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ASIC1 inhibition impairs the proliferation and migration of pancreatic stellate cells induced by pancreatic cancer cells

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Activated pancreatic stellate cells (PSCs) with an increased proliferation and migration ability are the partners in crime with pancreatic cancer cells. Acid-sensing ion channel 1 (ASIC1) is expressed in pancreatic cancer and PSCs, and especially, it mediates the activation of PSCs. However, whether ASIC1 is involved in pancreatic cancer cells-induced biological behavior re-educating of PSCs is unclear. In this study, the change of ASIC1 expression in PSCs and pancreatic cancer Panc-1 cells after in-direct co-culture was detected by western blotting, and the proliferation and migration of PSCs with ASIC1 knock-down under Panc-1 cells-conditioned medium (Panc-1-CM) was assessed. The results showed that pancreatic cancer cells induced ASIC1 overexpression, and the enhanced proliferation and migration of PSCs was weakened by ASIC1 inhibition. In addition, the extracellular signal-regulated kinase (ERK) expression in PSCs remained stable, but the phosphorylated ERK (p-ERK) expression in PSCs treated with Panc-1-CM increased, which was suppressed by ASIC1 knockdown. These results indicate that ASIC1 participates in the regulation of PSCs proliferation and migration induced by cancer cells via the ERK pathway, and ASIC1 inhibition may be beneficial to pancreatic cancer treatment.

Key words: ASIC1, pancreatic stellate cells, proliferation, migration

Pancreatic cancer is usually referred to as pancreatic ductal adenocarcinoma, which accounts for more than 85% of pancreatic cancer, and is extremely notorious for its high malignancy, therapeutic resistance, and dismal prognosis worldwide despite significant advances in molecular diagnosis and surgical skills in clinical practice. Mounting evidence proves that pancreatic stellate cells (PSCs) that transform from a resting state in the normal pancreas to an activated state in diseased pancreas under cancer cells and chemical or physical stimulation, interact closely with cancer cells as well as other stromal cells and eventually create a unique microenvironment to facilitate the development of malignant biological behaviors of pancreatic cancer [1, 2]. The activated PSCs, on the one hand, are responsible for the desmoplastic reactions by producing excessive extracellular matrix (ECM) components, which impedes drug delivery to the tumor, and on the other hand, they can support tumor metabolism via cancer cells-induced autophagic alanine secretion, or promote tumor metastasis through upregulating the expression of cytokines such as galectin-1, SDF-1, IL-6 [1, 3, 4]. Given the importance of PSCs, targeting PSCs has become a research hotspot in the field of pancreatic cancer therapy. Conversely, pancreatic cancer cells also have a very strong influence on the morphology and function of PSCs, including promoting PSCs activation, proliferation, autophagy, migration [5, 6]. Therefore, understanding of the interaction between cancer cells and PSCs contributes to reveal the underlying mechanism of pancreatic cancer progression and further adopt appropriate intervention measures.

ASIC1 belongs to the proton-gated ion channels, and is mainly expressed in the mammalian nervous system and several digestive system tumors such as pancreatic cancer, gastric cancer, hepatocellular cancer [7–9]. Interestingly, ASIC1 expression also occur in hepatic stellate cells and PSCs, and it mediates acidosis-induced fibrosis, a common non-physiological wound-healing process in liver and pancreatic diseases [10, 11]. In particular, ASIC1 regulates the acidity-induced epithelial mesenchymal transition (EMT) of pancreatic cancer cells and activation and autophagy of PSCs, and its expression level is inversely correlated with the tumor prognosis, suggesting its targeting value in pancreatic

cancer treatment [8, 11]. From the concerned results on the function of ASIC1 in pancreatic cancer, it can be inferred that ASIC1 not only is a receptor or responder of extracellular acidic pH, but also plays a certain special role in the interaction between cancer cells and PSCs. In this study, we try to investigate whether ASIC1 involves the biological behavior re-educating of PSCs under cancer cells-conditioned medium, and our results demonstrate that ASIC1 inhibition impairs cancer cells-induced proliferation and migration of PSCs, which may produce a positive effect on the treatment of pancreatic cancer.

Materials and methods

Reagents. Human pancreatic cancer Panc-1 cells and immortalized PSCs were purchased from Tongpai Biological Technology Co., Ltd. DMEM, fetal bovine serum (FBS), and Lipofectamine™ 2000 were obtained from Invitrogen. ASIC1 small interfering RNA (siRNA) with the following sequence: GCTGGAAATCATGCTGGACAT (ASIC1-sense) and GCATCAAAGTGCAGATCCATA (ASIC1-antisense), and the control siRNA were designed and synthesized by JRDUN Biotechnology Co., Ltd. Cell counting kit-8 (CCK-8) was purchased from Beyotime Institute of Biotechnology. Primary and secondary antibodies were purchased from Beijing Bioss Biological Technology Co., Ltd. Other commonly used agents were commercially available.

ASIC1 knockdown in PSCs. PSCs were cultured in DMEM containing 10% FBS in a 5% CO_2 incubator at 37 °C, and the medium was changed every two or three days. To observe the role of ASIC1 in PSCs, ASIC1 knockdown using siRNA was carried out. The purified ASIC1 siRNA or control siRNA were transfected into PSCs using Lipofectamine™ 2000 according to the manufacturer's instructions. The efficacy of the ASIC1 knockdown in PSCs was confirmed by western blotting (Figure 1A).

Indirect co-culture and the preparation of Panc-1-CM. Non-contacting co-culture transwell chambers were used to assess the interaction between Panc-1 cells and PSCs. PSCs were cultured on the bottom of the six-well transwell chamber and then the inserts containing Panc-1 cells were placed in the well. The cells were cultured for 3, 6, and 12 h in a serum-free DMEM condition, and then these two kinds of cells were collected respectively, and ASIC1 expression in Panc-1 cells and PSCs, α-SMA and Collagen I, two commonly used biomarkers of PSCs activation, were detected by western blotting.

To observe the effect of pancreatic cancer cells on the biological behaviors of PSCs, cancer cells conditioned medium was prepared according to the following procedures. Panc-1 cells were cultured in DMEM containing 10% FBS, and when cells reached about 80% confluence, the medium was replaced by serum-free DMEM followed by incubated for an additional 48 h. Then, the supernatant was

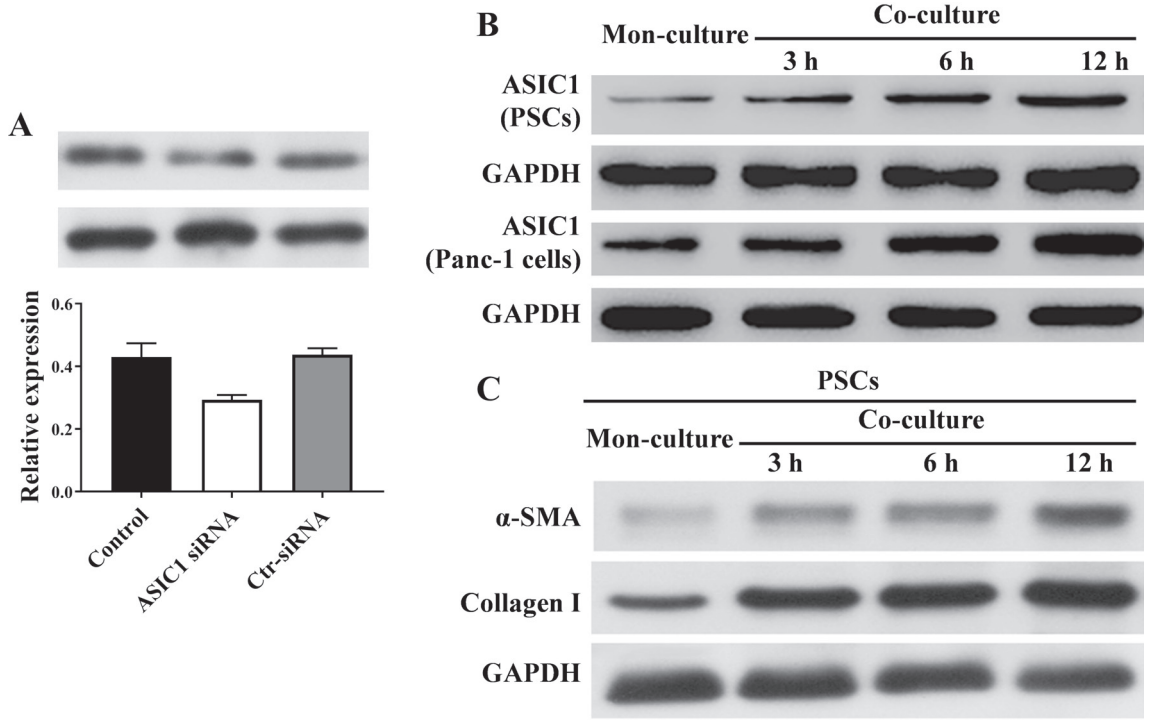

Figure 1. The interaction between Panc-1 cells and PSCs promoted ASIC1 expression and PSCs activation. A) The efficacy of ASIC1 knockdown in PSCs was confirmed by western blotting (n=3). B) Western blotting results of ASIC1 expression in PSCs and Panc-1 cells after in-direct co-culture for a different time and C) α-SMA and Collagen I expression in PSCs under different condition. Repeated experiments showed similar results.

collected, centrifuged at 10 000×g for 5 min at 4 °C, filtered using a 0.22 μm filter, designated as Panc-1-CM, and stored at –20 °C till use. To investigate the role of ASIC1 on PSCs proliferation and migration induced by Panc-1-CM, PSCs were divided into four groups firstly, that is, control group (cultured in serum-free DMEM), Panc-1-CM group, Panc-1-CM+ASIC1 siRNA group, and Panc-1-CM+Ctr-siRNA group. After receiving the corresponding treatment, PSCs were collected for the next experiments.

Cell proliferation assay. PSCs suspension was prepared in serum-free DMEM firstly, and then 2×10^3 cells (200 μl) per well were seeded into a 96-well plate. After cell adherence, the medium was removed and the new DMEM or Panc-1-CM was added. PSCs in four groups were sequentially cultured for 0, 24, and 48 h at 37 °C, respectively. At each time point, 20 μl of CCK-8 solution was added to each well, and 2 h later, the absorbance at 450 nm was measured with a microplate reader to represent the cell proliferation ability.

Wound-healing assay. Wound-healing or scratch assay *in vitro* is a simple and high cost-effective method to assess cell migration. For this assay, PSCs were seeded in a six-well plate with a 90% confluence, and then PSCs monolayers were manually scraped using a 200 μl sterile pipette tip. The cells were washed gently with PBS to remove the debris, followed by treatment with DMEM or Panc-1-CM at 37 °C. The digital photographs of PSCs were taken at 0, 24, and 48 h and the residual area of the wound in images was measured using ImageJ software. The ratio of the remaining area at different time points to the starting point was calculated to evaluate the migration of PSCs in a different group.

Western blotting. Besides ASIC1, α-SMA, and Collagen I, the expression of ERK and p-ERK also were detected using western blotting to observe whether they play a part in the process mentioned above. After various treatments, Panc-1 cells or PSCs were lysed on ice using RIPA lysis buffer to extract total protein. The extracts were centrifuged at 8 500×g for 20 min at 4 °C, and then the concentration was determined by BCA assay. Equal amounts of protein lysates were separated by 10% SDS-PAGE and transferred into the PVDF membrane. After blocked with 5% non-fat milk for 1 h and incubated overnight with the appropriate primary antibodies: ASIC1 (bs-2586R, 1:1000), α-SMA (bsm-52396R, 1:1000), Collagen I (bsm-52478R, 1:1000), ERK (bs-55069R, 1:1000), p-ERK (bs-3292R, 1:1000), GAPDH (bs-0755R, 1:1000), the membranes were incubated in secondary antibody solution (bs-40296G-HRP, 1:5000) at room temperature for 1 h, followed by reacted with enhanced chemiluminescence reagents for 5 min. At last, the specific protein bands of membranes were visualized by the ECL detection kit, and the densities of bands were quantified and analyzed via the ImageJ software.

Statistical analysis. The quantitative data were expressed as the mean ± SEM. Comparisons between two groups were carried out using a two-side and unpaired Student's t-test. The value of $p<0.05$ was considered statistically significant.

Results and discussion

The interaction between cancer cells and PSCs promotes the expression of ASIC1 and PSCs activation. The interaction between cancer cells and stromal cells (e.g. CAFs, PSCs, immune cells, endothelial cells) is a common and important phenomenon in pancreatic cancer, and the resultant effect promote tumor growth and metastasis [12, 13]. Previous studies have suggested that ASIC1 is expressed in both human pancreatic cancer cell lines (Panc-1, SW1990, BxPC-3, and AsPC) and PSCs, and the patients with a higher expression of ASIC1 have worse tumor differentiation, advanced TNM stage, lymph and distant metastasis, highlighting the potential value of ASIC1 as a thermotic target of pancreatic cancer [8, 11]. It is interesting to investigate the change of ASIC1 expression in cancer cells and PSCs after an in-direct co-culture. As shown in Figure 1B, the interaction caused ASIC1 expression increase in a time-dependent manner in both Panc-1 cells and PSCs, meaning that the malignant features of the tumor were reinforced. It has been reported that an acidic environment can upregulate ASIC1 expression in liver cancer cells, hepatic stellate cells, and PSCs [10, 11], but our results indicate that, besides acidity, pancreatic cancer cells and PSCs can promote ASIC1 expression for each other, despite through an uncertain mechanism.

Pancreatic cancer cells-induced PSCs activation occurs via a variety of ways, and the indirect co-culture and the use of cancer cell-conditioned medium can mimic the process comprehensively. As two commonly used biomarkers of PSCs activation, α-SMA and Collagen I both have an upregulated expression in a time-dependent manner due to the interaction (Figure 1C), meaning that Panc-1 cells effectively induce the development of the activated PSCs that have a distinct biological characteristic compared with quiescent PSCs, and in turn, can facilitate cancer cell viability and invasion. These results also point out that the expression of ASIC1 in PSCs is positively related to PSCs activation.

ASIC1 involves cancer cells-induced PSCs proliferation. Pancreatic cancer cells have a special ability to re-educate PSCs, which in turn facilitate the progression and metastasis of the tumor. PSCs activation caused by cancer cells was accompanied by ASIC1 expression increase, and increased proliferation and migration after PSCs activation has been confirmed by many studies [1, 5]. Therefore, the role of ASIC1 in these processes is worth investigating. Panc-1-CM containing various kinds of cytokines secreted by the cancer cells can represent the living environment of PSCs. As shown in Figure 2, exposure to Panc-1-CM for 24 h or 48 h significantly accelerated PSCs proliferation, but ASIC1 knockdown made this effect disappear completely and even inhibited PSCs proliferation, while Ctr-siRNA had no such effect, indicating that ASIC1 plays an important role in the enhanced proliferation of activated PSCs. ASIC1 inhibition induced-decreased proliferation in liver cancer SMMC-7721 and Huh-7 cells and lung cancer A549 cells has been reported

[14, 15], but in pancreatic cancer Panc-1 and BxPC-3 cells, ASIC1 knockdown has no obvious effect on cell viability [8, 14, 15]. Cancer cells stimulate proliferation and extracellular matrix production of PSCs, by which a fibrotic and hypoxic tumor microenvironment is generated, promoting the therapeutic resistance [16]. According to the present result, it is pretty well suspected that ASIC1 inhibition in PSCs helps to weaken the intractable tumor stroma and benefit the chemotherapy or radiotherapy of the tumor.

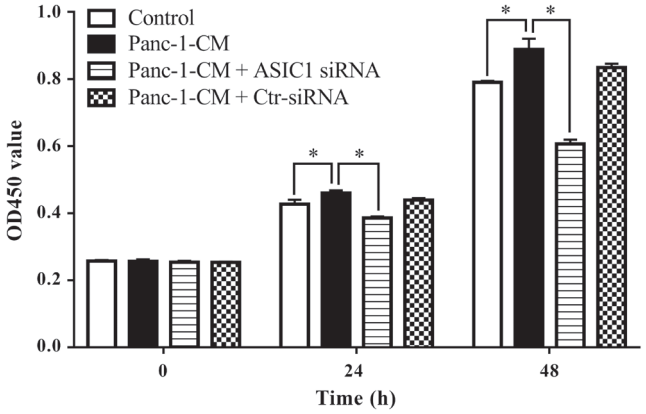

Figure 2. ASIC1 inhibition suppressed PSCs proliferation induced by Panc-1-CM. The cell viability of PSCs without or with ASIC1 knockdown in Panc-1-CM was detected using the CCK-8 assay (n=4, *p<0.05).

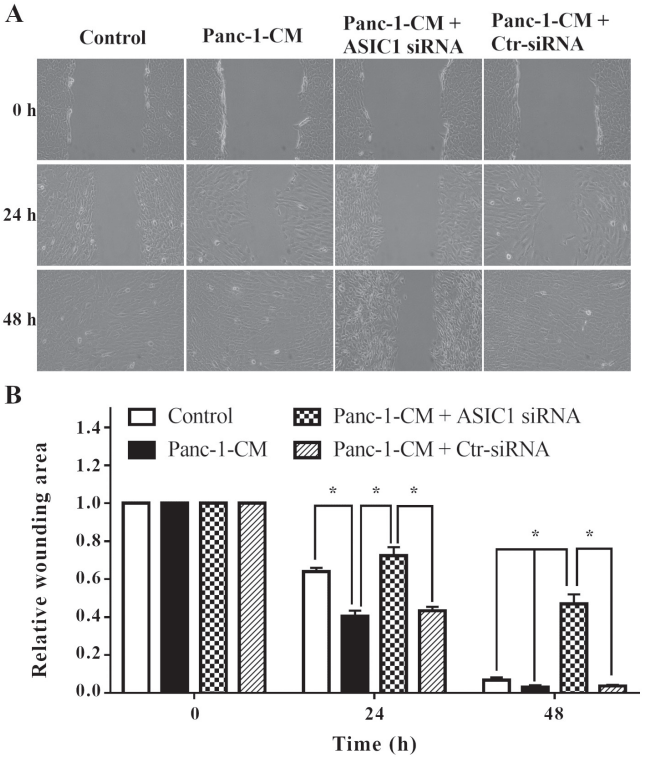

Figure 3. ASIC1 knockdown restricted PSCs migration induced by Panc-1-CM. A) Representative photos of wound-healing assay of PSCs without or with ASIC1 knockdown after Panc-1-CM treatment; B) Bar charts were quantification results of the wound-healing assay (n=3, *p<0.05).

ASIC1 knockdown inhibits PSCs migration. Metastasis is the predominant cause of cancer-associated death including pancreatic cancer, but the multi-phase process has not been completely understood because it involves cell migration, invasion into the blood or lymphatic vessels, tumor angiogenesis, and cell survival at distant sites [17]. Interesting research found that PSCs originating from the primary site of pancreatic cancer are present in metastatic nodules, meaning that the activated PSCs can migrate to distance under the induction of cancer cells [18]. In fact, the migration of activated PSCs in pancreatic cancer is of significance for the establishment of a pre-metastatic niche, where cancer cells can grow easily and enjoy an immune escape, and the migration restriction of PSCs might destroy the comfortable zone of cancer cell metastasis. Therefore, analysis of the mechanism and influence factors of PSCs migration can provide a valuable reference for pancreatic cancer therapy. In this study, the migration of PSCs in different conditions was tested by wound-healing assay. As shown in Figure 3, compared with DMEM, Panc-1-CM can significantly enhance the migration capability of PSCs, but this effect was reduced dramatically after the silencing of ASIC1 expression. Most notably, after 48 h of scraping, the wounds in the other three groups were almost completely healed but still obvious in the ASIC1 siRNA group, suggesting that ASIC1 plays a crucial role in the migration of activated PSCs. Knockdown of ASIC1 in pancreatic cancer cells can suppress liver and lung metastasis in animal tumor model [8], but the role of PSCs was not put into enough attention in this study. Our present results reveal that the strategy of targeting ASIC1 in both cancer cells and PSCs may produce a better anti-cancer effect.

Involvement of ERK pathway in ASIC1-mediated proliferation and migration of PSCs. As one of the mitogen-activated protein kinase (MAPK) family, ERK is responsible for the transmission of extracellular signals to intracellular targets and is related to many vital cellular functions including cell growth, differentiation, proliferation, migration, invasion, and stress response [19]. It has been reported that ASIC1 promotes hepatic stellate cells activation and breast cancer cell invasion and metastasis through ERK signaling pathway, but whether the ERK pathway involves ASIC1-mediated proliferation and migration of PSCs induced by cancer cells is still unknown [20, 21]. Our result from western blotting suggested that the total ERK was unaffected in all groups, but the expression of p-ERK was upregulated when PSCs were cultured in Panc-1-CM (Figure 4). In addition, it is ASIC1 siRNA rather than Ctr-siRNA that has a negative effect on the p-ERK expression of PSCs, meaning that ASIC1 inhibition may suppress the phosphorylation of ERK, and further influence the proliferation and migration of PSCs. Many external stimuli such as cytokines and growth factors can activate the ERK signaling pathway, resulting in downstream substrate phosphorylation

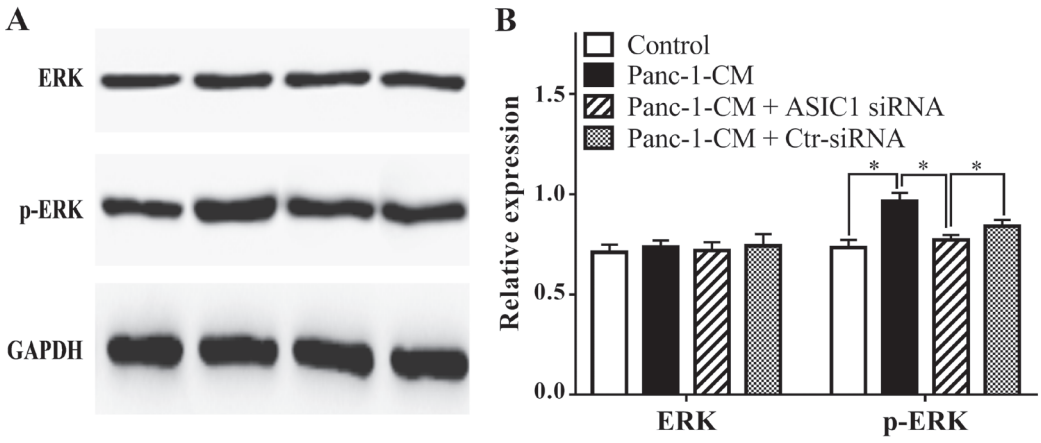

Figure 4. ERK signaling involves ASIC1-mediated biological behaviors induced by Panc-1-CM. A) Representative immunoblot images of ERK and p-ERK in PSCs with different treatment; B) Quantitative results of western blotting assay (n=3, *p<0.05).

and relative biological effect occurrence. It can be inferred that certain ingredients in Panc-1-CM can interact with ASIC1 and then activate ERK, leading to the active form of ERK, p-ERK expression increased, but this effect was blockaded when ASIC1 was absent in PSCs. However, the detailed molecular mechanism needs further exploration.

It is well known that pancreatic cancer cells can recruit PSCs and induce their activation via the secretion of a variety of cytokines and chemical factors. The activated PSCs as the accomplice of cancer cells display an enhanced proliferation and migration ability but how these processes happen is not very clear. Our present study suggests that ASIC1 plays a prominent part in the interaction between cancer cells and PSCs, and especially, ASIC1 inhibition in PSCs impairs its proliferation and migration induced by cancer cells via the suppression of the p-ERK pathway. Therefore, it can be assumed that the targeted inhibition of ASIC1 in PSCs might limit the tumor progression by destroying the tumor-promoting effect of PSCs. As for the next study, orthotopic pancreatic cancer xenografts mouse model should be established using the co-injection of Panc-1 cells and PSCs or PSCs with ASIC1 knockdown, and then the volume and metastasis of tumor should be observed to further assess the effect of targeting ASIC1 in PSCs on the malignant biological behaviors of pancreatic cancer *in vivo*.

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