LncRNA STXBP5-AS1 suppresses stem cell-like properties of pancreatic cancer by epigenetically inhibiting neighboring androglobin gene expression

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Abstract

Previous studies suggest the tumor suppressor role of long non-coding RNA (lncRNA) STXBP5-AS1 in cervical and gastric cancer, but its expression pattern and functional mechanism are still elusive in pancreatic cancer (PC). Relative expression of STXBP5-AS1 in PC both in vivo and in vitro was analyzed by real-time PCR. IC50 of Gemcitabine was determined by the MTT assay. Cell proliferation in response to drug treatment was investigated by colony formation assay. Cell apoptosis was measured by both caspase-3 activity and Annexin V/PI staining. Cell invasion capacity was scored by the transwell assay in vitro, and lung metastasis was examined with the tail vein injection assay. Cell stemness was determined in vitro by sphere formation and marker profiling, respectively, and in vivo by limited dilution of xenograft tumor incidence. Subcellular localization of STXBP5-AS1 was analyzed with fractionation PCR. Association between STXBP5-AS1 and EZH2 was investigated by RNA-immunoprecipitation. The binding of EZH2 on ADGB promoter was analyzed by chromatin immunoprecipitation. The methylation was quantified by bisulfite sequencing. We showed downregulation of STXBP5-AS1 in PC associated with poor prognosis. Ectopic STXBP5-AS1 inhibited chemoresistance and metastasis of PC cells. In addition, STXBP5-AS1 compromised stemness of PC cells. Mechanistically, STXBP5-AS1 potently recruited EZH2 and epigenetically regulated neighboring ADGB transcription, which predominantly mediated the inhibitory effects of STXBP5-AS1 on stem cell-like properties of PC cells. Our study highlights the importance of the STXBP5-EZH2-ADGB axis in chemoresistance and stem cell-like properties of PC.

Keywords: Long non-coding RNA, STXBP5-AS1, ADGB, Pancreatic cancer

Background

Pancreatic cancer (PC) is one of the most lethal human malignancies with poor prognosis [1]. There are 227,000 deaths claimed by this disease every year globally. Currently, surgical removal and chemotherapy are still the mainstay of clinical management options [2]. However, due to the lack of evident symptom and reliable early diagnosis, PC is frequently diagnosed at late and untreatable stage, which greatly contributes to the relatively unfavorable prognosis [3, 4]. On the other hand, assembled studies suggest the existence of specific
subpopulation in pancreatic tumor cells with characteristic features of self-renewal, differentiation and capability of driving tumor incidence and metastasis [5–7]. More importantly, the stem cell-like tumor cells are deemed as the major cause of resistance to both conventional chemotherapy and radiotherapy [8–10]. Therefore, insightful understanding into pathogenesis of PC and characterization of tumor stem cells involved in this disease is extremely critical in the search for early diagnostic marker and therapeutic targets.

Long non-coding RNA (lncRNA) is a class of RNA molecules with an average length of more than 200 nt and no protein coding potential [11]. Increasing evidences support the fundamental roles of lncRNA in multiple aspects of tumor biology in almost all human cancer types, including cell differentiation, proliferation, apoptosis, metastasis and cell stemness [12–15]. More recent investigations suggest the association between lncRNAs and PTEN via sponging miR-96-5p to reduce cervical cancer cell proliferation and invasion [16–18]. Here, we focused on a novel lncRNA, STXBPs-AS1, in PC, which was previously investigated in cervical and gastric cancers. Huang et al. first reported that STXBPs-AS1 suppressed cell proliferation, invasion and migration through blockading the PI3K/AKT pathway, which was predominantly mediated by negative regulation on STXBPs expression in non-small-cell lung carcinoma [19]. Subsequently, Cen et al. confirmed the involvement of STXBPs-AS1/PI3K/AKT in tumor suppressive effects in gastric cancer [20]. While in cervical cancer, Shao et al. suggested that STXBPs-AS1 functioned as a competing endogenous RNA to upregulate PTEN via sponging miR-96-5p to reduce cervical cancer cell proliferation and invasion [21]. Notably, Ham et al. found that both ginsenoside Rg3 and Korean red ginseng extracts were capable of epigenetically regulating STXBPs expression [22], therefore providing experimental evidences in support of the targetability of STXBPs by traditional Chinese medicine. Our study evidenced the anti-tumoral properties of STXBPs-AS1 in PC, suppression of which rendered drug resistance and stem cell-like features to PC cells. We further demonstrated the epigenetic regulation of ADGB by STXBPs-AS1 via interacting with and potently recruiting EZH2. Therefore, our data highlighted the critical contributions of STXBPs-AS1 in PC.

Materials and methods
Clinical samples
A total of 60 PC tumors with paired adjacent normal tissues were collected from Fujian Provincial Hospital, Shengli Clinical Medical College of Fujian Medical University, Fujian Medical University. Written consents were obtained from all enrolled patients, and approval from the Institutional Ethics Committee was received before initiation of this study. Diagnosis was confirmed by independent pathologists, and the specimens were immediately flash-frozen in liquid nitrogen.

Cell culture and treatment
PC cell lines (AsPC-1, SW1990, Capan-2, CFPAC-1, PANC-1 and Mia PaCa-2) and the normal human pancreatic ductal cell line hTERT-HPNE were ordered from the American Type Culture Collection (ATCC, VA, USA). All cancer cells were maintained in RPMI-1640 (Sigma, MO, USA) containing 10% fetal bovine serum (Invitrogen, CA, USA) and 1% antibiotics (penicillin–streptomycin, Hyclone, MA, USA). The hTERT-HPNE cells were cultured following the ATCC recommendation in 75% glucose-free DMEM (supplemented with L-glutamine and sodium bicarbonate, Sigma, MO, USA) and 25% M3 Base Medium (Incell, CA, USA). 5-Aza-CdR was purchased from Sigma (St. Louis, MO, USA), and cells were treated with the optimal concentration of 5-Aza-CdR (1 µM) for 72 h. Regular cell culture was performed in humidified CO2 (5%) incubator at 37 °C.

Gene overexpression and knockdown
STXBPs-AS1 and ADGB overexpression cell lines were established by infecting cells with lentivirus containing the STXBPs-AS1 (pSIN-STXBPs-AS1) and ADGB sequences (pSIN-ADGB), followed by puromycin selection to acquire stable overexpression cells. STXBPs-AS1 and EZH2 knockdown was achieved by transfecting siRNAs of the following sequences using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s instructions:

- si-STXBPs-AS1-1: GCAAGTTTGGTACGTATTAT.
- si-STXBPs-AS1-2: GGAATTTCTTTCTCCACAT.
- si-EZH2-1: GTTGAATGTCCTTGTTCAATA.
- si-EZH2-2: GAGCCTATTCTCGGTGTCA.

Real-time PCR
RNA was extracted with the TRIzol Reagent (Invitrogen, MA, USA) in accordance with the manufacturer’s manual. cDNA synthesis was conducted with 1 µg of RNA with cDNA Synthesis Kit (Takara, Ohtsu, Japan). Relative mRNA was quantified with SYBR Premix Ex Taq (TakaRa) on Applied Biosystems 7900 PCR System (Applied Biosystems, CA, USA). The quantification of gene level was calculated by the 2−ΔΔCT method, using GAPDH as the internal reference gene. The primer sequences were listed as below:

- STXBPs-AS1 F: 5′-AGGAATTTGCTCAGTGTTCGCTTGAT-3′
STXBPS-AS1  R:  5′-GAGATTAGGTGGGACGCTGC-3′;
GAPDH  F:  5′-ACGGATTTGCTGTATTGGGCG-3′;
GAPDH  R:  5′-GCTCTGGAGATGGTGGATGGG-3′;
Sox2  F:  5′-TGACCGCTAGCAGCTGAGC-3′;
Sox2  R:  5′-GCCCTGGAGTGGGAAGAAAGA-3′;
Bmi1  F:  5′-GCTTCAAGATGCCGGTTG-3′;
Bmi1  R:  5′-TTTCGTTGTGATGCATTTC-3′;
Lin28  F:  5′-AAAGGAGACAGGTGCTAC-3′;
Lin28  R:  5′-ATATGGCTGTGCCTGG-3′;
Nanog  F:  5′-AGTGGACAGGGAGATGGC-3′;
Nanog  R:  5′-AACCTTCTGGCTTACACG-3′;
ADGB  F:  5′-AGACCCCTCAGAAGTGACAG-3′;
ADGB  R:  5′-GCTTACAGGAGACAGACTCT-3′.

Sphera formation assay
400 cells were seeded into 6-well plate, and 50 cells were seeded into 24-well plate, followed by continuous culture for 10 days. Spheres were maintained in serum-free DMEM/F12 medium containing 2% B27 (Invitrogen, MA, USA) plus EGF (20 ng/ml), bFGF (20 ng/ml) and insulin (5 μg/ml from PeproTech, NJ, USA).

Cell viability and apoptosis
The indicated cells were prepared in 96-well plate (10³ cells/well) and treated with serial concentrations of Gemcitabine. After 48 h, cell viability was monitored by the MTT assay, and IC₅₀ value of Gemcitabine was determined with SPSS 23. To measure cell apoptosis, the indicated cells were treated with 100 ng/ml of Gemcitabine. After 48 h, single-cell suspension was prepared and stained with Annexin V-FITC-PI Apoptosis Detection Kit (Sigma, MO, USA) as suggested by the provider, and followed by FACS analysis on CytoFlex (Beckman Coulter, CA, USA).

Colony formation assay
Well-dispersed single cells were seeded into 6-well plate (500 cells/well) and subjected to drug treatment for 48 h at 37°C. Fresh medium was then replaced, followed by consecutive culture for another 10 days. Colonies were fixed with 3% formaldehyde briefly and stained with 0.5% crystal violet for 15 min (Sigma, MO, USA).

Transwell assay
Invasion capacity was assessed using the transwell chamber which was pre-coated with 1% Matrigel (BD Biosciences, CA, USA). Cells (10⁵/well) were seeded into insert and cultured in serum-free medium. The lower compartment was supplied with complete medium as chemo-attractant. After 12 h, the non-invaded cells were washed off and invaded cells were fixed with cold-methanol and stained with 0.25% crystal violet.

Lung colonization model
PANC-1 cells (either vector control or STXBPS-AS1-overexpressing) were prepared into single-cell suspension in PBS (1 × 10⁶ cells/ml), and i.v. injected into the lateral tail vein. After 21 days, all subject mice were sacrificed and lung macro-metastasis was examined with H&E staining. The animal study was approved by the Institutional Animal Care and Use Committee and in strict accordance with the NIH guideline.

Western blot
Cells were lysed in RIPA buffer on ice, and protein concentration was quantified by the BCA method (Sigma, MO, USA). 20 μg protein was resolved by SDS-PAGE and transferred onto PVDF membrane (Millipore, MA, USA). After brief blocking with 5% milk, the membrane was probed with primary antibodies: rabbit anti-Sox2 (#ab2748, Cell Signaling Technology, MA, USA), rabbit anti-Bmi1 (#ab6964, Cell Signaling Technology, MA, USA), rabbit anti-Lin28 (#3695, Cell Signaling Technology, MA, USA), rabbit anti-Nanog (#8822, Cell Signaling Technology, MA, USA), rabbit anti-ADGB (ab204085, Abcam, Cambridge, UK), rabbit anti-β-actin (#4970, Cell Signaling Technology, MA, USA) at 4°C overnight. After washing, membranes were hybridized with secondary antibodies for another hour. The blots were detected with ECL Kit (APPLYGEN, Beijing, China) and visualized on LI-COR system (Biosciences, Lincoln, NE, USA).

Xenograft tumor model
To evaluate the tumorigenic capacity, PANC-1 cells (control or STXBPS-AS1-overexpression, 2 × 10³, 2 × 10⁴, 2 × 10⁵, 2 × 10⁶, 2 × 10⁷ cells) were subcutaneously injected into nude mice (n=8 for each group). Tumor progression was continuously monitored for up to 2 weeks. All mice were then sacrificed, and xenograft tumor formation was validated by pathological examination.

Subcellular localization
PARIS Kit (Life Technologies, Carlsbad, CA, USA) was employed to fractionize cell nuclear and cytosol RNA. RNA was extracted and reversely transcribed as previously described. The relative distribution of STXBPS-AS1 was measured by real-time PCR. GAPDH and U6 were employed as reference for cytosol and nuclear localization, respectively.

RNA immunoprecipitation (RIP)
RIP assay was used to evaluate binding between STXBPS-AS1 and EZH2. The assay was performed with
the EZ-Magna RIP Kit (Millipore, MA, USA) following the manufacturer’s manual. Anti-EZH2 antibody and control IgG were obtained from Abcam. The immunoprecipitated RNA was recovered and further analyzed by qRT-PCR as previously described.

Chromatin immunoprecipitation (ChIP)
ChIP was conducted using EZ-Magna ChIP Chromatin Immunoprecipitation Kit (Millipore, MA, USA) according to the manufacturer’s recommendation. Chromatin cross-linked with 37% formaldehyde was ultrasonicated to generate DNA fragments with the average length of 500 to 1000 bp. The DNA species were precipitated with EZH2 antibody and recovered, which was further detected and quantified by qRT-PCR.

Bisulfite sequencing PCR (BSP)
DNA methylation of ADGB promoter was measured with commercially available kits. Genomic DNA from indicated cells was extracted with the DNeasy® Blood and Tissue Kit (Qiagen, CA, USA), which was followed by bisulfate modification. Bisulfite sequencing was then performed with EpiTect® Bisulfite Kit (Qiagen, CA, USA) in accordance with the provider’s instructions.

Statistical analysis
Data are reported as mean ± standard deviation (SD). The inter-group comparison was analyzed using Student’s t test or one-way ANOVA analysis with a post hoc test using SPSS 23.0. P < 0.05 was regarded as statistically significant.

Results
Decreased STXBPS-AS1 predicted poor prognosis in PC.
First of all, we analyzed a group of 60 PC patients, whose clinical–pathological features are listed in Additional file 1: Table S1. Analysis into these equally grouped patient samples, with respect to STXBPS-AS1 transcript levels, manifested favorable both overall and relapse-free survival linking to relatively high STXBPS-AS1 (Fig. 1a, b). Next, STXBPS-AS1 expression in lymph node metastasis (LNM) patients and lymph node metastasis-free (LNMF) patients were significantly decreased compared to respective adjacent normal tissues (ANT) (Fig. 1c). Furthermore, data from GEO datasets showed significant decreased STXBPS-AS1 in PC as compared to the benign tissues as well (Fig. 1d, GSE16515; Fig. 1e, GSE15471). We then determined the relative expression of STXBPS-AS1 in a panel of PC cells and found significant downregulation in all tested cancer cell lines in comparison with normal pancreatic duct cell line Htert-HPNE (Fig. 1f). The suppressive expression of STXBPS-AS1 was also noticed in Gemcitabine-resistant (GR) cells compared to parental ones in both PANC-1 and Mia PaCa-2 cell lines (Fig. 1g). The lower abundance of STXBPS-AS1 was especially characterized in sphere derived from these two cells (Fig. 1h), which might imply a possible correlation between down-regulated STXBPS-AS1 and cell stemness. Therefore, our data suggested that downregulation of STXBPS-AS1 in PC might be mechanistically associated with GR and tumor cell stemness, as well as poorer clinical outcome.

STXBPS-AS1 inhibited chemoresistance and metastasis of PC cells.
Next, we complemented GR cell lines derived from both PANC-1 and Mia PaCa-2 cells with ectopic STXBPS-AS1 (Fig. 2a). Drug resistance to Gemcitabine was significantly compromised by STXBPS-AS1 as indicated by reduction of IC50 value of Gemcitabine (Fig. 2b). The colony formation capacity of PANC-1/GR and Mia PaCa-2/GR cells was greatly inhibited by ectopic STXBPS6-AS1 (Fig. 2c). Concurrently, activation of capase-3 in response to Gemcitabine exposure was tremendously augmented by over-expression of STXBPS-AS1 in both GR cells (Fig. 2d). Correspondingly, remarkably increased cell apoptosis was observed STXBPS-AS1-proficient cells in comparison with parental ones upon treatment with 50 ng/ml Gemcitabine (Fig. 2e). In addition to GR cells, we generated STXBPS-AS1-overexpressing cells in naïve PANC-1 and Mia PaCa-2 cells as well (Fig. 2f). Cell invasion was evidently inhibited by ectopic STXBPS-AS1 in both PANC-1 and Mia PaCa-2 cells (Fig. 2g). More importantly, we provided evidences in support of the metastasis-inhibiting effect of STXBPS-AS1 in PANC-1 lung metastasis model. STXBPS-AS1-proficiency greatly suppressed lung metastatic loci establishment of tail vein-injected PANC-1 cells, as shown in the representative H&E staining of lung sections (Fig. 2h). Statistics suggested that lung metastasis occurred in 7 out 8 of vector control mice, while absent in only 1 out 8 STXBPS-AS1-complemented mice (Fig. 2i). Our data showed that STXBPS-AS1 significantly improved chemosensitivity of GR cells, whereas it greatly blockaded metastasis of naïve PC cells.

STXBPS-AS1 suppressed stemness of PC cells
Our preliminary data showed downregulation of STXBPS-AS1 in PC cell-derived spheres, which hinted the potential causal relation between STXBPS-AS1 and tumor cell stemness. To further clarify this issue, we over-expressed STXBPS-AS1 in both PANC-1 and Mia PaCa-2 cells, and examined the influence of STXBPS-AS1 on sphere formation. As shown in Fig. 3a, the sphere formation capacity was greatly compromised by STXBPS-AS1 in both cells. Molecular profiling of cell stemness
markers including Sox2, Bmi1, Lin28 and Nanog demonstrated remarkable reduction of all of four markers in response to STXBP5-AS1 overexpression in both PANC-1 and Mia PaCa-2 cells at the transcriptional level (Fig. 3b, c). The suppressed expression of Sox2, Bmi1, Lin28 and Nanog was validated at protein level by Western blot analysis (Fig. 3d). Most importantly, compromised stemness by STXBP5-AS1 was demonstrated by limiting dilution assay of xenograft tumor incidence. We noticed that STXBP5-AS1-proficiency greatly inhibited the incidence of PANC-1 cell-derived xenograft tumor, while total injected cell number was limited to $2 \times 10^6$ and less (Fig. 3e). Taken together, our data supported the suppressive effects of STXBP5-AS1 on stemness of PC cells both in vitro and in vivo.

**STXBP5-AS1 epigenetically regulated neighboring gene ADGB transcription by binding to EZH2**

Next, we sought to understand the molecular mechanism underlying the tumor-suppressor role of STXBP5-AS1 in
PC. We focused on the neighboring genes in view of the well-recognized mode of action of lncRNA in regulating adjacent genes. We found ADGB was greatly inhibited by STXBPS-AS1 in both PANC-1 and Mia PaCa-2 cells (Fig. 4a). In contrast, transcripts of ADGB were markedly up-regulated in STXBPS-AS1-depleted cells (Fig. 4b). The regulatory effects of STXBPS-AS1 on ADGB were further confirmed by Western blot analysis (Fig. 4c). To gain further insight into the regulatory mechanism, we then examined the subcellular localization of STXBPS-AS1 transcripts via fractionation PCR analysis. As suggested by Fig. 4d, e, the majority of STXBPS-AS1 from both PANC-1 and Mia PaCa-2 cells existed in the nuclear fraction with a minor proportion detectable in the cytoplasm, which indicated that STXBPS-AS1 exerted physiological roles predominantly in the nucleus. Multiple lncRNAs have been previously identified to be involved in complex with EZH2 and therefore in regulation of promoter methylation of target genes. Along this direction, we detected the enrichment of STXBPS-AS1 transcripts in EZH2 immunoprecipitated RNA species in both PANC-1 and Mia PaCa-2 cells (Fig. 4f). Meanwhile, direct association of EZH2 with ADGB promoter was demonstrated by ChIP assay as shown in Fig. 4g, which implicated the role of PRC2 complex in the epigenetic regulation of ADGB. The relative enrichment of ADGB promoter was significantly decreased by siRNA-mediated knockdown of STXBPS-AS1 in comparison with scramble control (Fig. 4h, i). The association of EZH2 with ADGB promoter therefore was greatly dependent on STXBPS-AS1. Consistently, over-expression of STXBPS-AS1 increased the enrichment of ADGB promoter in EZH2 immunoprecipitated complex, which suggested an enhancement of EZH2 binding to ADGB promoter (Fig. 4j, k). Consequently, methylation level of ADGB promoter region was tremendously decreased in response to STXBPS-AS1 knockdown, which was comparable with treatment by the DNA demethylating agent 5-Aza-CdR (Fig. 4l). STXBPS-AS1 overexpression oppositely increased methylation status of ADGB promoter, which was readily abrogated by simultaneous EZH2-knockdown or 5-Aza-CdR treatment (Fig. 4m). Summarily, we provided evidence that STXBPS-AS1 potently inhibited expression of neighboring ADGB via an epigenetic mechanism, specifically through complexation with EZH2.

STXBPS-AS1 inhibited stem cell-like properties of PC cells by suppressing ADGB expression

Next, we sought to clarify whether down-regulated ADGB mainly mediated the inhibitory effects of STXBPS-AS1 on cell stemness in PC cells. To this end, we established STXBPS-AS1-overexpressing and ADGB-overexpressing cells either individually or in combination in parental PANC-1 and Mia PaCa-2 cells (Fig. 5a, b), as well as in the respective GR cells (Fig. 5c, d). Consistent with previous observation, forced expression of STXBPS-AS1 greatly improved the sensitivity of GR cells, which was almost completely reversed by co-expression of ADGB (Fig. 5e). Likewise, colony formation capacity was compromized in response to ectopic STXBPS-AS1 in both PANC-1/GR and Mia PaCa-2/GR cells, while restored by complementation of ADGB (Fig. 5f). Caspase-3 activation and apoptotic index, which were greatly stimulated by introduction of STXBPS-AS1, were inhibited by ADGB overexpression as well (Fig. 5g, h). The invasive capacity compromised by STXBPS-AS1 was greatly recovered by simultaneous expression of ADGB (Fig. 5i). Similarly, ADGB complementation evidently restored the colony formation in STXBPS-AS1-proficient cells (Fig. 5j). At the molecular level, co-overexpression of ADGB in the context of ectopic STXBPS-AS1 expression up-regulated Sox2, Bim1, Lin28 and Nanog, which was significantly inhibited by STXBPS-AS1-overexpression alone (Fig. 5k, l). This change was also validated at the protein level by Western blot analysis (Fig. 5m). Therefore, our results supported that STXBPS-AS1 decreased the stem cell-like properties of PC cells mainly by epigenetic suppression of ADGB.
Discussion

Despite previously reported tumor suppressor roles in cervical cancer, gastric cancer and non-small-cell lung cancer \[19, 21\], the relative expression pattern and functional mechanism of STXBP5-AS1 in PC were still obscure currently. Here, we first characterized aberrant downregulation of STXBP5-AS1 in PC both in vitro and in vivo. Particularly, a potential association was observed between STXBP5-AS1 deficiency and cell stemness and drug resistance. In addition, high expression of STXBP5-AS1 was significantly enriched in the PC patients without LNM, and consequently associated with both overall and relapse-free survival clinically. In cell culture, overexpression of STXBP5-AS1 rendered
GR cells sensitivity to Gemcitabine and greatly inhibited the colony formation capacity, which was accompanied with caspase-3 activation and cell apoptosis induction. In parental PC cells, the invasive behavior was suppressed by ectopic STXBP5-AS1. This phenotype was validated in vivo with tail vein injection of both empty control and STXBP5-AS1-overexpressing PANC-1 cells into nude mice as well. Notably, we observed the sphere formation efficacy was significantly compromised by STXBP5-AS1 with concurrent down-regulation of stemness markers including Sox2, Bmi1, Lin28 and Nanog. The suppressed stem cell-like properties were especially validated by the limiting dilution of xenograft tumor incidence. Mechanistically, we showed
STXBP5-AS1 epigenetically inhibited neighboring ADGB expression through potently recruiting EZH2 to the ADGB promoter and therefore enhancing DNA methylation. Complementation with ADGB remarkably restored drug resistance and colony formation in STXBP5-AS1-proficient GR cells, and simultaneously suppressed apoptotic activation. In parental PC cells, ADGB overexpression rescued the compromised cell invasion and decreased cell stemness markers. In summary, we provided experimental data supporting the tumor suppressor function of STXBP5-AS1 in PC via inhibiting chemoresistance and stem cell-like...
STXBP5-AS1 inhibited stem cell-like properties of PC cells by suppressing ADGB expression. a, b The expression level of ADGB in PANC-1 and Mia PaCa-2 cells co-transfected with two empty pSin vector (pSin-vector + pSin-Ctr), STXBP5-AS1 overexpression plasmid and an empty pSin vector (pSin-STXBP5-AS1 + pSin-Ctr) or STXBP5-AS1 overexpression plasmid and ADGB overexpression plasmid (pSin-STXBP5-AS1 + pSin-ADGB) was determined by qRT-PCR and western blot. c, d The expression level of ADGB in PANC-1/GR and Mia PaCa-2/GR cells co-transfected with two empty pSin vector (pSin-vector + pSin-Ctr), STXBP5-AS1 overexpression plasmid and an empty pSin vector (pSin-STXBP5-AS1 + pSin-Ctr) or STXBP5-AS1 overexpression plasmid and ADGB overexpression plasmid (pSin-STXBP5-AS1 + pSin-ADGB) was determined by qRT-PCR and western blot. e MTT assay showed that overexpressing ADGB rescued the decreased Gemcitabine IC50 in PANC-1/GR and Mia PaCa-2/GR cells due to forced expression of STXBP5-AS1. f Colony formation assay showed overexpressing ADGB rescued the decreased cell survival of Gemcitabine-challenged (50 ng/ml) PANC-1/GR and Mia PaCa-2/GR cells due to forced expression of STXBP5-AS1. g, h Caspase-3 activity assay and FACS indicated that overexpressing ADGB rescued the increased apoptosis of Gemcitabine-challenged (50 ng/ml) PANC-1/GR and Mia PaCa-2/GR cells due to forced expression of STXBP5-AS1. i Transwell assay showed overexpressing ADGB rescued the decreased cell invasion of PANC-1 and Mia PaCa-2 cells due to forced expression of STXBP5-AS1. j Tumor sphere formation assay showed overexpressing ADGB rescued the decreased spheres of PANC-1 and Mia PaCa-2 cells due to forced expression of STXBP5-AS1. k-m qRT-PCR and western blot indicated that overexpressing ADGB rescued the decreased expression levels of stem markers (Sox2, Bmi1, Lin28 and Nanog) in PANC-1 and Mia PaCa-2 cells due to forced expression of STXBP5-AS1. The data represent the mean ± SD from three independent experiments. *P < 0.05; **P < 0.01, Student’s t test.
properties, which was greatly mediated by epigenetic silencing of its neighboring ADGB.

Emerging evidences have suggested the involvement of lncRNAs in complexation with EZH2 and exerted epigenetic regulation in human cancers. Our data confirmed this notion via demonstrating the direct interaction between STXBPS-AS1 and EZH2, which specified recruitment of EZH2 to the ADGB promoter and led to intensive DNA methylation and gene expression silencing. Our observations resembled multiple scenarios previously described. For instance, Liu et al. reported that LINC01088 enhanced cell proliferation through scaffolding EZH2 and inhibiting p21 in human non-small-cell lung cancer [23]. Song et al. showed that LINC01535 induced cervical cancer progression by specific targeting the miR-214/EZH2 feedback loop [24]. In colorectal cancer, Di et al. demonstrated that SNG14 facilitated distal metastasis via regulating EZH2-targeted EPHA1 [25]. Xu et al. suggested FOXD2-AS1 functioned as an oncogene in hepatocellular carcinoma by epigenetic suppression of CDKN1B (p27) with EZH2 [26]. Although we exhibited the direct association between STXBPS-AS1 with EZH2, the detailed complexation was still to be elaborated by structural analysis.

ADGB (androglobin) was firstly described as a chimeric globin in metazoans and preferentially expressed in mammalian testis with poorly understood physiologic functions [27]. Until recently, the study performed by Huang et al. proposed that ADGB knockdown in glioma cell lines significantly inhibited cell proliferation and stimulated apoptosis [28], suggesting the potential onco-genic properties of ADGB in this disease. Consistent with this work, our data exhibited that overexpression of ADGB in STXBPS-AS1-proficient GR cells greatly stimulated drug resistance and inhibited cell apoptosis, while rendered stem cell-like properties and invasive potential to parental PC cells in the context of STXBPS-AS1-overexpression. Notably, the intensive methylation was detected in the promoter region of ADGB indicated the possible efficacy of DNA demethylating agents for therapeutic purpose, which is definitely worthy of further investigations in the near future. In view of the critical contributions of ADGB to drug resistance of PC, we also raised the hypothesis that combinational administration of both Gemcitabine and 5-Aza-CdR would greatly benefit the intrinsic resistant patients clinically.

Noteworthily, despite the significant downregulation of STXBPS-AS1 characterized here in PC along with previous reports in both cervical and gastric cancers, the molecular mechanisms underlying this phenotype were still elusive currently. Another study limitation is that not all the clinical information was available in the public databases; therefore, the association of STXBPS-AS1 with survival cannot be analyzed. In the future, we would focus on this issue with the aid of bioinformatic analysis of publicly available tumor genome databases. In summary, we demonstrated that the STXBPS-AS1/EZH2/1 axis in PC contributed to the chemoresistance and stem cell-like features.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13148-020-00961-y.

Additional file 1: Table S1. Correlation of STXBPS-AS1 expression with clinicopathological features in 60 pancreatic cancer patients.

Abbreviations
LncRNA: Long non-coding RNA; PC: Pancreatic cancer; GR: Gemcitabine-resistant; RIP: RNA-immunoprecipitation; ChIP: Chromatin immunoprecipitation.

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None.

Authors’ contributions
Shi Chen, Long Huang, Ge Li, Funan Qiu, Yaodong Wang, Can Yang, Jingjing Pan, Zhangwei Wu conducted the experiments. Shi Chen, Jiagzhi Chen and Yifeng Tian prepared the main text with help of Long Huang, Ge Li, Funan Qiu and Yaodong Wang. Can Yang, Jingjing Pan and Zhangwei Wu prepared the figures. Shi Chen, Jiagzhi Chen and Yifeng Tian wrote the manuscript. All authors contributed to the content. All authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Ethics approval and consent to participate
The study was approved by the ethics commitment of Fujian Provincial Hospital, Shengli Clinical Medical College of Fujian Medical University, Fujian Medical University.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

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