Overexpression of Hedgehog Signaling Molecules and Its Involvement in the Proliferation of Endometrial Carcinoma Cells

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Abstract

Purpose: Research has revealed abnormal activation of the hedgehog pathway in human malignancies. The present study was undertaken to examine the expression and functional involvement of the hedgehog pathway in endometrial tissues.

Experimental Design: The expression of sonic hedgehog (Shh), patched (Ptch), Smoothened (Smo), and Gli1 was examined in various endometrial tissues and endometrial carcinoma cell lines. The effect of hedgehog signaling on the proliferation of endometrial carcinoma cell lines was also examined.

Results: The expression of Shh, Ptch, Smo, and Gli1 was very weak in normal endometrium, but was increased in endometrial hyperplasia and carcinoma stepwisely with significant differences. There was no marked difference in the expression of these molecules in carcinomas according to stages and histologic grades. Treatment with cyclopamine, a specific inhibitor of the hedgehog pathway, for endometrial carcinoma Ishikawa and HHUA cells suppressed growth by 56% and 67%, respectively, compared with the control. The addition of recombinant Shh peptide to HHUA cells enhanced their proliferation by 41%. The silencing of Gli1 using small interfering RNA (siGli1) resulted in the growth suppression and down-regulation of Ptch expression. In addition, the cyclopamine/siGli1-induced growth suppression was associated with the down-regulation of cyclins D1 and A and N-myc. No somatic mutations for *ptch* and *smo* genes were detected in the endometrial carcinoma cases examined.

Conclusions: The abnormal activation of this pathway is involved in the proliferation of endometrial carcinoma cells possibly in an auto-/paracrine fashion, suggesting the possibility of the hedgehog pathway being a novel candidate for molecular targeting.

Uterine endometrial carcinoma is one of the most common malignancies in the female genital tract (1). It accounts for 25% of all cancer-related deaths ascribed to the female genital tract in developed countries (2), and the number of patients with this tumor has recently been rapidly growing in Japan. Thus, understanding the growth characteristics of endometrial carcinoma is important for better management of this disease. Cell proliferation is, in general, thought to be evoked by extracellular signals such as hormones and growth factors. Endometrial carcinoma has been reported to have receptors for hormones and growth factors, such as transforming growth factor-α (TGF-α) and insulinlike growth factor (IGF), and the growth of endometrial carcinoma cells possessing these receptors is accelerated by these factors (3–5). In addition, we have reported the abnormal expression of cell cycle regulators in endometrial carcinoma, which possibly lead to the aggressive growth behavior of carcinoma cells (6, 7). However, the growth mechanism of this malignancy is not fully understood.

The hedgehog signaling pathway has important roles in organized cell growth and differentiation in a variety of embryonic tissues, including limbs, the nervous system, and digestive tract (8, 9). Hedgehog signaling is also involved in the maintenance of homeostasis in postembryonic tissues by regulating the fates of stem cells (10, 11). The mammalian hedgehog family of genes encodes several ligand proteins, including sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh), and their functional properties are highly conserved between organisms and expressed in a tissue-specific manner (10, 11). Hedgehog signaling is transduced by a transmembrane protein, Smoothened (Smo), the activity of which is suppressed by the membrane receptor patched (Ptch). When hedgehog ligands bind to Ptch, Smo is released from the inhibitory effect exerted by Ptch (8, 9). This leads to the

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activation and nuclear translocation of Gli transcriptional factors, which in turn promote the transcription of target genes like Ptch and bone morphologic protein (BMP-2; refs. 8, 9; Fig. 1). Recent studies have revealed that the activation of hedgehog signaling is implicated in the growth of various tumors, such as basal cell carcinoma of the skin (12), medulloblastoma (13, 14), and tumors of the lung (15), digestive tract (16, 17), and prostate (18, 19). In addition, a ligand-independent, constitutive activation of hedgehog signaling due to ptch or smo gene mutations has been reported in basal cell carcinoma (12, 20). However, the expression and functional involvement of hedgehog signaling molecules in endometrial tissues remain undetermined. In the present study, we examined the immunohistochemical expression of hedgehogs, Ptch, Smo, and Gli1 in normal and neoplastic endometrial tissues. The effect of hedgehog signaling on the proliferation of endometrial carcinoma cells and cultured normal endometrial glandular cells was examined using a specific hedgehog pathway inhibitor and recombinant Shh peptide. The somatic mutations of *ptch* and *smo* genes were also examined.

Materials and Methods

Immunohistochemistry

The formalin-fixed, paraffin-embedded tissue sections of 53 cases of normal endometrium (proliferative phase, 25 cases; secretory phase, 28 cases), 35 cases of endometrial hyperplasia (simple, 12 cases; complex, 11 cases; atypical, 12 cases), and 77 cases of endometrial carcinoma were used in the present study. The age of the 77 carcinoma patients ranged from 29 to 79 years. According to the Federation Internationale des Gynaecologistes et Obstetristes classification (1988), 47 patients had stage I, 10 had stage II, 19 had stage III, and 1 had stage IV tumors. Histologically, 72 had endometrioid adenocarcinomas, 3 had serous papillary adenocarcinomas, and 2 had clear cell adenocarcinomas. With regard to histologic grade, 43 tumors were grade 1, 16 were grade 2, and 18 were grade 3. Each tissue sample was used with the approval of the Ethics Committee of Shinshu University after obtaining written consent from the patients.

Immunostaining was done using primary goat polyclonal antibodies against Shh (N-19), Ihh (H-88), Dhh (H-85), Ptch (G-19), Smo (C-17), and Gli1 (N-16; Santa Cruz Biotechnology, Santa Cruz, CA), which were used at a dilution of 1:100. Indirect immunohistochemical staining was done by the avidin-biotin-peroxidase complex method using a Histofine SAB-PO detector kit (Nichirei, Tokyo, Japan) with microwave pretreatment, as previously described (7). The result of staining for Shh, Ptch, Smo, and Gli1 in each section was evaluated by two independent observers (Y. Feng and T. Shiozowa), and described as a "positivity index (PI)" which was calculated as follows: staining intensity (weak, 1; strong, 2) × percentage (calculated by the number of positively stained cells in arbitrarily selected 500 cells in each tissue section) of positive cells/2 (full score, 100). The results were classified as negative (−) when the PI was <5, weakly positive (+) when the PI was 6 to 50, and strongly positive when the PI was 51 to 100. Cases showing a PI for Shh, Ptch, Smo, or Gli1 of more than 5 were regarded as "positive" cases because the PIs of the majority of normal cases were <5.

Cell culture and chemicals

The endometrial carcinoma cell lines Ishikawa and Hec1A/B were kind gifts from Dr. H. Nishida at Tsukuba University (Tsukuba, Ibaragi) and Dr. H. Kuramoto at Kitazato University (Sagamihara, Kanagawa), respectively. The endometrial carcinoma cell line HHUA was purchased from the Riken Cell Bank (Saitama, Japan) with the permission of Dr. Ishiwata at the Ishiwata Laboratory (Mito, Japan). Endometrial carcinoma cell lines (KLE and RL95-2) were purchased from American Type Culture Collection (Rockville, MD). These cells were cultured in DMEM with 10% to 15% fetal bovine serum. Recombinant human Shh peptide was purchased from R&D Systems (Minneapolis, MN). A specific hedgehog signal inhibitor, cyclopamine (21), was purchased from Toronto Research Chemicals (North York, Canada) and its nonfunctional analogue, tomatidine hydrochloride, was purchased from Sigma-Aldrich (St. Louis, MO). The isolation and culture of

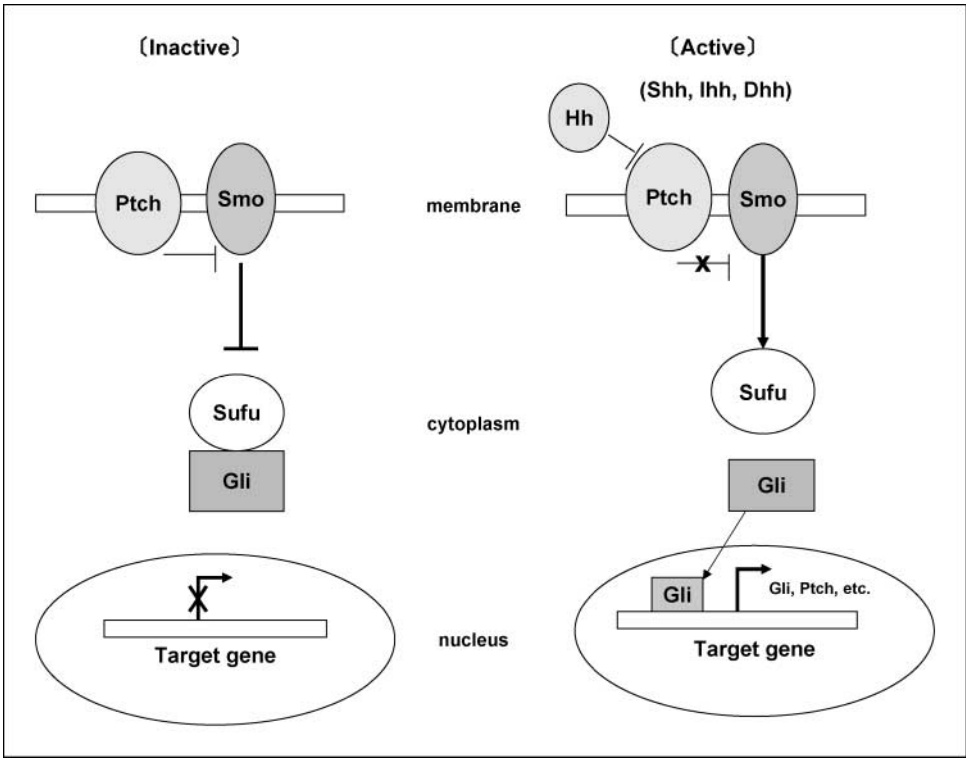

Fig. 1. Schematic demonstration of hedgehog signal pathway. When Ptch is free from ligands, Ptch suppresses the activation of downstream signals. When ligands bind to Ptch, the negative signal from Ptch is released and the pathway is activated. Then, the terminal transcription factor, Gli1, translocates to the nucleus, and the transcription of target genes can be initiated. Abbreviations: Hh, hedgehog; Shh, sonic hedgehog; Ihh, Indian hedgehog; Dhh, desert hedgehog; Ptch, Patched; Smo, Smoothened; Sufu, suppressor of fused; Gli1, glioma 1.

Table 1. Primers used for RT-PCR

	Primer	Size (bp)	Reference No.
Shh	Sense 5′-cgcacggggacagctcggaagt-3′ Antisense 5′-ctgcgcggccctcgtagtgc-3′	477	27
Ptch	Sense 5′-ggtggcacagtcaagaaca-3′ Antisense 5′-accaagagcgagaaatgg-3′	498	Design*
Smo	Sense 5′-ttaccttcagctgccacttctacg-3′ Antisense 5′-gccttggcaatcatcttgctcttc-3′	322	18
Gli1	Sense 5′-ttcctaccagagtcccaagt-3′ Antisense 5′-ccctatgtgaagccctattt-3′	185	Design*
Cyclin A	Sense 5′-attagtttacctggacccag-3′ Antisense 5′-cacaaactctgctacttctg-3′	444	39
Cyclin D1	Sense 5′-aggaacagaagtgcgaggagg-3′ Antisense 5′-gcacaagaggcaacgaaggt-3′	364	Design*
Cyclin E	Sense 5′-agttctcggctcgctccaggaaga-3′ Antisense 5′-tcttgtgtcgccatataccggtca-3′	475	39
p21	Sense 5′-gtgagcgatggaacttcga-3′ Antisense 5′-aatctgtcatgctggtctgc-3′	312	39
p27	Sense 5′-tagagctcgggccgtggct-3′ Antisense 5′-gtccattccatgaagtcagcgatatg-3′	873	40
N-myc	Sense 5′-gtcaccacattcaccatcac-3′ Antisense 5′-gggaaggcatcgtttgag-3′	335	41
C-myc	Sense 5′-gacatggtgaaccagagtttcat-3′ Antisense 5′-gttgctgatctgtctcaggactc-3′	690	Design*
Glyceraldehyde-3-phosphate dehydrogenase	Sense 5′-acgaccactttgtcaagctc-3′ Antisense 5′-ggtctacatggcaactgtga-3′	224	22

*Design: we designed the primer sequence.

normal endometrial glandular (NEG) cells were done as previously described (22). Only primarily cultured cells were used in all experiments. The NEG cells were cultured in Ham's F12 medium with 15% fetal bovine serum.

Reverse transcription-PCR and Western blotting

Six endometrial carcinoma cell lines (Ishikawa, HHUA, Hec1A, Hec1B, KLE, and RL95-2), NEG cells, and fresh tissue samples from four normal endometria and three endometrial carcinomas were subjected to reverse transcription-PCR (RT-PCR) and Western blotting to examine the expression mechanisms of Shh, Ptch, Smo, and Gli1. Total RNA was extracted using TRIzol reagent (Invitrogen, San Diego, CA), and RT-PCR was done using TaKaRa RNA PCR kit (Kakara Bio. Inc., Otsu, Japan). Primers used are listed in Table 1. Western blotting was done using proteins of total fractions and the primary antibodies for Shh, Ptch, Smo, and Gli1 which were the same as used in the immunostaining. Antibodies for cyclin D1 (C-20), cyclin A (H-432), cyclin E (H-145), p21 (H-164), and p27 (C-19) were purchased from Santa Cruz Biotechnology, and those for c-myc (OP-30) and N-myc (OP13) were from Oncogene (Boston, MA). Antibody for β-actin (A5441) was from Sigma-Aldrich. These antibodies were used in TBST containing 5% nonfat dry milk. Experimental procedures were done as previously described (22).

Cell proliferation assay

Cyclopamine treatment. Ishikawa or HHUA cells were seeded in a 96-well multiplate (2,000 cells per well). Twenty-four hours after the seeding, cyclopamine or tomatidine dissolved in ethanol was added to final concentrations of 5, 10, or 20 μmol/L. Cell proliferation was measured 48, 72, and 96 h after the addition of cyclopamine/tomatidine for endometrial carcinoma cells. NEG cells were seeded in a type IV collagen-coated 96-well multiplate (4,000 cells per well), and the agents were added at a concentration of 20 μmol/L. Cell proliferation was measured after 48 h because the viability of NEG cells was slightly unstable in cultures longer than 72 h. After the cell culture, WST-1 agent (Roche, Indianapolis, IN) was added according to the manufacturer's instructions and then measured at a wavelength of 450 nm with Multiscan JX (Thermo Labsystems, Vantee, Finland). In addition, the expression of Ptch and Gli1 mRNA/protein before and after cyclopamine treatment (20 μmol/L, 48 and 72 h) was examined using RT-PCR and Western blotting.

Shh treatment. HHUA cells were seeded in a 96-well multiplate (2,000 cells per well). Twenty-four hours after seeding, recombinant Shh dissolved in sterile PBS was added at concentrations of 50, 100, or 200 ng/mL daily. After 24, 48, 72, and 96 h of culture, viable cells were measured using the WST-1 assay. In addition, the expression of Ptch and Gli1 mRNA/protein before and after the addition of Shh (200 ng/mL, 48 and 72 h) was examined using RT-PCR and Western blotting. For NEG cells, cells were seeded in a type IV collagen-coated 96-well multiplate (4,000 cells per well), and Shh was added at a concentration of 200 ng/mL for 2 days.

Gli1 mRNA silencing. Silencing of the expression of Gli1, a terminal transcriptional factor of the hedgehog pathway, was done using small interfering RNA (siRNA) for Gli1. Primers were purchased from Ambion (Austin, TX) as listed in Table 2 and were transfected (250 nmol/L) into HHUA cells using OligofectAMINE (Invitrogen). Silencing efficiency was confirmed using RT-PCR and Western blotting for Gli1 mRNA/protein, 24 and 48 h after silencing.

RT-PCR for cell cycle regulators and myc. To examine the involvement of cell cycle regulators in hedgehog signaling-related growth, the expression of cyclins (D1, E, and A), p21, p27, c-myc, and N-myc was examined in HHUA cells before and after treatment of Shh, cyclopamine, and Gli1-silencing using RT-PCR. The procedure for RT-PCR was previously described in this section, and the primers used are listed in Table 1. All experiments were done thrice separately.

Mutational analysis

Mutations of the *ptch* gene were examined in above-mentioned 6 endometrial carcinoma cell lines and 57 cases of endometrial carcinoma tissues. In the 57 endometrial carcinoma cases, tissue DNA was extracted using microdissection as previously described (23). Mutations of the *ptch* gene were examined in exon 14, which includes a mutation "hotspot" spanning residues 663 to 694. Mutations of the

smo gene in the six endometrial carcinoma cell lines were examined in all codons except the first 60. Mutational analysis was done using direct sequencing as previously described (23). Primers used are listed in Table 2. Sequence data were analyzed using Basic Local Alignment Search Tool (BLAST) software located at the National Center for Biotechnology Information web site.

Statistical analysis. In immunostaining, values represent the mean ± SD in each category. Mann-Whitney's *U* test and the Kruskal-Wallis test were used to assess the difference. Spearman's rank correlation was used to evaluate the correlations. $P < 0.05$ was considered significant. These analyses were done using the StatView system (Abacus, Berkeley, CA).

Results

Immunohistochemistry

The expression of Shh, Ptch, and Smo was exclusively observed in the cytoplasm, and that of Gli1 was mainly observed in the cytoplasm and focally in the nucleus. The expression of these factors in the stroma was negligible in all cases examined. Representative photomicrographs are shown in Fig. 2. Results are summarized in Fig. 3 and Table 3.

Shh. The expression of Shh in the normal endometrial glands was only focally observed in the basal layer of the proliferative phase (Fig. 2A). The expression of Shh in the secretory phase was negative (Fig. 2B). The PI of Shh in all normal cases was 0.8 ± 3.2 (mean ± SD; Fig. 3). The expression of Shh was increased in hyperplasia (PI, 5.4 ± 9.7; Fig. 2C) and carcinoma (PI, 18.6 ± 20.7; Fig. 2D), with a significant difference ($P < 0.0001$; Fig. 3). The PI in atypical hyperplasia (9.4 ± 12.2) was larger than that of hyperplasia without atypia (3.7 ± 8.1); however, the difference was not significant. The total number of the Shh-positive endometrial carcinoma cases (PI > 5) was 43 (56%; grade 1, 24; grade 2, 8; grade 3, 11; Fig. 2D; Table 3). However, there was no marked difference in the PI or number of positive cases in endometrial carcinoma according to stage and histologic grade. The expression of Ihh and Dhh was negligible in all cases examined (data not shown).

Ptch. The expression of Ptch in the normal endometrial glands was negligible, with a PI of 0.1 ± 0.4 (Fig. 2E). The expression of Ptch was slightly higher in hyperplasia (PI, 0.4 ± 1.4) and markedly higher in carcinoma (PI, 15.0 ± 17.6; Fig. 2F), with a significant difference ($P < 0.0001$). There was no significant difference between the PI of atypical hyperplasia and that of hyperplasia without atypia. The total number of Ptch-positive carcinoma cases was 38 (49%; grade 1, 18; grade 2, 9; grade 3, 11). The PI for Ptch in stage III + IV patients (25.1 ± 19.9) was significantly higher than that of stage I + II patients (11.8 ± 15.5; $P = 0.0053$). There was no marked difference in the PI or number of positive cases in endometrial carcinoma according to the histologic grade.

Smo. The expression of Smo in the normal endometrial glands was focally observed in the proliferative phase with a PI of 0.7 ± 4.0 (Fig. 2G), which was nearly the same as that in hyperplasia (PI, 0.7 ± 0.3), but was markedly increased in carcinomas (PI, 5.0 ± 8.8; Fig. 2H), with a significant difference ($P < 0.0001$). There was no significant difference between the PI of atypical hyperplasia and that of hyperplasia without atypia. The total number of Smo-positive carcinoma cases was 17 (22%; grade 1, 9; grade 2, 7; grade 3, 1). The PI for Smo in grade 3 cases (0.6 ± 1.0) was significantly lower than that of grade 1 ($P = 0.0054$) or grade 2 ($P = 0.0004$).

Gli1. The expression of Gli1 in the normal endometrial glands was negative throughout the menstrual cycle (Fig. 2I and J). It was slightly increased in hyperplasia (PI, 0.1 ± 0.3) and markedly increased in carcinomas (PI, 6.7 ± 11.7; Fig. 2K and L), with a significant difference ($P < 0.0001$). There was no significant difference between the PI of atypical hyperplasia and that of hyperplasia without atypia. The total number of Gli1-positive carcinoma cases was 21 (27%; grade 1, 9; grade 2, 6; grade 3, 6). There was no marked difference in the PI or number of positive cases in endometrial carcinoma according to the histologic grade or stage. Three cases (grade 2, one case; grade 3, two cases) showed nuclear staining for Gli1 (Fig. 2L), one with diffuse, and two with focal nuclear staining.

Table 2. Primers used for direct sequencing of ptch and smo genes and Gli1 silencing

	Sequencing analysis		
	Primers	**Size (bp)**	**Reference**
ptc1	Sense 5′-gcccatgaaacgcagattacc-3′ Antisense 5′-tggaggctggagtcggagaa-3′	212	Design*
smo 1	Sense 5′-ctgagccactgcggccgg-3′ Antisense 5′-cggcacacagcaggggctg-3′	204	42
smo 2	Sense 5′-gtcgggcctccggaatgc-3′ Antisense 5′-gccgcgatgtagctgtgc-3′	381	42
smo 3	Sense 5′-ctcttcacagaggctgag-3′ Antisense 5′-cagggctttgaaggaagtg-3′	377	42
smo 4	Sense 5′-ggtttggtttgtggtcctc-3′ Antisense 5′-aggtaatgagcacaaagcc-3′	413	42
smo 5	Sense 5′-accatgctgcgcctgggc-3′ Antisense 5′-tgcaggagctcgtgccgc-3′	400	42
smo 6	Sense 5′-gcaagatgattgccaaggc-3′ Antisense 5′-agcgggcacacctccttc-3′	357	42
smo 7	Sense 5′-agagctgcagaagcgcct-3′ Antisense 5′-tcagaagtccgagtctgc-3′	375	42
Gli1 silencing	Sense 5′-gcgugagccugaaucugugtt-3′ Antisense 5′-cacagauucaggcucacgctt-3′		Ambion

*Design: we designed the primer sequence.

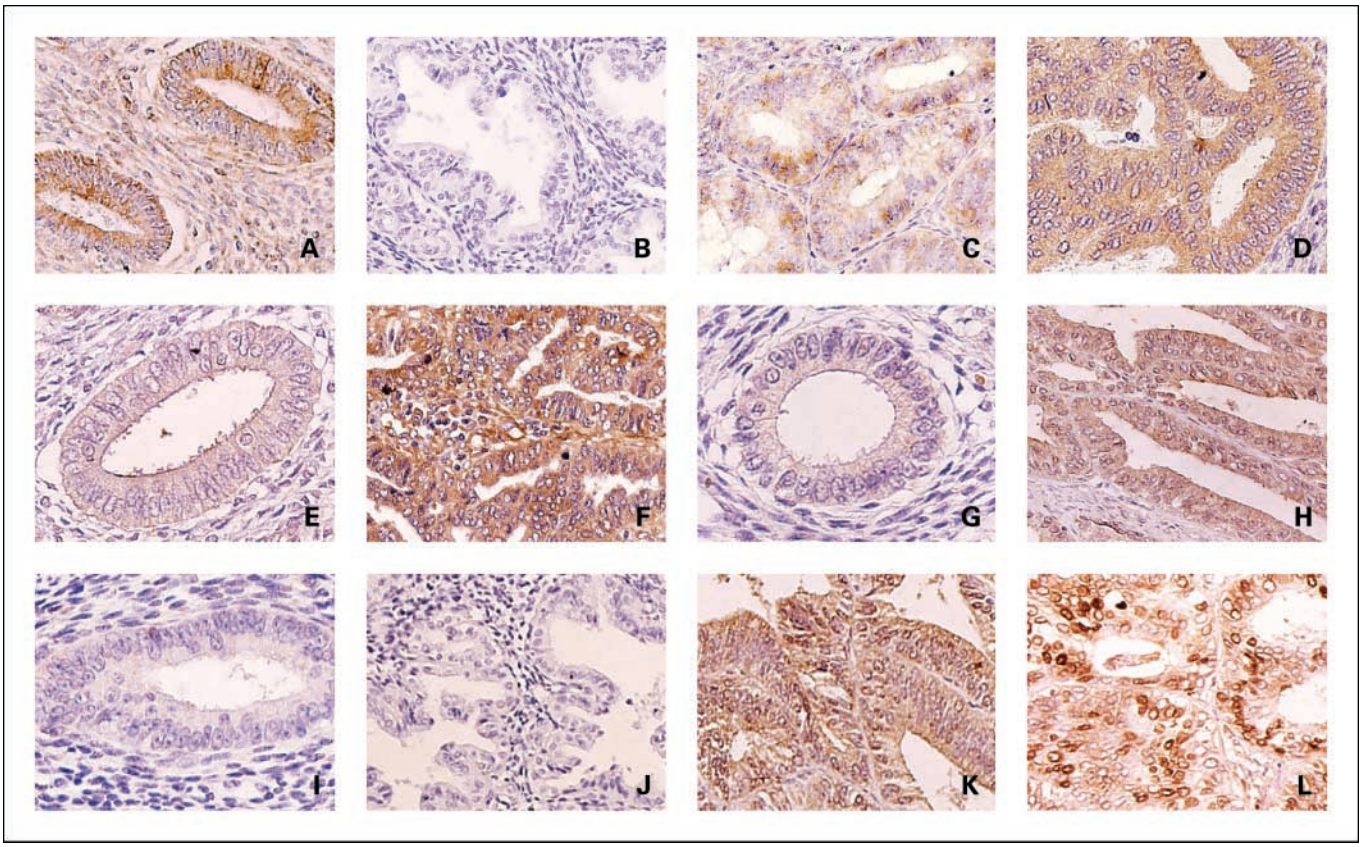

Fig. 2. Result of immunostaining for Shh (*A* to *D*), Ptch (*E* and *F*), Smo (*G* and *H*), and Gli1 (*I* to *L*) in normal, hyperplastic, and malignant endometria. The expression of Shh was focally observed in the cytoplasm of the glandular cells in the proliferative phase, with predominance in the basal layer (*A*, ×100), but was negative in the secretory phase (*B*, ×100). The expression of Shh was increased in atypical endometrial hyperplasia (*C*, ×100) and carcinoma (*D*, grade 1, ×100). The cytoplasmic expression of Ptch and Smo was weakly observed in proliferative phase endometrial glands (*E* and *G*, ×100) and was overexpressed in carcinomas (*F* and *H*, ×100). The expression of Gli1 was negative both in the proliferative (*I*, ×100) and secretory phases (*J*, ×100). The expression of Gli1 was increased in carcinoma in the cytoplasm (*K*, ×100) and nucleus (*L*, ×100).

Correlation of Shh expression with other molecules in endometrial carcinomas

The expression of Shh was positively and strongly correlated with the expression of Ptch, with a significant difference ($P < 0.0001$, $\rho = 0.544$). The expression of Shh was also positively and significantly correlated with Smo ($P < 0.0009$, $\rho = 0.349$) and Gli1 ($P = 0.042$, $\rho = 0.234$).

Expression of mRNA and protein of Shh, Ptch, Smo, and Gli1

Expression of Shh, Ptch, Smo, and Gli1 mRNAs and proteins was examined in surgically resected normal and malignant endometrial tissues and endometrial carcinoma cell lines and NEG cells using RT-PCR and Western blotting (Fig. 4). The expression of Shh, Ptch, and Smo mRNAs was weak in normal endometrial tissues, with a slight predominance in the proliferative phase in Shh, and that of Gli1mRNA was negative throughout the menstrual cycle. The expression of Shh, Ptch, Smo, and Gli1 mRNAs was increased in all three endometrial carcinomas (Fig. 4). These results were consistent with those obtained using immunostaining. The expression of Shh, Ptch, Smo, and Gli1 mRNAs was also observed in all six cell lines examined with a slight variation in the expression amount, indicating the activation of the hedgehog pathway in endometrial carcinoma. The expression of Shh and Gli1 proteins in normal endometrial tissues was also weak, but was increased in endometrial carcinoma tissues. The expression of Shh and Gli1 proteins was also observed in all six endometrial carcinoma cell lines. The pattern of protein expression for Shh and Gli1 was correlated well with that of their respective mRNA. In NEG

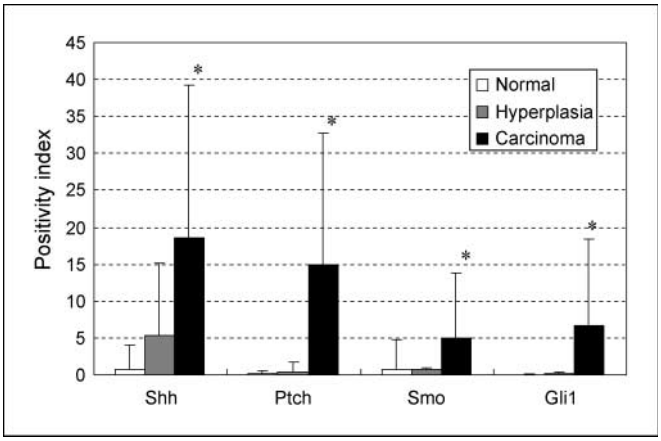

Fig. 3. Graphic demonstration of the PI for Shh, Ptch, Smo, and Gli1 in normal, hyperplastic, and malignant endometria. The expression of all four molecules was increased stepwisely along with the progression of diseases, with significant differences ($P < 0.05$).

Table 3. Results of immunostaining for Shh, Ptch, Smo, and Gli1 in endometrial carcinomas according to stage and histologic grade

	Stage		Histologic grade			Total (77)
	I + II (57)	III + IV (20)	1 (43)	2 (16)	3 (18)	
Shh PI	17.4 ± 21.2	21.9 ± 19.4	21.7 ± 23.7	12.8 ± 16.4	16.3 ± 15.3	18.6 ± 20.7
Cases, *n* (%)	31 (51)	12 (60)	24 (56)	8 (50)	11 (61)	43 (56)
Ptch PI	11.8 ± 15.5	25.1 ± 19.9*	11.8 ± 15.3	20.1 ± 22.9	18.1 ± 17.1	15.0 ± 17.6
Cases, *n* (%)	23 (40)	15 (75)	18 (42)	9 (56)	11 (61)	38 (49)
Smo PI	4.9 ± 8.6	5.2 ± 9.6	4.2 ± 6.6	12.3 ± 14.2	0.6 ± 1.0†	5.0 ± 8.8
Cases, *n* (%)	12 (21)	5 (25)	9 (21)	7 (43)	1 (6)	17 (22)
Gli1 PI	5.3 ± 9.9	10.6 ± 15.6	4.4 ± 8.1	9.5 ± 14.6	9.6 ± 15.4	6.7 ± 11.7
Cases, *n* (%)	14 (25)	7 (35)	9 (21)	6 (38)	6 (33)	21 (27)

NOTE: Cases, *n* (%): number of positive cases and percentage in the group. Each PI is indicated by the mean ± SD.
*Significantly different from that of stage I + II ($P < 0.05$).
†Significantly different from that of grade 1 or 2 ($P < 0.05$).

cells, the expression of Shh and Ptch mRNAs and Shh protein was weakly positive; however, that of other molecules was negative.

Growth inhibition of endometrial carcinoma cells by cyclopamine

The effect of cyclopamine, a specific inhibitor of the hedgehog signal pathway, on the proliferation of two endometrial carcinoma cell lines (Ishikawa and HHUA) and NEG cells was examined using WST-1 assay. Tomatidine was used as a negative control for cyclopamine. In Ishikawa cells, cyclopamine-induced growth suppression was observed as early as 48 h after the addition of cyclopamine in a dose-dependent manner. At 48 h after cyclopamine treatment, the proliferation of Ishikawa cells in three different cyclopamine concentrations (5, 10, and 20 μmol/L) was suppressed by 19%, 25%, and 31%, respectively, compared with the control, with a significant difference ($P < 0.05$; Fig. 5A). At 72 h, cyclopamine-induced suppression at the same cyclopamine concentrations was 14%, 35%, and 56%, respectively ($P < 0.05$). Likewise, cyclopamine suppressed the proliferation of HHUA cells after 72 h exposure at the same three cyclopamine concentrations by 12%, 29%, and 67%, respectively ($P < 0.05$). Cyclopamine treatment of HHUA cells was associated with the down-regulation of cyclin D1 and N-myc; however, no apparent changes were noted in other molecules examined (Fig. 5B). In NEG cells, cyclopamine or tomatidine treatment for 48 h suppressed the growth of NEG cells by 38% and 26%, respectively (Fig. 5A). The difference of proliferation between the control and those treated with cyclopamine was significant ($P < 0.05$); however, there was no significant difference between those treated with cyclopamine and tomatidine.

Growth stimulation of endometrial carcinoma cells by recombinant Shh

To examine the effect of Shh on the proliferation of endometrial carcinoma cells, HHUA cells were treated with recombinant Shh, and the effect of Shh was evaluated according to the expression of Ptch and Gli1 using RT-PCR and Western blotting because Gli1 is known to be a terminal transcriptional factor of the hedgehog pathway, and Ptch is an important target gene of Gli1 transcriptional factor (24). The results indicated that the expression of both Ptch and Gli1 was increased 48 and 72 h after the addition of Shh at 100 ng/mL concerning both mRNA and protein levels (Fig. 6A). The effect of Shh on the proliferation of HHUA cells was also examined

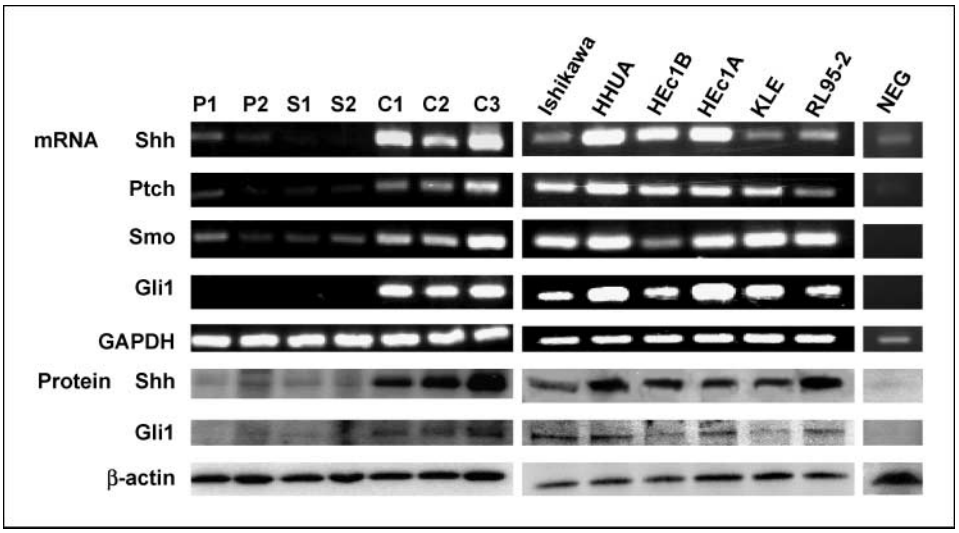

Fig. 4. Results of RT-PCR for Shh, Ptch, Smo and Gli1, and those of Western blotting for Shh and Gli1. (P1 and P2, proliferative phase; S1 and S2, secretory phase; C1 to C3, carcinoma) The expression of Shh, Ptch, and Smo mRNAs was weakly observed in normal endometria (P1, P2, S1, S2) with predominance in the proliferative phase (P1, P2) for Shh, but that of Gli1 was negative irrespective of the menstrual cycle. The expression of all these molecules was increased in carcinomas (C1 to C3). The expression of Shh and Gli1 proteins was correlated with that of mRNA. All six endometrial carcinoma cell lines expressed Shh, Ptch, Smo, and Gli1 mRNAs and Shh and Gli1 proteins. In normal endometrial glandular (NEG) cell, weak expression of Shh and Ptch mRNAs and Shh protein was noted.

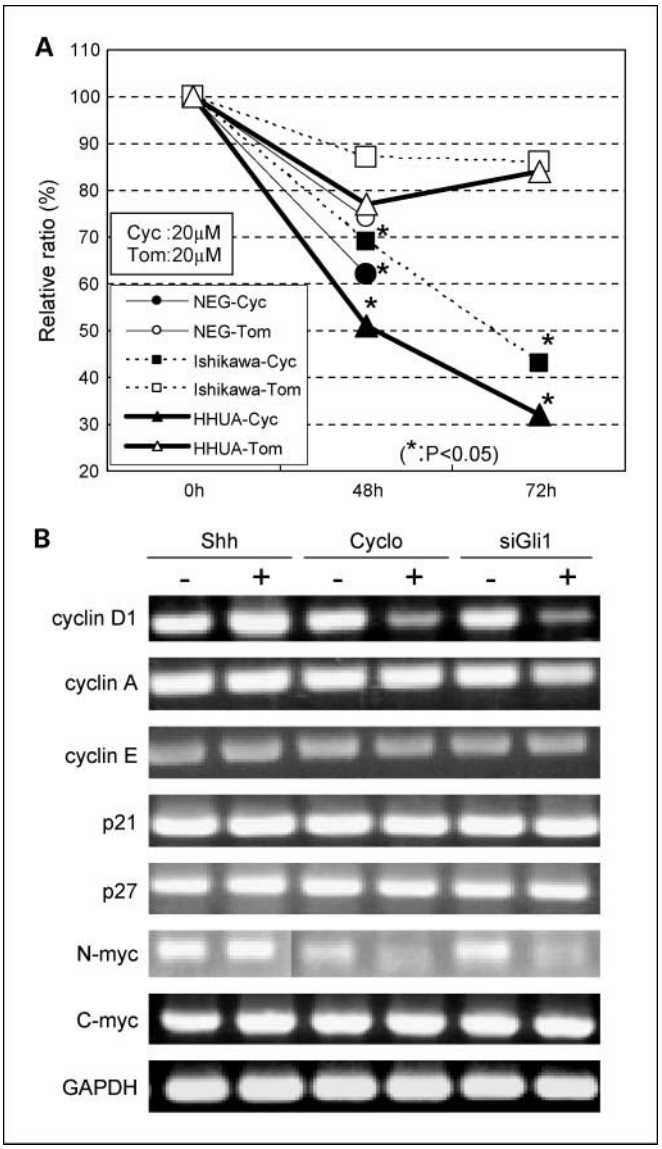

Fig. 5. *A,* effect of a hedgehog signal inhibitor, cyclopamine and its control homologue, tomatidine, on the proliferation of Ishikawa, HHUA, and NEG cells using WST-1 assay. Growth was indicated by relative ratio to the untreated controls. Addition of cyclopamine (20 μmol/L) to Ishikawa and HHUA cells for 48 and 72 h reduced the growth to the controls with significant differences. Such inhibitory effects were not evident when tomatidine (20 μmol/L) was added. Cyclopamine treatment for 48 h inhibited the growth of NEG cells with a significant difference; however, there was no significant difference in the growth inhibition between those treated by cyclopanine and tomatidine. *B,* changes in the expression of mRNA of various cell cycle regulators and myc in HHUA cells. Addition of Shh resulted in the increased expression of cyclin D1 mRNA. Treatment with cyclopamine resulted in the decreased expression of cyclin D1 and N-myc. Silencing of Gli1 resulted in the down-regulation of cyclins D1 and A and N-myc.

using the WST-1 assay. The result indicated that HHUA cell growth was stimulated compared with the control through 24 to 96 h dose dependently; the percentage increase compared with the control at 24, 48, 72, and 96 h incubation at 200 ng/mL was 13%, 38%, 37%, and 41%, respectively (Fig. 6B). The growth among the Shh-treated cells and control was significantly different with each incubation time (24 h, $P = 0.027$; 48 h, $P = 0.002$; 72 h, $P = 0.002$; 96 h, $P < 0.001$). The addition of Shh to HHUA cells induced a slight up-regulation of cyclin D1 (Fig. 5B). The addition of Shh increased the proliferation of NEG cell by 9% compared with the control after 48 h; however, the difference was not significant (Fig. 6C).

Silencing of Gli1

To further clarify the involvement of hedgehog signaling in the growth regulation of endometrial carcinoma cells, the effect of Gli1 silencing in HHUA cells using a small interference RNA for Gli1 was evaluated using WST-1. The blocked expression of Gli1 mRNA and protein was confirmed using RT-PCR and Western blotting (Fig. 7A). The silencing of Gli1 suppressed the proliferation of HHUA cells through 24 to 96 h, and by 56%

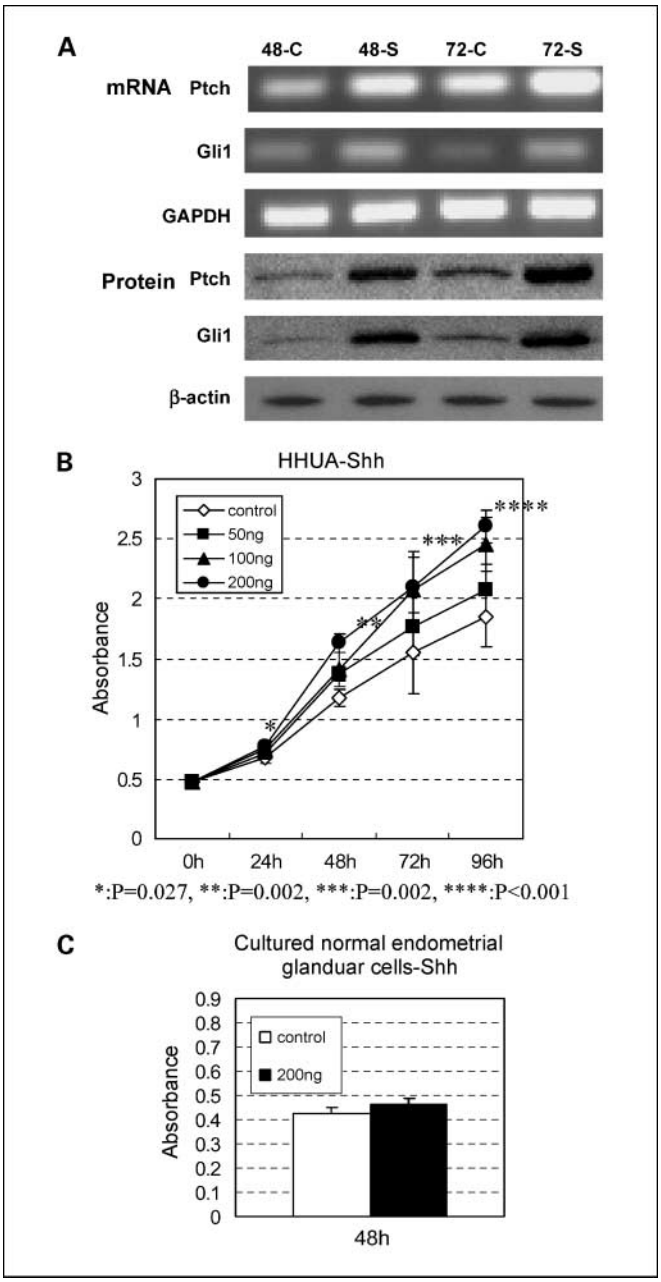

Fig. 6. Effect of recombinant Shh protein on the proliferation of HHUA cells (*A* and *B*) and NEG cells (*C*). C, control; S, Shh. *A,* Shh induced the expression of Ptch and Gli1 mRNA and protein expressions 48 and 72 h after the addition. *B,* Shh treatment for 24, 48, 72, and 96 h stimulated the proliferation of HHUA cells dose dependently, with significant differences. *C,* Shh treatment did not increase the growth of NEG cells significantly.

compared with the control at 96 h, with a significant difference ($P < 0.05$; Fig. 7B). Gli1 silencing resulted in the down-regulation of cyclins D1 and A and N-myc (Fig. 5B).

Mutational analysis

To examine the mechanism of the up-regulation of the hedgehog pathway in endometrial carcinoma, we examined somatic mutations of *ptch* and *smo* because loss of function mutations in the ptch gene and gain of function mutations in the *smo* gene have been reported. We examined the mutation in the *ptch* gene including a mutational hotspot located at exon 14 in a total of 57 cases of endometrial carcinomas and the above-mentioned six endometrial carcinoma cell lines. The results indicated that mutation was absent in all samples examined. We also examined mutation in the *smo* gene including exons 1 to 6 in the six endometrial carcinoma cell lines, and the results indicated no mutations.

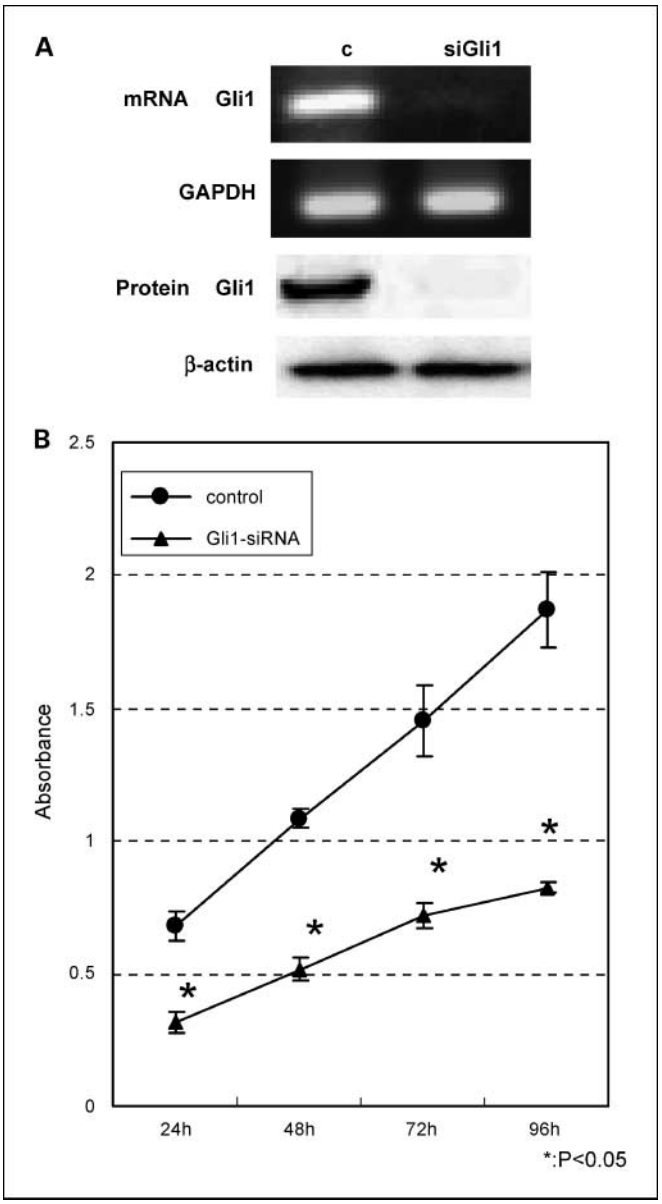

Fig. 7. Effect of siRNA for Gli1 on the proliferation of HHUA cells. *A,* silencing of Gli1 mRNA and protein was observed. *B,* Gli1-siRNA treatment reduced the proliferation of HHUA cells from 24 to 96 h after the silencing, with significant differences ($P < 0.05$).

Discussion

The present study showed that the expression of Shh in normal endometrial epithelia was only focally observed in the proliferative phase, and that of Ptch, Smo, and Gli1 was negligible throughout the menstrual cycle. Therefore, it seems less likely that these hedgehog signal-related molecules are directly involved in the growth or differentiation of normal endometrial glands. The expression of Shh was reportedly observed in the fundus of normal gastric glands, known as an active regeneration site (25). Although the present study showed weak and focal staining for Shh in the basal layer of proliferative phase glands, such a topological distribution in each gland was not evident. The present study also revealed that the immunohistochemical expression of Shh, Ptch, Smo, and Gli1 was increased in endometrial hyperplasia and carcinoma stepwisely with significant differences. In addition, analyses using Spearman's rank correlation revealed that the expression of Shh was positively correlated with the expression of Ptch, Smo, and Gli1, suggesting that the activation of this pathway is involved in the malignant transformation of a subset of endometrial carcinomas. Although the expression of Ptch was increased in advanced-stage patients and the expression of Smo was decreased in grade 3 tumors, the significances were not determined. Thus, we consider that there are no marked tendencies in the expression of these molecules among histologic grades and stages. Therefore, activation of the hedgehog pathway may represent a common event in malignant transformation among a subset of carcinomas expressing hedgehog-related molecules, rather than a specific step of tumor development. To our knowledge, this is the first report on the expression of hedgehog pathway molecules in endometrial carcinoma.

The overexpression of hedgehog signaling molecules in malignant tissues detected by immunostaining has been reported in the pancreas (17), breast (19), stomach (26), and colon (27). In breast carcinomas, almost all cases overexpressed Shh, Ptch, and Gli1 compared with the adjacent normal breast tissue, implying that hedgehog activation is a more constitutive phenomenon (19). In stomach and breast carcinomas, the expression of Gli1 was observed both in the cytoplasm and nucleus. In particular, all breast carcinoma cases showed nuclear expression of Gli1, with a predominance in estrogen receptor–positive cases, suggesting that the nuclear expression of Gli1 may be related to estrogen-induced carcinogenesis of this tumor (19). In the present study, we identified three cases with nuclear expression of Gli1, and two of the three cases were histologically grade 3 tumors. The expression of estrogen receptor in our cases was negative (data not shown); thus, the role of Gli1 in the nucleus remains undetermined in endometrial carcinoma.

More importantly, the present study showed the cyclopamine-induced growth suppression, and Shh-induced growth stimulation of endometrial carcinoma cells. In addition, silencing of Gli1 using siRNA suppressed the growth of endometrial carcinoma cells. These findings clearly indicated that the hedgehog pathway is involved in the proliferation of endometrial carcinoma cells. In tumors of the skin (12), brain

(28), pancreas (17), and others (15, 18, 27), blockade of hedgehog signaling results in growth suppression and apoptosis *in vitro* and sometimes *in vivo*. In addition, blockade of hedgehog signaling in prostatic tumors prevents the growth of primary tumors and metastasis (18). The present results are in line with these reports. In the present study, cyclopamine-induced growth suppression of HHUA cells was as high as 67%. In addition, it is noteworthy that only Shh was detected among the three hedgehog subtypes, and Shh actually induced the proliferation of endometrial carcinoma cells, indicating the specific role of Shh in this pathway. In contrast, in NEG cells, the expression of Shh and Ptch mRNA and Shh protein was weakly positive, and that of Smo and Gli1 mRNA was negative, being consistent with the results obtained with immunostaining. Cyclopamine treatment suppressed the growth of NEG cells; however, there was no significant difference in the suppression caused by tomatidine. Thus, we consider that the cyclopamine-induced growth suppression may be due to a pharmacologic effect because the growth of cultured NEG cells has been reportedly more susceptible to various cell culture conditions compared with that of established carcinoma cell lines (29, 30). In addition, Shh treatment did not significantly increase the growth of NEG cells, which may be explained by the lack of downstream effectors of this pathway. These data indicated that the growth of NEG cells was less dependent on hedgehog signals compared with endometrial carcinoma cells. Therefore, the growth of a subset of endometrial carcinomas may profoundly depend on Shh-related hedgehog signaling, suggesting the possibility of the hedgehog pathway as a novel therapeutic target.

The present study revealed that growth acceleration via the hedgehog pathway is associated with the up-regulation of cyclins D1 and A and N-myc. The increased expression of cyclin D1 is plausible because its expression is known to be induced by extracellular signals in the early G_1 phase (31). In animal cells, hedgehog-mediated growth activation has been reportedly exerted by the up-regulation of cyclins D and E (32); however, the involvement of cyclin E was not evident in the present study. More recently, phosphoinositide 3-kinase and Akt are reportedly essential for Shh signaling (33). Thus, terminal activation of the phosphoinositide 3-kinase/Akt pathway is likely to contribute to the up-regulation of cyclins after activation of the hedgehog pathway. In gastric carcinoma cells, Shh-induced cell proliferation was associated with reduced expression of p21 through a Gli1-dependent and p53-independent pathway (26). However, Shh-induced down-regulation of p21 was not observed in endometrial tissues. Therefore, growth machineries evoked by hedgehog activation may differ among cell types. The present study also revealed the cyclopamine-induced down-regulation and Shh-induced up-regulation of N-myc. Although the mechanisms are not fully understood, N-myc has been shown to be involved in the hedgehog-induced proliferation of neuronal precursors (34) and C-myc in hedgehog-related tumor formation (35).

Although the present study indicates the activation of the hedgehog pathway in endometrial carcinoma, molecular mechanisms leading to the up-regulation of Shh, Ptch, Smo, and Gli1 in malignant cells are not understood. Activation of the hedgehog pathway has been considered to be caused by two scenarios: ligand-independent and ligand-dependent mechanisms. The former is exemplified by brain and skin tumor as seen in hereditary Gorlin syndrome (12, 36), which harbors the loss of function *ptch* mutations. In addition, gain of function mutations of smo has been reported in sporadic basal cell carcinoma (12). This finding is supported by animal experiments showing that the *ptch* deficiency results in tumor formation (37). The latter includes malignant tumors of the digestive tract (16), pancreas (17), and lung (15), in which endogenous or exogenous ligands like Shh or Ihh are required for the activation of this pathway. Because endometrial carcinomas showed no mutations of *ptch* and *smo* genes in this study, this tumor is included in the latter. The mechanisms leading to the increased expression of Shh in malignant tissues have not been fully elucidated; however, recent studies have reported the methylation of the *shh* gene promoter in the regulation of the pathway (38). In endometrial carcinomas, tumor cells produce Shh, as shown by immunostaining and RT-PCR, and the expression of Shh was negative in the stroma. Collectively, these findings suggest an autocrine/paracrine mechanism in the proliferation of endometrial carcinoma cells.

In conclusion, the present study revealed the overexpression of hedgehog signal-related molecules in endometrial carcinomas. In addition, the activation of this pathway is deeply involved in the proliferation of endometrial carcinoma cells. These finding suggest the possibility of the hedgehog signaling pathway being a novel candidate for molecular targeting.

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