

Gli1 contributes to the invasiveness of pancreatic cancer through matrix metalloproteinase-9 activation

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The hedgehog (Hh) signaling pathway has been reported to be associated with the growth of pancreatic cancer, but its role in the invasive phenotype is poorly understood. Therefore, we investigated the role of the Hh pathway in pancreatic cancer cell invasiveness using a Matrigel invasion assay. Blockade of the Hh pathway by cyclopamine inhibited pancreatic cancer cell invasion in association with a decreased expression of matrix metalloproteinase (MMP)-9. By contrast, activation of the Hh pathway by the addition of exogenous Sonic hedgehog increased cell invasion and MMP-9 expression. Stable transfection of pancreatic cancer cells with *Gli1* increased their invasiveness, which was associated with activation of MMP-9. We also showed that inhibition of MMP-9 by small interfering RNA blocked the increased invasiveness of *Gli1*-transfected cells. Furthermore, inhibition of *Gli1* by small interfering RNA suppressed the invasiveness and MMP-9 expression of pancreatic cancer cells. Taken together, these findings suggest that members of the Hh pathway, especially *Gli1*, play an important role in the invasiveness of pancreatic cancer cells through the regulation of MMP-9 expression. (*Cancer Sci* 2008; 99: 1377–1384)

Pancreatic cancer is one of the most lethal malignancies that has yet to be successfully controlled with therapy. Although various therapies such as surgery, chemotherapy, and radiation therapy have been carried out, few patients survive for 5 years.^(1,2) One reason for this lethality is its invasive and metastatic character. A better understanding of the mechanisms that underlie the development of pancreatic cancer would help to identify novel molecular targets for treatment.

The Hh signaling pathway is crucial to growth and patterning in a wide variety of tissues, including the pancreas, during embryonic development.^(3–6) Of three Hh ligands, Shh, Ihh, Dhh, the former is reported to play an essential role in the development of pancreatic cancer as well as pancreatic organogenesis. The response to Shh is mediated by two transmembrane proteins, Smo and Ptch, and by downstream transcription factors that are members of the Gli family. Three Gli genes have been identified. *Gli2* and *Gli3* have distinct context-dependent repressor and activator functions.^(7–9) By contrast, *Gli1* is an activator of target genes, and is itself a transcriptional target of the Hh pathway.^(10–12) Recent studies have reported an association between Hh pathway activation and initiation of human tumors.⁽⁶⁾ Recent studies have also shown cell autonomous ligand-dependent activation of the Hh pathway in small cell lung cancer and carcinomas of the esophagus, stomach, biliary tract, and pancreas.^(13–15) It has been also shown that cyclopamine, a Smo antagonist, suppresses the growth of pancreatic cancer.^(14,15) These findings suggest that the Hh pathway could be a viable therapeutic target for the treatment of pancreatic cancer.

In the present study, we analyzed the relationship between Hh pathway activation, especially *Gli1*, and the invasive ability of pancreatic cancer cells.

Materials and Methods

Cell culture. Three human pancreatic ductal adenocarcinoma cell lines (AsPC-1, SUIT-2, and CFPAC-1) were maintained in RPMI-1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (Life Technologies), at 37°C in 5% CO₂.

Reagents and antibodies. Cyclopamine, purchased from Toronto Research Chemicals (Toronto, Canada), was diluted in 100% methanol. Mouse anti-Shh blocking antibody (5E1), developed by Thomas M. Jessell, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health & Human Development and maintained by The University of Iowa, Department of Biological Sciences (Iowa City, IA). Control mouse IgG was purchased from Sigma-Aldrich (St Louis, MO). rhShh NH₂-terminal peptide was purchased from R&D Systems (Minneapolis, MN). Recombinant human IL-1β was purchased from Sigma-Aldrich. Anti-*Gli1* (sc-6153) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MMP-9 (#3852) was purchased from Cell Signaling Technology (Danvers, MA).

Matrigel invasion assay and migration assay. The invasiveness of pancreatic cancer cells was assessed based on the invasion of cells through Matrigel-coated transwell inserts.⁽¹⁶⁾ In brief, the upper surface of a filter (pore size, 8.0 μm; BD Biosciences, Heidelberg, Germany) was coated with basement membrane Matrigel (BD Biosciences). Cells were suspended in RPMI-1640 with 10% fetal calf serum containing the desired dose of reagents. Then 0.8 × 10⁵ cells were added to the upper chamber and incubated for 16 h. After incubation, the filter was fixed and stained with Diff-Quik reagent (International Reagents, Kobe, Japan). Cells that had migrated from the upper to the lower side of the filter were counted under a light microscope (BX50; Olympus, Tokyo, Japan) at a magnification of ×100. Tumor cell invasiveness was defined as the total number of invaded cells. Each experiment was carried out in triplicate wells.

The migration ability of pancreatic cancer cells was assessed in the same way as described above, but non-Matrigel-coated transwell inserts were used.

Proliferation assay. To examine the proliferation of pancreatic cancer cells, we carried out a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay.⁽¹⁷⁾ Cells were incubated

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Abbreviations: Dhh, Desert hedgehog; GFP, green fluorescent protein; Hh, hedgehog; Ihh, Indian hedgehog; Ig, immunoglobulin; IL, interleukin; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; Ptch, Patched; rhShh, recombinant human sonic hedgehog; RNAi, RNA interference; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Shh, sonic hedgehog; si*Gli1*, small interfering RNA targeting *Gli1*; siRNA, small interfering RNA; Smo, smoothened.

under the same conditions as the Matrigel invasion assay, and their proliferation was assessed.

Real-time RT-PCR. For real-time RT-PCR, RNA (1 µg) was treated with DNase and reverse transcribed to cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Reactions were run with an iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) on a DNA Engine Opticon 2 System (MJ Research, Waltham, MA). The amount of each target gene in a given sample was normalized to the level of *β-actin* in that sample.

Gelatin zymography. Gelatinase secretion of cell culture supernatants was determined by SDS-PAGE zymography using gelatin as a substrate for MMP-2 and MMP-9, as described by Sehgal *et al.*⁽¹⁸⁾ In brief, tumor cells were seeded at 5×10^5 cells/6-well plate. After the cells reached 80% confluence, the medium was aspirated and 4 mL of fresh serum-free RPMI-1640 containing the desired dose of reagents was added to each well. Supernatants were collected after a 24-h incubation and concentrated to 50 µL using a membrane dialysis concentrator (Millipore, Bedford, MA). The same volume of concentrated supernatants was mixed with SDS sample buffer without prior denaturation and run on 10% SDS-PAGE gels containing 1 mg/mL gelatin. Gelatin Zymo MMP Marker (active-MMP-2, pro-MMP-2,-9; Life Laboratory Company, Yamagata, Japan) was used as a positive control. After electrophoresis, the gels were washed and stained in a solution of 0.25% Coomassie Brilliant Blue G-250 and destained with acetic acid and methanol. The gels were scanned using a GT-X800 scanner (Epson, Tokyo, Japan) and semiquantitative analysis was carried out with ImageJ version 1.33 software.

Transient transfection. pIRES2-hSHH-EGFP (referred to as pSHH-GFP) was kindly provided by Dr Aubie Shaw (Division of Urology, Department of Surgery, University of Wisconsin, MA⁽¹⁹⁾). Cells seeded in 6-well plates were transfected with 2 µg of pGFP or pSHH-GFP using TransFast reagent (Promega, Madison, WI) according to the manufacturer's instructions. Transfected cells were used for experiments 24 h after transfection.

Transfection and establishment of stable transfectants. CFPAC-1 cells were resuspended in Nucleofector Solution (Amaxa Biosystems, Cologne, Germany) followed by addition of 2.5 µg pcDNA3-Gli1 or pcDNA3 (a kind gift of Dr Y Sasaki, Riken, Kobe, Japan⁽⁷⁾). Cells were transfected by electroporation with a Nucleofector System (Amaxa Biosystems). G418 (Invitrogen, Carlsbad, CA) selection was started 24 h after transfection and maintained for 10–21 days; individual clones derived from G418-resistant single cells were pooled and grown for *Gli1* mRNA expression analyses. To confirm the levels of *Gli1* mRNA in the G418-resistant clones (pcDNA3-Gli1- and pcDNA3-transfected clones), real-time RT-PCR was carried out as described previously,⁽²⁰⁾ pcDNA3-Gli1 clones expressed 17.4-fold higher levels of *Gli1* mRNA than did AsPC-1 cells, which constitutively expressed *Gli1* mRNA. No clones obtained due to transfection with pcDNA3 expressed detectable levels of *Gli1* mRNA (data not shown).

RNA interference. siRNA for *MMP-9* (ON-TARGET^{plus} SMART pool, L-005970), siRNA for *Gli1* (ON-TARGET^{plus} SMART pool, L-003896) and negative control siRNA (ON-TARGET^{plus} siCONTROL non-targeting pool, D-001810) were purchased from Dharmacon RNA Technologies (Chicago, IL). Cells seeded in 6-well plates were transfected with 100 nM siRNA using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. Cells were used for experiments at the indicated hours after transfection.

Immunocytochemistry. Pancreatic cancer cells (5×10^5 /well) were seeded onto pre-underlaid poly L-lysine-coated cover glass (Asahi Techno Glass, Chiba, Japan) in 24-well plates, and were incubated overnight in 10% fetal bovine serum-RPMI. Immunostaining was carried out as previously described with some modification.⁽²¹⁾ In brief, cells were fixed in 4% parafor-

maldehyde followed by permeabilization with 0.2% Triton X-100, then incubated with primary followed by secondary antibodies. Cells were counterstained with 4',6'-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich). After mounting in Vectorshield Mounting Medium (Vector Laboratories, Burlingame, CA), samples were examined by fluorescence microscopy (Axio Imager.A1; Carl Zeiss, Oberkochen, Germany). Antibodies and dilutions used were as follows: rabbit anti-MMP-9 (1:100); goat anti-Gli1 (1:100); Alexa Fluor 488 chicken anti-rabbit IgG (1:400; Invitrogen); and Alexa Fluor 594 donkey anti-goat IgG (1:400; Invitrogen).

Statistical analysis. Mann-Whitney's *U*-test was used for statistical analysis. All results with a *P* < 0.05 were considered statistically significant.

Results

Inhibition of the Hh pathway suppressed the invasive ability of pancreatic cancer cells. We used two human pancreatic cancer cell lines, AsPC-1 and SUIT-2, in which constitutive activation of the Hh pathway has been confirmed.⁽²²⁾ Cyclopamine (10 µM) significantly suppressed *Gli1* mRNA expression, indicating inhibition of the Hh pathway, because *Gli1* is a transactivator of target genes and is itself a transcriptional target of the Hh pathway^(10–12) (Fig. 1a). When these cells were cultured with cyclopamine of various concentrations (5–10 µM) for 16 h, cyclopamine below 10 µM did not affect the proliferation of these cells (Fig. 1b). Cyclopamine (10 µM) did not affect the migration ability of either cell line (data not shown). However, the number of cells that invaded from the upper to the lower chamber through the Matrigel-coated filter was significantly decreased in a dose-dependent manner (Fig. 1c). We also confirmed the ligand dependency of Hh-related invasive ability using an anti-Shh blocking antibody (5E1). When incubated with 5 µg/mL 5E1, the number of invaded cells was significantly decreased (Fig. 1d), whereas the addition of 5E1 did not affect the proliferation of these cells during the Matrigel invasion assay (data not shown). These results suggest that Hh pathway inhibition suppresses the invasive ability of these cells in a ligand-dependent manner, independently of any inhibition of cell proliferation.

Activation of the Hh pathway enhanced the invasive ability of pancreatic cancer cells. First, both cell lines were transfected with pSHH-GFP to overexpress Shh or pGFP as a control, and the *Gli1* mRNA level was monitored by real-time RT-PCR. The transfection efficiency was over 70% in both cell lines (data not shown). Real-time RT-PCR revealed that cells transfected with pSHH-GFP expressed higher levels of *Gli1* mRNA than cells transfected with pGFP (Fig. 2a, left). pSHH-GFP transfection did not affect the proliferation of these cells during the 16-h cultivation period (data not shown). The Matrigel invasion assay showed that the number of pSHH-GFP transfected cells invading through the Matrigel-coated filter was much larger than the number of invading pGFP transfected cells (Fig. 2a, right).

Second, both cell lines were co-cultured with rhShh to activate the Hh pathway during the Matrigel invasion assay. The expression of *Gli1* mRNA was significantly increased by rhShh (Fig. 2b, left). rhShh did not affect proliferation of these cells during the Matrigel invasion assay (data not shown), but cells in which the Hh pathway was activated by rhShh invaded through the Matrigel-coated filter more than control cells (Fig. 2b, right).

Finally, we used our IL-1β-Hh pathway activation system reported previously.⁽²²⁾ In brief, when both types of cancer cell were treated with 5 ng/mL IL-1β, the Hh pathway was activated through increased Shh induction. Activation of the Hh pathway by IL-1β was verified by the detection of increased expression of *Gli1* mRNA using real-time RT-PCR (Fig. 2c, left). Although 5 ng/mL IL-1β did not significantly affect the proliferation of

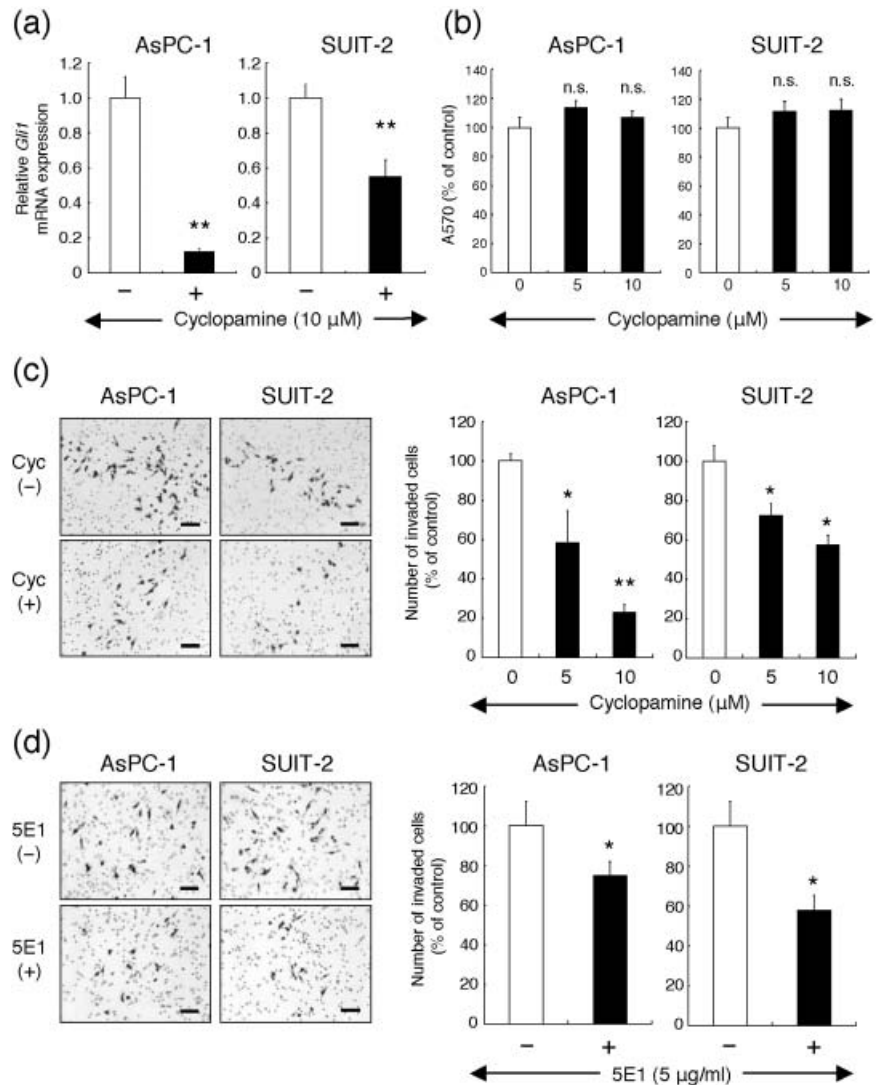


Fig. 1. Inhibition of the hedgehog pathway by cyclopamine (Cyc) in AsPC-1 and SUI-2 human pancreatic ductal adenocarcinoma cells. (a) AsPC-1 and SUI-2 cells were treated with 10 μ M Cyc for 12 h and relative *Gli1* mRNA expression was examined by real-time reverse transcription-polymerase chain reaction. *Gli1* mRNA expression was normalized to β -actin mRNA expression. (b) After incubation with the indicated dose of Cyc for 16 h, cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay detected at an absorbance at 570 nm. (c, d) AsPC-1 and SUI-2 cells were seeded into a Matrigel-coated invasion chamber with 5 or 10 μ M Cyc (c) or with 5 μ g/mL anti-sonic hedgehog antibody (5E1) or mouse immunoglobulin G (as a negative control) (d), and incubated for 16 h. The number of cells was counted under a light microscope. Magnification, \times 100; scale bars, 100 μ m; columns, means of three independent experiments; bars, SD. +, addition of cyclopamine (a,c) or 5E1(d); -, no addition of cyclopamine (a,c) or 5E1(d). * P < 0.05; ** P < 0.01. n.s., not significant.

these cells during a 16-h incubation period (data not shown), IL-1 β increased the number of cells that invaded to the lower chamber, and cyclopamine decreased the increased number of invaded cells in a dose-dependent manner in both cell lines (Fig. 2c, right).

These results confirmed that activation of the Hh pathway increases the invasive ability of pancreatic cancer cells in a ligand-dependent manner, independently of an enhancement of proliferation.

Hh pathway regulation affected MMP-9 expression of pancreatic cancer cells. These results strongly suggest that the Hh pathway contributes to the degradation of Matrigel, which includes type IV collagen. Therefore, we focused on the relationship between Hh pathway activity and MMP-2 and MMP-9 expression.

We carried out gelatin zymography to evaluate the enzymatic activity of MMPs. Inhibition of the Hh pathway by cyclopamine suppressed the enzymatic activity of both pro- and active-MMP-9 in both cell lines (Fig. 3a). However, we could find only pro-MMP-2, not active-MMP-2. Moreover, the expression of pro-MMP-2 was not suppressed significantly by cyclopamine. Then we carried out real-time RT-PCR to evaluate *MMP-9* mRNA expression. Inhibition of the Hh pathway by cyclopamine also suppressed *MMP-9* mRNA expression in both AsPC-1 and SUI-2 cells (Fig. 3b).

By contrast, activation of the Hh pathway by rhShh increased the enzymatic activity of both pro-MMP-9 and active-MMP-9

(Fig. 3c) in both cell lines. Here again, we could not find the active form of MMP-2, and the expression of pro-MMP-2 was not increased by rhShh. Real time RT-PCR showed that *MMP-9* mRNA expression was increased by rhShh (Fig. 3d). These results suggest a contribution of MMP-9 to the Hh pathway-related invasive ability of these cells.

Stable *Gli1*-expressing clones showed increased invasive ability mediated by MMP-9 activation. To better clarify the relationship between the Hh pathway, invasion and MMP-9 expression, we established stable *Gli1*-expressing clones using a human pancreatic cancer cell line (CFPAC-1) that has not been reported to express *Shh* mRNA,⁽²⁰⁾ as described in the Materials and Methods section. We used a clone (referred to as pcDNA-Gli1 cells) expressing 17.4-fold higher levels of *Gli1* mRNA than AsPC-1 cells. No clones obtained by transfection with pcDNA3 (referred to as pcDNA cells) expressed detectable levels of *Gli1* mRNA. No significant difference in cell proliferation was observed between pcDNA-Gli1 cells and pcDNA cells during the 16-h cultivation period (data not shown). In the Matrigel invasion assay, the number of invaded pcDNA-Gli1 cells was 4.2-fold higher than that of control pcDNA cells (Fig. 4a). On that occasion, zymography showed that both pro-MMP-9 and active-MMP-9 enzymatic activity of pcDNA-Gli1 cells were higher than that of pcDNA cells (Fig. 4b). Active-MMP-2 was not detected in CFPAC1 cells, and the expression of pro-MMP-2 was not significantly increased in pcDNA-Gli1 cells. Real time RT-

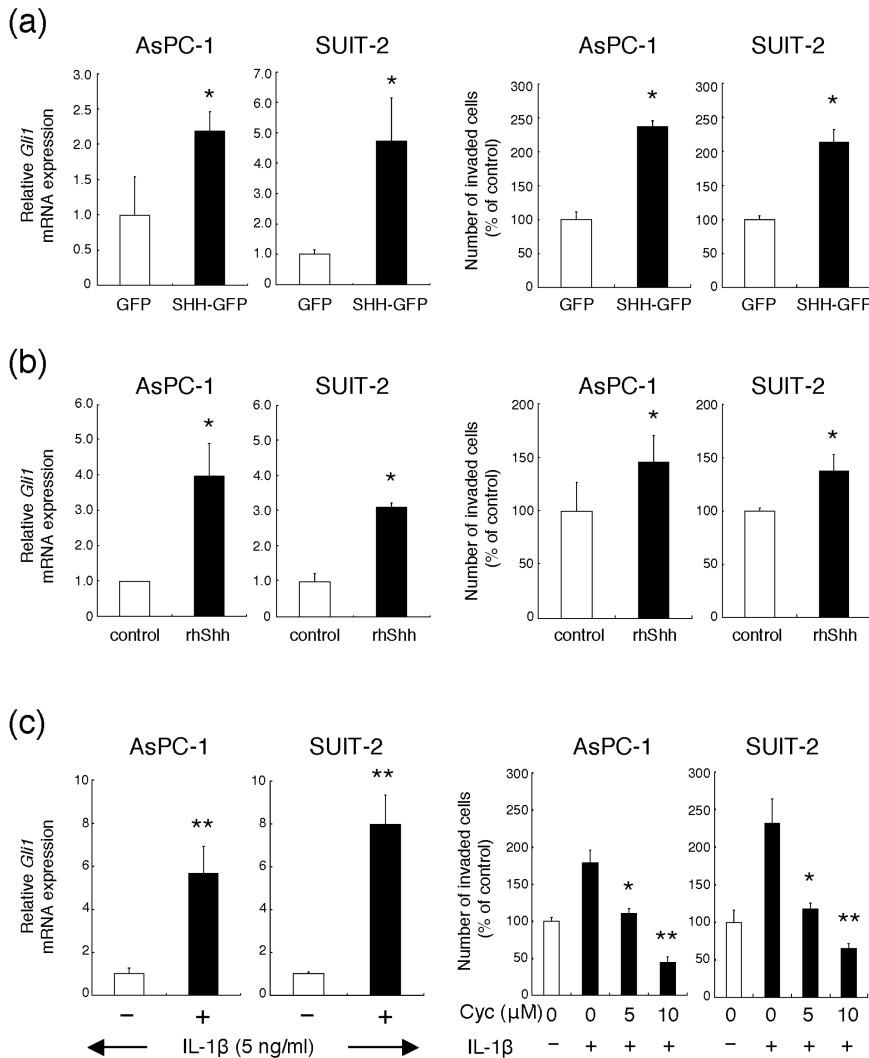


Fig. 2. Activation of the hedgehog pathway enhances the invasive ability of AsPC-1 and SUI2-2 human pancreatic ductal adenocarcinoma cells. (a) AsPC-1 and SUI2-2 cells were transfected with pIRES2-hSHH-EGFP (pSHH-GFP) or pGFP, then expression of *Gli1* mRNA relative to that of β -actin mRNA was assessed by real-time reverse transcription-polymerase chain reaction (left). After transfection, the cells were seeded into a Matrigel-coated invasion chamber and incubated for 16 h. Quantitation of the assay is shown in the graph (right). (b) AsPC-1 and SUI2-2 cells were treated with 0.4 μ g/mL recombinant human sonic hedgehog NH₂-terminal peptide (rhShh) for 4 h. Expression of *Gli1* mRNA was assessed (left). Cells were seeded into a Matrigel-coated invasion chamber with 0.4 μ g/mL rhShh and incubated for 16 h (right). (c) AsPC-1 and SUI2-2 cells were treated with 5 ng/mL interleukin-1 β (IL-1 β) for 6 h, then expression of *Gli1* was assessed (left). Cells were seeded into a Matrigel-coated invasion chamber with 5 ng/mL IL-1 β and 5 or 10 μ M cyclopamine (Cyc) for 16 h (right). Columns, means of three independent experiments; bars, SD. +, addition of IL-1 β ; -, no addition of IL-1 β . * P < 0.05; ** P < 0.01.

PCR showed that *MMP-9* mRNA expression in pcDNA-Gli1 cells was significantly higher than that in pcDNA cells (Fig. 4c).

RNAi for *MMP-9* suppressed the increased invasive ability of pcDNA-Gli1 cells. To confirm the role of *MMP-9* in the Hh pathway-related invasive ability of pancreatic cancer cells, we knocked down *MMP-9* expression in pcDNA-Gli1 cells and pcDNA cells by RNAi. Real-time RT-PCR showed that transfection of cells with siRNA targeting *MMP-9* (siMMP-9) resulted in a 90% or greater knockdown of *MMP-9* mRNA expression (Fig. 4d). Immunocytochemistry showed that siMMP-9 treatment decreased the *MMP-9* protein expression (Fig. 4e). We next carried out a Matrigel invasion assay using these siMMP-9-transfected cells. The number of siMMP-9 transfected cells that migrated through the Matrigel-coated filter was reduced by 70% in pcDNA-Gli1 cells and by 25% in pcDNA cells (Fig. 4f). These results showed that inhibition of *MMP-9* blocked the increased invasiveness of pcDNA-Gli1-transfected cells in the Matrigel invasion assay.

RNAi for *Gli1* suppressed the invasive ability of pancreatic cancer cells. We next focused on the role of *Gli1*, an activator of target genes and itself a transcriptional target of the Hh pathway, in the invasive ability of pancreatic cancer cells. To evaluate the role of *Gli1*, we knocked down *Gli1* expression of pancreatic cancer cells. Real-time RT-PCR showed that transfection of AsPC-1, SUI2-2 and pcDNA-Gli1 cells with siGli1 resulted in approximately 90% (in AsPC-1 and SUI2-2) and 60% (in

pcDNA-Gli1 cells) knockdown of *Gli1* mRNA expression (Fig. 5a). Immunocytochemistry showed that siGli1 treatment decreased the *Gli1* protein expression (Fig. 5b) and *MMP-9* mRNA expression was significantly decreased in all cells (Fig. 5c). Furthermore, in the Matrigel invasion assay, the number of invaded siGli1-transfected cells was decreased compared with the control siRNA-transfected cells (Fig. 5d).

Discussion

Recent increasing data indicate a contribution of the Hh pathway not only to cell proliferation, but also to the repair of several tissues.⁽²³⁾ For example, it has been shown that, during repair of bronchial epithelium, transient activation of Hh pathway occurs within the normally quiescent bronchial epithelium, and such a process might well occur during adult gut epithelial turnover.⁽¹³⁾ As we know, many kinds of molecules, including MMPs, take part in degradation of extracellular matrix are participating in tissue repair. This suggests that the Hh pathway might also contribute to cell invasion by mediating MMPs.

Based on these observations, we hypothesized that the Hh pathway plays an important role in the invasive ability of pancreatic cancer. In the present study, we selected two human cancer cells, AsPC-1 and SUI2-2. Blockade of the Hh pathway suppressed the invasive ability of both cell types (Fig. 1), but activation of the Hh pathway increased their invasive ability

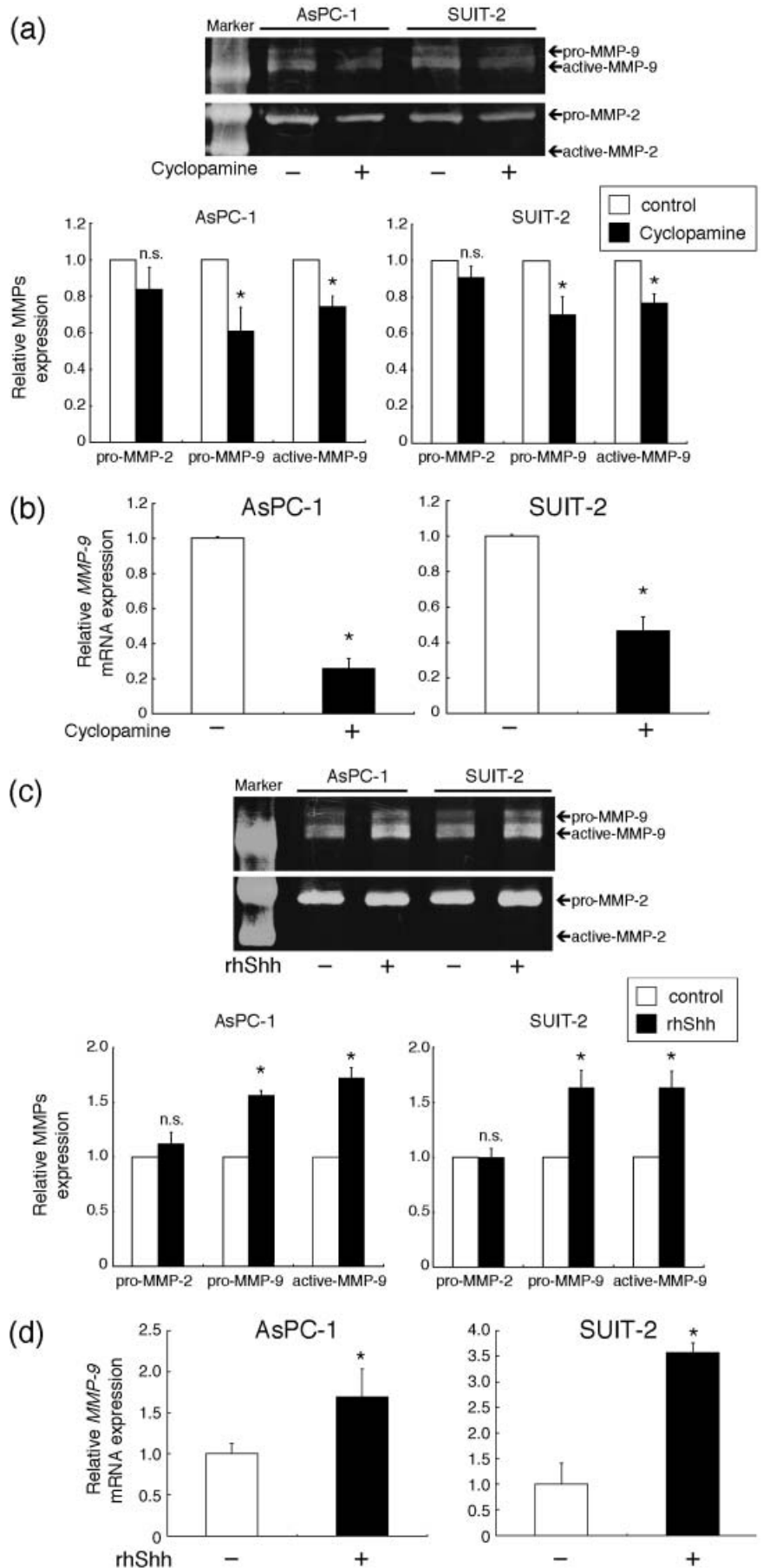


Fig. 3. Regulation of hedgehog pathway activation affects matrix metalloproteinase (MMP)-9 expression in AsPC-1 and SUI-2 human pancreatic ductal adenocarcinoma cells. (a) AsPC-1 and SUI-2 cells were incubated with 10 μ M cyclopamine for 24 h in serum-free medium, and MMP-2 and MMP-9 enzymatic activity was examined by gelatin zymography. (b) Cells were incubated with 10 μ M cyclopamine for 12 h, then expression of MMP-9 mRNA relative to that of β -actin mRNA was assessed by real-time reverse transcription-polymerase chain reaction. (c) Cells were incubated with 0.4 μ g/mL rhShh for 24 h in serum-free medium, and MMP-2 and MMP-9 enzymatic activity was examined. (d) Cells were incubated with 0.4 μ g/mL recombinant human sonic hedgehog NH₂-terminal peptide (rhShh) for 12 h, then expression of MMP-9 mRNA was examined. Band intensities of MMPs were quantified with ImageJ version 1.33 software. The control band intensity is indicated as 1.0. Marker indicates pro-MMP-9 and active-MMP-2. Columns, means of three independent experiments; bars, SD. n.s., not significant; * P < 0.05.

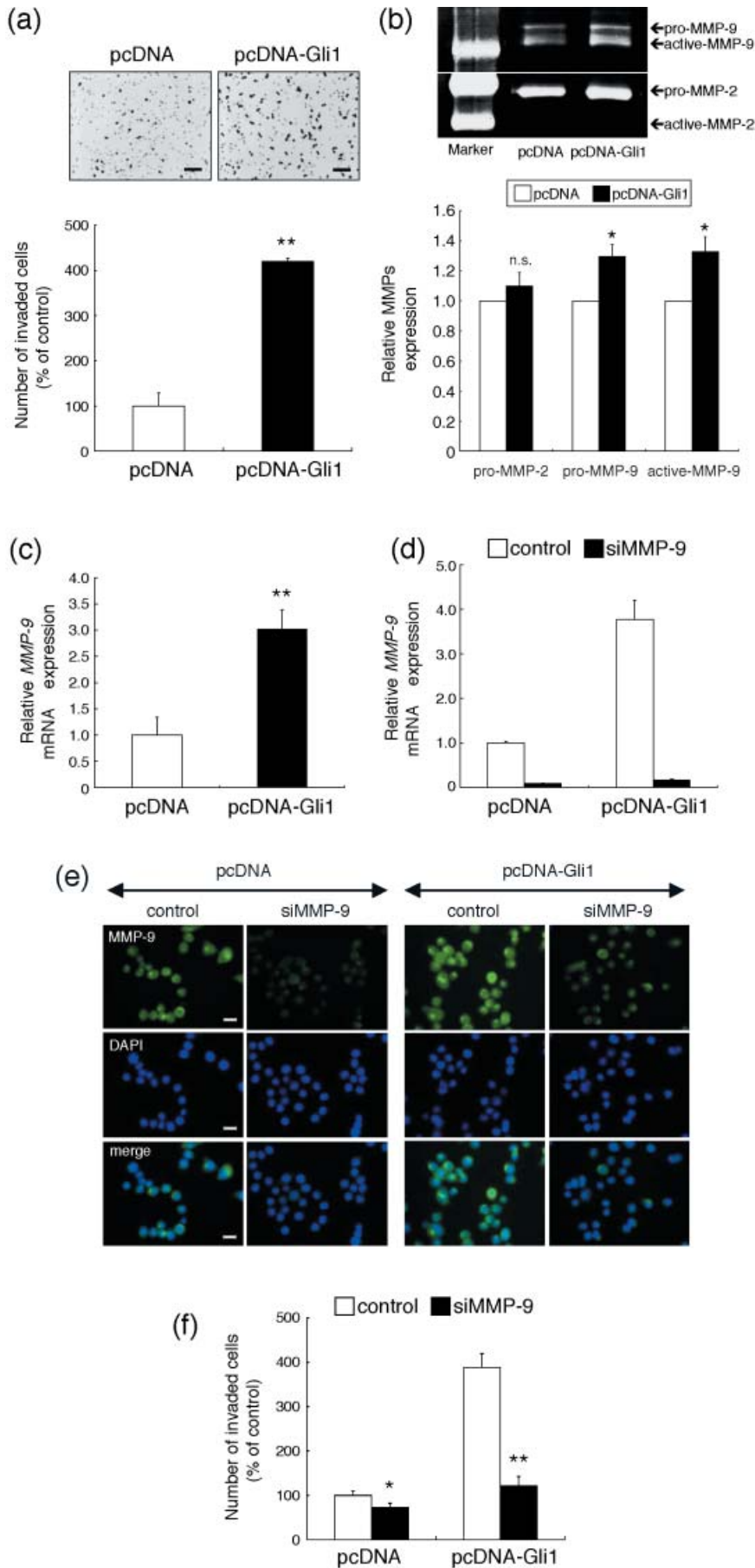


Fig. 4. CFPAC-1 human pancreatic ductal adenocarcinoma cells transfected with *Gli1* showed increased invasive ability. (a) CFPAC-1 cells were transfected with pcDNA-Gli1 or pcDNA, and stable transfectants were established. These clones were seeded into a Matrigel-coated chamber for 16 h. The number of cells was counted under a light microscope (top). Magnification: $\times 100$; scale bars, 100 μm . Quantitation of the assay is shown in the graph (bottom). (b) Both groups of transfected cells were incubated in serum-free medium, and the enzymatic activity of matrix metalloproteinase (MMP)-2 and MMP-9 was examined by gelatin zymography. Band intensities of MMP were quantified with ImageJ version 1.33 software. The control band intensity is indicated as 1.0. Marker indicates pro-MMP-9 and active-MMP-2. (c) Expression of *MMP-9* mRNA relative to that of β -actin mRNA was assessed by real-time reverse transcription-polymerase chain reaction. (d) pcDNA-Gli1- or pcDNA-transfected cells were transfected with small interfering RNA (siRNA) for MMP-9 (siMMP-9) or control siRNA. After transfection, the expression of *MMP-9* mRNA was assessed. (e) After transfection, the expression of MMP-9 protein was assessed by immunocytochemistry. MMP-9, green; 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI), blue. Magnification, $\times 400$; scale bars, 20 μm . (f) These cells were seeded into a Matrigel-coated invasion chamber for 16 h. Columns, means of three independent experiments; bars, SD. * $P < 0.05$; ** $P < 0.01$.

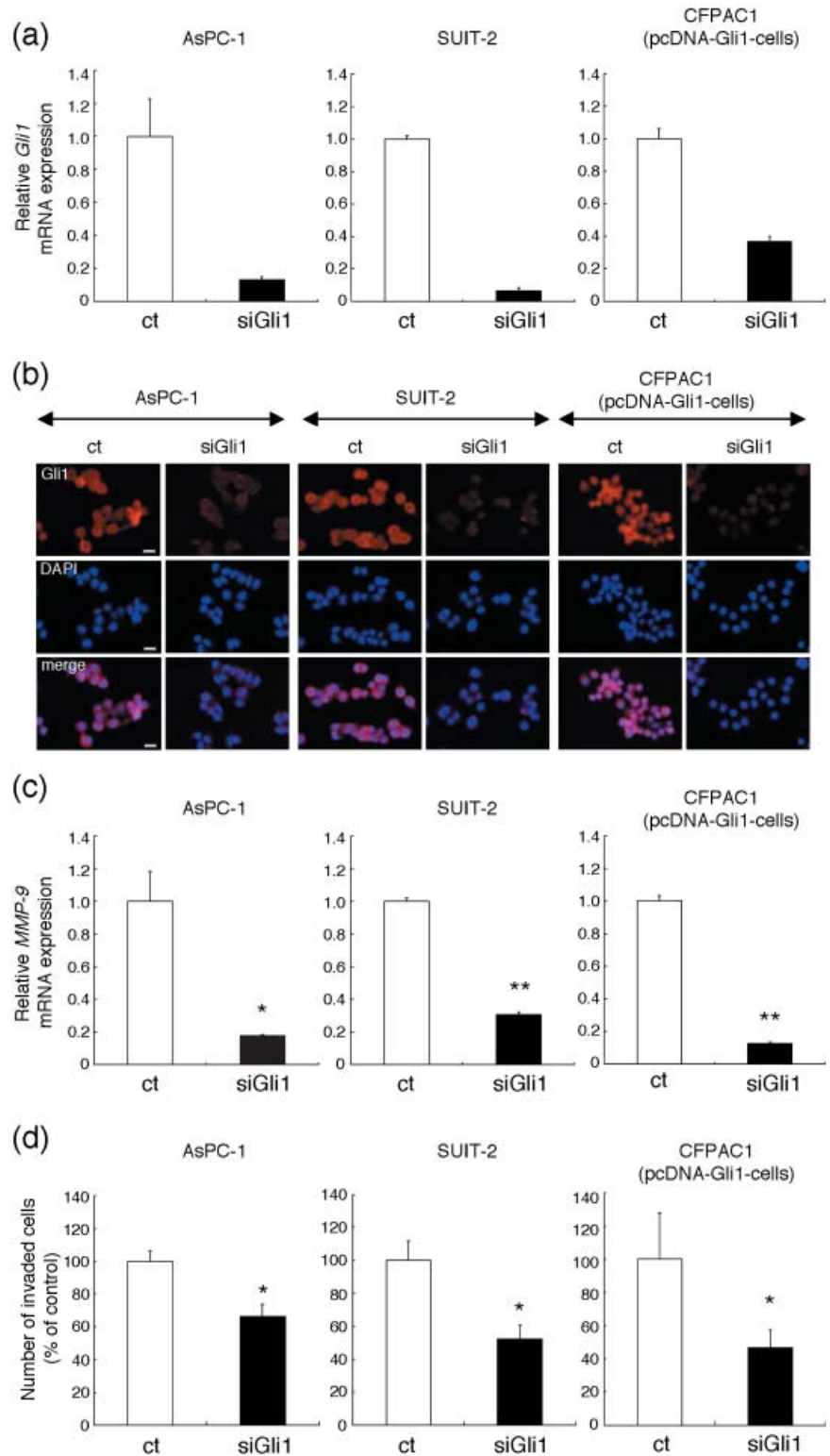


Fig. 5. RNA interference for *Gli1* inhibited the invasive ability of AsPC-1, SUIT-2, and pcDNA-Gli1-transfected CFPAC-1 human pancreatic ductal adenocarcinoma cells. (a and b) AsPC-1, SUIT-2, and pcDNA-Gli1-transfected CFPAC-1 cells (pcDNA-Gli1-cells) were transfected with small interfering RNA (siRNA) for *Gli1* (siGli1) or control siRNA (ct) for 48 h, and the expression of *Gli1* mRNA was assessed by real-time reverse transcription–polymerase chain reaction (RT-PCR). (a) The expression of *Gli1* protein was assessed by immunocytochemistry. *Gli1*, red; 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI), blue. Magnification, $\times 400$; scale bars, 20 μm (b). (c) Matrix metalloproteinase (*MMP-9*) mRNA relative to that of β -actin mRNA, was assessed by real-time RT-PCR. (d) After 48 h of transfection, these cells were seeded in a Matrigel-coated invasion chamber for 16 h. Quantitation of the assay is shown in the graph. Columns, means of three independent experiments; bars, SD. * $P < 0.05$; ** $P < 0.01$.

(Fig. 2). These findings indicate a close relationship between the Hh pathway and the invasive ability of pancreatic cancer cells.

How does the Hh pathway affect the invasive ability of pancreatic cancer cells? It is noteworthy that Hh pathway inhibition with cyclopamine affects neither the proliferation nor the migration ability of these cells, under the present experimental conditions (Fig. 1 and data not shown). These results indicate that the Hh pathway could play a relatively specific role in Matrigel degra-

ation. Most MMPs probably participate in the process of matrix degradation. The degradation of basement membrane type IV collagen is a critical early event in tumor invasion, suggesting that MMP-2 and MMP-9 might play a particularly important role.^(24,25) Therefore, we focused on MMP-2 and MMP-9. Real-time RT-PCR and gelatin zymography showed that cyclopamine and rhShh decreased and increased MMP-9 expression, respectively (Fig. 3). Furthermore, a *Gli1*-expressing

pancreatic cancer clones showed increased *MMP-9* mRNA expression and MMP-9 enzymatic activity (Fig. 4b,c), and the increased invasive ability of these cells was abolished by RNAi against MMP-9 (Fig. 4f). Thus, the Hh pathway contributes to the invasive ability of pancreatic cancer cells through MMP-9 induction.

It has been reported that aberrant activation of MMP-2 is more frequent in human pancreatic cancer than that of MMP-9.⁽²⁶⁾ Although we evaluated MMP-2 expression by gelatin zymography, we could detect only pro-MMP-2, not active-MMP-2. Moreover, the expression of pro-MMP-2 was not significantly correlated with Hh pathway activity. Considering these results we had come to a conclusion that the Hh pathway did not affect MMP-2 activity, at least not in the pancreatic cancer cells we used in this study.

It is important that stably *Gli1*-expressing pancreatic cancer cell clones (pcDNA-*Gli1* cells) showed a marked increase in their invasive ability (Fig. 4a) and increased active-MMP-9 expression (Fig. 4b), and that knockdown of *Gli1* resulted in suppression of the invasive abilities (Fig. 5d) and *MMP-9* mRNA expression (Fig. 5c). These data indicate that *Gli1* might play a crucial role both in the invasive ability and in the MMP-9 expression of these cells.

In our study, it is still unclear how the Hh pathway activates MMP-9 expression. Recently, it has been reported that the Hh pathway interacts with the Ras/MAPK pathway.⁽²⁷⁾ Because the MAPK pathway has been reported to regulate MMPs,⁽²⁸⁾ we

suggest that one of the possible mechanism of activating MMP-9 is the Ras/MAPK cascade.

Interestingly, Feldmann *et al.*⁽²⁹⁾ showed that the Hh pathway might play an important role in pancreatic cancer invasion and metastasis using a xenograft model. They focused on snail and E-cadherin as the key molecule of the Hh-related invasion, and suggested that epithelial-to-mesenchymal transition might participate in the Hh-related invasion in pancreatic cancer. In the present study, we also showed an important role for the Hh pathway in the invasive ability of pancreatic cancer cells. We focused on MMP-9 as the key molecule and suggested that degradation of the extracellular matrix also participates in the Hh-related invasion. We showed for the first time that MMP-9 plays an important role in the invasive ability mediated by the Hh pathway in pancreatic cancer.

We also showed that the Hh pathway, especially *Gli1*, contributes to pancreatic cancer invasion through MMP-9 induction. The results of the present study suggest that *Gli1* might become a new therapeutic target for inhibiting the invasion of pancreatic cancer cells.

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