Inhibition of hedgehog signaling reduces the side population in human malignant mesothelioma cell lines

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Deregulation of crucial embryonic pathways, including hedgehog signaling, has been frequently implicated in a variety of human cancers and is emerging as an important target for anticancer therapy. This study evaluated the potential anticancer effects of cyclopamine, a chemical inhibitor of hedgehog signaling, in human malignant mesothelioma (HMM) cell lines. Cyclopamine treatment significantly decreased the proliferation of HMM cells by promoting apoptosis and shifting the cell cycle toward dormant phase. The clonogenicity and mobility of HMM cells were significantly decreased by cyclopamine treatment. Treatment of HMM cells with cyclopamine significantly reduced the abundance of side population cells, which were measured using an assay composed of Hoechst 33342 dye staining and subsequent flow cytometry. Furthermore, the expression levels of stemness-related genes were significantly affected by cyclopamine treatment. Taken together, the present study showed that targeting hedgehog signaling could reduce a more aggressive subpopulation of the cancer cells, suggesting an alternative approach for HMM therapy.

INTRODUCTION

Human malignant mesothelioma (HMM) is a fatal tumor that is closely associated with exposure to asbestos fibers. Despite intensive efforts on asbestos reduction, the worldwide incidence of HMM is projected to increase over the next few decades. Recent studies have revealed that inhaled nanoparticles induce cellular responses similar to those of asbestos fibers, implying that HMM could be a potential consequence of nanoparticle inhalation. In addition to diagnostic difficulties, the relatively low efficacy of current therapies is attributable to the death of HMM patients shortly after the diagnosis. The prognosis of HMM is extremely poor, with a median survival of 6–13 months from diagnosis. Therefore, studies on HMM carcinogenesis with new perspectives are urgently needed to improve the clinical outcome of HMM patients.

Recent advances in cancer biology highlight the critical role of a rare subpopulation with stem cell-like features in tumor development and progression. This subpopulation of cancer cells, commonly referred to as cancer stem cells (CSCs) or tumor initiating cells, shares many properties with normal stem cells that are present in a variety of tissues. The CSC hypothesis provides important ramifications for cancer therapy. Because conventional chemotherapy targets rapidly dividing cells, tumors will ultimately relapse following an initial decrease in the tumor burden because of the continuous amplification of the surviving CSCs. Thus, eradication of CSCs from the tumor would lead to the complete cure of cancer patients. A potential strategy for removing the CSCs from the tumor is the disruption of the mechanisms that are responsible for the maintenance of CSCs. Published studies have reported that multiple signaling pathways involved in normal stem cell biology are frequently dysregulated in human cancers.

The hedgehog family of secreted proteins governs a wide variety of biological processes during embryonic development, adult tissue homeostasis and maintenance of stem cells. Altered hedgehog signaling has been reported in several types of cancer, such as breast cancer, prostate cancer, large B-cell lymphoma and malignant pleural mesothelioma. This pathway also has a crucial role in reversal of chemoresistance in some CSCs, such as CD34+ leukemic cells.

Many HMM cell lines reportedly contain a side population (SP) that is enriched with more aggressive cells with stem cell features. This study was conducted to investigate the expression profile of the key components of the hedgehog signaling cascade in selected HMM cell lines and to evaluate the anticancer effects of cyclopamine, a chemical inhibitor of the hedgehog signaling pathway. Treatment with cyclopamine significantly suppressed the aggressive features of the cancer cells and markedly reduced the percentage of SP cells in HMM cells, implicating the hedgehog pathway as a novel target for HMM therapy.

MATERIALS AND METHODS

Cell lines and culture

The cell lines used for the present study represented the sarcomatoid, epithelioid and biphasic types of HMM with different genetic alterations (Summarized in Table 1). The NCI-H513 (H513) and NCI-H2373 (H2373) were kindly provided by Dr R Kratzke (University of Minnesota), the MS1 cells were provided by Dr D Jablons (University of California San Francisco), and the LRK1A and REN cells were provided by Dr Albelda (University of Pennsylvania, Medical Center). The HMM cell lines NCI-H2452 (H2452), MSTO-211H (211H) and a SV40-transformed, non-malignant mesothelial cell line (MetSA) were purchased from American Type Culture Collection (Manassas, CA, USA, CRL-9444). All cell lines were cultured as described previously.
Chemicals and antibodies

Cyclopamine (Toronto Research Chemicals Inc., Toronto, Canada) was dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, St Louis, MO, USA) at 70 °C, and stored at −20 °C. Aliquots were heated to 70 °C prior to use. Tomatidine was bought from Sigma Aldrich Co. Antibodies against Patched (Ptc1), Sonic Hedgehog (Shh), Gli1, Gapdh, phosphorylated Akt and Akt, and secondary horseradish peroxidase-conjugated antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Smoothened (Smo) antibody was obtained from Pierce Biotechnology Inc. (Rockford, IL, USA). Propidium iodide (PI) was purchased from Sigma Aldrich Co. Fetal bovine serum was purchased from Mediatech Inc. (Herndon, VA, USA).

Western blot assay

Cellular lysates were prepared by re-suspending the human mesothelial cells in the mammalian protein extraction reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing protease (Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitors (Thermo Fisher Scientific Inc.) at 4 °C. Then, the lysates were sonicated, centrifuged at 23 000 g for 10 min, and concentrated using a Micron cellulose filter (Millipore, Billerica, MA, USA). The protein concentration was measured using Bio-Rad detergent-compatible protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Gel electrophoresis, membrane transfer, blocking and antibody binding were performed as described previously.15 Chemiluminescence was detected using enhanced chemiluminescent western blotting substrate (Thermo Fisher Scientific Inc.). As an internal loading control, the antibodies were stripped from the membrane with Restore western blot stripping buffer (Thermo Fisher Scientific Inc.) and re-probed with Gapdh antibody.

Cell proliferation assay

Cell growth was examined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Fitchburg, MA, USA) according to the manufacturer’s instructions with slight modifications.13 In brief, ~3000 cells were seeded onto flat-bottomed 96-well plates and pre-cultured overnight in reduced serum media. On the following day, the media was replaced by reduced serum media containing DMSO or various concentrations of cyclopamine and tomatidine. Each drug concentration was applied to triplicate wells containing cells. At the end of the incubation, 20 μl CellTiter 96 AQueous One solution was added to each well and the plate was incubated for 1 h at 37 °C. The absorbance of the well was read at 495 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Gel electrophoresis, membrane transfer, blocking and antibody binding were performed as described previously.15 Chemiluminescence was detected using enhanced chemiluminescent western blotting substrate (Thermo Fisher Scientific Inc.). As an internal loading control, the antibodies were stripped from the membrane with Restore western blot stripping buffer (Thermo Fisher Scientific Inc.) and re-probed with Gapdh antibody.

Scratch wound-induced migration assay

To investigate mobility, the HMM cells (10^5 cells per well) were grown for 24 h, and then wounded with a sterile yellow pipette tip, and then either left untreated or treated with 0, 10 and 20 μmol of cyclopamine or tomatidine. After 24 h, the wound gap was observed and cells were photographed using phase-contrast microscopy. The images were then analyzed using Image J software 1.45 s version (National Institutes of Health, Bethesda, Md, USA) to measure the width of the scratch. The relative migration distance was calculated by the following formula: relative migration distance [%] = (A − B)/A, where A is the width of the cell wound before incubation, and B is the width of the cell wound after incubation.16

Confluent monolayer of the human mesothelial cell lines were seeded onto 12-well plates (90 μl/well) and either undifferentiated or wounded with a sterile yellow pipette tip, and then either left untreated or treated with 0, 10 or 20 μmol of cyclopamine or tomatidine. After 24 h, the wound gap was observed and cells were photographed using phase-contrast microscopy. The images were then analyzed using Image J software 1.45 s version (National Institutes of Health, Bethesda, Md, USA) to measure the width of the scratch. The relative migration distance was calculated by the following formula: relative migration distance [%] = (A − B)/A, where A is the width of the cell wound before incubation, and B is the width of the cell wound after incubation.16

Clonogenicity assay

A total of 10^3 sorted cells per well were cultured in complete media with 10 μmol cyclopamine or tomatidine at 37 °C with 5% CO2 for 24 h and then transferred to complete media with no drugs. The cells were cultured in the media for 5 days. Then, the cells were fixed with 10% neutral-buffered formalin for 30 min, and stained with Diff Quik. The colonies were counted with a 10× objective using a microscope, and classified into three groups based on the number of cells within a colony: small (5–10 cells), medium (11–50 cells) and large (50 cells and more). The number of colonies was recorded for each category and the total number of colonies was also taken into account.

SP assay

The effects of cyclopamine and tomatidine on the SP fraction were evaluated using MS1 cells. The cells were pre-cultured in reduced serum media (0.5% fetal bovine serum) and then treated with DMSO, cyclopamine or tomatidine for 24 h, followed by SP assay as published.14 The percentages of SP cells in the treated samples were compared with the DMSO-treated control.

Cell cycle assay

Cultured cells were washed twice by centrifugation in phosphate-buffered saline at 4 °C. 1300 rpm for 3 min. After vortexing gently, the cell suspension was slowly added to 1 ml of 70% ethanol in a 1.5-ml e-tube and stored at −20 °C for 1 h. Following centrifugation at 1300 rpm, 4 °C for 3 min, the pellets were resuspended in 1 ml cold phosphate-buffered saline containing RNase A (50 μg ml−1) and incubated at 37 °C, 5% CO2 for 40 min. PI was diluted to 35 μg ml−1 with distilled water, and transferred to a glass tube. The tube was placed on ice and analyzed using a flow cytometer (BD AriaIII; BD Bioscience, San Jose, CA, USA).

Apoptosis assay

Cells were collected by trypsinization and washed with phosphate-buffered saline. Approximately 5 × 10^5 cells were resuspended in the binding buffer, Annexin V staining was accomplished using Annexin V Apoptosis Detection Kit FITC (eBbioscience, San Diego, CA, USA) according to the manufacturer’s instruction and a published protocol.14 Primers for all genes analyzed are listed in the Table 2. Cycle conditions were 90 °C for 5 min, 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Fold increases or decreases in gene expression were determined by quantitation of complementary DNA generated from cDNA by QIAGEN SYBR Green PCR Kit (Takara) according to the manufacturer’s instructions. Relative expression was calculated as 2−ΔΔCt values for each gene and was normalized to GAPDH expression.14

Quantitative real-time RT-PCR

Table 1. Summary of morphologic subtypes and known genetic alterations in human mesothelial cell lines used for this study

| Cells | Morphology | Genetic alterations |
|-------|------------|---------------------|
| NCI-H513 | Epithelioid | NF2mt, INK/ARF<sub>HD</sub>, p53<sub>mt</sub> |
| MS1<sup>a</sup> | Biphasic | INK/ARF<sub>PP</sub>, p53<sub>PP</sub> |
| MSTD-211H<sup>b</sup> | Biphasic | NF2<sub>PP</sub>, INK/ARF<sub>PP</sub>, p53<sub>mt</sub> |
| NCI-H2452<sup>a</sup> | Biphasic | NF2<sub>PP</sub>, INK/ARF<sub>PP</sub>, p53<sub>mt</sub> |
| REN<sup>a</sup> | Epithelial | P53<sub>mt</sub> |
| LRK1A<sup>a</sup> | Epithelial | P53<sub>mt</sub> |
| NCI-H2373<sup>a</sup> | Sarcomatoid | NF2<sub>PP</sub>, INK/ARF<sub>PP</sub> |
| Met5A<sup>a</sup> | Epithelioid | SV40 Tag |

Abbreviations: HD, homozygous deletion; mt, mutant; wt, wild type.

<sup>a</sup>Human malignant mesothelioma cell line. <sup>b</sup>SV40-transfected non-transformed mesothelial cell line.

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RESULTS

Hedgehog signaling was activated in multiple HMM cell lines. In seven HMM cell lines and one mesothelial cell line (Met5A), the expression pattern of hedgehog signaling components was determined using western blot assay (Figure 1). Ptch1, which functions as a receiving receptor for members of the hedgehog protein family, was marginally expressed in the Met5A, H513 and MS1 cells (data not shown). In contrast, Smo was clearly detected in all the cell lines examined, with very high expression in Met5A, MS1 and LRK1A cell lines. In some cell lines, two different Smo were detected on the immunoblot as an 86 kDa band of expected molecular size and a strong 120 kDa band corresponding to the fully glycosylated form. The 45 kDa precursor of Shh was detected in all cell lines, whereas the 19 kDa active form of Shh was highly expressed in MS1 cells and weakly expressed in H513 cells. Gli1 was clearly detected in Met5A, 211H and MS1. The Gli1 was also detected in H513 when a higher amount of protein (40 μg) was used for western blot assay.

Inhibition of hedgehog signaling in HMM cell lines by cyclopamine

Treatment with cyclopamine for 48 h reduced the expression of hedgehog-related genes such as GLI1, PTCH1, SHH and SMO in mesothelial cell lines, indicating the inhibition of hedgehog signaling (Figure 2). However, treatment with tomatidine, a chemical with similar structure to cyclopamine, did not affect the gene expression profile of hedgehog-related genes (data not shown).

Suppressed proliferation of HMM cells was induced by cyclopamine and tomatidine

Because earlier studies have suggested that bovine serum albumin present in fetal bovine serum may interfere with the growth inhibitory effects of cyclopamine, the effects of cyclopamine was determined in reduced serum media (0.5% fetal bovine serum). Cyclopamine treatments for 24 h significantly reduced cell proliferation in a dose-dependent manner (Figure 3).

Table 2. Primer sequences of the cancer-related genes used for quantitative real-time PCR

| Classification         | Genes     | Forward primers                      | Backward primers                      |
|------------------------|-----------|--------------------------------------|---------------------------------------|
| Drug transporter protein | ABCG2     | 5′-GGGTTCTCTTCTCTTCTGACGACC-3′       | 5′-TGTTGAGATTGACCAACAGACC-3′          |
| Stemness-related gene   | OCT4      | 5′-ACATGTGTAAGCTGCGGCC-3′            | 5′-GTGTGACATGCTGCGTTG-3′              |
|                        | SOX2      | 5′-CGATGCGGCAAGAAAGACTT-3′           | 5′-CAACCTCTGCAAAGCTCC-3′              |
| Stemness-related gene   | NANO5     | 5′-TTCACTCTGAGACACTGCTG-3′           | 5′-CTGCCTAGTACGCTCACA-3′              |
|                        | GLI1      | 5′-TTCTACCCAGAGTCTCCAGAAGT-3′        | 5′-CTCTATGGAAGGCCTTATT-3′             |
|                        | PTC1      | 5′-GTGGAGAGAAACACACAG-3′             | 5′-AGGGTCAGAGTCAGTGA-3′               |
|                        | SHH       | 5′-CGCAGGGGACAGCTCGGAAGT-3′          | 5′-CTGGGCAACCTCGTACTG-3′              |
|                        | SMO       | 5′-TTACCTCTACGCTGCACTTCTACG-3′       | 5′-GGCTGGGCAATCTCGTCTC-3′             |
Compared with the DMSO-treated cells, the cell proliferation in MS1 and H513 cells treated with 20 μM cyclopamine for 48 h was reduced by ~ 21% and 43%, respectively (data not shown). Cell proliferation was also significantly reduced in MS1 and H513 cells that were treated with tomatidine. However, the degree of reduction was much less than that of cyclopamine-treated cells (data not shown).

Scratch wound-induced migration assay
Cell mobility is an important trait that distinguishes benign tumor from their malignant counterparts. Inhibition of cell mobility and invasion may restrain metastasis and neoangiogenesis of tumors. The wound-induced migration assay showed that the gap between cell layers after scratching was occupied by the migrating cells from both ends. Migration capacity of MS1 cells treated with 10 and 20 μM cyclopamine was more significantly reduced than the migration capacity of untreated cells (Figure 4).

Clonogenicity assay
The ability of a single cell to grow into a colony is a feature of malignant cells. The total number of colonies generated from the cells treated with 10 μM cyclopamine was similar to that from the cells treated with untreated cells (Figure 5). MS1 cells treated with 10 μM cyclopamine had an increased number of small colonies and a decreased number of medium and large colonies, compared with vehicle-treated MS1 cells. In contrast, treatment of MS1 cells with 10 μM tomatidine markedly reduced the number of total colonies with similar proportions of each colony.

Marked reduction of mesothelial SP percentage was induced by cyclopamine but not by tomatidine
To determine the effects of cyclopamine on the abundance of mesothelial SP cells, HMM cells were treated with cyclopamine or tomatidine, followed by SP assay. The SP region was defined as published, and it was clearly delineated as a tail located at the lower left corner of the flow cytometric histogram (Figures 6a and c). The SP fractions of MS1 cells treated with 10 and 20 μM cyclopamine for 24 h were significantly decreased (Figure 6b). In contrast, the abundance of SP fractions was not significantly changed in the MS1 cells treated with 10 and 20 μM tomatidine for 24 h (Figure 6d).

Cell cycle analysis
Following the treatment of MS1 cells with cyclopamine or tomatidine, flow cytometry was performed to evaluate the distribution of cell cycles in G0, G1, G2, S and M phases (Figure 7).
By treatment of MS1 cells with 20 μM cyclopamine, the percentage of cells in G0 and G1 stages were increased from 47.55 to 59.9%, and the percentage of cells in the G2 and M stages was significantly reduced from 29.9 to 19.1%. In contrast to cyclopamine, treatment with tomatidine did not alter the distribution of the cell cycle. These results indicated that cyclopamine promotes the shifting of the cells from the proliferative phase to the resting phase.

**Apoptosis assay**

The relative proportions of apoptotic cells following treatment with cyclopamine and tomatidine were quantitatively assessed by flow cytometry. In the early stage of apoptosis, phosphatidylserine is translocated from the inside of the cell membrane to the outside. Early apoptotic cells can be detected using Annexin V, a calcium-dependent phospholipid-binding protein associated with a high affinity for phosphatidylserine. PI is an intercalating agent used as a DNA stain to evaluate DNA content in cell cycle analysis. It stains cells with damaged membrane integrity and can be used to differentiate necrotic, apoptotic and normal cells. Thus, cells stained with Annexin V and PI could be classified as necrotic cells (Q1, Annexin−/PI+), late apoptotic cells (Q2, Annexin+/PI+), intact cells (Q3, Annexin−/PI−), and early apoptotic cells (Q4, Annexin+/PI−).

Cyclopamine increased apoptotic cells of MS1 from 1.5 to 2.8% in the early apoptosis, and from 1.2 to 1.6% in the late apoptosis (Figure 8a). The early and late apoptosis of MS1 cells increased in a concentration-dependent manner. However, tomatidine decreased apoptotic cells from 2.9 to 0.7% in Q4, and increased from 1.0 to 3.3% in Q2 (Figure 8b). In summary, these results indicate that the inhibitory effects on cell growth observed in response to cyclopamine in MS1 cells may be associated with induction of apoptotic cell death.

**Gene expression analysis**

Expression levels of genes related to oncogenic signaling pathways including hedgehog signaling were determined by quantitative real-time RT-PCR. The expression levels of hedgehog signaling genes such as GLI1, PTCH, SHH and SMO were significantly decreased in a concentration-dependent manner by treatment with cyclopamine. The expression levels of the stemness genes, including OCT4, NANOG and SOX2, were also determined (Figure 9). In MS1 cells, treatment with 10, 20 μM cyclopamine increased the expression levels of SOX2 and decreased that of OCT4. NANOG was not significantly altered in MS1 cells by the cyclopamine treatment. Expression of a drug transporter gene, ABCG2, was decreased and increased by treatment with 10 and 20 μM cyclopamine, respectively.

**DISCUSSION**

The expression analysis of its key players illustrated that hedgehog signaling is activated in the selected HMM cell lines. Consistent with previous studies, treatment with cyclopamine significantly affected the cancer cell phenotype, including survival,
proliferation and migration capacity of HMM cells. Furthermore, cyclopamine induced marked reduction of SP fraction that is reportedly enriched with more aggressive and chemoresistant subpopulation.14 Cyclopamine has been reported to suppress the growth of many cancer cell lines by inhibition of hedgehog pathway.21

Cyclopamine is a lipophilic compound extracted from the lily Veratrum californicum, and antagonizes SMO activity by binding to its heptahelial bundle when cells are treated.9 Thus, it can block hedgehog signaling pathway, causing both downregulation of proliferation and initiation of apoptosis, with consequent reduction in tumor growth.10 In the present study, tomatidine was selected as an experimental control for cyclopamine. Tomatidine showed nonspecific effects on the expression results of hedgehog signaling genes. Tomatidine decreased cell proliferation less than cyclopamine and did not significantly affect SP of HMM cells. A recent study showed that GDC-0449, a SMO inhibitor, suppressed survival of HMM cells in vitro by targeting hedgehog signal transduction.22

HMM is notoriously refractory to traditional therapeutic modalities.23 Despite the aggressive therapeutic approach, survival rate of HMM patients is dismal.4 Although surgery and radiation therapy have a role in the treatment of HMM, systemic chemotherapy is the only treatment option for the majority of HMM patients. The current standard regimens for HMM chemotherapy include cisplatin and pemetrexed. Cisplatin kills the neoplastic cells by interacting with the DNA-forming DNA adducts, and pemetrexed is an antifolate agent affecting DNA synthesis, incurring apoptosis and cell cycle arrest.24 The cytotoxicity of the anticancer effects is also responsible for undesirable side effects. In addition, rapid development of drug resistance against anticancer drugs is the major obstacle for successful chemotherapy against HMM.5 Combined treatment with drugs differing in their action mechanisms may enhance their efficacies with much lower doses of each drug than single-agent treatment. Furthermore, combined treatment may help to overcome drug resistance and to avoid potential serious side effects by simultaneous targeting of multiple mechanisms. Therefore, combined treatment with cisplatin, pemetrexed and cyclopamine would be more effective than treatment with pemetrexed and cisplatin alone. Cyclopamine additively enhanced the effects of cisplatin (unpublished data). Further studies are warranted for the pharmacokinetic interactions of the drug combinations of pemetrexed, cisplatin and cyclopamine.

Figure 7. Cell cycle assay. MS1 cells were treated with cyclopamine or tomatidine and flow cytometry was performed to evaluate the distribution of cell cycles in G0, G1, G2, S and M phases. (a) DNA flow cytometry (FCM) histograms of PI-stained MS1 cells treated with vehicle (left panel) or with 20 μM cyclopamine (right panel). Percentages of G0 and G1 stages were increased, and those of G2 and M stages were significantly reduced in the MS1 cells treated with 20 μM cyclopamine. (b) DNA-FCM histograms of PI-stained MS1 cells treated with vehicle (left panel) or with 20 μM tomatidine (right panel). Treatment with tomatidine did not significantly alter the distribution of the cell cycle.
Even within a single tumor, there is a striking variability among the individual cancer cells. This ‘intratumoral heterogeneity’ has long been recognized with regard to biological and molecular properties. As such, the emergence of a drug-resistant cell population in tumors underlies the failure of various cancer therapies. Therefore, gaining a better understanding about the mechanism(s) regulating the intratumoral heterogeneity linked to therapeutic resistance is highly relevant to clinical applications.

HMMs are known to be extremely heterogeneous with respect to the cellular and molecular phenotypes, stages and prognostic factors. Recently, we demonstrated the cellular heterogeneity in HMM cell lines using SP assay. The mesothelial SP is a distinct cell fraction with properties known for stem cells including self-renewal, enhanced chemoresistance, overexpression of stemness genes and tumorigenic capacity. Our results indicate that the SP...
in HMM cell lines may be enriched for the mesothelial CSCs, which may be a feasible target to achieve complete cure for HMM patients. The present study demonstrated that treatment with cyclopa mine decreased SP fraction markedly in HMM cell lines. Expression of the stemness gene OCT4 was significantly decreased. One study reported that sulforaphane, a component of dietary cruciferous vegetables, inhibits the messenger RNA expression of Oct4 in pancreatic CSCs orthotopic tumors by hedgehog blockade.27 The results suggest that inhibition of the hedgehog pathway may be a potential strategy to target more aggressive subpopulation cells in HMM.

The mechanism underlying the reduction of SP abundance in HMM cells is unclear. Previous studies have shown that ABCG2 is a direct transcriptional target of hedgehog signaling.11 In the present study, however, ABCG2 expression level was variably affected by cyclopa mine treatment of HMM cell lines. Although not proven, this observation may be attributable to the composition of cell subpopulation. Because SP cells in HMM cell lines is a minor population that constitutes <3% of the tumor,14 the changes of ABCG2 in whole-cell population that was measured might not properly reflect the changes of ABCG2 in the SP cells. Alternatively, the expression level of ABCG2 might not be altered, but its activity could be severely affected by cyclopa mine treatment. Another potential expla nation is that SP cells are selectively killed by cyclopa nine treatment. Determining the underlying mechanism warrants further studies.

It is intriguing that the degree of hedgehog signaling inhibition in HMM cell lines may be dependent on genetic backgrounds. Previous studies have shown that cyclopa mine decreases the levels of phosphorylated (activated) Akt, a known antiapoptotic protein.18,28 In contrast to MS1 cells with wild-type p53, the expression levels of hedgehog component along with phosphoryl ated Akt were not reduced by cyclopa mine treatment in HS13 cells harboring mutations in p53. One of the functions of Akt is inhibiting a pre-apoptotic protein, BAX, which is triggered by p53 and induces apoptosis. The present study showed that apoptosis was induced in MS1 cells treated with cyclopa mine. The phosphoinositide 3-kinase-dependent Akt activation is known to be critical for sonic hedgehog signaling, indicating the synergistic role of phosphoinositide 3-kinase for hedgehog signaling in various biological processes, such as cell proliferation and survival. Therefore, the essential role of phosphoinositide 3-kinase/Akt in mammalian hedgehog signaling would be their potential use as therapeutic targets in the treatment of hedgehog-dependent malignancies.5,29 Interactions between hedgehog signaling and phosphoinositide 3-kinase-Akt pathways in HMM cell lines require further studies.

CONCLUSIONS
The present study showed that inhibition of hedgehog path way markedly reduced the SP fraction in HMM cell lines, supporting the future clinical investigation of the hedgehog signaling as a novel target for HMM therapy, with the aim of increasing the efficacy while minimizing the toxicity of anticancer drugs.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (Grant #: 2012R1A2A1A0384777) and by BK21 PLUS Program for Creative Veterinary Science Research.

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ACKNOWLEDGEMENTS
This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (Grant #: 2012R1A2A1A0384777) and by BK21 PLUS Program for Creative Veterinary Science Research.

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