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An alternative splicing isoform of eukaryotic initiation factor 4H promotes tumorigenesis *in vivo* and is a potential therapeutic target for human cancer

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Deregulation of protein synthesis plays a critical role in cell transformation. Several translation initiation factors (eIFs) have been implicated in malignant transformation; thus, suppression of eIFs could be a potential cancer therapy if cancer cells are selectively killed without damaging healthy cells. One of the potential molecular targets is a cancer-specific splicing variant. We have previously shown that one of the splicing variants of eIF4H (isoform 1) was overexpressed in primary human colorectal cancer. Our study aimed to explore whether eIF4H isoform 1 contributes to carcinogenesis and could be an efficient molecular target for human cancer therapy. We found that its overexpression in immortalized mouse fibroblasts, NIH3T3 cells, generated tumors in nude mice. Conversely, suppression of eIF4H isoform 1 expression using specific siRNA inhibited the proliferation of colon cancer cells *in vitro* and subcutaneously implanted tumor *in vivo*. Strikingly, eIF4H isoform 1 specific siRNA showed no effect on the growth of immortalized human fibroblasts. More interestingly, ectopic expression of eIF4H isoform 1 greatly increased the cyclin D1 level. On the other hand, cyclin D1 decreased by shRNA-mediated suppression of eIF4H isoform 1. Moreover, cotransfection of eIF4H isoform 1 siRNA and cyclin D1 expression plasmid was able to reverse the growth suppression effect of eIF4H isoform 1 knockdown. These results suggest that eIF4H isoform 1 plays an important role in carcinogenesis through the activation of oncogenic signaling and could be a promising molecular target for cancer therapy.

There is increasing evidence that deregulation of protein synthesis is associated with cell transformation and the malignant phenotype.[1–4] Protein synthesis is primarily regulated at the step of ribosome recruitment to the 5′-mRNA terminus and this association is mediated by a trimeric complex, termed eIF-4F, which consists of the large scaffolding protein eIF4G, the RNA helicase eIF4A, and the cap binding protein eIF4E. Several eIFs have been demonstrated to be involved in carcinogenesis. Overexpression of eIF4E, a subunit of the eIF-4F complex, has been observed in many solid tumors and tumor cell lines[1–5] and, in experimental models, it markedly alters cellular morphology, enhances proliferation and induces cellular transformation, tumorigenesis and metastasis.[6–8] Other members of the eIF-4F complex are also implicated in malignant transformation. For example, eIF4G is overexpressed in squamous cell lung carcinomas[9,10] and its overexpression in NIH3T3 cells leads to anchorage-independent growth of cells and tumor formation in nude mice.[11] eIF4A, an ATP-dependent RNA helicase, is also overexpressed in human melanoma cells and primary hepatocellular carcinomas.[12,13] eIF4H was first identified to stimulate translation in rabbit reticulocytes.[14] It has been recognized that eIF4H stimulates protein synthesis by enhancing the helicase activity of eIF4A by increasing the processivity of eIF4A.[14–16] We have previously reported that eIF4H was overexpressed in most human colorectal cancer tissues[17]; thus, it has become evident that control of mRNA translation plays a critical role in carcinogenesis. A likely mechanism of cellular transformation by eIFs has been suggested as increased translational efficiency of the mRNA responsible for the control of cell growth or

Key words: alternative splicing, translation initiation factor, therapeutic target

Abbreviations: CHAPS: 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DTT: dithiothreitol; eIFs: eukaryotic initiation factors; FACS: fluorescence-activated cell sorting; IMDM: iscove's modified dulbecco's media; MTS: 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; RNAi: RNA interference; RPMI-1640: Roswell Park Memorial Institute media; shRNA: short hairpin RNA; TdT: terminal deoxynucleotidyl transferase; Tet-Off system: tetracycline-off system; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

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apoptosis, although the precise mechanism of how these factors are involved in carcinogenesis remains to be established.

Alternative splicing has been observed in many cancer-associated genes, suggesting that it might have a key role in carcinogenesis.[18–21] Hence, cancer-specific spliced isoforms may be potential tools as tumor markers or for molecular treatments that can be designed to recognize only the variant form. The *eIF4H* gene is known to produce two splice variants, isoform 1 and 2 (exon 5 is alternatively spliced),[22] which generate two protein products, 27 kDa and 25 kDa. Between them, overexpression of isoform 1 is much more prominent than isoform 2 in colon cancers. In this regard, eIF4H isoform 1 might be a cancer-driving splice variant and therefore a promising therapeutic target.

In our work, we investigated whether the alternative splicing form of eIF4H contributes to cell proliferation and carcinogenesis. We showed that overexpression of eIF4H isoform 1 induces tumor formation in nude mice and its suppression inhibited cell proliferation *in vitro* and *in vivo*. The effect of eIF4H isoform 1 on proliferation is likely due to the upregulation of an oncogenic factor cyclin D1. Our results suggest that eIF4H isoform 1 might become an attractive target of therapeutic intervention for colon cancer.

Material and Methods

Human tissue samples

Tissues from 10 patients with primary colorectal and 20 patients with esophageal cancer (Table 1) were resected surgically in the Department of Frontier Surgery, Chiba University Hospital. The ethics committee of the Graduate School of Medicine, Chiba University approved the protocol. Written informed consent was obtained from each patient before operation. The percentage of tumor cells in the tissues was 50–80% in all cases. The excised samples were obtained within 1 hr after the operation from tumor tissues and the corresponding nontumor tissues of the same patients 5–10 cm from the tumor. All excised tissues were placed immediately in liquid nitrogen and stored at −80°C until analysis.

Cell culture

Two human colon cancer cell lines, LOVO and RKO, two human lung fibroblast cell lines, MRC5 and WI38, and a mouse fibroblast, NIH3T3, were purchased from RIKEN Cell Bank (Tsukuba, Japan). The tumor cells, LOVO and RKO, were grown in RPMI-1640, and immortalized cells, MRC5, WI38 and NIH3T3, were grown in IMDM medium, both supplemented with 10% fetal bovine serum (FBS), 1% (v/v) penicillin and streptomycin (100 U/ml) (all from Invitrogen, Carlsbad, CA), and maintained at 37°C in a 5% CO_2-95% air atmosphere.

Real-time quantitative PCR

Total RNA was extracted from tumor and nontumor tissues with an RNeasy Mini kit (Qiagen, Tokyo, Japan). Total RNA from the nucleus and cytoplasm of colon cancer cells was extracted with a Protein and RNA Isolation System (PARIS) Kit (Applied Biosystems Japan, Tokyo, Japan). cDNA was synthesized from total RNA with the first-strand cDNA synthesis kit for RT-PCR (Roche, Mannheim, Germany). Real-time quantitative PCR of eIF4H cDNA using the LightCycler

Table 1. Clinical features of patients with esophageal cancer and colorectal cancer

No.	Age	Sex	Location	Stage[1]	Histological type
Patients with esophageal cancer					
1	66	M	Mt/Ut	2a	Mod[2]
2	53	M	Ae	3	Mod[2]
3	67	M	Mt/Ut	4b	Well[2]
4	64	M	Ae	2a	Mod[2]
5	55	M	Mt	2a	Mod[2]
6	56	M	Ut/Mt	4b	Mod[2]
7	67	M	Lt	1	Mod[2]
8	63	M	Mt/Lt	4b	Mod[2]
9	62	M	Lt	3	Well[2]
10	55	M	Lt	2a	Well[2]
11	73	M	Lt	4a	Mod[2]
12	63	M	Lt/Ae	3	Mod[2]
13	71	M	Lt/Ae	3	Mod[2]
14	63	M	Lt/Ae	4b	Well[2]
15	64	M	Mt	3	Mod[2]
16	45	M	Lt/Ae/Mt	4b	Poor[2]
17	54	M	Ae	4a	Mod[2]
18	54	M	Lt/Ae	3	Well[2]
19	58	M	Lt/Ae	2a	Combined[2]
20	67	M	Lt/Ae	2a	Mod[2]
Patients with colorectal cancer					
6R	57	M	S	2	Well[3]
27R	51	M	R	4	Mod[3]
29R	51	M	R	3b	Mod/Poor[3]
34R	59	M	R	3a	Well/Mod/Poor[3]
35C	72	F	S	2	Well[3]
36C	67	M	T	3a	Mod/Poor[3]
37C	49	M	R	3a	Well[3]
112	72	M	S	3b	Mod[3]
117	49	M	Ce	4	Mod[3]
118	85	M	S	2	Well[3]

[1]Stage is described according to Union Internationale Contre le Cancer (UICC) TNM Classification (Fifth Edition, 1997). [2]Well, well-differentiated squamous cell carcinoma; Mod, moderately differentiated squamous cell carcinoma; Poor, poorly differentiated squamous cell carcinoma; Combined, combined squamous cell carcinoma with basaloid carcinoma. [3]Well, well-differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Poor, poorly differentiated adenocarcinoma.Abbreviations: Ut: upper thoracic esophagus; Mt: middle thoracic esophagus; Lt: lower thoracic esophagus; Ae: abdominal esophagus; S: sigmoid colon; R: rectum; T: transverse colon; Ce: cecum.

instrument (Roche) was carried out in 20 μl of reaction mixture containing 2 μl of 10× LightCycler DNA Master SYBR Green I (FastStart Taq DNA polymerase, deoxynucleotide triphosphate, buffer, SYBR Green I), 3.0 mM $MgCl_2$ and 0.5 μM each of forward (5′-GGAAGCCTTGACATACGAT-3′), and reverse primer (5′-CATCCCTGAAGCCAGAAT-3′) in a LightCycler capillary. Real-time quantitative PCR of cyclin D1 cDNA was carried out in 20 μl of reaction mixture containing 2 μl of 10× LC FastStart DNA Master Hybridization Probes, 4.0 mM $MgCl_2$ and 0.5 μM each of forward (5′-CCC CAA CAA CTT CCT GTC CTA-3′) and reverse primer (5′-CTC CAG CAG GGC TTC GAT-3′) 0.3 μM of Fluorescein Probe (5′-AGG CAG TCC GGG TCA CAC TTG ATC ACT-3′-Fluorescein), 0.4 μM of LCRed Probe (5′-LCRed640-5′-TGG AGA GGA AGC GTG TGA GGC GGT A-3′-phosphorylation) in a LightCycler capillary. Serial dilutions of template cDNA were made for PCRs to optimize PCR products within the linear range. LightCycler software version 3.3 (Roche) was used for analysis of quantitative PCR.

Western-blot analysis

Frozen tissue samples were solubilized in lysis buffer (7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.1 M dithiothreitol (DTT), 2% IPG buffer (Amersham Pharmacia Biotech, Buckinghamshire, UK), 40 mM Tris) using a Polytron homogenizer (Kinematica, Switzerland) followed by centrifugation (100,000*g*) for 1 hr at 4°C. Cultured cells were solubilized in SDS sample buffer (62.5 mM Tris-Cl pH6.8, 2% SDS, 20% glycerol, 0.2% bromophenol blue, 0.025% β-mercaptoethanol) and heated for 5 min at 100°C. After passing through 27G needles, the samples were centrifuged for 15 min at 15,000*g*. A 30-μg sample of total protein from each lysate was loaded and separated by electrophoresis on 10–20% gradient gels (Bio-Rad, Hercules, CA). The proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) in a tank-transfer apparatus (Bio-Rad), and the membranes were blocked with 5% skim milk in PBS. Antibodies used include eIF4H (prepared by Japan Bio Services, Saitama, Japan diluted 1:500), cyclin D1 (Sigma, Japan; 1:1,000), ornithine decarboxylase (ODC; Sigma; 1:200), Bcl-XL (Sigma; 1:1,000), Bcl-2 (Sigma; 1:1,000) and c-Myc (Oncogene Research Products, La Jolla, CA 1:500). Goat anti-rabbit IgG horseradish peroxidase (Bio-Rad) diluted 1:3,000, mouse anti-rabbit IgG horseradish peroxidase (Bio-Rad) diluted 1:1,000 and rabbit anti-goat IgG horseradish peroxidase (Cappel, West Chester, PA) diluted 1:500 in blocking buffer were used as secondary antibodies. Antigens on the membrane were detected with enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech).

Plasmid transfection and establishment of eIF4H isoform 1 overexpressed stable NIH3T3 Tet-Off cell line

The Tet-off system utilizes the tetracycline-dependent transcriptional repression activity of tTA protein (BD Biosciences Clontech, Palo Alto, CA). The eIF4H isoform 1 cDNA, driven by a tTA-repressible CMV promoter element in a TRE-Tight plasmid (pTRE-Tight/eIF4H isoform 1), was constructed as follows. Full-length eIF4H isoform 1 was amplified by PCR using HeLa cDNA library as a template and cloned into the TRE-Tight vector plasmid (BD Biosciences Clontech). Plasmids were purified with an Endofree® Plasmid Maxi Kit (Qiagen) and the DNA sequences were verified. To generate the NIH3T3 Tet-Off stable cell line, pTet-Off plasmid (BD Biosciences Clontech) was transfected as described previously.[23,24] Briefly, NIH3T3 cells were plated in 6-well plates in IMDM containing 10% FBS without antibiotics one day before transfection so that they were at 70–90% confluence at the time of transfection. On the day of transfection, 4 μg plasmids and 10 μl Lipofectamine 2000™ (Invitrogen) were diluted in 250 μl Opti-MEM I Reduced-Serum Medium (Invitrogen), respectively. After 5-min incubation at room temperature, the diluted plasmids and Lipofectamine 2000™ were combined and then further incubated for 20 min at room temperature. Then, DNA-Lipofectamine 2000™ complexes were then added to each well and cells were incubated for 48 hr at 37°C in a CO_2 incubator. NIH3T3 cells, 5×10^4, transfected with pTet-Off plasmid were transferred to 10-cm dishes 48 hr after transfection, and 400 μg/ml geneticin (Invitrogen) and 1 μg/ml doxycycline (Dox) were added to complete medium containing IMDM, 10% FBS, 1% penicillin-streptomycin. The complete medium with geneticin was replaced every 4 days and fresh Dox was added every 2 days until geneticin-resistant colonies began to appear. At least 30 clones were screened to find the clone with the highest induction in the absence of Dox and with the lowest background. Once the NIH3T3 Tet-Off stable cell line was obtained, the eIF4H isoform 1-overexpressed stable NIH3T3 Tet-Off cell line was generated by transfecting pTRE-Tight/eIF4H isoform 1 plasmid and linear hygromycin marker (0.2 μg) into the cells. Stable clones were selected by adding 400 μg/ml hygromycin and 1 μg/ml Dox to the medium as described above.

Gene knockdown using siRNA and cotransfection of cyclin D1 expression plasmid

siRNA targeting eIF4H RNA were generated to reduce eIF4H expression. The target sequence for eIF4H siRNA was 5′-AACCCACAGAAGAGGAAAGAG-3′ (si102), which is common to two splice variants of eIF4H, and two eIF4H isoform 1 specific siRNA were 5′-AATGGGTAGCTCTCGA GAATC-3′ (si103), 5′-AATCTAGAGGTGGATGGGATT-3′ (si104) (Japan Bio Services) and eIF4H isoform 2-specific siRNA was 5′-AUG ACA GAG GCU UCA GGG A-3′ (iGENE, Tokyo, Japan). Blast analysis (http://www.ncbi.nlm.nih.gov/BLAST/) did not reveal overlapping regions between target sequences and other human genes. Cyclin D1 expression plasmid (CCND1 Human cDNA Clone) was purchased from Origene (Rockville, MD). Transfection of siRNA and plasmid was performed using lipofectamin 2000™ as described above.

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Establishment of eIF4H isoform 1 stable knockdown colon cancer cell line

The eIF4H shRNAs, siRNA oligonucleotides connected by a spacer sequence, were cloned into a pBAsi-hU6 Neo plasmid (Takara Bio; Japan), where eIF4H shRNAs were driven by a hU6 promoter. The resulting plasmids, pBAsi-hU6-eIF4H shRNAs, were then transfected into LOVO cells and eIF4H isoform 1 stable knockdown clones, sh103-1, sh103-2 and sh104, were selected by adding 400 μg/ml geneticin, as described above.

Proliferation assays

Viable cell number was assessed according to 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) dye absorbance following the manufacturer's instructions (Promega, Madison, WI). Absorbance was measured using a Wallac 1420 ARVOsx Multilabel Counter (Perkin-Elmer, Tokyo, Japan). Experiments were repeated in eight parallel studies.

Cell cycle analysis

Cells were fixed in 70% ethanol and treated with 50 μg/ml propidium iodide (Wako, Japan) in the presence of 200 μg/ml RNase A. Then, cells were subjected to cell cycle analysis using fluorescence activated cell sorting (FACS) Caliber cytometer (Becton Dickinson, San Jose, CA). At least 10,000 cells were counted for each sample, and data were analyzed with a Cell Quest program (Becton Dickinson, San Jose, CA).

TUNEL assay

Apoptotic cells were detected by the TUNEL assay according to the manufacturer's instructions (Apoptosis Detection System, Fluorescein; Promega). Briefly, cells cultured in 6-well plate were fixed with paraformaldehyde at 4°C for 10 min on ice and permeabilized with 0.5% Triton-X-100 solution in PBS for 5 min. After washing with PBS twice, apoptotic cells were visualized through the detection of internucleosomal fragmentation of DNA using *in situ* nick-end labeling with terminal deoxynucleotidyl transferase (TdT) and FITC-labeled dUTP (MEBSTAIN Apoptosis Kit; Medical & Biological Laboratories, Japan).

Subcutaneous injection of the cells and tumor formation in nude mice

Male BALB/c nude mice, 4–5 weeks of age, were subcutaneously injected with 1×10^6 LOVO cells or 6×10^5 NIH3T3 cells into the left leg. Tumor size was calculated using the formula $(ab^2)/2$, where a and b represent the larger and smaller diameters, respectively, and was monitored every 3 days.

Results

eIF4H isoform 1 was overexpressed in gastrointestinal cancers

eIF4H gene is known to produce two splice variants, isoform 1 and 2 (exon 5 is alternatively spliced), which generate two protein products, 27 kDa and 25 kDa. Between them, not isoform 2, but isoform 1 expression, was significantly increased in colorectal cancers.[17] We then investigated if this was true in other gastrointestinal cancers, such as esophageal cancer. Detailed information on the esophageal cancer and colorectal cancer cases is shown in Table 1. Total protein lysates were prepared from 20 matched samples of the tumor (T) and adjacent nontumor tissue (N) and the expressions of eIF4H isoforms were examined with anti-eIF4H antibody. We found that eIF4H isoform 1 protein, but not isoform 2, was greatly increased in most esophageal cancers compared with the corresponding nontumor tissues (Figs. 1*a* and 1*b*), although there were no striking correlations between patient's pathological data and the eIF4H isoform 1 expression level (Table 1).

We therefore decided to focus on eIF4H isoform 1 in further studies. To examine if the increase of eIF4H isoform 1 occurred at the mRNA level, a set of primers that specifically amplifies isoform 1 was designed and real-time quantitative RT-PCR was carried out using total RNA extracted from tumor and nontumor tissues of 20 esophageal and 10 colorectal cancer patients (Fig. 1*c*). The eIF4H isoform 1 mRNA level was significantly elevated in the tumors, indicating that overexpression of the eIF4H isoform 1 is regulated at the mRNA level, such as elevated transcription, mRNA stability or de-regulation of alternative splicing.

Overexpression of eIF4H isoform 1 in NIH3T3 cell leads to tumor formation in nude mice

To directly investigate the transforming potential of eIF4H isoform 1, we created NIH3T3 cell lines that overexpress eIF4H isoform 1 using a Tet-Off gene expression system. The NIH3T3 cell line, which expresses the tetracycline-controlled transactivator, was stably trasfected with the eIF4H isoform 1 expression plasmid under the control of the tetracycline-responsive promoter so that eIF4H isoform 1 was expressed only in the absence of doxycycline (Dox). The cells were then screened for inducible eIF4H isoform 1 protein expression by immunoblotting with anti-eIF4H antibody. Three stable clones, no. 9, 10 and 306, were obtained, in which eIF4H isoform 1 expression was greatly induced by Dox removal from the media (Fig. 2*a*). Strikingly, subcutaneous injection of these stable clones overexpressing eIF4H isoform 1 into nude mice formed tumors after 3.5 weeks, whereas control mice injected with NIH3T3 cells did not generate tumors (Figs. 2*b* and 2*c*). These results suggest that eIF4H isoform 1 possesses transforming activity *in vivo*.

Suppression of eIF4H isoform 1 inhibits proliferation of colon cancer cell lines

To further examine the involvement of eIF4H isoform 1 in cell proliferation and carcinogenesis, RNA interference (RNAi) was used to suppress eIF4H expression. Several siRNA were designed to target different regions of the eIF4H open reading frame (Supporting Information Figure 1). Among them, siRNA (si103 and si104) suppressed only isoform 1

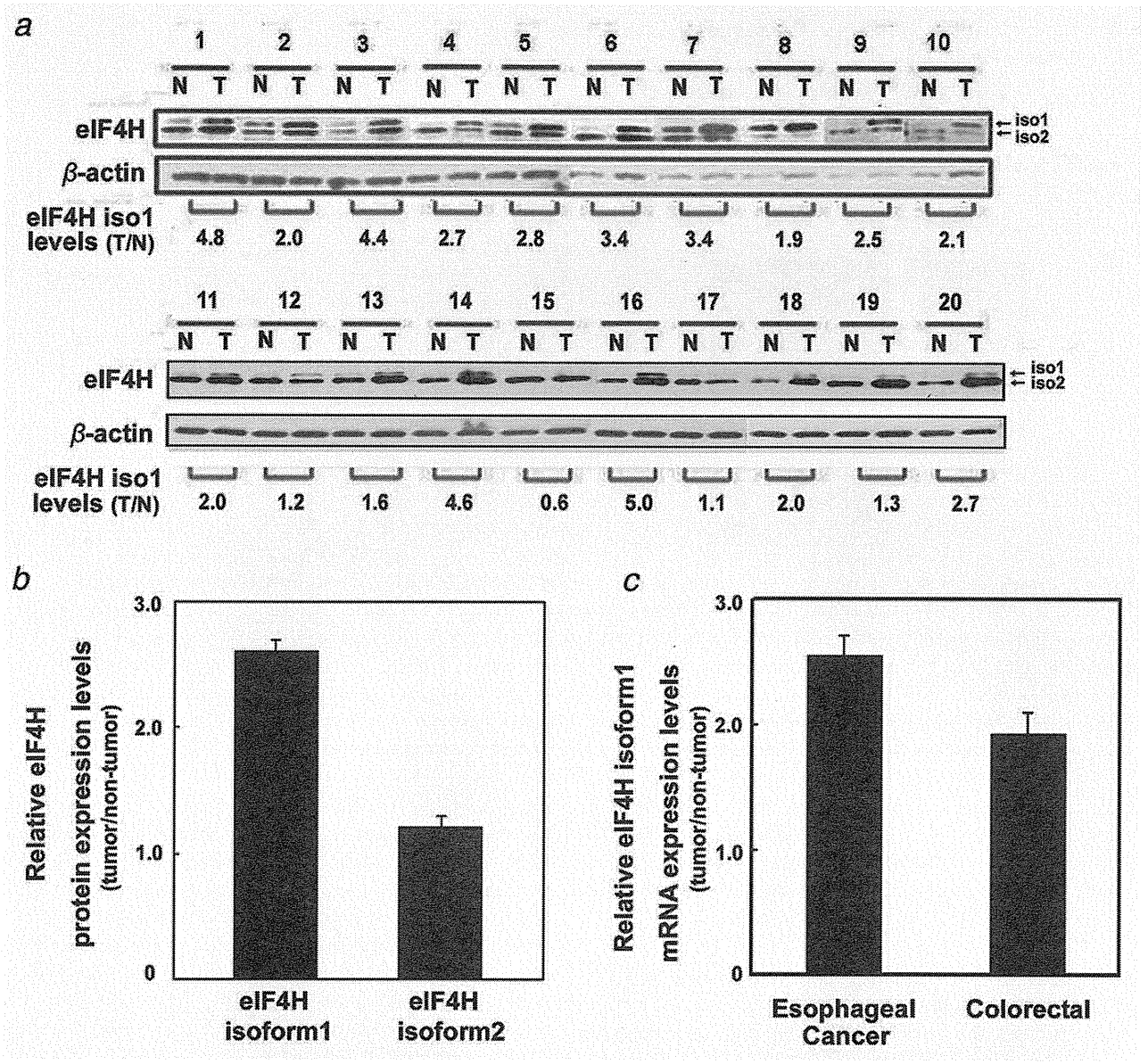

Figure 1. eIF4H isoform 1 was overexpressed in primary esophageal cancers tissues. (*a*) Total protein lysates were prepared from 20 matched samples of the tumor (T) and adjacent nontumor tissues (N). Equal amounts of protein from each pair were resolved on 10–20% gradient polyacrylamide gel and immunoblotted with anti-eIF4H and β-actin antibodies. Upper band: isoform 1; lower band: isoform 2. (*b*) The intensity of each band in (*a*) was measured with NIH Image and the mean ± SD of the relative eIF4H protein levels between tumor and nontumor tissue of 20 esophageal cancer patients normalized with β-actin were calculated. (*c*) Total RNA was prepared from matched samples of tumor and adjacent nontumor tissue of esophageal cancer and colorectal cancer patients, and real-time PCR was performed to examine the eIF4H isoform 1 mRNA level. Mean ± SD of relative eIF4H mRNA levels between tumor and nontumor tissue of 20 esophageal and 10 colorectal cancer patients normalized with β-actin were calculated.

and si201 suppressed only isoform 2 expression 48 hr after siRNA treatment in two colon cancer cell lines, LOVO and RKO (Figs. 3*a* and 3*b*); therefore, these three eIF4H siRNA (si103, 104 and 201) were used for further analysis.

To investigate the effect of eIF4H suppression on cell growth, the MTS assay was performed 48 hr after siRNA treatment of LOVO and RKO (Figs. 3*c* and 3*d*). Surprisingly, the suppression of eIF4H isoform 1 alone, using si103 and si104, strongly decreased the viable cell number by 30–50% as compared with the control. A similar decrease of the cell number was observed in RKO cells. These results imply that eIF4H isoform 1 plays an important role in the proliferation of colon cancer cells. Next, we examined if the suppression of eIF4H isoform 2 inhibits the proliferation of colon cancer

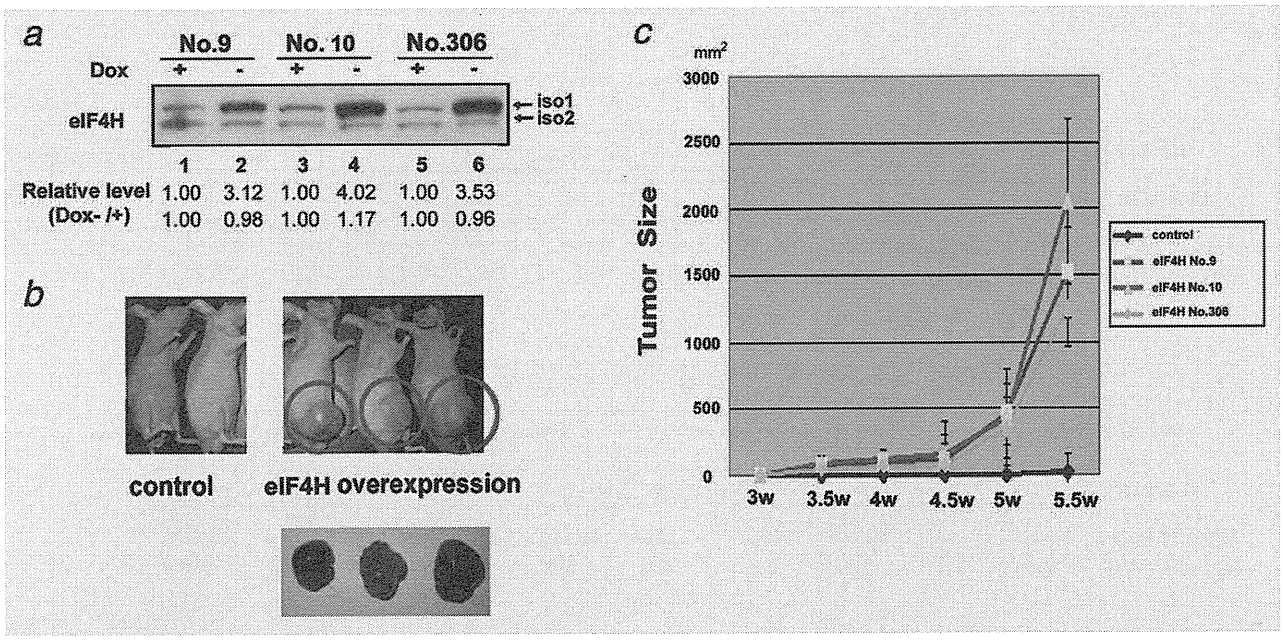

Figure 2. Overexpression of eIF4H isoform 1 led to tumor formation *in vivo*. (*a*) NIH3T3 stable cell lines overexpressing eIF4H isoform 1 (clone no. 9, 10 and 306) were generated using a Tet-Off gene expression system, as described in "Material and Methods" section, and the overexpression of eIF4H isoform 1 was confirmed by Western blotting. eIF4H isoform 1 was overexpressed in the absence of doxycycline (Dox) (lanes 2, 4 and 6) compared with in the presence of Dox (lanes 1, 3 and 5) in stable cell lines. (*b*) eIF4H isoform 1-overexpressing cells (6×10^5 cells) were subcutaneously injected into nude mice and tumor formation was examined for 5.5 weeks. (*c*) NIH3T3 stable clones overexpressing eIF4H isoform 1, no. 9, 10 and 306, were injected into six mice, respectively. Tumor size was measured every 3 days and the mean ± SD of six mice from three independent experiments are shown.

cells using isoform 2-specific siRNA (si201). As expected, si201 did not affect the cell number of LOVO and RKO, in great contrast to si103 and 104 (Figs. 3*c* and 3*d*). These results further supported the eIF4H isoform 1-specific effect on the cell proliferation of colon cancer cells.

Suppression of eIF4H isoform 1 induces apoptosis of colon cancer cell lines

To understand the mechanism of the inhibitory effect on proliferation by eIF4H isoform 1 siRNA, flow cytometric analysis was performed. The treatment of cells with si103 caused a 3-fold increase in the sub-G1 cell population and a decrease in the S phase compared with control cells, which suggests that the growth inhibition of cells by RNAi is due to the induction of apoptosis (Fig. 4*a*). This apoptosis was further confirmed by TUNEL assay (Fig. 4*b*). Both LOVO and RKO treated with si103 showed 60–70% of TUNEL-positive cells compared with about 20% in untreated control cells (Student's *t*-test, $p < 0.05$) (Fig. 4*c*). These results suggest that inhibition of apoptosis might be involved in the transforming activity of eIF4H isoform 1.

Suppression of eIF4H isoform 1 inhibited tumor growth in nude mice

Next, we investigated whether the suppression of eIF4H isoform 1 could also inhibit tumor growth *in vivo*. For the experiment, we created LOVO cell lines that were stably transfected with short hairpin RNA (shRNA) expression plasmids, sh103 and sh104, corresponding to si103 and si104 used in the above experiment. Several clones, sh103-1, 103-2 and 104, showed remarkable reduction of eIF4H isoform 1 compared with control cells (Fig. 5*a*, upper panel). These clones were injected subcutaneously into nude mice and the tumor size was monitored. Strikingly, the tumors formed in mice injected with control LOVO cells grew rapidly, whereas tumors were greatly reduced in mice injected with shRNA stably expressed clones (Figs. 5*a* and 5*b*). These results indicate that eIF4H isoform 1 contributes to the development of colon tumors.

Suppression of eIF4H isoform 1 showed no effect on the growth of immortalized human fibroblasts

The above results have raised the question of whether the decrease of the cell number by eIF4H isoform 1 knockdown is specific to cancer cells or is a more general feature; if it is unique to cancer cells it might be a potential target for cancer therapy. To address this question, we examined the effect of eIF4H suppression on the proliferation of immortalized human fibroblast cell MRC5 using the same siRNA shown in Figure 3. The expression of eIF4H isoforms, especially isoform 1, was greatly suppressed 48 hr after siRNA transfection (Fig. 5*c*). In great contrast with colon cancer cells, there was

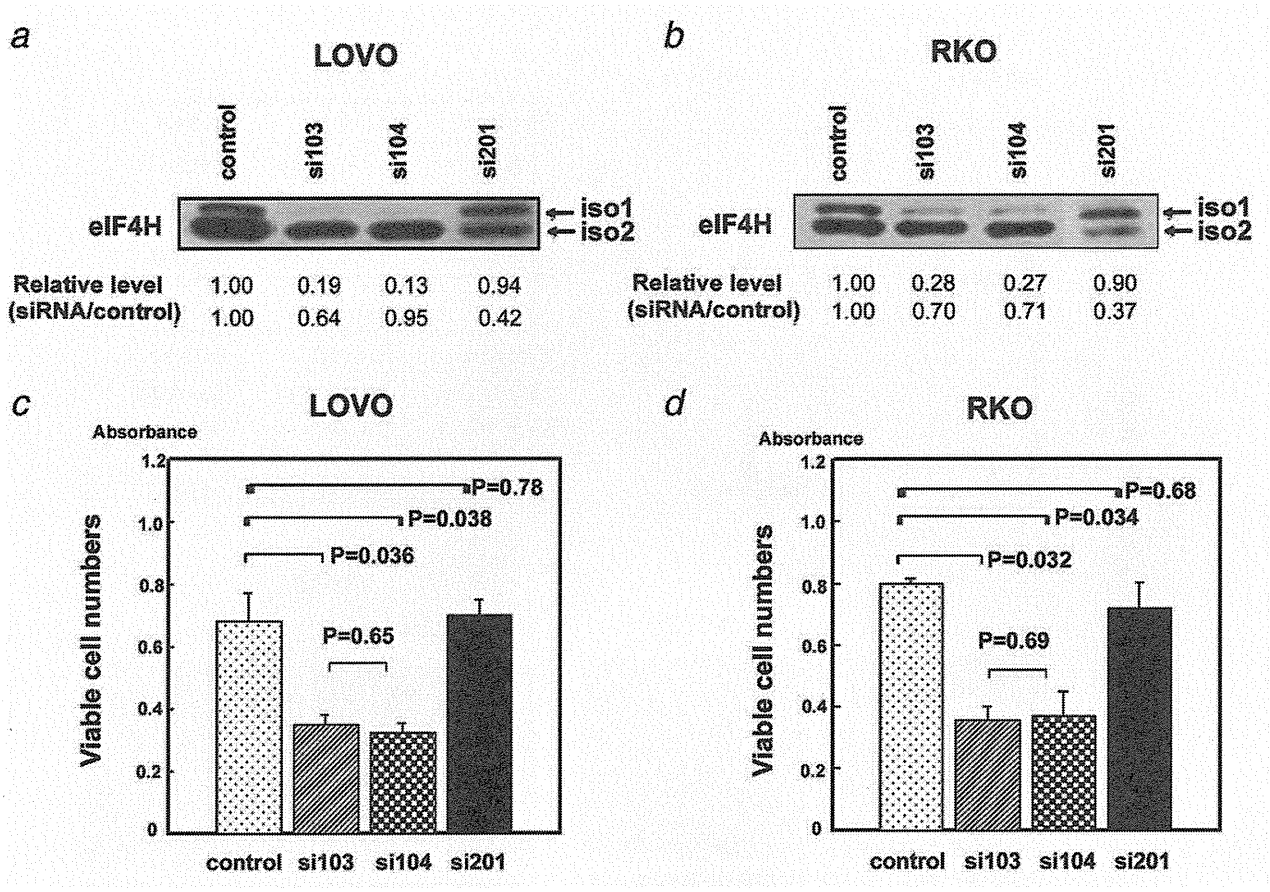

Figure 3. Suppression of eIF4H isoform 1, and not isoform 2, inhibited the growth of colon cancer cell lines. (*a*, *b*) 20 pmol of siRNA (isoform 1 specific: si103, 104; isoform 2 specific: 201) for eIF4H were transfected into LOVO and RKO cells, and the expression of eIF4H isoforms was examined by Western blotting 48 hr after siRNA treatment. The intensity of each band was measured with NIH Image and the relative level of eIF4H isoforms between control and siRNA-treated cells normalized with β-actin was calculated. (*c*, *d*) Viable cell numbers 48 hr after eIF4H siRNA treatment were assessed by MTS assay. Data represent the mean ± SD of eight independent experiments and statistical analysis was performed by Student's *t*-test.

no effect of RNAi on the cell growth of MRC5 (Fig. 5*d*). A similar result was observed in WI38 immortalized lung fibroblast cells (data not shown). Thus, eIF4H isoform 1 might be a suitable therapeutic target for colon cancer.

Possible mechanism of cellular transformation caused by eIF4H isoform 1

What is the mechanism of transformation induced by eIF4H isoform 1 expression? One possible mechanism is that eIF4H isoform 1 leads to the overproduction of potentially oncogenic proteins that stimulate cell proliferation or inhibit apoptosis, such as c-Myc, cyclin D1, VEGF, ODC, Bcl-2 and Bcl-X_L. mRNA of the genes are known to have long and structurally complex 5′-untranslated regions and may require high levels of eIFs, including eIF4H, for their translation. To examine the effect of eIF4H isoform 1 overexpression on the expression of oncogenic proteins, immunoblotting was performed using extracts from NIH3T3 cell lines overexpressing eIF4H isoform 1. Surprisingly, the level of cyclin D1 greatly increased in eIF4H isoform 1-overexpressing cells compared with control cell lines, whereas ODC, Bcl-2 and Bcl-X_L showed only a marginal effect (Figs. 6*a* and 6*b*, Supporting Information Figure 2). Next, we tested whether the suppression of eIF4H isoform 1 decreases the cyclin D1 level. As expected, eIF4H isoform 1-suppressed LOVO cells, sh103-1 and sh103-2, showed marked reduction of cyclin D1 expression (Figs. 6*c* and 6*d*). c-Myc level increased in only one of the eIF4H isoform 1 overexpressing clones and did not decrease in eIF4H stable knockdown cells, which indicates that eIF4H overexpression is not involved in the regulation of c-Myc expression. These results indicate that upregulation of cyclin D1 is involved in the transforming activity of eIF4H isoform 1.

If cyclin D1 upregulation is a direct mechanism of the transforming activity of eIF4H, ectopic expression of cyclin D1 in eIF4H knockdown cells would suppress the inhibitory effect on cell proliferation. To test this, we cotransfected eIF4H isoform 1 siRNA (si104) and cyclin D1 expression

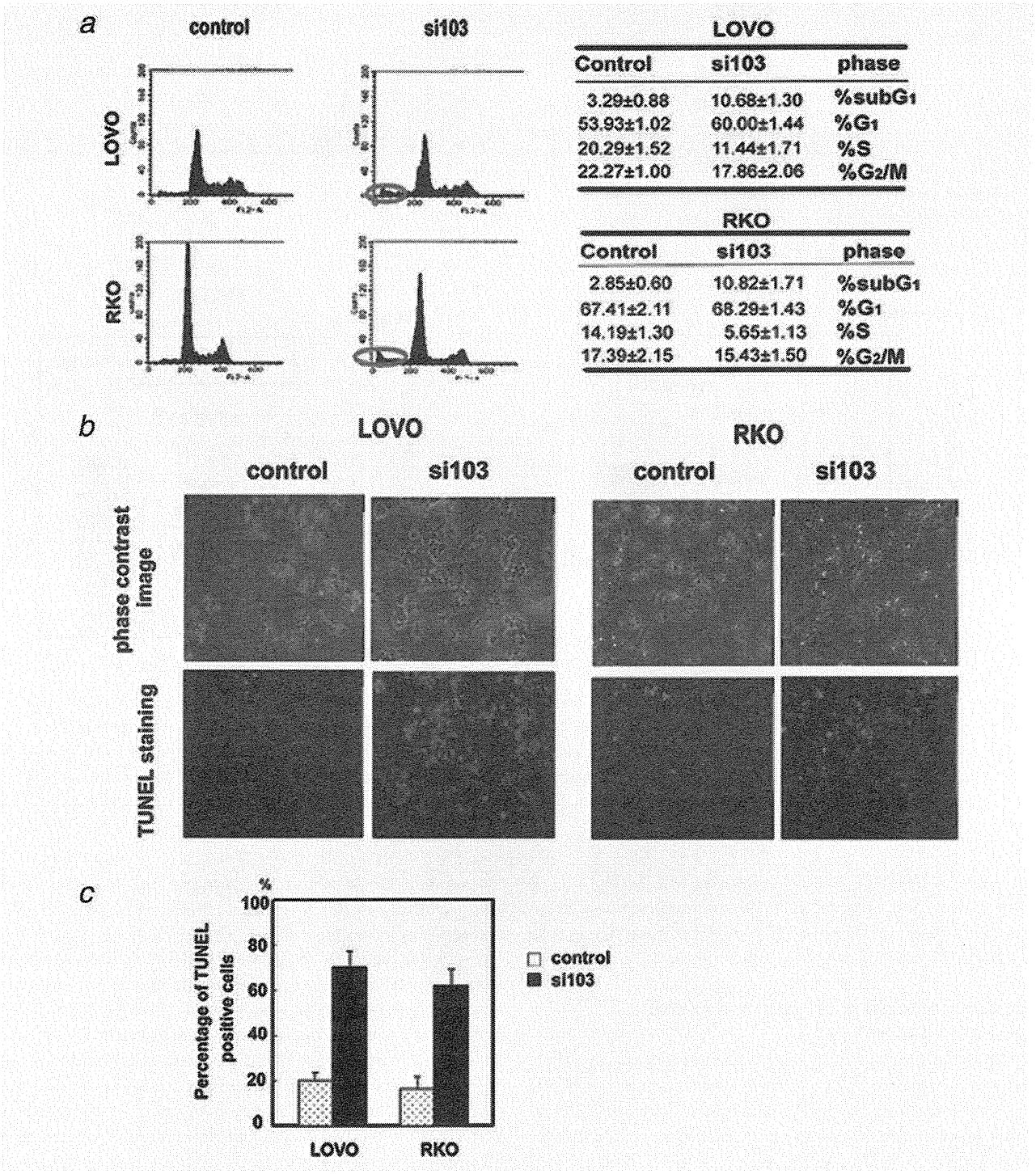

Figure 4. Suppression of eIF4H isoform 1 induces apoptosis of colon cancer cell lines. LOVO and RKO cells were treated with 20 pmol of si103, siRNA specific to eIF4H isoform 1, for 48 hr and FACS (*a*) and TUNEL (*b*) analysis was performed. (*a*) FACS analysis was repeated three times and the percentage (mean ± SD) of the cell population in each phase of the cell cycle is shown in the right table. (*b*) TUNEL analysis of control or si103-treated colon cancer cells (LOVO and RKO) showed apoptotic cells. (*c*) The number of TUNEL-positive cells was counted from five different fields. The percentage (mean ± SD) was calculated.

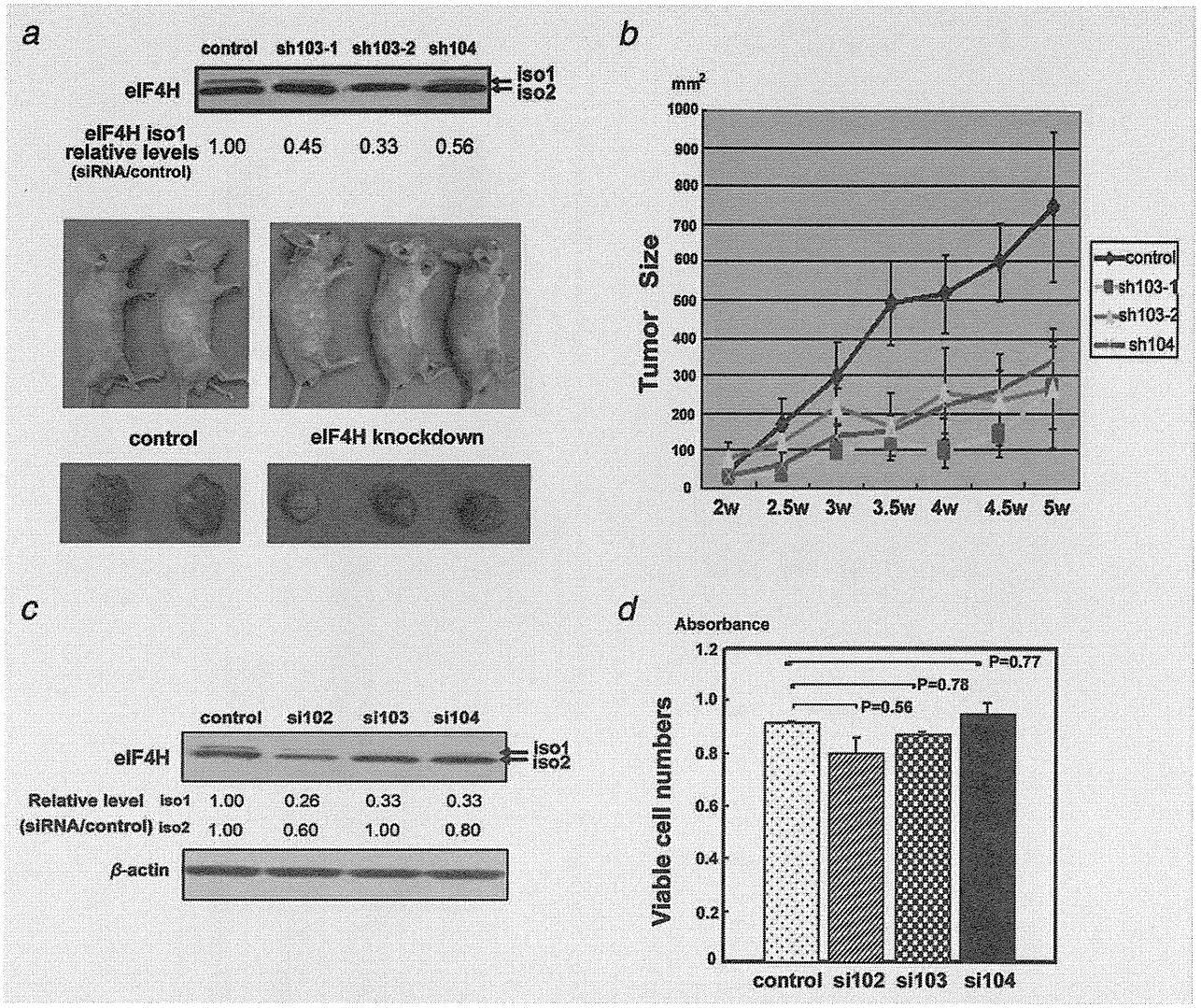

Figure 5. Suppression of eIF4H isoform 1 inhibited tumor growth in nude mice, but showed no effect on the proliferation of immortalized fibroblasts. (*a*) shRNAs (sh103, 104) specific for eIF4H isoform 1 were transfected in LOVO cells and stable knockdown cell lines of eIF4H isoform 1 were generated. sh103-1 and 103-2 are different stable clones generated by sh103 transfection. The suppression of eIF4H isoform 1 in each clone was confirmed by Western blotting. The intensity of each band was measured with NIH image and the relative level of eIF4H isoform 1 between control and stable knockdown cell lines normalized with β-actin was calculated. eIF4H isoform 1 stable knockdown LOVO cells (1×10^6 cells) were subcutaneously injected into nude mice and tumor formation was examined for 5 weeks (lower panel). (*b*) A control and three stable clones, sh103-1, 103-2 and 104, were injected into six mice, respectively. Tumor size was measured every 3 days and tumor growth curves of the mean ± SD of six mice from two independent experiments are shown. (*c*) 20 pmol of siRNA (si102, 103 and 104) for eIF4H were transfected into MRC5 cells and the expression of eIF4H isoforms was examined by Western blotting 48 hr after siRNA treatment. The intensity of each band was measured with NIH image and the relative level of eIF4H isoforms between control and siRNA-treated cells normalized with β-actin was calculated. si102 suppressed both isoform 1 and 2 of eIF4H, whereas si103 and 104 suppressed only isoform 1. (*d*) Viable cell numbers 48 hr after eIF4H siRNA treatment were assessed by MTS assay. Data represent the mean ± SD of eight independent experiments and statistical analysis was performed by Student's *t*-test.

plasmid into a colon cancer cell line, RKO, and measured the viable cell numbers by MTS assay. Expression of the cyclin D1 plasmid was confirmed by Western blotting (Fig. 5*e*). Cotransfection of si104 with control plasmid decreased the cell number, as shown in Figure 3, whereas cotransfection of cyclin D1 expression plasmid reversed the growth suppression effect of si104 (Fig. 5*f*). This result further supports our hypothesis that eIF4H isoform 1 plays an important role in carcinogenesis through the activation of cyclin D1.

The expression of cyclin D1 is well known to be regulated by another eukaryotic translation initiation factor, eIF4E. Regulation is thought to occur at the translational level;

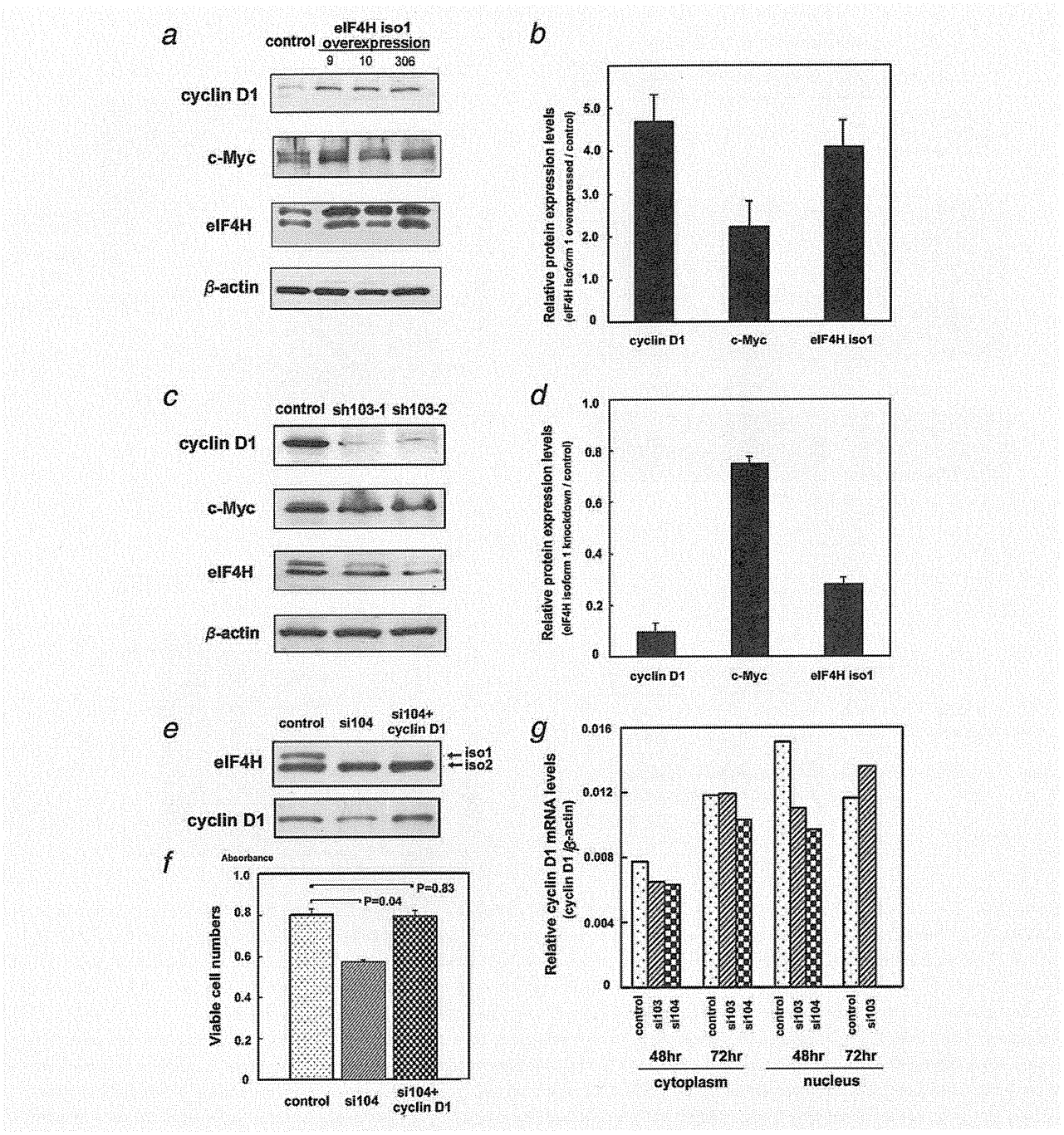

Figure 6. Cyclin D1 upregulation is involved in the transforming activity of eIF4H isoform 1. (*a*) Expressions of cyclin D1 and c-Myc in three different stable clones overexpressing eIF4H isoform 1, no. 9, 10 and 306, were examined with Western blotting. (*b*) The intensity of each band was measured with NIH image and the relative expression levels of each protein normalized with β-actin in eIF4H isoform 1-overexpressing cells compared with control cells are shown. Means and SD values were calculated from the results of the different clones. (*c*) Expression of cyclin D1 and c-Myc was examined in eIF4H isoform 1 stable knockdown LOVO cell lines. (*d*) Data represent the means and SD values calculated from the results of two different clones. (*e*) 50 pmol of eIF4H isoform 1-specific siRNA (si104) with 25 ng of control or cyclin D1 expression plasmid was transfected into RKO cells and the expression of eIF4H isoforms (upper panel) and cyclin D1 (lower panel) was examined by Western blotting 72 hr after siRNA treatment. (*f*) Viable cell numbers 72 hr after transfection were assessed by MTS assay. Data represent the mean ± SD of eight independent experiments and statistical analysis was performed by Student's *t*-test. (*g*) Control or eIF4H isoform 1 siRNA (si103, 104; 50 pmol) was transfected to RKO cells. Total RNA from nucleus and cytoplasm of the cells was extracted 48 and 72 hr after transfection and real-time PCR was performed to examine the cyclin D1 mRNA level. Relative cyclin D1 mRNA levels normalized with β-actin were calculated. Nuclear mRNA level 72 hr after si104 treatment is not shown.

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however, recent reports have demonstrated that cyclin D1 mRNA is also controlled at the level of mRNA export from the nucleus, and this transport of cyclin D1 mRNA to the cytoplasm is important for transformation activity.[25–27] Thus, we investigated whether eIF4H also regulates cyclin D1 at the mRNA level. RKO cells were treated with eIF4H siRNA (si103 and 104), and then nuclear and cytoplasmic cyclin D1 mRNA were evaluated by real-time quantitative RT-PCR. Cyclin D1 mRNA levels did not alter either in the nucleus or cytoplasm of siRNA-treated cells (Fig. 6*g*). Although it is possible that eIF4H could control other processes on cyclin D1 mRNA, regulation of cyclin D1 expression at the protein level seems to be the main mechanism, as the decrease of cyclin D1 expression after eIF4H isoform 1 downregulation is more pronounced at protein level.

Discussion

In our study, we investigated whether an alternative splicing form of eIF4H, which is overexpressed in gastrointestinal cancers, contributes to cell proliferation and carcinogenesis. We found that the overexpression of eIF4H isoform 1 in immortalized mouse fibroblasts developed tumors in nude mice. Conversely, silencing of the eIF4H isoform 1 inhibited the proliferation and induced apoptosis of colon cancer cell lines. It is striking that the growth suppression is specific to eIF4H isoform 1 siRNA and not isoform 2. Suppression of eIF4H isoform 1 also inhibited tumor growth in nude mice, although it showed no effect on the proliferation of immortalized human fibroblasts. Moreover, eIF4H isoform 1 upregulated the cyclin D1 level, a protein involved in cell proliferation. Thus, eIF4H isoform 1 stimulates cell proliferation and inhibits apoptosis, both of which are a possible mechanism of the transforming activity of eIF4H isoform 1. These observations provided strong evidence that eIF4H isoform 1 is a novel oncogene and might be a promising molecule target for colon cancer therapy.

Recently, many studies have argued that eIFs play an important role in carcinogenesis.[4,28] eIF4E, an mRNA 5′-cap-binding protein, has been identified to play a pivotal role in the tumorigenesis and metastatic progression of various cancers. Another subunit of eIF-4F complex eIF4G, a scaffolding protein, is also implicated in malignant transformation.[11,29] In our work, we demonstrated that another translation initiation factor, eIF4H, is involved in tumorigenesis, which suggests that translation initiation is a key event for the development of cancer. A mechanism suggested to contribute to tumorigenesis is the translational de-repression of structurally burdened mRNA known to encode proto-oncogenes, *e.g.*, cyclin D1, c-Myc, ODC, FGF and VEGF, through increased levels of eIFs.[30–32] Cyclin D1 was one of the first discovered targets of eIF4E. It has been reported that eIF4E regulates the amount of cyclin D1 not only by translation initiation but by nuclear export of its mRNA.[25–27] Thus, we investigated whether eIF4H isoform 1 is also involved in the nuclear export of cyclin D1 mRNA. Unlike eIF4E, eIF4H isoform 1 showed no effect on the nuclear export (Fig. 6*g*), which suggests that upregulation of cyclin D1 by eIF4H isoform 1 occurs at the translational level.

We also found that suppression of eIF4H isoform 1 induced the apoptosis of colon cancer cells. The induction of apoptosis is associated with the downregulation of translation rates, and underlying changes to the translation mechanism during apoptosis have been elucidated recently.[33] Apoptosis-associated modifications include the specific fragmentation of translational mechanism, such as eIF4G, eIF4B and eIF3j, and alterations in the phosphorylation states of initiation factors, such as eIF2α, eIF4E and eIF4E-BP1,[34] which might change the translation rates of pro- or antiapoptotic proteins. Moreover, eIF4E mediates *myc*-dependent apoptosis by inhibiting mitochondrial cytochrome *c* release through an increase in Bcl-X_L mRNA translation.[35] One explanation for apoptosis occurring by the suppression of eIF4H isoform 1 is that low levels of eIF4H decrease the translational efficiency of antiapoptotic factors, although knockdown of eIF4H isoform 1 showed no effect on Bcl-2 or Bcl-X_L levels (data not shown). Further investigation is needed to clarify the precise mechanism of the induction of apoptosis.

Pre-mRNA splicing is a sophisticated and ubiquitous nuclear process, which is a natural source of cancer-causing errors in gene expression. A large number of cancer-related genes that exhibit alternative splicing have been characterized, including CD44, WT1, BRCA1, MDM2, FGFR and kallikrein family members.[19] As well as being associated with cancer, the nature of alternative gene products is usually consistent with an active role in cancer; for example, several splicing variants of HDM2, the human homolog of MDM2, increase p53 levels and enhance p53 transcriptional activity.[36] Our recent data also showed that a splicing variant of *c-myc* suppressor FIR was frequently found in colon cancer tissues and promoted tumor development by disabling FIR repression to sustain high levels of *c-myc*[37]; therefore, the alternative spliced isoform plays an important role in carcinogenesis and eIF4H isoform 1 is one of these.

In our study, we showed that only eIF4H isoform 1 and not isoform 2 possesses transforming activity. An important question is why only one isoform has oncogenic potential. eIF4H is thought to stimulate protein synthesis by enhancing the helicase activity of eIF4A. The most likely explanation of eIF4H isoform 1-specific activity is that the isoform 1-specific exon 5 causes conformational change of the protein and enhances its helicase activity, which increases the cyclin D1 level, although this was not confirmed. Another possibility is that eIF4H isoform 1 specifically binds to other oncogene products or tumor suppressor proteins and enhances/inhibits their activities. Further studies are needed to elucidate the mechanism of the isoform 1-specific oncogenic potential of eIF4H.

Drugs that can selectively kill pathogenic cells without damaging healthy cells are so-called "magic bullets."[38] Our

observation showed that selective suppression of a splicing variant of eIF4H inhibited the proliferation of colon cancer cells and had no effect on immortalized human fibroblasts. Consequently, eIF4H isoform 1 would be an ideal therapeutic target for colon cancer.

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RESEARCH ARTICLE Open Access

Adhesion molecule periplakin is involved in cellular movement and attachment in pharyngeal squamous cancer cells

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Abstract

Background: We previously reported that periplakin (PPL) is downregulated in human esophageal cancer tissues compared to the adjacent non-cancer epithelium. Thus PPL could be a useful marker for detection of early esophageal cancer and evaluation of tumor progression, but largely remains unknown in this field. To investigate PPL involvement in carcinogenesis, tumor progression, cellular movement or attachment activity, siRNAs against PPL were transfected into pharyngeal squamous cancer cell lines and their effects on cellular behaviours were examined.

Results: PPL knockdown appeared to decrease tumor cell growth together with G2/M phase accumulation in cells attached to a culture dish. However, the extent of cell growth suppression, evaluated by the number of cells attached to the culture dish, was too distinctive to be explained only by cell cycle delay. Importantly, PPL knockdown suppressed cellular movement and attachment to the culture dish accompanied by decreased pAktSer473 phosphorylation. Additionally, LY294002, a PI3K inhibitor that dephosphorylates pAktSer473, significantly suppressed D562 cell migration. Thus PPL potentially engages in cellular movement al least partly via the PI3K/Akt axis.

Conclusions: PPL knockdown is related to reduced cellular movement and attachment activity in association with PI3K/Akt axis suppression, rather than malignant progression in pharyngeal cancer cells.

Background

Pharyngeal and esophageal cancers are some of the most malignant gastrointestinal tumors [1]. We previously reported that a cell adhesion molecule, periplakin (PPL), is significantly downregulated in human esophageal cancers. Immunohistochemical staining has also revealed that PPL changes its cellular localization as well as its expression levels with cancer progression [2]. In addition, PPL expression has been associated with nodal metastasis [3]. These findings indicate that PPL plays an important role in the development of esophageal and pharyngeal squamous cell carcinoma; however, the precise mechanism remains largely unknown.

PPL is a member of the plakin family comprising desmoplakin, envoplakin, plectin, and bullous pemphigoid antigen 1, which have various functions in connecting cytoskeleton elements to form intercellular junction complexes [4]. Plakin families also function as "molecular bridges" of cells that link the intracellular cytoskeleton and cell-cell junctions. For example, plectin, one of the most well-studied plakins, directly interacts with signal transductions [5,6]. PPL also plays a role as a localization signal in oncogenic serine/threonine protein kinase Akt/protein kinase B (PKB)-mediated signaling in human cancer cell lines [7]. Here, Akt/PKB is a downstream effector of phosphatidylinositol 3' kinase (PI3K) and functions as a critical regulator of cell survival and proliferation. Furthermore, activation of the PI3K/Akt/PKB pathway is emerging as a central feature of epithelial-mesenchymal transition (EMT), which is believed to be a crucial event in tumor development [8].

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In this study, we examined changes in cell growth, morphology, migration, and attachment activity following siRNA against PPL to investigate the involvement of PPL in tumor development. We found that PPL knockdown was related to a reduction in cellular movement and attachment activity, and was accompanied by PI3K/Akt axis suppression in pharyngeal cancer cells; it was potentially related to EMT promotion.

Results

Knockdown of PPL expression reduced the number of cells attached to the culture dish

Since PPL expression is decreased in esophageal carcinoma tissues [2], we tried to determine the effects of PPL downregulation on cancer cell activities. First, endogenous PPL expression in hypopharyngeal squamous carcinoma (D562 and FaDu) and esophageal squamous carcinoma (YES5, TE2, TE4, TE9, TE11, and TTn) cells was examined (Figure 1A). D562 cells were selected because siRNA against PPL was most efficient among these cell lines (Figure 1B, data not shown). In D562 cells, PPL siRNA inhibited cell growth and reduced the number of cells attached to the culture dish (Figure 1C).

Knockdown of PPL expression increased the proportion of G2/M phase cells attached to the culture dish

So why was the number of attached cells to the culture dish reduced after PPL downregulation? To determine the mechanism behind this phenomenon, we first analyzed the cell cycle populations after PPL siRNAs were transfected into D562 cells. Compared to control (GL2) siRNA, cultures treated with PPL siRNA had a decreased G1 (2N) and an increased late S and G2/M cell population (Figure 2A). To verify this result, we further developed a double channel, BrdU and 7-amino-actinomycin D (7-AAD), flow cytometry protocol. By plotting BrdU incorporation (S phase cells) on the y-axis and 7-AAD (DNA content) on the x-axis, the cells could be divided into 3 populations: G1, S, and G2/M (Figure 2B, right). Thus, knockdown of PPL reduced the S population and increased the G2/M population (Figure 2C). In particular, the G2/M population increased 12.9% in the control cell culture and 15.6% in the PPL siRNA cell culture, a 20.9% increase (average of siRNA1 and siRNA2). These data suggest that PPL siRNA decreased cellular proliferation of D562 cells, at least partly by delay in the G2/M phase of cells attached to the culture dish. However, to determine the effect of PPL knockdown on cell growth more precisely, cells detached from the culture dish will need to be examined along with cells attached to the culture dish.

Knockdown of PPL reduced cell adhesion activity

Although siRNA against PPL increased the G2/M phase population, the decrease in the number of cells grown on the culture dish was more remarkable (Figure 1C). Nonetheless, PPL siRNA did not increase apoptosis when this was assessed by an Apopercentage assay™ (Biocolor Ltd., Newtownabbey, Northern Ireland, UK; data not shown). How then should we explain the reduction in the number of attached cells? Possible explanations include cell growth suppression, cell cycle retardation, or detachment from the culture dish. Therefore, we next examined the effects of PPL downregulation on adhesion to the extracellular matrix (ECM) by an adhesion assay. Cells transfected with PPL siRNA decreased their attachment to ECM compared with controls (Figure 3A). In order to confirm this result, we performed an ethylenediaminetetraacetic acid (EDTA) assay in which EDTA was used to gently block adhesion of D562 cells to ECM during a 1-h incubation at 37°C. Interestingly, PPL siRNA-treated and control cells lost cell-cell and cell-substrate adhesion and changed from elongated to spherical shape (Figure 3B). However, 6 h after the release of EDTA, control cells recovered their ability to adhere to ECM and began to reproliferate (Additional file 1). In contrast, PPL siRNA-treated cells remained spherical and did not proliferate, suggesting that PPL siRNA significantly attenuated cellular adhesion to ECM (Additional file 2). These data suggest that knockdown of PPL decreases cellular adhesion to ECM in D562 cells.

Knockdown of PPL reduces cell migration activity

Cell migration is essential for the invasion and metastasis of cancer cells. Thus, to evaluate the effects of PPL downregulation, a wound-healing assay was also performed using D562 cells. PPL siRNA-treated cells showed a remarkable delay in wound closure compared with control cells (Figure 4A and 4B). This delay in wound closure could be caused by disturbance of cellular proliferation or migration. To discriminate between these possibilities, we used time-lapse and video microscopy after transfection of PPL siRNA to compare and record cellular movement and migration (Figure 4C and 4D). As expected, PPL siRNA-treated cells showed a significant decrease in motility and a shorter tracking migration-length than controls (Additional file 3 and Additional file 4). Moreover, we injected YES5 cells (low PPL expression) and D562 cells (high PPL expression) beneath the right thigh of nude mice and monitored subsequent tumor growth. No tumor growth was observed in YES5 mice, while some tumors were observed in D562 mice (Additional file 5). These results indicate that YES5 could not migrate into mice tissues

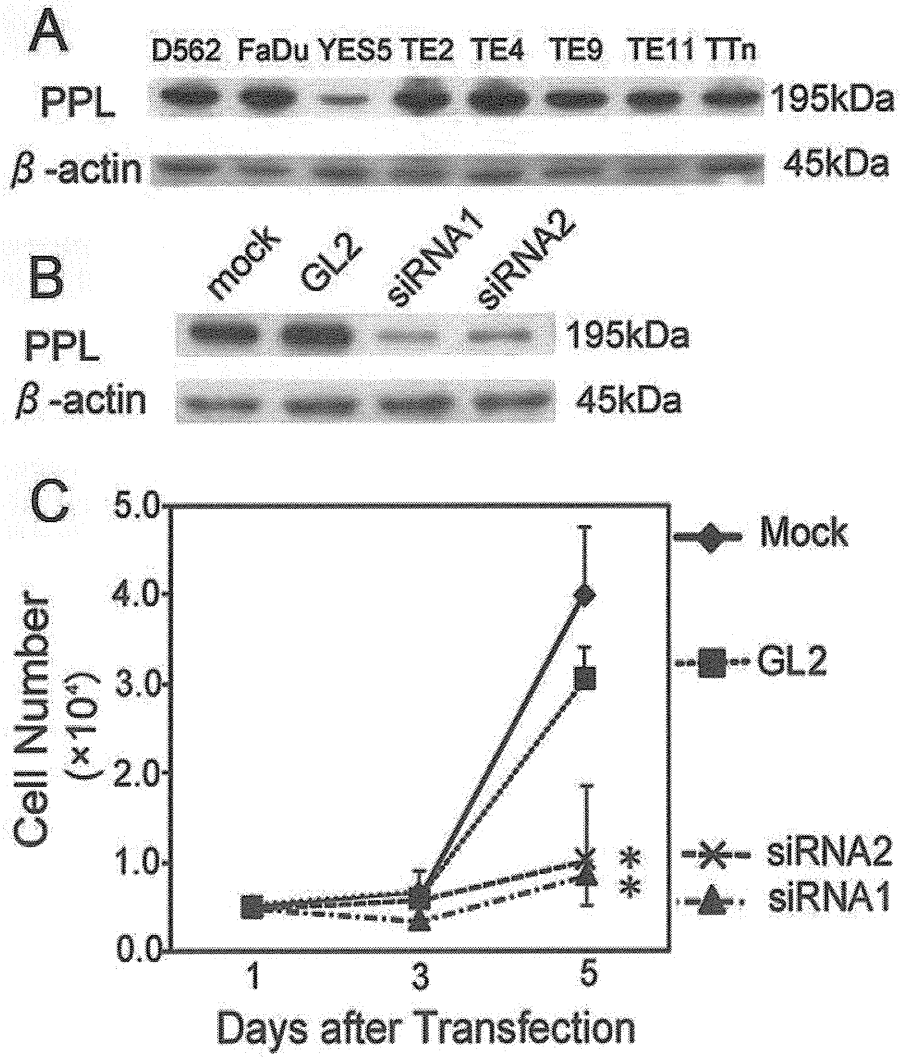
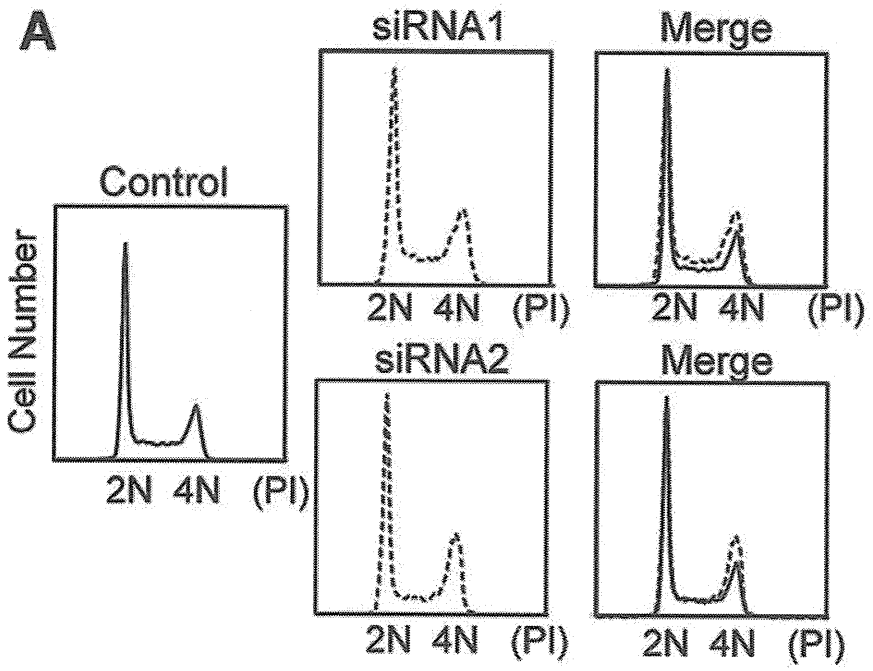


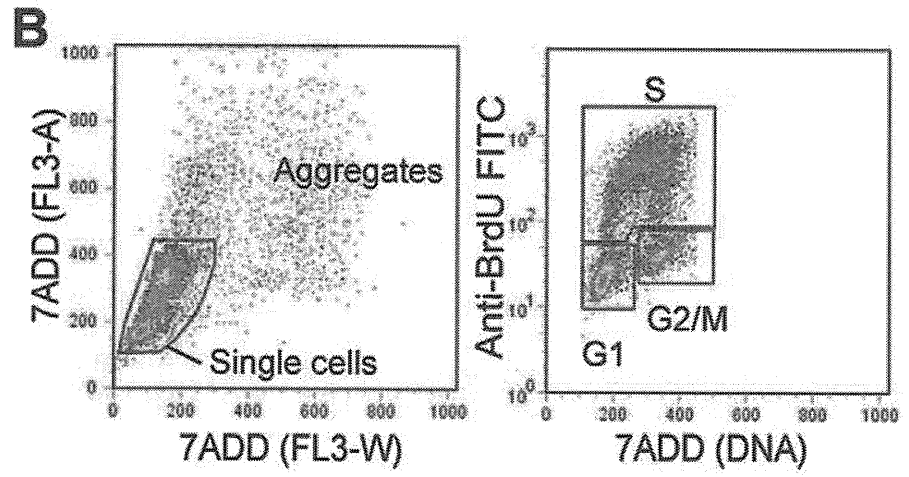
Figure 1 Downregulation of PPL by siRNA decreased the number of attached D562 cells. (A) PPL expression was detected in human pharyngeal (D562 and FaDu) or esophageal (YES5, TE2, TE4, TE9, TE11, and T.Tn) cell lines by western blotting. (B) siRNA1 and siRNA2 against PPL was shown in D562 cells. Total cell extracts were collected 72 h after siRNA treatment, and 5 μg protein aliquots were blotted with antibodies against the indicated proteins. siRNA (GL2) against firefly luciferase was used as a control. β-actin was the internal control. (C) Downregulation of PPL inhibited cell growth and decreased the number of attached cells. The number of viable cells was measured at different times by trypan blue exclusion. Values represent the mean ± S.E. from 3 separate experiments. Asterisks (*) indicate a p value less than 0.01 based on a t-test using Day 5 data.

owing to its slow proliferation or weak adhesiveness to ECM.

Knockdown of PPL decreases cellular migration via the PI3K/Akt axis

Finally, we examined changes in the expression of phospholipid signaling proteins that regulate cell migration because PPL has been reported to play a role as a scaffold in Akt/PKB signaling in human cancer cell lines [7]. The mitogen-activating protein kinase (MAPK) pathway was also examined because PI3K/Akt/PKB and the pathway are activated by G protein-coupled receptors (GPCRs) that induce signaling events [9-11], and PPL can also interact with a wide range of GPCRs and modulate their functions [12-14]. As expected, protein expression of pAktSer473 was drastically downregulated by PPL siRNA (Figure 4E). PPL also regulates keratin organization and epithelial migration, participating in the accumulation of keratin filaments to form thick cables [15,16]. In our study, PPL siRNA reduced not only the migration of assembled cells but also that of single D562 cells with pAktSer473 suppression. These data indicate that knockdown of PPL significantly decreases cellular migration in D562 cells, at least partly





C

	G1	S	G2/M
mock	39.6±4.20	45.1±3.17	12.9±1.25
GL2	40.6±4.50	44.2±4.70	12.9±1.29
siRNA1	39.9±3.79	42.7±4.23	15.1±1.53 *
siRNA2	38.6±2.04	43.4±2.49	16.0±1.65 *

Figure 2 siRNA against PPL delayed the cell cycle at the G2/M phase. D562 cells were transfected with control siRNA or siRNA 1 against PPL. (A) Downregulation of PPL delayed cell cycle progression during the S and G2/M phases. Cells were harvested after 72 h, fixed, and processed as described in "Materials and Methods." DNA content was measured by flow cytometry after propidium iodide staining. (B) (C) Flow cytometry analysis of cell cycle parameters was performed after double staining for BrdU incorporation (S phase cells) and 7-AAD (DNA content). To measure direct cell cycle distributions in cell cycle phases, exponentially proliferating cells were labeled with 10 μM BrdU for 1 h and processed for 2-color FACS, as described in "Materials and Methods." Values represent the mean ± S.E. from 5 separate experiments. Asterisks (*) indicate a p value less than 0.05 based on Student's t-test.

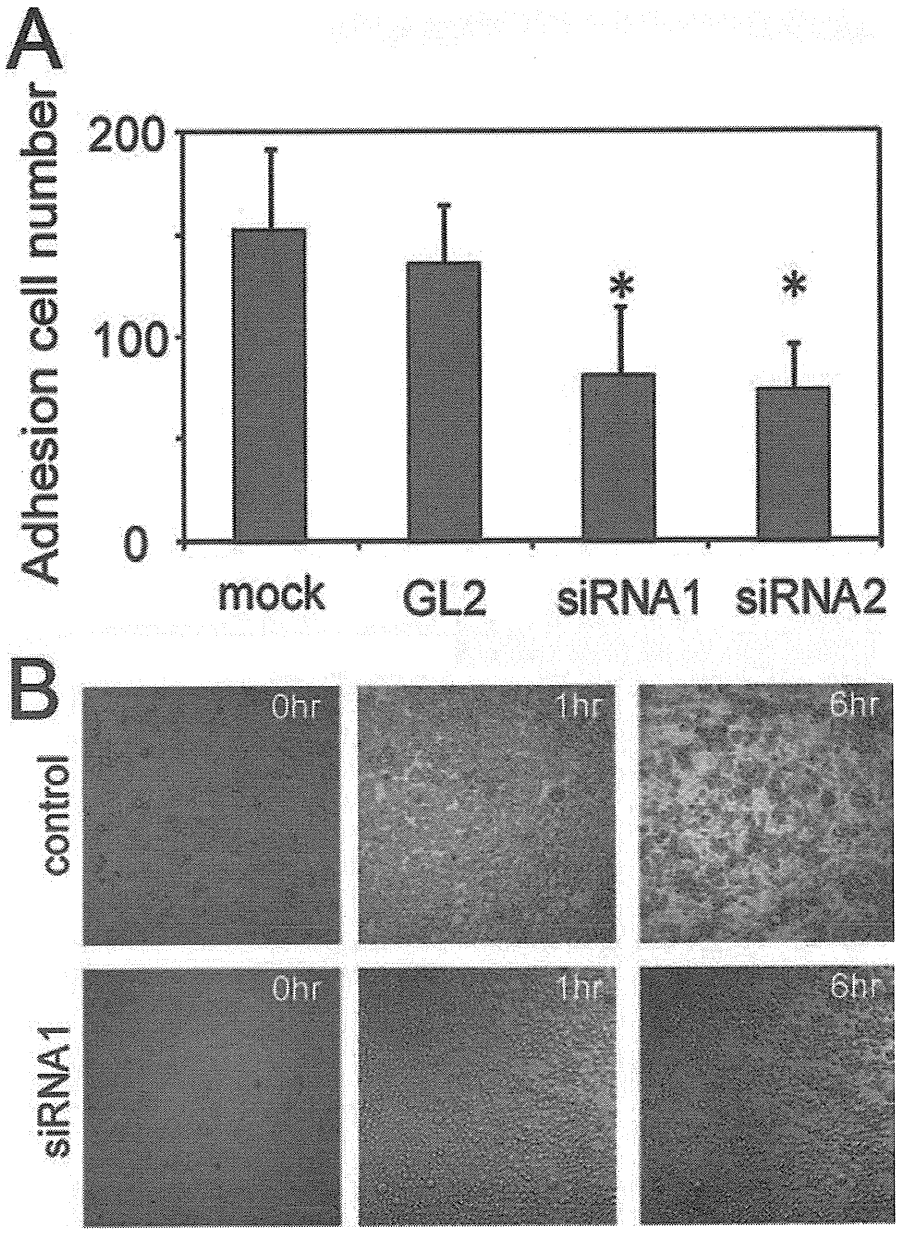


Figure 3 siRNA against PPL reduces adhesion to ECM in D562 cells. D562 cells were transfected with either control or PPL siRNA. (A) Effect of downregulation of PPL by siRNA on the adhesive characteristics of D562 cells. After 48 h, cells were trypsinized and 2×10^4 cells were resuspended in a 24-well plate coated with 200 μg of Matrigel. The plates were incubated at 37°C and adherent cells were counted. Four random fields were counted in 2 separate wells and the results were averaged. Values represent the mean ± S.E. from 3 separate experiments. Asterisks indicate a p value less than 0.05 based on Student's t-test. (B) Snapshot from a time-lapse recording of 2 transfected wells. D562 cells were transfected with either control siRNA (Additional file 1) or PPL siRNA (Additional file 2). Cells were grown to confluence in glass-bottomed dishes and EDTA (2.5 mM final concentration) was added. Changes in the morphology of the culture were recorded by live imaging. Time-lapse recordings were made every 5 min for 6 h. Bar: 200 μm.

via the PI3K/Akt axis. Recent reports have indicated that the PI3K/Akt axis is a central feature of EMT and that Akt-induced EMT involves downregulation of E-cadherin [8,17]. As stated above, our results also showed that siRNA against PPL suppressed pAktSer473 and reduced cellular attachment activity.

PPL expression was different among normal, atypical epithelium and cancer of the pharyngeal mucosa

If PPL expression relates to cellular migration, movement or invasion, PPL expression should be different among normal, atypical epithelium and cancers. Under this hypothesis, we immunohistochemically examined

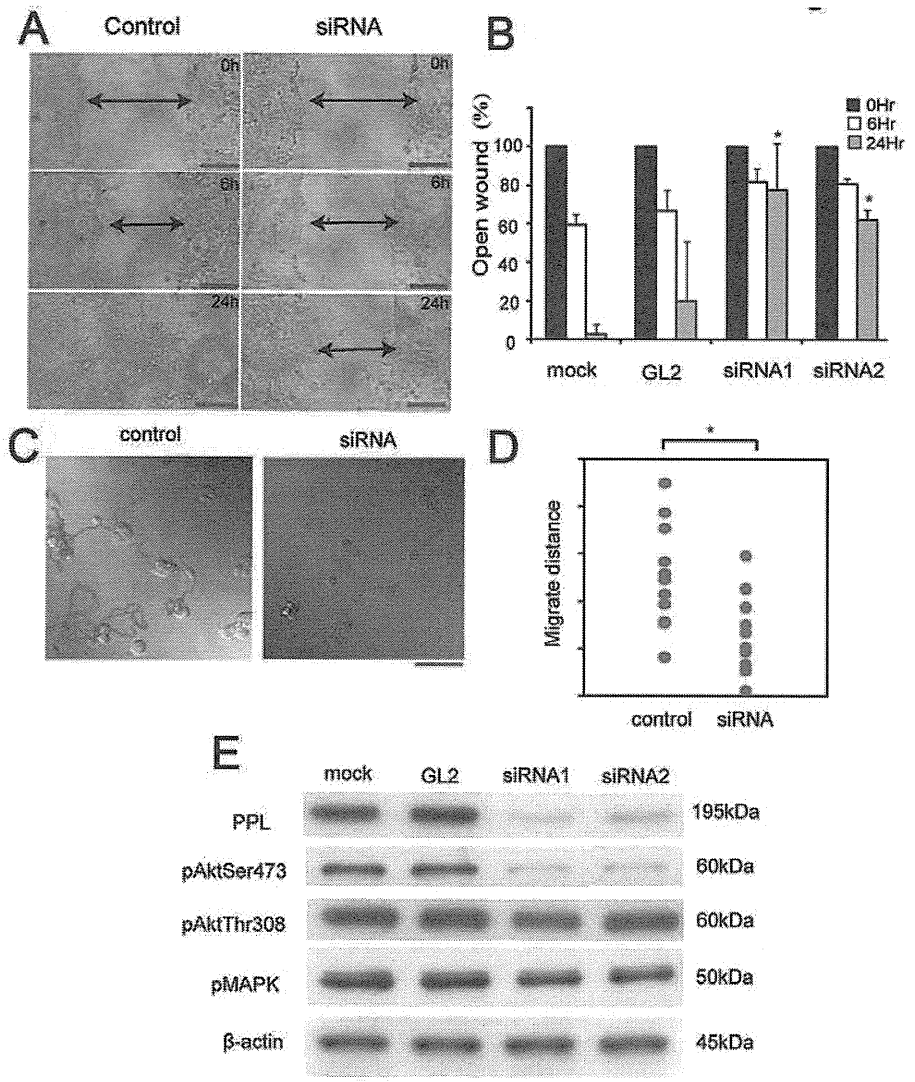


Figure 4 siRNA against PPL reduces cell migration in D562 cells. D562 cells were transfected with control and PPL siRNA. (A), (B) Quantification of wound closure in control and PPL siRNA. Each cell was grown to confluence and then wounded by scraping. For each wound, the width of the wound at 0 h was designated as 100%, and the subsequent timepoints in the graph show the relative width of the open wound. Values represent the mean ± S.E. from 3 separate experiments. Asterisks indicate a p value less than 0.01 based on Student's t-test. Bar: 200 μm. (C), (D) Paths of 2 transfected cells from a time-lapse recording. D562 cells were transfected with either control or PPL siRNA. After 24 h, cells were seeded on glass-bottomed dishes and incubated for another 24 h. Time-lapse recordings were made every 5 min for 24 h. Migration distance was measured using 10 control and 10 PPL siRNA-transfected cells. Bar: 100 μm. Asterisks indicate a p value less than 0.05 based on Student's t-test. (E) Downregulation of PPL resulted in reduced pAkt expression. The expression of PPL and β-actin was analyzed by western blotting with total cell extracts from transient transfections with control siRNA (GL2) and PPL siRNA 1 and 2.

PPL expression in excised human normal, atypical epithelium and in cancer of the pharyngeal mucosa (Figure 5). Interestingly, PPL was expressed in the upper layer of the squamous epithelium in normal hypopharyngeal mucosa (Figure 5A, arrows), whereas PPL expression was rare in atypical hypopharyngeal epithelium (Figure 5B, arrows). Note that in both the non-keratinized (Figure 5C, asterisk) and keratinized cancer areas (Figure 5C, arrows), PPL expression was scarce. Further experiments are needed to determine how PPL relates to EMT, including investigations of whether PPL knockdown affects the expression of mesenchymal markers, such as E-cahderin, fibronectin and/or N-cadherin.

Discussion

In this study, we revealed that siRNA against PPL significantly reduced the cellular motility and adhesion activity. In addition, PPL downregulation significantly