Role of p16$^{\text{INK4a}}$ in the inhibition of DNA synthesis stimulated by HGF or EGF in primary cultured rat hepatocytes

Mizuho Harashima$^{1}$, Taiichiro Seki$^{1}$, Toyohiko Ariga$^{1}$, and Shingo Niimi$^{2}$

$^{1}$Department of Nutrition and Physiology, Nihon University College of Bioresource Sciences, Kameino Fujisawa 252-8510, Japan and
$^{2}$Division of Medical Devices, National Institute of Health Science, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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ABSTRACT

In the present study, we investigated the role of p16$^{\text{INK4a}}$ in the inhibition of DNA synthesis stimulated by hepatocyte growth factor (HGF) or epidermal growth factor (EGF) using RNA interference in primary cultured rat hepatocytes. The transfection of small interfering RNAs targeting p16$^{\text{INK4a}}$ reduced the corresponding mRNA and protein expression by more than approximately 90% and 50%, respectively, at 24 h after transfection. In the cells transfected with p16$^{\text{INK4a}}$ small interfering RNA, control, HGF, and EGF-stimulated DNA synthesis as assessed by $[^3]H$thymidine incorporation increased by approximately 1.5-fold, 1.6-fold, and 1.7-fold, respectively, compared with that in the control small interfering RNA-transfected cells. These findings indicate that p16$^{\text{INK4a}}$ plays a significant role in the inhibition of DNA synthesis.

It is now well established that control of the cell cycle is accomplished through sequential assembly, activation, and destruction of specific protein complexes containing cyclins and cyclin-dependent kinase (Cdk) partners (4). The D-type cyclins and their catalytic subunits Cdk4 and Cdk6 play a key role in cell progression through G1 phase when triggered by growth factors. Cyclin-Cdk complex assembly and enzymatic activities are regulated by a number of small proteins, termed Cdk inhibitors. Two different families of Cdk inhibitors have been described in mammalian cells that differ in structure, mechanism of inhibition, and Cdk target specificity. The Ink family of Cdk inhibitors includes the tumor suppressor protein p16$^{\text{INK4a}}$, as well as p15$^{\text{INK4b}}$, p18$^{\text{INK4c}}$, p19$^{\text{INK4d}}$, which appear to specifically target the G1 phase cyclin D-Cdk4/Cdk6 complexes (15, 16). The structurally and functionally distinct Cip/kip family comprises three proteins: p21 (also known as Cip1, Waf1, Sdi1) (3, 5, 11, 21), p27$^{\text{Kip1}}$ (14, 19), and p57$^{\text{Kip2}}$ (7, 9). The Cdk inhibitors bind to cyclin/Cdk complexes and inhibit their activities with different efficacy (5, 9).

In terms of biological functions, different Cdk inhibitors appear to participate in inhibition of DNA synthesis following stimulation by growth factors. However, the role of Cdk inhibitors in the inhibition could vary depending on the balance between the expression level of Cdk inhibitors and cyclin/Cdk complexes. There is one report showing that treatment of cultured rat hepatocytes with p21 antisense oligonucleotides resulted in approximately 1.4-fold increase of DNA synthesis stimulated by EGF compared with that in mismatch p21 oligonucleotide-treated cells during the first 52 h of culture (6). However, it remained to be elucidated whether p16$^{\text{INK4a}}$, an important Cdk inhibitor, plays a role in the inhibition of the DNA synthesis, although the specific involvement of p16$^{\text{INK4a}}$ in liver cancer is indicated both by frequent deletion of the locus in hepatocarcinoma (8, 17) and by a negative correlation between p16$^{\text{INK4a}}$ expression level and hepatocarcinoma susceptibility in rats (12, 13). In the present study, we investigated the role of p16$^{\text{INK4a}}$ in the inhibition of DNA synthesis stimulated by hepatocyte growth factor (HGF) or epidermal growth factor...
O2 air. The 6-cm dish and 24-well microplate were precoated with 0.03% collagen type-1 AC in a humidified chamber at 37°C. Primer sequences for p16 INK4a
GGT ATT TGC GGT ATC TAC TCT C-3' and 5'-GGC ATC ACA GAG CTG TTA TTG CTC-3'. p16 INK4a siRNA was purchased from Thermo Fisher Scientific (Hudson, NH, USA) as the ON-TARGET plus SMART pool. The targeted sequences were as follows: J-097994-09, GAUGAUG GGCAACGUCAAA; J-097994-10, CAACGCGAG ACUAGCAUAU; J-097994-11, AGGCAUAACUU UGCUCUAA; and J-097994-12, CCAAGCGAGGC CUAAGUU. The sequences for control siRNA were as follows: 5'-ACU CUA UCU GCA CGC UGA CUU-3' and 5'-P G UCA GCC AGG AGA AGT G-3'. No homology between any relevant mammalian gene and the control siRNA was observed. After 20 h of cell culture, the medium was replaced with WE containing aprotinin (1 μg/mL) and bovine serum albumin (BSA) (1 mg/mL) immediately prior to transfection. Transfection with siRNA was performed using siFactor (B-bridge International, Inc., Cupertino, CA, USA), in accordance with the user guidelines. p16 INK4a siRNA (80 μL; 20 μM) and control siRNA (80 μL; 20 μM) were diluted with OPTI-MEM (Invitrogen) to a final volume of 400 μL. siFactor (32 μL) was also diluted in OPTI-MEM to a final volume of 400 μL, then suspended and incubated at room temperature for 5 min. Next, the diluted siRNA was combined with siFactor, and the mixture was incubated at room temperature for 20 min. The mixture was added to the cultures (6-cm dish). Twenty-four-well microplates were used for the measurement of DNA synthesis, and the volume of each solution per well was scaled down to 1/10. Total RNA and cell lysates were prepared from the cells transfected with control or p16 INK4a siRNAs and cultured for 24 h. The details of transfection used for measurement of DNA synthesis and the time schedule are described below.

Cell lysates were prepared using a modified method reported by Ueberham et al. (20). The cells were washed once with phosphate-buffered saline, followed by buffer A (10 mM Tris-HCl [pH 7.5], 5 mM EDTA) twice. The cells were then harvested, suspended 20 times, and sonicated four times for 15 s each time after the addition of 1/5 [vol/vol] of buffer A containing 5% NP-40 and 0.5% SDS and 1/100 [vol/vol] of a protease inhibitor cocktail (SIGMA, St. Louis, MO, USA). After centrifugation at 100,000 × g, the supernatant was stored at −70°C.

An equal amount of supernatant protein from each experiment (approximately 36 μg) was subjected to SDS-PAGE on a 15% gel and electroblotted to a PVDF membrane (GVHP; Millipore, Billerica, MA, USA). After blocking the membrane with 5% skimmed milk, a Western blot analysis was performed using mouse anti-human p16 INK4a monoclonal antibody (1 : 500) (ab54210; Abcom, Cambridge, UK), and rabbit anti-rat β-actin antibody (1 : 500) (Biolegend Inc., San Diego, CA, USA). Detection was performed using the ECL Plus Western blotting detection system (GE Healthcare, Piscataway, NJ, USA).
INK4a inhibits DNA synthesis by the siRNAs on DNA synthesis stimulated by HGF (20 ng/mL) or EGF (2 ng/mL). HGF and EGF stimulated DNA synthesis by approximately 6-fold and 7-fold, respectively, in hepatocytes treated with control siRNA (Fig. 3). The stimulations by HGF or EGF were enhanced to approximately 1.6-fold and 1.7-fold by treatment with p16INK4a siRNAs. The control was also enhanced to approximately 1.5-fold by the treatment.

In the present study, we showed that suppression of p16INK4a expression by RNA interference (RNAi) using the siRNAs significantly increased the HGF or EGF-stimulated DNA synthesis, indicating that p16INK4a played a role in the inhibition of DNA synthesis. The extent of decrease by p16INK4a expression was approximately 30% in HGF and 40% in EGF-stimulated DNA synthesis. On the other hand, the extent of decrease by p21 expression was approximately 30% in EGF-stimulated DNA synthesis (6).

![Fig. 1 Suppression of p16INK4a mRNA level with RNAi.](image)

USA). Intensity of each band was measured over a proportional range. A computer-assisted analyzer was used to quantitatively analyze intensity, with intensity of the p16INK4a band normalized to intensity of the β-actin.

After 20 h of culture, the medium was replaced with WE containing aprotinin (1 μg/mL) and 0.1% BSA, and EGF (2 ng/mL) or HGF (20 ng/mL) was added. After 2 h, the mixture of siRNA and siFector (80 μL), prepared as described above, was added to each well. After another 24 h, [3H]thymidine (0.53 μCi) and thymidine (17.2 ng) were added. The cells were then cultured for another 24 h. [3H]thymidine incorporation was measured as described previously (10). The radioactivity in the hot-trichloroacetic acid soluble fraction was calculated in terms of dpm/mg protein. Cell protein was measured by a previously described method (2), using BSA as a standard.

Data were analyzed by Student’s t-test, and P values less than 0.05 were considered to be statistically significant.

At first, we attempted to suppress p16INK4a expression by siRNA. p16INK4a mRNA expression was markedly reduced by treatment with p16INK4 siRNAs, compared with control siRNAs, 1 day after transfection, with an inhibition of approximately more than 90% (Fig. 1). Furthermore, the p16INK4a protein level was also reduced by treatment with the level in control siRNA-treated cells, with an inhibition of approximately 50% (Fig. 2).

Next, we examined the effect of the suppression of p16INK4a expression by the siRNAs on DNA synthesis stimulated by HGF (20 ng/mL) or EGF (2 ng/mL). HGF and EGF stimulated DNA synthesis by approximately 6-fold and 7-fold, respectively, in hepatocytes treated with control siRNA (Fig. 3). The stimulations by HGF or EGF were enhanced to approximately 1.6-fold and 1.7-fold by treatment with p16INK4a siRNAs. The control was also enhanced to approximately 1.5-fold by the treatment.

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Therefore, the roles of p16\(^{\text{INK4a}}\) and p21 expression may be similar in HGF and EGF-stimulated DNA synthesis. As the hepatocytes normally exist in non-divided state in rat liver in vivo, p16\(^{\text{INK4a}}\) and p21 may act as a brake, protecting the hepatocytes from excess growth-stimulating signal to maintain the homeostasis. On the other hand, there is a report on a divided state in rat liver synthesis. As the hepatocytes normally exist in non-

**Fig. 3** Enhancement of control, EGF or HGF-dependent increase of DNA synthesis by RNAi targeting p16\(^{\text{INK4a}}\). The data are expressed as the mean ± S.D. of 3 experiments using duplicate wells in each experiment. \(*P < 0.01\), compared with the value of cells transfected with control siRNA.

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