Int J Clin Exp Med 2017;10(2):2634-2640
www.ijcem.com /ISSN:1940-5901/IJCEM0028406

Original Article

Netrin-1 up-regulates SENP1 expression in pancreatic cancer cells through activation of NF-kappB

Anan Liu, Rui Liu, Ligang Shi, Danlei Chen, Liang Tang, Jun Chen, Chenghao Shao

Department of General Surgery, Changzheng Hospital, Second Military Medical University, Shanghai, China

Received March 16, 2016; Accepted July 4, 2016; Epub February 15, 2017; Published February 28, 2017

Abstract: Aim: Netrin-1 displays proto-oncogenic activity in several cancers, which might due to the ability of this secreted cue to stimulate survival when bound to its receptors. SENP1 encodes a cysteine protease that specifically targets members of the small ubiquitin-like modifier (SUMO) protein family. Methods: In the present study, we determined the role of Netrin-1 on SENP1 expression in pancreatic cancer cells. Results: Our results revealed that Netrin-1 overexpression promoted while its knockdown inhibited SENP1 expression in two pancreatic cancer cells. At the molecular level, we found that Netrin-1 promoted SENP1 expression through activation of NF-κB signaling pathway. Conclusion: our data suggest a previous unknown Netrin-1/SENP1 network in pancreatic cancer cells.

Keywords: Netrin-1, SENP1, NF-κB, pancreatic cancer

Introduction

Netrin-1, a diffusible and laminin-related protein, was initially identified as neuronal guidance cues during development of the nervous systems [1, 2]. Netrin-1 exerts its biological effects through binding to two families of receptors: deleted in colorectal cancer (DCC) and uncoordinated-5 homolog (UNC-5H), a group of the four UNC5H1, UNC5H2, UNC5H3, and UNC5H4 receptors [3]. Subse-quent studies demonstrate that Netrin-1 is up-regulated in several types of cancer tissues and either knockdown of its expression or inhibition of its receptors result in the tumor regression [4-7]. Besides, plasma netrin-1 levels are also significantly increased in patents with liver, breast, meningioma, prostate and glioblastoma cancers as compared to normal subjects, suggesting that plasma netrin-1 may be a potential diagnostic biomarker for human cancers [8].

In pancreas, it has been that netrin-1 increases the migration of fetal islet cells and of a ductal cell line, mainly via a chemokinetic effect [9, 10]. Besides, netrin-1 expression was also significantly up-regulated in the pancreatic cancer tissues while its knockdown inhibited tumor cell metastasis and invasion *in vivo* [10], suggesting that netrin-1 might be an important regulator of pancreatic tumor progression.

SUMO (small ubiquitin-like modifier) modification of protein substrates is a dynamic process that modulates the target protein's expression, function, and/or subcellular location. SUMOylation is regulated by SUMO-specific activating (E1), conjugating (E2), and ligating (E3) enzymes and reversed by a family of sentrin/SUMO-specific proteases (SENPs) [11, 12]. SENP1 is involved in the pathogenesis of tumorigenesis and its ex-pression levels are elevated in several types of cancers, such as thyroid adenocarcinoma and prostate cancer [13, 14]. However, the molecular mechanisms underlying the dysregulation of SENP1 remain largely unexplored. In the present study, we investigate the roles of Netrin-1 on the regulation of SENP1 in pancreatic cancer cells.

Materials and methods

Cell cultures

The pancreatic cancer cell line PANC-1 and CFPAC-1 cells were purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CAS, Shanghai), and cultured in Dulbecco modified Eagle's medium

(DMEM, Gibco, USA) supplemented with 10% fetal calf serum (Gibco, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin(Gibco, USA). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

RNA isolation and real-time PCR

Total RNAs were isolated from cells by TRIzol reagent (Invitrogen, USA), and reverse transcriptions were performed by Takara RNA PCR kit (Takara, Dalian, China), following the manufacturer's instructions. In order to determine the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Dalian, China) on an ABI 7500 machine.

Western blot analysis

Cells after different treatments were lysed with RIPA buffer. An equal amount of protein was subjected to 8% SDS-PAGE, and separated proteins were transferred to nitrocellulose membranes. The membranes were blo-cked in 10% skim milk for 2 hours at room temperature. The immunoblots were incu-bated overnight at 4°C with antibodies. Next day, the membranes were incubated with a horseradish peroxidase-conjugated secon-dary antibody (Santa Cruz Biotechnology) for 2 hours at room temperature. The immun-oreactive bands were detected with chemilminescence substrate kit (ProteinSimple, Santa Clara, CA) under the Fluor Chem FC2 system. Antidodies were purchased from Abcam (anti-βactin, anti-SENP1, anti-p65 and anti-GAPDH)

Small interfering RNA (siRNA)

Cells were transfected with siRNA targeting the Netrin-1, p65 gene or a negative control (QIAGEN, Valencia, CA) using Lipofectamine 2000 (Invitrogen, USA) as described by the manufacturer's instructions. Cells were harvested 24 and 48 hr after transfection for RNA and protein analysis, respectively.

Dual luciferase reporter assays

Human SENP1 promoter was amplified from the human genomic DNA template and inserted into PGL4.15 basic vector (Promega, USA). Mutant p65 binding motif was generated using a PCR mutagenesis kit (Toyobo) with a primer (mutation sites underlined): 5'-CGGACTTACG-AAAGGAAACCATCAGTAA-3' and a rev-erse complement primer. All the transient transfections were performed by Lipofec-tamine 2000 (Invitrogen), according to the manufacturer's instructions. For luciferase reporter assays, PANC-1 cells were seeded in 24-well plates and transfected with the indicated plasmids. Cells were harvested 36 hr after transfection. Luciferase activities were measured using the Dual Luciferase Repo-rter Assay System (Promega).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assay kits were used (Upstate, USA). In short, PANC-1 cells were fixed with 1% formaldehyde to cross-link the proteins and DNA, followed by sonication in an ultrasound bath on ice. DNA was sheared to fragments at 200-1000 bp using sonication. The chromatin was then incubated and precipitated with p65 antibody or IgG as a control. The immunoprecipitated DNA frament were detected using real-time PCR analysis.

Statistical analysis

Statistical analysis was performed with SPSS version 13.0 software. Numerical data are expressed as mean ± SEM. Statistical signifcance is shown as *(P<.05), **(P<.01), or ***(P<.001).

Results

Netrin-1 overexpression increases SENP1 expression in pancreatic cancer cells

Firstly, we selected two cell lines (PANC-1 and CFPAC-1) to investigate the roles of Netrin-1 overexpression. As shown in **Figure 1A-D**, forced overexpression of Netrin-1 led to a dramatic increase of SENP1 mRNA and protein levels.

Inhibition of Netrin-1reduces SENP1 expression in pancreatic cancer cells

Next, Netrin-1 small interfering RNA oligos (siRNA) were used to silence its expression in pancreatic cells. As expected, knockdown of Netrin-1 repressed SENP1 expression in both two cell lines (**Figure 2A-D**).

Netrin-1 activates NF-κB signaling pathway

Next, we tried to seek the molecular mechanisms of induction of SENP1 by Netrin-1. As shown in **Figure 3A** and **3B**, Netrin-1 overex-

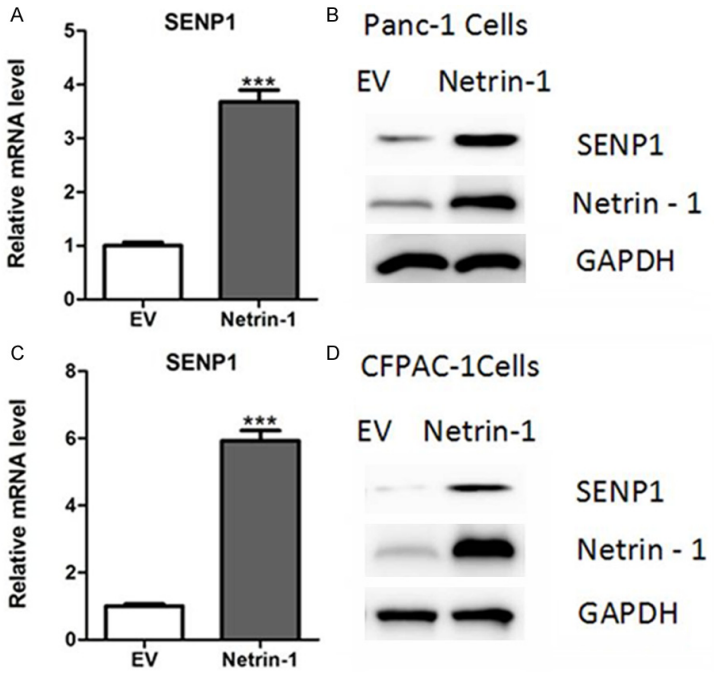

Figure 1. Netrin-1 overexpression increases SENP1 expression in pancreatic cancer cells. (A), (B) Real-time PCR and western blot analysis of SENP1 mRNA (A) and protein (B) levels in Panc-1 cells overexpressing empty vector (EV) or Netrin-1. (C), (D) Real-time PCR and western blot analysis of SENP1 mRNA (C) and protein (D) levels in CFPAC-1 cells overexpressing empty vector (EV) or Netrin-1. ***P<0.001.

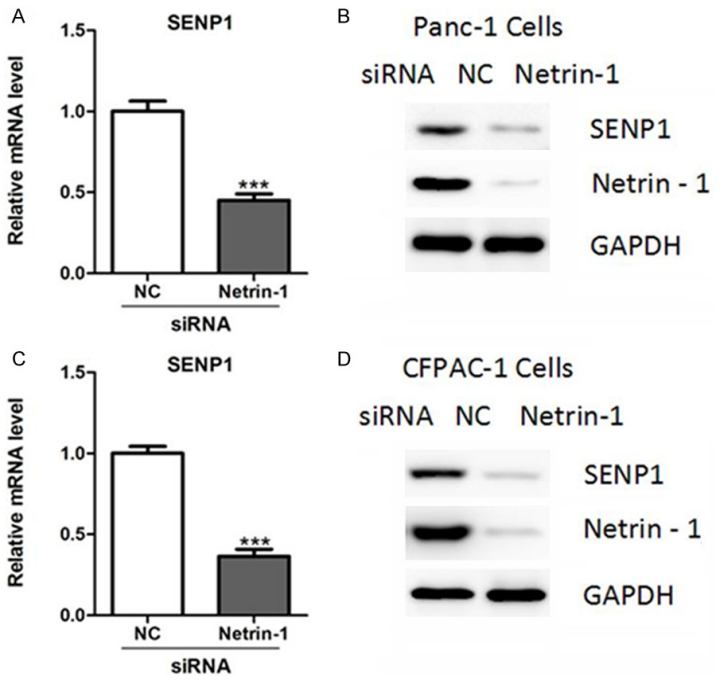

Figure 2. Netrin-1 deficiency reduces SENP1 expression in pancreatic cancer cells. (A), (B) Real-time PCR and western blot analysis of SENP1 mRNA (A) and protein (B) levels in Panc-1 cells transfected with siRNA oligos targeting Netrin-1 or negative control (NC). (C), (D) Real-time PCR and western blot analysis of SENP1 mRNA (C) and protein (D) levels in CFPAC-1 cells transfected with siRNA oligos targeting Netrin-1 or negative control (NC). ***P<0.001.

pression activated the phosphorylation and acetylation of p65, suggesting the activation of NF-κB signaling [15]. Besides, Netrin-1 overexpression treatment also increased mRNA and protein levels of IkBa (**Figure 3C-F**), whose expression is induced by NF-κB signaling [16].

Netrin-1 regulates SENP1 expression through NF-κB signaling

We next test whether NF-κB signaling is required for the effect of Netrin-1 on SENP1 expression. As shown in **Figure 4A**, BAY-117082, a specific and potent inhibitor of NF-κB, could reverse the effect of Netrin-1 overexpression on the mRNA and protein levels of SENP1 in PANC-1 cells (**Figure 4A**, **4B**), suggesting that Netrin-1-indu-ced SENP1 expression was me-diated by NF-κB signaling. Mor-eover, ce-lls were infected with small interfering RNA targeting p65, which significantly inhi-bits endogenous p65 expression (**Figure 4C**). The inhibition of endogenous p65 also significantly inhibited the ability of Netrin-1 to up-regulate SENP1 (**Figure 4D**, **4E**), demonstrating that NF-κB signaling plays an indispensable role in Netrin-1 induced SENP1 expression.

Given that these results suggested that NF-κB signaling regulated SENP1 expression at the trascription level, we ex-amined whether p65 could bind with SENP1 promoter. Seque-nce scanning of the 5' flanking element of the SENP1 gene was performed using an online promoter analysis system (http://www.cbil.upenn.edu/cgi-

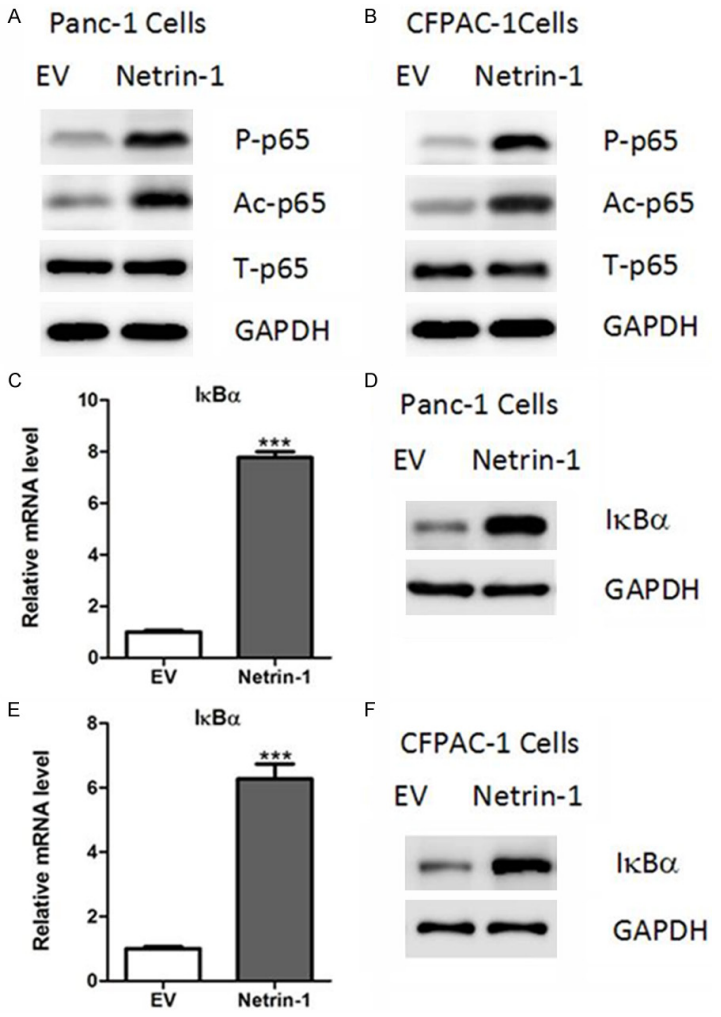

Figure 3. Netrin-1 overexpression activates NF-kB signaling pathway. (A), (B) Western blot analysis of phosphorylated (P) and acetylated (Ac) of p65 in Panc-1 (A) and CFPAC-1 (B) cells overexpressing empty vector (EV) or Netrin-1. Total (T) p65 and GAPDH were employed as loading controls. (C), (D) Real-time PCR and western blot analysis of IkBa mRNA (C) and protein (D) levels in Panc-1 cells overexpressing empty vector (EV) or Netrin-1. (E), (F) Real-time PCR and western blot analysis of IkBa mRNA (E) and protein (F) levels in CFPAC-1 cells overexpressing empty vector (EV) or Netrin-1. ***P<0.001.

bin/tess/tess). Scanning the proximal promoter of SENP1 reve-aled a potential motif similar to an NF-κB consensus site located at position -153 bp, relative to the start site of transcription (**Figure 5A**). Luciferase reporter assays revealed that overexpression of p65 could enhance the promoter activity, but not the binding site mutant ones (**Figure 5B**). Besides, mutation of this binding site also attenuated the roles of Netrin-1 overexpression (**Figure 5C**). Moreover, Chromatin immunoprecipitation (ChIP) analysis was performed to determine the association of NF-κB to the SENP1 promoter in vivo. As shown in **Figure 5D**, p65 was found to be associated with the SENP1 promoter in PANC-1 cells, which was further enhanced by Netrin-1 overexpression (**Figure 5D**).

Discussion

The pathogenesis of pancreatic cancer has not been studied extensively, and the molecular events underlying the initiation and progression of pancreatic cancer are largely unknown [17]. Therefore, understanding of its genetic mechanisms is pressing for the development of targeted and effective treatment of the disease. In the present study, we tried to explore the roles of Netrin-1 and its molecular mechanisms in pancreatic cancer cells. Netrin-1 was shown to promote SENP1 expression in PANC-1 and CFPAC-1 cells, whereas its deficiency using siRNA oligos led to a reduced expression of SENP1. Moreover, we found that Netrin-1 activates NF-κB signaling, which plays an indispensable role in Netrin-1 induced SENP1 expression. There-fore, our data add a novel mechanism of Netrin-1 in the progression of pancreatic cancer cells.

SENP1 has been shown to play a critical role in the induction of cell proliferation. For example, overexpression of SENP1 enhances the key cell cycle regulator cyclin D1, which has been shown to also facilitate prostate cell proliferation [18]. Besides, SENP1 is essential for HIF1α stability under hypoxic conditions in SENP1-deleted embryos and mouse embryonic fibroblast cells [19]. Ove-rexpression of SENP1 in an adult mouse model significantly enhances expression of nuclear HIF1α, which is, in turn, required for initiation of the angiogenic switch [20]. Also, SENP1 overexpression initiates the HIF1α pathways in mice as indicated by the elevation of HIF1α-regulated VEGF [21]. While SENP1 expression is highly up-regulated in multiple tumors and directly correlates with cancer

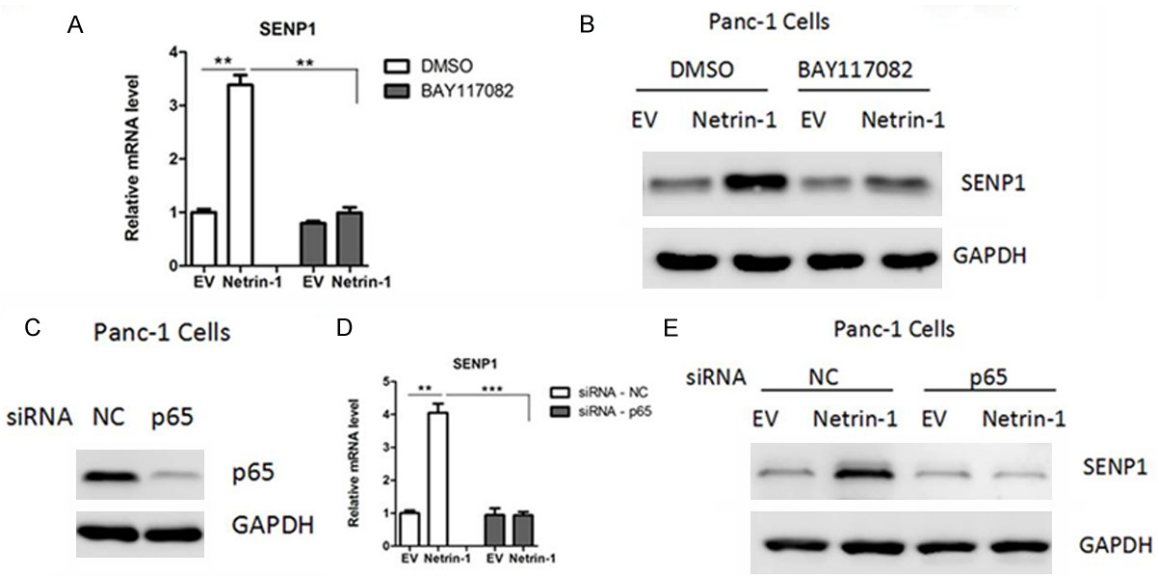

Figure 4. Block of NF-kB signaling attenuates the roles of Netrin-1 on SENP1 expression. (A), (B) Real-time PCR and western blot analysis of SENP1 mRNA (A) and protein (B) levels in PANC-1 cells overexpressing empty vector (EV) or Netrin-1. Cells were pre-treated with BAY117082 (5 mm) or vehicle control (DMSO) for 6 hr. (C) Western blot analysis of p65 protein levels in PANC-1cells transfected with siRNA oligos targeting p65 or negative control (NC). (D), (E) Real-time PCR and western blot analysis of SENP1 mRNA (D) and protein (E) levels in PANC-1 cells overexpressing empty vector (EV) or Netrin-1. Cells were pre-transfected with siRNA oligos targeting p65 or negative controls (NC) for 24 hr. **P<0.01, ***P<0.001.

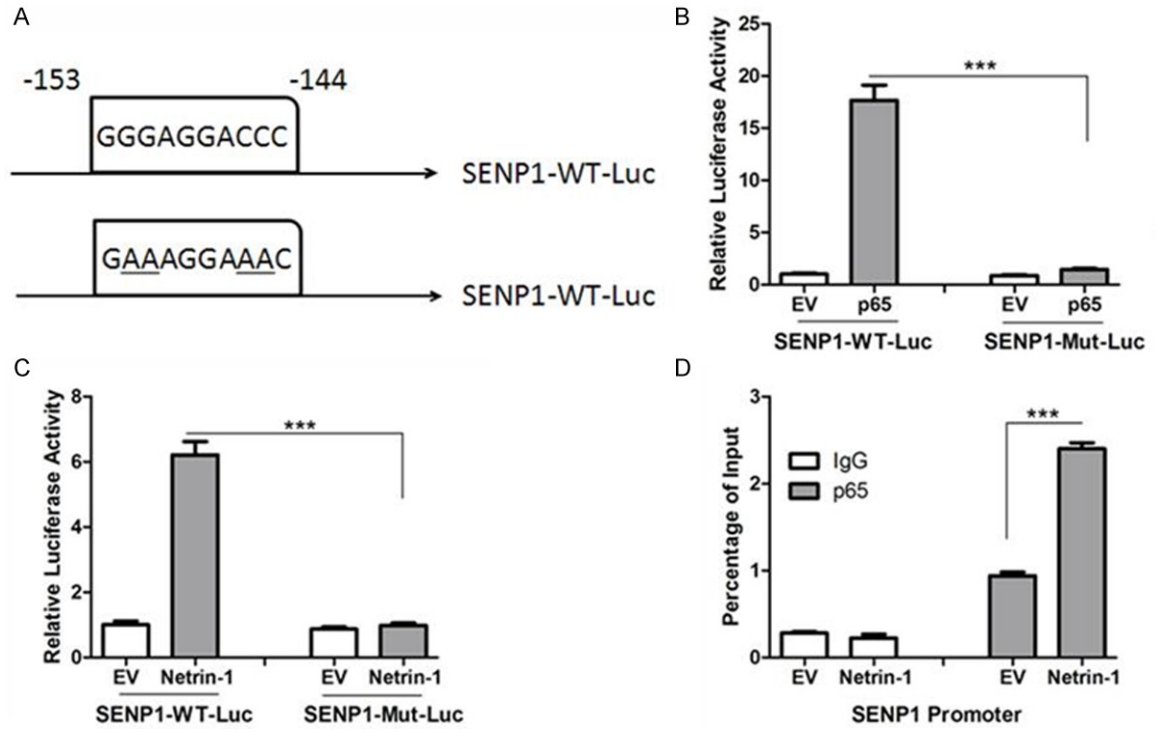

Figure 5. NF-κB binds and transcriptionally regulates the SENP1 promoter. A. Schematic illustration of the human SENP1 promoter containing a putative NF-κB consensus binding sequences upstream of the transcription start site. B, C. PANC-1 cells were co-transfected with the indicated plasmids for 36 hr, and the luciferase activity was measured. D. ChIP assays showed bound of p65 on the promoter region of SENP1 in PANC-1 cells transfected with Netrin-1 or empty vector (EV). ***P<0.001.

aggressiveness and recurrence [13, 14, 20, 22, 23], however, the roles of SENP1 in pancreatic cancer remain poorly defined. Thus, it will be interesting to further investigate the functions of SENP1 to regulate cell proliferation, invasion and angiogenesis in pancreatic cancers.

In summary, our studies demonstrate a previous unknown Netrin-1/SENP1 network in pancreatic cancer cells, suggesting that the abnormal function of Netrin-1 could be an important mechanism for the initiation and progression of pancreatic NETs.

Disclosure of conflict of interest

None.

Address correspondence to: Chenghao Shao, Department of General Surgery, Changzheng Hospital, Second Military Medical University, Shanghai, China. Tel: +86-013817701771; E-mail: xianguihu_123@sina.com

References

[1] Ko SY, Dass CR, Nurgali K. Netrin-1 in the developing enteric nervous system and colorectal cancer. Trends Mol Med 2012; 18: 544-554.
[2] Gershon MD. Developmental determinants of the independence and complexity of the enteric nervous system. Trends Neurosci 2010; 33: 446-456.
[3] Rajasekharan S, Kennedy TE. The netrin protein family. Genome Biol 2009; 10: 239.
[4] Round J, Stein E. Netrin signaling leading to directed growth cone steering. Curr Opin Neurobiol 2007; 17: 15-21.
[5] Nikolopoulos SN, Giancotti FG. Netrin-integrin signaling in epithelial morphogenesis, axon guidance and vascular patterning. Cell Cycle 2005; 4: e131-135.
[6] Hebrok M, Reichardt LF. Brain meets pancreas: netrin, an axon guidance molecule, controls epithelial cell migration. Trends Cell Biol 2004; 14: 153-155.
[7] Ramkhelawon B, Yang Y, van Gils JM, Hewing B, Rayner KJ, Parathath S, Guo L, Oldebeken S, Feig JL, Fisher EA, Moore KJ. Hypoxia induces netrin-1 and Unc5b in atherosclerotic plaques: mechanism for macrophage retention and survival. Arterioscler Thromb Vasc Biol 2013; 33: 1180-1188.
[8] Ramesh G, Berg A, Jayakumar C. Plasma netrin-1 is a diagnostic biomarker of human cancers. Biomarkers 2011; 16: 172-180.
[9] De Breuck S, Lardon J, Rooman I, Bouwens L. Netrin-1 expression in fetal and regenerating rat pancreas and its effect on the migration of human pancreatic duct and porcine islet precursor cells. Diabetologia 2003; 46: 926-933.
[10] Dumartin L, Quemener C, Laklai H, Herbert J, Bicknell R, Bousquet C, Pyronnet S, Castronovo V, Schilling MK, Bikfalvi A, Hagedorn M. Netrin-1 mediates early events in pancreatic adenocarcinoma progression, acting on tumor and endothelial cells. Gastroenterology 2010; 138: 1595-1606, 1606, e1-8.
[11] Geiss-Friedlander R, Melchior F. Concepts in sumoylation: a decade on. Nat Rev Mol Cell Biol 2007; 8: 947-956.
[12] Gareau JR, Lima CD. The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. Nat Rev Mol Cell Biol 2010; 11: 861-871.
[13] Jacques C, Baris O, Prunier-Mirebeau D, Savagner F, Rodien P, Rohmer V, Franc B, Guyetant S, Malthiery Y, Reynier P. Two-step differential expression analysis reveals a new set of genes involved in thyroid oncocytic tumors. J Clin Endocrinol Metab 2005; 90: 2314-2320.
[14] Wang Q, Xia N, Li T, Xu Y, Zou Y, Zuo Y, Fan Q, Bawa-Khalfe T, Yeh ET, Cheng J. SUMO-specific protease 1 promotes prostate cancer progression and metastasis. Oncogene 2013; 32: 2493-2498.
[15] Zhu X, Liu Q, Wang M, Liang M, Yang X, Xu X, Zou H, Qiu J. Activation of Sirt1 by resveratrol inhibits TNF-alpha induced inflammation in fibroblasts. PLoS One 2011; 6: e27081.
[16] Park SY, Lee SW, Baek SH, Lee CW, Lee WS, Rhim BY, Hong KW, Kim CD. Suppression of PU.1-linked TLR4 expression by cilostazol with decrease of cytokine production in macrophages from patients with rheumatoid arthritis. Br J Pharmacol 2013; 168: 1401-1411.
[17] Werner J, Combs SE, Springfeld C, Hartwig W, Hackert T, Buchler MW. Advanced-stage pancreatic cancer: therapy options. Nat Rev Clin Oncol 2013; 10: 323-333.
[18] Cheng J, Bawa T, Lee P, Gong L, Yeh ET. Role of desumoylation in the development of prostate cancer. Neoplasia 2006; 8: 667-676.
[19] Cheng J, Kang X, Zhang S, Yeh ET. SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. Cell 2007; 131: 584-595.
[20] Bawa-Khalfe T, Cheng J, Lin SH, Ittmann MM, Yeh ET. SENP1 induces prostatic intraepithelial neoplasia through multiple mechanisms. J Biol Chem 2010; 285: 25859-25866.
[21] Xu Y, Zuo Y, Zhang H, Kang X, Yue F, Yi Z, Liu M, Yeh ET, Chen G, Cheng J. Induction of SENP1 in endothelial cells contributes to hypoxia-driven VEGF expression and angiogenesis. J Biol Chem 2010; 285: 36682-36688.

[22] Chen CH, Chang CC, Lee TH, Luo M, Huang P, Liao PH, Wei S, Li FA, Chen RH, Zhou XZ, Shih HM, Lu KP. SENP1 deSUMOylates and regulates Pin1 protein activity and cellular function. Cancer Res 2013; 73: 3951-3962.

[23] Bawa-Khalfe T, Yeh ET. SUMO Losing Balance: SUMO Proteases Disrupt SUMO Homeostasis to Facilitate Cancer Development and Progression. Genes Cancer 2010; 1: 748-752.