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Forskolin, a Hedgehog signal inhibitor, inhibits cell proliferation and induces apoptosis in pediatric tumor cell lines

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Abstract. The Hedgehog (Hh) signaling pathway regulates the development of many organs in mammals. Recent studies have indicated that the activation of the Hh signaling pathway contributes to the growth of various adult cancers. However, little is known about its role in the development of pediatric malignancies. The present study was undertaken to examine the expression and functional involvement of Hh signal transcription factors in pediatric tumor cells in order to determine their potential as therapeutic targets. We utilized real-time RT-PCR to investigate the expression of Glioma-associated oncogene homolog 1 (*Gli1)* in various pediatric tumor cell lines, including rhabdomyosarcoma, neuroblastoma and hepatoblastoma. The mRNA expression of *Gli1* was markedly increased in rhabdomyosarcoma (RMS-YM, RD, RH30) cell lines, and moderately increased in neuroblastoma (NB19) and hepatoblastoma (Huh6) cell lines. The proliferation of these cell lines was dose dependently inhibited by Forskolin, a specific Hh signal inhibitor. In addition, Forskolin-induced growth suppression was associated with the down-regulation of *C-Myc*. Moreover, the blockade of Hh signaling with Forskolin enhanced cell apoptosis in a dose-dependent manner. These results demonstrated that Hh signal activation frequently occurs in neuroblastoma, hepatoblastoma and rhabdomyosarcoma cell lines. The inhibition of Hh signaling suppressed proliferation and increased apoptosis in these tumor cells. These findings suggest that the Hh signaling pathway plays an important role in tumorigenesis and is a potential molecular target of new treatment strategies for these pediatric malignant tumors.

Introduction

The hedgehog (Hh) signaling pathway is crucial to growth and differentiation in a wide variety of tissues during embryonic development (1,2). Vertebrate organisms possess three Hh proteins, namely, Sonic Hh (Shh), Indiana Hh (Ihh) and Desert Hh (Dhh), which all bind to the same receptor, Patched 1 (Ptch1). Without ligand stimulation, Ptch1 inhibits the activity of the seven-pass transmembrane protein Smoothened (Smo), which results in the inactivation of Hh signaling. The Hh pathway is activated through Shh, which represses the negative modulation of the pathway executed by Ptch on Smo. Upon Hh binding, this negative modulation is relieved and Smo transduces the signal to the ultimate effectors of the pathway, the zinc-finger transcription factors Glioma-associated oncogene homolog (Gli)1, Gli2 and Gli3 (3).

The Hh signaling pathway has recently been shown to be involved in the development of various malignant tumors. Constitutive activation of the Hh pathway has been observed in several types of tumors, such as basal cell carcinoma of the skin (BCC), cerebellar medulloblastoma and cancers of the stomach, colon, pancreas, lung and prostate (4-11). Interestingly, it has been reported that the plant-derived teratogenic steroidal alkaloid Cyclopamine, which inhibits the Hh pathway by antagonizing Smo (12,13), suppresses the growth of malignant tumors, including pancreatic, breast and gastric cancers (7,9,14,15). Although these observations suggest that the Hh pathway may be involved in tumor development and progression, the biological significance of Hh pathway activity in pediatric malignancies is not clear. Only a few reports on rhabdomyosarcoma have been published (10). Recently, we examined the expression of the Hh signaling mediators Shh, Path1 and Gli1 using immunostaining in various pediatric tumors, and found that the Hh signals were present in various pediatric malignancies (16). These findings suggest that the activation of the Hh signaling pathway contributes to the development of various pediatric malignancies. We therefore hypothesized that the inhibition of the Hh pathway may suppress the growth of pediatric tumors in which Hh signaling is activated. We subsequently investigated the inhibitory effects of Forskolin, an Hh pathway inhibitor, on pediatric tumor cell lines.

The proper function of the Hh pathway involves several kinases, including protein kinase A (PKA), which has been implicated in the modulation of several steps of Hh signal transduction (17). PKA has been shown to be an antagonist of Hh signaling in several vertebrate systems, and this antagonism has been ascribed to its role in regulating the processing of Gli-related proteins to the repressor form (18).

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Forskolin stimulates PKA activity by increasing intracellular cyclic AMP; therefore, it inhibits the intercellular signaling of the Hh pathway. Forskolin has been shown to inhibit Shh signaling in several developing systems, including the mesoblastic segment, brain, feather bud, tooth and testis (19-23). To address the need for broadly active downstream inhibitors of Hh signaling, we targeted Forskolin as an antagonist of the Gli family of transcriptional effectors, which constitutes the final stage in the Hh pathway.

In this study, we initially analyzed Hh pathway activation in various pediatric tumor cell lines, and revealed that the Hh pathway is constitutively activated in these cell lines. We subsequently investigated the effects of the Hh pathway inhibitor Forskolin on cell proliferation and cell death in order to determine whether the Hh pathway could be a therapeutic target for the treatment of pediatric malignant tumors.

Table I. Gene-specific primers used for semi-quantitative RT-PCR.

Primer	Sequence (5'-3' orientation)
GAPDH-F	TGAAAGTGCTGTCTCCATGC
GAPDH-R	ACCTTTGGTGAGACCTGTGG
Gli1-F	TGCCTTGTACCCTCCTCCCGAA
Gli1-R	GCGATCTGTGATGGATGAGATTCCC
Smo-F	ATCTCCACAGGAACTGGTTCGG
Smo-R	AAAGTGGGGCCTTGGGAACATG
Shh-F	CGCACGGGGACAGCTCGGAAGT
Shh-R	CTGCGCGGCCCTCGTAGTGC
Ptch1-F	CGGCGTTCTCAATGGGCTGGTTTT
Ptch1-R	GTGGGGCTGCTGTCTCGGGTTCG

Materials and methods

Cell lines. Eight cell lines derived from human pediatric malignant tumors, namely, Huh6 (hepatoblastoma), Huh7 and HepG2 (hepatocellular carcinoma), NB1 and NB19 (neuroblastoma), RMS-YM and RD (embryonal type rhabdomyosarcoma) and RH30 (alveolar type rhabdomyosarcoma), were used. Caco2 (colorectal cancer) and HEK293 (primitive renal cell) were used as controls. The colonic cell line Caco2 was used as a negative control, since it has been reported that the Hh pathway is not activated in colonic carcinomas (26). The cell lines were provided by Riken BRC (Ibaraki, Japan) or ATCC (VA, USA). Cells were cultured in Dulbecco's modified essential medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics.

RNA extraction and cDNA preparation. Total RNA was extracted from the tumor cell lines using TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration of total RNA was measured by NanoDrop 1000 (Thermo Scientific Co., Yokohama, Japan). Reverse transcription of 1 μg of total RNA was performed using Oligo dT primers (Invitrogen Co.) and reverse transcriptase (Toyobo Co., Osaka, Japan) to create cDNA, which was then stored at -80˚C.

mRNA expression of target genes. The mRNA expression of Hh signal component genes was evaluated by RT-PCR using the PrimeScript RT-PCR kit (Takara Co., Shiga, Japan) according to the manufacturer's protocol. PCR amplifications of the human Hh signaling related genes *Gli1*, *Smo*, *Shh*, *Ptch1* and housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were performed with 50 ng of cDNA of the cell lines using the forward and reverse primers listed in Table I. PCR reactions were performed in 20 μl of the final reaction mixture for 28-35 cycles consisting of 30 sec at 58˚C and extension for 1 min at 72˚C. The individual cycle number for each gene was defined by predetermining the linear range of the PCR.

Inhibition of Hh signaling using Forskolin. To test the inhibitory effects of Forskolin (Sigma Aldrich Co., Tokyo, Japan) on the Hh pathway, the Hh signal-activated cell lines Huh6, HepG2, NB19, RMS-YM, RD and RH30 were cultured for 24 or 48 h in medium in the presence of 50, 100, 200, 300 or 400 mM Forskolin dissolved in DMSO. In the control samples, DMSO alone was added.

Expression of downstream genes. The expression of Hh signaling pathway downstream genes *Gli1* and *c-Myc* were evaluated by real-time PCR, performed using the ABI PRISM 9700 Sequence Detector System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The primer and probe mixtures for *Gli1*, *c-Myc* and *GAPDH* were purchased from Perkin-Elmer Applied Biosystems, and the PCR was performed according to the manufacturer's protocol. For quantification, *GAPDH* transcript served as the control and each sample was normalized to its *GAPDH* transcript level. The relative levels of mRNA expression were expressed as the ratio to the expression levels of the control samples.

Analysis of cell proliferation and apoptosis. Cell proliferation was assessed by a WST-1 assay (Dojindo Co., Kumamoto, Japan). The absorbency of the treated samples against a blank control was measured using a microplate reader (Immuno Mini NJ-2300, Nippon InterMed, Tokyo, Japan) under 414 nm as a detection wavelength and 630 nm as a reference wavelength.

To assess the influence of Hh inhibitor Forskolin on cell survival, the apoptotic features of *Gli1*-activated cell lines were evaluated. Cells were grown to confluence in 96-well plates and incubated with DMEM containing 0.5% FBS and Forskolin at a concentration of 100 μM for 48 h. Apoptosis was evaluated using the Cell Death Detection ELISA Kit (Roche Applied Science, Basel, Switzerland) according to the manufacturer's protocol. This kit is a photometric enzyme-immunoassay for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) after apoptotic cell death.

Comparison of the inhibitory effect of Cyclopamine and Forskolin. To compare the inhibitory effect of Cyclopamine and Forskolin on cell proliferation, the Hh signal-activated cell lines Huh6, HepG2, NB19, RMS-YM, RD and RH30

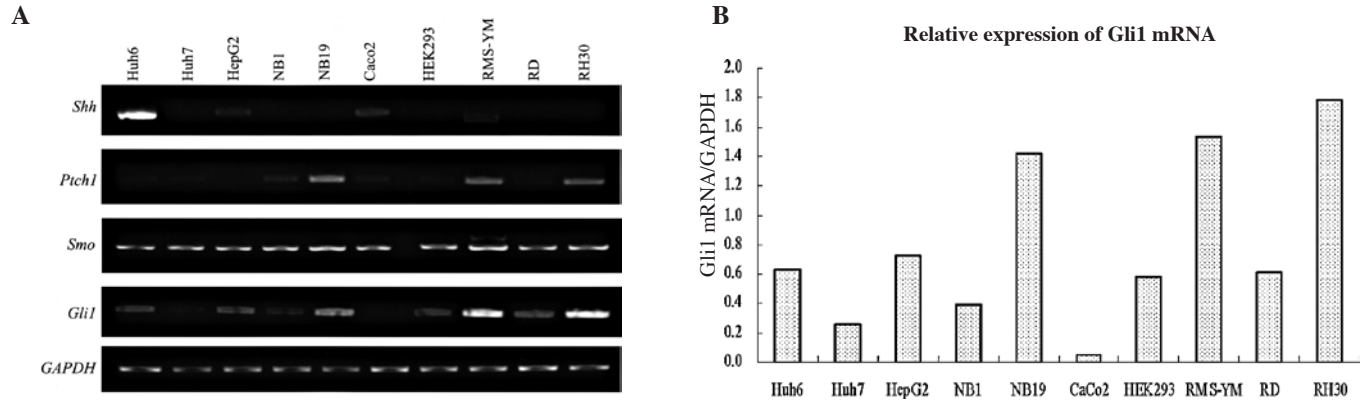

Figure 1. (A) The mRNA expression of Hedgehog signaling molecules *Shh*, *Ptch1*, *Smo* and *Gli1* in 10 cell lines using RT-PCR. *Shh* was expressed in Huh6, HepG2, Caco2 and RMS. *Ptch1* expression was higher in NB19, RMS and RH30 than in other cell lines. The expression of *Smo* was detected in all cell lines. (B) Relative mRNA expression of Gli1 in 10 cell lines evaluated by real-time PCR. *Gli1* was expressed at various levels in all cell lines except Caco2.

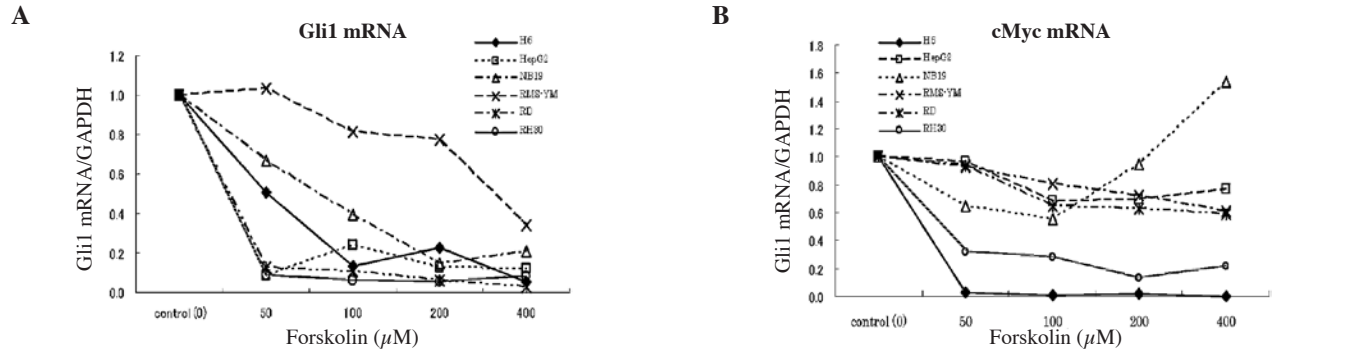

Figure 2. Inhibition of *Gli1* (A) and *cMyc* (B) mRNA expression by Forskolin. Six cell lines with high *Gli1* expression were cultured in the presence of 50-400 μM Forskolin. Real-time RT-PCR in each cell line revealed the inhibitory effects of Forskolin. (A) *Gli1* mRNA levels were significantly reduced by Forskolin in a dose- and time-dependent manner. (B) *c-Myc* mRNA levels were significantly decreased by Forskolin in a dose- and time-dependent manner in all cell lines except NB19.

were cultured for 48 h in medium in the presence of 5 mM Cyclopamine or 100 mM Forskolin dissolved in DMSO. Cell proliferation was assessed by the WST-1 assay. DMSO alone was added in the negative control samples.

Statistical analysis. Experiments were performed at least three times and representative results are presented. Data are presented as the mean with standard deviation for each parameter. Statistical analysis was performed using an unpaired Student's t-test, and a P-value of <0.05 was considered statistically significant.

Results

Expression of Hh pathway components in each cell line. To analyze whether Hedgehog signaling is present in pediatric malignancies, we initially examined the expression of the Hh signaling molecules *Shh*, *Ptch1*, *Smo* and *Gli1* in 10 cell lines using RT-PCR (Fig. 1A). *Shh* was expressed in Huh6, HepG2, Caco2 and RMS. Although various levels of *Ptch1* expression were observed in all the cell lines, *Ptch1* expression was most intense in NB19, RMS and RH30. The expression of *Smo* was detected in all cell lines. *Gli1*, the transcription factor which is also considered to be a downstream target of Hh signaling, was expressed at various levels in every cell line except Caco2 (Fig. 1B).

Inhibition of the Hh signaling pathway by Forskolin. To explore the potential therapeutic utility of Hh pathway blockade, 6 cell lines with intensive *Gli1* expression were cultured in the presence of 50-400 μM Forskolin. Real-time RT-PCR in each cell line demonstrated the inhibitory properties of Forskolin in a dose- and time-dependent manner. *Gli1* mRNA levels were significantly reduced by Forskolin from the starting concentration of 50 μM at 24 h, indicating strong pathway inhibition (Fig. 2). We also evaluated *c-Myc* mRNA levels, since *c-Myc* is downstream of the Hh signal pathway. *c-Myc* mRNA levels were significantly decreased in response to Forskolin in a dose- and time-dependent manner in all cell lines except NB19.

Effect of Forskolin on cell proliferation and apoptosis. Cell proliferation was significantly suppressed by Forskolin administration from 24 to 96 h in NB19, RMS, RD and RH30. Proliferation of Huh6 was suppressed at 24 h, but recovered at 48 h. HepG2 proliferation was not affected by Forskolin (Fig. 3).

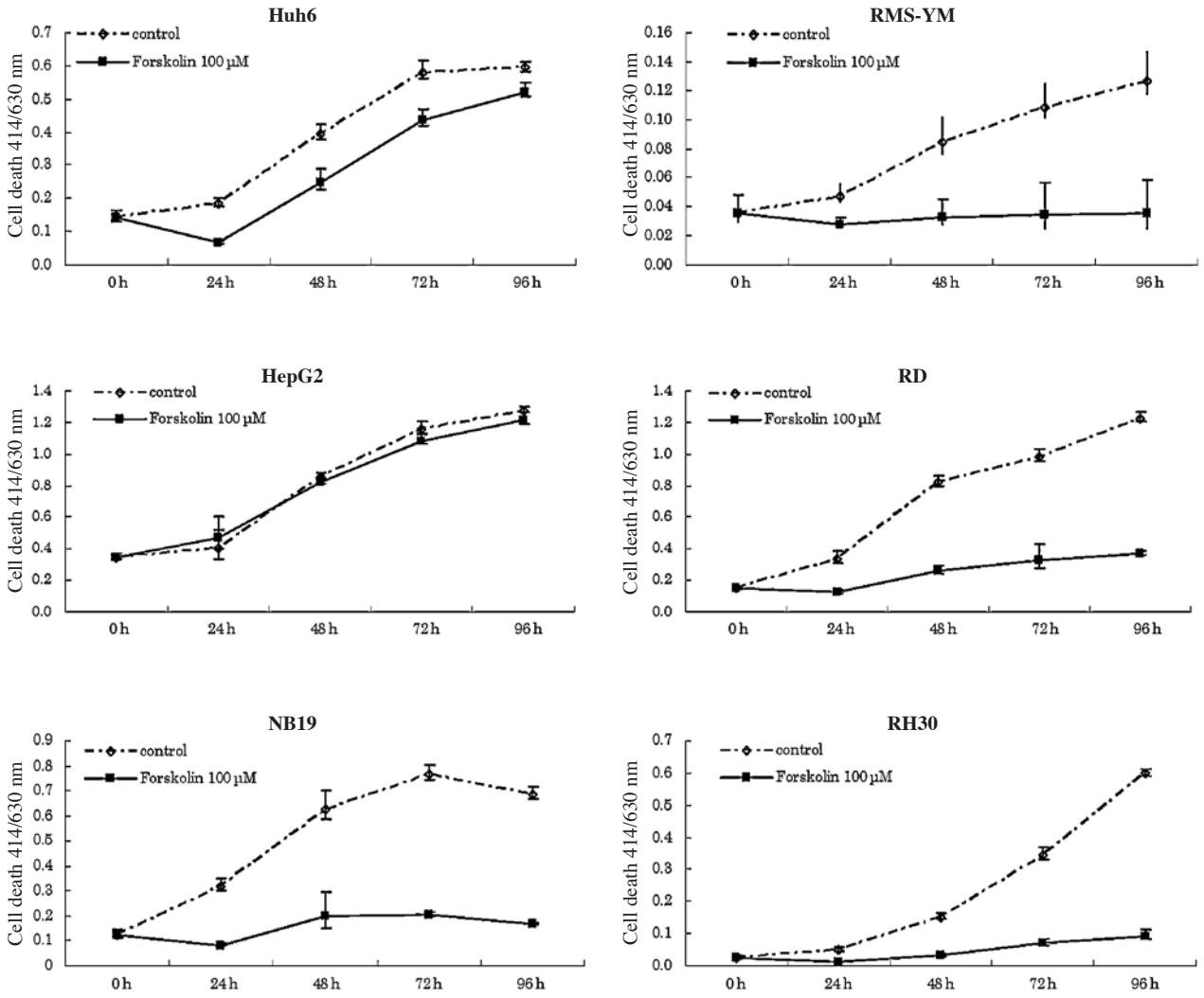

Figure 3. Effect of Forskolin on cell proliferation. Cell proliferation assessed by WST-1 assay was significantly suppressed by Forskolin administration from 24 to 96 h in NB19, RMS, RD and RH30 (P<0.05). Proliferation of Huh6 was suppressed at 24 h, but had recovered at 48 h.

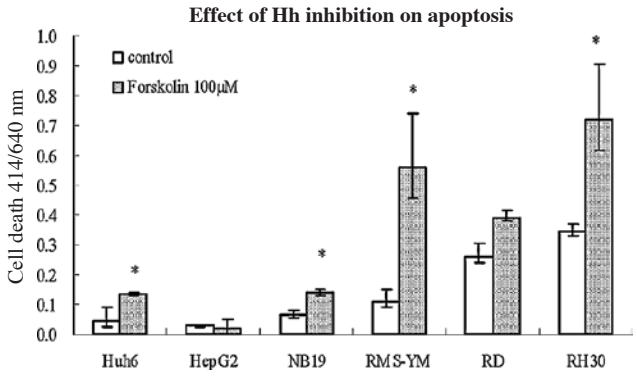

Figure 4. Effect of Forskolin on apoptotic cell death. Apoptotic cell death as assessed by the Cell Death Detection ELISA Kit was significantly increased by the administration of Forskolin in Huh6, NB19, RMS-YM, RD and RH30; however, Forskolin had no effect on apoptosis in HepG2 (P<0.005).

Apoptic cell death significantly increased as a result of Forskolin administration in Huh6, NB19, RMS-YM, RD and RH30; however, Forskolin had no effect on apoptosis in HepG2 (Fig. 4).

Comparison of the inhibitory effects of Cycropermine and Forskolin. Cell proliferation of Huh6, HepG2, NB19, RMS-YM, RD and RH30 was significantly reduced by Forskolin at 24 h in comparison to the control samples. Moreover, Forskolin was significantly more effective than Cyclopamine in regard to the inhibition of cell proliferation (Fig. 5).

Discussion

A few reports have described the expression of Hh pathway components in pediatric tumors. Tosler *et al* analyzed Ptch1 and Gli1 mRNA expression in 43 sporadic rhabdomyomas and rhabdomyosarcomas and observed that Ptch1 (43/43) and Gli1 (41/43) were overexpressed in these tumors (10). Mehdi *et al* investigated the activation of the Hh pathway in glioblastoma, neuroblastoma and medulloblastoma. They observed the presence of Hh signaling in glioblastoma and medulloblastoma and to a lesser extent in neuroblastoma (24). Eichenmuller *et al* reported the frequent occurrence of *Gli1* and *Ptch1* overexpression and HHIP promoter methylation in early childhood hepatoblastoma (25). We recently

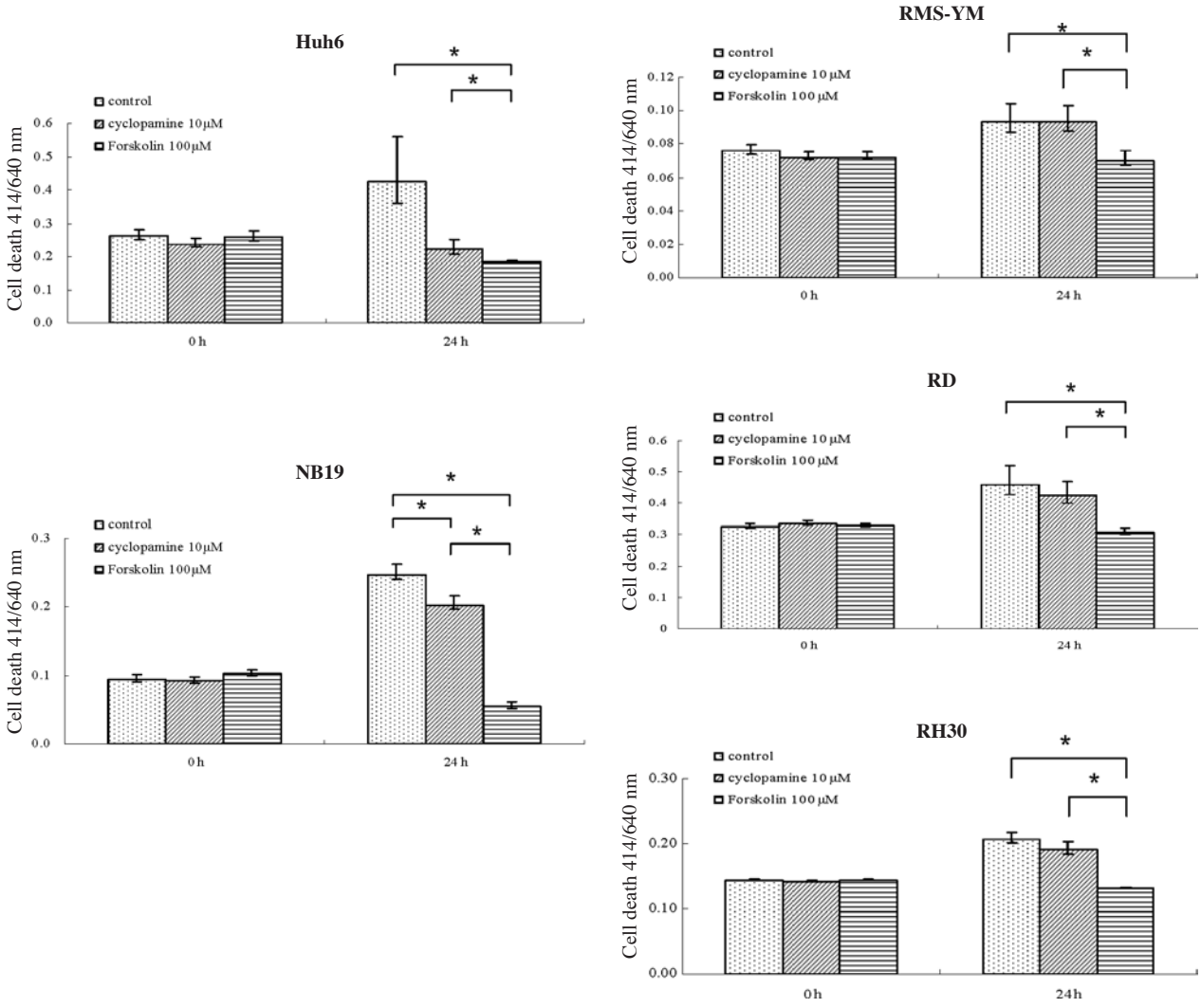

Figure 5. Comparison of the inhibitory effect of Cyclopamine and Forskolin on cell proliferation. Cell proliferation of NB19, RMS-YM, RD and RH30 was significantly reduced by Forskolin at 24 h as compared with the control samples, whereas only the proliferation of NB19 was reduced by Cyclopamine. Cell proliferation was more effectively inhibited by Forskolin than by Cyclopamine.

investigated Shh, Ptch1 and Gli1 expression using immunohistochemistry in various pediatric tumors and cell lines, including hepatoblastoma, neuroblastoma and rhabdomyosarcoma. Shh and its receptor Ptch were expressed in most of the examined pediatric tumors, and Gli1 was expressed in almost 70% of the tumors (16). In the present study, all of the pediatric tumor cell lines showed various levels of *Ptch1* and *Smo* expression, and *Gli1* was expressed at various levels in every cell line except Caco2 (Fig. 1A and B). These data suggest that the Hh signaling pathway is broadly present and activated in various pediatric tumors and cell lines; furthermore, Hh signaling may be associated either with tumor development or maintenance. These findings also indicate that the Hh pathway may be a novel therapeutic target in the treatment of pediatric malignancies displaying an overexpression of the Hh pathway. Other authors have proposed that Forskolin inhibits Hh pathway activation via direct interaction with the Gli family of transcriptional effectors (26,27). To determine whether activated Hh signaling is essential for the proliferation of pediatric tumor cells, we treated the cell lines with Forskolin.

When pediatric tumor cells were treated with 100 μmol/l Forskolin, the proliferation of hepatoblastoma (Huh6), neuroblastoma (NB19) and rhabdomyosarcoma (RMS-YM, RD, RH30) cells was significantly decreased in a dose- and time-dependent manner (Fig. 3). These results suggest that the Hh pathway contributes to cell proliferation in neuroblastoma, hepatoblasatoma and rhabdomyosarcoma. These data provide evidence that blockade of the Hh pathway effectively suppresses tumor cell growth.

Cyclopamine, a plant-derived teratogenic steroidal alkaloid, is often used to suppress tHh signaling. It blocks Hh signal transduction by direct binding to Smo (30). It has been reported that Cyclopamine suppresses the growth of adult malignant tumors, including pancreatic, breast and gastric cancers *in vitro* and *in vivo* (7,9,14,15,29-32). In this study, we used Forskolin as an inhibitor of Hh signaling instead of Cyclopamine. Cyclopamine inhibits the transmembrane protein Smo, whereas Forskolin inhibits Gli protein, the downstream intra-cellular signaling mediator. Therefore, we hypothesized that Forskolin inhibits Hh signaling more specifically and directly than Cyclopamine. In fact, the inhibi-

tion of cell proliferation of NB19, RMS-YM, RD and RH30 cells was more effective by Forskolin than by Cyclopamine, as shown in Fig. 5.

Previous studies reported that the inhibition of Hh signaling induces apoptosis in cancer cells with activated Hh signaling (33-35); therefore, we investigated whether the inhibition of Hh signaling by Forskolin induces apoptosis in pediatric tumor cells. We determined that apoptotic cell death was significantly increased in hepatoblastoma (Huh6), neuroblastoma (NB19) and rhabdomyosarcoma (RMS-YM, RD, RH30) cells. Our data clearly demonstrate that Forskolin-induced growth inhibition of *Gli1*-activated pediatric tumor cells is mediated not only by the reduction of proliferation but also by the induction of apoptotic cell death.

Forskolin is reported to suppress Hh signaling by the inhibition of the Gli family of intracellular signal mediators. Three vertebrate Gli genes (Gli1, -2 and -3) have been identified (4). Gli2 and -3 have distinct, context-dependent repressor and activator functions, and Gli3 is cleaved into a repressor form in the absence of Hh signaling (11,36,37). In contrast, Gli1 is a strong positive activator of downstream target genes and is itself a transcriptional target of Hh signaling (38). Gli1 is considered to be a marker of Hh pathway activation (4,39-42). Therefore, Forskolin suppressed the Hh signaling pathway by controlling the elevated expression of Gli1 in the cell lines with high *Gli1* expression.

The mechanisms of the activation of Hh signaling in malignant tumors remain unknown. The first evidence linking Hh pathway activity to human cancer was the identification of germ-line mutations of Ptch1 in Gorlin syndrome, a rare autosomal disease associated with an increased incidence of basal cell carcinoma (BCC), medulloblastoma and rhabdomyosarcoma (2,43,44). In addition, somatic mutations of several components of the Hh pathway, including Ptch1 and Smo, have been detected in many sporadic BCCs and medulloblastomas (45-48). To date, no mutation of Hh pathway components has been reported in pediatric tumors. It is necessary to further analyze the mutation status of Ptch, Smo and other signaling molecules in pediatric malignancies in order to determine whether the mutations of these genes are associated with tumorigenesis.

Although the treatment of pediatric tumors has dramatically improved over the past 20 years, the prognosis of patients with high-risk or recurrent tumors is still poor. Therefore, the development of new treatment strategies is essential. Many factors may contribute to the poor prognosis of pediatric tumors; however, the lack of understanding of the molecular pathways involved in tumor progression has limited the development of effective treatments. In this study, the data clearly demonstrated that Forskolin inhibited cell growth and induced apoptosis in Gli1-positive pediatric tumor cell lines, suggesting that Gli1 may be a key factor in the tumorigenesis of pediatric malignancies. Forskolin may therefore be a candidate therapeutic agent for Gli1-elevated malignancies. At present, there is no clinically available treatment that specifically targets the Hh signaling pathway. Further research should be conducted to demonstrate that Hh pathway inhibition is a useful therapeutic strategy in pediatric malignancies. We plan to investigate the tumor-suppressing effects of Forskolin *in vivo* using a xenograft model of pediatric tumors.

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