G Protein-Coupled Receptor GPR87 Promotes the Expansion of PDA Stem Cells through Activating JAK2/STAT3

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Cancer stem cells are the main reason for drug resistance and tumor relapse, and screening the targets for cancer stem cells is essential for tumor therapy. Here, we studied the role and regulatory mechanism of a G protein-coupled receptor named as G protein-coupled receptor 87 (GPR87) in the expansion of pancreatic ductal adenocarcinoma (PDA) stem cells. We found that GPR87 was an independent prognostic factor for PDA patients: patients with high GPR87 had a poor outcome. GPR87 significantly promoted the sphere formation ability, increased side population (SP) cell number, increased the expression of PDA stem cell markers, and increased the tumor initiation ability, suggesting that GPR87 promotes the expansion of PDA stem cells. Mechanism analysis suggested that signal transducer and activator of transcription 3 (STAT3) directly bound to the promoter of GPR87 to increase GPR87 expression; inversely, GPR87 also activated STAT3. Further analysis suggested that GPR87 activated Janus kinase 2 (JAK2), which can activate STAT3, inhibiting JAK2 activation in GPR87-overexpressing PDA cells, which significantly inhibited the expansion of PDA stem cells; these findings suggested that GPR87, JAK2, and STAT3 formed a positive feedback loop increasing PDA stem cell population. In PDA specimens, GPR87 expression is positively correlated with the phosphorylation level of STAT3 and JAK2, confirming GPR87 promoted PDA stem cell expansion through activating JAK2/STAT3. In summary, we found that GPR87, together with JAK2 and STAT3, formed a positive feedback loop to promote the expansion of PDA stem cells.

INTRODUCTION
Pancreatic ductal adenocarcinoma (PDA) is one of the most lethal malignancies, with high metastatic ability and recurrence rate. Surgical resection and first-line drug gemcitabine plus albumin-bound paclitaxel particles (nab-paclitaxel) could not effectively cure PDA, and the 5-year survival rate is only about 6%1,2. So, it is important to elucidate the key regulatory mechanisms driving tumor metastasis and recurrence.

G protein-coupled receptors (GPCRs) are the largest family of signaling membrane proteins in eukaryotic, which consists of over 800 proteins;3 they are key targets for pharmaceutical design. Many pharmacologists and structural biologists are developing drugs targeting GPCRs,4–6 and many drugs targeting GPCRs have been approved by the US Food and Drug Administration (FDA), such as Northera and Zontivity.7 G Protein-Coupled Receptor 87 (GPR87) is a GPCR, and it has been demonstrated to regulate the progression of several tumors.8 GPR87 is an independent prognostic factor for bladder cancer intravesical recurrence;9 it promotes bladder cancer cell proliferation and inhibits apoptosis.10 Knockdown of GPR87 increases the sensibility to DNA damage, and enhances p53 stabilization and activation.11 Recently, a group reported that GPR87 promotes PDA proliferation, angiogenesis, gemcitabine resistance, and tumorigenicity through activating the nuclear factor-kB (NF-kB) pathway,12 but the researchers do not study the role of GPR87 in PDA stem cell expansion, and the regulatory mechanism of GPR87 in PDA progression is still to be explored. In this study, we studied the role of GPR87 in the expansion of PDA stem cells and found that GPR87 promotes the expansion of PDA stem cells through...
activating Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3).

RESULTS

GPR87 Is a Poor Prognostic Factor for PDA
To investigate the importance of GPR87 in the expansion of PDA stem cells, we studied its expression in PDA cells and tissues; quantitative PCR (qPCR) and western blot showed that GPR87 was upregulated in PDA cells compared with non-malignant human pancreatic cell line hTERT-HPNE (Figure 1A). We used microarray data (GEO: GSE57495) to analyze GPR87 expression in PDA tissues; GPR87 was also significantly upregulated in PDA tissues compared with normal pancreas (Figure S1A), and high GPR87 expression was associated with significantly poorer prognosis (Figure S1B). Gene Set Enrichment Analysis (GSEA) suggested that GPR87 expression was positively correlated with gene signatures upregulated in PDA, and data were downloaded from GEO: GSE71729 and The Cancer Genome Atlas (TCGA) (Figure S1C). We used a cohort of 121 PDA tissues to confirm the above results, and found PDA tissues from patients with a short survival time (<1 year) had high GPR87 expression, whereas PDA tissues from patients with a long survival time (≥1 year) had low GPR87 expression (Figure 1B), and high expression was associated with poor outcome (Figure 1C). A multivariate analysis of survival based on Cox proportional-hazard model suggested that high GPR87 expression was an independent prognostic factor for PDA patients (Figure 1D). These results suggested that GPR87 was upregulated in PDA cells and tissues, and is a poor prognostic factor for PDA patients.

GPR87 Promotes the Expansion of PDA Stem Cells
Sphere formation assay, side population (SP) assay, CSCs biomarker expression assay, and tumor initiation assay in vivo are often used to
analyze CSCs. According to the above results (Figure 1A), GPR87 was low expression in SW1990 and Panc10.05, whereas it was high expression in BXPC-3 and Capan-2, so we overexpressed GPR87 in SW1990 and Panc10.05, and downregulated GPR87 in BXPC-3 and Capan-2. Sphere formation assay suggested that GPR87 overexpression increased sphere size, and GPR87 knockdown inhibited sphere size (Figure 2A). In three consecutive passages, GPR87 overexpression cells were capable of generating sphere in suspension culture at a density of 500 cells/mL with high frequency, about 6%, and the sphere generation frequency of GPR87 knockdown cells was significantly reduced, about 1.6% (Figure 2B). We also found with the extension of cultural time that GPR87 overexpression significantly increased the cell number in the sphere, whereas GPR87 knockdown significantly reduced the cell number in the sphere (Figure 2C). These results suggested that GPR87 promoted the sphere generation. SP assay suggested GPR87 overexpression increased SP-positive population, and its knockdown reduced SP positive population; this finding also suggested that GPR87 promoted the expansion of PDA stem cells. Finally, we examined the effect of GPR87 on the expression of markers for PDA stem cells. CD133, EpCAM, CD24, CD44, and MET have been reported to be the markers for PDA stem cells, and qPCR analysis found that GPR87 overexpression increased CD133, epithelial cell adhesion molecule (EpCAM), CD24, CD44, and MET expression, whereas GPR87 knockdown inhibited their expression. Together, these findings suggested that GPR87 promoted PDA expansion.

We used tumor initiating in vivo to further confirm that GPR87 promotes the expansion of PDA stem cells. We carried out tumor formation in vivo using limiting dilutions of GPR87 overexpression and knockdown cells (100,000 cells, 10,000 cells, and 1,000 cells); as shown in Figure 3A, tumor size correlated with the number of GPR87-overexpressing PDA cells injected. Remarkably, 1,000 GPR87 knockdown cells failed to generate tumors, and 1,000 GPR87 overexpression cells still generate tumors (Figure 3A). The volume of tumors generating GPR87 overexpression PDA cells was larger than tumors generating GPR87 knockdown PDA cells (Figure 3B). These in vivo experiments also demonstrated that GPR87 promoted the expansion of PDA stem cells.

STAT3 Upregulates GPR87 Expression

To analyze the upstream regulators for GPR87, we used GSEA to find the correlation signaling, and found that GPR87 expression positively correlated with the expression of STAT3-regulated gene signatures in PDA using three databases, respectively (Figure 4A); analysis of the GPR87 promoter region using the CONSITE program predicted three typical STAT3-responsive elements (SREs; Figure 4B). Chro-matin immunoprecipitation (ChIP)-qPCR assays showed that endogenous STAT3 proteins bound to the first SRE (SRE1) in the GPR87 promoter (Figure 4C). In addition, to validate whether the SREs in the GPR87 promoter were responsive to STAT3, we cloned an SRE1 fragment into the pTAL-luciferase reporter (pTAL-luc) and observed a consistent and dose-dependent induction of luciferase activity upon IL-6 treatment (Figure 4D). Furthermore, qPCR and western blot analysis suggested that GPR87 dramatically increased following activation of STAT3 through treating with IL-6, whereas inhibition of STAT3 activity through treating with the JAK2/STAT3 pathway inhibitor AG490 decreased GPR87 expression at the levels of mRNA and protein (Figure 4E); AG490 is a JAK2 inhibitor and inhibits the JAK2/STAT3 pathway. Taken together, these results indicate the STAT3-induced GPR87 expression through direct targeting of the GPR87 promoter.

GPR87 Activates STAT3 through Interacting with JAK2

We have demonstrated that STAT3 induced GPR87 expression, but regarding whether GPR87 could regulate STAT3 activity, western blot analysis found that GPR87 overexpression promoted the phosphorylation of STAT3, whereas its knockdown inhibited the phosphorylation of STAT3 (Figure 5A), suggesting GPR87 could activate STAT3; this phenotype was also demonstrated in tumors derived from GPR87 overexpression or knockdown cells (Figure 3C). JAKs are protein tyrosine kinases; they are activated through cytokine-induced phosphorylation, either as heterodimeric receptor-JAK complexes or as homodimeric receptor-JAK2 complexes. Activated JAKs phosphorylated STATs to regulate cell growth, differentiation, proliferation, and CSCs. We also found that GPR87 overexpression increased the phosphorylation of JAK2, and its GPR87 knockdown inhibited the phosphorylation of JAK2 (Figure 5A); this result argued that GPR87 increased JAK2 activity, and this phenotype was also demonstrated in tumors derived from GPR87 overexpression or knockdown cells (Figure 3C). So, we conferred that GPR87 increased STAT3 activity through activating JAK2. Western blot analysis suggested inhibition of JAK2 activity suppressed GPR87 expression, and the phosphorylation level of STAT3 was reduced (Figure 5B). This finding suggested that GPR87 activated STAT3 activity through activating JAK2. Then we determined whether GPR87 interacted with JAK2, and immunoprecipitation (IP) analysis suggested that GPR87 interacted with JAK2 (Figure 5C). To determine which domain(s) of JAK2 interacted with GPR87, we constructed three truncated JAK2 fragments: JH1, JH2, and JH3-7, representing the three major functional regions of JAK2 (Figure 4D). We co-transfected hemagglutinin (HA)-tagged JH1-, JH2-, and JH3-7-overexpressing vector with FLAG-tagged GPR87 overexpressing vector into BXPC-3 cells, and IP using anti-FLAG antibody analysis suggested that GPR87 interacted with the JH3-7 region (Figure 5E). Together, GPR87 activated STAT3 through interacting with JAK2.

We further examined whether GPR87 promoted the expansion of PDA stem cells through activating JAK2, and we inhibited JAK2 activity either by inhibiting JAK2 expression or treating with JAK2 inhibitor AG490 in GPR87-overexpressing cells. As shown in Figures S2A–S2C and S2F, suppression of JAK2 activity in GPR87-overexpressing cells resulted in a marked reduction in sphere-forming ability. PDA stem cell markers expression analysis suggested that CD133, EpCAM, CD24, CD44, and MET were downregulated (Figure S2D), and SP population analysis suggested the percentage of the SP-positive population was also reduced when JAK2 activity was inhibited in GPR87-overexpressing cells (Figure S2E). Tumor
Figure 2. GPR87 Promotes the Expansion of PDA Stem Cells In Vitro

(A) Representative images showing sphere formation ability after GPR87 overexpression or knockdown.

(B) Sphere formation assay for GPR87 overexpression of knockdown after three consecutive passages.

(C) Sphere formation assay for the fold change in the number of cells per sphere after GPR87 overexpression or knockdown.

(D) SP population assay showing that overexpressing GPR87 promoted the SP cells, whereas silencing of GPR87 attenuated the SP cells in the indicated cells.

(E) qPCR analysis of the expression of PDA stem cell markers, including CD133, EpCAM, CD24, CD44, and MET, in the indicated cells. Error bars represent the means ± SD of three independent experiments. *p < 0.05.
initiating in vivo suggested 1,000 JAK2 activity inhibition cells with GPR87 overexpression failed to generate tumors, but 1,000 GPR87 overexpression cells still generate tumors (Figure S2F). These results suggested that GPR87 promoted the expansion of PDA stem cells through activating JAK2.

GPR87 Expression Positively Correlates with STAT3 and JAK2 Activation in PDA Tissues

Finally, we examined whether GPR87 expression was positively correlated with the phosphorylation of STAT3 and JAK2; as shown in Figures 6A and 6B, correlation studies showed that GPR87 expression positively correlated with the phosphorylation levels of JAK2 and STAT3 in PDA specimens (p < 0.001). These results were further confirmed in eight freshly collected PDA specimens, in which GPR87 expression positively correlated with the phosphorylation level of STAT3 (p = 0.002, r = 0.832) and JAK2 (p = 0.028, r = 0.718) and STAT3 transcriptional activity (p = 0.007, r = 0.829; Figures 6C and 6D). These results suggested that GPR87 expression positively correlated with STAT3 and JAK2 activation in PDA tissues, confirming that GPR87 promoted the expansion of PDA stem cells through forming a positive feedback loop with JAK2 and STAT3.

DISCUSSION

In the present study, we found that GPR87 was upregulated in PDA cells and tissues. It is a poor prognostic for PDA patients, and functional analysis suggested that GPR87 promoted the expansion of PDA stem cells. STAT3 could bind to the promoter of GPR87 to promote GPR87 expression, and previous studies demonstrate that STAT3 activity is regulated by JAK2. We also found that GPR87 increased STAT3 activity through interacting with JAK2. Inhibition of JAK2 in GPR87-overexpressing cells suppressed the expansion of PDA stem cells, suggesting that GPR87 promoted the expansion of PDA stem cells through activating JAK2. We confirmed this conclusion through detecting GPR87 expression and the phosphorylation levels of JAK2 and STAT3, and found GPR87 expression was positively correlated with JAK2 and STAT3 phosphorylation.

A previous report has suggested that GPR87 was upregulated in pancreatic cancer cells and tissues, and patients with high GPR87 expression had a short survival time. We also found that GPR87 was upregulated in PDA cells and tissues, and further confirmed that GPR87 was an independent prognostic factor for PDA patients. They have found that GPR87 promoted PDA cell proliferation, angiogenesis, and tumorigenesis, and increased the resistance to gemcitabine-induced apoptosis through activating the NF-kB pathway, but they do not study the detailed regulatory mechanism. In our study, we mainly focused on the role of GPR87 in PDA stem cells, and found that STAT3 directly bound to the promoter of GPR87 and promoted its expression. JAK2 is critical for STAT3 activation; JAK2 interacted with GPR87 to activate STAT3, and they formed a positive feedback pathway to promote the expansion of PDA stem cells (Figure 6E). Activated STAT3 is upregulated in PDA cells and
it can promote MMP7, Mcl-1, and other targets to initiate PDA and to promote PDA metastasis, suggesting that STAT3 plays a critical role in PDA stem cells expansion. GPR87 was a new target for STAT3, increasing the expansion of PDA stem cells. We also found that GPR87 not only activated STAT3, but also activated JAK2. JAK2 is critical for STAT3 activation, and inhibition of JAK2 in PDA reproduces phenotypes caused by inhibition of STAT3. Further analysis argued that GPR87 interacted with JAK2, and inhibition of JAK2 in GPR87-overexpressing PDA cells inhibited the expansion of PDA stem cells, confirming that GPR87 activated STAT3 through activating JAK2. These findings suggested that GPR87 was important for PDA therapy.

JAK2 promotes the expansion of many kinds of tumor; for example, increased JAK2 signaling activates adenosine deaminase ADAR1, and ADAR1 promotes leukemia stem cell (LSC) expansion through let-7 pri-microRNA editing and Lin28B upregulation. IL-6 activates the JAK2/STAT3 pathway to promote the expansion of liver CSCs, and long noncoding RNA (lncRNA) DILC inhibits liver CSC expansion through blocking the interaction of the IL-6 promoter and lncRNA DILC and then IL-6 autocrine. We found that GPR87 promotes the expansion of PDA stem cells through activating the JAK2 and STAT3 pathway, confirming that JAK2 also regulated CSCs in PDA.

Conventional cancer therapy kills only non-CSCs. Tumors still relapse easily; for example, antiangiogenic agents sunitinib and bevacizumab promote the expansion of breast CSCs through the generation of tumor hypoxia microenvironment, and patients treated with them easily relapse. With a combination of drugs killing non-CSCs with drugs targeting CSCs, tumors will eradicate. Now the effect of drugs for PDA therapy is poor, so developing new targets is urgent. GPR87 not only promoted proliferation and angiogenesis, but also promoted the expansion of PDA stem cells, suggesting it is a good target for PDA therapy. GPR87 has been found to regulate the growth and metastasis of CD133+ hepatocellular carcinoma stem cells, so it might also be a good target for other tumors.

In conclusion, we found a new target for PDA therapy: GPR87 promoted the expansion of PDA stem cells through interacting with JAK2, activating JAK2/STAT3.

**MATERIALS AND METHODS**

**Cell Culture**

The non-malignant human pancreatic cell line hTERT-HPNE and PDA cell lines Panc03.27, Capan-2, Capan-1, SW1990, HPAFII, Panc10.05, BXPC-3, and CFPAC-1 were obtained from ATCC (Manassas, VA, USA). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (HyClone).

**Tissue Specimens and Immunocytochemistry**

A total of 121 paraffin-embedded, archived PDA specimens collected between 2000 and 2006 were provided by the Affiliated Hospital of Guizhou Medical University. Samples were obtained from surgically
resected tumors in patients clinically and histopathologically diagnosed with PDA, and were confirmed by pathological review. This research was approved by the institutional research ethics committee of the Affiliated Hospital of Guizhou Medical University. The detailed clinicopathological characteristics were shown in Table S1. Immunocytochemistry (IHC) was performed according to previous reports.18

Western Blot and qPCR Analysis
Western blot analysis was performed using anti-GPR87 antibody (1:500, ab77517) from Abcam; anti-phosphorylated (p)-STAT3 (Tyr705, 1:2,000, #9145), anti-STAT3 (1:1,000, #12640), anti-p-JAK2 (Tyr1007-1008, 1:1,000, #3776), anti-JAK2 (1:1,000, #3230), and anti-p-Tyr-100 (1:2,000, #8954) antibodies from Cell Signaling Technology; and anti-FLAG (1:1,000, F7425) and anti-HA (1:2,000, H6908) antibodies from Sigma-Aldrich. Membranes were stripped and re-probed with an anti-β-Actin (1:1,000, SAB4301137; Sigma-Aldrich) or anti-GAPDH (1:1,000, SAB2701826; Sigma-Aldrich) antibody for loading controls. RNA was isolated using TRNZol Reagent (DP405; Tiangen), cDNA was synthesized using FastKing one-step RT-PCR Kit (KR123; Tiangen), and qPCR was performed using FastFire qPCR PreMix (SYBR Green, FP207; Tiangen) on a CFX 96 Touch (Bio-Rad).

Microarray Data Process and Visualization
Microarray data (GEO: GSE57495 and GSE57495) were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/).
Figure 6. Clinical Relevance of GPR87-Induced Activation of STAT3 in Human PDA

(A) The expression levels of p-STAT3 and p-JAK2 were associated with the expression of GPR87 in 121 primary human PDA specimens. Two representative cases are presented. (B) Percentage of samples showing low or high p-STAT3 (Tyr705) or p-JAK2 (Tyr1007-1008) expression in 121 primary human PDA specimens relative to the levels of GPR87. (C and D) Western blot (C) and correlation analyses (D) of GPR87 expression with the levels of p-STAT3, p-JAK2, and STAT3 transcriptional activity in eight freshly collected human PDA samples. β-Actin served as loading control. E) The schematic diagram of GPR87 regulating the expansion of PDA stem cells.
Microarray data extracts were performed on MeV 4.6 (https://sourceforge.net/projects/mev-tm4/files/mev-tm4/MeV%204.6/). GSEA was performed using GSEA 2.0.9 (https://www.broadinstitute.org/gsea/).

**Plasmids Construction**

The coding sequence of GPR87 was amplified using PCR and subcloned into pSin EF2 lentiviral vector for expression of recombinant FLAG-tagged GPR87. Truncated JAK2 fragments were also cloned into pSin EF2. Short hairpin RNAs (shRNAs) targeting GPR87 and JAK2 were cloned into the pSuper Retro viral vector. To generate luciferase reporters, we cloned 3× SREs into the pTAL-Luc vector. Transfection of short interfering RNAs (siRNAs) into the nontargeting FLAG-tagged GPR87. Truncated JAK2 fragments were also subcloned into pSin EF2 lentiviral vector for expression of recombinant FLAG-tagged GPR87.

**IP**

A total of 3 × 10^7 BXPC-3 cells transfected with designated plasmids were lysed in a buffer containing 150 mM NaCl, 10 mM HEPES (pH 7.4), and 1% Nonidet P-40 (NP-40). Lysates were cleared by centrifugation at 4°C at 12,000 rpm for 5 min. IP was performed using Anti-FLAG M2 Magnetic Beads (Sigma-Aldrich) at 4°C. Immunocomplexes were washed seven times with IP wash buffer (150 mM NaCl, 10 mM HEPES [pH 7.4], 0.1% NP-40), and eluted with 2 × 200 μL of 1 M glycine (pH 3.0). Samples were denatured and then resolved by SDS-PAGE.

**Sphere Formation Assays**

A total of 5 × 10^3 cells were seeded in six-well ultra-low cluster plates, whereas 10 or 20 cells were seeded in 24-well ultra-low cluster plates. Cells were cultured for 10 days in DMEM/F12 serum-free medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 20 ng/mL epidermal growth factor (EGF), 20 ng/mL basic fibroblast growth factor (bFGF; PeproTech), 0.4% BSA (Sigma-Aldrich), and 5 μg/mL insulin for sphere formation.

**Flow Cytometric Analysis**

Cells were trypsinized and resuspended at a concentration of 1 × 10^6 cells/mL in DMEM containing 2% FBS. Cells were then preincubated for 30 min at 37°C in the presence or absence of 100 μM verapamil (Sigma-Aldrich) to inhibit ABC transporters. Subsequently, cells were incubated for 90 min at 37°C with 5 μg/mL Hoechst 33342 (Sigma-Aldrich). This was followed by a 10-min incubation on ice and a wash with ice-cold PBS prior to flow cytometry analysis. Data were analyzed using Summit 5.2 software (Beckman Coulter).

**Tumor Xenografts**

SW1990 and BXPC-3 were transfected with luciferase-expressing vector and constructed a stable cell line. Nude mice were randomly assigned to six groups (n = 5 per group). An indicated number of cells expressing luciferase (1 × 10^3, 1 × 10^4, or 1 × 10^5) was inoculated subcutaneously into nude mice, and in vivo bioluminescence imaging was performed using Caliper IVIS imaging systems. The mice were sacrificed 31 days after inoculation, and the tumors were excised and subjected to pathologic examination. All experiments involving mice were conducted in accordance with standard operating procedures approved by the University Committee on the Use and Care of Animal at the Guizhou Medical University.

**ChIP**

A total of 1 × 10^7 SW190 cells were collected for ChIP assay, and ChIP was performed using Magna ChIP HiSens Chromatin Immunoprecipitation Kit (Millipore) according to the manufacturer’s protocol. The precipitated DNA specimens were analyzed using qPCR.

**Statistics**

All statistical analyses with the exception of microarray data analysis were performed using the SPSS 21.0 (IBM) statistical software package. To establish a correlation between GPR87 expression and clinicopathologic features of PDA, we used Pearson’s χ^2 test. Kaplan-Meier curves for both GPR87-high and GPR87-low patients were plotted, and statistical differences were compared using a log-rank test. Univariate and multivariable survival analyses were performed using Cox regression analysis. Student’s t test was performed for comparisons between two groups. Bivariate correlations between study variables were calculated by Pearson’s correlation coefficients. Data represent the means ± SD. A p value less than 0.05 was considered statistically significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2020.01.006.

**AUTHOR CONTRIBUTIONS**

J.J. and C.S. designed experiments. J.J., C.Y., X.G., H.Z., S.T., K.C., and Z.H. performed experiments and analyzed data. C.Y. and X.G. provided resources. J.J. and C.S. wrote the paper.

**CONFLICTS OF INTEREST**

The authors declare no competing interests.

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