

Small-interfering RNA-mediated silencing of the MAPK p42 gene induces dual effects in HeLa cells

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Abstract. The genesis and progression of cervical cancer involve the mutation or deviant expression of numerous genes, including the activation of oncogenes (Ha-ras, C-myc, C-erbB2 and Bcl-2) and inactivation of tumor-suppressor genes (p53 and Rb). Previous studies showed that small-interfering RNAs (siRNAs) targeting the MAPK p42 gene partly inhibit proliferation and increase apoptosis in human cervical carcinoma HeLa cells. Results of a microarray analysis showed that MAPK p42 siRNA inhibited cell growth through the regulation of cell cycle control and apoptosis and induced interferon-like response in HeLa cells. In order to confirm the dual effects of MAPK p42 siRNA, we compared the roles of siRNA and U0126, an inhibitor of MAPK p42, in HeLa cells. Short 21-mer double-stranded/siRNAs were synthesized to target MAPK p42 mRNA in HeLa cells. The siRNAs were transfected into HeLa cells using Lipofectamine. The cells were treated with siRNA or U0126 at different concentrations for a period of 48 h. The biological effect of siRNA and U0126 on HeLa cells was measured by MTT and flow cytometry. MAPK1, NUP188, P38, STAT1, STAT2, PML and OAS1 were analyzed by real-time quantitative PCR. HeLa cell growth was inhibited by siRNA or U0126, and the effect of siRNA inhibition was greater than that of U0126. Cell cycle phases were different for siRNA or U0126, but HeLa cell growth was arrested at the S phase by siRNA and at G1 phase by U0126. A down-regulation in MAPK p42 expression by

siRNA and up-regulation by U0126 were noted. The results of real-time quantitative PCR showed that P38 was up-regulated and NUP188 was down-regulated by siRNA in comparison with the control groups, and the results were consistent with those of U0126. Expression levels of STAT1, STAT2, PML and OAS1 induced by siRNA differed from those induced by U0126. siRNA-mediated silencing and deactivation induced by U0126 in MAPK p42 led to growth inhibition in the HeLa cells. The effects of siRNA on HeLa cell growth were different from those of U0126. Dual effects of MAPK p42 siRNA-2 on HeLa cell growth were noted: one consisted of a specific effect induced by siRNA-mediated p42 MAPK silencing and the other exhibited a non-specific interferon-like response.

Introduction

RNA interference (RNAi) is a natural protective mechanism that functions in various organisms (1-4). Small-interfering RNAs (siRNAs) (21-23 nt) are thought to be generated from stretches of double-stranded RNA by Dicer, a conserved member of the RNase III gene family. siRNAs are then incorporated into a large multiprotein RNA-inducing silencing complex. In the siRNA-mediated mRNA degradation pathway, the antisense strand of the siRNA molecule is used to target the cognate mRNA for degradation. This process involves specific base pairing between the antisense strand of the siRNA and the target mRNA, endonucleolytic cleavage of the mRNA strand across the middle of the siRNA strand and subsequent degradation of the unprotected mRNA (5). RNAi provides a revolutionary tool to identify gene functions (6,7) and opens new possibilities for therapeutic interventions (8-10). In early reports, 21 nt siRNA was thought to be too small to activate PKR, a kinase that senses double-stranded RNA (11). However, other reports maintain that the transfection or expression of even short duplexes or hairpins is able to induce a subset of markers of innate immune response (12,13).

The extracellular signal-regulated kinase (ERK)/MAPK pathway is constitutively active in several human malignancies, and it is critical for the induction of cell proliferation, differentiation and cell survival. Four major groups of MAPKs in mammalian cells are found: ERK, c-jun NH2-terminal kinase/stress-activated protein kinase, p38 and extracellular

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Table I. The primer sequences of eight genes for real-time quantitative PCR.

Gene Bank no.	Gene	Primer sequence	PCR product length (bp)	Temperature (°C)
NM_002534	OAS1	Forward: 5'-CCAGGAAATTAGGAGACAGC-3' Reverse: 5'-GAGCGAACTCAGTACGAAGC-3'	165	88.0
NM_002675	PML	Forward: 5'-AGTCGGTGCGTGAGTTCCT-3' Reverse: 5'-GGAACATCCTCGGCAGTAG-3'	110	88.9
NM_015354	NUP188	Forward: 5'-GCACTCTGCTGCTTATCC-3' Reverse: 5'-CTTGGCCTTGGTCTTCTC-3'	137	88.6
NM_139014	p38	Forward: 5'-GCAGGAGCTGAACAAGACAATC-3' Reverse: 5'-TTTCGCATGAATGATGGACTG-3'	169	88.4
AA195999	MAPK1	Forward: 5'-CCCAAATGCTGACTCCAAAGC-3' Reverse: 5'-GCTCGTCACTCGGGTCGTAAT-3'	131	84.8
BC002704	STAT1	Forward: 5'-TGCTCCTTTGGTTGAATCCC-3' Reverse: 5'-GGAATTTTGAGTCAAGCTGCTG-3'	92	90.7
NM_005419	STAT2	Forward: 5'-GTGGTTCAGGAAAGGGCAG-3' Reverse: 5'-GGAGGGTGTCCGTTTTTCAG-3'	123	87.9
NM_000094	COL7A1	Forward: 5'-GTGTTGCTGCGTGACTTGG-3' Reverse: 5'-AACAGAAGCGTCAGTGCGAG-3'	114	90.3
NM_002046	GAPDH	Forward: 5'-AGTTAGCCGCATCTTCTTTTGC-3' Reverse: 5'-CAATACGACCAAATCCGTTGACT-3'	100	87.8

signal regulated kinase-5 (ERK5, also known as Big MAP kinase-1) (14-17). ERK is mainly activated by mitogenic stimuli such as growth factors and hormones to induce cell proliferation. Previous studies showed that siRNA targeting for the MAPK p42 gene partially inhibits proliferation and increases apoptosis in human cervical carcinoma HeLa cells (18,19). Results of a microarray analysis showed that MAPK p42 siRNA inhibited cell growth through the regulation of cell cycle control and apoptosis and induced an interferon-like response in HeLa cells (20). In order to confirm the specific effects of siRNA on MAPK p42 and the non-specific interferon-like response effect, the roles of siRNA and U0126, an inhibitor of MAPK p42, were compared in HeLa cells.

Materials and methods

siRNA synthesis. siRNAs were designed using RNAi target finder (http://www.ambion.com/techlib/misc/siRNA_finder.html). The two sets of siRNA sequences were: siRNA-1 (negative control) sense CUCUACGUAAGAUCAGCUUU and antisense AGCUGGAUCUUACGUAGAGUU, bearing no homology with any known relevant human genes; and siRNA-2 sense AGCAAUAGUUCUAGCUUUU and antisense AAGCUAGGAACUAAUUGCUUU. siRNA were synthesized and purified by means of the Silencer™ siRNA Construction kit (Ambion, Austin, TX, USA).

Cell culture and transfection. Human HeLa cells (5.0×10^4 cells/ml) were cultured in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum containing 2.0 mmol/l glutamine and 20 µg penicillin-streptomycin/ml in 5% CO₂ at 37°C, and allowed to adhere for 24 h. HeLa cells were transfected by siRNA, using

Lipofectamine 2000 (Invitrogen) or treated with U0126 at different concentrations. Two days after transfection and treatment, cells were analyzed for MTT, cell cycle and real-time quantitative PCR. All tests were repeated five times.

MTT assay for cell viability. Cells (1.0×10^4 /well) were cultured in 96-well plates. After 24 h, the cells were incubated with siRNA for the indicated times at 37°C in 5% CO₂. Then, 20 µl/well of MTT solution (5 mg/ml) was added, and cells were incubated for another 4 h. The supernatants were removed, and formazan crystals were solubilized in 200 µl of dimethylsulfoxide. Finally, optical density was determined at 490 nm by a POLARstar⁺Optima (BMG Labtechnologies).

Cell cycle analysis by flow cytometry. DNA content per duplicate was analyzed using a FACSCalibur FCM (Becton-Dickinson, Mountainview, CA, USA). The adherent cells were harvested by brief trypsinization, washed with PBS, fixed in 70% ethanol, stained with 20 µg/ml propidium iodide containing 20 µg/ml RNase (DNase-free) for 30 min and analyzed by flow cytometry. The populations of G0/G1, S and G2/M cells were quantified.

Real-time quantitative PCR using SYBR-Green I. The message RNA levels of seven genes, i.e., mitogen-activated protein kinase 1 (MAPK1), signal transducer and activator of transcription 1/2 (STAT1/2), cyclin-dependent kinase (PML), nucleoporin 188 (NUP188), 2',5'-oligoadenylate synthetase (OAS1) and p38 were measured in siRNA and control samples by real-time quantitative PCR (Table I). The reaction was performed using the ABI 7000 real-time PCR detection system (ABI PRISM, USA) with SYBR-Green I.

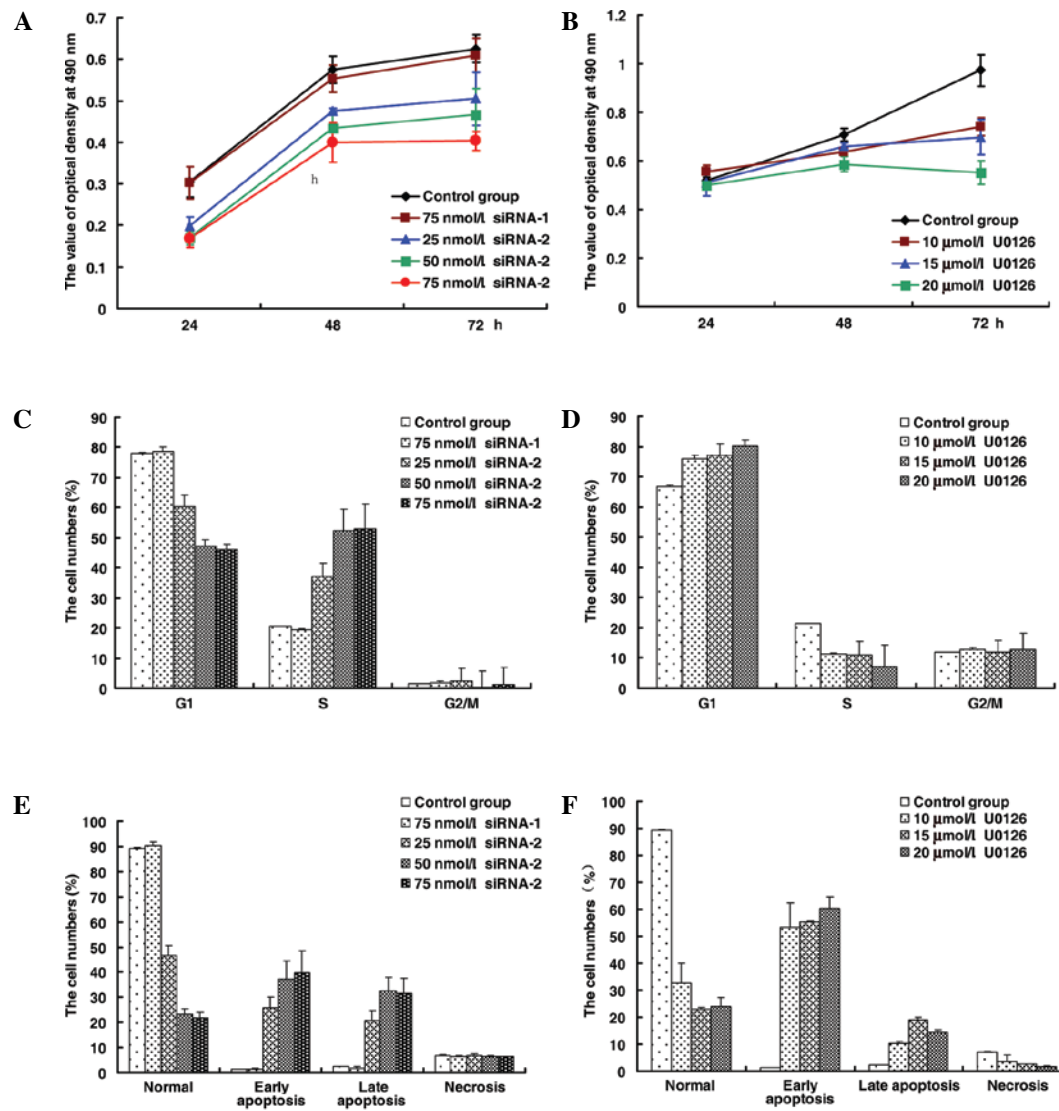


Figure 1. Inhibition of siRNA-2 and U0126 on cell growth in HeLa cells. Growth inhibition induced by (A) siRNA-2 and (B) U0126 treatment. Cell cycle arrest induced by (C) siRNA-2 and (D) U0126 treatment for 24 h. Apoptosis induced by (E) siRNA-2 and (F) U0126 treatment for 24 h. Results are shown as the means \pm SD of five independent experiments.

Simultaneously, GAPDH was used in all of the specimens as the reference, and the quantitative analysis of message RNA levels was normalized by GAPDH. The fold change of the gene expression between siRNA and the control group was calculated for each pair sample using $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{\text{gene}} - Ct_{\text{GAPDH}})_{\text{siRNA}} - (Ct_{\text{gene}} - Ct_{\text{GAPDH}})_{\text{control}}$. $2^{-\Delta\Delta Ct} > 2$ was calculated to be gene overexpression (14).

Statistical analysis. Data were expressed as means \pm SD and analyzed by SPSS10.0 software. $P < 0.05$ was considered to be statistically significant.

Results

Effects of siRNA and U0126 on the inhibition of HeLa cell growth. HeLa cells were exposed to siRNA or U0126 at different concentrations at different times. U0126 significantly inhibited the growth of HeLa cells at certain concentrations (Fig. 1B), and the inhibition ratio of U0126 reached 17.1% at 20 $\mu\text{mol/l}$ for 48 h. In comparison with the control group and

negative siRNA-1 (Fig. 1A), siRNA-2 was found to inhibit the proliferation of HeLa cells. The inhibition ratio of siRNA-2 reached the highest level of $\sim 30.4\%$ at 75 nmol/l for 48 h. The cell cycle of HeLa cells was arrested at the G1 phase by U0126 (Fig. 1C) and at the S phase by siRNA-2 (Fig. 1D). The effects of siRNA-2 were greater than those of U0126 on the apoptosis of HeLa cells in that U0126 induced early apoptosis (Fig. 1F), while siRNA-2 increased late apoptosis (Fig. 1E).

Effects of siRNA and U0126 on the expression of MAPK p42, NUP188 and p38. To evaluate the effects of siRNA-2 on MAPK p42 silencing, real-time quantitative PCR was used. The results showed that the expression of MAPK p42 was inhibited ~ 60 and 70% , respectively, by siRNA-2 in comparison with the control group and negative siRNA-1 (Fig. 2A). By contrast, U0126 induced MAPK p42 expression (Fig. 2B). The results showed that inhibition of the MAPK p42 activity by U0126 led to an increase in MAPK p42 expression in compensation.

NUP188 was down-regulated and p38 was up-regulated by siRNA-2 and U0126. The consistency in the result between

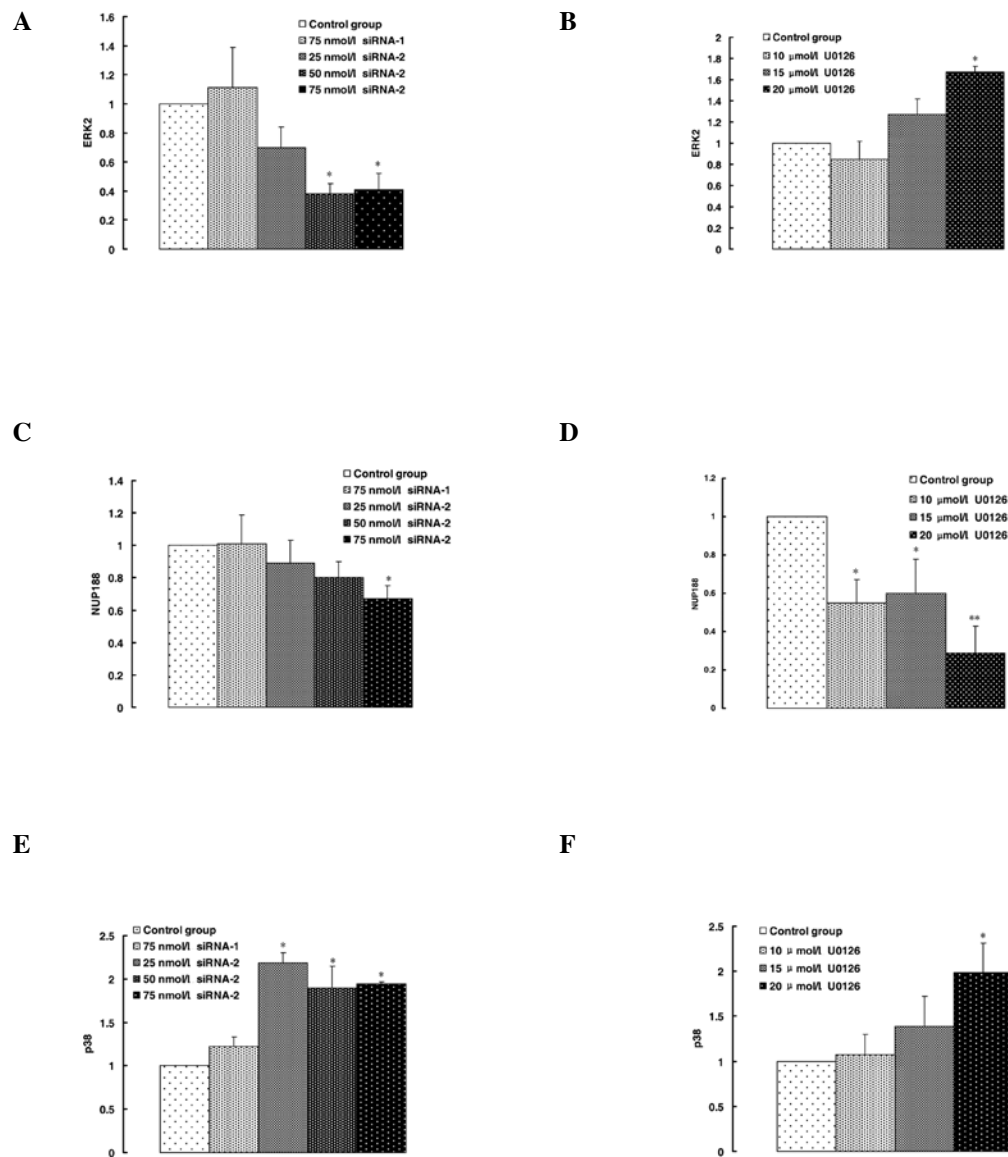


Figure 2. Effects of siRNA-2 and U0126 on the expression of ERK2, NUP188 and p38 in HeLa cells. ERK2 expression in HeLa cells treated with (A) siRNA and (B) U0126. NUP188 expression in HeLa cells treated with (C) siRNA and (D) U0126 in HeLa cells. The p38 expression in HeLa cells was treated with (E) siRNA and (F) U0126. Results are shown as the means \pm SD of three independent experiments. * P <0.05 and ** P <0.01 vs. the control group.

siRNA-2 and that of U0126 showed that the down-regulation or inhibition in MAPK p42 activity led specifically to the response of the expression in NUP188 (Fig. 2C and D) and p38 (Fig. 2E and F).

Effects of siRNA and U0126 on the expression of interferon-like response genes. The OAS1 gene is a member of the OAS family of interferon-induced antiviral enzymes. siRNA-2 and negative siRNA-1 induced the overexpression of OAS1 in HeLa cells in comparison with the control group (Fig. 3A); U0126 inhibited the expression of OAS1 (Fig. 3B). However, in addition to the treatment of siRNA-2 at 25 nmol/l, other siRNA-2 groups inhibited the expression of OAS1 in comparison with negative siRNA-1. The mode of expression of the PML gene was consistent with that of OAS1 induced by siRNA-2 (Fig. 3C). These results showed that the up-regulation of the expression in OAS1 and PML was a non-specific response to siRNA-2. STAT1 and STAT2 are involved in interferon (IFN)

signaling pathways and play a key role in promoting apoptosis in a variety of cell types. STAT1 and STAT2 expression was slightly down-regulated by U0126 (Fig. 4B and D), but up-regulated significantly by siRNA-2 and negative siRNA-1 (Fig. 4A and C).

Discussion

The overexpression and activation of ERK has been documented in leukemia, renal cell carcinoma, breast cancer and several ovarian cancer cell lines (21-23). The suppression of MAPK p42 expression and activity by siRNA or U0126 resulted in the inhibition of HeLa cell growth. Results showed that MAPK p42 is involved in HeLa cell growth. Steinmetz *et al* also demonstrated that silencing of the ERK1/2 protein expression using RNAi led to the complete suppression of HeyC2 and SKOV3 cell proliferation (24). Tamemoto *et al* found that 44- and 42-kDa MAPKs exhib-

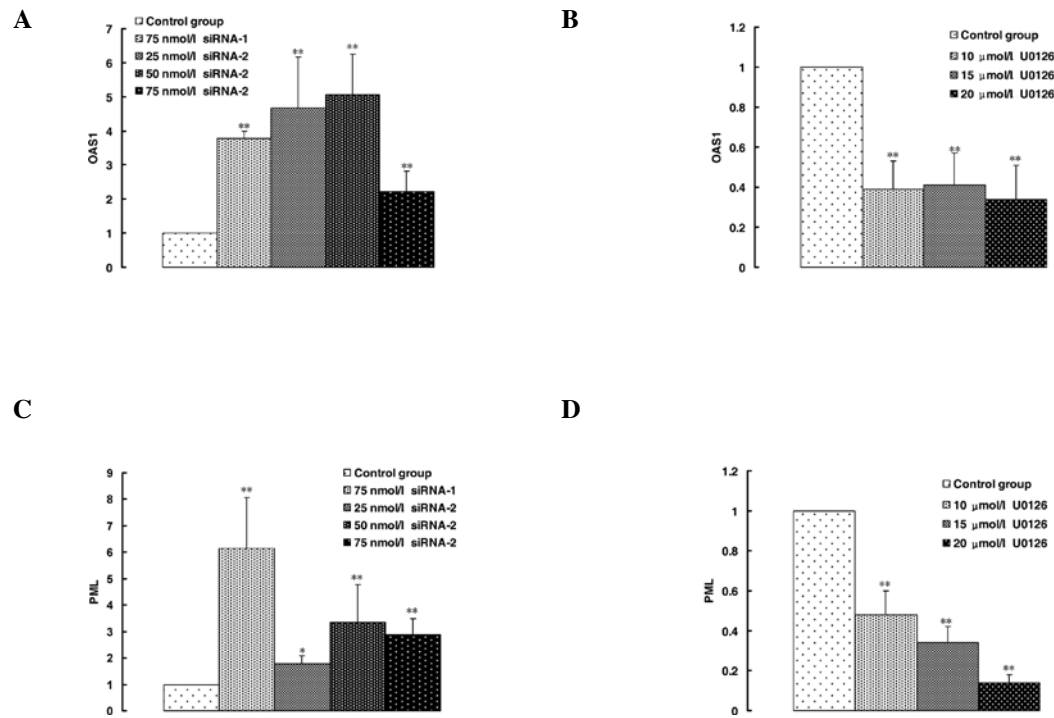


Figure 3. Effects of siRNA-2 and U0126 on the expression of the PML and OAS1 genes in HeLa cells. OAS1 expression caused by (A) siRNA and (B) U0126 treatment in HeLa cells. PML expression caused by (C) siRNA and (D) U0126 when applied in HeLa cells. Results are shown as the means \pm SD of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. control group.

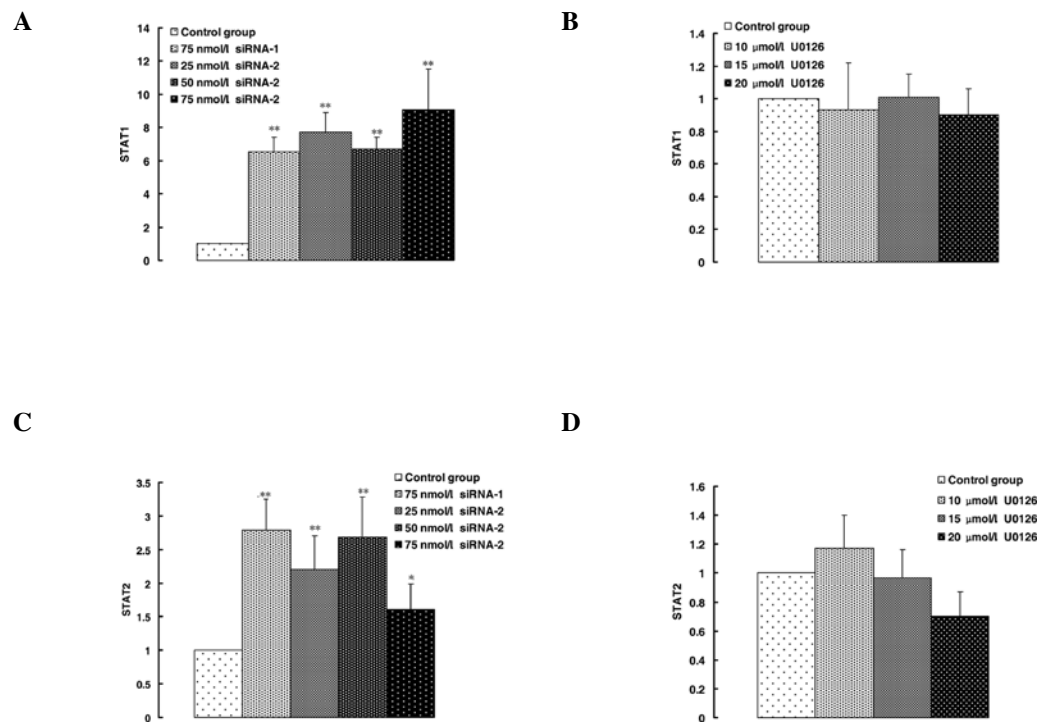


Figure 4. Effects of siRNA-2 and U0126 on the expression of STAT1 and STAT2 genes in HeLa cells. STAT1 expression caused by (A) siRNA and (B) U0126 treatment in HeLa cells. STAT2 expression caused by (C) siRNA and (D) U0126 treatment in HeLa cells. Results are shown as the means \pm SD of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. the control group.

ited activities in the G1 through S and G2/M phases and were activated biphasically in the G1 phase and around the M phase (25). Our results showed that the cell cycle was arrested at the G1 phase by U0126 and at the S phase by

siRNA-2, suggesting different cell proliferation suppression effects between U0126 and siRNA-2.

The 21 nt siRNA targeting MAPK p42 induced the down-regulation of MAPK p42 in comparison with the control group

and negative siRNA-1, while U0126 induced MAPK p42 expression, suggesting that siRNA-mediated silencing of the MAPK p42 gene was a specific effect of siRNA. A decrease in MAPK p42 occurred along with an increase in MAPK p38, another protein of the MAPK pathway (26,27). This increase was thought to be responsible for the progression of apoptosis. Our results were similar in that the decrease in MAPK p42 expression induced by siRNA or the decrease in MAPK p42 activity induced by U0126 caused a slight increase in MAPK p38 expression (Fig. 2E and F). NUP188 is a type of nucleoporin (Nup). Approximately 30 types of Nup family nucleoside transporters can construct a nuclear pore complex in the membrane of a cell nucleus. This complex is an important component involved in the nucleocytoplasmic transport of biomacromolecules, but its mechanism remains unknown. NUP188 was down-regulated by siRNA-2 and U0126. The consistency between the result of siRNA-2 and U0126 showed that the down-regulation or inhibition of activity of MAPK p42 led particularly to a response of expression of NUP188 (Fig. 2C and D).

dsRNA structures greater than 30 bp were found to stimulate the IFN pathway mediated in part by the activation of the dsRNA-dependent protein kinase R (PKR), which represented a host response to viral infection (28,29). Several genes were activated in the IFN pathway, including the member of the OAS family, STAT1/2 and PML (30,31). It was thought that 21 nt siRNAs were too short to induce interferon expression (12). It was possible to administer naked, synthetic siRNA to mice and down-regulate an endogenous or exogenous target without inducing an interferon response (32). However, previous studies found that the transfection of siRNA resulted in an interferon-mediated activation of the Janus kinase/STAT pathway and the global up-regulation of interferon-stimulated genes (12,13,33). In order to confirm the interferon responses of siRNA-2, STAT1/2, PML and OAS1 were detected by real-time PCR. Differential effects of siRNA and U0126 on the expression of interferon-like response genes were noted. The inhibition of MAPK p42 activity by U0126 induced the down-regulation of OAS1 and PML (Fig. 3B and D) in HeLa cells, but the knockdown of MAPK p42 by MAPK p42 siRNA caused the up-regulation of OAS1 and PML levels (Fig. 3A and C), which were lower than those induced by negative siRNA. The effects of U0126 on the expression of STAT1 and STAT2 were slight (Fig. 4B and D) and MAPK p42 siRNA promoted the expression of STAT1 and STAT2 (Fig. 4A and C). PML, STAT1 and STAT2 were proven to be involved in the inhibition of cell growth (34-36).

In conclusion, MAPK p42 siRNA, not only specifically knocked down MAPK p42 and increased p38 expression in comparison with the small-molecule MEK inhibitor U0126, but also non-specifically stimulated the interferon responses which increased the expression of pro-apoptotic genes including PML, STAT1 and STAT2, ultimately triggering HeLa cell apoptosis. Our results suggest that siRNA-mediated down-regulation of MAPK p42 is an attractive strategy for cancer gene therapy.

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