

RNA interference against granulin-epithelin precursor prevents hepatocellular carcinoma growth: Its application as a therapeutic agent

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Abstract. Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world. However, little is known regarding the molecular mechanism of HCC development and progression and effective therapeutic methods. Recently, the granulin-epithelin precursor (GEP) was reported as a novel growth factor that can control HCC cell proliferation. Using the CAPSID program, we designed three small interfering RNAs (siRNAs) targeting the *GEP* gene (GEP-siRNA1, 2 and 3) and examined their tumor regression and suppression effects on cell proliferation. GEP-siRNA1 exhibited the strongest anti-proliferative effect among the GEP-siRNAs, in a time-dependent manner. To increase the biostability of the siRNA, we also constructed a short hairpin RNA (shRNA) using an H1/TO promoter with the same sequence of GEP-siRNA1 (GEP-shRNA). GEP-shRNA decreased the expression levels of GEP and tumor cell growth via cell cycle arrest at the G2/M stage and down-regulation of the cell proliferation proteins cyclin D1 and α -tubulin. Furthermore, GEP-shRNA inhibited tumor growth significantly after intratumoral injection into tumor-bearing Balb/C nude mice. Taken together, these results represent the first therapeutic application of RNA interference to GEP, which is a promising target molecule for HCC treatment, as an approach for the suppression of HCC cell proliferation.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignant liver cancer in adults and is a highly prevalent disease worldwide, with most patients presenting a highly advanced disease status and thus poor prognosis (1). HCC is also known as the third most common cause of cancer-related deaths among patients with cirrhosis in Europe and the USA (2-5). The most relevant risk factor for HCC is liver injury from diverse causes, which induces hepatic cirrhosis in most patients; these causes include chronic hepatitis C (HCV) infection, hepatitis B (HBV) infection, alcoholic cirrhosis, and nonalcoholic steatohepatitis (6). Furthermore, the increasing incidence of obesity and diabetes, which was also identified as an independent risk factor for chronic liver disease and HCC, is becoming a major issue in the treatment of HCC (7). Despite advances in many aspects of HCC treatment, including liver transplantation and surgical resection, over two-thirds of HCC patients present with advanced disease and do not benefit from these treatment modalities (7). To date, surgical liver transplantation is considered the only curative treatment for HCC and only one chemotherapeutic agent is approved by the United States Food and Drug Administration for the treatment of advanced HCC (8). However, most HCC patients are not candidates for liver transplantation or surgical resection because of their advanced disease status (9) and the majority of HCC patients represents a significant medical need for more effective systemic therapy options. Furthermore, as HCC tumor tissues have high chemoresistance, systemic chemotherapy is not a promising treatment. In addition, the molecular pathogenesis of HCC remains poorly understood and its clinical course varies widely (10). Thus, many recent investigations have indicated that molecularly targeted therapies are an encouraging trend in the management of HCC (10,11).

The granulin-epithelin precursor (GEP), also termed progranulin, acrogranin, and PC-derived growth factor (PCDGF), is a 68.5-kDa pluripotent glycoprotein and an obscure cysteine-rich novel growth factor (12). GEP is structurally composed of seven and one-half granulin/epithelin module (GEM) repeats (12). The GEMs are separated by a 6-kDa peptide fragment and are made up of four β hairpins stacked onto each other in a twisted ladder-

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Abbreviations: HCC, hepatocellular carcinoma; GEP, granulin-epithelin precursor; siRNA, small interfering RNA; shRNA, short hairpin RNA; RNAi, RNA interference; HCV, hepatitis C virus; HBV, hepatitis B virus; PCDGF, PC cell-derived growth factor; GEM, granulin-epithelin module; CAPSID, convenient application program for siRNA design

Key words: HCC, granulin-epithelin precursor, siRNA, shRNA, cell proliferation protein

like formation (13). Generally, GEP affects many biological features, such as development, cell growth and proliferation, host defense, and wound healing (14). In addition, overexpression of GEP is associated with cancer progression (15). Strong GEP expression is highly associated with HCC growth with a large tumor size and GEP is considered as an important factor in hepatocarcinogenesis and a promising factor that can be used as a tumor marker or as a therapeutic target molecule (15).

After the first report of strong GEP expression from HCC tissues, antisense GEP and a monoclonal antibody to GEP were used to block the expression of GEP in an HCC mouse model (15,16), which demonstrated that GEP agents can be used as safe anti-HCC therapies that delay tumor growth. However, the effect of antisense GEP was not so effective in a highly overexpressed GEP system, which suggests that this mechanism is only effective for intermediate-to-low levels of GEP expression (15,16).

Recently, the examination of RNA interference (RNAi) using small interfering RNA (siRNA) and short hairpin RNA (shRNA) in a preclinical cancer study yielded promising results, including an efficient inhibitory effect on tumor angiogenesis, tumor cell growth, tumor invasion, and chemoresistance (17,18). Therefore, we designed three different types of siRNAs (GEP-siRNAs) and one shRNA (GEP-shRNA) against GEP using the CAPSID (Convenient Application Program for siRNA Design) software. The cell-growth inhibitory effect of the siRNA and shRNA molecules constructed in this study was tested on HCC. They induced the inhibition of tumor-cell proliferation and a decrease in tumor size via cell-cycle arrest at the G2/M stage. In conclusion, GEP-siRNA and GEP-shRNA seem to be effective therapeutic agents for HCC.

Materials and methods

Cell culture and GEP cloning. The HCC cell lines Hep3B, Huh7, and skHep1 were cultured in Dulbecco's modified Eagle's medium (Hyclone, Rockford, IL, USA) supplemented with heat-inactivated 10% fetal bovine serum (Hyclone), 50 U/ml penicillin, and 50 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were maintained at 37°C in a humidified chamber with 5% CO₂. For the cloning of *GEP*, the full-length *GEP* cDNA cloned in pAcGP67-A (provided by the 21C Frontier Human Gene Bank, Daejeon, Republic of Korea) was used as a template for the fusion of *GEP* and human *IgG FC* (which was used as a tag) into pcDNA3.1(+) (pcDNA-FC-GEP) (Invitrogen). The pcDNA-FC-GEP was obtained from the subcloning of full-length *GEP* using *Hind*III and *Xho*I enzymatic digestion. The detected plasmid clone is shown in Fig. 1.

Design of siRNAs and shRNA. The cDNA sequence of *GEP* was obtained from GenBank (access number BC010577). Three short interfering RNAs (siRNAs) targeting *GEP*, GEP-siRNA1 (5'-AAGGACACUUCUGCCAUGAUAC-3'), GEP-siRNA2 (5'-AAGAGAGAUGUCCCCUGUGAUAA-3'), and GEP-siRNA3 (5'-AAUCUAAGGCCUUCUCCCCUGUCAGA-3'), were designed using the CAPSID program (<http://cms.ulsan.ac.kr/capsid>). The HCC cell lines were used to examine the interfering effect of the three siRNA duplexes. GEP-shRNA containing GEP-siRNA1 sequences was constructed using an H1/TO promoter. A luciferase-gene-specific shRNA (Luciferase-shRNA) obtained from Genolution Pharmaceuticals Inc. (Seoul,

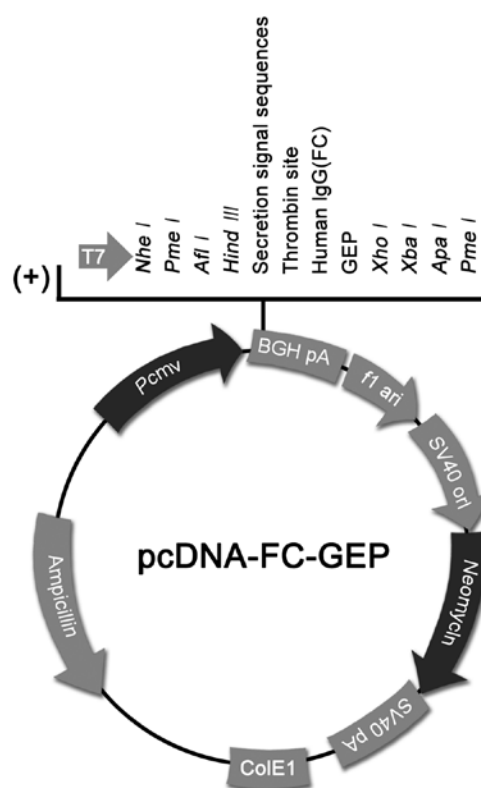


Figure 1. Construction of pcDNA-FC-GEP.

Republic of Korea) was used as a negative control. Transfection of the GEP-siRNAs and GEP-shRNA was performed using a Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Human IgG ELISA. The level of human IgG FC expression measured by an ELISA kit (Koma Biotech, Seoul, Republic of Korea) was used to monitor the GEP expression in the supernatants of Hep3B cells transfected with pcDNA-FC-GEP.

Cell proliferation and viability assay. A cell proliferation assay was performed to count viable cells using a hemacytometer. HCC cells grown in 24-well plates (1x10⁴ cells/well) were transfected with 2 µg of GEP-shRNA or Luciferase-shRNA using Lipofectamine 2000. Cells were harvested 1, 3, and 5 days after transfection and viable cells were counted by trypan blue exclusion and counted using a hemacytometer. In addition, cell viability was assessed by measuring the optical density at 490 nm using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Real-time RT-PCR. Total RNA was extracted from Hep3B cells using TRIzol (Invitrogen). Total RNA samples (2 µg) were used in real-time RT-PCR experiments. First-strand cDNA was synthesized using Oligo-dT and RT&GO™ (Q-Biogene Inc., Montreal, Canada) according to the manufacturer's instructions. The primer pairs used for the amplification of specific cDNAs were as follows: GEP, 5'-TCCACGTGCTGTGTTATGGT-3' (sense) and 5'-CTGCCCTGTTAGTCCTCTGG-3' (antisense); 18S rRNA, 5'-AACTTTCGATGGTAGTCGCCG-3' (sense)

and 5'-GGATGTGGTAGCCGTTTCTCA-3' (antisense). Real-time RT-PCR amplification mixtures (25 μ l) contained 500 ng of template cDNA, 12.5 μ l of SYBR Green I Master Mix buffer (Applied Biosystems, Foster City, CA, USA), and 1 μ l of forward and reverse primers. The MyiQ Single-Color Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used to perform real-time quantitative RT-PCR.

Tumorigenicity assay using tumor-bearing Balb/C nude mice. Male Balb/C mice (4 weeks old) were obtained from Orient Bio Inc. (Seongnam, Republic of Korea), housed in special pathogen-free units, and maintained in the animal facility at the Catholic University according to the institutional guidelines. An *in vivo* tumorigenesis assay performed to assess the effect of the transfectants was modified from the previously reported method (19,20). In brief, Hep3B cells (1×10^7 cells) were injected subcutaneously into the femoral region of mice. Tumor formation was confirmed after 4 weeks of injection. All tumor-bearing mice were divided into two groups, one injected with GEP-shRNA and the other with Luciferase-shRNA (which was used as a negative-control group). Mice were administered intratumoral injections of 30 μ g of GEP-shRNA in 30 μ l of PBS or 30 μ g of Luciferase-shRNA in 30 μ l of PBS every 4 days. Tumor dimensions after transfection were measured every day for 31 days using a digital caliper and tumor volume was calculated using the formula $V = \pi/6 \times \text{larger diameter} \times \text{smaller diameter}^2$ (2,19,20).

Western blot assay. Total protein was extracted from transfected Hep3B cells using M-PER buffer (Pierce, Rockford, IL, USA) containing Protease Inhibitor Cocktail™ (Roche Molecular Biochemicals, Indianapolis, IN, USA). Protein concentrations were determined using the BCA protein assay kit (Sigma-Aldrich Corp., St. Louis, MO, USA). Equal quantities of protein (30 μ g) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and were electrophoretically transferred onto a nitrocellulose membrane. The blots were probed with the appropriate antibodies against GEP (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin D1 (1:2,000, Santa Cruz Biotechnology), α -tubulin (1:4,000, Abfrontier, Seoul, Republic of Korea), and β -actin (1:4,000, Cell Signaling Technology, Danvers, MA, USA), followed by incubation with the corresponding secondary antibodies, and were developed using an ECL solution (Amersham Biosciences, Piscataway, NJ, USA). The level of β -actin expression was used as an internal control. The band densities were quantified using the ImageJ program (National Institute of Health, Bethesda, MD, USA).

Cell cycle analysis. Cell cycle analysis was performed using a flow cytometer. Hep3B cells were transfected with GEP-shRNA and Luciferase-shRNA using Lipofectamine 2000. Five days after transfection, cultures were trypsinized and centrifuged to collect the cells. Collected cells were then stained using propidium iodide (40 μ g/ml; Sigma) in PBS after washing twice with PBS. Cell cycle data were collected using a Beckman Coulter FC500 instrument (Beckman Coulter Inc., Brea, CA, USA).

Statistical analysis. The significance of differences between values was determined using the Student's t-test with the SAS 9.1 software (SAS Institute Inc., Cary, NC, USA). All data are

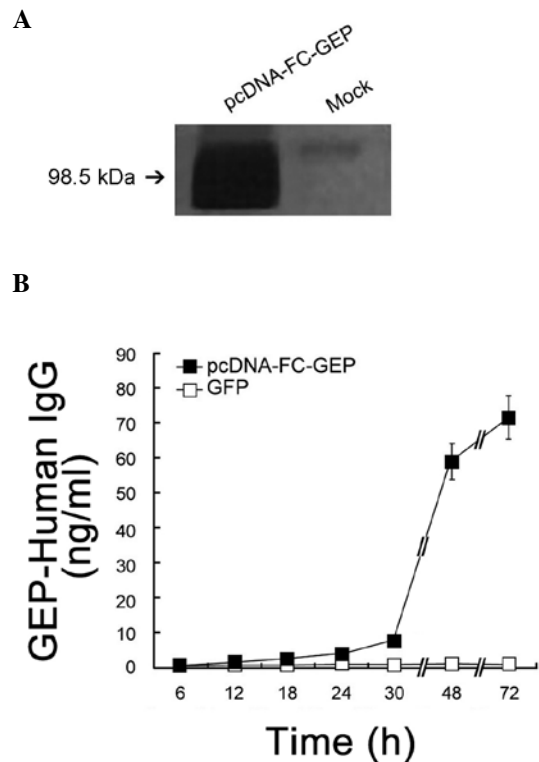


Figure 2. Expression of FC-tagged GEP in Hep3B cells. (A), Western blot analysis using an anti-GEP antibody to detect the 98.5 kDa band of the FC-tagged GEP protein in pcDNA-FC-GEP-transfected Hep3B cells. Non-transfected Hep3B cells were used as a mock. (B), The level of secreted FC in pcDNA-FC-GEP-transfected Hep3B culture medium, which is representative of the level of GEP expression because FC was fused to GEP, was detected using ELISA. GFP-transfected cells (GFP) were used as a control group.

expressed as the mean \pm SD of triplicates. p-values <0.05 were considered statistically significant.

Results

Detection of FC-tagged GEP secretion. Transfection of 2 μ g of pcDNA-FC-GEP into Hep3B cells using Lipofectamine 2000 led to the expression of a 98.5-kDa FC-GEP protein compared with non-GEP-transfected Hep3B cells (mock) (Fig. 2A). We also confirmed the secretion of GEP in the supernatant of pcDNA-FC-GEP-transfected Hep3B cells using an ELISA, which can detect the amount of FC fused with GEP. The expression of the GEP protein increased gradually after 24 h of transfection (up to 72 h), with a dramatic increase at 48 h after pcDNA-FC-GEP transfection compared with in GFP-transfected Hep3B cells (Fig. 2B).

Design of three GEP-siRNAs and their inhibitory effect on GEP expression in Hep3B cells. Three structurally different GEP-siRNAs were designed using the CAPSID program and were used to check the applicability of RNAi as a therapeutic agent for HCC. The three regions of the GEP-siRNAs were designed using GEP sequences encoding stem-loop structures, which suggest the presence of biologically active regions (Fig. 3A). We compared the GEP-suppression effect of all three GEP-siRNAs to select the most effective RNA interfering sequence. The three GEP-siRNAs yielded a similar GEP-suppression effect at 1 day after transfection. However,

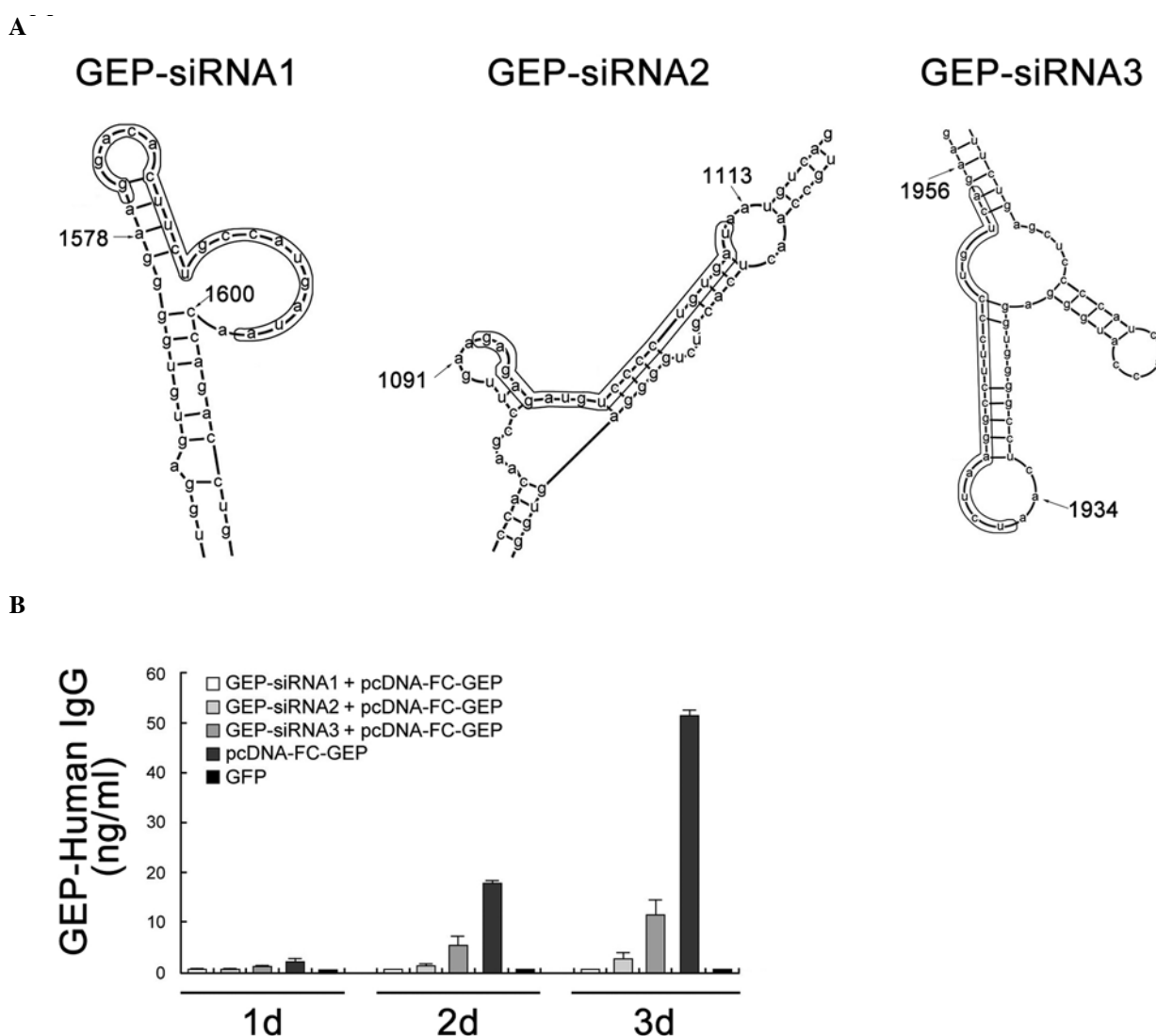


Figure 3. Design of GEP small interference RNAs (GEP-siRNAs) using the CAPSID program and their inhibitory effect on GEP expression in GEP-siRNA-transfected Hep3B cells. (A), Three different structures of GEP-siRNAs designed using CAPSID. The box in stem-loop structures indicates the sequences selected for the siRNAs against GEP. (B), GEP-siRNAs suppressed GEP secretion, as measured using ELISA. GEP-siRNA1 yielded the most significant GEP-suppression effect in Hep3B cells, in a time-dependent manner, compared with GEP-siRNA2 and GEP-siRNA3. All groups were cotransfected with pcDNA-FC-GEP (2 μ g) and GEP-siRNAs (100 pmol). GFP transfection was used as a negative control.

compared with GEP-siRNA1, GEP-siRNA2 and GEP-siRNA3 exhibited a weak GEP-suppression effect 2 and 3 days after transfection, respectively (Fig. 3B). These results led us to select GEP-siRNA1 as the most effective GEP-suppressing agent among the three siRNAs.

GEP-shRNA effectively suppressed GEP expression in Hep3B cells. To increase the stability and handling efficiency of the agent for the application both in *in vitro* and *in vivo* experiments, an shRNA plasmid containing the GEP-siRNA1 sequence (termed GEP-shRNA) was constructed. To investigate the GEP-silencing effect of GEP-shRNA in Hep3B cells, four different components, GEP-shRNA, GEP-siRNA1, pcDNA-FC-GEP, and GFP (which was used as a mock), were transfected into Hep3B cells, individually. Using an ELISA assay to detect FC levels secreted to the cell culture medium, we confirmed the presence of a GEP-suppression effect in GEP-shRNA-transfected cells that was similar to that observed in GEP-siRNA1-transfected cells (Fig. 4A). In addition, over 0.5 μ g of GEP-shRNA was sufficient

to achieve suppression of GEP expression (Fig. 4B). Moreover, GEP-shRNA down-regulated the level of the endogenous GEP mRNA in Hep3B cells by 74% ($74 \pm 4\%$, $p < 0.05$) (Fig. 4C).

GEP-shRNA-mediated growth inhibition and cell cycle arrest in Hep3B cells. To test the cell growth-inhibition effect of GEP-shRNA, we measured cell proliferation using a hemacytometer and analyzed cell viability using a commercially available kit to detect the amount of viable GEP-shRNA-transfected cells in three different HCC cell lines (Hep3B, Huh7, and skHep1 cells). At 1, 3, and 5 days after transfection, the number of GEP-shRNA-transfected cells counted using trypan blue staining was significantly decreased compared with pcDNA-FC-GEP-transfected cells ($p < 0.01$ in Hep3B, $p < 0.001$ in Huh7, and $p < 0.01$ in skHep1 cells) (Fig. 5A). Moreover, a cell viability determined by an OD values yielded similar results with the cell-counting data shown in Fig. 5A (Fig. 5B). Interestingly, cell cycle analysis showed significant differences in the number of GEP-shRNA-transfected Hep3B cells of

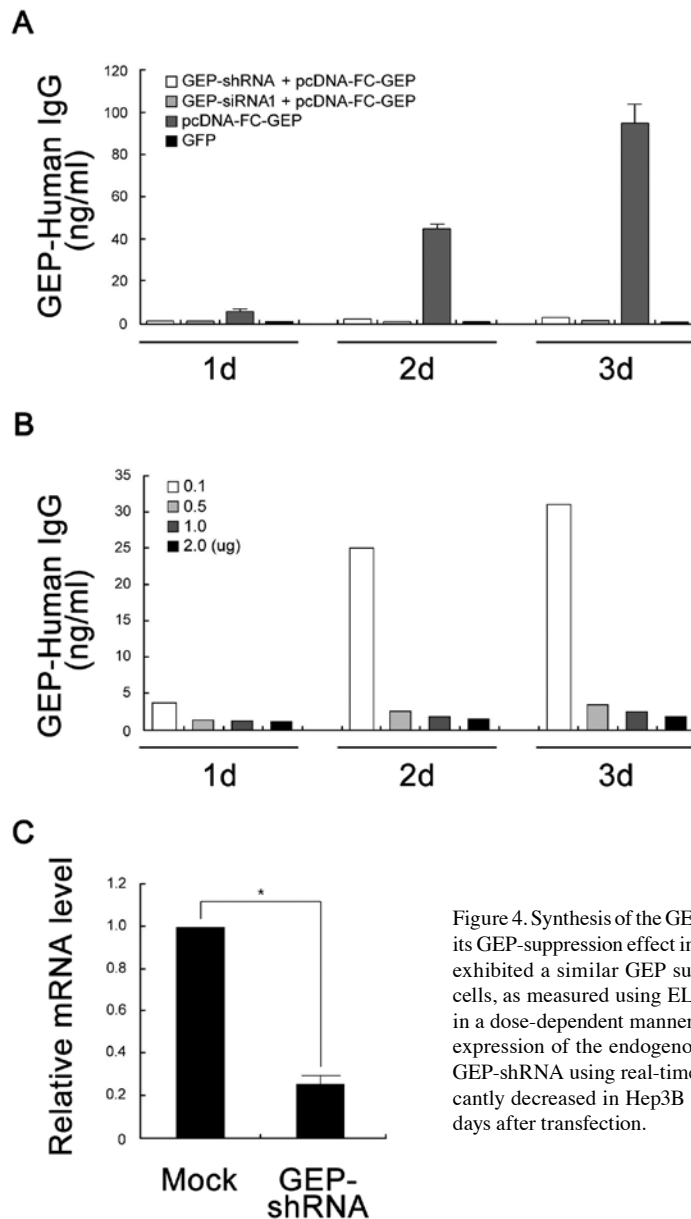


Figure 4. Synthesis of the GEP-shRNA containing GEP-siRNA1 sequences and its GEP-suppression effect in Hep3B cells. (A), GEP-shRNA and GEP-siRNA1 exhibited a similar GEP suppression effect in pcDNA-FC-GEP-transfected cells, as measured using ELISA. (B), The expression of GEP was suppressed in a dose-dependent manner by GEP-shRNA. (C), The analysis of the level of expression of the endogenous GEP mRNA in Hep3B cells transfected with GEP-shRNA using real-time RT-PCR revealed that this molecule was significantly decreased in Hep3B cells (* $p < 0.05$) after GEP-shRNA transfection. d, days after transfection.

their G0/G1, S, and G2/M phases compared with the control cells (Luciferase-shRNA-transfected Hep3B cells); namely, compared with the control group, significantly small number of GEP-shRNA-transfected cells were in the G0/G1 phase ($58.16 \pm 1.08\%$ vs 69.54%) and significantly large number of GEP-shRNA-transfected cells were in the S phase ($20.64 \pm 1.92\%$ vs 15.67%) and in the G2/M phase ($19.82 \pm 1.92\%$ vs 14.25%) (Fig. 6A and B). These cell cycle data suggest that GEP-shRNA arrests the cell cycle at the G2/M phase.

Expression levels of cyclin D1 and α -tubulin in Hep3B cells.

The expression level of the cell-proliferative proteins, α -tubulin and cyclin D1, was detected using Western blot analysis. As shown in Fig. 6C and D, the level of expression of these two proteins was significantly decreased in GEP-shRNA-transfected cells compared with the Luciferase-shRNA-transfected Hep3B cells. These results indicate that GEP-shRNA suppresses the expression of cell-proliferative proteins, thereby reducing the cell proliferation.

Regression of tumor volume by GEP-shRNA injection into tumor-bearing Balb/C nude mice. As shown in Fig. 7A, tumors continued to grow in control mice injected with Luciferase-shRNA and the maximum mass of excised tumors reached $1,373 \pm 250 \text{ mm}^3$ at 31 days after injection. In contrast, the volume of the tumors in the GEP-shRNA-treated group was significantly smaller than that detected in the Luciferase-shRNA-treated group ($p < 0.05$) (Fig. 7B and C). Therefore, the down-regulation of GEP expression by GEP-shRNA can be a promising therapeutic treatment for HCC.

Discussion

HCC is a highly proliferative and vascularized tumor and its progress is closely related to angiogenesis and vascular remodeling (21). At present, a humanized anti-VEGF monoclonal antibody (22) and Sorafenib (23), a small tyrosine kinase inhibitor targeting VEGF receptors, are under consideration as efficient HCC chemotherapeutic agents, as they reduce angiogenesis,

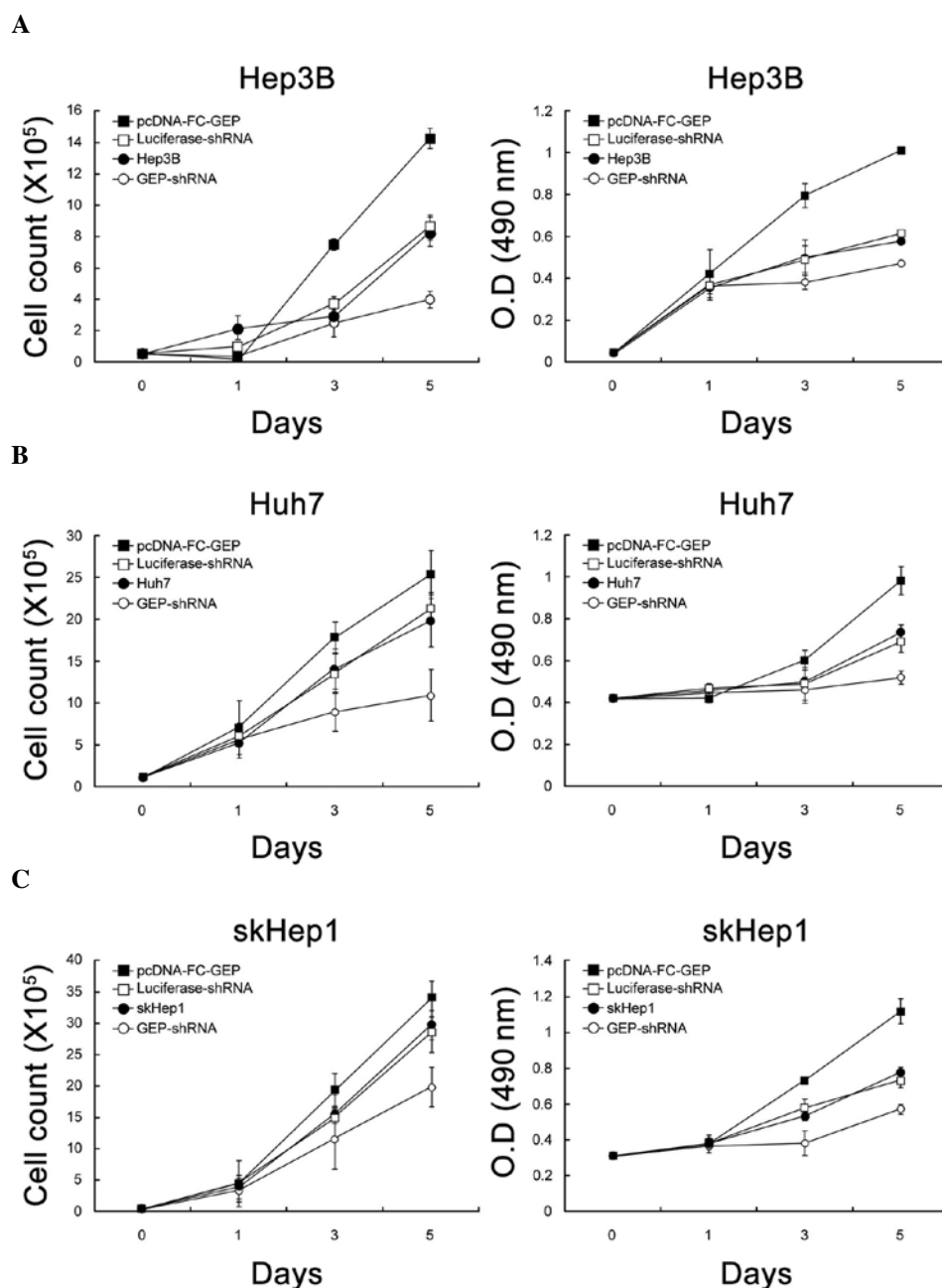


Figure 5. Suppression of GEP expression by GEP-shRNA led to a reduction of the proliferation and viability of HCC cell lines. (A), A cell-proliferation assay was performed at 1, 3, and 5 days after transfection by counting cells using trypan blue staining. (B), Cell viability was measured at 1, 3, and 5 days after GEP-shRNA transfection using an MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). All experiments were performed independently three times. pcDNA-FC-GEP was transfected as a positive control. An shRNA targeting the luciferase gene (Luciferase-shRNA) was used as a negative control. GEP-shRNA, HCC cells transfected with GEP-shRNA; Hep3B, Huh7, and skHep1, non-treated HCC cells used in mock experiments.

thus reducing cancer cell proliferation in HCC tumorigenesis. However, no significant increase in survival rate or therapeutic effect was demonstrated during those trials (24). Another study reported that Sorafenib improves the median survival time of patients diagnosed with advanced HCC by nearly 3 months (8); however, severe side effects such as significant risk of bleeding, were also reported (6). HCC is relatively resistant to systemic therapy (25). RNAi methods can be used to overcome this chemotherapeutic resistance, as they use a naturally occurring mechanism of sequence-specific gene-expression silencing (26). Furthermore, the novel finding of GEP overexpression in highly proliferative HCC tumor tissues provides a promising

therapeutic target for HCC via the reduction of cell proliferation (15,16). A growing number of studies dedicated to GEP report this molecule as a promising target molecule to cure HCC.

In this study, we examined the GEP-suppression effect of three different types of GEP-siRNAs and one shRNA. Specifically, the GEP-shRNA designed using the GEP-siRNA1 sequences yielded a decrease in the level of expression of the endogenous GEP mRNA and in tumor cell growth via the reduction of the cell-proliferative proteins α -tubulin and cyclin D1 in GEP-shRNA-transfected Hep3B cells (Figs. 4-6). Moreover, GEP-shRNA yielded a significant regression of tumor volume in tumor-bearing Balb/C mice (Fig. 7).

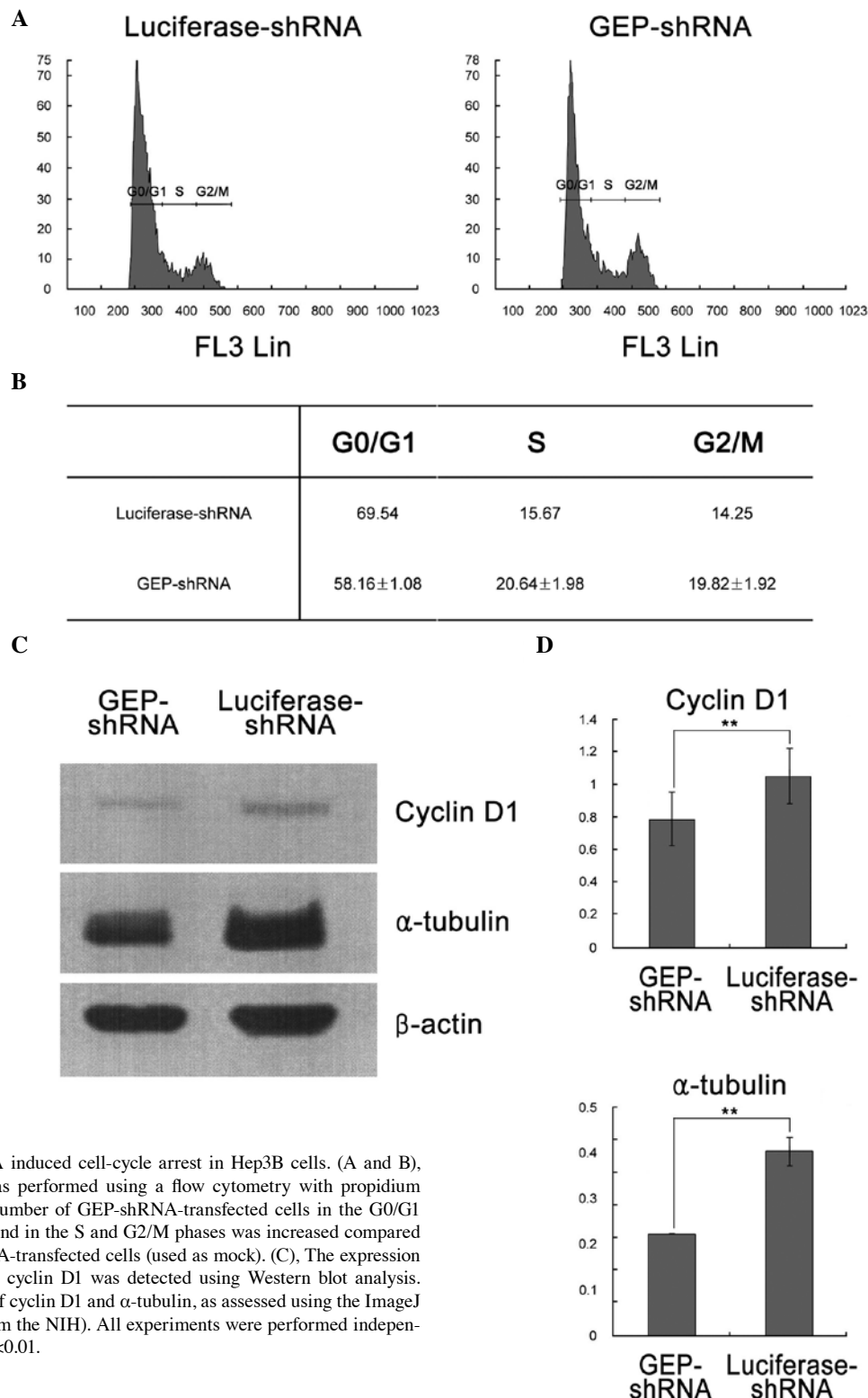


Figure 6. GEP-shRNA induced cell-cycle arrest in Hep3B cells. (A and B), Cell-cycle analysis was performed using a flow cytometry with propidium iodide staining. The number of GEP-shRNA-transfected cells in the G0/G1 phase was decreased and in the S and G2/M phases was increased compared with Luciferase-shRNA-transfected cells (used as mock). (C), The expression level of α -tubulin and cyclin D1 was detected using Western blot analysis. (D), Relative amount of cyclin D1 and α -tubulin, as assessed using the ImageJ program (obtained from the NIH). All experiments were performed independently three times. ** $p < 0.01$.

All GEP-siRNAs analyzed in this study yielded an inhibitory effect on GEP secretion, as assessed using an ELISA assay, because of the presence of tight stem-loop structures in all siRNA sequences (Fig. 3). A previous report showed that siRNA sequences located in stem-loop structures of RNA may be more functional during mRNA suppression (27). Our result (Fig. 3) showing that GEP-siRNA1 exhibited the strongest inhibitory effect on GEP secretion in Hep3B cells indicates that the GEP-siRNA1 oligonucleotide has a specific sequence

that is more favorable for the blocking GEP expression. The GEP-shRNA designed using the sequence of GEP-siRNA1 yielded a similar blocking effect of GEP secretion (Fig. 4). GEP-shRNA transfection also demonstrated that GEP is positively associated with cell proliferation (Fig. 5), which is in accordance with the GEP-expression-blocking pattern reported by Cheung *et al* (15). Therefore, we concluded that the proliferation of HCC cells was markedly inhibited by GEP-shRNA. Furthermore, compared with previous results showing limited

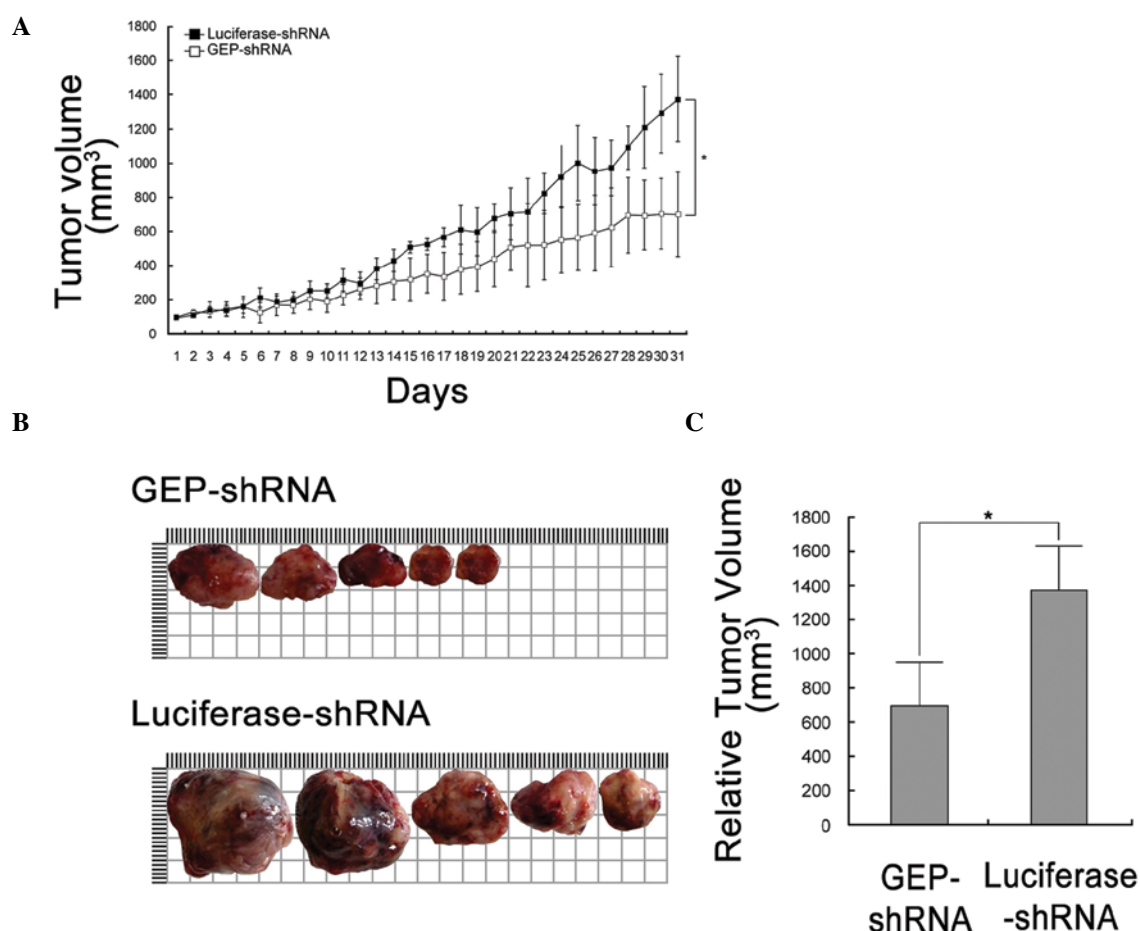


Figure 7. Tumor-suppressing effect of GEP-shRNA in a tumor-bearing mouse model. (A), The suppression of GEP expression by GEP-shRNA led to a decrease in tumor volume in a Hep3B xenograft mouse model for 31 days. Hep3B cells were inoculated subcutaneously into the femoral region. Tumor volume was measured using a digital caliper. The GEP-shRNA-injected group (five mice) showed a significant delay of tumor growth compared with the Luciferase-shRNA-injected group (five mice). (B), Tumors extracted at 31 days after transfection. (C), The relative tumor volume observed 31 days after GEP-shRNA transfection was significantly decreased compared with that of the Luciferase-shRNA-transfected group (used as mock; * $p < 0.05$).

regression in cell proliferation after the use of antisense GEP (15), the concentration of 0.5 μ g of GEP-shRNA can be considered as an intermediate-to-moderate level of treatment to achieve regression of cell proliferation (Fig. 4).

The cell cycle analysis led to the detection of a significant difference in the number of GEP-shRNA-transfected Hep3B cells in the G0/G1, G2/M, and S phases compared with control cells (Fig. 6B). The number of GEP-shRNA-transfected cells in the G2/M and S phases was increased and in the G0/G1 phase was decreased compared with the control group. We also investigated cell proliferation and cell cycle-related protein expression in Hep3B cells. Interestingly, we found that the α -tubulin and cyclin D1 proteins were down-regulated in GEP-shRNA-transfected Hep3B cells (Fig. 6C and D). α -tubulin plays a pivotal role in the G2 phase of the cell cycle (28). Consequently, the dysfunction or dysregulation of α -tubulin protein can lead to a G2 cell-cycle arrest (29). Moreover, it was previously reported that cyclin D1 is a key regulator of cell proliferation (30). Therefore, the reduction of GEP could arrest the cell cycle via the down-regulation of cyclin D1 and α -tubulin. Taken together, our data imply that the reduction of GEP may prevent HCC growth via tumor cell cycle arrest.

GEP overexpression is closely associated with HCC development, e.g., in terms of large tumor size, which is related with

a late stage of HCC (31). In this work, the tumorigenic effect of GEP was tested using a tumor-bearing athymic nude mouse model. We found that the treatment with GEP-shRNA led to a comparable reduction of tumor volume (1.9-fold decrease compared with that of observed in Luciferase-shRNA-treated mice; Fig. 7) after 31 days of GEP-shRNA treatment, which shows a similar antitumorigenic effect observed in mice treated with antisense GEP and anti-GEP monoclonal antibody for the management of HCC (15,16).

However, still improvement of the GEP-shRNA is required for the clinical applications including the development of an effective delivery system of shRNA into tumors. To overcome this problem, we are developing a new viral vector system and cell permeable peptide sequences fused to the shRNA. We are in the process of studying a novel method to ameliorate the effectiveness of this delivery system.

In summary, this is the first report of targeting the GEP using RNAi (either siRNA or shRNA) for HCC treatment in association with a cancer cell-proliferation inhibitory effect. We concluded that GEP plays a pivotal role in hepatocarcinogenesis by clearly demonstrating that GEP-shRNA effectively inhibited tumor growth *in vitro* and *in vivo* via cell cycle arrest. Therefore, GEP-shRNA may be a safe and useful treatment for HCC therapy.

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