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Original Article

Goosecoid promotes pancreatic adenocarcinoma metastasis through TGF-β signaling

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Article Info

Abstract

Goosecoid (GSC), translated from a homeobox gene, is a protein that participates in metastasis of various cancers. Pancreatic adenocarcinoma (PAAD) is one of the deadliest malignancies associated with a poor diagnosis and prognosis. To develop new treatment target or biomarker for PAAD, this study intended to assess the effects and the molecular mechanism of GSC on PAAD metastasis. The expressive discrepancy of GSC in PAAD and normal tissues/cells was compared by both the quantitative PCR and western blot. The effects of GSC silencing and GSC over-expression on PAAD cells and TGF-β signaling were proved by wound-healing assay, cell counting kit-8, Transwell assay and western blot. From the results, GSC mRNA and protein levels were enriched in PAAD cancer tissues and cells. GSC silencing prohibited metastasis of PAAD cells including the ability to invade, migrate and epithelial-mesenchymal transition (EMT), whereas GSC upregulation stimulated these cells behaviors above. GSC silencing reversed the effects on cellular processes induced by activation of the TGF-β pathway. Furthermore, silencing of GSC postponed tumor growth in xenograft model. In summary, GSC was abundantly expressed in PAAD, which activated the TGF-β pathway to enhance cell metastasis and tumor development.

Keywords: Cell metastasis, GSC, Pancreatic adenocarcinoma, Tumor development, TGF-β signaling.

1. Introduction

Pancreatic adenocarcinoma (PAAD) is a noteworthy malignant neoplasm with an overall dismal prognosis in the digestive system, it typically appears as an unexpected aggressive tumor behavior [1]. To date, it still contributes to the fourth global death toll related to cancer in males and females [2]. Recently, the incidence and death rate of PAAD have kept rising [3], causing heavy societal and public health burden worldwide. The poor clinical outcomes of pancreatic adenocarcinoma can be partly ascribed to the development of chemo-resistance and metastasis [4]. PAAD is treacherous and asymptomatic in its early stage because of the specific position of the pancreas, this concealed location causes delayed diagnosis until the metastasis stage [5]. Once diagnosed, it easily becomes advanced. The inadequacy of sensitive molecular targeted markers for pancreatic adenocarcinoma impedes early diagnosis. Therefore, focusing on the molecular mechanisms of PAAD metastasis and searching for new biomarker strategies will improve the prognosis and survival of patients with PAAD.

The goosecoid (GSC) gene, a conservative transcription factor, which initially recognized as the paired-like homeobox gene in the Spemann organizer. GSC is primarily expressed in the primitive streak and is crucial for the creation of gastrulation, endosperm, and mesendoderm morphogenesis [6]. GSC distinctly induces cell morphogenetic movement and encourages cell migration when ectopically expressed in the embryo of X.laevis [7], which implies GSC may be involved in cell adhesion, intercellular signaling and cell movement in human beings. GSC is related to a worse metastasis of hepatocellular carcinoma [8] and prostate cancer [9]. However, the relationship of GSC between pancreatic adenocarcinoma and the underlying mechanisms mastering the behavior of pancreatic adenocarcinoma is still unsettled. Based on the previous study on the close correlation between epithelial-mesenchymal transition (EMT) and the metastasis of pancreatic adenocarcinoma, we anticipated GSC may potentially activate the metastasis of pancreatic adenocarcinoma by EMT.

Transforming growth factor beta (TGF-β) signaling is widely demonstrated to be vital for various cancers based on the abnormal TGF-β enrichment microenvironment around tumor [10]. Furthermore, it has been revealed to

facilitate the proliferation, invasion and metastasis of pancreatic cancer [11]. GSC has close relations with TGF-β signaling, for TGF-β and its subsequent signaling are necessary for Spemann organizer formation and can increase GSC expression in embryonic cells [11]. TGF-β signaling promotes cancer and induces GSC expression in human cancer cells [12].

To date, the effects of GSC in pancreatic adenocarcinoma have not been completely explored. We aimed to investigate if GSC contributes to pancreatic adenocarcinoma on invasion and metastasis. We hypothesized that GSC can promote the invasion and metastasis of pancreatic adenocarcinoma by mediating the TGF-β signaling pathway. This study may offer a novel therapeutic target or prognosis biomarker for metastatic PAAD.

2. Materials and methods
2.1. Bioinformatic forecast
The differential expression of GSC in different pathological stages of PAAD, the differential gene expression of GSC in pancreatic tumor and non-tumor tissues, and the survival of PAAD patients with high or low GSC were predicted using the GEPIA database version 2.0 (http://gepia2.cancer-pku.cn/#index).

2.2. Tissue sample collection
A total of 33 patients with pancreatic adenocarcinoma from November 2017 to April 2020 were prospectively enrolled in this study. All subjects were eligible and diagnosed by pathological examination by Xi'an No.3 Hospital, the Affiliated Hospital of Northwest University. Written informed consents were all collected from subjects. Pair-matched tumor tissues and adjacent non-tumor tissues (size≥0.5cm×0.5cm×0.5cm) were obtained from surgery. The tissues were immediately frozen at -80°C until subsequent extraction.

2.3. Cell culture and transfection
The human PAAD cell lines (AsPC-1, CFAPC-1 and PANC-1) and normal cells hTERT-HPNE (American type culture collection) were cultured at Dulbecco's modified eagle medium (DMEM) containing 1% penicillin & streptomycin (Gibco) and 10% fetal bovine serum (Gibco) at 37°C with 5% CO_2 in a humidified incubator.

The down-expression and overexpression of GSC were achieved using inhibitors and expression vectors from Genepharma (Shanghai). Small interfering RNA targeting GSC (si-GSC) and si-negative control (NC) were constructed. To obtain the plasmid that expresses extra GSC in AsPC-1 and CFAPC-1 cells, the sequence of GSC or TGF-βRII was cloned into pEGFP-C1 (Clontech), while pEGFP-NC with low homology to any known sequence were constructed as negative control. All siRNA and vectors mentioned above were confirmed by sequencing.

siRNAs or pEGFP-C1 vectors mentioned above were transfected into AsPC-1 and CFAPC-1 cells ($4×10^6$) respectively with the aid of Lipofectamine 3000 (Invitrogen) for 48 hours. Cells were collected and re-suspended for the assay below.

2.4. Real-time quantitative PCR (qPCR)
With the TRIzol reagent (Yeasen), total RNAs were extracted from tissues or cells. Using the miScript II RT kit (Qiagen). Following RNA concentration and purity assay ($OD_{260/280}$ was between 1.8~2.0), 1 µg of RNA was reversely transcribed to complementary DNA. Using the Light-Cycle PCR system (Roche) and miScript SYBR Green PCR kit (Qiagen), RNA contents were evaluated by qRT-PCR. Primer sequences used in qRT-PCR were described previously [13]. Relative gene expression was normalized to GAPDH endogenous control.

2.5. Cell counting kit-8 (CCK-8)
CCK-8 kit (Dojindo) was utilized as a classical assay for cell viability. Following a 24-hour incubation, $1×10^3$ cells were planted onto 96-well plate and treated with the CCK-8 for 4 hours. The optical density was measured with a Bio-Rad microplate reader at 450 nm.

2.6. Cell invasion assay
24-well transwell chambers were used 24 hours after siRNA or pEGFP vector transfection, $2×10^5$ cells in FBS-free DMEM medium were seeded to the upper Transwell chambers (BD Biosciences), which precoated with 50µL Matrigel in advance. The solution in bottom chambers was DMEM adding 10% FBS. After being cultivated at 37°C for 24h,0.5% crystal violet, as chromogenic reagent, was cultivated with the cells adhered to the submembrane. The cell invasion rate was measured by the microplate reader (Bio-Rad) at 450 nm.

2.7. Cell invasion assay
Wound-healing was conducted to measure cell movements.$2×10^5$ of each cell were seeded in the well of a 6-well plate. Following 24-hour cultivation at 37°C in DMEM medium containing 10% FBS, straight line was scratched into the cell mono-layer. cultures were cultivated at 37°C for 48h after twice washing with DMEM medium. Under a microscope, the width of the wound was measured after the scratch, 24 and 48 hours later.

2.8. Western blot
RIPA lysis buffer (Beyotime) was used to extract the total cell lysate in AsPC-1 and CFAPC-1 cells. The protein concentration was measured using the BCA kit (Beyotime). 10% SDS-PAGE was used to separate about 25 µg protein. The electrophoretic bands were transferred to PVDF membranes (Membrane solutions). The membrane was incubated overnight at 4°C with primary antibodies as mouse anti-human GSC IgG (Abcam) 1:500, rabbit anti-human Vimentin, E-cadherin, N-cadherin, SMAD2, p-SMAD2, and β-actin (Cell Signaling Technologies). Then, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG F(ab′)2 antibody for 2h at 25°C. The electrophoretic bands were recorded using Gel Doc EZ Gel imaging system (Bio-Rad).

2.9. *In vivo* study
4-5 weeks nude mice (male, BALB/c; SLAC) were maintained in SPF environment. The xenograft tumor model for metastasis was generated by subcutaneously injecting $2×10^5$ AsPC-1 cells into the inguinal folds of nude mice. The mice were randomly divided into different groups and transfected with si-GSC and si-NC. The tumor volume was measured every week (tumor volume = $\frac{L×W^2}{2}$, where L is the tumor's largest diameter, and W is the lowest diameter). Four weeks later, the tumors were collected and weighed.

2.10. Statistical analysis

Data were acquired from experiments in triplicate and the averages were shown. GraphPad Prism 8.0 software was assisted in to analysis. Comparisons between tumors and adjacent non-tumor tissues were carried out by the Wilcoxon signed rank test, and comparisons between multiple groups were carried out by one-way ANOVA. A two-sided p-value of less than 0.01 was considered statistically significant.

3. Results
3.1. GSC is up-regulated in pancreatic adenocarcinoma

The GSC expression was predicted by GEPIA2. The data collected from 179 pancreatic cancer tissues and 171 normal tissues showed that GSC was up-regulated in pancreatic tumor tissues compared to the normal pancreatic tissues (Fig. 1A). The GSC expression increased in the late-stage of pancreatic adenocarcinoma (Fig. 1B). High GSC exhibit a poorer survival of pancreatic adenocarcinoma than that of low GSC from Kaplan–Meier survival prediction (Fig. 1C). We evaluated GSC relative mRNA and protein expression in 33 human tissues and confirmed that pancreatic cancer had elevated GSC expression compared to the normal tissues (Fig. 1D-E). The GSC mRNA and protein levels in the human pancreatic adenocarcinoma cells AsPC-1, CFAPC-1 and PANC-1 were all significantly higher than in the normal cell line hTERT-HPNE (Fig. 1F-G).

3.2. GSC overexpression promotes PAAD cell metastasis

Afterwards we explored the role of GSC in pancreatic adenocarcinoma cells AsPC-1 and CFAPC-1 by forced expression of GSC. The GSC levels were confirmed to increase both in AsPC-1 and CFAPC-1 cells with pEGFP-GSC compared to that with pEGFP-NC by PCR and western blot (Fig. 2A-B). CCK-8 assay showed that GSC overexpression increased cell viability of AsPC-1 and CFAPC-1 (Fig. 2C-D). GSC overexpression could promote both cell invasion, migration and wound healing of AsPC-1 and CFAPC-1 (Fig. 2E-F). Besides, GSC
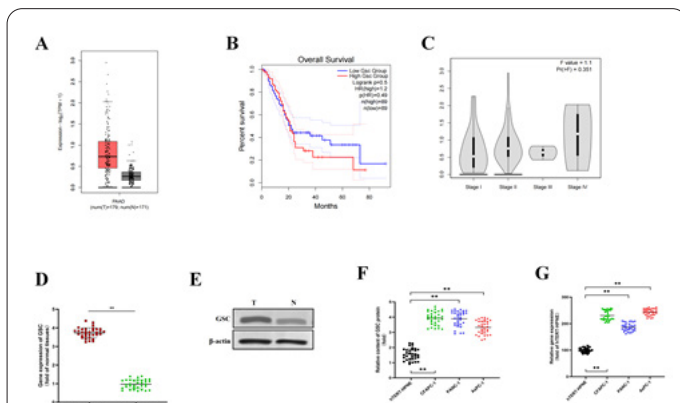

Fig. 1. GSC is up-regulated in pancreatic adenocarcinoma. (A) GEPIA prediction of GSC RNA content in PAAD and normal tissues. (B) GEPIA prediction of GSC expression in the early and late stage of PAAD. (C) Kaplan–Meier survival prediction of the relationship between GSC and PAAD prognosis. (D) qPCR and (E) western blot results of GSC level in tumor and adjacent non-tumor tissues from patients with pancreatic adenocarcinoma. (F) qPCR and (G) western blot results of GSC expression in normal cells (hTERT-HPNE) and tumor cells (AsPC-1, CFAPC-1 and PANC-1). **P<0.01.

overexpression resulted in increasing of EMT-associated markers N-cadherin and Vimentin levels and decreasing of E-cadherin (Fig. 2G). According to the results, GSC may play a key role in the cell metastasis of PAAD.

3.3. GSC knockdown suppresses the metastasis of PAAD cells

To investigate the effect of GSC in PAAD by ectopic and endogenous knockdown of GSC, si-GSC and si-NC were transfected into AsPC-1 and CFAPC-1 cells. The results of PCR and western blot tests verified that si-GSC satisfied the knockdown purpose (Fig. 3A-B). GSC knockdown
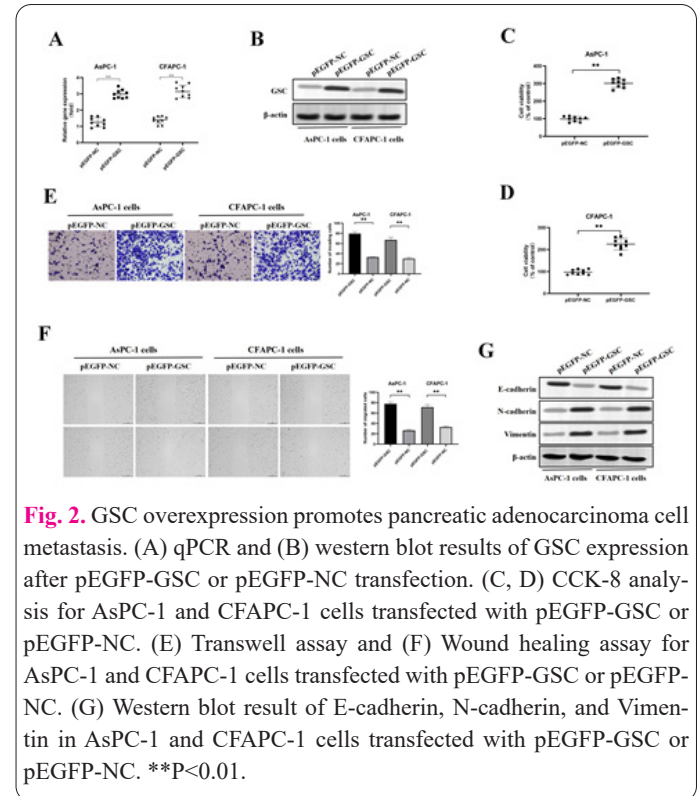
Fig. 2. GSC overexpression promotes pancreatic adenocarcinoma cell metastasis. (A) qPCR and (B) western blot results of GSC expression after pEGFP-GSC or pEGFP-NC transfection. (C, D) CCK-8 analysis for AsPC-1 and CFAPC-1 cells transfected with pEGFP-GSC or pEGFP-NC. (E) Transwell assay and (F) Wound healing assay for AsPC-1 and CFAPC-1 cells transfected with pEGFP-GSC or pEGFP-NC. (G) Western blot result of E-cadherin, N-cadherin, and Vimentin in AsPC-1 and CFAPC-1 cells transfected with pEGFP-GSC or pEGFP-NC. **P<0.01.
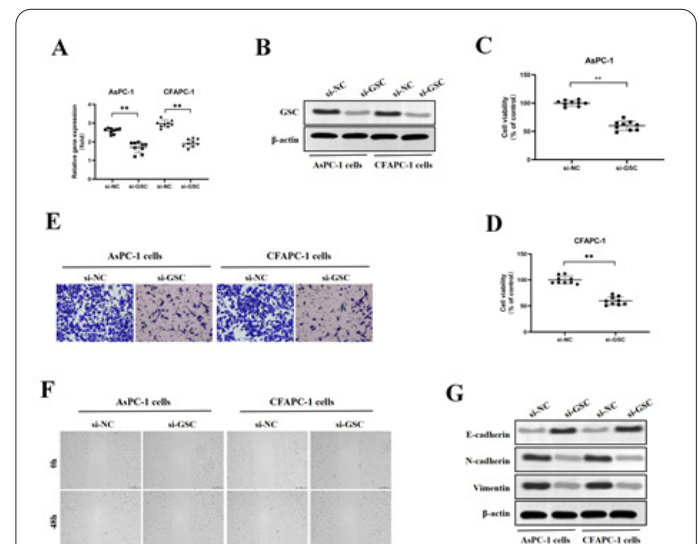
Fig. 3. GSC knockdown suppresses the metastasis of pancreatic adenocarcinoma cells. (A) qPCR and (B) western blot results of GSC expression after si-GSC or si-NC transfection. (C, D) CCK-8 analysis for AsPC-1 and CFAPC-1 cells transfected with si-GSC or si-NC. (E) Transwell assay and (F) Wound healing assay for AsPC-1 and CFAPC-1 cells transfected with si-GSC or si-NC. (G) Western blot of E-cadherin, N-cadherin, and Vimentin in AsPC-1 and CFAPC-1 cells transfected with si-GSC or si-NC. **P<0.01.

reduced cell viability of AsPC-1 and CFAPC-1 (Fig. 3C-D). Furthermore, GSC silencing prevented cell invasion, migration and wound healing of AsPC-1 and CFAPC-1 (Fig. 3E-F). GSC downregulation resulted in decreased levels of Vimentin and N-cadherin and increased amounts of E-cadherin protein (Fig. 3G).

3.4. GSC activates the TGF-β pathway in PAAD cells and promotes the metastasis of PAAD via the TGF-β signaling

After confirming the effect of GSC on PAAD cell lines by overexpression and knockdown of GSC, we anticipated this effect was mediated by the activation of the TGF-β pathway. We artificially induced TβRII expression in AsPC-1 and CFAPC-1 cells (Fig. 4A). Knockdown of GSC decreased the protein levels of p-SMAD2 compared the negative control, while rescued the increasing effect by TβRII overexpression, and knockdown of GSC did not affect SMAD2 (Fig. 4A). In addition, GSC overexpression augmented the production of of p-SMAD2 and did not affect SMAD2 levels (Fig. 4B).

TβRII overexpression in AsPC-1 and CFAPC-1 cells exhibited more cell viability compared to cells with pEGFP-NC (Fig. 4C-D). Furthermore, GSC silencing rescued the increase of cell viability by TβRII overexpression. The upregulation of cell invasion, migration and wound healing induced by TβRII overexpression were partly abolished by GSC silencing (Fig. 4E-F). The results of western blot indicated that GSC downregulation alleviated the increasing effect on Vimentin and N-cadherin, and also alleviated the attenuation effect on E-cadherin protein by TβRII overexpression (Fig. 4G).

### 3.5. GSC Silencing inhibits PAAD growth *in vivo*

A PAAD xenograft mice model was established to verify the ability of GSC *in vivo*. The GSC level in tumor tissues decreased compared to negative control in xenografts (Fig. 5A-B). GSC Knockdown significantly inhibited tumor growth (Fig. 5C-E).

4. Discussion

Despite the rapid breaking-thorough in clinical technology, PAAD is associated with a poor diagnosis and prognosis among malignant tumors. Given that the 2020-year diagnosis rate of PAAD (11.0/100000) approximates the death rate (13.2/100000) from SEER Statistics, PAAD is still one of the deadliest malignancies. Novel tactics and better understanding of PAAD are in urgent need.

Rising research has indicated that GSC plays a critical role in multiple cancer progression. GSC has been reported to correlate with metastasis of hepatocellular carcinoma [14]. Moreover, GSC could regulate the progression of prostate cancer [15]. GSC could potentially guide ovarian serous carcinoma treatment by indicating drug response and poor prognosis [16]. But, regrettably, the relationship between GSC and PAAD is unclear. We forecasted that high levels of GSC exhibit a markedly poorer survival of pancreatic adenocarcinoma based on the bioinformatic analysis by GEPIA. We experimentally verified the hypothesis on clinical tissues and cell levels, the results both showed that GSC is up-regulated in pancreatic cancer, which suggested that GSC could be the candidate biomarker of diagnosis and poor prognosis in PAAD.

Then we focused on whether and how GSC affected
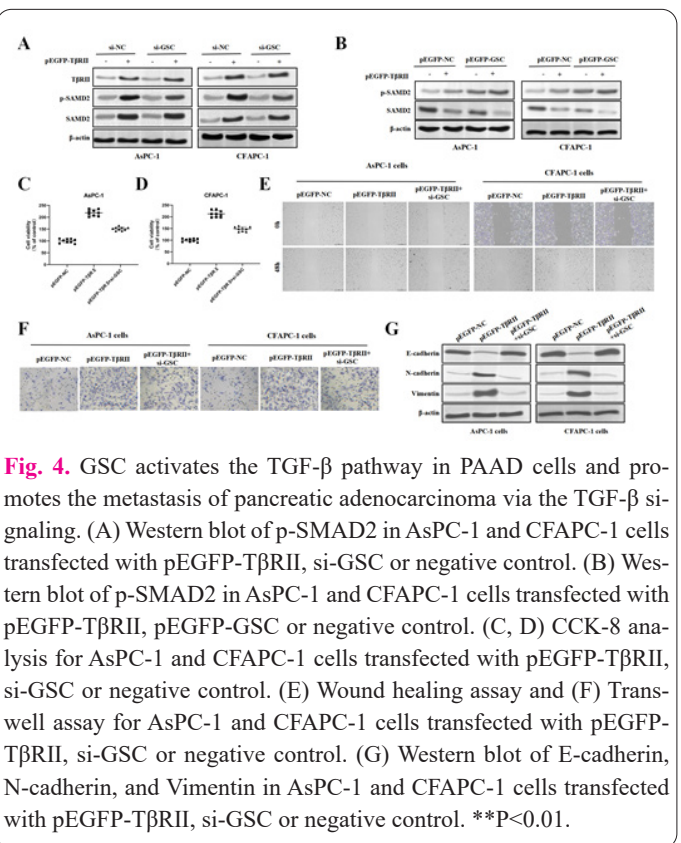
Fig. 4. GSC activates the TGF-β pathway in PAAD cells and promotes the metastasis of pancreatic adenocarcinoma via the TGF-β signaling. (A) Western blot of p-SMAD2 in AsPC-1 and CFAPC-1 cells transfected with pEGFP-TβRII, si-GSC or negative control. (B) Western blot of p-SMAD2 in AsPC-1 and CFAPC-1 cells transfected with pEGFP-TβRII, pEGFP-GSC or negative control. (C, D) CCK-8 analysis for AsPC-1 and CFAPC-1 cells transfected with pEGFP-TβRII, si-GSC or negative control. (E) Wound healing assay and (F) Transwell assay for AsPC-1 and CFAPC-1 cells transfected with pEGFP-TβRII, si-GSC or negative control. (G) Western blot of E-cadherin, N-cadherin, and Vimentin in AsPC-1 and CFAPC-1 cells transfected with pEGFP-TβRII, si-GSC or negative control. **P<0.01.
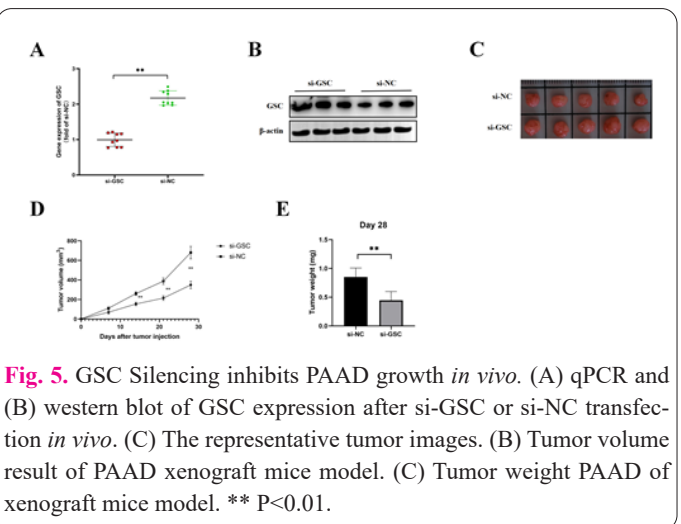
Fig. 5. GSC Silencing inhibits PAAD growth *in vivo*. (A) qPCR and (B) western blot of GSC expression after si-GSC or si-NC transfection *in vivo*. (C) The representative tumor images. (B) Tumor volume result of PAAD xenograft mice model. (C) Tumor weight PAAD of xenograft mice model. ** P<0.01.

PAAD metastasis. GSC silencing managed to suppress the proliferation, migration and invasion of PAAD at cellular levels, whereas GSC overexpression stimulated this cellular progression conversely. GSC silencing significantly inhibited tumor growth in xenograft PAAD model, suggesting that GSC, as conserved organizer genes, may function cancer-promoting similarly in human bodies. Collectively, the results described here strongly support that GSC is an oncogene in PAAD, offering a better understanding of GSC and the pathogenesis mechanisms responsible for PAAD.

The EMT is the key mechanism for invasion and metastasis of PAAD, which frequently results in cancer-related deaths [16]. We observed significant suppression of EMT by GSC in PAAD cells by up-regulating Vimentin and N-cadherin, and down-regulating E-cadherin. Those three cytoskeletal proteins are identified at the heart of EMT-mediated metastasis [17]. Echoing previous study [14], GSC may serve as a potential transcription factor

with the metastatic capability for EMT.

The TGF-β pathway is widely considered a therapeutic target in the regulation of tumor cell survival, migration, apoptosis and EMT [18, 19], including pancreatic adenocarcinoma [20]. TGF-β activators currently serve as anti-cancer therapeutic targets [21]. Therefore, exploring new targets related to the TGF-β signaling is of significance. TGF-β signaling can be commonly triggered by the interaction of TGF-β ligand and TGF-β type II serine/threonine receptor (TGFBR2), which phosphorylates the cytoplasmic effectors SMAD2 [22]. The transcription of TGF-β target genes is regulated by phosphorylated SMAD2. In this study, we demonstrated that GSC activated the TGF-β pathway by phosphorylating SMAD2.GSC silencing could reverse the effects of activation of TGF-β pathway on cell migration, invasion and EMT. Besides, GSC downregulation alleviated the effects on Vimentin, N-cadherin and E-cadherin by TβRII overexpression, suggesting GSC knockdown diminished the maintenance of the TGF-β induced EMT. Thus, the evidence supported the conclusion that GSC promoted the metastasis of pancreatic adenocarcinoma via the TGF-β pathway. Our study outlined GSC has a hopeful prospect as a treatment target or prognosis biomarker for pancreatic adenocarcinoma.

To date, our understanding of the mechanisms of GSC driving PAAD metastasis is limited. The major insufficiency is that we only explore the effects of GSC on phosphorylation of SMAD2. The molecule mechanisms of GSCs to interact with TGF-β pathway and other regulators will be our further research direction, such as TGF-β related protein ubiquitination or combination.

5. Conclusion

In summary, GSC was abundantly expressed in PAAD, which activated the TGF-β pathway to enhance cell metastasis *in vitro* and tumor development *in vivo*. According to the results, GSC may be a new target for pancreatic cancer therapy.

Conflict of interests
The authors declare no competing interests.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
We have received approval from the Ethics Committee of Northwest University and Xi'an No.3 Hospital, the Affiliated Hospital of Northwest University.

Informed consent
We have received informed consent from the Ethics Committee of Northwest University and Xi'an No.3 Hospital, the Affiliated Hospital of Northwest University.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions
MY and LR contributed to the study conception and design. Experimental operation, data collection and analysis were performed by MY, JW, CW, LZ, XZ and GS. The first draft of the manuscript was written by MY and all authors commented on previous versions of the manuscript.

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