with scrambled or ITGα1-siRNAs for 48 h. Exogenous rDUSP28 (25 μg/mL) was pretreated for 1 h. Migrated cells were evaluated using the Transwell-assay for a 6 h ($n = 3$; Tukey's *post-hoc* test was used to detect significant difference in ANOVA, $p < 0.0001$; asterisks indicate a significant difference compared with 0% inhibition, $^{***}P < 0.001$). **(F)** Capan-1 and CFPAC-1 cells were transfected with scrambled or ITGα1-siRNAs for 48 h. Exogenous rDUSP28 (25 μg/mL) was pretreated for 1 h. Invasive cells were evaluated using the Transwell-assay for a 24 h ($n = 3$; Tukey's *post-hoc* test was used to detect significant difference in ANOVA, $p < 0.0001$; asterisks indicate a significant difference compared with 0% inhibition, $^{***}P < 0.001$). **(G)** Capan-1 cells were transfected with scrambled or ITGα1-siRNAs for 48 h. Exogenous rDUSP28 (25 μg/mL) was treated for 6 h and the cell lysates were subjected to Western blot using antibodies specific for phospho-CREB, total CREB, phospho-AKT, total AKT, phospho-ERK1/2, total ERK1, and GAPDH. **(H)** Kaplan-Meier plot of the median survival for pancreatic cancer patients included dependent on differential expressions of ITGα1 (*P* value was calculated using Log-rank (Mantel-Cox) Test, respectively).

3.5. A novel molecular partner of DUSP28

We have previously investigated that ITGα1 was highly expressed in various human pancreatic cancer cells according to preliminary study (Supplementary Fig. 5) and DUSP28 affected the critical functions in human pancreatic cancer [20,34]. Based on these features, we demonstrated the correlation between cellular DUSP28 and ITGα1 expression. Various integrin mRNAs were highly variable in pancreatic cancer and normal pancreas according to GEO public database analysis (Supplementary Figs. 6A–B) and ITGα1 was specifically regulated by DUSP28 expression at both mRNA and protein levels, but not other integrins (ITGs). Silencing DUSP28 expression resulted in reduction of ITGα1 expression at both mRNA and protein levels in Panc-1 cells, which positively express DUSP28. (Supplementary Figs. 6C–D). Enhanced wild-type DUSP28 expression upregulated ITGα1 expressions at both mRNA and protein levels in SNU-213 cell, which almost negatively express DUSP28, but not in mutant DUSP28-C103S overexpression. The mutated DUSP28-C103S transfection had little activity compared to those of DUSP28-WT transfection as previously reported [35]. To validate the correlation of ITGα1 as an interaction partner of DUSP28 in human pancreatic cancer cell, we examined the IP analysis following exogenous rDUSP28 treatment in SNU-213 cells. ITGα1 was precipitated by exogenously treated rDUSP28 in SNU-213 cells compared to which of normal mouse IgG antibody (nmIgG) (Fig. 5A, Supplementary Figs. 6A and 6B). Transfection of si-ITGα1 decreased both mRNA and protein levels of ITGα1 in Capan-1 cells (Fig. 5B Left and Right). Silencing of ITGα1 significantly diminished migration, invasion, and viability in Capan-1 cells (Fig. 5C and D). In particular, transfection of si-ITGα1 inhibited migration, invasion, and viability even stimulated by exogenous rDUSP28 treatment in Capan-1 and CFPAC-1 cells compared to those of scrambled si-RNA transfection (Fig. 5E and F, and Supplementary Fig. 7). Inhibition of ITGα1 expression was also remarkably decreased the phosphorylation of CREB, AKT, and ERK1/2 induced by exogenous rDUSP28 treatment compared to those in scrambled si-RNA transfected cells, respectively (Fig. 5G). According to the GSE57495 data-set, low ITGα1 expression significantly improved median survival by 391 days compared to that of a high level of ITGα1 (Fig. 5H). Low DUSP28 expression also improved median survival by 254 days compared to that of a high level of DUSP28, though the difference was not significant (Supplementary Fig. 8). These results indicated that rDUSP28 aggravates malignancy of human metastatic pancreatic cancer *via* interaction with oncogenic ITGα1.

3.6. Efficacious detection of scattered DUSP28

We explored whether sDUSP28 can be detected in whole blood samples as a means of diagnosing pancreatic cancers. To demonstrate the detection of sDUSP28 in whole blood, we prepared xenograft models using Capan-1 (n = 4) and SNU-213 (n = 4) cells and validated sandwich ELISA using whole blood samples of Capan-1 and SNU-213 xenograft models. sDUSP28 was specifically detected in whole blood samples of the Capan-1 xenograft models in a volume-dependent manner, but not in whole blood samples of the SNU-213 xenograft model and non-tumor bearing mice (Normal) (Fig. 6A). The detection of sDUSP28 in the Capan-1 model's blood samples was also significant using Western blot compared to whole blood samples of the SNU-213 xenograft model and non-tumor bearing mice (Fig. 6B). sDUSP28 could be significantly detected in whole blood samples from pancreatic cancer patients using sandwich ELISA with a success rate of 90% (9/10) compared to sixteen healthy donors (Fig. 6C and Supplementary Fig. 9). Western blot (Top) and IP analysis (Bottom) produced similar results using whole blood samples of pancreatic cancer patients and healthy donors (Fig. 6D).

Collectively, these results indicate that sDUSP28 is a critical regulator responsible for metastatic pancreatic cancer malignancy cooperating with ITGα1 and associated angiogenesis. Moreover, sDUSP28 might be a potential biomarker for selection of malignant metastatic pancreatic cancer (Fig. 6E).

4. Discussion

Understanding the mechanism underlying malignant metastatic pancreatic cancer features has become increasingly important due to the worst clinical outcome. Survival rates of metastatic pancreatic cancer patients have not improved during the past decades [4]. This grave lack of response might originate from the strong resistance to chemotherapies, tenacious metastasis to different sites and difficulty in early diagnosis [33,36,37].

Presently, metastatic pancreatic cancer cells specifically scattered a soluble form of DUSP28 compared to other pancreatic cancer cells. Treatment of recombinant DUSP28 (rDUSP28) induced the malignancy of human metastatic pancreatic cancer cells and pro-angiogenic effects of HUVECs through the activation of intracellular signaling pathway *in vitro* and *in vivo*. In addition, ITGα1 acted as the molecular interaction partner of rDUSP28 in human metastatic pancreatic cancer cells. Notably, sDUSP28 was readily detected in whole blood samples of xenograft models and pancreatic cancer patients.

Several DUSPs have been reported as positive regulators of malignant cancer features including apoptosis, proliferation, cell cycle, migration, and chemo-resistance apart from the functions of classical DUSPs. DUSP3 is up-regulated in cervical and prostate cancers, and functions as an inhibitor of apoptosis [38,39]. DUSP12 is also amplified in a variety of cancers including neuroblastoma, retinoblastoma, intracranial ependymoma, and chronic leukemia, and participates in cell cycle regulation, inhibition of apoptosis, and activation of migration [40]. Selective over-expression of DUSP23 in MCF-7 human breast cancer cells induces cell proliferation, while knock-down of DUSP23

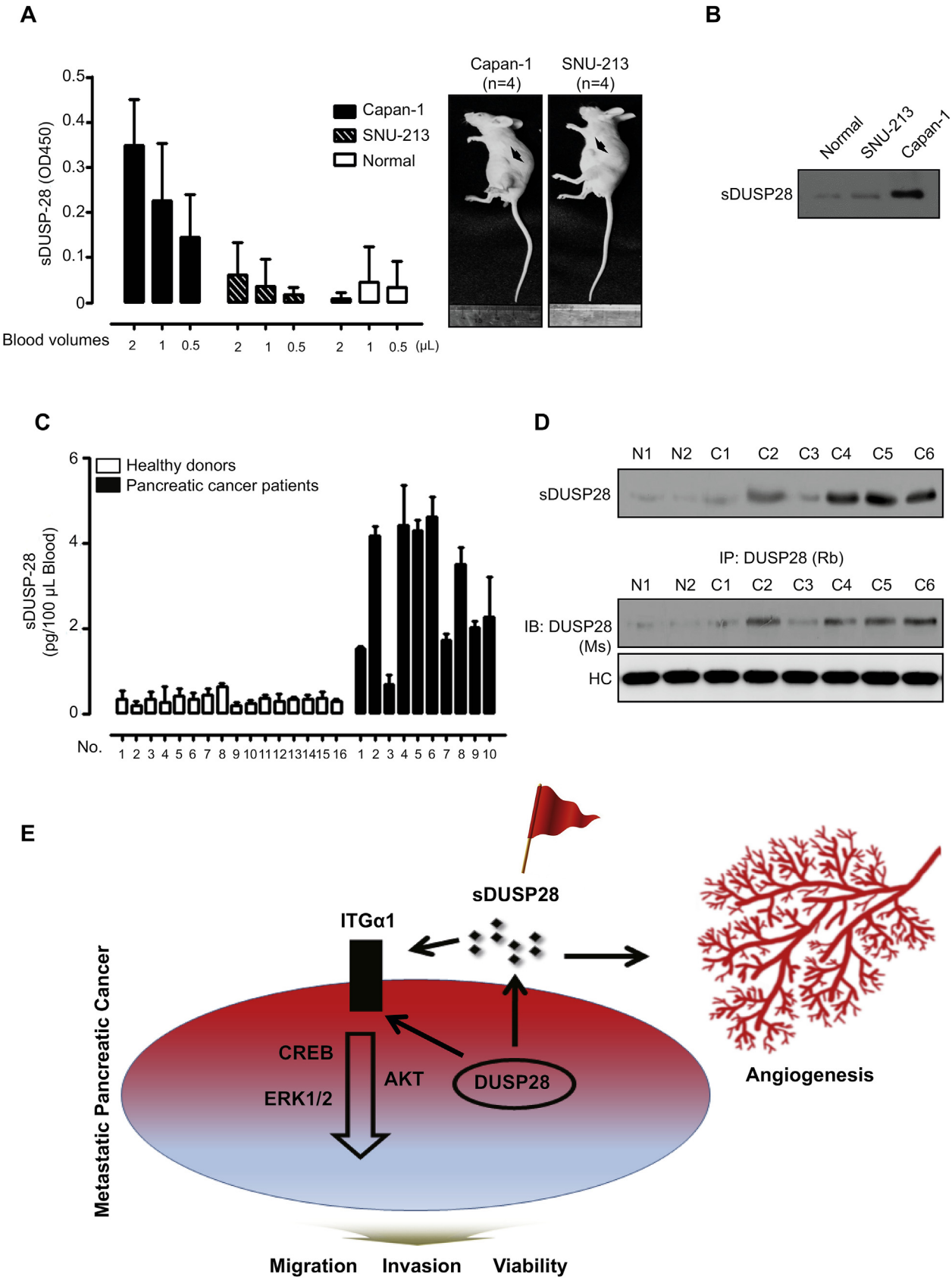

Fig. 6. Detection of scattered DUSP28 (sDUSP28). (A) sDUSP28 was selected by a sandwich ELISA using the varying volumes of whole blood samples from Capan-1 and SNU-213 xenograft models (Normal means non-tumor bearing nude mice). **(B)** sDUSP28 was detected by Western blot analysis using same volumes of whole blood samples from Capan-1 and SNU-213 xenograft models (normal, non-tumor bearing nude mice). **(C)** Concentrations of sDUSP28 were analyzed by a sandwich ELISA using whole blood samples from sixteen health donors (N1-N16) and ten pancreatic cancer patients (C1-C10). **(D)** sDUSP28 was detected by Western blot (Top) and IP (Bottom) analysis using same volumes of whole blood samples from two health donors (N1 and N2) and six pancreatic cancer patients (C1-C6). **(E)** Graphical abstract about the functions of scattered DUSP28 in human metastatic pancreatic cancers.

expression decreases cell proliferation [41]. In addition, enhanced expression of DUSP26 promotes colony formation, while si-DUSP26 transfection reduces cell proliferation [42]. In agreement with previous studies, DUSP28 was uniquely expressed in metastatic pancreatic cancer cells as a secreted form, and aggravated pancreatic cancer malignancy through the activation of the autocrine MAPK signaling pathway *in vitro* and *in vivo*. Treatment of rDUSP28 also significantly stimulated the angiogenesis *in vitro* and *in vivo*, indicating that scattered DUSP28 aggravate the malignancy of metastatic pancreatic cancer *via* induction of dual action mechanisms, autocrine and paracrine signaling pathway, simultaneously. In addition, conditioned medium from si-DUSP28 transfection retarded the basal features of human metastatic pancreatic cancer cells and HUVECs, compared to scrambled siRNA transfected cells. These observations indicate that decent targeting of scattered DUSP28 might be an advantageous therapeutic strategy for blockade of metastatic pancreatic cancer. However, as is the case for most effective therapies, successful targeting of scattered DUSP28 in metastatic pancreatic cancer will be seriously considered by mining therapeutic reagents, such as therapeutic antibody or small inhibitor. Although many DUSPs display enhanced expression and function as oncogenic molecules in various cancers, there are conflicting reports as classical functions of DUSPs [43–46]. Interestingly, different groups have opposing conclusions regarding DUSP26 expression in the same neuroblastoma cell types [47,48]. Current gaps in knowledge on the controversial functions and expressions of DUSPs should be narrow down. Additionally, scattered DUSP28 signaling has a bi-directional interaction with multiple other pathway or molecules, which include candidate therapeutic targets. Understanding these relationships will significantly stimulate our cogitation to design rational combination therapy.

Recent work has demonstrated that targeting ITGα1 induced cytotoxicity of pancreatic cancer cells and reduced expansion capacity of collagen *in vitro*, indicating inhibition of ITGα1 sensitizes pancreatic cancer cells to gemcitabine-induced cytotoxicity [49]. Another report demonstrated that enhanced DUSP12 induced cellular migration and protection from apoptosis through the upregulation of ITGα1 and the hepatocyte growth factor receptor, c-met [40]. In agreement with previous works, silencing ITGα1 significantly decreased migration, invasion, and viability of human metastatic pancreatic cancer cells. However, we revealed that ITGα1 cooperated with exogenous rDUSP28 just like interaction between growth factor and its receptor to promote metastatic pancreatic cancer malignancy via activation of MAPK signaling pathway such as CREB, AKT, and ERK1/2. Furthermore, we demonstrated that enhanced ITGα1 expression was critical for prognosis of human pancreatic cancer for the first time. Most notably, DUSP28 and ITGα1 formed a uniquely functional autocrine loop by transcriptional regulation that affects the pancreatic cancer features. Given that DUSP28 is endogenously overexpressed in pancreatic cancers compared with normal pancreas [16,20,34], our present results clearly suggest that this autocrine loop might be involved in autonomous pancreatic cancer malignancy, implying that DUSP28 might be an executor of the acquired autocrine loop. Together with our results verifying that ITGα1 promotes the malignancy of pancreatic cancer cooperating with sDUSP28, the correlation of ITGα1-sDUSP28 should be further investigated for understanding and inhibiting malignant pancreatic cancer.

Since detection of pancreatic cancer at the earliest stage is the best path to a cure, validation of a truly effective predictive biomarker for diagnosis remains the major challenge for pancreatic cancer. Investigations of existing and new biomarkers to predict prognosis and to stratify therapy are needed. Despite such efforts, the various candidate biomarkers have limited clinical efficiency in diagnosis of pancreatic cancer due to the difficulty in accessing the pancreas, low specificity, and inefficient sensitivity [9]. Currently, CA19-9 is the most promising serum biomarker in pancreatic cancer. However, even CA19-9 is not the perfect biomarker due to its varied sensitivity and specificity [50]. The present data implicate scattered DUSP28 as a potentially suitable biomarker for metastatic pancreatic cancer. Scattered DUSP28 can be specifically detected in cultured supernatant of metastatic pancreatic cancer cells, Capan-1 and CFPAC-1 compared to other six pancreatic cancer cells. We extended the detection to whole blood samples of metastatic pancreatic cancer xenograft models and pancreatic cancer patients. In particular, scattered DUSP28 was specifically detected with a significant success rate in whole blood samples of pancreatic cancer patients, especially metastatic pancreatic cancer patients (C4, C5, and C6 in Fig. 6C) using easy-to-do techniques. The encouraging results strongly suggest scattered DUSP28 as a critically promising biomarker for tremendous metastatic pancreatic cancer. However, further researches concerning the detection capability of scattered DUSP28 in early-stage pancreatic cancer are necessary to conclusively determine the therapeutic utility of sDUSP28. To actualize the “bench to clinic” of scattered DUSP28 as an effective biomarker for pancreatic cancer patients, it will be also necessary to establish and develop the medical examination more easily and inexpensively.

In summary, the present study provides the rationale for development of effective inhibitors against scattered DUSP28 for blockade of human metastatic pancreatic cancer and scattered DUSP28 might be a promising biomarker for diagnosis of metastatic pancreatic cancer.

List of abbreviation

CA19-9, cancer antigen 19-9; CD31, cluster of differentiation 31; CREB, cAMP response element binding protein 1; DUSP28, dual specificity phosphatase 28; ELISA, emzyme-linked immunosorbent assay; ERK1/2, extracellular signal-regulated kinase 1/2; FAK, focal adhesion kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cell; IHC, immunohistochemistry; IP, immune precipitation; mitogen-activated protein kinase, MAPK; proliferating cell nuclear antigen, PCNA; siRNA, small interfering RNA.

Authors contributions

JW. Lee designed the study, performed the experiments, and drafted the manuscript. J. Lee arranged all data-sets. JW. Lee and J.H. Kim supervised the project. All authors discussed data and read the manuscript.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.006.

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