Role of Hedgehog Signaling in Ovarian Cancer

Resham Bhattacharya,1 Junhye Kwon,1 Bushra Ali,1 Enfeng Wang,1 Sujata Patra,1 Viji Shridhar,2 and Priyabrata Mukherjee1,3

Abstract

Objective: In humans, several distinctive cancers result from mutations that aberrantly activate hedgehog (HH) signal transduction. Here, we investigate the role of HH signaling in ovarian cancer.

Experimental Design: We assessed the expression of different components of hedgehog pathway in primary tumor samples and cell lines. We used specific hedgehog pathway blocker to study the effect on clonal growth and proliferation of ovarian cancer cell both in vitro and in vivo.

Results: We show that the up-regulation of several HH pathway components is a common feature of primary ovarian tumors and cell lines. However, expression of PATCHED1 (PTCH1), a direct transcriptional target of the HH pathway, is down-regulated in ovarian cancer in direct contrast to the expression observed in other adult solid tumors. Cyclopamine, a specific HH pathway inhibitor, inhibits the proliferation and clonal growth of ovarian tumor cells in vitro and arrests ovarian tumor growth in vivo. Expression of BMI-1, a polycomb gene, is down-regulated in ovarian cancer cells following cyclopamine treatment. Overexpression of PTCH1 phenocopied the effects of cyclopamine; it down-regulated BMI-1 and reduced clonal growth in ovarian cancer cell lines. Furthermore, knocking down BMI-1 using small interfering RNA also inhibited the clonal growth of all the ovarian cancer cell lines tested.

Conclusions: In brief, the constitutive low-level expression of PTCH1 contributes to proliferation and clonal growth of ovarian cancer cells by an aberrant HH signal. Because the HH pathway can be inhibited by specific inhibitors, these findings point toward possible new treatments to inhibit ovarian cancer growth.

Ovarian cancer has few symptoms early in its course; therefore, the majority of patients are diagnosed with advanced-stage disease. It has a 5-year survival rate of only 20% to 25% (1). Despite the initial response to surgical debulking of the tumor and the front-line chemotherapy with carboplatin and paclitaxel, most tumors eventually develop the drug resistance, causing patients to succumb to their disease (2, 3). Thus, it is the most lethal of the gynecologic malignancies (3).

Hedgehog (HH) proteins form a small and highly conserved family of intercellular signaling molecules. In Drosophila, the HH protein plays several distinct roles in the development including gonadal development and function (4). Mammals have three HH proteins: sonic HH (Shh), Indian HH (Ihh), and desert HH (Dhh). All of these proteins show the conservation of structure with the single HH proteins in Drosophila (5). The different HH proteins are implicated in the etiology of several human congenital malformations and cancers (6–9). The 12-transmembrane protein PATCHED1 (PTCH1) acts as a common HH receptor. In the absence of HH ligand, PTCH1 inhibits the signaling of the seven-transmembrane G-protein-coupled protein SMOOTHEENED (SMO; ref. 10). In humans, the germ-line mutations in PTCH1 result in the development of medulloblastoma and basal cell carcinoma, suggesting that its role in inhibiting HH signaling is associated with a critical function as a tumor suppressor (11).

HH binding inactivates the PTCH1 function, making SMO available to initiate the intracellular signaling. Signaling by SMO transduces HH signals using the Gli family of transcription factors as downstream effectors (12–14). HH signaling has not been observed in the mature vertebrate ovary where proliferation of germ cells no longer occurs (15). In contrast, HH was found to act specifically on the stem cells in the Drosophila ovary (4).

Recent studies suggest that multipotent somatic stem cells may be present in the ovary and that these cells are responsible for the regulated repair of the surface epithelium after the ovulatory rupture and possibly the generation of oocyte nurse cells for folliculogenesis (16–18). However, under abnormal conditions that lead to the aberrant activation of HH signaling, repeated asymmetric self-renewal of somatic stem cells lead their immediate progenitors to accrue mutations over time, which might ultimately lead to their transformation into the

Authors' Affiliations: Departments of 1Biochemistry and Molecular Biology, 2Experimental Pathology, and 3Biomedical Engineering, College of Medicine, Mayo Clinic, Rochester, Minnesota

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Requests for reprints: Priyabrata Mukherjee or Resham Bhattacharya. Departments of Biochemistry and Molecular Biology and Biomedical Engineering, College of Medicine, Mayo Clinic, Rochester, MN 55905. Phone: 507-284-8963; Fax: 507-284-1767; E-mail: Mukherjee.Priyabrata@mayo.edu or bhattacharya.resham@mayo.edu.

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cancer stem cells and malignant progression (12, 17). Recently, a side population of cells has also been characterized in ovarian cancer that possesses stem cell character (17). Furthermore, loss of heterozygosity in ovarian tumors at the PTCH1 locus (chromosome 9q22-31) is a frequent and early event in ovarian cancer (19). However, a role for PTCH1 as a tumor suppressor in ovarian cancer has not been defined.

Here, we determine the role of HH signaling in ovarian cancer. Our observations suggest that HH signaling is active in human ovarian cancer and responsible for clonal growth and proliferation in ovarian cancer cells.

**Materials and Methods**

**Reagents.** [3H]thymidine was from Amersham Biosciences. The antibodies were purchased from the following companies: SMO (SC13943), PTCH1 (SC6149), and Gli-1 (SC6153) were from Santa Cruz Biotechnology; BMI-1 (37-5400) was from Zymed; and 5E1 antibody was obtained from Developmental Studies Hybridoma Bank, University of Iowa.

**Translational Relevance**

This work has direct clinical implications in the treatment of ovarian cancer. The HH pathway has been implicated in the development of several cancers including basal cell carcinoma and medulloblastoma. Currently, an analogue of cyclopa-mine, GDC-0449 (HH antagonist) is being tested in phase II clinical trials in metastatic colorectal cancer. Here, we show that all the components of the HH pathway are present in ovarian cancer. We further show that this pathway is active and can be blocked by cyclopa-mine and 5E1 (Shh-inhibiting antibody). Cyclopa-mine causes a decrease in cancer cell proliferation in vitro and inhibits the tumor growth in vivo. Furthermore, in contrast to other solid tumors, levels of PTCH1 in ovarian cancer are low and overexpression results in decreased clonal growth. Therefore, our work suggests that, in the future, inhibition of HH pathway may be a potential therapeutic target in ovarian cancer.

Fig. 1. Signaling components of the HH pathway are expressed in primary ovarian tumors. Total RNA was collected from 19 primary tumor samples and 10 pooled normal ovarian samples and subjected to real-time PCR using primers for PTCH1 (A), Gli-1 (B), SMO (C), Shh (D), and β-actin. Mean of three individual experiments.
Cell culture. OVCAR-5 cells were purchased from the American Type Culture Collection and grown in DMEM with 10% fetal bovine serum and 1% antibiotic (penicillin/streptomycin) according to the provider’s recommendation. OV-167, OV-202, and OSE (tsT) cell lines were established and grown in MEM supplemented with 10% and 20% fetal bovine serum, respectively, and 1% antibiotic (penicillin/streptomycin) as described previously (20).

Cell proliferation assay. Ovarian cells ($2 \times 10^4$) were seeded in 24-well plates and cultured for 24 h. After 24 h, the cells were treated with respective concentrations of cyclopamine. For Shh-N monoclonal antibody, 5E1, treatment, antibody was added 4 h after plating the cells. Forty-eight hours later, $1 \mu$Ci [3H]thymidine was added; 4 h later, cells were washed with chilled PBS, fixed with 100% cold methanol, and collected for measurement of TCA-precipitable radioactivity. Experiments were repeated at least three times each time in triplicate and assay was done as described previously (21).

RNA collection, cDNA synthesis, and real-time PCR analysis. Ovarian cells ($5 \times 10^3$) were cultured in 60 mm dish. After 24 h, the cells were treated with cyclopamine. RNA was extracted after 48 h using RNeasy Kit (Qiagen). cDNA was prepared with SuperScript III (Invitrogen) using standard protocol. Primers for real-time PCR for human PTCH1 (A0225A), Gli-1 (PPIH00153A), and Shh (02405A) were obtained from Super Array. Real-time PCR was done as described below. An ABI System Sequence Detector 7500 (Applied Biosystems) was used with the following regimen of thermal cycling: stage 1: 1 cycle, 15 min at 95°C and stage 2: 40 cycles, 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Similarly, in case of primary tumors, total RNA was extracted using RNeasy kit (Qiagen). However, in case of normal samples, cDNA synthesized from 10 normal ovarian cell brushings from patients without cancer was pooled.

Relative mRNA levels were calculated with respect to the OSE (tsT) cell line or normal ovarian-pooled samples as follows. Real-time PCR for the housekeeping gene, $\beta$-actin, was done for each test sample along with target gene of interest (e.g., Gli-1). To normalize the value of Gli-1 for each reaction condition, the value of $\beta$-actin at that condition was deducted and the resulting value was designated as $D$. Therefore, $D = CT(\text{Gli-1 sample}) - CT(\text{$\beta$-actin sample})$. To calculate the relative expression first $\Delta D$ was determined by deducting the $D$ value of the control sample from the treated sample. Therefore, $\Delta D = D(\text{Gli-1 value for OVCAR-5}) - D(\text{Gli-1 value for OSE}).$ Finally, relative RNA amount in comparison with the control was calculated by using the formula: $2^{-\Delta D}$. Average and SD for three experiments, each sample in triplicate, were calculated (22).

Western blot experiments. Harvested ovarian cancer cells, both treated and nontreated, were washed in PBS and lysed in ice-cold radioimmunoprecipitation assay buffer with freshly added 0.01% protease inhibitor cocktail (Sigma) and incubated on ice for 30 min.
Cell debris was discarded by centrifugation at 13,000 rpm for 10 min at 4°C and the supernatant (30-50 µg protein) was run on an SDS-PAGE.

**Transfection experiments.** The ovarian cells OSE (tsT), OVCAR-5, OV-167, or OV-202 were grown in their respective antibiotic free medium for 2 days before electroporation. Subsequently, the cells were trypsinized and resuspended in cytomix buffer at 4°C to 10^6/mL [10 mmol/L K_2HPO_4/KH_2PO_4 (pH 7.6), 120 mmol/L KCl, 25 mmol/L HEPES, 0.15 mmol/L CaCl_2, 5 mmol/L MgCl_2, 2 mmol/L EGTA]. These cells were then electroporated (325 V) with 5 µg PTCH1 overexpression vector (gift from Dr. Neil Watkins, Johns Hopkins University) or control green fluorescent protein plasmid using the Bio-Rad Gene Pulser. Forty-eight hours after transfection, the cells were lifted and plated for proliferation or clonogenic assay. Small interfering RNA (siRNA) experiments were done similarly with electroporation done with 2 ng siRNA per sample.

**Clonogenic assays.** Clonogenic assays were done as follows: OSE (tsT), OV-167, OV-202, and OVCAR-5 cells were grown in their corresponding medium containing appropriate amount of serum and antibiotic with a concentration of 400, 100, 50, and 200 cells/mL, respectively. The cells were treated with cyclopamine at 5 µmol/L. Colonies were counted after staining the cells with 0.1% crystal violet within 5 to 10 days after plating the cells. However, in case of PTCH1 overexpression and BMI-1 knockdown experiments, cells were grown for 2 days in antibiotic-free medium before transfection. Forty-eight hours after transfection, cells cultured in antibiotic-free medium were used for clonogenic assay and assays were done in antibiotic-free medium.

**In vivo nude mice experiments.** OVCAR-5 cells (2 x 10^6) were injected subcutaneously in one flank of 4- to 6-week-old female nude mice. After 21 days, when the tumors were ~80 mm^3 in size, intraperitoneal administration of cyclopamine (50 mg/kg/d) began and was continued until day 18. Mice in the treatment group (n = 8) received cyclopamine (supplied by Infinity Pharmaceuticals) in cyclodextrin and the control group (n = 8) received only the vehicle. In brief, the vehicle was prepared by mixing a 10% solution of 2-hydroxypropyl-β-cyclodextrin with 0.1 mol/L sodium citrate in a phosphate buffer. Tumors were measured twice a week. Tumor volume was measured by the formula: V = ab^2 / 2, where V is the tumor volume, a is the length, and b is the width of the tumor.


**Results and Discussion**

Components of the HH signaling pathway are expressed in primary ovarian tumors and ovarian cancer cell lines. Because loss of heterozygosity at 9q22-31, the locus for the PTCH1 gene, is a frequent event in ovarian cancer (19), we wanted to determine if the HH pathway is active in primary human tumors. To that end, we performed real-time PCR from cDNAs isolated from 19 primary human tumor tissues to look for different components of the HH pathway and compared them with the normal ovarian tissue controls (n = 10). Unexpectedly, expression of PTCH1, a commonly used marker of canonical HH pathway activation, was up-regulated by ≥5-fold in only 1 primary tumor sample when compared with the normal control (Fig. 1A). By contrast, the up-regulation in expression (≥5-fold) of all of the other components of the HH pathway were far more frequent compared with the normal controls: Gli-1 (9 of 19; Fig. 1B), SMO (9 of 19; Fig. 1C), and Shh (10 of 19; Fig. 1D). This transcriptional analysis suggested that HH signaling could be active in primary ovarian tumors.

Because expression of these genes in primary tumors could originate from tumor cells or benign stroma, we studied their expression in ovarian tumor cell lines (Fig. 2). Again, in contrast to a single report thus far (23), relative mRNA expression of PTCH1 was lower compared with OSE (tsT; nonmalignant ovarian surface epithelial cells) in each of the cancer cell lines tested (Fig. 2A). Western blot analysis showed that PTCH1 protein levels in all of the three human ovarian cancer cell lines were also low compared with OSE (tsT; Fig. 2A, inset).

Interestingly, HH pathway activation is usually associated with increased expression of PTCH1. In case of ligand-dependent activation, the inhibitory effect of PTCH1 on SMO is relieved on ligand binding to PTCH1 (7, 8, 24). However, for the ligand-independent activation, particularly in the case of medulloblastoma, this is caused either by mutations that render SMO insensitive to regulation by PTCH1 or by mutational inactivation of PTCH1 (24). Therefore, down-regulation of PTCH1 in all of the human ovarian cancer cell lines suggest, at least in vitro, a potential for the involvement of ligand-independent activation of SMO leading to activation of HH signaling. By contrast, we observed increased Gli-1 mRNA and protein expression in these cells (Fig. 2B and inset of Fig. 2B). Gli-1 and Gli-2 are the prime transcriptional effectors of HH signaling. Constitutive activation of at least one of them is critically important for tumor growth. Thus, higher Gli-1 expression in primary tumors is suggestive of persistent activation of HH signaling in ovarian cancer (25, 26). Similarly, increased expression of both mRNA and protein of SMO and Shh were observed compared with their respective normal controls (Fig. 2C, inset, and Fig. 2D, inset, respectively). These data differ from reports of HH pathway expression in other adult solid tumors, in which coexpression of Shh, PTCH1, and Gli-1 is associated with HH pathway activation (22).

Inhibition of HH signaling by cyclopamine results in inhibition of proliferation of ovarian cancer cell lines in vitro and tumor growth in vivo. To test the role of HH pathway activity in tumor growth, we used cyclopamine, a plant derivative that specifically inhibits HH signaling at the level of SMO (7, 8, 22, 27). Figure 3A shows the effect of cyclopamine on proliferation of all the human ovarian cancer cell lines used in the present study. By 48 h, cyclopamine treatment resulted in a 70% to 90% inhibition in proliferation of all the human ovarian cancer cell lines tested, with maximum inhibition observed at the highest dose (5 μmol/L). However, normal epithelial cells, OSE (tsT), were least sensitive to cyclopamine, suggesting preferential activation of HH signaling in ovarian cancer. This is also corroborated by the low expression of HH components in the OSE (tsT) cells compared with the malignant cells. The Gli-1 transcript levels were also decreased 60% to 80% with the increasing concentrations of cyclopamine (Fig. 3B). Gli-1 at the protein level was also down-regulated by the cyclopamine treatment in a dose-dependent manner (Fig. 3C). However, the effect of cyclopamine on Gli-1 in OSE (tsT) cells is less pronounced. These results correlate with the inhibition in proliferation observed in these cell lines with the same concentrations of cyclopamine and are consistent with a pathway-specific effect of cyclopamine at the level of SMO. In accordance with the in vitro result, we also found significant inhibition (P < 0.05) in tumor growth in a mouse xenograft model generated with OVCAR-5 cells on cyclopamine treatment (Fig. 3D).

Because Shh expression was detected in all the ovarian cancer cell lines both at the transcript and at the protein levels (Fig. 4), we wanted to determine the requirement for HH ligand in ovarian cancer cell growth. Because the level of Shh in OSE (tsT) is very low, we did not perform this experiment with this particular cell line. We incubated the ovarian cancer cells with a 5E1-Shh-N monoclonal antibody that inhibits the binding of Shh to PTCH1 (22) and determined the proliferation after 48 h by [3H]thymidine incorporation. We observed a significant dose-dependent decrease in proliferation of OV-202, OVCAR-5, and OV-167 cell lines (Fig. 4). These data suggest that the expression of Shh contributes to the proliferation of ovarian cancer cells through sustained HH pathway activation.
HH signaling maintains clonal growth in ovarian cancer cells. Cancer cells express transcripts indicative of a progenitor cell type (22) and retain intrinsic stem cell hierarchies (28–30). Here, we investigated the possibility that ovarian cancer cells might retain clonogenic property and that HH signaling might be responsible for it. Recently, it was reported that, in the cerebellum, BMI-1 is regulated by Shh, thus providing a direct link between the polycomb gene and the HH signaling pathway (31, 32). Moreover, BMI-1 is overexpressed in several human cancers such as colorectal cancer, liver cancer, and non-small cell lung cancer (31, 32). Therefore, we wanted to determine whether a relationship existed between BMI-1 and HH signaling in ovarian cancer. Hence, we studied the expression of BMI-1 in ovarian cancer cell lines. Increased expression of BMI-1 protein was found in ovarian cancer cell lines compared with the OSE (tsT) cells (Fig. 5A). Treatment with cyclopamine resulted in a dose-dependent decrease in BMI-1 protein, suggesting that the BMI-1 is under the direct control of the HH pathway (Fig. 5A). To further confirm that HH signaling maintains clonal growth, we performed the clonogenic assay. Clonogenic assays in several SMO-expressing ovarian cancer cell lines revealed that cyclopamine effectively inhibited the clonal growth in vitro (Fig. 5B). These data directly implicate the HH pathway in maintenance of clonal growth and suggest the involvement of BMI-1 in ovarian cancer.

To further investigate the implication of low PTCH1 expression in ovarian cancer cells, we overexpressed PTCH1 in all of the ovarian cancer cell lines tested. Overexpression of PTCH1 resulted in significant reduction of BMI-1 levels as detected by the Western blot (Fig. 5C). Because BMI-1 is required for the clonal growth and down-regulated after PTCH1 overexpression, we wanted to determine whether the overexpression of PTCH1 also resulted in the inhibition clonal growth. As expected, overexpression of PTCH1 inhibited clonal growth of all of the ovarian cancer cell lines tested (Fig. 5D). To further confirm the role of BMI-1 in ovarian cancer, we studied

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**Fig. 5.** BMI-1 expression and clonogenic growth assay of ovarian cancer cells. A, expression of BMI-1 at the protein level in different ovarian cancer cells and decrease in expression after treatment with different doses of cyclopamine. Cells were treated with different concentrations of cyclopamine for 48 h. After cyclopamine treatment, cells were lysed in ice-cold radioimmunoprecipitation assay buffer with freshly added 0.01% protease inhibitor cocktail (Sigma) and incubated on ice for 30 min. Cell debris was discarded by centrifugation at 13,000 rpm for 10 min at 4 °C and the supernatant was run on an SDS-PAGE. B, effect of cyclopamine (5 μmol/L) on clonogenic growth of different ovarian cancer cells. C, effect of PTCH1 overexpression on BMI-1 protein expression as measured by Western blot analysis. D, effect of PTCH1 overexpression on clonogenic growth in different ovarian cancer cells. Significant reduction in clonogenic growth was observed on PTCH1 overexpression.
the clonal growth of ovarian cancer cells after knocking down BMI-1 with siRNA. Significant knockdown of BMI-1 was confirmed by the Western blot analysis compared with the control scrambled siRNA-transfected cells (Fig. 6A). Similar to PTCH1 overexpression, BMI-1 knockdown also resulted in inhibition of clonal growth in ovarian cancer cells as measured by clonogenic assay (Fig. 6B). These data suggest that the low PTCH1 expression in ovarian cancer is associated with the increased expression of BMI-1 resulting in increased clonogenicity of ovarian cancer cells. However, the regulation of BMI-1 by PTCH1, as observed in ovarian cancer, although interesting, is not the current focus of this report and a subject of future investigation.

HH signaling controls many aspects of vertebrate development. In humans, tumors such as medulloblastoma and basal cell carcinoma are associated with germ-line or somatic mutations in PTCH1 and exhibit clonally deregulated, ligand-independent HH pathway activation (22). In adult solid tumors derived from the foregut endoderm, evidence of HH pathway activation and/or dependence is associated with variable expression of the HH ligand and autocrine HH signaling (22, 33).

Here, we report the overexpression of Gli-1, SMO, and Shh in both primary ovarian tumors and cell lines. However, the expression of PTCH1 was universally low. Our data support the conclusion that, in ovarian cancer, HH signaling is active at the level of SMO and is driven in part by the expression of the HH ligand. By definition, ligand responsiveness must be associated with some level of functional PTCH1 expression. We speculate that the autonomous activation of the HH pathway in ovarian cancer results from a combination of high levels of HH ligand expression and low levels of PTCH1 that are unable to suppress the signaling in response. The PTCH1 locus (9q22-31; ref. 19) is a frequent target of heterozygous deletion in ovarian cancer, raising the possibility that epigenetic events might be involved in the silencing of the remaining wild-type allele. We think this is unlikely given that the PTCH1 is still expressed and functional in ovarian cancer. Furthermore, increased expression of BMI-1 in ovarian cancer cells and its down-regulation by cyclopamine suggest that the HH signaling might be involved in the maintenance of clonal growth in ovarian cancer. This idea is further supported by the inhibition of clonal growth of ovarian cancer cells by cyclopamine. Furthermore, the inhibition in clonogenicity was observed either by knocking down BMI-1 or by the overexpression of PTCH1 that led to the down-regulation of BMI-1. All these evidences support the direct involvement of HH signaling in ovarian cancer and the maintenance of the clonogenicity through the polycomb gene BMI-1. Therefore, we speculate that the ovarian cancer represents a unique and a complex milieu where high Shh and low PTCH1 expression leads to both paracrine and autocrine activation of the HH pathway to maintain the clonogenic property through the polycomb gene BMI-1. However, we cannot completely rule out the possibility of the involvement of HH unrelated pathways such as RAS and transforming growth factor-β, which may activate the signaling downstream of SMO (26).

Conclusion. In conclusion, it can be stated that the up-regulation of the HH pathway was observed in primary ovarian tumors and all of the human ovarian cancer cell lines tested. The HH pathway helps to maintain the clonal growth of human ovarian carcinoma-derived cell lines. Treatment with cyclopamine, a specific inhibitor of the HH pathway, results in the inhibition of proliferation and clonal growth of all of the ovarian cancer cell lines in vitro and arrests the tumor growth in vivo. Overexpression of PTCH1 down-regulates BMI-1 and inhibits the clonogenic capacity. Similarly, knocking down of BMI-1 by siRNA also inhibits clonogenic potential of ovarian cancer cells. All these evidences directly implicate HH signaling in ovarian tumor growth and maintenance of the clonogenic character through the polycomb gene BMI-1. In future, the HH antagonist may be used to inhibit ovarian tumor growth by inhibiting both the clonal growth and the proliferation of ovarian cancer cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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