# Effects and mechanisms of blocking the hedgehog signaling pathway in human gastric cancer cells

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**Abstract.** Excessive activation of the hedgehog (Hh) signaling pathway is important in a variety of human cancer cell types, including gastric cancer. However, the underlying mechanisms of the Hh signaling pathway in inducing gastric tumorigenesis and its downstream target genes are largely unknown. In the present study, the inhibitory effect of cyclopamine on the Hh signaling pathway was investigated in the human gastric cancer AGS cell line. It was identified that cyclopamine treatment inhibited the proliferation, migration and invasion of the AGS cells in a dose- and time-dependent manner, and resulted in the downregulation of a number of key Hh signaling pathway-associated factors [glioma-associated oncogene homolog 1, C-X-C chemokine receptor type 4 and transforming growth factor (TGF)-β1] at the RNA and protein levels. Furthermore, the secretion of TGF-β1 was significantly reduced following the administration of cyclopamine to the AGS cells. The results of the present study provided insight into the mechanisms by which the Hh signaling pathway regulates gastric cancer formation and identified the Hh signaling pathway as a potential novel therapeutic target in human gastric cancer.

## Introduction

The hedgehog (Hh) signaling pathway is important in embryonic cell differentiation, tissue development and organ formation (1-4). In mammals, sonic Hh (Shh), the glycoprotein ligand of Hh, binds to the transmembrane receptors Patched (Ptch) 1 and 2 to activate the Hh signaling pathway and relieve its suppression of the transmembrane protein Smoothened (Smo). Subsequently, the activated Smo protein induces nuclear translocation of a family of transcription factors, including glioma-associated oncogene homolog (Gli) 1, 2 and 3, to activate specific downstream target genes (2,5,6). Following maturation, Smo proteins are suppressed and the pathway is inactivated; however, if excessive activation mutations in the *Smo* gene and loss of function mutations in the *ptch* gene occur, Smo activity is not suppressed, and full-length Gli proteins are translocated to the nucleus. In the nucleus, Gli proteins activate downstream genes, such as c-myc and vascular endothelial growth factor (VEGF), resulting in excessive cell proliferation or tumorigenesis. Previous studies have identified that the Hh signaling pathway is involved in inducing cancer, including skin cancer (7), medulloblastoma (8), and lung (9,10), gastrointestinal (11-13), breast (14), prostate (15), ovarian (16) and endometrial cancer (17), in various mammalian systems. In addition, it has been demonstrated that inhibiting the Hh signaling pathway with a ligand-blocking antibody or Smo inhibitor, such as cyclopamine, may lead to the inhibition of the growth of tumor tissue (18,19).

Previous studies have indicated that the proliferation, migration and invasion of gastric cancer cells are associated with excessive Hh signaling. A study conducted in 90 gastric cancer patients identified that 70% of the collected gastric samples exhibited high Shh, Ptch1 and Gli1 expression levels (63/90 samples) (20). Additionally, excessive overexpression of Shh has been detected in intestinal metaplasia and stomach adenoma (21). A number of studies have also determined that the Hh signaling pathway appears to directly participate in cell proliferation and migration in the majority of gastric cancer cell lines, including the AGS, MKN1, MKN7, MKN45 and MKN74 cell lines (22,23).

Although the Hh signaling pathway is critical in inducing gastric tumorigenesis, the underlying cellular and molecular mechanisms are largely unknown. In the present study, cyclopamine was used to specifically block the Hh signaling pathway in the human gastric cancer AGS cell line, and its effect on cell proliferation, migration and invasion were evaluated in a dose- and time-dependent manner. Furthermore, the mechanism of this inhibition was investigated by examining the protein and RNA expression levels of key factors associated with the Hh signaling pathway, Gli1, C-X-C chemokine receptor type (CXCR) 4 and transforming growth factor (TGF)-β1, as well as determining the rate of TGF-β1 protein secretion in the AGS cells.

## Materials and methods

*Cell culture and treatment*. The human gastric carcinoma AGS cell line was obtained from the Shanghai Institute of Biological

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Sciences, Chinese Academy of Science (Shanghai, China). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA) and 100 U/ml penicillin/streptomycin. Various concentrations of cyclopamine (2.5, 5, 10, 20, 40 and 80 $\mu$M; EMD Millipore, Billerica, MA, USA) were added to the medium and the cells were maintained at 37˚C in a humidified atmosphere containing 5% $CO_2$ for 24, 48 or 72 h.

*Cell proliferation assay*. The AGS cells were plated at a concentration of $2.5x10^4$ cells/ml culture medium in 96-well plates and treated with the abovementioned concentrations of cyclopamine, in triplicate. After 24, 48 and 72 h, the number of viable cells were determined by performing an MTT assay (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. Briefly, cells were seeded at $1x10^4$ cells/well in 96-well plates overnight, then the cells were treated with cyclopamine for 24, 48 or 72 h. Subsequently, 20 $\mu$l MTT solution was added and after 4 h the medium was gently aspirated and 150 $\mu$l DMSO (Sigma-Aldrich) was added to each well to dissolve any formazan crystals. The plate was shaken for 10 min to allow for complete solubilization. Cell viability was determined spectrophotometrically by measuring the absorbance at 490 nm using a 96-well plate reader (Multiskan MK3, Thermo Fisher Scientific, Waltham, MA, USA) and the results were calculated as the mean of eight wells per group. Each experiment was performed in 8 wells a minimum of three times independently.

*Apoptosis assay*. After 24 h in culture with 0, 40 and 80$\mu$M cyclopamine, $1x10^6$ gastric cancer cells were washed twice with phosphate-buffered saline (PBS) and resuspended in binding buffer (10 mM HEPES/NaOH, 140 mM NaCl and 2.5 mM $CaCl_2$). Fluorescein isothiocyanate Annexin V (BD Biosciences, Franklin Lakes, NJ, USA) was added at a final concentration of 1 mg/ml, followed by 10 mg/ml propidium iodide. The mixture was incubated for 10 min in the dark at room temperature and subsequent cell counting was conducted using a FACScan™ flow cytometer with CellQuest™ software (BD Biosciences).

*Matrigel invasion assay*. A migration assay was performed using a quantitative cell migration assay kit (ECM500; EMD Millipore), according to the manufacturer's instructions. Briefly, serum-free RPMI-1640 medium (200 $\mu$l) was added to the extracellular matrix layer in the upper chamber and allowed to hydrate for 1-2 h at ambient temperature. The cells were dislodged following brief trypsinization, dispersed into homogeneous single-cell suspensions, washed and resuspended in serum-free medium at a concentration of $5x10^5$ cells/ml. The cell suspension (100 $\mu$l) was applied to the surface and allowed to adhere for 1 h at 37˚C, and 500 $\mu$l migration medium containing 0, 2.5, 5 or 10 $\mu$M cyclopamine was added to the bottom chamber. After 24 h of incubation at 37˚C in an atmosphere of 5% $CO_2$ in air, cells within the inserts were removed from the upper membrane surface using a moist cotton-tipped swab. Invasive cells on the lower membrane surface, which had migrated through the polycarbonate membrane with a precoated thin layer of basement membrane matrix, were fixed in 100% ethanol and were rinsed with PBS. After being air-dried and photographed, the cells in the upper chamber were stained with crystal violet (AppliChem GmbHm, Darmstadt, Germany) for 20 min and dissolved in 10% acetic acid. Finally, the optical density was read at an absorbance of 560 nm on a standard microplate reader (Multiskan MK3, Thermo Fisher Scientific).

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. Total RNA was isolated from $2x10^6$ AGS cells treated with 0, 2.5, 5 or 10 $\mu$M cyclopamine for 24 h using TRIzol® reagent (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. First strand complementary (c)DNA synthesis and amplification were performed using a Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific), and the qPCR was performed using an iQ5 Multicolor Real-Time PCR Detection system (Bio-Rad Laboratories, Hercules, CA, USA) in 96-well plates. The PCR was run in a 20-$\mu$l reaction containing 1 $\mu$l DNA template, 0.2 $\mu$l Taq polymerase, 2 $\mu$l dNTPs, 0.2 $\mu$l each primer and 2 $\mu$l 10X Taq buffer. The mixture was incubated at 95˚C for 5 min, followed by 25 cycles at 95˚C for 40 sec, 58˚C for 40 sec and 72˚C for 1 min, with a final extension at 72˚C for 10 min. Cycle threshold values were obtained using ABI PRISM® 7000 software (Applied Biosystems, Foster City, CA, USA) and the fold change of relative mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: Forward, 5'-TCCTTTGGGGTCCAGCCTTG-3' and reverse, 5'-ATGCCTGTGGAGTTGGGGCT-3' for Gli1; forward, 5'-TGGAGCTGGTGAAGCGGAAG-3' and reverse, 5'-TTTCCACCATTAGCACGCGG-3' for TGF-β1; forward, 5'-TCAGTCTGGACCGCTACCTG-3' and reverse, 5'-CCACCCACAAGTCATTGGGG-3' for CXCR4; and forward, 5'-AGGTCGGAGTCAACGGATTTG-3' and reverse, 5'-GTGATGGCATGGACTGTGGT-3' for GAPDH.

*Western blot analysis*. Whole-cell collection of AGS cells treated with 0, 2.5, 5 or 10 $\mu$M cyclopamine for 24 h was conducted using radioimmunoprecipitation assay buffer [50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate and 1% sodium deoxycholate (pH 7.4)] supplemented with protease inhibitor. Following protein concentration determination using a Bio-Rad protein assay kit (Bio-Rad Laboratories), the protein lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Hybond™-P; GE Healthcare Life Sciences, Chalfont, UK). The membranes were blocked with PBS containing 0.2% Tween 20 and 5% skimmed dry milk, and incubated with primary rabbit anti-human polyclonal antibodies against Gli1 (1:1,000, cat. no. AB3444, Millipore) and CXCR4 (1:500, cat. no. AB1846, Millipore), rabbit monoclonal antibody against TGF-β1 (1:1,000, cat. no. 3709, Cell Signaling Technology, inc., Beverly, MA, USA) and β-actin (1:500, cat. no. sc-130656, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) followed by a horseradish peroxidase-labeled goat anti-rabbit secondary antibody IgG-HRP (1:5,000, cat. no. sc-2004, Santa Cruz Biotechnology, Inc.). Finally, X-ray film was used to image the western blots and determine protein expression levels.

*TGF-β1 quantification*. After 24 h of cell culture in various concentrations of cyclopamine, the quantity of TGF-β1 released into the culture supernatant was measured using an ELISA kit (Fujirebio Diagnostics, Inc., Malvern, PA, USA), according to

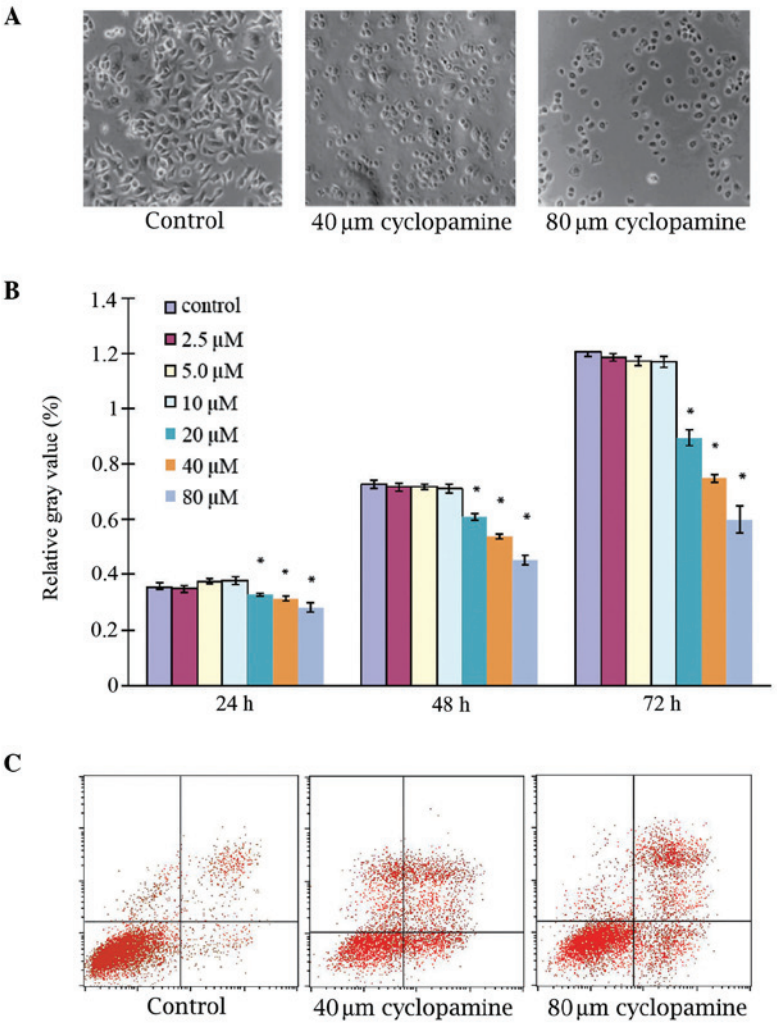


Figure 1. Effect of cyclopamine on AGS cell proliferation and apoptosis. (A) Microscopy images of AGS cells treated with 0, 40 and 80 $\mu$M cyclopamine for 48 h (magnification, x100). (B) AGS cells were treated with various concentrations of cyclopamine for 24, 48 and 72 h. AGS cell proliferation analyzed by performing an MTT assay demonstrated that at 20-80 $\mu$M cyclopamine, cell proliferation decreased in a concentration-dependent manner. *P<0.05. (C) Evaluation of apoptosis by annexin V-fluorescein isothiocyanate/propodium iodide staining and flow cytometry in AGS cells treated with 0, 40 and 80 $\mu$M cyclopamine for 24 h. Error bars represent the standard error of the mean.

manufacturer's instructions. Absorbance was determined at a wavelength of 490 nm.

*Statistical analysis*. Data were analyzed using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). All the results were obtained in triplicate and are presented as the mean ± standard error of the mean. Comparisons were made by one-way analysis of variance or Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Cyclopamine inhibits the proliferation of AGS cells*. Examination of the effects of cyclopamine administration on AGS cell proliferation identified that the untreated AGS cells grew as an adherent monolayer, established cylindrical shapes and exhibited nuclei that were located at the proximal pole of the cell bodies (Fig. 1A). Upon AGS cell treatment with 40 and 80 $\mu$M cyclopamine for 48 h, cell growth was markedly inhibited, exhibiting a diminished three-dimensional appearance and increased inter-cellular gaps. Quantitative measurement identified that cyclopamine inhibited the growth of the AGS cells in a dose-dependent manner; however, when the AGS cells were treated with 2.5, 5 and 10 $\mu$M cyclopamine for 24, 48 or 72 h, the proliferation rates were not significantly different to those under control conditions (P>0.05), indicating that cyclopamine at a concentration range of 2.5-10 $\mu$M may not affect cell proliferation. However, cyclopamine significantly inhibited cell proliferation at higher concentrations (20, 40 and 80 $\mu$M; P<0.05; Fig. 1B).

*Cyclopamine induces apoptosis in AGS cells*. Examination of the effects of cyclopamine on AGS cell apoptosis was conducted by flow cytometric analysis. Annexin staining was used to determine the effect on apoptosis 24 h after the treatment of the cancer cells with 40 or 80 $\mu$M cyclopamine (Fig. 1C; Table I). Under controlled conditions (untreated), no increase in AGS cell apoptosis was observed, however, the administration of cyclopamine appeared to induce significant apoptosis in the AGS cells. The early and late apoptotic rates were 2.34±0.90

Table I. Percentage of cell apoptosis induced by 40 and 80 $\mu$M cyclopamine.

| Apoptosis | Control | 40 $\mu$mol/l | 80 $\mu$mol/l |
|---|---|---|---|
| Early | 2.34±0.90 | 13.53±1.27[a] | 20.89±7.72[a,b] |
| Late | 4.05±0.87 | 16.12±1.63[a] | 22.06±0.98[a,b] |

[a]P<0.05 vs. control. [b]P<0.05 vs. 40 $\mu$M.

Table II. Effect of cyclopamine administration on TGF-β1 secretion in AGS cells.

| Cyclopamine, $\mu$mol/l | TGF-β1, $\mu$g/l |
|---|---|
| 0.0 (control) | 5.935±0.825 |
| 2.5 | 5.268±0.638 |
| 5.0 | 3.527±0.539[a] |
| 10.0 | 1.947±0.635[b] |

[a]P<0.05 and [b]P<0.01 vs. control. Differences were analyzed by performing a Student's t-test. TGF, transforming growth factor.

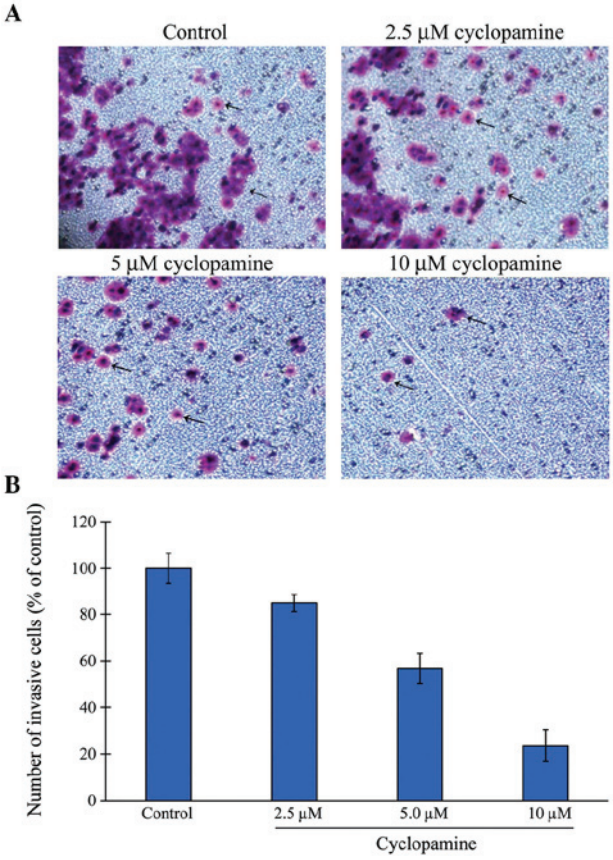


Figure 2. Cyclopamine inhibits AGS cell invasion. (A) Invasion of AGS cells through Matrigel in the Transwell assay. Arrows indicate invaded AGS cells (magnification, x100). (B) AGS cell invasion in response to a 24-h treatment with cyclopamine at concentrations of 2.5, 5 and 10 $\mu$M. Data from three separate experiments are combined and expressed as an invasion index, whereby the degree of invasion in untreated AGS cells was set at 100%. Differences were analyzed by one-way analysis of variance. Error bars represent the standard error of the mean.

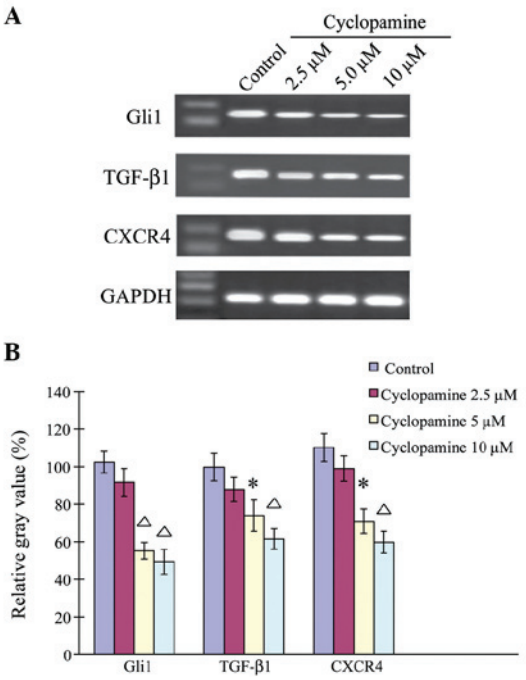


Figure 3. (A) Reverse transcription-polymerase chain reaction demonstrated that cyclopamine downregulated mRNA expression levels in the AGS cells. (B) Quantification of the gel identified that Gli1, TGF-β1 and CXCR4 expression levels were significantly downregulated in the AGS cells treated with 5 or 10 $\mu$M cyclopamine for 24 h. Differences were analyzed by one-way analysis of variance. *P<0.05; ΔP<0.01. Error bars represent the standard error of the mean. Gli1, glioma-associated oncogene homolog; TGF, transforming growth factor; CXCR4, C-X-C chemokine receptor type.

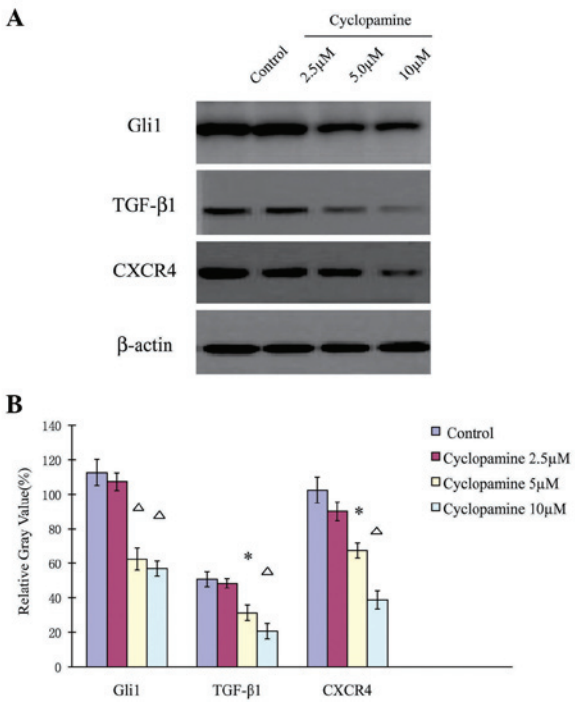


Figure 4. (A) Western blotting demonstrated that Gli1, TGF-β1 and CXCR4 protein expression levels were downregulated in the AGS cells treated with cyclopamine for 24 h. (B) Quantification of the western blotting revealed that treatment of the AGS cells with 5 or 10 $\mu$M cyclopamine significantly reduced the expression levels of all three proteins. Differences were analyzed by one-way analysis of variance. *P<0.05; ΔP<0.01. Error bars represent the standard error of the mean. Gli1, glioma-associated oncogene homolog; TGF, transforming growth factor; CXCR4, C-X-C chemokine receptor type.

and 4.05±0.87%, respectively, for the control group. After 24 h of cyclopamine treatment, the proportion of early and late apoptotic cells was increased in a dose-dependent manner and was significantly higher than that of the control group (P<0.05).

*Cyclopamine reduces motility and invasiveness of AGS cells.* The ability to invade a reconstituted basement membrane is an important phenomenon that distinguishes cancer cells from other cell types (20). Thus, the effect of cyclopamine on cellular motility and invasion of the AGS cells was evaluated by treatment with doses of cyclopamine low enough to not affect AGS cell proliferation and apoptosis. The cancer cells were untreated or treated with cyclopamine at concentrations of 2.5, 5 and 10 $\mu$M, and maintained for 24 h. As hypothesized, the AGS cells demonstrated a moderate rate of invasion under the control conditions, however, upon cyclopamine treatment, baseline invasion was diminished. A dose-response effect was observed such that 10 $\mu$M resulted in the least degree of invasion (Fig. 2).

*Cyclopamine downregulates Hh-associated genes in AGS cells.* The effects of cyclopamine on gene regulation were then examined in the AGS cells (Fig. 3). The AGS cells were treated with 2.5, 5 and 10 $\mu$M cyclopamine for 24 h. Quantitative measurement showed that cyclopamine downregulated the genes in the AGS cells in a dose-dependent manner. When the AGS cells were treated with 2.5 $\mu$M cyclopamine for 24 h, the gene expression levels of Gli1, TGF-β1 and CXCR4 were similar to those under control conditions (P>0.05). When the AGS cells were treated with 5 or 10 $\mu$M cyclopamine, the Gli1, TGF-β1 and CXCR4 gene expression levels were significantly downregulated (P< 0.05).

*Cyclopamine downregulates Hh-associated proteins in AGS cells.* Consistent with its effect on mRNA expression level, cyclopamine additionally reduced Hh-associated protein expression levels in a dose-dependent manner (Fig. 4). When the AGS cells were treated with 2.5 $\mu$M cyclopamine for 24 h, the protein expression levels of Gli1, TGF-β1 and CXCR4 were similar to those under control conditions (P>0.05). However, the AGS cells that were treated with 5 or 10 $\mu$M cyclopamine exhibited significantly downregulated Gli1, TGF-β1 and CXCR4 protein expression levels (P<0.05).

*Cyclopamine inhibits TGF-β1 secretion in AGS cells.* Following the observations that cyclopamine appears to inhibit cancer cell invasion and downregulate the mRNA and protein expression levels of Shh-associated genes, the effect of cyclopamine on the TGF-β signaling pathway in the AGS cells was examined in attempt to elucidate the mechanism of these observations. As indicated in Table II, when the AGS cells were treated with 2.5 $\mu$M cyclopamine for 24 h, the quantity of TGF-β1 identified in the collected supernatant was similar to that observed under control conditions (P>0.05). However, in the AGS cells treated with 5 and 10 $\mu$M cyclopamine, TGF-β1 secretion was significantly reduced (P<0.05).

## Discussion

The Hh signaling pathway was initially recognized for its role in modulating embryonic cell proliferation and differentiation (1-4); however, more recently, it has been demonstrated that Hh is important in the proliferation of various types of cancer cells, including lung, pancreatic and gastric cancer cells (12,14,16,24-26).

While the mechanisms of the Hh signaling pathway in promoting gastric tumorigenesis and regulating downstream target genes are largely unknown, various lines of evidence indicate that a number of key factors, such as TGF-β1 and CXCR4, are actively involved. Previous studies have demonstrated that TGF-β mRNA is overexpressed in gastric carcinoma (27,28), and that the Hh pathway may promote cancer cell mobility via activation of the TGF-β/activin receptor-like kinase-Smad3 pathway in gastric cancer cell lines, such as MKN-28 (29). In addition, it has previously been demonstrated that TGF-β may induce cancer migration via the c-Jun N-terminal kinase or extracellular signal-regulated kinase pathways (30). The chemokine receptor, CXCR4, and its cognate ligand, C-X-C ligand type 12, are expressed in various types of tissue and have been proposed as regulators of the directional trafficking and invasion of tumor cells, such as breast, endometrial and prostate cancer cells (31-34). Furthermore, CXCR4 is expressed in gastric carcinoma, as well as gastric cancer cell lines, and appears to be highly associated with lymph node metastasis and a high tumor stage (35).

The present study demonstrated that by blocking the Hh signaling pathway with cyclopamine, the proliferation and migration of gastric cancer AGS cells could be significantly reduced. Furthermore, it was identified that the mRNA and protein levels of Gli1, TGF-β1 and CXCR4 were coordinately downregulated in the cyclopamine-treated AGS cells, and that the quantity of TGF-β1 secreted into the culture supernatant was significantly reduced following a 24-h treatment with 5 and 10 $\mu$M cyclopamine. These findings are in agreement with a number of previously conducted studies (25,36,37). To further elucidate the role of Hh as an important regulator in AGS cells, the present study demonstrated that the Hh signaling pathway appears to regulate tumor invasion and metastasis via TGF-β1 and CXCR4. Furthermore, the current study demonstrated that blocking the Hh signaling pathway downregulated TGF-β1 and CXCR4 expression, thus, inhibiting human gastric cancer cell invasion and metastasis. In conclusion, the present study identified that blocking the Hh signaling pathway by cyclopamine administration may serve as a potential therapeutic strategy for the prevention and treatment of gastric cancer invasion in human cancer patients.

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