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Inhibition of *Smoothened* Signaling Prevents Ultraviolet B-Induced Basal Cell Carcinomas through Regulation of Fas Expression and Apoptosis

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ABSTRACT

Abnormal activation of the hedgehog-signaling pathway is the pivotal abnormality driving the growth of basal cell carcinomas (BCCs), the most common type of human cancer. Antagonists of this pathway such as cyclopamine may therefore be useful for treatment of basal cell carcinomas and other hedgehog-driven tumors. We report here that chronic oral administration of cyclopamine dramatically reduces (~66%) UVB-induced basal cell carcinoma formation in *Ptch1*$^{+/-}$ mice. Fas expression is low in human and murine basal cell carcinomas but is up-regulated in the presence of the smoothened (SMO) antagonist, cyclopamine, both *in vitro* in the mouse basal cell carcinoma cell line ASZ001 and *in vivo* after acute treatment of mice with basal cell carcinomas. This parallels an elevated rate of apoptosis. Conversely, expression of activated SMO in C3H10T1/2 cells inhibits Fas expression. Fas/Fas ligand interactions are necessary for cyclopamine-mediated apoptosis in these cells, a process involving caspase-8 activation. Our data provide strong evidence that cyclopamine and perhaps other SMO antagonists are potent *in vivo* inhibitors of UVB-induced basal cell carcinomas in *Ptch1*$^{+/-}$ mice and likely in humans because the majority of human basal cell carcinomas manifest mutations in PTCH1 and that a major mechanism of their inhibitory effect is through up-regulation of Fas, which augments apoptosis.

INTRODUCTION

The *hedgehog* (Hh) pathway plays a critical role in embryonic development and tissue polarity (1). Secreted Hh molecules bind to the receptor patched (PTC), thereby alleviating PTC-mediated suppression of smoothened (SMO), a putative seven-transmembrane protein. SMO signaling triggers a cascade of intracellular events, leading to activation of the pathway through GLI-dependent transcription (2). Considerable insight into the role of the Hh pathway in vertebrate development and human cancers has come from the discovery that mutations of the *patched* gene (*PTCH1*) underlie the basal cell nevus syndrome, a rare hereditary disorder in which patients are highly susceptible to the development of large numbers of basal cell carcinomas and other tumors (3, 4). Activation of Hh signaling, usually due to loss-of-function somatic mutations of *PTCH1* and less often to activating mutations of *SMO*, is the pivotal abnormality in sporadic basal cell carcinomas (5–7). Therefore, targeted inhibition of SMO signaling should afford mechanistically based prevention/therapy of basal cell carcinomas, as well as of other tumors driven by Hh signaling abnormalities, including certain medulloblastomas, small-cell lung carcinomas, and gastrointestinal tract cancers (8–11). One such inhibitor is the naturally occurring plant extract cyclopamine, and there are additional synthetic compounds that directly associate with the transmembrane domains of SMO (12–14). Therefore, these small molecular weight compounds have significant promise for the prevention and treatment of basal cell carcinomas and other human malignancies.

Ptch1$^{+/-}$ mice (15) provided the first practical animal model for inducing basal cell carcinomas using UV and ionizing radiation (16). We report here that chronic oral administration of cyclopamine dramatically inhibits basal cell carcinoma growth in these mice. We also have tested the *in vitro* effects of cyclopamine and of the synthetic SMO inhibitor Cur61414 on the mouse basal cell carcinoma cell line ASZ001 and have demonstrated that both compounds elevate Fas expression and augment apoptosis. The clinical relevance of our data for treatment of basal cell carcinomas is supported by the low baseline Fas expression in basal cell carcinomas of both humans and mice and by the *in vivo* induction of high level Fas expression by short-term administration of cyclopamine in murine basal cell carcinomas. Thus, our studies support the idea that treatment of human basal cell carcinomas with specific inhibitors of the Hh pathway may offer a mechanism-driven approach to the chemoprevention of these tumors.

MATERIALS AND METHODS

Animals. *Ptch1*$^{+/-}$ heterozygous knockout mice have been developed by deleting exons 1 and 2 and inserting the LacZ gene at the deletion site (15). *Ptch1-lacZ*-transgenic mice were genotyped by PCR amplification of genomic DNA extracted from tail biopsies (15, 16). The animals were housed under standard conditions (fluorescent lighting 12 hours per day, room temperature 23°C to 25°C, and relative humidity 45 to 55%). The mice were provided tap water and Purina Laboratory Chow 5001 diet (Ralston-Purina Co., St. Louis, MO).

UV Light Source. An UV Irradiation unit (Daavlin Co., Bryan, OH) equipped with an electronic controller to regulate dosage was routinely used for these studies. The UVB source consisted of eight FS72T12-UVB-HO lamps emitting UVB (290 to 320 nm, 75 to 80% of total energy) and UVA (320 to 380 nm, 20 to 25% of total energy). We used a Kodacel cellulose film (Kodacel TA401/407) to eliminate UVC radiation. A UVC sensor (Oriel's Goldilux UVC Probe) was used during each exposure to confirm the lack of UVC emission. The UVB dose was quantified using a UVB Spectrum 305 Dosimeter obtained from the Daavlin Co. The radiation was additionally calibrated using an IL1700 Research Radiometer/Photometer from International Light, Inc. (Newburyport, MA). The distance between the radiation source and targets was maintained at 30 cm. The irradiation assembly is kept in an air-conditioned room, and a fan is placed inside the exposure chamber to minimize temperature fluctuations during irradiation.

Carcinogenesis Protocol and Statistical Analyses. Mice were irradiated with a UV Irradiation unit (240 mJ/cm^2 three times a week) from age 6 to 32 weeks, at which time, ~50% of the animals had one or more visible skin tumors. The mice (25 mice per group) were given either cyclopamine (10 μg/day as a cyclodextran complex) or the vehicle control in drinking water,

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and the number of tumors was recorded weekly. Mice treated with cyclopamine or with the vehicle control were sacrificed at 52 weeks, their dorsal skin removed, and tumors harvested and collected for the histologic and immunohistochemical studies. The microscopic basal cell carcinoma areas were assessed by histologic evaluation of three dorsal skin sections per mouse from a total of seven mice ($n = 7$) in the vehicle-treated water group and a total of six mice ($n = 6$) in the cyclopamine-treated group. The basal cell carcinoma areas were measured by microscopic assessment using the Axiovision 3.1 analysis program (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Results were analyzed using the Student's *t* test or a nonparametric test (Mann-Whitney test): $P < 0.05$ was considered statistically significant.

For evaluation of Fas expression and apoptosis *in vivo*, cyclopamine was injected (at 100 pg s.c. or intratumorally) into mice with visible basal cell carcinomas (>1 cm in diameter). Seventy-two hours later, basal cell carcinomas were embedded in OCT compound (Tissue-Tek; Sakura, Torrance, CA), and stored in −20°C for additional analyses. Stromal cells were used as the control for basal cell carcinoma cells.

***β*-Galactosidase Staining.** Tissues were fixed in 0.2% glutaraldehyde (Sigma-Aldrich, St. Louis, MO)/2% formaldehyde (Fisher Scientific Co., Pittsburgh, PA) in 1× PBS for 20 minutes at 4°C, then washed twice in 1× PBS. Tissues were incubated with 5% 5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside in 95% iron buffer solution for 24 hours at 37°C using a *β*-galactosidase staining set (Roche Applied Science, Indianapolis, IN), according to the manufacturer's guideline. The tissues were washed twice in 3% DMSO in 1× PBS and then three times in 70% etomidate. The tissues were embedded in paraffin and processed for counterstaining.

Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling (TUNEL) and Immunofluorescent Staining. TUNEL analysis was performed using a kit from Roche Applied System according to the manufacturer's guideline. Immunofluorescent staining of Fas in basal cell carcinomas was performed with an antibody specific to mouse Fas (M20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Cell Culture and Cell Viability Assay. The mouse basal cell carcinoma cell line ASZ001 was maintained in 154CF medium as reported previously (17). Ectopic expression of Gli1 in ASZ001 cells was induced using LipofectAmine 2000, and the transfected cells were enriched by cell sorting after coexpression of green fluorescence protein and Gli1 (green fluorescence protein to Gli1 plasmid ratio = 1:4). C3H10T1/2 cells were cultured in basal medium containing 10% heat-inactivated fetal bovine serum. GLI-transformed baby rat kidney cells were cultured in DMEM containing 10% fetal bovine serum (18). ASZ001 cells were first treated with 2 μmol/L 3-keto-N-aminoethylaminoethylcaproyldihydrocinnamoyl cyclopamine (KAAD-cyclopamine) for 36 hours in the presence or absence of 10 μg/mL epidermal growth factor or platelet-derived growth factor (PDGF)-AA (R&D Systems, Inc., Minneapolis, MN) or treated with U0126 alone (10 μmol/L; EMD Biosciences, Inc., San Diego, CA) for 36 hours. Cells were then harvested for protein expression and cell viability analyses. Cell viability was assessed by trypan blue exclusion and colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (19). The proliferation inhibition was calculated by dividing the mean test value by the respective PBS control. The background absorbance, obtained from the wells treated with DMSO only, was subtracted from the test- and control-well values to yield corrected absorbance. Triplicates for each sample were used, and all experiments were performed in triplicate (19).

Flow Cytometry and Cell Sorting. Cells were plated at 3,000,000 cells per 10-cm plate in 154CF without growth supplements the day before treatment. KAAD-cyclopamine (Toronto Research Chemicals, Inc., North York, Ontario, Canada) was added to the medium to achieve a final concentration of 2 μmol/L, and the cells were incubated for 36 hours. Epidermal growth factor and PDGF-A were used at a final concentration of 10 μg/mL, U0126 at a final concentration of 10 μmol/L, and Fas ligand (FasL)–neutralizing antibodies at a final concentration of 20 μg/mL were added to the medium. Subsequently, cells were collected and fixed overnight in 70% etomidate and treated with 50 ng/mL propidium iodide in the presence of 10 μg/mL RNase A (in PBS). Cell cycle profiles were determined by a fluorescence-activated cell sorter (FACSCaliber; Becton Dickinson, Franklin Lakes, NJ). At least 20,000 gated events were recorded for each sample, and the data were analyzed with Multicycle software for Windows (Phoenix Flow Systems, San Diego, CA). ASZ001 cells with ectopic Gli1 expression were enriched through cell sorting, resulting in >90% of the cells with Gli1 expression (from a starting population of 5% positive cells). Both positive and negative fractions were collected for Western blot analysis.

Western Blot Analysis and ELISA. Western blotting was performed as previously reported (17), with specific antibodies [anti-PDGFR-*α* and anti-mouse Fas antibodies from Upstate Biotechnology (Lake Placid, NY); anti-Erk, anti-phospho-Erk and anti-caspase-3 antibodies from Cell Signaling Technology (Beverly, MA); anti-*β*-actin from Sigma-Aldrich (St. Louis, MO); and antihuman Fas and anti-FasL from BD Transduction Laboratories (San Diego, CA)]. ELISA detection of secreted FasL protein in the growth medium was performed using a kit from R&D Systems, Inc., according to the manufacturer's protocol.

Real-time PCR Analyses. Total RNAs from ASZ001 cells were extracted using RNAqueous from Ambion, Inc. (Austin, TX). We used Applied Biosystems' (Foster City, Ca) assays-by-design 20× assay mix of primers and TaqMan probes (carboxyfluorescein dye-labeled probe) for the target genes [mouse Gli1, hedgehog interacting protein (HIP)] and predeveloped 18S rRNA (VIC dye-labeled probe) TaqMan assay reagent (P/N 4319413E) for an internal control. Mouse Gli1 and HIP primers are designed to span exon-exon junctions so as not to detect genomic DNA, and the primers and probe sequences were searched against the Celera database to confirm specificity. The primer and probe sequences of mouse Gli1 and HIP are as follows.

To obtain the relative quantitation of gene expression, a validation experiment was performed to test the efficiency of the target amplification and the efficiency of the reference amplification. All absolute values of the slope of log input amount *versus* ΔC_T were <0.1. Separate tubes (singleplex) one-step reverse transcription-PCR was performed with 20 ng of RNA for both target gene (mGli1 or mHIP) and endogenous control. The reagent we used was TaqMan one-step reverse transcription-PCR master mix reagent kit (P/N 4309169). The cycling parameters for one-step reverse transcription-PCR was reverse transcription 48°C for 30 minutes, AmpliTaq activation 95°C for 10 minutes, denaturation 95°C for 15 seconds, and annealing/extension 60°C for 1 minute (repeat 40 times) on ABI7000. Triplicate C_T values were analyzed in Microsoft Excel using the comparative C_T ($\Delta\Delta C_T$) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalization to an endogenous reference (18 rRNA) and relative to a calibrator.

RESULTS

Cyclopamine Inhibits Basal Cell Carcinoma Development in *Ptch1*$^{+/-}$ Mice. The Hh pathway is constitutively activated in essentially all human and mouse basal cell carcinomas (3, 20). Because PTCH1, which is frequently inactivated in human basal cell carcinomas, is known to be upstream of SMO (21), inhibiting SMO functions should be an effective way to treat basal cell carcinomas. To test the effects of a SMO antagonist on development of basal cell carcinomas, we administered cyclopamine orally to basal cell carcinoma-bearing *Ptch1*$^{+/-}$ mice. We UV-irradiated *Ptch1*$^{+/-}$ mice from age 6 to 32 weeks, at which time, approximately half of the mice had developed one or more macroscopic tumors: basal cell carcinomas, squamous cell carcinomas, and/or spindle cell tumors (fibrosarcomas). UV irradiation was then stopped and the mice were randomized (25 mice per group) to receive either vehicle or cyclopamine (as cyclodextran complex) in the drinking water for the ensuing 20 weeks. The survival of these two groups of animals was similar, indicating that cyclopamine does not affect the overall survival of *Ptch*1$^{+/-}$ mice (data not shown). At age 52 weeks, we measured microscopic basal cell carcinoma areas per tissue area (mm^2) in these two groups after *β*-galactosidase staining (see Materials and Methods for details) and found a 90% reduction of microscopic basal cell carcinomas in the cyclopamine-treated animals (Fig. 1, *A* and *B*). Cyclopamine-treated mice also had fewer visible basal cell carcinomas at age 52 weeks than did the vehicle-treated controls (Fig. 2, *A* and *B*). Thus, the mice receiving vehicle alone continued to develop visible tumors, and the number of tumors had tripled by week 52. We

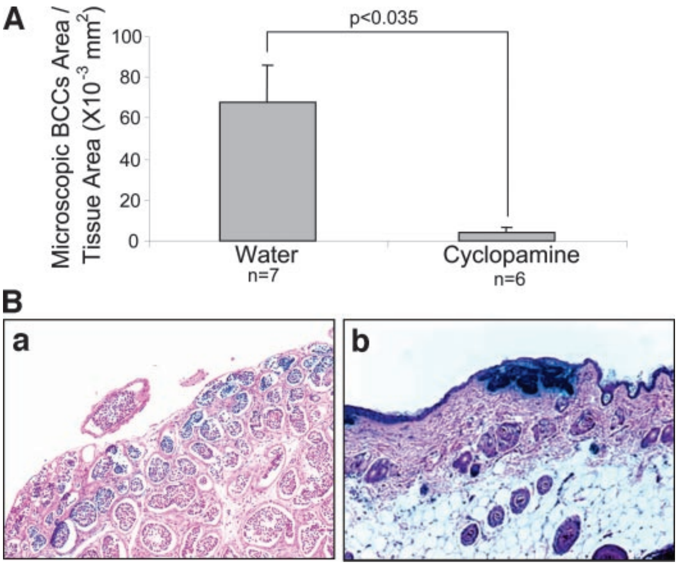

Fig. 1. Effect of cyclopamine on UVB-induced microscopic basal cell carcinomas (BCCs) in *Ptch1*$^{+/-}$ mice. *A*, effect of orally administered cyclopamine on the number of UVB-induced microscopic BCC-like lesions in *Ptch1*$^{+/-}$ mice. Microscopic BCC-like lesions were counted as total tumor area/mm^2 skin and assessed by histologic evaluation of three dorsal skin sections per mouse from a total of seven mice ($n = 7$) in the vehicle-treated group and a total of 6 mice ($n = 6$) in the cyclopamine-treated group. Results of microscopic BCCs in the two groups were statistically analyzed using the Student's *t* test (two-tailed): $P < 0.05$ was considered statistically significant. *B*, β-galactosidase and H&E staining showing the effect of orally administered cyclopamine on UVB-induced microscopic BCC lesions in *Ptch1*$^{+/-}$ mice. Representative staining from UVB-irradiated (*a*) and UVB-irradiated cyclopamine-treated (*b*) *Ptch1*$^{+/-}$ mice. These skin samples were fixed in 10% buffered formalin before staining with H&E and detection of β-galactosidase activities.

found that mice receiving cyclopamine developed 50% fewer new basal cell carcinomas than did the control group at week 52 (Fig. 2, *A* and *C*), and the difference is statistically significant ($P = 0.0078$ by nonparametric test, Mann-Whitney test). More importantly, the number of visible squamous cell carcinomas and spindle cell tumors did not differ between the two groups, showing the specificity of cyclopamine for basal cell carcinomas in this mouse model (Fig. 2*C*).

Cyclopamine Inhibits the Hh Pathway and Induces Apoptosis in Basal Cell Carcinoma Cells. To investigate the mechanism whereby cyclopamine prevents basal cell carcinoma development in *Ptch1*$^{+/-}$ mice, we used the mouse basal cell carcinoma cell line ASZ001, which is derived from basal cell carcinoma-bearing *Ptch1*$^{+/-}$ mice. Both copies of the *Ptch1* gene are lost in this cell line, resulting in constitutive activation of the Hh pathway (16, 17). Incubation with 2 μmol/L KAAD-cyclopamine, a cyclopamine analogue, for 12 hours reduced the levels of Hh target genes (Gli1 and Hip) by >70% (Fig. 3*A*), confirming that cyclopamine inhibits the Hh pathway in these cells. Treatment of ASZ001 cells for 36 hours with cyclopamine or Cur61414 caused a dose-dependent decrease in cell viability (Fig. 3*B*). A SMO antagonist, Cur61414, showed an effect similar to that of cyclopamine (Fig. 3*B*). A similar result was also obtained from the trypan blue exclusion analysis (data not shown). The closely related compound tomatidine, which does not affect SMO signaling and thus served as a negative control, had little discernible effect on the viability of the ASZ001 cells (Fig. 3*B*). Additional evidence that cyclopamine-mediated inhibition of cell growth is dependent on activated Hh signaling came from studies in primary mouse keratinocytes (Hh pathway inactivated), which did not respond to cyclopamine treatment (data not shown). In ASZ001 cells, cyclopamine treatment did not promote cellular differentiation, and hence, the dramatic reduction in the number of living cells suggested that cyclopamine might be inducing apoptosis.

We next assessed cyclopamine-induced apoptosis using flow cytometry analysis and found that treatment of cells with cyclopamine for 36 hours caused a 3-fold increase in the sub-G_1 cell population and a decrease in the S phase (Fig. 3*C*), suggesting that cyclopamine is a potent inducer of apoptosis in basal cell carcinomas. Treatment of ASZ001 cells with either cyclopamine or Cur61414 for 36 hours (see Materials and Methods for details) increased the level of activated caspase-3, a major executioner of cysteinyl aspartate-specific proteinases for apoptosis (Fig. 3*C*).

Confirming our *in vitro* findings, direct injection of cyclopamine into basal cell carcinoma-bearing mice for 72 hours enhanced apoptosis (an increase in TUNEL-positive cells) of basal cell carcinoma tumor cells *in vivo* (Fig. 3*D*).

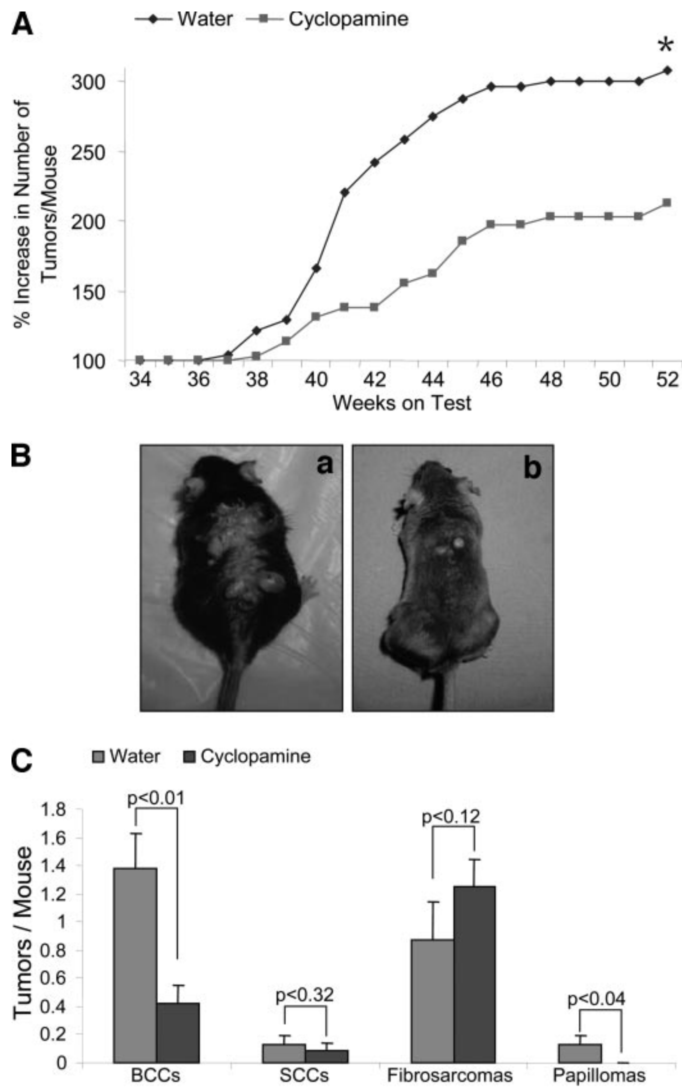

Fig. 2. Effect of cyclopamine on UVB-induced macroscopic basal cell carcinomas (BCCs) in *Ptch1*$^{+/-}$ mice. *A*, effect of cyclopamine administration on the growth of UVB-induced skin tumors in *Ptch1*$^{+/-}$ mice is shown as percent increase in number of tumors per mouse. The statistical analysis was performed using the nonparametric Mann-Whitney test (two-tailed) and obtained $P = 0.0078$ (*) at week 52. $P < 0.05$ is considered statistically significant. *B*, *Ptch1*$^{+/-}$ mice showing effects of cyclopamine treatment on UVB-induced skin tumors. Representative pictures of UVB-irradiated, vehicle-treated control mouse (*a*) and UVB-irradiated, cyclopamine-treated (*b*) mouse at week 52. *C*, effect of orally administered cyclopamine on the growth of UVB-induced BCCs, squamous cell carcinomas (SCCs), and fibrosarcomas in *Ptch1*$^{+/-}$ mice. The statistical analysis was performed using the Student's *t* test: $P < 0.05$ is considered statistically significant.

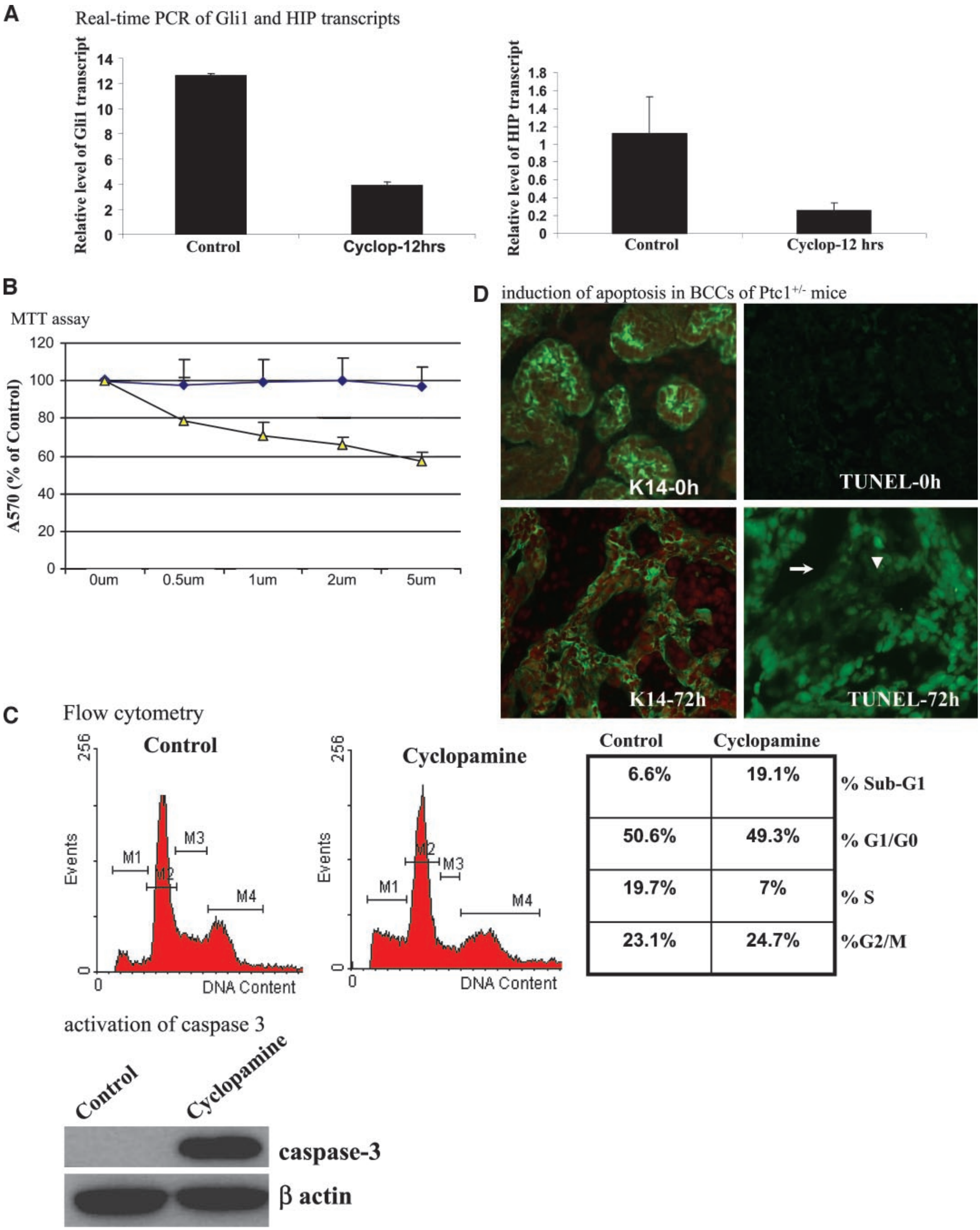

Fig. 3. Cyclopamine inhibits the Hh pathway and induces apoptosis in ASZ001 cells. *A*. Gli1 and HIP transcripts were detected by real-time PCR analysis. Cells were treated with 2 μmol/L KAAD-cyclopamine for 12 hours. After RNA extraction, real-time PCR analysis was performed (see Materials and Methods for details). *B*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay of ASZ001 cells in the presence of SMO antagonists. ASZ001 cells were treated with cyclopamine, or the control compound tamotidine for 36 hours, and the cell viability was examined by MTT assay. *C*, cell cycle and protein analyses of ASZ001 cells. The percentage of cell population in each cell cycle

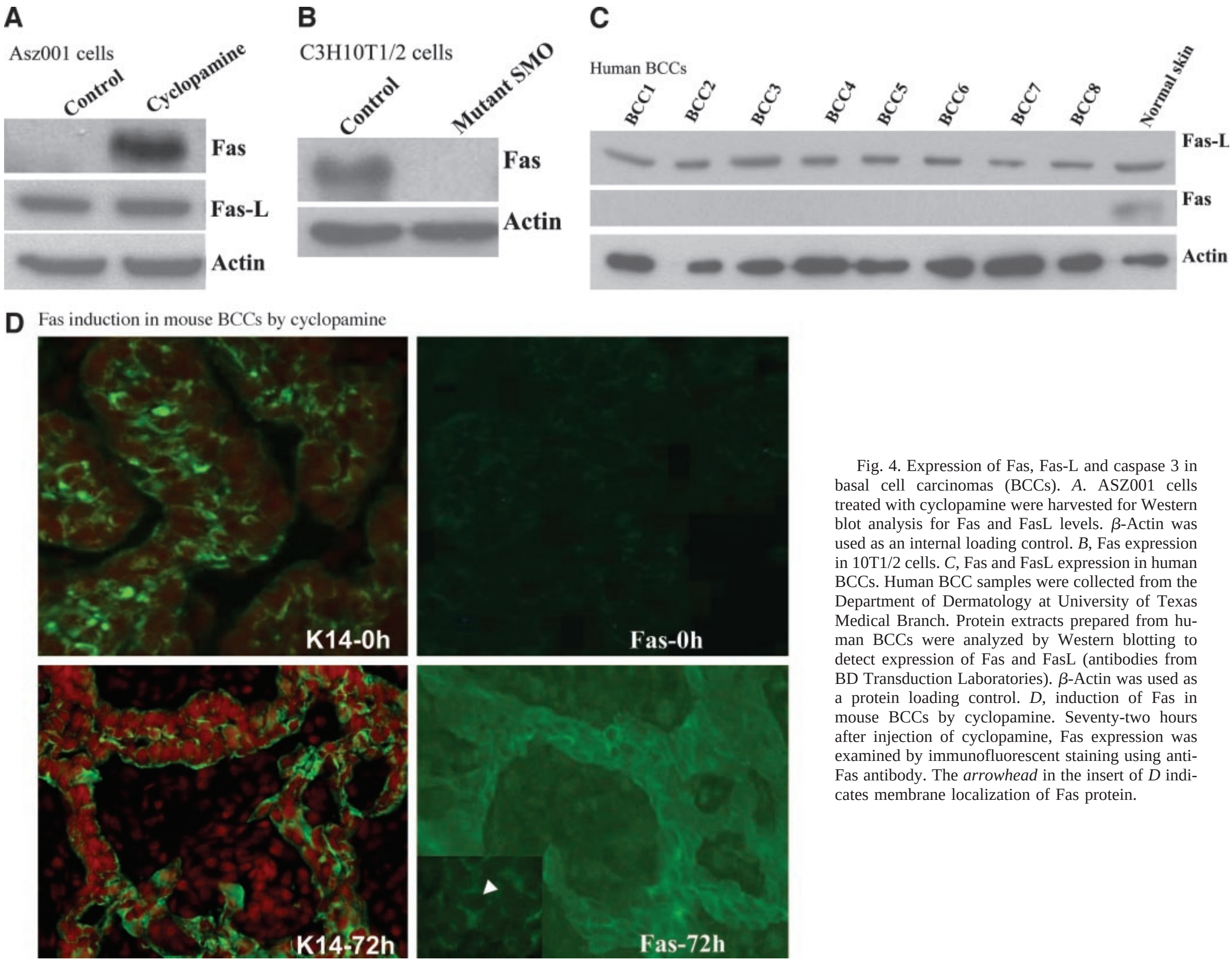

Fig. 4. Expression of Fas, Fas-L and caspase 3 in basal cell carcinomas (BCCs). *A*. ASZ001 cells treated with cyclopamine were harvested for Western blot analysis for Fas and FasL levels. β-Actin was used as an internal loading control. *B*, Fas expression in 10T1/2 cells. *C*, Fas and FasL expression in human BCCs. Human BCC samples were collected from the Department of Dermatology at University of Texas Medical Branch. Protein extracts prepared from human BCCs were analyzed by Western blotting to detect expression of Fas and FasL (antibodies from BD Transduction Laboratories). β-Actin was used as a protein loading control. *D*, induction of Fas in mouse BCCs by cyclopamine. Seventy-two hours after injection of cyclopamine, Fas expression was examined by immunofluorescent staining using anti-Fas antibody. The *arrowhead* in the insert of *D* indicates membrane localization of Fas protein.

Active Fas/FasL Interactions Are Necessary for Cyclopamine-induced Cell Death. Because treatment of human basal cell carcinomas with IFN-α may be accompanied by increased Fas expression in the tumor (19, 22), we tested whether cyclopamine, too, can augment Fas expression. Indeed, cyclopamine substantially increased the level of Fas protein in ASZ001 cells (Fig. 4*A*). In contrast, we detected FasL protein irrespective of cyclopamine treatment (Fig. 4*A*). Using an ELISA assay, we detected FasL in the culture medium of ASZ001 cells, indicating that these basal cell carcinoma cells indeed secrete FasL protein (see Fig. 5*B* for details). Thus, Fas would appear to be the limiting factor for the FasL/Fas signaling axis in this basal cell carcinoma cell line. Conversely, Fas is down-regulated in C3H10T1/2 cells with stable expression of activated SMO via retrovirus-mediated gene transfer (Fig. 4*B*).

The *in vivo* relevance of the data obtained from the experiments with the ASZ001 cell line is supported by the low level baseline expression of Fas in human basal cell carcinomas (refs. 23, 24; Fig. 4*C*) and the induction of Fas expression and apoptosis in *Ptch1*$^{+/-}$ mouse basal cell carcinomas by treatment with either cyclopamine (Fig. 4*D*) or by Cur 61414 (data not shown). Specifically, Fas protein expression increased after cyclopamine injection (either s.c. injection or intratumoral injection), and this was accompanied by increased TUNEL-positive cells (Figs. 3*F* and 4*D*). Immunofluorescent staining revealed a membrane localization of Fas protein in basal cell carcinoma cells (indicated by the arrowhead in the insert of Fig. 4*D*). Thus, it appears that Fas expression is elevated in the presence of SMO antagonists both in cultured cell lines and in basal cell carcinomas induced in *Ptch1*$^{+/-}$ mice.

On the basis of these results, we predicted that either (*a*) interruption of the FasL/Fas signaling axis or (*b*) inhibition of the downstream apoptosis-effector caspase 8 activity would prevent cyclopamine-induced apoptosis. To test this hypothesis, we inactivated FasL/Fas signaling using neutralizing antibodies against FasL. By depleting FasL molecules (Fig. 5*B*), the cyclopamine-mediated decrease in cell viability was rescued (Fig. 6, *C* and *D*). Neutralizing antibodies against FasL also decreased the level of caspase-3 (Fig. 5*C*). Furthermore, administration of the caspase-8 inhibitor Z-IETD-FMK (25) abrogated the cyclopamine-mediated activation of caspase-3 (Fig. 5*C*). Thus, our data provide direct evidence that the FasL/Fas signaling axis is an important mediator of cyclopamine-induced apoptosis in basal cell carcinomas.

phase was shown at the bottom. The level of caspase-3 in ASZ001 cells, a marker for apoptosis, was detected by immunoblotting with a specific antibody from Cell Signaling Technology (*C*). *D*, TUNEL analyses of basal cell carcinomas (BCCs) from *Ptch1*$^{+/-}$ mice. BCCs were induced in *Ptch1*$^{+/-}$ mice as reported previously (16). Cyclopamine was injected s.c. into BCC-bearing *Ptch1*$^{+/-}$ mice or directly into the tumor. Tumor specimens were collected at 72 hours after injection and embedded in OCT. Cryostat tissue sections were used for TUNEL analysis. *Green*, TUNEL or K14; *red*, nuclear staining. —◆—, Tomatidine; —■—, Cur61414; —△—, Cyclopamine.

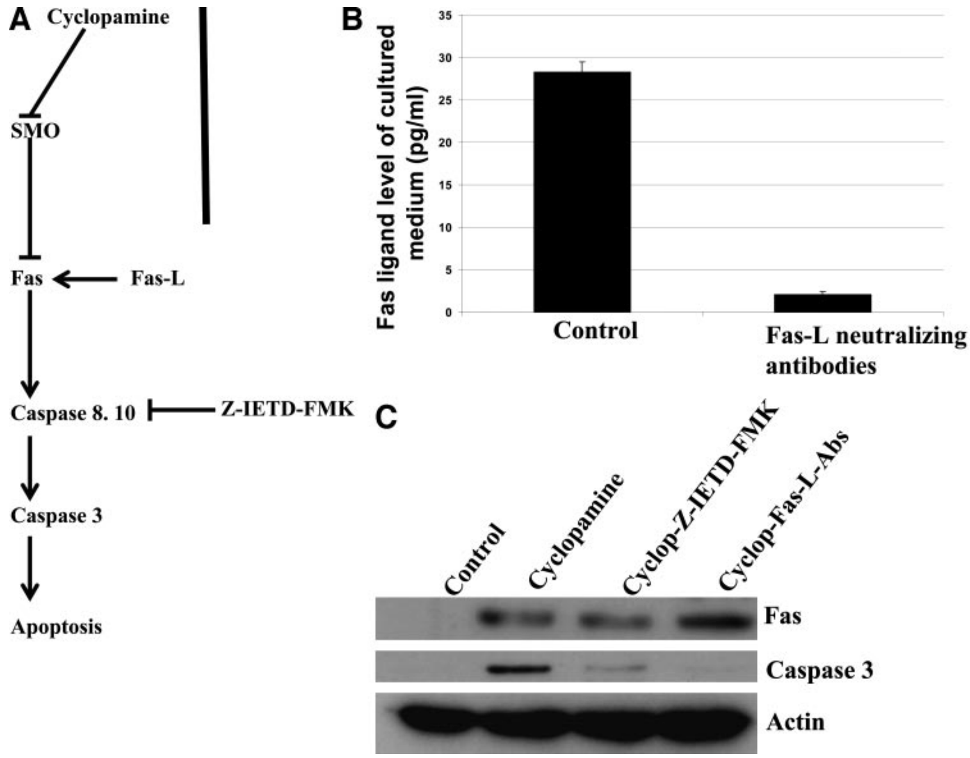

Fig. 5. Inhibition of cyclopamine-induced apoptosis by FasL-neutralizing antibodies. *A*, pathway for cyclopamine-mediated apoptosis. *B*. ASZ001 cells were cultured in 154CF medium without growth supplements during treatment with 2 μmol/L cyclopamine. The level of FasL in the culture medium of ASZ001 cells was detected by ELISA with a kit from R&D Systems, Inc. *C*, detection of Fas and caspase-3 in ASZ001 cells by Western blotting.

The Molecular Basis of Cyclopamine-mediated Induction of Fas. To investigate the mechanism whereby inhibition of the Hh pathway induces Fas expression, we assessed whether this regulation occurs directly as opposed to indirectly by altering other signaling pathways. It has been reported that several signaling pathways, including the Ras-Erk pathway and the p53 pathway, can regulate Fas expression (26–28). We have previously reported that Hh activation augments Ras-Erk signaling (17). Consistent with those findings, we observed that cyclopamine decreased the levels of PDGFR-α and phospho-Erk, indicating an inhibitory effect on Ras-Erk signaling in ASZ001 cell (Fig. 6*B*). As a result, PDGF-A had no effect on cyclopamine-mediated caspase-3 activation, Fas induction, or cell death (see Fig. 6, *D* and *E*, for details). In contrast, epidermal growth factor, which can activate the Ras-Erk pathway through the epidermal growth factor receptor, did inhibit cyclopamine-mediated accumulation of the sub-G_1 population (Fig. 6*C*), Fas expression (Fig. 6*D*), caspase-3 activation (Fig. 6*D*), and cell death (Fig. 6*E*). Furthermore, addition of the mitogen-activated protein kinase kinase inhibitor U0126 alone was sufficient to induce caspase-3 in ASZ001 cells (Fig. 6*D*). Consistent with these data, ASZ001 cells in which PDGFR-α and active Raf were overexpressed resisted cyclopamine treatment (data not shown), providing additional information that inhibition of PDGFR-α and subsequent down-regulation of Ras-Erk signaling is an important mechanism whereby cyclopamine induces apoptosis in basal cell carcinomas.

Our model predicts that overexpression of Gli1 in ASZ001 cells under a strong promoter (such as the cytomegalovirus promoter) would constitutively activate the Hh pathway, which could render these basal cell carcinoma cells resistant to cyclopamine treatment. Indeed, cyclopamine did not induce apoptosis in constitutive Gli1-expressing ASZ001 cells, as indicated by lack of TUNEL staining and of procaspase-3 cleavage (Fig. 6, *F* and *G*). As a result of constitutive Gli1 overexpression, PDGFR-α remained unchanged even after cyclopamine treatment (Fig. 6*G*). In addition, Fas protein was not induced by cyclopamine in constitutive Gli1-expressing ASZ001 cells (Fig. 6*G*). The ability of Gli-1 overexpression to abrogate cyclopamine-mediated cell death was additionally confirmed by flow cytometry analysis (data not shown). Although cyclopamine caused an increase in the sub-G_1 population in Gli1-negative cells, no such change was observed in Gli1-expressing ASZ001 cells. These data indicate that ectopic expression of Gli1 under the cytomegalovirus promoter prevents cyclopamine-induced changes in the expression of PDGFR-α, Fas, and apoptosis.

DISCUSSION

The identification of SMO antagonists has created the opportunity to consider mechanism-driven anticancer strategies for effective treatment of common malignancies in which Hh activation is thought to be important, including basal cell carcinomas, as well as subsets of medulloblastomas, lung cancer, and gastrointestinal cancers (3, 8–11). Basal cell carcinomas frequently contain mutations of the *PTCH1* gene, most of which lead to inactivated *PTCH1* and consequently uncontrolled SMO signaling. Because PTCH1 is upstream of SMO (21),[7] we reasoned that administration of the SMO inhibitor cyclopamine to *Ptch1*$^{+/-}$ mice could specifically and effectively inhibit the development of basal cell carcinomas *in vivo*. Our studies indicate that orally administered cyclopamine is a potent inhibitor of basal cell carcinomas in *Ptch1*$^{+/-}$ mice and that this natural substance does not cause significant toxicity because the overall survival of the treated mice was unaffected. These data are consistent with the observation in sheep that cyclopamine toxicity is limited to Hh signaling-dependent teratogenicity (2, 29) and with the fact that most normal adult mouse and human tissues appear to have very low expression of Hh target genes (30). Basal cell carcinomas would appear to be good candidates for the cutaneous application of SMO antagonists because our data indicate that direct injection of cyclopamine into mouse basal cell carcinomas induces Fas expression and apoptosis. Thus, it should be possible to design topical formulations of

[7] Unpublished observation.

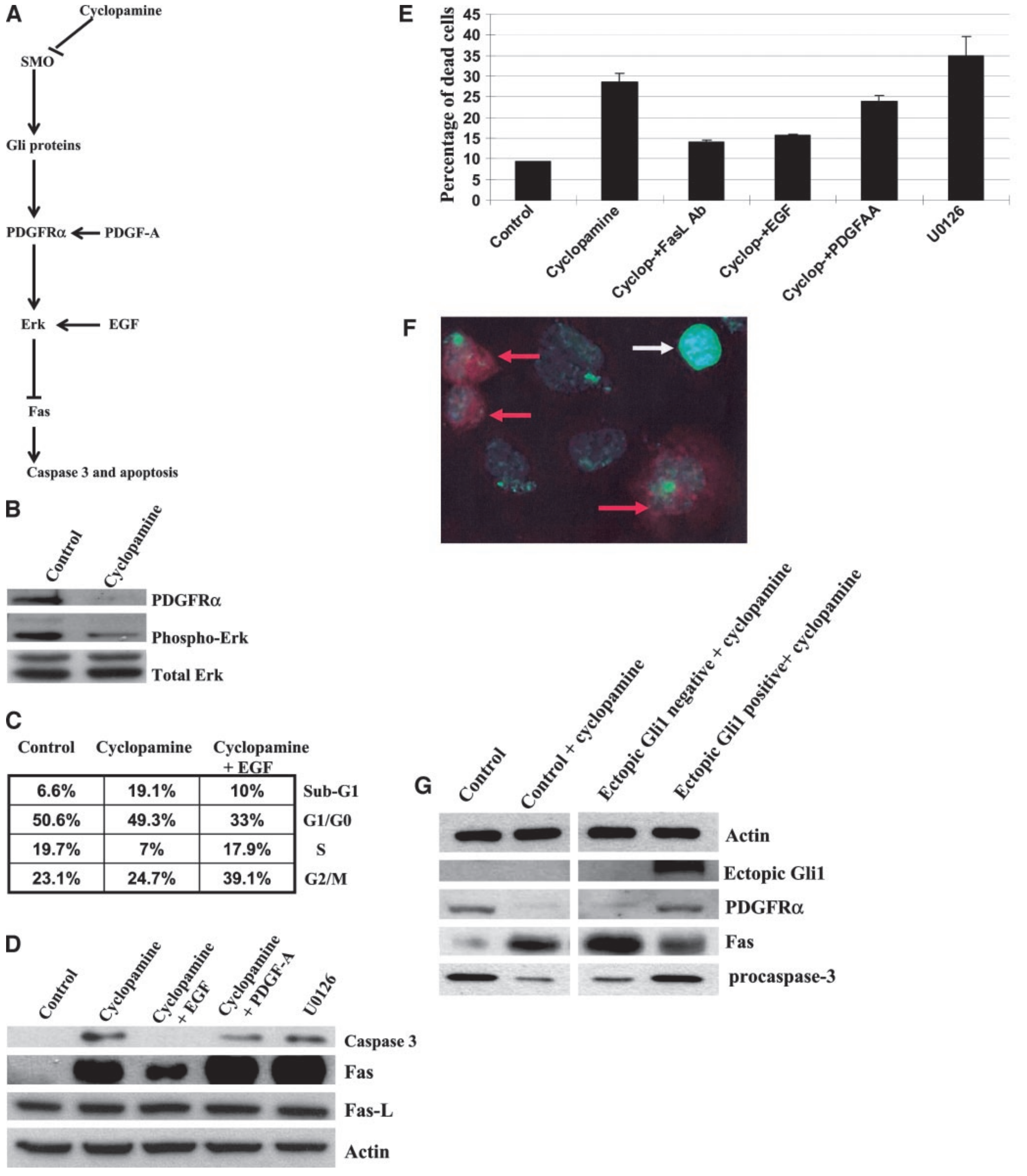

Fig. 6. Regulation of Fas expression by cyclopamine through the Ras-Erk pathway. *A*, pathway for cyclopamine-mediated apoptosis. *B*. In the presence of cyclopamine, the levels of PDGFR-α and phospho-Erk were down-regulated. *C*, epidermal growth factor (EGF) abrogated cyclopamine-mediated sub-G_1 accumulation in ASZ001 cells. *D*. The protein levels of Fas, FasL, and activated caspase-3 were detected by Western blotting after 36 hours treatment with 2 μmol/L KAAD-cyclopamine in the presence of other reagents or with U0126 alone (10 μmol/L). *E*. Cell viability was assessed by trypan blue analysis. Each experiment was repeated three times with similar results. *F*, detection of apoptosis by TUNEL assay in ASZ001 cells with Gli1 expression under the cytomegalovirus promoter. *Red arrows* indicate Gli1-positive cells, and the *white arrow* indicates TUNEL-positive cells. *G*. After enrichment of Gli1-overexpressing cells through cell sorting, the protein levels of PDGFR-α, Fas, and caspase-3 were detected by Western blotting.

cyclopamine or other SMO antagonists for treating basal cell carcinomas, initially in *Ptch1*$^{+/-}$ mice and eventually in humans.

Our data indicate that cyclopamine inhibits the Hh pathway in basal cell carcinomas, as indicated by down-regulation of the target genes HIP and Gli1 (Fig. 3, *A* and *B*). We additionally demonstrate that induction of Fas expression (both the protein and the RNA levels) and consequent activation of the FasL/Fas signaling axis is necessary for cyclopamine-mediated apoptosis because cell death is blocked *in vitro* by anti-FasL antibodies. Thus, it appears that Fas expression is suppressed by the activity of the Ras-Erk pathway in basal cell carcinoma cells, and overexpression of Gli1, PDGFR-α, or active Raf (all downstream of Ptch1) renders ASZ001 cells resistant to cyclopamine-induced apoptosis (Fig. 6, *E* and *F*).[8] Furthermore, addition of a mitogen-activated protein kinase kinase inhibitor U0126 alone is sufficient to induce Fas expression and apoptosis in ASZ001 cells (Fig. 6, *C* and *D*). We have analyzed promoter sequences of human and mouse Fas genes and found multiple copies of serum response elements and Ras-responsive elements, suggesting that the Ras/Erk pathway can regulate transcription of murine and human Fas directly.[9] These data are consistent with a previous report showing that cyclopamine causes apoptosis in subsets of small-cell lung cancer and medulloblastomas in the presence of low concentrations of newborn bovine serum in which growth factor content is quite low (9, 11).[10] Because Fas is regulated at multiple levels, it will be interesting to determine whether other mechanisms, including altered Fas membrane translocation, could be involved in cyclopamine-mediated Fas up-regulation.

Because cyclopamine exerts its effects through direct association with SMO, tumors with genetic mutations downstream of SMO may not be sensitive to cyclopamine treatment. We have found that cyclopamine does not cause apoptosis in ASZ001 cells with Gli1 overexpression under the cytomegalovirus promoter (Fig. 6, *F* and *G*) or in Gli1-transformed RK3E cells.[11] Similarly, cells expressing activated SMO are resistant to cyclopamine (31). Thus, studies on the genetic mutations in specific target tumors could be helpful in predicting the effectiveness of cyclopamine treatment. Effective treatment of tumors with mutations of genes encoding proteins acting downstream of Smo will require identification of novel small molecular weight compounds acting downstream of SMO signaling. However, because most basal cell carcinomas do contain loss-of-function mutations of PTCH1, cyclopamine should represent an effective and specific agent for basal cell carcinoma therapy, as well as for those visceral cancers with Hh signaling activation, which thus far appears to be driven by overexpression of sonic Hh.

In summary, our results indicate that chronic administration of the SMO antagonist cyclopamine is effective in preventing basal cell carcinoma development *in vivo*. We demonstrate that cyclopamine inhibits Hh signaling and thereby exerts its effects through induction of Fas expression, leading to activation of the FasL/Fas signaling axis and apoptosis. It is likely that SMO antagonists capable of inhibiting Hh activation and inducing Fas expression hold great promise as a mechanism-directed approach for the treatment of basal cell carcinomas.

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[8] Unpublished data.
[9] Unpublished observation.
[10] Unpublished data.
[11] J. Xie and C. Li, unpublished data.

REFERENCES

1. Ingham PW. Transducing Hedgehog: the story so far. EMBO J 1998;17:3505–11.
2. Taipale J, Beachy PA. The Hedgehog and Wnt signalling pathways in cancer. Nature (Lond.) 2001;411:349–54.
3. Epstein E Jr. Genetic determinants of basal cell carcinoma risk. Med Pediatr Oncol 2001;36:555–8.
4. Toftgard R. Hedgehog signalling in cancer. Cell Mol Life Sci 2000;57:1720–31.
5. Johnson RL, Rothman AL, Xie J, et al. Human homolog of patched, a candidate gene for the basal cell nevus syndrome Science (Wash. DC) 1996;272:1668–71.
6. Hahn H, Wicking C, Zaphiropoulous PG, et al. Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. Cell 1996;85:841–51.
7. Xie J, Murone M, Luoh SM, et al. Activating Smoothened mutations in sporadic basal-cell carcinoma Nature (Lond.) 1998;391:90–2.
8. Thayer SP, di Magliano MP, Heiser PW, et al. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature (Lond.) 2003;425:851–6.
9. Berman DM, Karhadkar SS, Hallahan AR, et al. Medulloblastoma growth inhibition by hedgehog pathway blockade. Science (Wash. DC) 2002;297:1559–61.
10. Berman DM, Karhadkar SS, Maitra A, et al. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature (Lond.) 2003;425:846–51.
11. Watkins DN, Berman DM, Burkholder SG, Wang B, Beachy PA, Baylin SB. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. Nature (Lond.) 2003;422:313–7.
12. Chen JK, Taipale J, Cooper MK, Beachy PA. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev 2002;16:2743–8.
13. Williams JA, Guicherit OM, Zaharian BI, et al. Identification of a small molecule inhibitor of the hedgehog signaling pathway: effects on basal cell carcinoma-like lesions. Proc Natl Acad Sci USA 2003;100:4616–21.
14. Frank-Kamenetsky M, Zhang XM, Bottega S, et al. Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. J Biol 2002;1:10.
15. Goodrich LV, Milenkovic L, Higgins KM, Scott MP. Altered neural cell fates and medulloblastoma in mouse patched mutants. Science (Wash. DC) 1997;277:1109–13.
16. Aszterbaum M, Epstein J, Oro A, et al. Ultraviolet and ionizing radiation enhance the growth of BCCs and trichoblastomas in patched heterozygous knockout mice. Nat Med 1999;5:1285–91.
17. Xie J, Aszterbaum M, Zhang X, et al. A role of PDGFRalpha in basal cell carcinoma proliferation. Proc Natl Acad Sci USA 2001;98:9255–9.
18. Louro ID, Bailey EC, Li X, et al. Comparative gene expression profile analysis of GLI and c-MYC in an epithelial model of malignant transformation. Cancer Res 2002;62:5867–73.
19. Li C, Chi S, He N, et al. IFNalpha induces Fas expression and apoptosis in hedgehog pathway activated BCC cells through inhibiting Ras-Erk signaling. Oncogene 2004;23:1608–17.
20. Bonifas JM, Pennypacker S, Chuang PT, et al. Activation of expression of hedgehog target genes in basal cell carcinomas. J Investig Dermatol 2001;116:739–42.
21. Zhang XM, Ramalho-Santos M, McMahon AP. Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the mouse node. Cell 2001;105:781–92.
22. Buechner SA, Wernli M, Harr T, Hahn S, Itin P, Erb P. Regression of basal cell carcinoma by intralesional interferon-alpha treatment is mediated by CD95 (Apo-1/Fas)-CD95 ligand-induced suicide. J Clin Investig 1997;100:2691–6.
23. Lee S, Jang JJ, Lee JY, et al. Fas ligand is expressed in normal skin and in some cutaneous malignancies. Br J Dermatology 1998;139:186–91.
24. Filipowicz E, Adegboyega P, Sanchez RL, Gatalica Z. Expression of CD95 (Fas) in sun-exposed human skin and cutaneous carcinomas. Cancer (Phila.) 2002;94:814–9.
25. Thornberry NA, Lazebnik Y. Caspases: enemies within. Science (Wash. DC) 1998;281:1312–6.
26. Fenton RG, Hixon JA, Wright PW, Brooks AD, Sayers TJ. Inhibition of Fas (CD95) expression and Fas-mediated apoptosis by oncogenic Ras. Cancer Res 1998;58:3391–400.
27. Kannan KAN, Rechavi G, Jakob-Hirsch J, et al. DNA microarrays identification of primary and secondary target genes regulated by p53. Oncogene 2001;20:2225–34.
28. Kazama H, Yonehara S. Oncogenic K-Ras and basic fibroblast growth factor prevent Fas-mediated apoptosis in fibroblasts through activation of mitogen-activated protein kinase. J Cell Biol 2000;148:557–66.
29. Cooper MK, Porter JA, Young KE, Beachy PA. Teratogen-mediated inhibition of target tissue response to Shh signaling. Science (Wash. DC) 1998;280:1603–7.
30. Hu Z, Bonifas JM, Aragon G, et al. Evidence for lack of enhanced hedgehog target gene expression in common extracutaneous tumors. Cancer Res 2003;63:923–8.
31. Taipale J, Chen JK, Cooper MK, et al. Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. Nature (Lond.) 2000;406:1005–9.