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Hedgehog inhibitor decreases chemosensitivity to 5-fluorouracil and gemcitabine under hypoxic conditions in pancreatic cancer

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Pancreatic cancer is one of the deadliest types of cancer. Previously, we showed that hypoxia increases invasiveness through upregulation of *Smoothened (Smo)* transcription in pancreatic ductal adenocarcinoma (PDAC) cells. Here, we first evaluated whether hypoxia-induced increase in *Smo* contributes to the proliferation of PDAC cells. We showed that Smo, but not Gli1, inhibition decreases proliferation significantly under hypoxic conditions. To further investigate the effects of Smo on PDAC growth, cell cycle analysis was carried out. Inhibition of Smo under hypoxia led to G_0/G_1 arrest and decreased S phase. As 5-fluorouracil (5-FU) and gemcitabine, which are first-line drugs for pancreatic cancer, are sensitive to S phase, we then evaluated whether cyclopamine-induced decreased S phase under hypoxia affected the chemosensitivity of 5-FU and gemcitabine in PDAC cells. Cyclopamine treatment under hypoxia significantly decreased chemosensitivity to 5-FU and gemcitabine under hypoxia in both *in vitro* and *in vivo* models. In contrast, cis-diamminedichloroplatinum, which is cell cycle-independent, showed significant synergistic effects. These results suggest that hypoxia-induced increase of Smo directly contributes to the proliferation of PDAC cells through a hedgehog/Gli1-independent pathway, and that decreased S phase due to the use of Smo inhibitor under hypoxia leads to chemoresistance in S phase-sensitive anticancer drugs. Our results could be very important clinically because a clinical trial using Smo inhibitors and chemotherapy drugs will begin in the near future. (*Cancer Sci* 2012; 103: 1272–1279)

Pancreatic cancer is one of the deadliest types of cancer, with an overall 5-year survival rate of <5% when all stages are combined.[1] One reason for its lethality is that chemotherapy is largely ineffective. The exact molecular mechanisms responsible for this dismal clinical course are unclear. Better understanding of the mechanisms that underlie the development of pancreatic cancer could identify novel molecular targets for treatment.

The Hh signaling pathway is crucial to growth and patterning in a wide variety of tissues during embryonic development, including the pancreas.[2] Recent studies have reported an association between Hh pathway activation and initiation of human tumors.[3] Of the Hh inhibitors, many Smo inhibitors, including cyclopamine, IPI-926, GDC-0499, LDE225, BMS-833923, XL-139, PF-0449913, SANT 74-75, and SANT 1-4 have been developed. Some of these drugs are under clinical study,[4–11] however, combination therapies with Smo inhibitors and other chemotherapy drugs have not been fully evaluated.

Tumor hypoxia is found in regions that are distant from the supporting tumor vasculature.[12] Understanding this hypoxic microenvironment is important for therapeutic approaches for pancreatic cancer. Recently, we showed that hypoxia mediates *Smo* transcription in PDAC cells.[13] This discovery led to the next question: how does upregulation of *Smo* under hypoxic conditions affect the proliferation and chemosensitivity in PDAC cells? The negative impact of hypoxia on the efficacy of chemotherapy has been known for several decades. However, the molecular basis of hypoxia-mediated chemotherapy failure has only recently been reported. In these studies, a contribution of HIFs to drug resistance has been observed in a wide spectrum of neoplastic cells.[14–18] Many signaling pathways, including PI3k, MAPK, HER2, tyrosine kinase, EGFR, and COX2, are reported to induce chemoresistance through HIF-1 activity.[19–25]

Olive *et al.*[26] showed that inhibition of Hh signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. However, in the present study, we show, for the first time, that Smo inhibitor induces G_0/G_1 arrest with decreased S phase in PDAC cells under hypoxic conditions, and that cyclopamine-induced decrease in S phase may lead to impaired chemosensitivity. We should be cautious about combination therapy with cyclopamine and 5-FU or gemcitabine in the future clinical use in pancreatic cancer.

Materials and Methods

Cell culture and reagents. Two human pancreatic ductal cell lines, ASPC-1 and SUIT-2, were maintained in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FCS (Life Technologies, Grand Island, NY, USA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). For normoxic conditions, cells were cultured in 5% CO_2 and 95% air. For hypoxic conditions, cells were cultured in 1% O_2, 5% CO_2, and 94% N_2 using a multigas incubator (Sanyo, Tokyo, Japan). Cells were counted by a Coulter counter (Beckman Coulter, Fullerton, CA, USA). 5-Fluorouracil, gemcitabine, and CDDP were purchased from Kyowa Hakko Kirin (Tokyo, Japan), Eli Lilly (Indianapolis, IN, USA), and Bristol Myers Squibb (New York, NY, USA), respectively. Cyclopamine, purchased from Toronto Research Chemicals (Toronto, Canada), was diluted in 99% ethanol.

Flow cytometry. Cells (3.0×10^5 cells/well) were plated in 6-well plates and treated with appropriate combinations of drugs. Adherent and detached cells were harvested by trypsinization, and fixed in ice-cold 75% ethanol for at least 1 h. Cell pellets were washed twice with cold PBS and incubated for 30 min at room temperature in 1 mL PBS containing 50 μg propidium iodide (Sigma-Aldrich, St. Louis, MO, USA), 0.1% Triton X-100, 1 mM/L EDTA, and 0.5 mg ribonuclease A (Sigma-Aldrich). After staining, samples were analyzed using FACScan (BD Biosciences, San Jose, CA, USA) at 20 000 events per sample. Data from flow cytometry were analyzed

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with the ModFit LT (Verity Software House, Topsham, ME, USA) and CellQuest (BD Biosciences) software programs. Fragmented apoptotic nuclei were recognizable by their sub-diploid (sub-G_1) DNA content. The percentage of sub-G_1 cells was recorded for each sample.

RNA interference. Small interfering RNA for Smo (On-Targetplus Smart pool, L-005726), siRNA for Gli1 (On-Targetplus Smart pool, L-003896), siRNA for HIF-1α (On-Targetplus Smart pool, L-004018), and negative control siRNA (On-Targetplus siControl non-targeting pool, D-001810) were purchased from Dharmacon RNA Technologies (Chicago, IL, USA). Cells (0.2×10^6 cells/well) seeded in 6-well plates were transfected with 100 nM siRNA using Lipofectamine RNAiMax Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were used for experiments 2 days after transfection.

Immunoblotting. Whole-cell extraction was carried out with M-PER Reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Protein concentration was determined with Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA), and whole-cell extract (50 μg) was separated by electrophoresis on SDS-polyacrylamide gel, and transferred to Protran nitrocellulose membranes (Whatman, Dassel, Germany). Blots were then incubated with anti-cyclin D1 (1:200, SC-450; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p21 (1:200; BD Biosciences), anti-CDK4 (1:200, SC-601; Santa Cruz Biotechnology), and anti-α-tubulin (1:1000; Sigma-Aldrich) primary antibodies overnight at 4°C. Blots were then incubated in HRP-linked secondary antibody (Amersham Biosciences, Piscataway, NJ, USA) at room temperature for 1 h. Immunocomplexes were detected with ECL plus Western Blotting Detection System (Amersham Biosciences) and visualized with a Molecular Imager FX (Bio-Rad). α-Tubulin was used as a protein loading control.

Fluorescence immunohistochemistry. Slides were deparaffinized with xylene and rehydrated with alcohol. Antigen retrieval was achieved by microwaving in Target Retrieval Solution (pH 6.0; Dako, Tokyo, Japan) for 10 min. The sections were rinsed with PBS and blocked using skim milk (Megmilk Snow Brand Co. Ltd, Tokyo, Japan) for 10 min at room temperature. Sections were incubated with anti-CA-9 (carbonic anhydrase-IX, 1:200; Novus Biologicals, Littleton, CO, USA) and anti-cyclin D1 antibodies (1:200, SC-450; Santa Cruz Biotechnology) at 4°C overnight. Primary antibodies were then visualized by incubating cells with Alexa 488 conjugated chicken anti-rabbit (1:1000; Invitrogen) and Alexa 594 conjugated goat anti-mouse (1:1000; Invitrogen) for 1 h at 37°C. After incubation with secondary antibodies, sections were rinsed three times with PBS. The cells were counterstained with DAPI (Sigma-Aldrich) then mounted by VectaShield (Vector

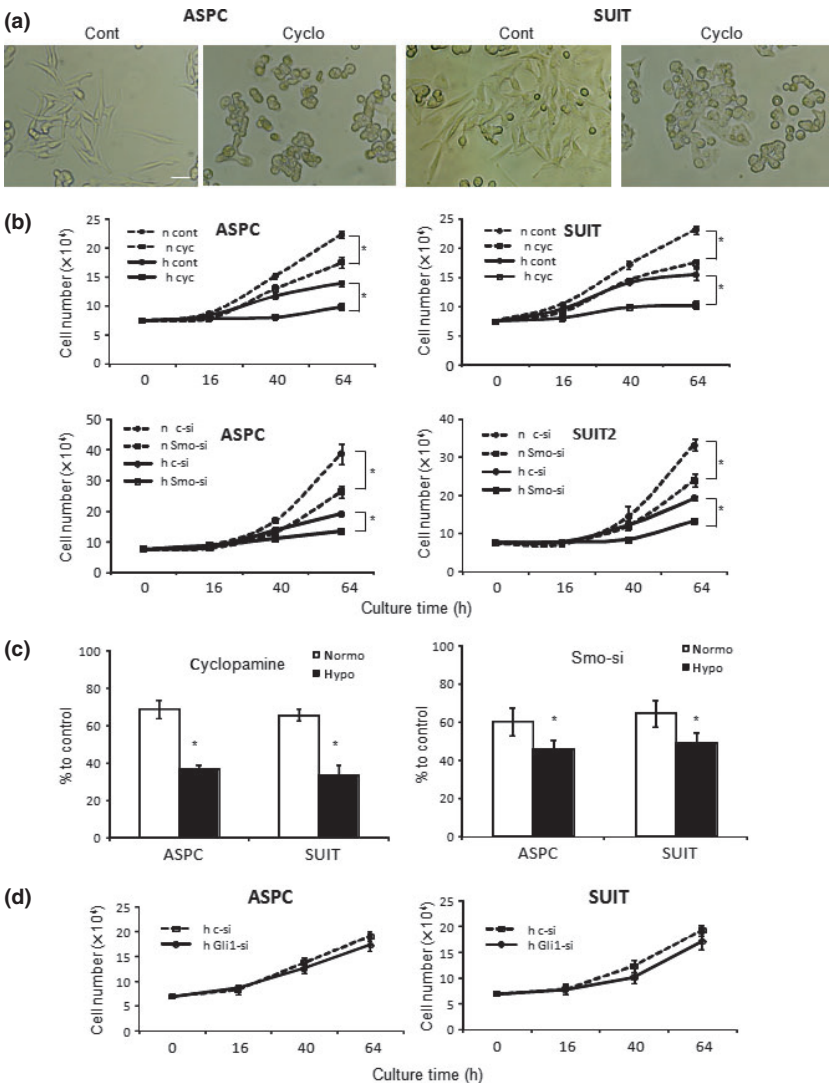

Fig. 1. Smooothened (Smo) is required for proliferation of pancreatic cancer cells. (a) Representative pictures of cell morphology in ASPC-1 (ASPC) and SUIT-2 (SUIT) pancreatic ductal adenocarcinoma (PDAC) cells 24 h after treatment with 10 μM cyclopamine (cyclo) under hypoxia. Bar = 20 μm. (b) Cyclopamine-treated (10 μM) PDAC cells (cyc) and *Smo* siRNA-transfected PDAC cells (7×10^4/well) (Smo-si) were plated in 6-well plates under normoxic (n) and hypoxic (h) conditions. After the indicated culture period, cells were harvested and cell numbers were counted by Coulter counter. (c) Proliferation rates under normoxic (normo) and hypoxic (hypo) conditions were examined after 64 h culture. Bar shows mean ± SD. $*P < 0.05$. (d) *Gli1* siRNA-transfected PDAC cells (7×10^4/well) (Gli1-si) were plated in 6-well plates. After the indicated culture period, cells were harvested and counted by Coulter counter. Bar shows mean ± SD. Cont, control; c-si, control si-RNA-transfected cells. $*P < 0.05$.

Laboratories, Burlingame, CA, USA). The samples were examined by fluorescence microscopy (Carl Zeiss, Tokyo, Japan). Detection of apoptotic cells in tumor from the mouse xenograft model was carried out using an *In situ* Apoptosis Detection Kit (Takara Bio, Tokyo, Japan).

Xenograft tumor experiments. For the tumor experiments, 1×10^6 ASPC-1 cells in 50 μL RPMI medium were injected s.c. into four BALB/c female nude mice (4–6 weeks old) in each group. All animals were obtained from Charles River Laboratory (Wilmington, MA, USA) and maintained in standard conditions according to institutional guidelines. These animal experiments were approved by the Ethics Committee in Kyushu University (Fukuoka, Japan) (Inspection No. A23-051-3). Primary tumor size was measured every 2 days with calipers; approximate tumor weights were determined using the formula $0.5ab^2$, where b is the smaller of the two perpendicular diameters. After the tumors had grown to an average size of 110 mm^3, mice with tumors were injected s.c. with 0.1 mL vehicle (triolein : ethanol, 4:1) or cyclopamine (triolein : cyclopamine; 1 mg/body) once a week (on days 0 and 8). From 3 days after cyclopamine was first given, injections of 5-FU (30 mg/kg) or equivalent normal saline by i.p. injection was carried out three times a week. All animals were killed 5 weeks after tumor cell injection.

Statistical analysis. Student's *t*-test was used for statistical analysis. $P < 0.05$ was considered significant.

Results

Increase of Smo under hypoxic conditions is required for proliferation of pancreatic cancer. We proposed that hypoxia upregulates Smo transcription, and that it leads to increased invasiveness of PDAC cells under hypoxic conditions.[13] These observations led to the next question: does upregulation of Smo affect the proliferation of PDAC cells under hypoxia? We used cyclopamine to suppress Smo. Cyclopamine-treated cells were round in shape, whereas control cells showed spindle-like shapes in both cell lines (Fig. 1a). Cyclopamine-treated cells were dose-dependently less proliferative than control cells in hypoxic conditions in the two cell lines (Fig. S1). We chose 10 μM cyclopamine concentration in this experiment. Cyclopamine-treated cells showed significantly lower

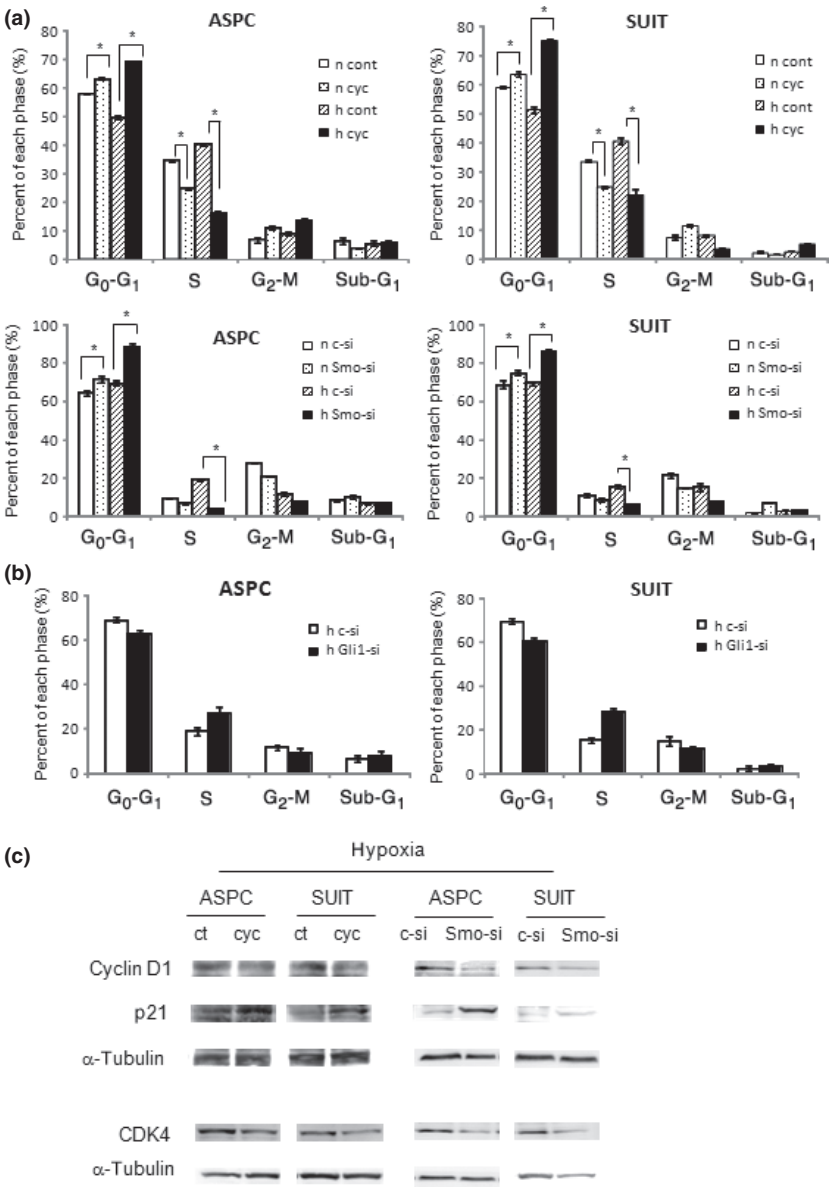

Fig. 2. Cell cycle arrest at G_0/G_1 with the decrease of S phase is induced by Smoothened (Smo) inhibition under hypoxic conditions in ASPC-1 (ASPC) and SUIT-2 (SUIT) pancreatic ductal adenocarcinoma (PDAC) cells. (a) Cell cycle analysis in cyclopamine-treated (10 μM) (cyc) PDAC cells or *Smo* siRNA-transfected PDAC cells (Smo-si) was carried out under normoxia (n) and hypoxia (h) by FACS. cont, control. (b) Cell cycle analysis in *Gli1* siRNA-transfected PDAC cells (Gli1-si) was carried out under normoxia (n) and hypoxia (h) by FACS. The graph shows the mean ± SD. $*P < 0.05$. (c) Cyclin D1, CDK4, and p21 expressions in cyclopamine-treated PDAC cells or *Smo* siRNA-transfected PDAC cells under hypoxia were estimated by Western blot. α-Tubulin was used as the loading control. c-si, control si-RNA-transfected cells; ct, control.

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proliferative ability than control cells under normoxic and hypoxic conditions (Fig. 1b, upper panels). To evaluate the Smo inhibitory effect using another Smo inhibitor, PDAC cells were transfected with siRNA targeting Smo. Transfection of siRNA targeting Smo significantly reduced mRNA expression of Smo by 70% or more on day 2 (data not shown). PDAC cells transfected with Smo siRNA also had significantly lower proliferative ability than control cells under normoxic and hypoxic conditions in both cell lines (Fig. 1b, lower panels). Next, the proliferation rate was determined by calculating the increased cell numbers in cyclopamine-treated or Smo siRNA-transfected cells/increased cell number in control cells after 64 h culture. The proliferation rate under hypoxic conditions was significantly lower than that under normoxic conditions (Fig. 1c). In contrast, transfection of Gli1 siRNA, which resulted in 70% knockdown of Gli1, did not affect the proliferation of PDAC cells (Fig. 1d). These results suggest that upregulation of Smo under hypoxic conditions significantly affects the proliferative activity of PDAC cells through a Hh–Gli1-independent pathway.

Cell cycle arrest at G_0/G_1 and decreased S phase is induced by Smo inhibition under hypoxic conditions. Next, to analyze the mechanism of the significant inhibitory effect of proliferation in Smo-inhibited PDAC cells under hypoxic conditions, we investigated the cell cycle of Smo-inhibited PDAC cells in both normoxic and hypoxic conditions. Cyclopamine treatment under hypoxic conditions showed a significant increase of cells in G_0/G_1 phase (approximately 20%) and fewer cells in S phase (20–30%) in ASPC-1 and SUIT-2 cell lines compared with control. In contrast, cyclopamine-treated cells under

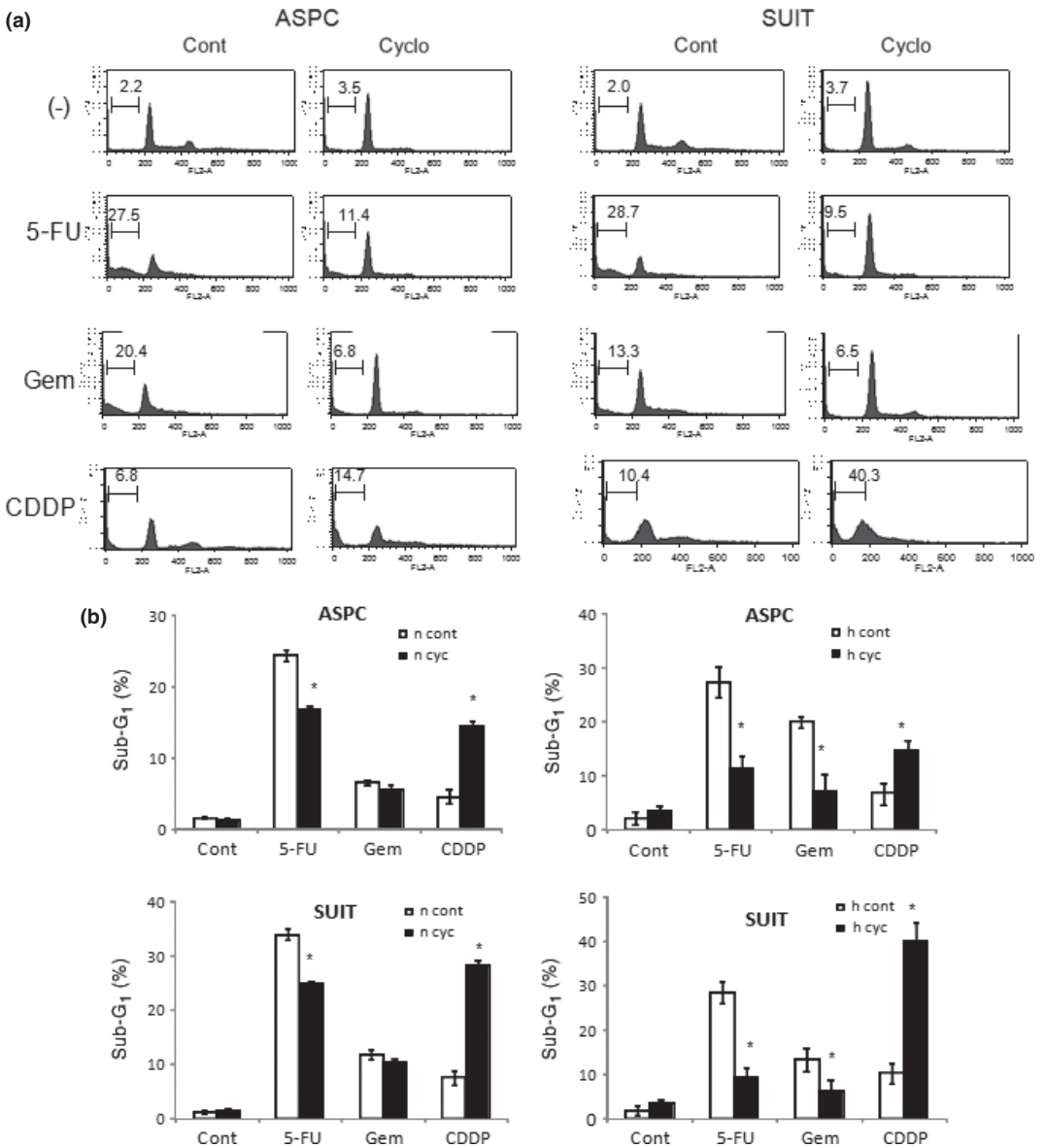

Fig. 3. Cyclopamine treatment under hypoxic conditions inhibits chemosensitivity in pancreatic ductal adenocarcinoma cell lines ASPC-1 (ASPC) and SUIT-2 (SUIT). Twenty-four hours after treatment with 10 μM cyclopamine (cyclo), 100 μg/mL 5-fluorouracil (5-FU), 100 μg/mL gemcitabine (Gem), and 500 μM cis-diamminedichloroplatinum (CDDP) was added to the culture. After an additional 48 h, cells were harvested and the sub-G_1 population was estimated by FACS. (a) Histogram shows representative results in cells treated with 5-FU, Gem, and CDDP under hypoxic conditions. The value in each upper left corner shows the mean percentage of sub-G1 population. (b) Summary of the experiment under normoxic (n) and hypoxic (h) conditions showing the mean ± SD. cont, control. *$P < 0.05$.

normoxic conditions revealed only a 5% increase of cells in G_0/G_1 phase and a 10% decrease of cells in S phase (Fig. 2a, upper panels). Cells transfected with Smo siRNA also showed a significant increase of cells in G_0/G_1 phase (approximately 20%) and significantly fewer cells in S phase (10–15%) under hypoxic conditions in both cell lines compared with control, however, those cells under normoxic conditions revealed only a 5% increase in G_0/G_1 phase and no change in S phase (Fig. 2a, lower panels). Transfection of Gli1 siRNA did not induce G_0/G_1 arrest or a decreased S-phase population under hypoxic conditions (Fig. 2b). To confirm the cell cycle results, we analyzed cell cycle-related proteins cyclin D1, CDK4, and p21. Consistent with Fig. 2(a), cyclin D1 and CDK4 decreased and p21 increased in Smo-inhibited PDAC cells under hypoxic conditions in both cell lines (Fig. 2c). These results suggest that G_0/G_1 arrest by Smo-inhibited PDAC cells may lead to the significant suppression of proliferation under hypoxic conditions.

Cyclopamine treatment inhibits chemosensitivity in PDAC cells under hypoxia. Fluorouracil is commonly used in pancreatic cancer and is principally effective on cancer cells in S phase. We estimated how the decreased S phase that was induced by Smo inhibition under hypoxic conditions affects chemosensitivity. In this experiment, we used the sub-G_1 population as an index of apoptotic cells. Cyclopamine treatment under hypoxia showed almost 60% and 70% decreases in 5-FU sensitivity in the ASPC-1 and SUIT-2 cell lines, respectively; cyclopamine treatment under normoxic conditions revealed only 33% and 25% decreases in 5-FU sensitivity in the ASPC-1 and SUIT-2 cell lines, respectively (Fig. 3a,b). Because gemcitabine is also often used in pancreatic cancer and is also effective on cancer cells in S phase, we confirmed chemosensitivity to gemcitabine when Smo was inhibited under hypoxia. Cyclopamine treatment under hypoxia showed almost 60% and 50% decreases in gemcitabine sensitivity in ASPC-1 and SUIT-2 cell lines, respectively, however, there was no significant difference in gemcitabine sensitivity between control and cyclopamine-treated cells under normoxia (Fig. 3b). Reduction of chemosensitivity to 5-FU and gemcitabine under hypoxic conditions was also examined in a time-course experiment by counting cell numbers. The number of viable cells was time-dependently less in both groups in the two cell lines, and the number of viable cells in cyclopamine- and 5-FU/gemcitabine-treated cells was significantly higher than that in 5-FU/gemcitabine-treated cells under hypoxic conditions (Fig. S2). Conversely, CDDP, which is cell cycle-independent, did show significant synergistic effects with chemosensitivity under normoxic and hypoxic conditions in both cell lines (Fig. 3b). These results suggest that the decreased S phase from Smo inhibition under hypoxia might play important roles in decreased chemosensitivity to S phase-sensitive drugs, 5-FU and gemcitabine. We confirmed whether the sub-G_1 represents apoptotic cells using annexin V and propidium iodide staining by FACS (Fig. S3).

Hypoxia inducible factor-1α did not affect chemosensitivity of cyclopamine-treated PDAC cells under hypoxia. Hypoxia inducible factor-1α is an important transcriptional factor produced under hypoxia; reportedly, HIFs contribute to drug resistance in many kinds of carcinomas. We next evaluated whether HIF-1α affects the decreased S phase in cyclopamine-treated PDAC cells and chemosensitivity under hypoxic conditions using HIF-1α siRNA. Transfection of siRNA targeting HIF-1α significantly reduced mRNA expression of HIF-1α by 80% or more (data not shown). The number of cells in G_0/G_1 phase decreased and the number in S phase increased significantly in HIF-1α siRNA-transfected PDAC cells compared with control under hypoxic conditions in both cell lines (Fig. 4a). Even when HIF-1α is knocked down by HIF-1α siRNA transfection, G_0/G_1 increases and S phase decreases significantly by cyclopamine treatment (Fig. 4a). These results suggest that HIF-1α is independent of the increased cell numbers in G_0/G_1 phase and decreased cell numbers in S phase in Smo-inhibited PDAC cells under hypoxia. In the chemosensitivity analysis, as we expected, knockdown of HIF-1α improved chemosensitivity both in control and cyclopamine-treated cells in both cell lines. However, cyclopamine-treated cells still showed chemoresistance to 5-FU and gemcitabine even in HIF-1α siRNA-transfected cells under hypoxia in both cell lines (Fig. 4b). These

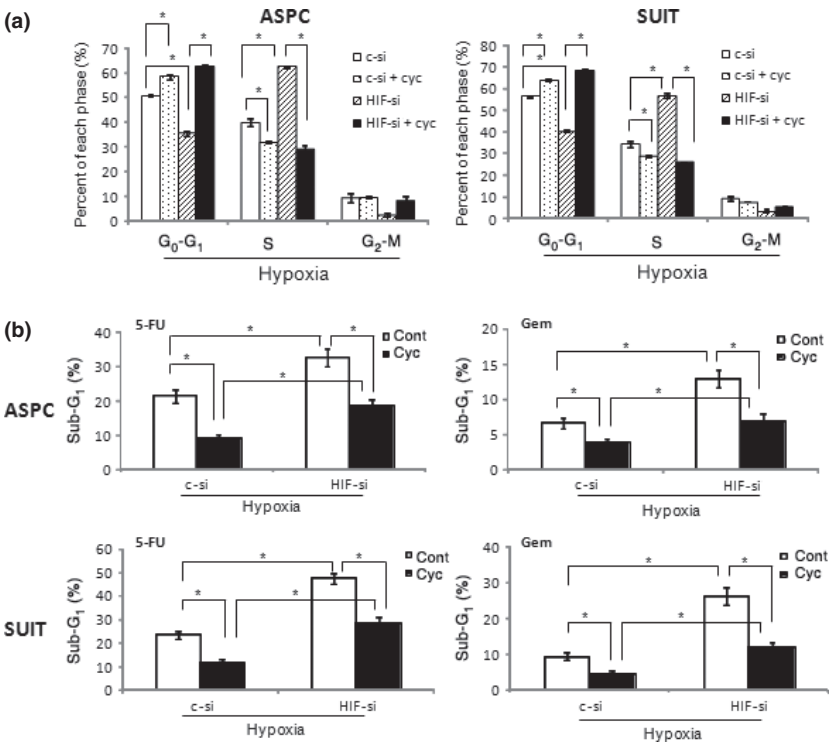

Fig. 4. Hypoxia inducible factor (HIF)-1α does not improve cyclopamine-induced chemoresistance in ASPC-1 (ASPC) and SUIT-2 (SUIT) pancreatic ductal adenocarcinoma (PDAC) cell lines under hypoxic conditions. (a) Cell cycle analysis of HIF-1α siRNA-transfected PDAC cells (HIF-si) in the absence or presence of 10 μM cyclopamine (+cyc) was carried out under hypoxic condition by FACS. (b) Twenty-four hours after the treatment with 10 μM cyclopamine in HIF-1α siRNA-transfected PDAC cells, 100 μg/mL 5-fluorouracil (5-FU) and 100 μg/mL gemcitabine (Gem) was added to the culture. After an additional 48 h, cells were harvested and the sub-G_1 population was estimated by FACS. The graph shows the mean ± SD. *$P < 0.05$.

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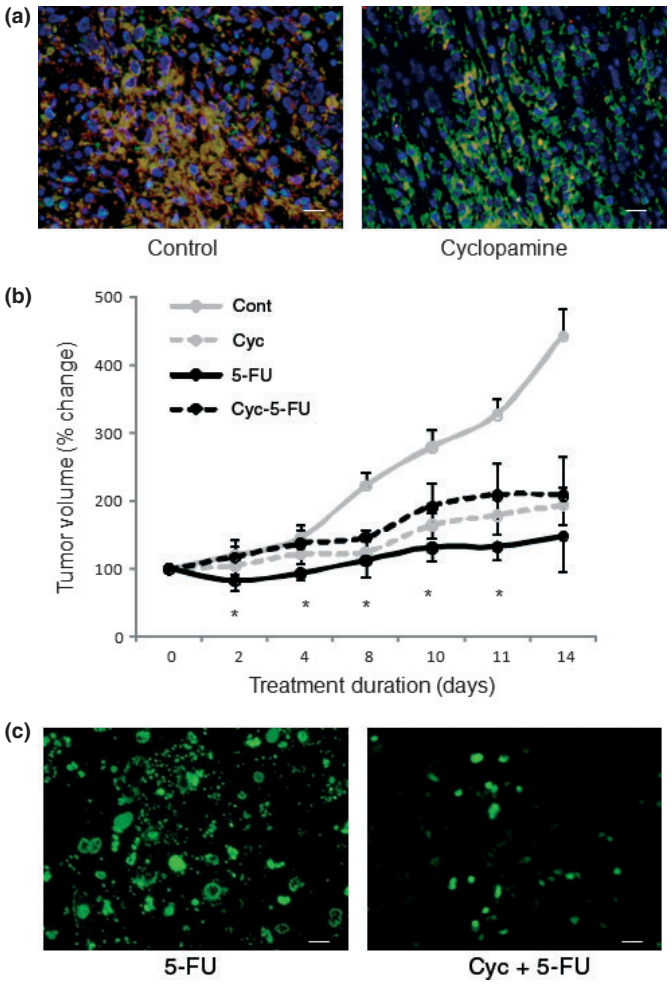

Fig. 5. Cyclopamine treatment inhibited chemosensitivity to 5-fluorouracil (5-FU) *in vivo*. Mice bearing tumors were injected s.c. with 0.1 mL vehicle (triolein : ethanol, 4:1) or cyclopamine (triolein : cyclopamine, 1 mg/body) once a week (on days 0 and 8). From 2 days after the first injection of cyclopamine (day 0), injection of 5-FU (30 mg/kg) or equivalent normal saline by i.p. injection was given three times a week. (a) Representative photographs of immunofluorescent staining with CA-9 (green) and cyclin D1 (red) in cyclopamine-treated or non-treated ASPC-1 pancreatic tumor obtained from mouse xenograft model. Bars = 40 μm. CA-9/cyclin D1 double positive cells turned yellow. (b) Tumor volume was estimated at the indicated days. cyc, cyclopamine-treated pancreatic tumor cells; cont, non-treated pancreatic tumor cells. $*P < 0.05$, comparing 5-FU group with 5-FU/cyclopamine group. (c) Apoptotic cells in tumor from xenograft model were examined by the TUNEL method. Apoptotic cells were labeled by FITC. Representative pictures are shown. Bar = 30 μm.

results suggest that HIF-1α does not contribute to cyclopamine-induced impairment of chemosensitivity under hypoxia.

Cyclopamine treatment inhibits chemosensitivity to 5-FU *in vivo*. Next we confirmed the *in vitro* results using a mouse model. The protein CA-9 was used as a marker for hypoxia.[27] Tumors from cyclopamine-injected mice showed significantly decreased cyclin D1 in hypoxic areas compared with control, resulting in a significant decrease of overlap with CA-9 and cyclin D1 compared with control (Fig. 5a). This suggests that G_0/G_1 arrest and decreased S phase were induced in these tumors.

Tumor volume in mice injected with cyclopamine and 5-FU was significantly higher than in those injected with 5-FU alone (Fig. 5b). In addition, a significantly higher number of apoptotic cells was detected in tumors from mice injected with 5-FU alone compared to mice treated with cyclopamine and 5-FU (Fig. 5c). These results suggest that combination therapy with 5-FU and cyclopamine does not bring favorable antitumor effects in pancreatic cancer.

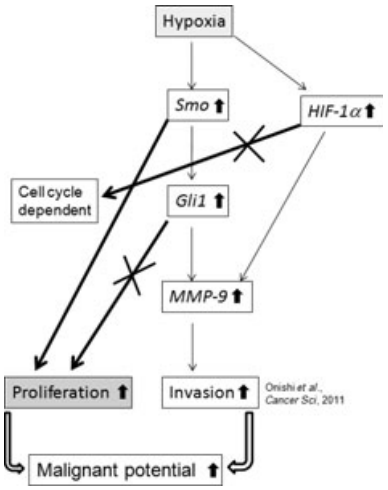

Fig. 6. Schematic figure of findings. New findings in the present study (bold line) overlapped with our previous findings. HIF, hypoxia inducible factor; Smo, smoothened.

Discussion

Pancreatic tumors show high levels of hypoxia.[28] Previously, we showed that hypoxia increased invasiveness through the upregulation of *Smo* transcription in pancreatic cancer.[13] Proliferation as well as invasion is a major factor for cancer progression. In the present study, we showed that hypoxia-induced increase of Smo affects the proliferation of PDAC cells through a Hh–Gli1-independent and HIF-1α-independent pathway. We also showed for the first time that Smo inhibition under hypoxic conditions led to the increase in cell numbers in G_0/G_1 phase and decrease in cell numbers in S phase (Fig. 2). Hirotsu *et al.*[29] showed that cyclopamine treatment promotes G_1 arrest under normoxic conditions in osteosarcoma cells. Arrest at G_0/G_1 with decreased S phase is consistent with the result that Smo-inhibited PDAC cells showed a lower proliferation ability under hypoxia. Figure 6 summarizes the pathway of pancreatic cancer to accumulate malignant potential, overlapping with our previous findings.[13]

5-Fluorouracil and gemcitambine are generally used in pancreatic cancer patients. These two drugs are S phase-sensitive. Next, we evaluated whether cyclopamine-induced decrease of S phase under hypoxic conditions affects chemosensitivity. As we hypothesized, both 5-FU and gemcitabine with prior cyclopamine treatment significantly decreased chemosensitivity under hypoxia, whereas CDDP, which is cell cycle-independent, showed a significant synergistic result (Fig. 3). These results suggest that decreased S phase due to the use of Smo inhibitor under hypoxia may play a pivotal role in decreased chemosensitivity. It also suggests that CDDP would be useful combined with cyclopamine in pancreatic cancer. As Thayer *et al.*[30] showed that cyclopamine induced apoptosis in PDAC cells, we also showed decreased proliferation and decreased chemosensitivity in cyclopamine-treated PDAC cells, even under normoxic conditions. One reason could be that cyclopamine treatment induces G_0/G_1 arrest with decreased S phase even under normoxic conditions because the Hh pathway in PDAC cells activates even under normoxic conditions.[31]

However, the inhibition rate of proliferation, chemosensitivity, and the percentage of accumulation of G_0/G_1 phase under hypoxic conditions were much higher than those under normoxic conditions. We suggest that these increases may be due to the hypoxia-induced increase of Smo.

Reportedly, HIFs contribute to drug resistance[14–18] and another pathway to chemoresistance under hypoxia still remains unclear. In the present study, chemosensitivity in *HIF-1α* siRNA-transfected cells has been significantly improved compared with control cells (Fig. 4b). These results support the notion that HIF-1α decreases chemosensitivity to CDDP, 5-FU, and vincristine under hypoxia, as many authors suggest.[14–18] We think that one reason is the significant S phase increase by the transfection of *HIF-1α* siRNA. However, cyclopamine treatment induced decreased S phase, even in *HIF-1α* siRNA-transfected cells under hypoxia; *HIF-1α* siRNA transfection did not improve cyclopamine-induced chemoresistance under hypoxia. So we think that HIF-1α did not affect either the cyclopamine-induced decreased S phase or chemosensitivity under hypoxia. This may be one reason why there was little difference in chemosensitivity between normoxia and hypoxia. We tested other pathways, such as the MAPK and PI3k pathways, but still did not detect the signaling pathway that contributed to Smo-inhibition-induced S phase decrease and chemoresistance under hypoxia. Therefore, the mechanism of cyclopamine-induced chemoresistance under hypoxia remains unclear.

Hedgehog inhibitors, especially many kinds of Smo inhibitors, are under clinical trial. To get a strong antitumor effect, combination therapy with cyclopamine and other chemotherapy drugs may be recommended. However, the clinical effect of combined use of Smo inhibitor with other chemotherapy drugs is unknown. Our results suggest that Smo can be a therapeutic target for pancreatic cancer because hypoxia-induced increase in Smo can induce invasiveness and proliferation. However, Smo inhibitors may increase chemoresistance in some chemotherapy drugs in hypoxic PDAC cells. We consider our results to be of considerable clinical significance, because a clinical trial using Smo inhibitors and chemotherapy drugs will begin in the near future. Therefore, we would like to report our preliminary study as soon as possible.

Acknowledgments

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Disclosure Statement

The authors declare no conflict of interest in this work.

Abbreviations

5-FU	5-fluorouracil
CDDP	cis-diamminedichloroplatinum
Hh	Hedgehog
HIF	hypoxia inducible factor
PDAC	pancreatic ductal adenocarcinoma
Smo	Smoothened

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Proliferation in cyclopamine-treated ASPC-1 (ASPC) and SUIT-2 (SUIT) pancreatic ductal adenocarcinoma (PDAC) cells showed dose-dependent decrease.

Fig. S2. Viable cells number in cyclopamine (cyc)-treated and 5-fluorouracil (5-FU)/gemcitabine (gem)-treated cells was significantly higher than that in 5-FU/gemcitabine treated cells.

Fig. S3. Cyclopamine treatment (cyclo) inhibits the induction of apoptosis in 5-fluorouracil (5-FU) and gemcitabine (Gem) but not cis-diammined-ichloroplatinum (CDDP)-treated ASPC-1 (ASPC) and SUIT-2 (SUIT) pancreatic ductal adenocarcinoma (PDAC) cells under hypoxic conditions.

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