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Original Articles

HIF-1α mediates tumor-nerve interactions through the up-regulation of GM-CSF in pancreatic ductal adenocarcinoma

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ABSTRACT

Perineural invasion (PNI) is a typical pathological feature of pancreatic ductal adenocarcinoma (PDAC). PNI is associated with poor prognosis of PDAC patients, however, the underlying molecular mechanisms in the development of PNI have not been fully revealed. In this study, we found that the expression of GM-CSF and HIF-1α were dramatically increased in PDAC cells. The overexpression of HIF-1α and GM-CSF closely correlated with increased occurrence of PNI and cancer-related pain and shortened overall survival in PDAC patients. GM-CSF expression in PDAC cells was mediated by HIF-1α through direct binding to the hypoxia response element in the GM-CSF promoter. The activated HIF-1α can up-regulate GM-CSF expression and secretion, which promoted the migration of Schwann cells in tumor microenvironment. Furthermore, GM-CSF overexpression could rescue the inhibition of Schwann cells migration by HIF-1α knockdown. Moreover, HIF-1α inhibition with PX478 drastically reduced the expression level of GM-CSF and decreased the PNI in a PDAC xenograft mouse model. Overall, our results demonstrated that the HIF-1α/GM-CSF pathway is involved in the tumor-nerve interactions and promotes the occurrence of PNI. The blockade of this signal might help to inhibit PDAC progression.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the leading cause of cancer-related deaths worldwide [1,2], with a typical pathological feature of perineural invasion (PNI). Tumor cell neurotropism is considered as a major cause for recurrence after curative resection and neuropathic pain, and could also serve as an important prognostic factor for PDAC patients [3]. A positive correlation between hypoxia microenvironment and intrapancreatic neuropathy/PNI has been reported in PDAC patients [4]. However, the underlying molecular mechanisms for the development of PNI have not been fully revealed.

The presence of hematopoietic factors in the tumor microenvironment can promote tumor progression [5,6]. Recently, a notable finding indicated that the tumor-derived granulocyte- and granulocyte-macrophage-colony-stimulating factors (G-CSF/GM-CSF) played an important role in the incidence of hypersensitive pain in the tumor-affected regions [7]. In a mouse model of bone tumor-induced pain, neuron specific downregulation of GM-CSF receptors (GM-CSFR) reduces tumor-induced hypersensitive pain [8]. Protein and mRNA analyses revealed that GM-CSFR was also expressed in the sensory nerves of peripheral tissues, including pancreatic cancer [9]. In this study, we found that hematopoietic colony stimulating factors (GM-CSF) were expressed at a high level in pancreatic cancer biopsy specimens, however, the role of tumor-derived GM-CSF in inducing PNI remains unknown.

Hypoxia is a common condition in the microenvironment of solid tumors including PDAC. Hypoxia-inducible factor (HIF) plays crucial roles in the pathogenesis and progression of PDAC, especially in metastasis [10–12]. HIF-1α also closely relates to cancer progression [13–15] through the chemokine induction to attract different immune cells and thus affect the anti-tumor immune response [16–19]. Our previous studies have shown that HIF-1α up-regulates the expression of CX3CR1 on the surface of pancreatic cancer cells which enhances the chemotactic migration of tumor cells to CX3CL1-secreting nerve fibers

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[20], however, whether the hypoxic microenvironment regulates perineural invasion by regulating tumor-derived secretory factors in PDAC is still unclear.

In this study, we aimed (i) to analyze whether the expression of GM-CSF is associated with PNI in PDAC patients; (ii) to investigate the regulation mechanism of HIF-1α on GM-CSF expression in PDAC cells; (iii) to explore the function of HIF-1α/GM-CSF signal in the interaction between tumor cells and Schwann cells in vitro and in vivo. This study reports for the first time that HIF-1α promotes the secretion of GM-CSF in PDAC tumor cells, and further induces the migration of Schwann cells to tumor cells and mediates tumor-neural interactions in PDAC. Our findings indicate that the blockade of HIF-1α/GM-CSF signal might help to inhibit perineural invasion and PDAC progression.

2. Materials and methods

2.1. Human tissues and clinical cohort

Eight pairs of fresh PDAC tissues and noncancerous tissues, and 70 formalin-fixed, paraffin-embedded (FFPE) sections of PDAC specimens were collected from patients who underwent a radical surgery without preoperative radiation or chemotherapy at the Tianjin Medical University Cancer Institute and Hospital. Fresh tissue fragments were immediately frozen in liquid nitrogen at the time of surgery. Normal FFPE pancreatic tissues were obtained from patients who underwent distal pancreatectomy for benign diseases. Experienced pathologists provided detailed pathological diagnosis. Another cohort of 185 pancreatic adenocarcinoma patients with mRNA expression profiling were included from The Cancer Genome Atlas (TCGA) database. All aspects of the study were approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital.

2.2. Cell culture and transfection

Human PDAC cell lines (PANC-1, MIA-PaCa-2, CFPAC-1 and SW1990), mouse PDAC cell line (Pan02) and Schwann cells were cultured using DMEM with 10% FBS. Cells were seeded in plates and performed transient transfection using Lipofectamine 2000 according to the instructions. For stable transfection, lentivirus-mediated plasmid was done using the pCDH-cDNA system following the manufacturer's instructions.

2.3. Western blot analysis

Cells were lysed in RIPA buffer supplemented with a proteinase inhibitor cocktail (Sigma). Total proteins were separated by SDS-PAGE gels and transferred to PVDF membrane. After blocking, the membranes were incubated overnight at 4 °C with primary antibodies against GM-CSF (1:1000, proteintech, 17762-1-AP), HIF-1α (1:1000, abcam, ab113642), or β-Tublin (1:5000, Ray Antibody, RM2003), respectively. The next day, after incubation with secondary antibodies, specific proteins were visualized using an Enhanced Chemiluminescence Detection Reagent (Pierce).

2.4. Reverse transcription PCR

Total RNA was isolated using TRIzol Reagent (15596018, Invitrogen, USA) and the first-strand cDNA synthesis was performed using the First-Strand Synthesis System for reverse transcription PCR. The mRNA levels of target genes were normalized to β-actin. After the PCRs were completed, the cycle threshold (CT) data were calculated, and the mean CT value was determined from triplicate PCR reactions. Each experiment was repeated independently at least three times.

2.5. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using a commercial kit (Upstate Biotechnology) according to the instructions. Primers flanking the HRE of the VEGF promoter were used as a positive control. The primers are indicated in Supplementary Table S1.

2.6. Luciferase reporter assay

Genomic DNA fragments of the human GM-CSF gene relative to the transcription initiation sites were inserted into the pGL3-Basic vector (denoted as pGL3-GM-CSF). Point mutation was introduced into HIF-1α-binding sites by specific primers. All constructs were sequenced to confirm their identity. The luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) as previously described [20,21].

2.7. Enzyme-linked immunosorbent assay (ELISA)

PDAC cells were cultured for 72 h and the conditioned media was collected after centrifugation at 700*g* for 5 min at 4 °C. GM-CSF protein was quantified using ELISA kit (R&D Systems) according to the manufacturer's instructions.

2.8. Wound healing assay and transwell migration assay

A wound healing assay was performed when the cells attached. A 10 μL pipette tip was used to create a linear region devoid of cells. The remaining cells were cultured in conditioned media collected from PDAC cells. The wound closure was monitored at 0, 12 and 24 h after scraping. Transwell migration assays were performed with 8-μm pore inserts in a 24-well chamber. Briefly, 1×10^5 Schwann cells were seeded into the upper chamber with serum-free medium, and transfected PDAC cells was cocultured in the lower chamber as a chemoattractant. Nonmigratory cells on the upper chamber were removed slightly after 24 h of incubation. The membranes were then fixed with methanol and stained with a three-step stain set (Thermo Scientific).

2.9. Determination of PNI and immunohistochemistry (IHC) assays

H&E slides were reviewed to evaluate the degree of perineural invasion, which is defined as the presence of cancer cells in the perineurium of nerve fascicles as previously described [20]. Specimens were cut into 4-μm thick, deparaffinized and rehydrated. Heat-mediated antigen retrieval in citrate buffer was carried out followed by 3% hydrogen peroxide. After blocking, the sections were incubated with primary antibody (GM-CSF, 1:100, proteintech; HIF-1α, 1:200, abcam; S-100, 1:100, proteintech) at 4 °C overnight. The next day, the sections were incubated with secondary antibody. Diaminobenzidine was used as a chromogen substrate. Sections were then counterstained with hematoxylin, dehydrated and cover slipped. Based on the intensity of staining and the extent of staining, the expression of HIF-1α or GM-CSF was quantified. Intensity of staining was scored as 0 = negative; 1 = low; 2 = medium; 3 = high, respectively. Extent of staining was scored as 0 = 0% stained; 1 = 1–25% stained; 2 = 26–50% stained; 3 = 51–100% stained, respectively. The final score was determined by multiplying the two scores, ranging 0–9. A multiplied score ≤ 3 was recorded as low staining (+), 4–6 as medium staining (++) and > 6 as high staining (+++), respectively.

2.10. Animal experiments

All animal studies were conducted under an approved protocol by Tianjin Cancer Institute and Hospital in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals. Cells were harvested, washed, re-suspended at

10^7 cells/ml, and injected subcutaneously into the right flank of nude mice. Tumors were measured and the volume was calculated as previously described [22]. At the 18th day after inoculation, tumors were harvested. Part of tumor was fixed by formalin and embedded by paraffin, and the remaining was used for protein or RNA extraction. The HIF-1α inhibitor (PX478) was delivered via p.o. gavage at 30 mg/kg every other day after inoculation.

2.11. Statistical analysis

Analyses were performed using SPSS 21.0 statistical analysis software. All data were derived from at least three independent experiments. The results are displayed as means ± SEM. Spearman's rank correlation coefficient test was carried out for testing the association between ordinal variables. Statistical analyses were performed using Student's *t*-test or ANOVA test. Survival curves were estimated with the Kaplan-Meier method and compared using the log-rank test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. GM-CSF overexpression in tumor cells is associated with poor prognosis in patients with PDAC

GM-CSF has been shown to play an important role in various immunological and physiological processes [23–26]. However, its expression pattern in PDAC has not been described yet. In this study, the expression of GM-CSF was explored in two PDAC cohorts. There were 38 men and 32 women with a median age of 59 years (range 36–79 years) in our cohort. GM-CSF was found to be mainly expressed in the cytoplasm (Fig. 1A). Compared to normal pancreatic tissues, the expression of GM-CSF was dramatically increased in PDAC specimens (Fig. 1B). The correlation between GM-CSF expression and clinicopathologic parameters were exhibited in Table 1. GM-CSF expression was positively correlated with degree of pathological differentiation ($P = 0.044$) and TNM stage ($P = 0.008$). We further investigated GM-CSF expression in fresh PDAC tissue and paired adjacent normal tissue. Despite the individual variations, both the GM-CSF protein and mRNA were dramatically overexpressed in PDAC tissues (Fig. 1C and D). Importantly, PDAC patients with high GM-CSF protein expression had significantly worse overall survival than those with low GM-CSF expression (Fig. 1E, $P < 0.0001$), suggesting that GM-CSF play an important role in PDAC progression. The same results were found from another pancreatic adenocarcinoma cohort from TCGA. As shown in Fig. 1F and G, the expression of GM-CSF mRNA showed a significantly increase in pancreatic adenocarcinoma patients and corelated to overall survival. These above results showed that GM-CSF was significantly upregulated in PDAC, and was related with the poor prognosis of patients.

3.2. GM-CSF overexpression correlates to PNI in PDAC

PNI often accompanies by neuropathic pain, and has a significant impact on the survival and life quality of pancreatic cancer patients [3]. Since GM-CSF signaling was reported to play an important role in the incidence of tumor-evoked pain and tumor-nerve interactions [8,27], we investigated the correlation between GM-CSF and PNI in PDAC. The expression of GM-CSF and S-100 (an indicator of nerve cells) was examined in consecutive PDAC sections. IHC results showed that the GM-CSF expression positively correlated with S-100 expression (Fig. 2A). And the symptom of neuropathic pain occurred more in PDAC patients with high GM-CSF protein expression (Fig. 2B, $P = 0.001$). Furthermore, GM-CSF had higher expression in PNI patients as compared to the non-PNI patients (Table 1, $P = 0.001$), indicating the intense relationship between PNI and GM-CSF expression. To further verify this relationship, mice models were conducted. Pan02 cells with stably overexpression of GM-CSF were used for the subsequent animal experiments (Supplementary Fig.S1A and S1B). Mice in Group G were inoculated with Pan02 cells with stably overexpression of GM-CSF, and mice transplanted with control Pan02 cells were recorded as Group N. The overexpression of GM-CSF did not affect the tumor size (Supplementary Fig.S1C and S1D). We then evaluated the expression of GM-CSF in tumors by PCR, Western blot and IHC assay. As expected, the protein and mRNA level of GM-CSF in tumors of Group G were significantly higher than that of Group N (Fig. 2C–E). The injection of tumor cells could induce leg paralysis in mice of Group G compared with that of Group N (Fig. 2E), indicating more aggressive properties due to the overexpression of GM-CSF. Moreover, tumors with overexpressed GM-CSF had more S-100-positive cells infiltration, demonstrating that GM-CSF expression is strongly associated with PNI in the tumor tissues (Fig. 2E). The above results indicated a positive correlation between GM-CSF and PNI in PDAC tissues. However, the underlying mechanism needs further exploration.

3.3. GM-CSF overexpression in PDAC cells promotes the migration of Schwann cells

As an exocrine cytokine, GM-CSF exhibits pleiotropic functions on different target cells [26,28]. To explore whether the overexpressed tumor-derived GM-CSF could affect the migration of Schwann cells, we first identified its basic expression in different PDAC cell lines (Supplementary Fig.S2). GM-CSF was ectopically overexpressed in two PDAC cell lines with low endogenous GM-CSF levels (PANC-1 and MIA PaCa2). We also knock down GM-CSF expression in two PDAC cell lines with high endogenous GM-CSF levels (CFPAC-1 and SW1990). Both the mRNA and protein expression of GM-CSF were augmented by the overexpression plasmid and were markedly inhibited by siRNAs (Fig. 3A–C). ELISA assays indicated the same change of GM-CSF excretion in the supernatant of PDAC cells upon plasmid or siRNA transfection (Fig. 3D and E).

To further investigate the migration ability of Schwann cells in conditions with different GM-CSF expression level, wound healing and transwell assays of Schwann cells were performed with conditioned media collected from PDAC cells after transfection. As a result, Schwann cells showed a stronger migration ability in a microenvironment with higher expression of GM-CSF. Meanwhile, the migration ability was strongly inhibited when Schwann cells were cultured with medium from PDAC cells transfected with GM-CSF siRNA (Fig. 3F–I). Furthermore, GM-CSF did not affect the proliferation of Schwann cells (Supplementary Fig.S3). These results suggest that the tumor-derived GM-CSF could act as a chemoattractant for Schwann cells and promotes the migration of Schwann cells, but whether the promotion is caused by GM-CSF directly needs exploration. Since GM-CSF functions by the direct interaction with GM-CSFR, we then knock down GM-CSFR in Schwann cells by using specific siRNAs (Supplementary Fig.S4A and S4B). As expected, the promotion disappeared after GM-CSFR knockdown in Schwann cells (Supplementary Fig.S4C and S4D), indicating that the enhanced migration of Schwann cells was directly regulated through tumor derived GM-CSF.

3.4. HIF-1α expression is positively correlated with GM-CSF expression in PDAC tissues

Our previous study identified HIF-1 as a critical transcriptional factor in the progression of pancreatic cancer [29]. Thus, we further speculated whether the hypoxic environment affects the expression of GM-CSF in PDAC cells. As a result, the expression level of HIF-1α and GM-CSF in PANC-1 cells were increased when cultured in hypoxia condition compared with that in normoxia condition, followed by the enhanced migration of Schwann cells in hypoxia condition, indicating that the hypoxia microenvironment could regulate the expression of GM-CSF and the migration of Schwann cells through the overexpression

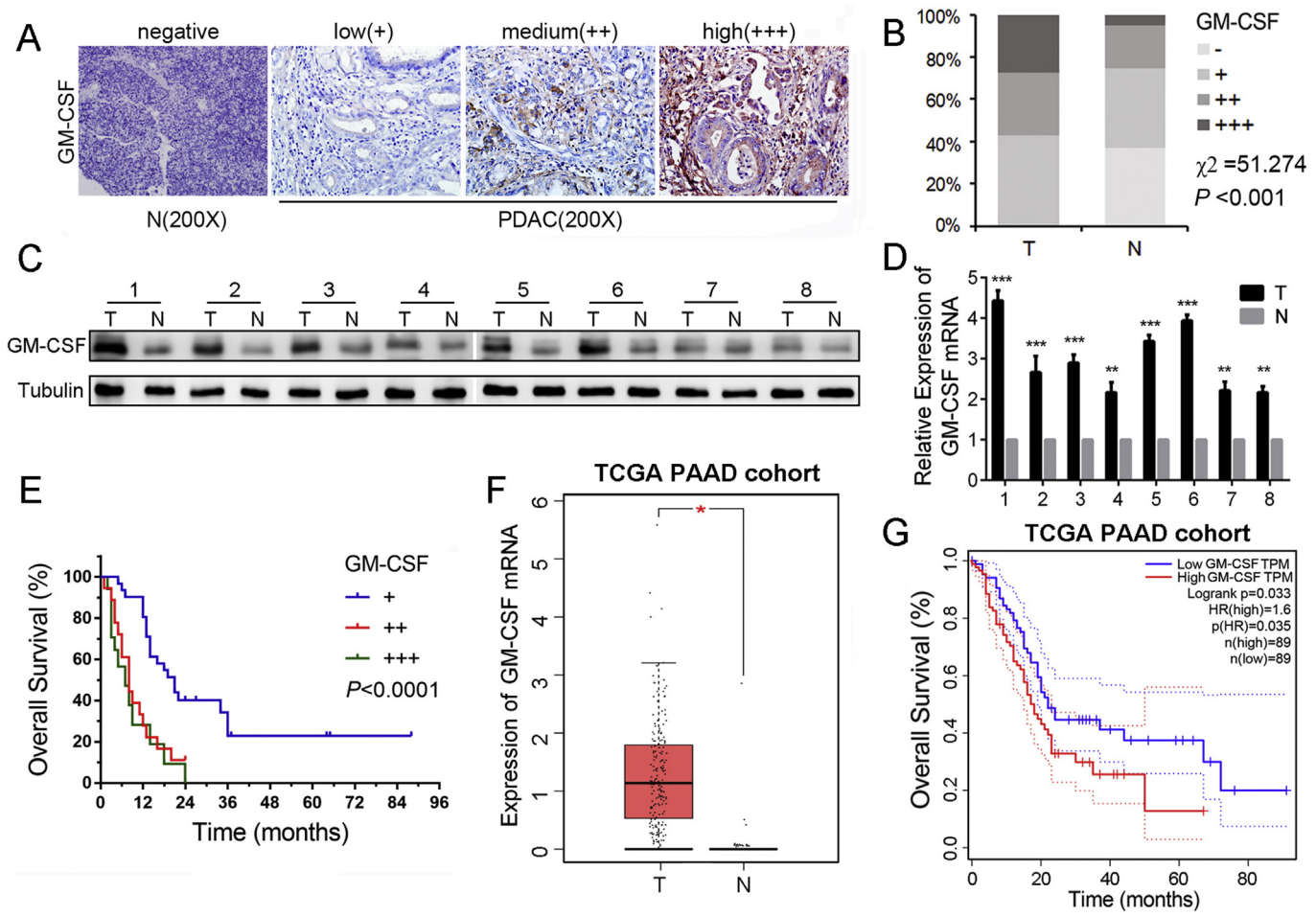

Fig. 1. GM-CSF is overexpressed in PDAC. (A) IHC staining of GM-CSF in normal pancreatic tissues and PDAC tissues (200×). **(B)** IHC scores of tumor tissues and normal tissues (n = 70). **(C and D)** Western blot and PCR analysis of GM-CSF levels in eight pairs of PDAC tumorous and matched adjacent nontumorous tissues. **(E)** Survival curves according to the GM-CSF status in our PDAC cohort. **(F)** The mRNA levels of GM-CSF in tumor tissues and normal tissues of the TCGA pancreatic adenocarcinoma (PAAD) cohort (n = 185). **(G)** Survival curves according to the GM-CSF status in TCGA PAAD cohort. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; T, tumor; N, normal.

Table 1
Correlations between clinicopathological features and HIF-1α or GM-CSF expression in PDAC.

	HIF-1α			r	*P*-value	GM-CSF			r	*P*-value
	+	++	+++			+	++	+++		
Age (years)	59 (36–79)			4.710[a]	0.194	59 (36–79)			6.138[a]	0.052
Sex				0.078	0.520				0.001	0.995
Male	13	16	9			19	9	10		
Female	14	11	7			15	10	7		
Tumor size(cm)	3.30 ± 1.63	4.69 ± 1.80	5.10 ± 1.79	6.392[b]	0.003	4.02 ± 1.63	4.58 ± 1.99	4.40 ± 1.87	0.584[b]	0.561
Histological grade				0.202	0.094				0.242	0.044
G1	14	12	5			18	6	7		
G2	7	6	3			10	5	1		
G3	6	9	8			6	8	9		
Lymph node metastasis				0.347	0.003				0.259	0.030
N 0	19	13	4			22	8	6		
N 1	8	14	12			12	11	11		
pTNM stage				0.416	< 0.001				0.316	0.008
I	3	0	0			2	0	1		
II	21	18	7			26	14	6		
III	3	9	9			6	5	10		
Perineural invasion (PNI)				0.433	< 0.001				0.380	0.001
–	14	7	0			16	4	1		
+	13	20	16			18	15	16		

[a] Kruskal-Wallis test; b, ANOVA test.

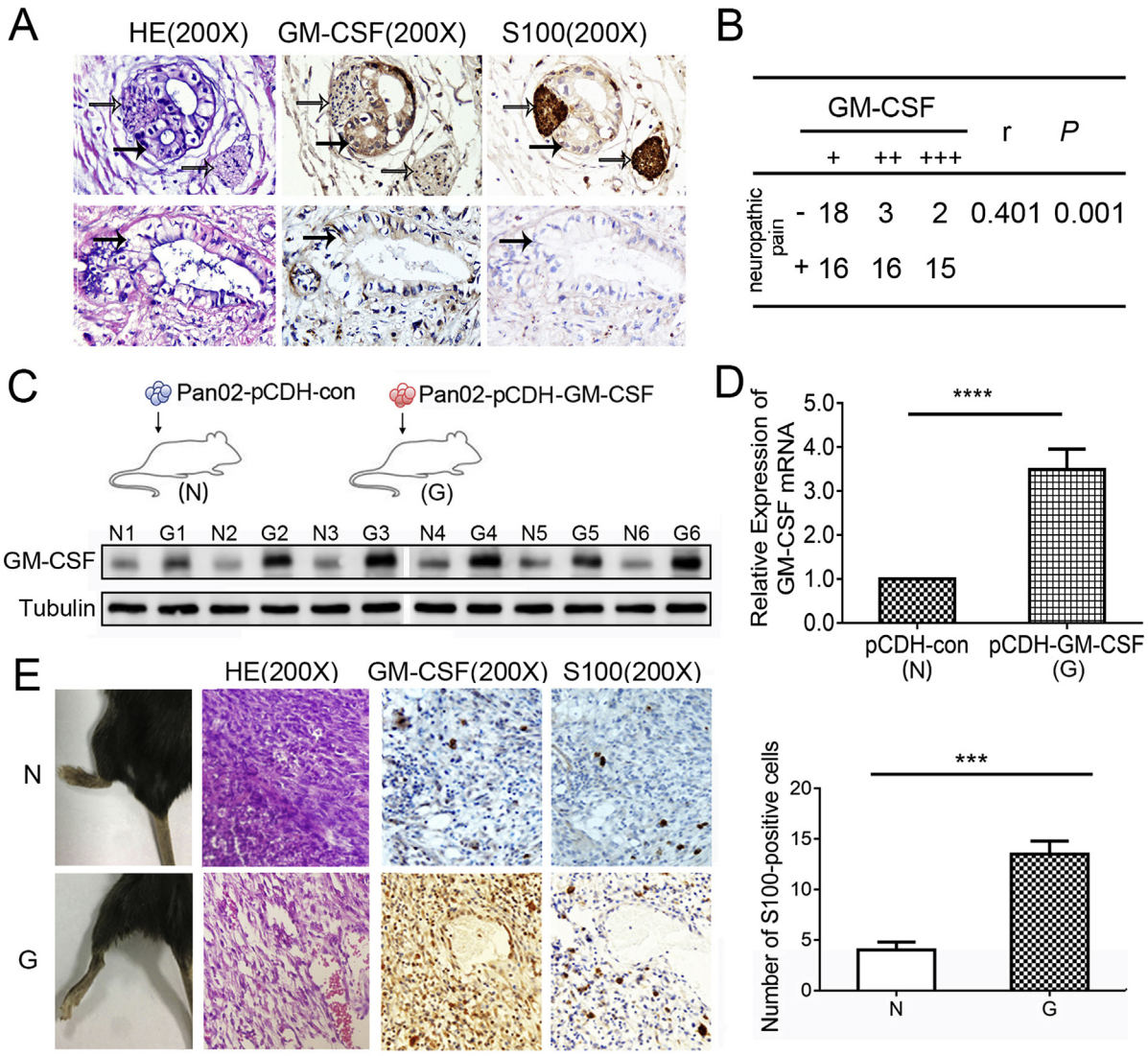

Fig. 2. GM-CSF overexpression correlates to PNI in PDAC. (A) The HE staining and IHC staining of GM-CSF and S-100 in consecutive PDAC sections (200×). The arrows indicate tumor cells, and the open arrows indicate nerve fibers. **(B)** The correlation between GM-CSF expression and the occurrence of neuropathic pain in PDAC patients. **(C–E)** Pan02 cells overexpressing GM-CSF (Group G) or control Pan02 cells (Group N) were subcutaneously implanted into the mice. Western blot (C), PCR (D), HE (E, 200×) and IHC (E, 200×) analysis showed that both the protein and mRNA levels of GM-CSF were upregulated in Group G than that of Group N. The injection with Pan02 cells overexpressing GM-CSF induced leg paralysis in Group G and corelated to more infiltration of S100-positive cells. ***, $P < 0.001$; ****, $P < 0.0001$.

of HIF-1α (Supplementary Fig.S5).

Subsequently, we evaluated the effects of HIF-1α on GM-CSF expression in PDAC. In accordance with previous studies, HIF-1α expression was significantly increased in both FFPE PDAC specimens and fresh PDAC tissues (Supplementary Fig.S6A-C), and correlated to poor prognosis of PDAC patients (Supplementary Fig.S6D). IHC staining of consecutive PDAC tissue sections showed tumor cells around nerve fibers were overexpressed with HIF-1α and GM-CSF compared to specimen with no nerve infiltration (Fig. 4A), indicating an important role of HIF-1α and GM-CSF in the tumor-nerve interactions. Furthermore, spearman analysis showed that the expression of GM-CSF was significantly associated with HIF-1α expression (Fig. 4B, $P = 0.006$). The incidence of PNI was also higher in tumors with higher HIF-1α expression (Table 1, $P < 0.001$). The above results demonstrated that HIF-1α upregulation in hypoxia condition is positively correlated with GM-CSF expression and PNI in PDAC. However, HIF-2α which is also upregulated in hypoxia environment did not affect the expression of GM-CSF in PDAC cells (Supplementary Fig.S7). Thus, HIF-1α was chosen for deep exploration in tumor-nerve interactions in PDAC.

3.5. Validation of HIF-1α as a potential upstream regulator of GM-CSF

Whether HIF-1α regulates the transcription of GM-CSF was then investigated in different PDAC cell lines. When HIF-1α was overexpressed in PANC-1 cells and MIA PaCa2 cells, GM-CSF mRNA and protein expression was markedly increased (Fig. 4C and D). Moreover, specific siRNAs targeting HIF-1α or lentivirus were used to effectively knock down HIF-1α expression. Knockdown of HIF-1α expression decreased GM-CSF mRNA and protein expression in CFPAC-1 cells and SW1990 cells (Fig. 4C and E).

Since HIF-1α is widely known as a transcription factor, we searched for the promoter region of human GM-CSF gene and identified two HREs with binding possibility (Fig. 4F). ChIP assay was performed to investigate whether or not HIF-1α directly binds to GM-CSF promoter. In chromatin fractions pulled down by an anti−HIF−1α antibody, only the HRE of GM-CSF promoter located at −322 to −326 was detected,

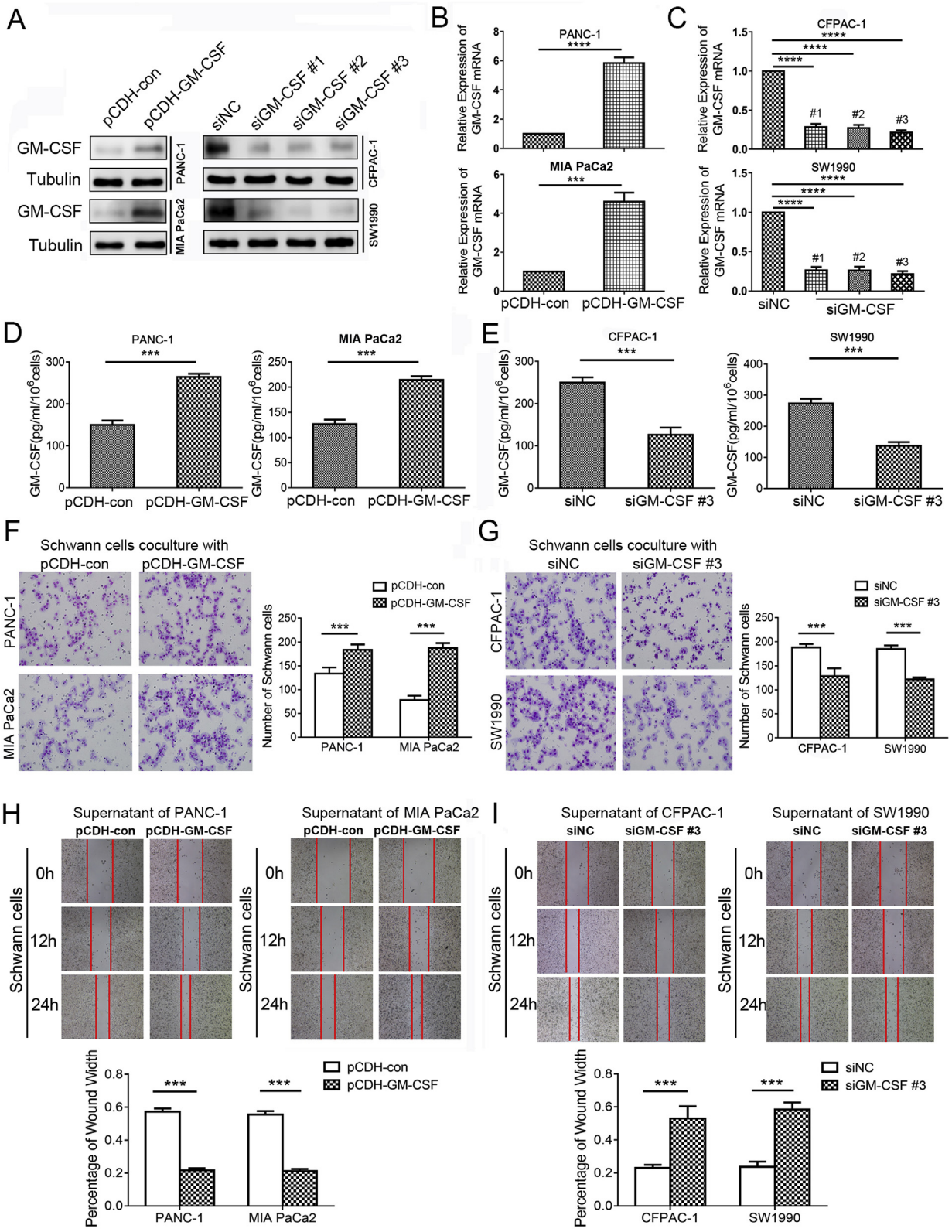

Fig. 3. The up-regulation of GM-CSF promotes Schwann cell migration. (A–C) The protein levels (A) and RNA levels (B and C) of GM-CSF in PDAC cells after transfection with plasmids or siRNAs. **(D and E)** ELISA assays of GM-CSF levels secreted by PDAC cells after transfection. **(F and G)** Transwell assays of Schwann cells cocultured with transfected PDAC cells. **(H and I)** Wound healing assays of Schwann cells cultured with the supernatant of transfected PDAC cells. ***, $P < 0.001$; ****, $P < 0.0001$.

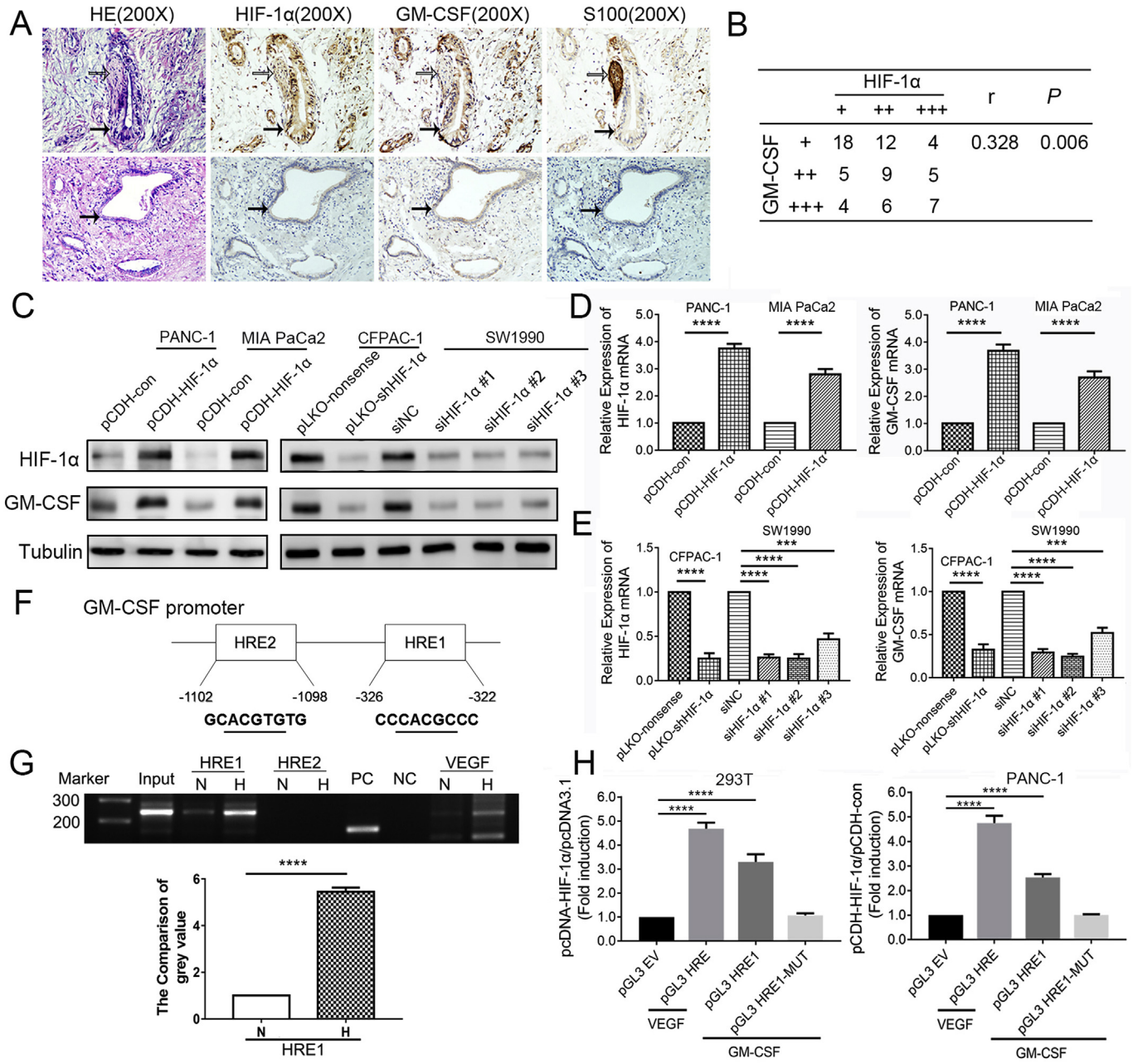

Fig. 4. GM-CSF is a novel HIF-1α target gene. (A) IHC assays for HIF-1α, GM-CSF and S100 in consecutive PDAC sections (200×). The arrows indicate tumor cells, and the open arrows indicate nerve fibers. **(B)** The correlation between GM-CSF expression and HIF-1α levels. **(C–E)** Western blot (C) and PCR (D and E) analysis of GM-CSF levels and HIF-1α levels in PDAC cells after the up-regulation or down-regulation of HIF-1α. **(F)** The DNA sequence of the GM-CSF promoter. Two HRE sites are located at the different sites. **(G)** Chromatin immunoprecipitation analysis of HIF-1α binding to GM-CSF promoter in PANC-1 cells. The PCR products of VEGF promoter were used as positive control. N, normoxia; H, hypoxia; PC, positive control; NC, negative control. **(H)** luciferase analysis in 293T cells or PANC-1 cells. These two cell lines overexpressing HIF-1α (pCDH−HIF−1α) or control vector (pCDH-con) were transfected with pGL3-GM−CSF−promoter, pGL3-GM−CSF−mutated-promoter, pGL3-VEGF-promoter, or pGL3-empty vector. After transfection for 48 h, cells were subjected to dual luciferase analysis. Results were expressed as a fold induction relative to the cells transfected with the control vector after normalization to Renilla activity. ****, $P < 0.0001$.

and this phenomenon was more evident in hypoxia condition (Fig. 4G). To further determine whether the binding of HIF-1α could activate GM-CSF promoter, we constructed a full-length GM-CSF luciferase promoter vector (containing HRE, −322 to −326) and co-transfected this reporter construct with or without HIF-1α cDNA into 293T cells or PANC-1 cells. Luciferase analyses showed that HIF-1α overexpression (pCDH−HIF−1α) significantly increased GM-CSF promoter activity in these two cell lines, however, the transactivation of GM-CSF promoter was almost abolished when the HIF-1α binding site was mutated instead (Fig. 4H). The HRE of the VEGF promoter were used as a positive control. Taken together, these results suggest that HIF-1α directly regulates GM-CSF expression through transactivation.

3.6. HIF-1α overexpression promotes Schwann cell migration through GM-CSF

The excretion levels of GM-CSF by PDAC cells after HIF-1α up-regulation or down-regulation were examined by ELISA assays. Higher extracellular expression of GM-CSF was detected when the expression of HIF-1α was increased in PDAC cells, whereas the extracellular GM-

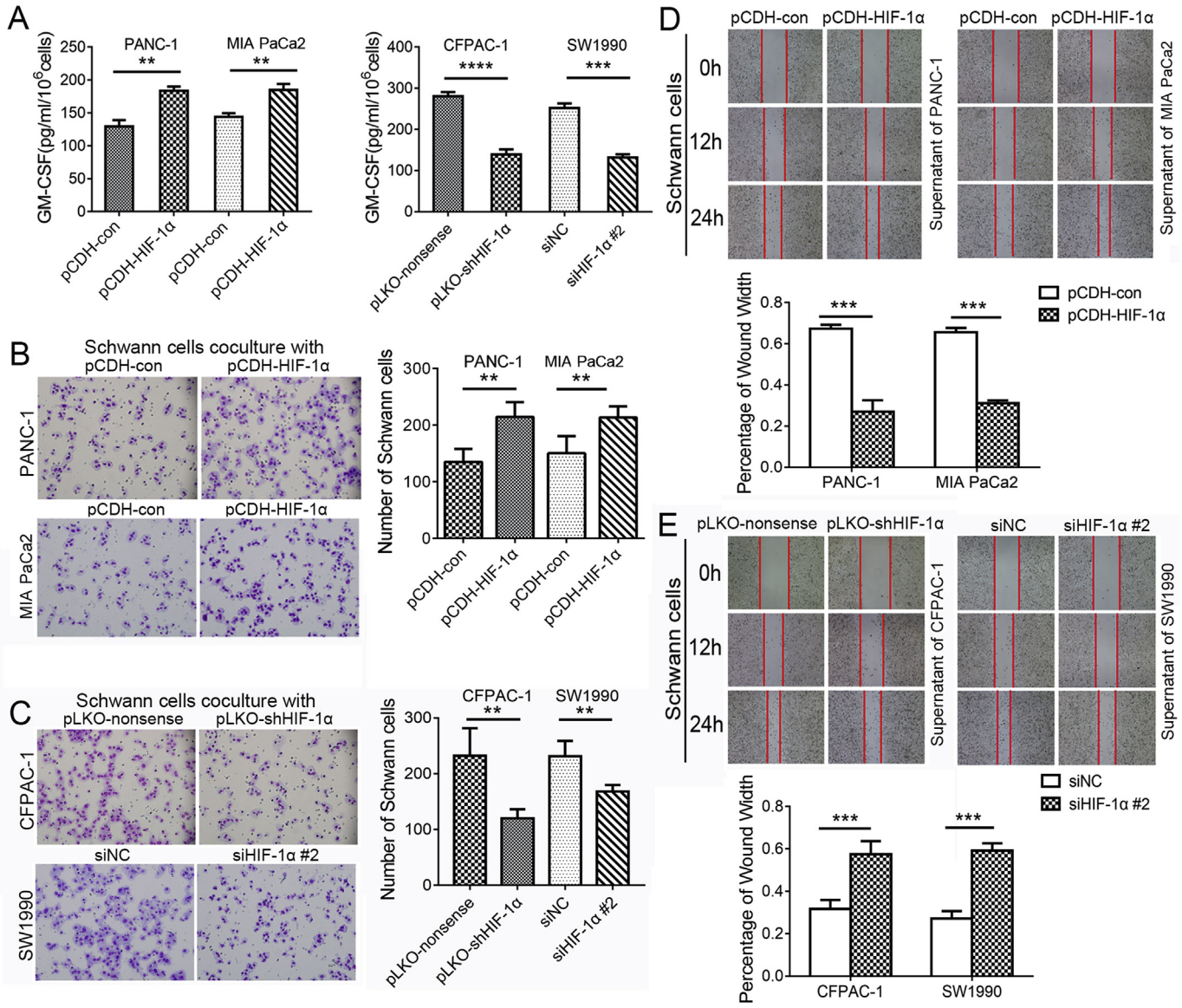

Fig. 5. HIF-1α overexpression promotes Schwann cell migration through GM-CSF. (A) ELISA assays of GM-CSF levels secreted by PDAC cells after the up-regulation or down-regulation of HIF-1α. **(B and C)** Transwell assays of Schwann cells cocultured with different PDAC cells. **(D and E)** Wound healing assays of Schwann cells cultured with the supernatant of different PDAC cells. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

CSF was lower in cells with down-regulated HIF-1α (Fig. 5A). Migration assays were performed to further validate the role of HIF-1α/GM-CSF signal in the tumor-nerve interactions. As shown in Fig. 5B– E, Schwann cells cocultured with PDAC cells overexpressing HIF-1α showed stronger migration ability, while downregulated HIF-1α of tumor cells might inhibit Schwann cell migration significantly. No difference was discovered among the proliferate ability of Schwann cells cultured in the supernatant from PDAC cells with HIF-1α overexpressing or control cells (Supplementary Fig.S8).

Since the role of GM-CSF depends on the expression of GM-CSFR in Schwann cells, we further investigated whether the expression of GM-CSFR had also changed. The results showed that there was no significant difference regrading to the expression level of GM-CSFR in Schwann cells after HIF-1α up-regulation and down-regulation in PDAC cells (Supplementary Fig.S9A and S9B).

3.7. GM-CSF overexpression can rescue the inhibition of Schwann cell migration by HIF-1α knockdown in vitro and in vivo

To understand the role of GM-CSF in tumor-derived HIF-1α-mediated Schwann cell migration, we constructed an GM-CSF overexpressed CFPAC-1 cell line on the basis of HIF-1α knockdown. As shown in Fig. 6A-D, GM-CSF overexpression at least partially rescued the inhibitory effect of HIF-1α knockdown on Schwann cell migration, suggesting that GM-CSF was involved in tumor-derived HIF-1α-mediated Schwann cell migration. Furthermore, we inhibited GM-CSF expression in HIF-1α overexpressed MIA PaCa2 cells (Supplementary Fig.S10). The results showed that down-regulated GM-CSF could partially suppress the migration of Schwann cells due to the overexpression of HIF-1α in MIA PaCa2 cells.

Next, we investigate the effects of HIF-1α inhibitor on tumor-nerve interactions in mouse models. When the growth of tumor reached to 100 mm^3, saline or PX478 (HIF-1α inhibitor, 30 mg/kg) were orally administered to mice every other day after inoculation. As shown in Fig. 6E, mice in Group A were implanted with control Pan02 cells and

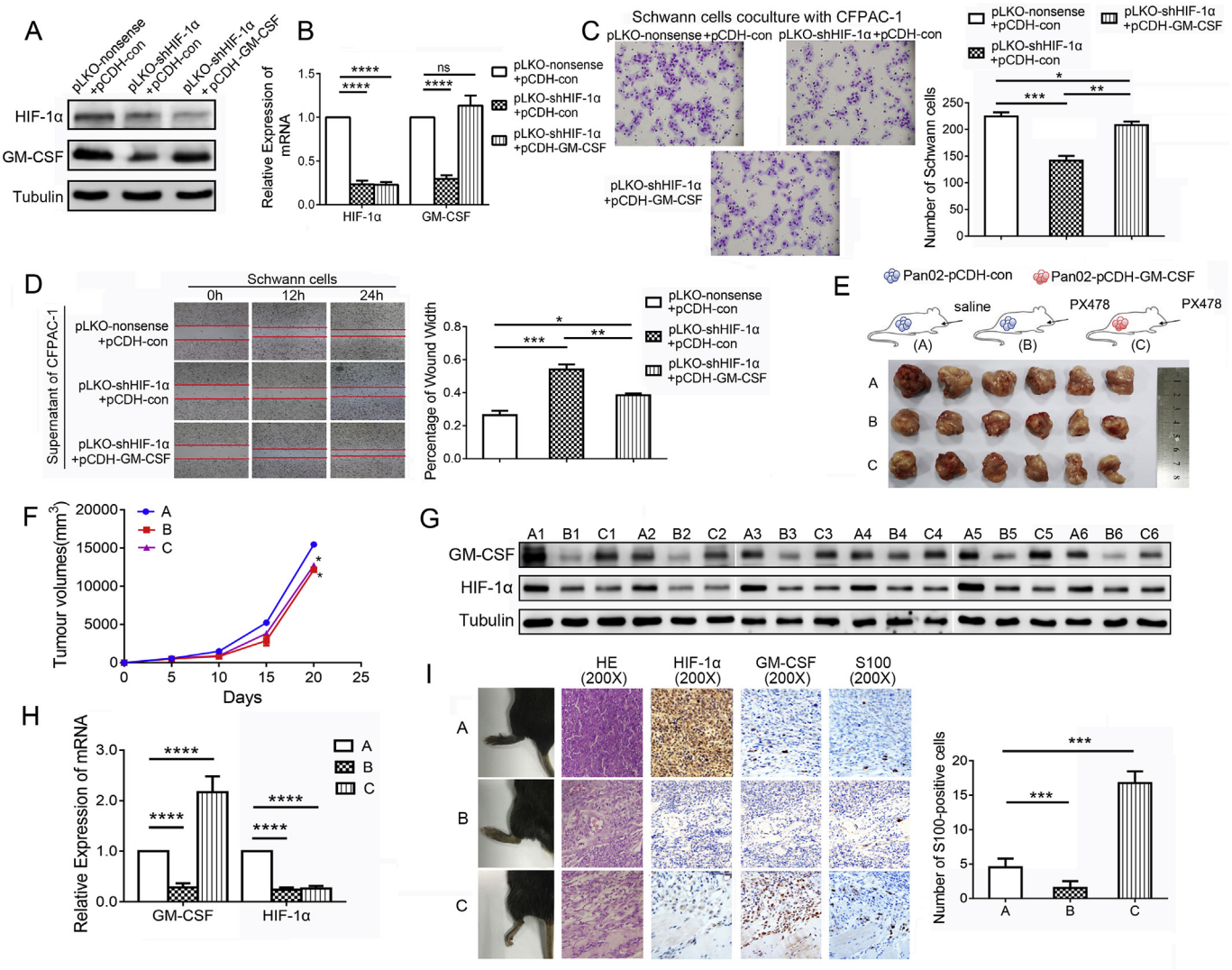

Fig. 6. GM-CSF overexpression is able to rescue the inhibition of Schwann cell migration by HIF-1α knockdown in vitro and in vivo. (A–D) CFPAC-1 cells were co-transfected with pLKO-shHIF-1α and pCDH-GM-CSF plasmids and assessed by Western blot (A), PCR (B), transwell analysis (C) and wound healing analysis (D). **(E–I)** Pan02 cells overexpressing GM-CSF or control Pan02 cells were subcutaneously implanted into the mice, which were treated with saline or PX478 (HIF-1α inhibitor) subsequently. Mice in Group A were injected with control Pan02 cells and treated with saline, mice in Group B were injected with control Pan02 cells and treated with PX478, and mice in Group C were injected with Pan02 cells overexpressing GM-CSF and treated with PX478. Representative figure of tumors formed were shown (E) and the volumes of all primary tumors are expressed as mean value (F). Western blot (G), PCR (H), HE (I, 200×) and IHC (I, 200×) analysis were performed in tumors of Group A, Group B and Group C. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

subsequently treated with saline, mice in Group B were injected with control Pan02 cells and subsequently treated with PX478, and those inoculated with Pan02 cells overexpressing GM-CSF and subsequently treated with PX478 were recorded as Group C. After the treatment with PX478, the average tumor volume reduced in Group B and Group C compared to that of Group A (Fig. 6E and F), and both HIF-1α expression and GM-CSF expression in tumors were inhibited significantly (Fig. 6G and H, Group A vs. Group B). The exogenous overexpression of GM-CSF could induce leg paralysis in mice of Group C even when they were treated with HIF-1α inhibitor simultaneously (Fig. 6I). And the IHC staining of consecutive tumor sections showed that the amounts of nerves decreased after PX478 treatment (Fig. 6I, Group A vs. Group B), whereas increased when the tumor was GM−CSF−overexpressed (Fig. 6I, Group A vs. Group C). These in vivo results suggested that HIF-1α is a critical regulator for GM-CSF overexpression, which then mediate the tumor-nerve interaction in PDAC.

4. Discussion

PDAC is one of the solid tumor that accompanied with high PNI incidence. It usually manifests as hypersensitive pain, which involved the malfunction of various molecules. Aside from the strong neurotropic characteristics of PDAC cells and the close proximity of the pancreas to several neural plexuses, the reason for the preponderance of PNI in PDAC remains largely to be elucidated. Our study revealed a novel mechanism of tumor-nerve interactions in PDAC. GM-CSF expression was found to be up-regulated in PDAC tissues and had positive correlation with PNI. Subsequent mechanistic studies demonstrated that elevated HIF-1α expression in PDAC activated GM-CSF mRNA transcription and protein expression, which further promoted Schwann cell migration. Moreover, HIF-1α inhibition with PX478 drastically reduced GM-CSF protein expression and decreased the amount of nerve fibers in PDAC xenograft mouse model. These results suggested a critical role of the HIF-1α/GM-CSF signal in the tumor-nerve interactions of PDAC.

Although conflicting evidence has been published [30–33], the

major opinion is that the infiltration and growth of cancer cells within these surrounding nerves could serve as an indicator for aggressive behavior of pancreatic cancer. As discovered in prostate cancer, tumor cells have a propensity to travel towards nerves, while nerves can preferentially grow towards tumor cells in response to certain signals secreted by cancer cells [34]. On the basis of previously identified neurotropism of pancreatic cancer cells, our study indicates that nerves can migrate towards cancer cells via the attraction by tumor-derived GM-CSF, demonstrating the crosstalk signaling between cancer cells and nerve fiber in PDAC.

PNI contributes to the hypersensitive pain which is experienced by the majority of pancreatic cancer patients. The function of GM-CSF signaling in sensory neurons was revealed by an attenuation of tumor-evoked pain following a sensory nerve-specific knockdown of GM-CSFR. Our study discovered that overexpressed GM-CSF induced the migration of nerve cells and is significantly correlated with the suffering of pain in PDAC patients. This result indicated that GM-CSF signaling might be a therapeutic target in the pain control of PDAC patients. Although previous studies have demonstrated both pro- and anti-tumor properties of GM-CSF [35], the majority of them indicated a promotive role of GM-CSF on tumor growth and progression [36–39]. As a secretory factor, high expression of GM-CSF deriving from pancreatic cancer cells increases the recruitment of myeloid-deriving suppressor cells, which suppresses T cell immunity and promotes tumor development [40]. In our study, we further elucidated the role of GM-CSF in PDAC, which is induction of nerve cells migration.

Tumor hypoxia is known to play an important role in genetic instability, tumor cell invasiveness and metastasis, chemoresistance, which all contributes to an adverse clinical outcome [41]. As a master regulator in response to hypoxia [42], HIF-1α has been identified to regulate several genes in pancreatic cancer [10,20,21,29] and could also recruit macrophage to activate pancreatic stellate cells [17]. This study demonstrated that HIF-1α could directly activate the transcription and expression of GM-CSF, and further mediate tumor-nerve interaction in PDAC. The HIF-1α inhibitor could not only suppress tumor growth, but also reduce the migration of nerve cells which might decrease the incidence of PNI and relieve the patients from neuropathic pain. On the basis of our result, we suggested that agents targeting the HIF-1α/GM-CSF signaling pathways may have the potential to inhibit PNI and help to alleviate the pain suffering in PDAC patients.

In conclusion, we demonstrated that GM-CSF was upregulated in PDAC patients, particularly in those with cancer-relating pain. Overexpressed GM-CSF suggests a poor clinical prognosis in our cohort. As the regulation of tumor-nerve interaction by HIF-1α/GM-CSF signal has been identified both in vivo and in vitro, targeting this signal pathway might be promising in the future treatment of pancreatic cancer.

Author contributions

Study supervision and funding support: Jihui Hao, He Ren and Xiuchao Wang.

Haotian Wang and Rujiang Jia contributed equally to this study. Jihui Hao, He Ren and Xiuchao Wang conceived the idea and designed the study. Haotian Wang, Rujiang Jia and Xiuchao Wang performed immunohistochemistry studies. Yan Sun and Xiuchao Wang analyzed the immunohistochemistry data. Tiansuo Zhao, Hongwei Wang, Zengxun Li, Bodong Zhou, Chungen Lan and Xin Li supplied study material or patients. Haotian Wang, Bodong Zhou, Liangliang Wu and Zengxun Li performed mouse experiments. Haotian Wang, He Ren and Xiuchao Wang performed statistical analysis. Haotian Wang, Xiuchao Wang, He Ren and Jihui Hao wrote the manuscript. All authors reviewed and approved the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.036.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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