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# Down regulation of *c-Myc* and induction of an angiogenesis inhibitor, thrombospondin-1, by 5-FU in human colon cancer KM12C cells

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**Abstract**

5-FU is commonly used for treatment of various solid tumors including colon carcinoma. We have previously demonstrated that Egr-1 induced by 5-FU enhanced TSP-1 expression in human colon cancer KM12C cells. In this study, a Genechip analysis of KM12C cells treated with 5-FU revealed down-regulation of 924 genes and up-regulation of 460 genes. The decreased expression of *c-Myc* mRNA and phosphorylated c-Myc were detected and confirmed by RT-PCR and immunoblotting. Since 5-FU induced the expression of TSP-1, we examined the effect of c-Myc on the *TSP-1* promoter. Deletion of the *TSP-1* promoter region in which binding sites for c-Myc reside had no effect on the *TSP-1* promoter activity induced by 5-FU. Meanwhile, 5-FU dose-dependently decreased the expression of *miR-17-92* cluster. These findings suggest that 5-FU decreased the expression of *c-Myc* and consequently *miR-17-92* cluster and increased the expression of *TSP-1* mRNA.


*Keywords:* 5-Fluorouracil; Genechip analysis; Thrombospondin-1; c-Myc

*Abbreviations:* TSP-1, thrombospondin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 5-FU, 5-fluorouracil; Egr-1, early growth response factor-1.

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## 1. Introduction

5-FU is commonly used either alone or in adjuvant regimens for the treatment of solid tumors [1,2]. UFT (a prodrug of 5-FU) inhibits tumor-induced angiogenesis, possibly prolonging survival or contributing to the anti-metastatic effects in UFT adjuvant chemotherapy [3]. However, despite their extensive use in clinical practice, the molecular basis for the anti-tumor effect of 5-FU has not been fully elucidated. We have previously shown that 5-FU up-regulated the expression of TSP-1 in KM12C cells. 5-FU also induced Egr-1, a transcription factor that was suggested to play an important role in the regulation of angiogenesis, growth, and apoptosis. Egr-1 augmented *TSP-1* promoter activity, and subsequently the production of *TSP-1* mRNA and protein in KM12C cells [4]. Furthermore, our recent results have shown that 5-FU-based drugs have antiangiogenic function through up-regulation of TSP-1 in colorectal cancer xenografts [5].

Numerous *in vitro* and *in vivo* experiments have been carried out to identify multiple mechanisms by which TSP-1 inhibits angiogenesis. These mechanisms are broadly characterized as direct effects on endothelial cells and indirect effects on the various growth factors, cytokines and proteases that regulate angiogenesis [6]. However, the molecular basis for the TSP-1 induction by 5-FU is as yet unknown.

The microarray analysis to determine gene expression profiles is a valuable tool for understanding the regulation of biological systems and the effect of various stresses. A number of studies suggested that DNA microarrays are useful for predicting the sensitivity of cancer cells to 5-FU treatment [7–9].

In this study, we used Genechip to analyze the effect of 5-FU on transcriptional profile of KM12C cells. The expression of 924 genes was attenuated and the expression of 460 genes in KM12C cells was augmented by 5-FU. The expression of *c-Myc* that is involved in the regulation of TSP-1 expression [10–12] was decreased by 5-FU. The *miR-17-92* cluster was recently reported to be upregulated by the proto-oncogene *c-Myc* [13]. Our results suggested that the down-regulation of *c-Myc* increased the stability of *TSP-1* mRNA through the decreased expression of *miR-17-92* cluster.

## 2. Materials and methods

### 2.1. Reagents and antibodies

5-FU was provided by Taiho pharmaceutical Co., Ltd. (Tokyo, Japan). An anti-phospho-c-Myc antibody was obtained from Cell Signaling Technology. An antibody α-HMGB1 was generously provided by Dr. Kawahara (Kagoshima University, Kagoshima, Japan).

### 2.2. Cell lines and culture conditions

KM12C human colon cancer cell with mutant p53 were provided by Dr. Kiyoshi Morikawa (Iwamizawa Worker's Compensation Hospital, Hokkaido, Japan), and grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 2 mM L-glutamine in a humidified atmosphere of 5% $CO_2$ at 37 °C.

### 2.3. Genechip analysis

KM12C cells were treated with 1 μM 5-FU for 4 days. Extraction of total RNA from the cells using the RNeasy mini kit (Qiagen) was carried out according to the manufacturer's instructions. The integrity of the RNA was confirmed with an Agilent Bioanalyser using the RNA 6000 Nano kit (Agilent). We used a Genechip® Expression 3′ Amplification One-Cycle Target Labeling kit (Affymetrix) to label the RNA following the manufacturer's protocol. The cRNA was hybridized to Affymetrix Human U133_Plus_2 arrays according to the manufacturer's protocol. Using the Fluidics Station (Affymetrix), the biotin-labeled cRNA was revealed by successive reactions with biotinylated anti-streptavidin antibody and streptavidin R-phycoerythrin conjugate. The arrays were finally scanned in an Affymetrix/Hewlett Packard Genechip Scanner 3000. Raw data were then imported into GeneSpring software (Silicon Genetics). We performed a per-chip (the expression of each probe set in each chip divided by the median of the chip) and a per-gene (each gene divided by the mean of all the samples) normalization using the GeneSpring software. Then we compared the global gene expression profile between KM12C and KM12C + 5-FU to screen genes affected by 5-FU.

### 2.4. RNA isolation and cDNA synthesis

KM12C cells were treated with various concentrations of 5-FU for various periods as described. The total RNA from the cultured cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA (2 μg) was reverse-transcribed using a ReverTra Ace kit (Toyobo, Osaka, Japan).

### 2.5. Semiquantitative reverse-transcription (RT)-PCR

The expression levels of *c-Myc* and *TSP-1* were detected by RT-PCR. Primer sequences were as follows: forward primer 5′-AGAATGCTGTCCTCGCTGTT-3′ and reverse primer 5′-TTTCTTGCAGGCTTTGGTCT-3′ for *TSP-1*; forward primer 5′-AAGGCTCTCCTCTG CTTAG-3′ and reverse primer 5′-CTCTCCTCGTCG CAGTAGAAATAC-3′ for *c-Myc*; forward primer 5′-T CAACGGATTTGGTCGTATT-3′ and reverse primer 5′-CTGTGGTCATGAGTCCTTCC-3′ for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### 2.6. Quantitative real-time PCR

The expression levels of *c-Myc* and *miR-17-92* primary mRNA level were determined by real-time PCR (PRISM 7900HT, Applied Biosystems) using Taqman® probe (Applied Biosystems) or SYBR® Green I (Takara, Osaka, Japan) technology following the manufacture's protocol. The sequence of the primer/probe sets used for this analysis is as follows: primary *miR-17-92*, forward primer 5′-C AGTAAAGGTAAGGAGAGCTTCATCTG-3′, reverse primer 5′-CATACAACCACTAAGCTAAAGAATAA TCTGA-3′ and probe 6-FAM-TGGAAATAAGATCAT CATGCC CACTTGAGAC-(TAMRA) (Applied Biosystems). Human *GAPDH* (Applied Biosystems, #4310884E) was used for normalization. The sets of primers for *TSP-1*, *c-Myc* and *GAPDH* were same as the semiquantitative RT-PCR indicated above. The expression levels were measured using SYBR® Green I kit. Human *GAPDH* was used for normalization. Target gene expression was quantified by the comparative cycle threshold method according to the manufacturer's instructions.

### 2.7. Protein extraction and immunoblotting

For separation of the nuclear protein fractions from human KM12C cells treated with 5-FU at indicated times and concentrations, the treated KM12C cells were washed twice with ice-cold PBS and then resuspended in 200 μL buffer A [10 mM Hepes–KOH (pH 7.8), 10 mM KCl, 0.1 mM EGTA (pH 8.0), 1% NP-40, 1 mM sodium orthovanadate, 0.5 mM APMSF, 1 mM DTT, 2 μg/mL aprotinin and 2 μg/mL leupeptin]. Following incubation on ice for 15 min, the cells were disrupted by passage through a 29-gauge needle 40 times, and then were centrifuged at 750*g* for 10 min at 4 °C. This pellet containing the nuclei was washed in buffer A without NP-40, and resuspended in 100 μL nuclear lysis buffer (Buffer C) [50 mM Hepes–KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 20% glycerol, 1 mM sodium orthovanadate, 0.5 mM APMSF, 1 mM DTT, 2 μg/mL aprotinin and 2 μg/mL leupeptin], incubated for 30 min at 4 °C, and centrifuged at 20,000*g* for 20 min. The supernatant was used as the nuclear protein fraction, 100 μg of this fraction was subjected to SDS–PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane and blocked with 3% skimmed milk containing 350 mM NaCl, 10 mM Tris–HCl (pH 8.0) and 0.05% Tween. The blotted membranes were incubated overnight at 4 °C with a 500-fold diluted antibody against phospho-c-Myc or a 200-fold diluted antibody against α-HMGB1. After incubation, membranes were washed and incubated with a 3000-fold diluted horseradish peroxidase-conjugated goat anti-rabbit antibodies (GE Health Science) for 1 h at room temperature. Membranes were then washed and developed using the ECL detection system (GE Health Science).

### 2.8. TSP-1 promoter–reporter constructs

Four deletion mutants of pGL3-TSP-1, namely (−1290/+750), (−1123/+750), (−767/+750) and (−43/+750) were prepared as described previously [14].

The pGL3-TSP-1(−2033/+750ΔE-box) plasmid was derived from pGL3-TSP-1 (−2033/+750) plasmid, deleting the *NheI–BoxI* region including c-Myc binding sites.

### 2.9. Transient transfection and luciferase assay

KM12C cells were pretreated with various concentrations of 5-FU for 3 days before transfection. Cells were cotransfected with 0.5 μg of *TSP-1* promoter luciferase plasmid DNA or empty vector pGL3-basic (control group) and 25 ng phRL-TK vector (Promega) using 2.5 μL Lipofectamine (Invitrogen) according to the manufacture's instructions. The luciferase activity of transient transfectants was measured using the Dual-Luciferase assay protocol (Promega).

### 2.10. Statistical analysis

Statistical comparisons were performed using the Student's *t*-test. Quantitative data were expressed as the means ± SD. $P < 0.05$ was considered significant.

## 3. Results

### 3.1. Microarray analysis of 5-FU-regulated genes

To obtain more available information concerning the induction of TSP-1 by 5-FU [7,8], the change of gene expression in KM12C cells treated with 1 μM ($IC_{50} = 4.2$ μM) 5-FU for 4 days was examined using Genechip assay. Among approximately 21,159 genes tested by the Affymetrix array, 924 were significantly downregulated while 460 were upregulated by more than twofold above the control as shown in a scatter plot of the genes (Fig. 1a). Concerning the genes

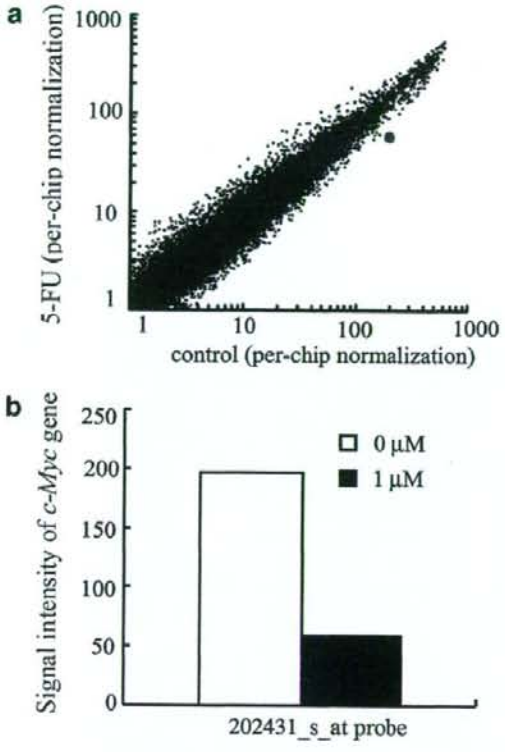


Fig. 1. Effect of 5-FU on global gene expressions in KM12C cells. (a) The scatter plot shows mean signal intensities of each gene probe using the data obtained from all microarrays. Statistical analysis identified 460 upregulated genes and 940 downregulated genes. *c-Myc* is shown as a blue dot (below diagonal line). (b) The signal intensity of *c-Myc* gene was downregulated by 1 μM of 5-FU. Genechip analysis was performed using 202431_s_at probe. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

involved in angiogenesis, the expression levels of interleukin 8 (IL-8), fibroblast growth factor receptor 3, heparanase, fetal hypothetical protein and tumor necrosis factor α-induced protein 2 in 5-FU-treated KM12C cells were more than twofold higher than those in the untreated cells. On the other hand, the expression levels of vascular endothelial growth factor A (VEGFA), thrombospondin-2 (TSP-2), angiopoietin 2 and transforming growth factor β receptor II in the 5-FU-treated cells were decreased to 50% or less of those in the untreated cells (Supplement Table). Tumor angiogenesis can be modulated by tumor suppressor genes, oncogenes and other related genes [15]. The *TSP-1* gene is down-regulated by *c-Myc* [10,11]. *c-Myc* gene expression in 5-FU-treated cells was decreased to 30% of control as evaluated by the Affymetrix microarray (Fig. 1b).

### 3.2. Effect of 5-FU on the expression of c-Myc and TSP-1 in KM12C cells

To confirm our Genechip data, we performed semi-quantitative RT-PCR, real-time RT-PCR and immunoblotting to determine the expression level of *c-Myc* gene. The expression of *c-Myc* mRNA was decreased by 5-FU in a dose-dependent manner (Fig. 2a and b). Moreover, phospho-c-Myc protein was also decreased by 5-FU in a dose-dependent manner (Fig. 2c). On the other hand, the expression of *TSP-1* mRNA was increased during the treatment with 5-FU at 1 and 2 μM (Fig. 2a).

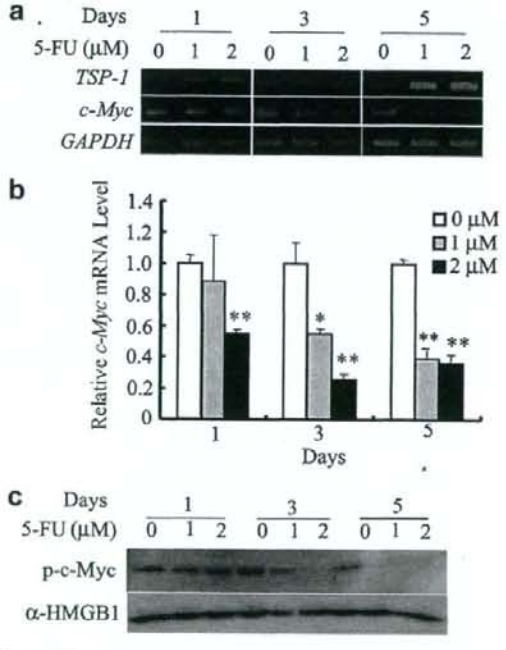


Fig. 2. Effect of 5-FU on the expression of *c-Myc* and *TSP-1* in KM12C cells. (a) and (b) Cells were treated with 5-FU at 1 and 2 μM for 1, 3 and 5 days, and the expression of *TSP-1* and *c-Myc* mRNA was analysed by RT-PCR. (a) RNA was isolated and semiquantitative RT-PCRs were performed with primers specific for the indicated genes. (b) Relative expression levels of *c-Myc* mRNA in KM12C cells were measured by real-time PCR. The expression of *GAPDH* gene was used to normalize the values of *c-Myc*. (Columns) Representative triplicate independent experiments; bars, SD. $^{*}P < 0.05$, $^{**}P < 0.01$, statistically significant differences. (c) KM12C cells were treated as described above. The same amount of nuclear protein (100 μg) was subjected to 9.4% SDS–PAGE followed by immunoblotting to determine the expression of phospho-c-Myc (p-c-Myc). α-HMGB1 was used as a loading control.

### 3.3. Effect of c-Myc on the transcriptional regulation of the TSP-1 gene induced by 5-FU

Tikhonenko and his colleagues suggested that the *TSP-1* promoter is a target for repression by Myc [10]. We thus investigated whether 5-FU enhances *TSP-1* promoter activity by decreasing c-Myc. For promoter assay, a series of *TSP-1* promoter deletion mutants were prepared, as shown in Fig. 3a and used for the luciferase reporter assay. The 5′-flanking region up to −2033 from the transcription initiation site contained several putative binding sites for known transcription factors, including two E-box-like elements (Fig. 3a). Myc in combination with MAX bound to the E-box-like element (−273/−268) in the *TSP-1* promoter [11]. Deletion of the two E-box-like elements did not affect the *TSP-1* promoter activity induced by 5-FU (Fig. 3b). These findings strongly suggested that c-Myc does not act as a transcription factor for the enhanced expression of TSP-1 by 5-FU.

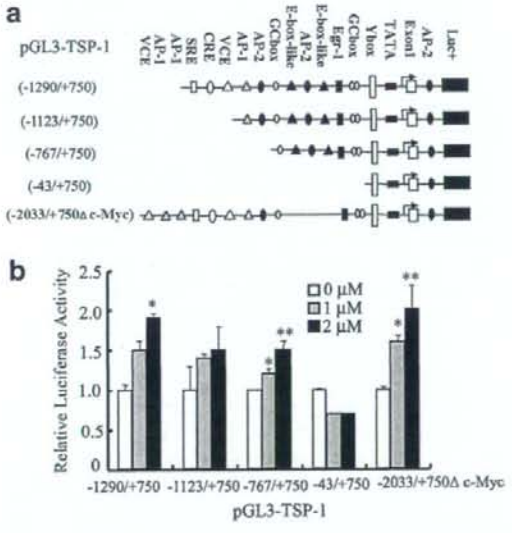


Fig. 3. Effect of 5-FU on the *TSP-1* promoter activity. (a) Schematic representation of a series of *TSP-1* mutant promoter constructs. The c-Myc-binding site (E-box-like) is indicated by the closed triangle. (b) KM12C cells were pretreated for 3 days with 5-FU and were transiently transfected with these TSP-1 promoter constructs using Lipofectamine. The cells were further incubated with or without 1 and 2 μM 5-FU for 2 days, harvested and their luciferase activities were determined. The luciferase activities were corrected for differences in transfection efficiency among wells as estimated with the *Renilla* luciferase activities. (Columns) Representative triplicate independent experiments; bars, SD. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from untreated cells transfected with the same constructs.

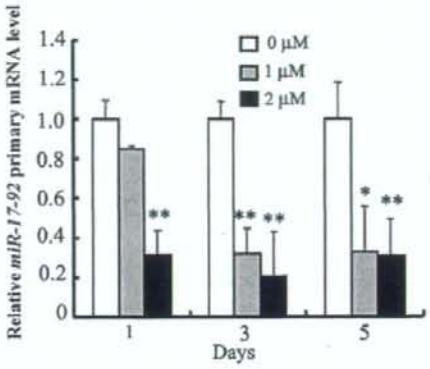


Fig. 4. Effect of 5-FU on *miR-17-92* primary mRNA. Cells were treated with 1 and 2 μM 5-FU for 1, 3 and 5 days and the levels of *miR-17-92* mRNA were measured by real-time PCR. The expression of the *GAPDH* gene was used to normalize the values of *miR-17-92*. (Columns) Representative triplicate independent experiments; bars, SD. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from untreated cells.

### 3.4. Effect of 5-FU on expression of miR-17-92 clusters in KM12C cells

Since c-Myc controls the transcription of *miR-17-92* cluster that affects tumor neovascularization by downregulating TSP-1 [13], we examined the effect of 5-FU on expression of *miR-17-92* cluster. We determined the levels of *miR-17-92* cluster primary transcript using real-time PCR. The expression levels of *miR-17-92* clusters primary transcript were decreased with increasing concentrations of 5-FU up to 2 μM (Fig. 4).

## 4. Discussion

In the past several years, powerful new technologies including DNA microarrays have emerged in the field of molecular biology which may help to decipher the molecular machinery of tumors and cells in previously unseen detail [16,17]. We have used Genechip assay to acquire the more information involved in the effect of 5-FU on TSP-1 expression. 5-FU affected the transcription levels of numerous genes (Fig. 1a). The biological functions for these genes were involved in angiogenesis, apoptosis, cell cycle, signal transduction as well as 5-FU metabolism. The acquisition of an angiogenic phenotype by tumor cells involves the up-regulation of pro-angiogenic factors and down-regulation of endogenous inhibitors, like TSP-1 and TSP-2 [6,15]. Among genes for pro-angiogenic factors, expression of *VEGFA* was decreased, but that of

*IL-8* was increased. Concerning genes involved in suppression of angiogenesis, *TSP-1* but not *TSP-2* was increased (Supplement Table). TSP-1 is an endogenous angiogenesis inhibitor that plays a pivotal role in the regulation of angiogenesis. VEGF is the major tumor angiogenesis factor. The increased expression of *TSP-1* (Fig. 2a), together with the decreased expression of VEGF might suppress angiogenesis and tumor progression. TSP-1 expression was also induced by docetaxel, paclitaxel, cyclophosphamide and trastuzamab [18–20]. In our recent study, we demonstrated that a sub-MTD (maximum tolerated dose) of 5-FU-based agent, S-1, suppressed tumor growth. A significant reduction in the number of microvessels was seen in xenografts treated with S-1. Our results suggested that TSP-1 induced by S-1 contributed to the antiangiogenic effect of S-1 [5]. We were thus interested in the molecular basis for the induction of TSP-1 by 5-FU. Tumor angiogenesis can be modulated by tumor suppressor genes, oncogenes and other related genes. The *TSP-1* gene is down-regulated by oncogenes including *jun*, *src*, *c-Myc* and *ras*, and up-regulated by the tumor suppressor gene, *p53* [15]. In our study, the expression level of *jun* mRNA in 5-FU treated cells was similar to that in untreated cells, and the expression levels of *src* and *ras* were low (data not shown). Since the expression level of *c-Myc* in the 5-FU-treated cells was decreased to 30% of that in the control cells (Fig. 1), we focused our study on c-Myc. Using real-time RT-PCR and semiquantitative RT-PCR, we confirmed the decreased expression of *c-Myc* gene in 5-FU-treated cells (Fig. 2a and b). Watnick and his colleagues demonstrated that phosphorylation of c-Myc is sufficient to confer an angiogenesis phenotype by suppressing the expression of TSP-1, even in the absence of c-Myc overexpression [12]. We therefore examined the effect of 5-FU on the level of phosphorylated c-Myc, and found that phosphorylated c-Myc was also decreased in the 5-FU-treated cells. 5-FU might have increased the expression TSP-1 by decreasing the phosphorylated c-Myc (Fig. 2c).

c-Myc has been shown to behave as a transcription repressor and can disrupt transcription of *TSP-1* by binding to *TSP-1* promoter and result in *TSP-1* silencing [10]. Recently, a number of studies have shown that c-Myc decreased *TSP-1* mRNA half-life [21,22]. c-Myc activation decreased TSP-1 expression by increasing the turnover of, or by decreasing the stability of *TSP-1* transcripts [11,12]. In accordance with the previous studies, we found that the deletion of c-Myc binding sites in *TSP-1* promoter had no effect on the *TSP-1* promoter activity induced by 5-FU in KM12C cells (Fig. 3). These results suggested that down-regulation of c-Myc by 5-FU caused the stabilization of *TSP-1* mRNA and consequently the augmentation of TSP-1 expression.

MicroRNAs (miRNAs) are 21–23 nucleotide RNA molecules that regulate the stability or translational efficiency of target mRNAs [23]. More than half of miRNAs are located at sites in the human genome that are frequently amplified, deleted, or rearranged in cancer, suggesting that abnormalities of miRNAs play an important role in cancer pathogenesis [24]. Several experiments and clinical analysis suggest that miRNAs may function as a novel class of oncogenes or tumor suppressor genes. One of the miRNAs, *miR-17–92* functions as an oncogene in humans and other animal models [25]. The *miR-17-92* cluster was recently reported to be upregulated by the proto-oncogene *c-Myc* [13,26]. This miRNA also augmented tumor neovascularization by downregulating TSP-1 expression [13]. We found that the expression levels of *miR-17-92* clusters primary transcript were decreased in the cells treated with 5-FU (Fig. 4). These results together with the previous findings suggested that 5-FU elongated the *TSP-1* mRNA half-life by down-regulating *c-Myc* and consequently *miR-17-92* cluster. Further study is needed to elucidate how *miR-17-92* cluster regulates *TSP-1* mRNA half-life. Our findings that *c-Myc* plays a role in 5-FU-induced TSP-1 expression has several implications. Deregulation of c-Myc is often observed in various cancers and occurs as a consequence of activation of one or more signalling pathways, such as mitogen activated protein kinases (MAPK), phosphatidylinositol-3 kinase (PI3K), wnt-TCF/LEF pathway, and signal transducer and activator of transcription (STAT) pathways [27]. In response to signals from the cellular environment, c-Myc can orchestrate the many biological activities attributed to c-Myc, including proliferation, growth, transformation, apoptosis, genomic instability, angiogenesis and blockage of differentiation. The combined down-regulation of an oncogene, *c-Myc*, and up-regulation of an anti-angiogenic factor, TSP-1, will be an effective therapeutic strategy for cancer. Further study is needed to elucidate the molecular basis for the down-regulation of *c-Myc* by 5-FU, and to know the down-regulation of *c-Myc* and up-regulation of TSP-1 are involved in the antitumor activity of 5-FU.

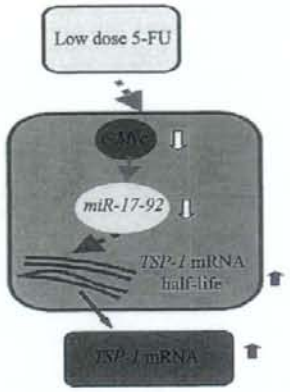


Fig. 5. Schematic pathway for 5-FU-induced TSP-1 expression in KM12C cells. In this model, downregulation of *c-Myc* by 5-FU in an unknown pathway resulted in the attenuation of *miR-17-92*. The attenuation of *miR-17-92* might be involved in the elongation the half-life of *TSP-1* mRNA.

The present study supports the ideas that expression of TSP-1 is regulated at the post-transcriptional step. A schematic representation of a proposed mechanism of TSP-1 up-regulation by 5-FU in KM12C cells is shown in Fig. 5. Our findings demonstrate the effect of 5-FU on TSP-1 expression through down-regulation of *c-Myc* and *miR-17-92* cluster in KM12C cells. A better understanding of the regulatory mechanisms of TSP-1 by 5-FU might provide new approaches to treatment of colon cancer.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2008.04.045.

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# Molecular Basis for the Induction of an Angiogenesis Inhibitor, Thrombospondin-1, by 5-Fluorouracil

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## Abstract

**5-Fluorouracil (5-FU) is one of the most commonly used anticancer drugs in chemotherapy against various solid tumors. 5-FU dose-dependently increased the expression levels of intrinsic antiangiogenic factor thrombospondin-1 (TSP-1) in human colon carcinoma KM12C cells and human breast cancer MCF7 cells. We investigated the molecular basis for the induction of TSP-1 by 5-FU in KM12C cells. Promoter assays showed that the region with the Egr-1 binding site is critical for the induction of *TSP-1* promoter activity by 5-FU. The binding of Egr-1 to the *TSP-1* promoter was increased in KM12C cells treated with 5-FU. Immunofluorescence staining revealed that 5-FU significantly increased the level of Egr-1 in the nuclei of KM12C cells. The suppression of Egr-1 expression by small interfering RNA decreased the expression level of TSP-1. Furthermore, 5-FU induced the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and heat shock protein 27 (HSP27). Blockade of the p38 MAPK pathway by SB203580 remarkably inhibited the phosphorylation of HSP27 induced by 5-FU and decreased the induction of Egr-1 and *TSP-1* by 5-FU in KM12C cells. These findings suggest that the p38 MAPK pathway plays a crucial role in the induction of Egr-1 by 5-FU and that induced Egr-1 augments *TSP-1* promoter activity, with the subsequent production of TSP-1 mRNA and protein.** [Cancer Res 2008;68(17):7035–41]

## Introduction

5-Fluorouracil (5-FU) is a commonly used anticancer drug in chemotherapy against various solid tumors (1). Recent clinical studies have shown that UFT (a prodrug of 5-FU, Tegafur, combined with uracil in a 1:4 molar ratio) is an active oral chemotherapeutic agent in postoperative adjuvant settings for completely resected early-stage lung, gastric, colorectal, and breast cancer that does not exhibit any remarkable toxicity (2). UFT can achieve a higher maximum plasma 5-FU level for a longer period by inhibiting 5-FU degradation, thereby enhancing its antitumor effect (3). Angiogenesis is an important therapeutic target for a variety of malignant tumors. UFT-containing long-term chemotherapy significantly improved patient survival (4). The antiangiogenic effect of UFT might contribute, at least in part, to its clinical efficacy. Recently, we examined the antitumor and antiangiogenesis activities of the 5-FU–based drug, S-1 (1 mol/L teagaful, 0.4 mol/L 5-chloro-2,4-dihydroxypyridine, and 1mol/L potassium oxonate), at a sub–maximum tolerated dose (sub-MTD) on human colorectal cancer xenografts. The up-regulation of thrombospondin-1 (TSP-1), as well as down-regulation of microvessel formation, has been shown (5). However, the molecular basis for the suppression of angiogenesis by 5-FU has not been fully elucidated (6).

TSP-1 has been shown to inhibit angiogenesis by inhibiting endothelial cell migration, inducing endothelial cell apoptosis, directly interacting with vascular endothelial growth factor (VEGF), and inhibiting matrix metalloproteinase-9 activation (7). In addition, TSP-1 may inhibit angiogenesis by decreasing the level of circulating endothelial cell progenitors (8). However, the molecular basis for TSP-1 induction by 5-FU and other anticancer agents is unknown (9).

In the present study, we found that 5-FU induced TSP-1 in human colon carcinoma KM12C cells. A transcription factor, Egr-1, was also induced by 5-FU and bound to the promoter of *TSP-1*, enhancing its transcription and the subsequent production of TSP-1 protein. Moreover, we present the evidence that p38 mitogen-activated protein kinase (MAPK) plays an important role in 5-FU–induced Egr-1 transactivation.

## Materials and Methods

**Reagents and antibodies.** 5-FU was provided by Taiho Pharmaceutical Co., Ltd. SB2003580 was obtained from Calbiochem. An antibody against Egr-1 was purchased from Santa Cruz Biotechnology. Mouse monoclonal antibodies against α-tubulin and TSP-1 were purchased from Oncogene and NeoMarkers, respectively. Anti–heat shock protein 27 (HSP27) G31 monoclonal and anti–phosphorylated HSP27 antibodies were obtained from Cell Signaling Technology.

**Cell lines and cell cultures.** KM12C human colon cancer cells were provided by Dr. Kiyoshi Morikawa (Iwamizawa Worker's Compensation Hospital), LOVO human colon cancer cells were purchased from Dainippon Seiyaku Co., Ltd., and MCF7 breast cancer cells were obtained from National Cancer Institute. The cells were grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum.

**RNA isolation and cDNA synthesis.** KM12C cells were treated with various concentrations of 5-FU for various periods, as described. The total RNA from the cultured cells were isolated using TRIzol (Invitrogen),

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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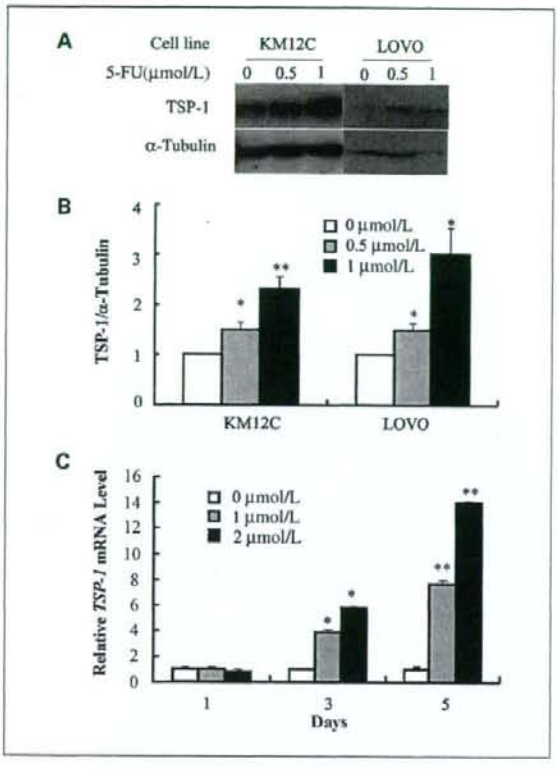


**Figure 1.** TSP-1 and *TSP-1* mRNA expression induced by 5-FU in human colon cancer cell lines. *A*, effect of 5-FU on TSP-1 protein levels in human colon cancer cell lines. Cells were treated with 5-FU at 0.5 and 1 μmol/L for 4 d. An immunoblot analysis was performed using an antibody against TSP-1. α-Tubulin was used as a loading control. *B*, quantification of relative TSP-1 protein levels. The staining intensities of the bands for TSP-1 and α-tubulin were quantified using NIH image. Protein levels of TSP-1 were normalized to α-tubulin protein levels. Expression levels of TSP-1 are shown relative to that in the untreated cells. *Columns*, average of three independent experiments; *bars*, SD. *, $P < 0.05$, **, $P < 0.01$, significantly different from untreated cells. *C*, effect of 5-FU on *TSP-1* mRNA levels in KM12C cells. Cells were treated with 5-FU at 1 and 2 μmol/L for 1, 3, and 5 d. The relative expression levels of *TSP-1* mRNA in KM12C cells were measured using real-time PCR. The expression of the *GAPDH* gene was used to normalize the values of *TSP-1*. Data are expressed relative to the *TSP-1* mRNA 5-FU–untreated cells at day 1 (considered 1). *Columns*, average of three independent experiments; *bars*, SD. *, $P < 0.05$; **, $P < 0.01$, significantly different from untreated cells on the same day.

according to the manufacturer's instructions. RNA (2 μg) was reverse-transcribed using a ReverTra Ace kit (Toyobo).

**Reverse transcription–PCR.** The expression levels of *Egr-1* and *TSP-1* were detected using reverse transcription–PCR (RT-PCR). Primer sequences are given in Supplementary Table S1.

**Real-time RT-PCR quantification.** Expression levels of *TSP-1* and *Egr-1* were determined using real-time PCR (PRISM 7900HT, Applied Biosystems), according to the manufacture's protocol. The sets of primers and TaqMan probes for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *TSP-1* and *Egr-1* (4310884E, Hs00170236, and Hs00152928, respectively) were purchased from Applied Biosystems. Human *GAPDH* was used for normalization. Target gene expression was quantified using the comparative cycle threshold method, according to the manufacturer's instructions.

**Protein extraction and immunoblotting.** KM12C and LOVO cells were plated at a density of $1 \times 10^6$ per well in a six-well plate and were treated for 4 d in the absence or presence of 5-FU (0.5 and 1 μmol/L). The cells were harvested and resuspended in lysis buffer [10 mmol/L Tris-HCl (pH 7.5), 25 mmol/L NaCl, 1 mmol/L EDTA, 0.25% Triton X-100, 2 μg/mL aprotinin, 0.5 mmol/L (*p*-amidinophenyl) methanesulfonyl fluoride, 1 mmol/L DTT, and 2 μg/mL leupeptin]. After lysis, the cell debris was removed by centrifugation at 14,000 × *g* for 15 min at 4°C.

The proteins in the whole cell lysate (200 μg) were separated using SDS-PAGE. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane and reacted with primary antibodies against TSP-1, Egr-1, phosphorylated HSP27, α-tubulin, and HSP27. After incubation, membranes were washed and incubated with antimouse or antirabbit secondary antibodies (GE Health Science). The membranes were developed using the enhanced chemiluminescence detection system (Amersham Biosciences).

**Inhibition of *Egr-1* expression by *Egr-1* small interfering RNA.** *Egr-1*–specific small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology. Transfections (40 nmol/L *Egr-1* siRNA) were accomplished using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. As a control, the cells were treated with an equal amount of *GFP* (eGFP) siRNA (Ambion). After transfection, the cells were exposed to 5-FU (1 and 2 μmol/L) for 5 d and then harvested, and the effect of the siRNA on the expression of *TSP-1* and *Egr-1* was assessed using real-time PCR, RT-PCR, and immunoblotting, as described above.

***TSP-1* promoter-reporter constructs.** Three deletion mutants of pGL3-TSP-1, namely (−1,210/+750), (−267/+750), and (−71/+750), were prepared as described previously (10).

The pGL3–TSP-1 (−2,033/+750) plasmid was constructed as follows. The *TSP-1* promoter, ranging from −2,033 to +750, was amplified using PCR with KOD plus polymerase (Toyobo) from the genomic DNA of KM12C cells using a sense primer (5′-CGGCTAGCCGTCTGCAGAGGCATTCCTACAATCCCTA-CAATCCCTCAGC-3′) and an antisense primer (5′-CCGCTCGAGATCCTG-TAGCAGGAAGCACAAGAGCCGAGG-3′). These primers contained an *Nhe*I or an *Xho*I site at the 5′ end, respectively.

The pGL3–TSP-1 (−2,033/+750 *ΔEgr-1*) plasmid was derived from pGL3–TSP-1 (−2,033/+750) plasmid, deleting the *Sty*I-*Not*I region, including the *Egr-1* binding site.

**Transient transfection and dual luciferase reporter assay.** KM12C cells were plated at a density of $1 \times 10^5$ per well in 24-well plates and pretreated with various concentrations of 5-FU for 3 d before transfection. Transfection and dual-luciferase assay were performed as described previously (11).

**Chromatin immunoprecipitation assay.** Cells treated with 5-FU, as described above, were fixed with 1% formaldehyde for 10 min at 37°C to cross-link protein to DNA. A chromatin immunoprecipitation (ChIP) assay was carried out using a ChIP assay kit (Upstate Biotechnology), according to the manufacturer's instructions. The soluble DNA fraction was mixed with an anti–Egr-1 antibody or nonimmunized mouse IgG (Santa Cruz Biotechnology), and the precipitated DNA was amplified with primers for the *TSP-1* promoter [5′-AACGAATGGCTCTCTTGGTGT-3′ (sense) and 5′-CTTTCCAGCTAGAAAGTAAG -3′ (antisense)].

**Confocal fluorescence microscopy.** KM12C cells ($7.5 \times 10^4$) were cultured in the medium with or without 2 μmol/L 5-FU for 5 d on coverslips, fixed with 3% formaldehyde in PBS for 10 min at room temperature, and permeabilized with 100% methanol for 10 min. Cells were incubated with an antibody against Egr-1 at 4°C overnight. After washing thrice in PBS, the cells were incubated with 200-fold diluted Alexa Fluor 546–labeled antirabbit IgG (Invitrogen). Nuclei were stained by incubating cells with 6 μmol/L 4′,6-diamidino-2-phenylindole (DAPI). The cells were observed using confocal fluorescence microscopy (FV500, Olympus Corporation).

**Statistical analysis.** Statistical comparisons were performed using the Student's *t* test. Quantitative data were expressed as the means ± SD. $P < 0.05$ was considered significant.

## Results

**Expression of TSP-1 protein induced by 5-FU in human colon cancer cell lines.** Our recent results have shown that the

5-FU–based drug, S-1, at sub-MTD concentration has antiangiogenic function through up-regulation of TSP-1 in colorectal cancer xenografts (5). To investigate possible mechanisms underlying the specificity of this effect, human colon cancer cells were treated with 5-FU at concentrations near or below $IC_{50}$ for 4 days (Supplementary Fig. S1). The expression of TSP-1 in two human colon cancer cell lines was then determined by immunoblot analysis using an antibody against TSP-1. When KM12C and LOVO cells were treated with 5-FU at 1 μmol/L, the expression levels of TSP-1 were 3-fold and 2-fold increased, compared with counterpart untreated cells, respectively (Fig. 1*A* and *B*). Meanwhile, treatment of the cells with 10 μmol/L 5-FU for 1 day also increased the expression of TSP-1 protein in KM12C cells (data not shown). In this study, we focused on the molecular basis for the induction of TSP-1 by low-dose 5-FU. Because the TSP-1 protein level in KM12C cells treated with 5-FU was considerably higher than that in 5-FU–treated LOVO cells, we used KM12C cells for further study.

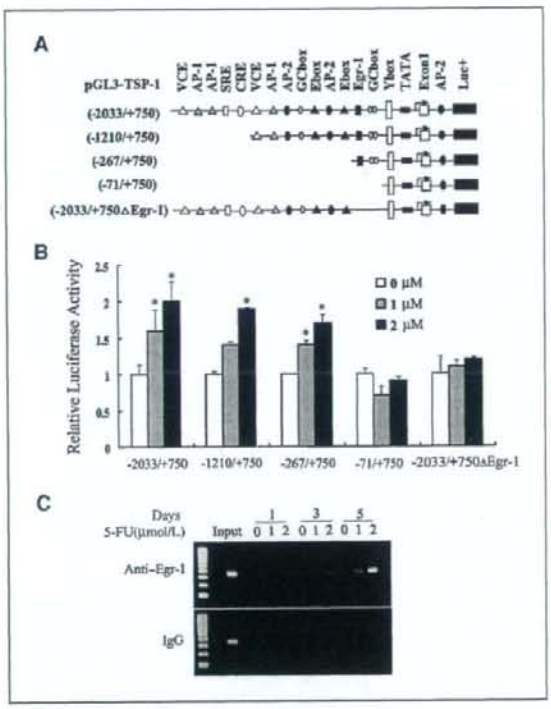


**Figure 2.** Effect of 5-FU on *TSP-1* promoter activity. *A*, schematic representation of a series of *TSP-1* promoter deletion constructs. The Egr-1–binding site is indicated by the closed square. *B*, KM12C cells were pretreated for 3 d with 5-FU, and these constructs were transiently transfected to the cells using Lipofectamine. The cells were further incubated with or without 2 μmol/L 5-FU for 48 h and harvested, and their luciferase activities were determined. The luciferase activities were corrected for differences in transfection efficiency among wells, as estimated using the *Renilla* luciferase activities. *Columns*, representative of triplicate independent experiments; *bars*, SD. *, $P < 0.05$, significantly different from untreated cells transfected with the same construct. *C*, binding of Egr-1 to *TSP-1* promotor in KM12C cells. KM12C cells were treated with 5-FU at 1 and 2 μmol/L for 1, 3, and 5 d. The cells were fixed with formaldehyde to form a DNA-protein complex and were subjected to a ChIP assay, as described in the Materials and Methods. PCR for the core promoter region of the *TSP-1* gene was performed using DNA extracted from the DNA-protein complex immunoprecipitated using an anti–Egr-1 antibody or nonimmune IgG.

**Effect of 5-FU on the expression of the *TSP-1* gene in KM12C cells.** The levels of *TSP-1* mRNA in KM12C cells incubated in the absence or presence of 5-FU were determined using real-time RT-PCR. Treatment of the KM12C cells with 5-FU at 1 and 2 μmol/L for 1, 3, and 5 days increased the *TSP-1* mRNA levels in a dose-dependent and time-dependent manner compared with those in untreated cells (Fig. 1*C*). Furthermore, the expression level of *TSP-1* mRNA in human umbilical vein endothelial cells treated with 5-FU was ~2-fold higher than in the untreated cells (data not shown). These data suggested that 5-FU enhanced TSP-1 protein expression in both cancer and endothelial cells by activating transcription of *TSP-1* gene.

**Identification of the transcriptional regulatory element necessary for transcriptional activation of the *TSP-1* gene by 5-FU.** To investigate which transcriptional regulatory elements in the *TSP-1* promoter contribute to the transcriptional activation of the *TSP-1* gene by 5-FU, we made wild-type and various deletion constructs of the *TSP-1* promoter (Fig. 2*A*). The 5′-flanking region up to −2,033 from the transcription initiation site contained several putative binding sites for known transcription factors, including an Egr-1 site and Sp-1 sites (GC boxes; Fig. 2*A*). The longest construct (−2,033/+750) showed the highest promoter activity among the constructs in the cells treated with 5-FU, and the activity was considerably decreased when the constructs lacked the region including both the Egr-1 and Sp-1 binding sites (Fig. 2*B*). These findings suggested that Egr-1 or Sp-1 transcription factors might enhance the expression of the *TSP-1* gene.

***TSP-1* promoter and its *in vivo* Egr-1 recruitment.** DNA-damaging agents can up-regulate the expression of the tumor suppressor gene *Egr-1* in both normal and cancer cells (12), whereas Sp-1 plays a role in the EGF-induced activation of the *TSP-1* gene (10). We thus focused our study on Egr-1. To confirm the recruitment of Egr-1 to the *TSP-1* promoter *in vivo*, a ChIP assay was performed (Fig. 2*C*). The Egr-1 recruitment was dose-dependently and time-dependently enhanced by 5-FU in KM12C cells. This enhancement was hardly detected when nonimmune IgG was used (Fig. 2*C*, *bottom*). These results indicated the enhanced binding of Egr-1 to the *TSP-1* promoter in cells treated with 5-FU.

**Effect of 5-FU on Egr-1 expression in KM12C cells.** The expression of *Egr-1* mRNA was also verified using RT-PCR and real-time PCR (Fig. 3). It was dose-dependently and time-dependently increased by 5-FU (Fig. 3*A* and *B*). To identify the subcellular localization of Egr-1 induced by 5-FU, Egr-1 was observed using confocal fluorescence microscopy. Egr-1 was mainly localized in the nuclei of KM12C cells treated with 5-FU but was not detected in the control cells (Fig. 3*C*).

**Effect of *Egr-1* knockdown on the induction of *TSP-1* by 5-FU.** To confirm that Egr-1 is involved in the enhanced expression of TSP-1 by 5-FU, *Egr-1* siRNAs were used to knockdown the expression of *Egr-1*. The induction of *Egr-1* mRNA and protein expression by 5-FU was considerably suppressed by *Egr-1* siRNA, but not by *GFP* siRNA. *Egr-1* knockdown resulted in the decreased expression of *TSP-1* mRNA and protein (Fig. 4*A–D*). These results show that Egr-1 is required for the 5-FU–mediated induction of TSP-1.

To examine whether Egr-1 is required for the 5-FU–mediated induction of TSP-1 in other tumor cells, we determined the expression levels of Egr-1 and TSP-1 in MCF7 cells. When MCF7 cells were treated with 1 and 2 μmol/L 5-FU at concentrations near $IC_{50}$ (Supplementary Fig. S2*A*), the expressions of Egr-1 and

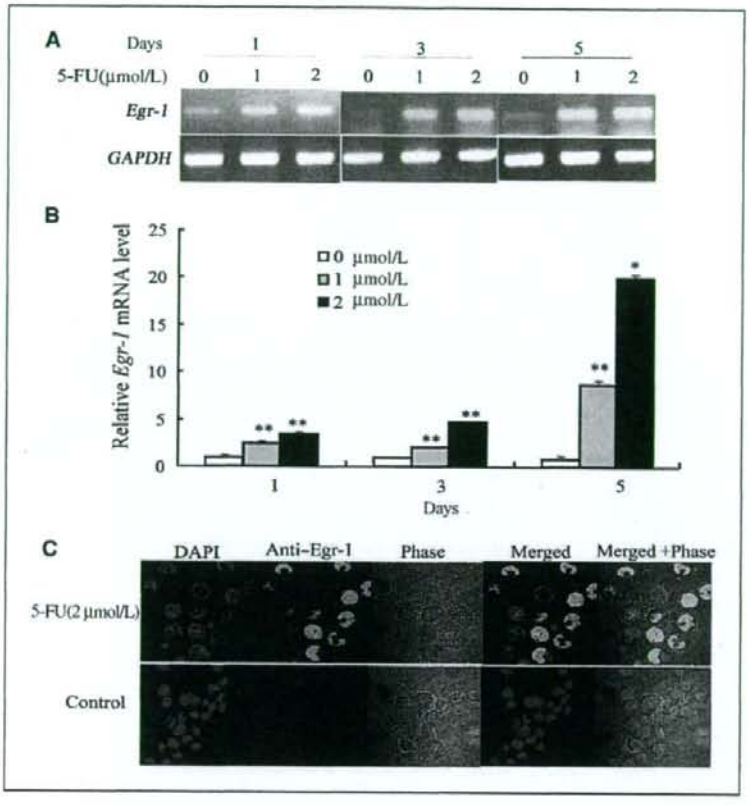


**Figure 3.** Effect of 5-FU on Egr-1 expression in KM12C cells. *A* and *B*, cells were treated with 5-FU at 1 and 2 μmol/L for 1, 3, and 5 d, and the *Egr-1* mRNA was analyzed using RT-PCR. *A*, semiquantitative RT-PCRs performed with primers specific for the indicated genes. *B*, relative expression levels of *Egr-1* mRNA in KM12C cells were measured using real-time PCR. The expression of the *GAPDH* gene was used to normalize the values of *Egr-1*. Data are expressed relative to the *Egr-1* mRNA level in untreated cells at day 1 (considered 1). *Columns*, representative of triplicate independent experiments; bars, SD. *, $P < 0.05$; **, $P < 0.01$, significantly different from untreated cells. *C*, nuclear localization of Egr-1 protein induced by 5-FU in KM12C cells. The intracellular localization of Egr-1 was analyzed using confocal fluorescence microscopy. The cells were treated with 2 μmol/L 5-FU for 5 d and stained with DAPI (*blue*) and an anti–Egr-1 antibody (*green*). *Top*, KM12C cells treated with 2 μmol/L 5-FU; *bottom*, untreated cells.

TSP-1 were also increased compared with those of untreated cells (Supplementary Fig. S2*B–D*). TSP-1 protein levels in the 5-FU–treated MCF7 cells were suppressed when Egr-1 was down-regulated by *Egr-1* siRNA (data not shown). These results suggest that our findings are not limited to KM12C cells.

**Effect of 5-FU on the activation of the p38 MAPK pathway.** Several studies have indicated the activation of one or more members of the MAPK family of intracellular signaling kinases by cytotoxic agents (13). Among them, p38 often transmits the signal generated by diverse stimuli (14). The p38 MAPK inhibitor SB203580 attenuated the TSP-1 up-regulation induced by trastuzumab and transforming growth factor-β1 (TGF-β1; refs. 10, 15). We thus focused our study on p38 MAPK. To examine whether 5-FU is involved in the activation of the MAPK pathways, the effect of 5-FU on the phosphorylation of proteins involved in p38 MAPK pathways was studied. The treatment of KM12C cells with 1 and 2 μmol/L 5-FU for 5 days increased the phosphorylation of p38, whereas the expression level of p38 remained unchanged (Fig. 5*A*). This indicated that the p38 MAPK pathway is activated by 5-FU.

**Effect of p38 MAPK inhibitor on 5-FU–induced Egr-1 and *TSP-1* expression.** To confirm that the activation of p38 MAPK contributes to 5-FU–induced Egr-1 and TSP-1 expression, we examined the effect of a p38 MAPK inhibitor on the expression of Egr-1 and TSP-1. As shown in Fig. 5, the p38 MAPK inhibitor SB203580 remarkably suppressed the expression of *Egr-1*, *TSP-1* mRNA, and Egr-1 (Fig. 5*B–D*). The p38 MAPK signaling pathway might play an important role in the 5-FU–induced expression of Egr-1 and *TSP-1* in KM12C cells. In accordance with these findings, SB203580 attenuated the 5-FU–induced phosphorylation of HSP27, one of the downstream molecules of p38 MAPK. However, it had no effect on 5-FU–induced HSP27 expression (Fig. 5*D*).

## Discussion

Previous studies indicated that TSP-1 induced by low-dose cyclophosphamide is implicated in the suppression of tumor growth (16). Furthermore, our recent study showed that 5-FU–based drugs have antitumor function partially through up-regulation of TSP-1 in colorectal cancer xenografts (5). In accordance with these results, we also found that 5-FU enhanced the expression of TSP-1 in human colon cancer (KM12C and LOVO cells) and breast cancer MCF7 cells (Fig. 1 and Supplementary Fig. S2*B* and *D*). The expression level of *TSP-1* mRNA in human umbilical vascular endothelial cell was also increased by 5-FU (data not shown). Various extracellular stimuli and compounds altered *TSP-1* gene expression (17). It is generally accepted that TSP-1 expression levels are tightly regulated at the transcriptional level. Donoviel and colleagues (18) identified the *TSP-1* promoter region and found that the 5′-flanking region between −234 and +750 was important for the basal transcriptional activity (19). The promoter region of mouse *TSP-1* contains one Egr-1 binding site, and *TSP-1* transcription is enhanced by

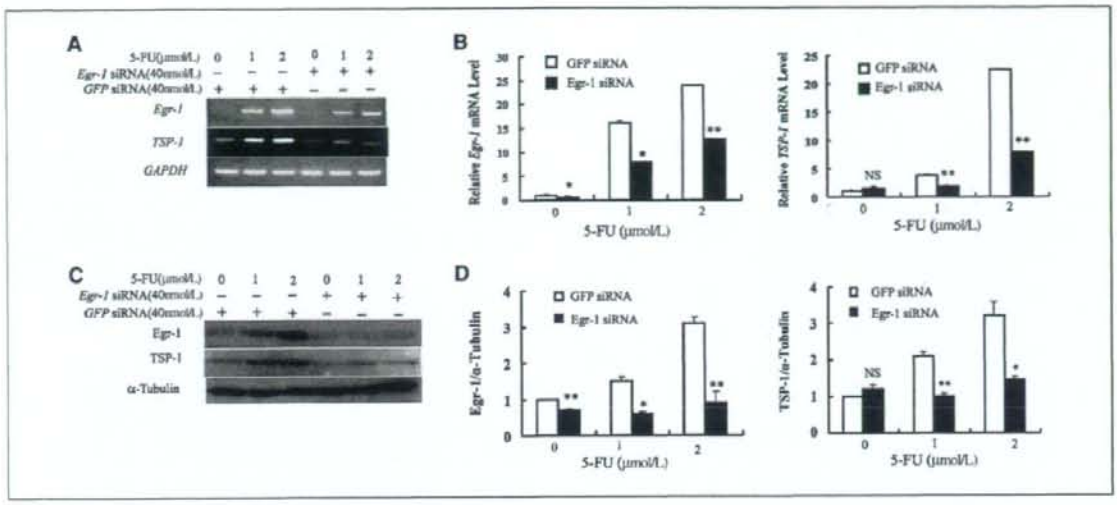


**Figure 4.** Effect of *Egr-1* knockdown on the induction of *TSP-1* in KM12C cells treated with 5-FU. KM12C cells were transfected with 40 nmol/L *Egr-1* siRNA for 6 h and then subjected to 5-FU at 2 μmol/L for 5 d. *A*, semiquantitative RT-PCRs performed with primers specific for the indicated genes. *B*, the levels of *Egr-1* (*left*) and *TSP-1* (*right*) mRNA were measured using real-time RT-PCR. The *GAPDH* gene was used to normalize the values of *TSP-1* and *Egr-1* mRNAs. Data are expressed relative to the *Egr-1* or *TSP-1* mRNA level in *GFP* siRNA-transfected and 5-FU–untreated cells. *Columns*, an average of three independent experiments; *bars*, SD. *, $P < 0.05$; **, $P < 0.01$, significantly different from *GFP* siRNA-transfected cells treated with the same concentrations of 5-FU. *C*, the expression level of Egr-1 and TSP-1 in 5-FU–treated KM12C cells transfected with *GFP* or *Egr-1* siRNA was analyzed. The cell lysates were subjected to SDS-PAGE, and the expressions of Egr-1 and TSP-1 were detected using immunoblotting with the antibodies indicated in Materials and Methods. *D*, Egr-1 (*left*) and TSP-1 (*right*) protein levels were determined as described in Fig. 1*B*. Data are expressed relative to the levels of Egr-1 or TSP-1 in *GFP* siRNA-transfected and 5-FU–untreated cells. *Columns*, an average of three independent experiments; *bars*, SD. *, $P < 0.05$; **, $P < 0.01$, significantly different from *GFP* siRNA-transfected cells untreated with 5-FU.

Egr-1 (20). In human hepatic HuH-7 cells, the *TSP-1* promoter region between −267 and −71 contained two GC boxes to which Sp-1 bound. These boxes were found to be responsible for the promoter activity enhanced by EGF (10). Consistent with these studies, we also showed that *TSP-1* promoter region (−267/−71) is needed for the augmentation *TSP-1* promoter activity by 5-FU, and the deletion of the region in which the Egr-1 and SP-1 binding sites reside almost completely blocked the *TSP-1*

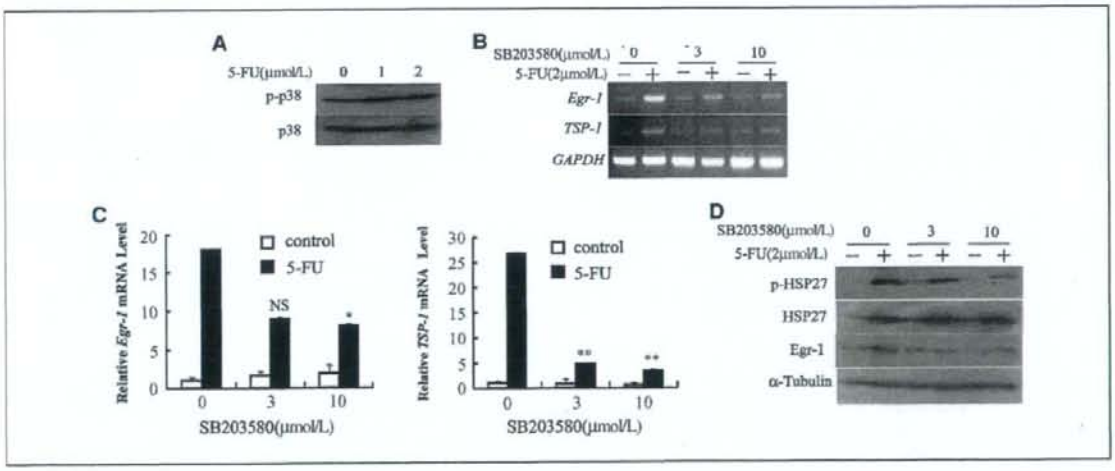


**Figure 5.** Activation of p38 MAPK pathway by 5-FU and the effect of the activated p38 MAPK pathway on the 5-FU–induced expression of *TSP-1* mRNA levels and Egr-1 expression in KM12C cells. *A*, KM12C cells were exposed to 1 or 2 μmol/L of 5-FU for 5 d, and an immunoblot analysis was performed using an antibody against phosphorylated p38 kinase. The blot was reprobed with an antibody against p38. KM12C cells were treated as described above, and 5-FU–induced *Egr-1* and *TSP-1* mRNA levels were measured using semiquantitative RT-PCR and real-time RT-PCR. *B*, semiquantitative RT-PCRs performed with primers specific for the indicated genes. *C*, relative expression levels of *Egr-1* (*left*) and *TSP-1* (*right*) mRNAs in KM12C cells were measured using real-time RT-PCR. The expression of the *GAPDH* gene was used to normalize the values of *Egr-1* and *TSP-1*. Data are expressed relative to the *Egr-1* or *TSP-1* mRNA in the untreated cells. *Columns*, an average of three independent experiments; *bars*, SD. *, $P < 0.05$; **, $P < 0.01$, significantly different compared with the cells treated with 2 μmol/L 5-FU alone. *D*, KM12C cells were exposed to 2 μmol/L 5-FU with or without MAPK inhibitor for 5 d, and an immunoblot analysis was performed using the indicated antibodies. α-Tubulin was used as an internal control.

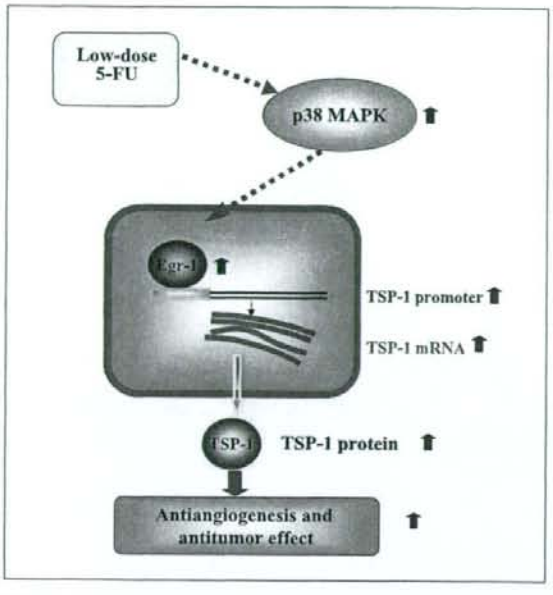


**Figure 6.** Schematic pathway for 5-FU–induced TSP-1 expression in KM12C human colon cancer cells. In this model, 5-FU causes the activation of the p38 MAP kinase pathway, leading to the increased expression of Egr-1. Egr-1 binds to the promoter of *TSP-1* and enhances the transcription of the *TSP-1* gene and subsequently the expression of Egr-1.

promoter activity (Fig. 2*B*). Furthermore, the increased binding of Egr-1 to the *TSP-1* promoter in cells treated with 5-FU was also observed (Fig. 2*C*).

Egr-1 is a Cys2-His2–type zinc-finger transcription factor and binds to GC-rich, *cis*-acting promoter elements, controlling the expression of a wide variety of pathogenesis-relevant genes, encoding growth factors, cytokines, receptors, adhesion molecules, and proteases, many of which are involved in angiogenesis, tumorigenesis (21), the response to ischemia (22), and the progress of several vascular diseases (23). A number of reports also indicate that *Egr-1* acts as a tumor suppressor gene. Egr-1 is down-regulated in several types of neoplasia, as well as an array of tumor cell lines. Egr-1 is induced very early in the apoptotic process, where it mediates the activation of downstream regulators, such as p53 (24). Egr-1 also activates phosphatase and tensin homologue tumor suppressor gene during UV irradiation (25), suppressing the growth of transformed cells in both soft agar and athymic nude mice (26). Sustained Egr-1 expression may cause the induction of multiple pathways of antiangiogenesis, growth arrest, and apoptosis induction in proliferating cells leading to preferential inhibition of angiogenesis and tumor growth. Egr-1 possesses a strong inhibitory effect on the angiogenic activity of VEGF *in vivo* (27). Our results showed that Egr-1 induced by 5-FU was needed for the increased expression of TSP-1 in KM12C cells treated with 5-FU (Figs. 3 and 4).

MAPK pathways have been implicated in the response to chemotherapeutic drugs (28). c-Jun amino-terminal kinase and p38 kinase are important for controlling cell growth and apoptosis in response to chemical stress, radiation, and growth factors (29). Our results showed that 5-FU activated p38 MAPK (Fig. 5*A*). Activated p38 MAPK phosphorylates MAPKAP kinase 2, which in turn phosphorylates HSP27 (30). SB203580 attenuated the 5-FU–enhanced phosphorylation of HSP27, indicating that SB203580 effectively inhibited the activation of p38 pathway in 5-FU–treated KM12C cells. SB203580 suppressed the expression of both *Egr-1* and *TSP-1* mRNAs, suggesting that the activation of the p38 MAPK pathway by 5-FU is responsible for the induction of Egr-1 and TSP-1 (Fig. 5*B–D*). Trastuzumab also stimulated sustained p38 activation, and SB203580 attenuated the TSP-1 up-regulation induced by trastuzumab (15). SB203580 partially inhibited TGF-β1–induced TSP-1 expression (10). These findings suggest that the activation of p38 MAPK plays an important role in the induction of TSP-1 by some anticancer agents and growth factors. Further study is needed to determine the detailed mechanisms underlying the regulation of the p38 MAPK pathway by 5-FU.

Several transcription factors are regulated by p38 MAPK, and this kinase is involved in the control of the expression of various genes. *In vitro* studies show that the transcription factor ATF2 is phosphorylated and activated by p38 MAPK. In addition, p38 MAPK activates the Elk-1, CHOP, MEF2C, and SAP-1 transcription factors (31). Our finding that 5-FU activated p38 MAPK and then increased the expression of Egr-1 may be useful for elucidating the molecular basis for the chemopreventive and antitumor effects of 5-FU and its prodrugs.

HSP27 is a molecular chaperone that is constitutively expressed in several mammalian cells, particularly during pathologic conditions. This protein protects cells against toxicity mediated by aberrantly folded proteins or oxidative inflammatory conditions. In addition, this protein has antiapoptotic properties and is tumorigenic when expressed in cancer cells (32). Some anticancer agents, particularly cisplatin (33), vincristine, and colchicines (34), also enhanced HSP27 expression. It is as yet unknown whether the induced HSP27 affects the antitumor activity of these anticancer agents.

A schematic representation of a proposed molecular basis for the up-regulation of TSP-1 by 5-FU in KM12C cells is shown in Fig. 6. Our findings show that 5-FU activated p38 MAPK and then up-regulated Egr-1 expression, resulting in the expression of an endogeneous antiangiogenic factor, TSP-1. Recently, we have found that the expression of *VEGF* mRNA was suppressed by 5-FU using Genechip analysis (11). VEGF is produced in varying quantities in tumors and seems to be an important modulator of TSP function. Relative ratios of TSPs to VEGF might determine whether vessels regress or proliferate (17). Further study is needed to elucidate whether TSP-1 induced by 5-FU is involved in the antitumor effect of 5-FU. A better understanding about the mechanisms of the antiangiogenic and the antitumor effect of 5-FU might provide new approaches for the treatment of colon and breast cancers.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

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# Growth inhibitory effects of pegylated IFN-α2b and 5-fluorouracil in combination on renal cell carcinoma cell lines *in vitro* and *in vivo*

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**Abstract.** We investigated the effects of pegylated IFN-α2b (PEG-IFN-α2b) alone and PEG-IFN-α2b plus 5-fluorouracil (5-FU) *in vitro* on the proliferation of renal cell carcinoma (RCC) cell lines. After the transplantation of RCC cells into nude mice, we administered IFN (PEG-IFN-α2b or IFN-α2b) alone, 5-FU alone, or IFN (PEG-IFN-α2b or IFN-α2b) plus 5-FU; and investigated tumor volume, tumor weight, the numbers of apoptotic cells and artery-like blood vessels, relative mRNA expression levels of enzymes which relate to 5-FU metabolism, angiogenesis factor, and type I interferon receptor. RCC cells *in vitro* were generally and relatively resistant to the anti-proliferative effects of PEG-IFN-α2b, but the addition of 5-FU augmented IFN-induced anti-proliferative effects with the induction of apoptosis. PEG-IFN-α2b *in vivo* presented stronger anti-tumor effects than IFN-α2b, and its combination with 5-FU augmented the effects. The significant anti-tumor effect of the combination treatment was the increase in apoptotic cell number, but there were no significant differences in the suppression of angiogenesis, expression of IFN receptor, and the actions of metabolic enzymes of 5-FU. In conclusion, PEG-IFN-α2b presents stronger anti-tumor effects than non-pegylated IFN, and the effects are augmented in the combination with 5-FU. Our findings suggest the clinical usefulness of PEG-IFN-α2b in the treatment of RCC.

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*Key words:* renal cell carcinoma, pegylated interferon-α2b, 5-fluorouracil, combination therapy, apoptosis

## Introduction

Renal cell carcinoma (RCC) is highly resistant to conventional chemotherapy. The objective response rate is 6-9% for vinblastine and 5-8% for 5-fluorouracil (5-FU) (1). The response rates of treatment regimens using interleukin-2 are 6-31% (2), and the therapeutic response rates of interferon (IFN)-α are 4-33% in patients with metastatic RCC (3). The response rates of immunochemical therapies that utilize chemotherapeutic agents with IFN-α or interleukin-2 range between 8 and 39% (4). Immunochemical therapy is the best treatment for advanced RCC, but potential synergetic effects of the medicines as well as their mechanisms remain to be elucidated.

Wadler and Wienik (5) for the first time proposed a combination therapy of IFN-α and 5-FU in 1988 in their study using colon cancer cell lines. Later, this combination therapy was applied to various types of human malignancies including RCC and hepatocellular carcinoma (HCC). 5-FU has two major anti-tumor mechanisms: one involves its active metabolite 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), inhibiting the activity of thymidylate synthase (TS) and consequently DNA synthesis; the other is related to the incorporation of 5-FU metabolite into RNA and DNA, thereby disrupting normal RNA processing and function. The sensitivity of cancer cells to 5-FU is often influenced by the enzymes affecting 5-FU metabolism, including TS, dihydropyrimidine dehydrogenase (DPD), orotate phosphoribosyl transferase (OPRT), thymidine phosphorylase (TP), uridine phosphorylase (UP) and thymidine kinase (TK).

PEG-IFN-α2b, a new interferon, is a covalent conjugate of recombinant IFN-α2b with monomethoxy polyethylene glycol (PEG) in a 1:1 molar ratio that produces a 31,000-Da molecule (6). PEG conjugation increases the size of the molecule, therefore, the absorption of the pegylated molecule is slower, its serum half-life is longer, and its rate of clearance from the plasma is lower than that of the unmodified molecule. PEG-IFN-α2b thereby increases patient exposure

to IFN-α2b and requires less frequent administration (6). Clinical trials in chronic hepatitis C patients have suggested that PEG-IFN-α preparations produce more potent therapeutic effects than IFN-α preparations (6-10). Yano *et al* (11) examined the *in vitro* and *in vivo* anti-tumor effects of PEG- and non-PEG-IFN-α2b on human liver cancer cells, and they reported that the anti-tumor effect of PEG-IFN-α2b was significantly more potent than that of non-PEG-IFN-α2b. In addition, Motzer *et al* (12) conducted a phase I study of PEG-IFN-α2b on advanced renal cancer patients, and reported that partial response was obtained in 5 (19%) patients. Yet, there have been few basic studies evaluating the efficacy of PEG-IFN-α2b on RCC *in vitro* and *in vivo*.

Our current study examined the *in vitro* and *in vivo* anti-tumor effects of PEG-IFN-αb, IFN-α2b, 5-FU, and the combination of one of the two IFNs and 5-FU, on RCC cell lines, using PEG-IFN-α2b concentrations close to the clinical dosage. We also examined the effects of the therapies on apoptotic cells, artery-like blood vessels, the enzymes affecting 5-FU metabolism, vascular endothelial growth factor (VEGF), and type I IFN receptor subunits in human RCC tumors which were developed in nude mice.

## Materials and methods

*Cell lines and cell culture*. This study used 8 human RCC cell lines. KRC/Y (13) was established in our laboratory. KUR11 and KURM were donated by Professor K. Itoh of the Department of Immunology at our University. Caki-1, Caki-2, and ACHN were purchased from American Type Culture Collection. VMRC-RCW was purchased from Japan Health Sciences Foundation. OS-RC-2 was purchased from Riken Cell Bank (Tsukuba, Japan).

Culture medium for KRC/Y consisted of Dulbecco's modified Eagle's medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with heat-inactivated (56°C, 30 min) 5% fetal bovine serum (FBS, Bioserum, Vic, Australia), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL/Life Technologies Inc., Gaithersburg, MD). Culture medium for Caki-1, Caki-2, VMRC-RCW and ACHN consisted of modified Eagle's medium (Gibco); the medium for KUR11, KURM and OS-RC-2 consisted of RPMI-1640; and each medium was supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in an atmosphere of 5% $CO_2$ in air at 37°C. 5-FU was purchased from Kyowa Hakko K.K. (Tokyo, Japan).

*IFN and reagents*. PEG-IFN-α2b (PEG Intron®) and IFN-α2b (Intron® A) were provided by Schering-Plough K.K. (Osaka, Japan). The specific activity of PEG-IFN-α2b was $6.4 \times 10^7$ IU/mg protein and that of IFN-α2b was $2.6 \times 10^8$ IU/mg protein.

Rat antibody against mouse endothelial cells (anti-CD34, clone MEC14.7) was purchased from Serotec Co., Oxford, UK; and mouse monoclonal antibody against human α-smooth muscle actin (SMA) that cross-reacts with mouse α-SMA (clone 1A4), from Immunon (Pittsburgh, PA).

*Effects of PEG-IFN-α2b on the proliferation of RCC cell lines in vitro*. The effects of PEG-IFN-α2b or 5-FU on cell proliferation were examined in colorimetric assays by using 3-(4,5-dimethylthiazol-2yl-yl-)-2,5-diphenyl tetrazolium bromide (MTT) cell growth assay kits (Chemicon International Inc.) as described elsewhere (14). Briefly, the RCC cells ($1.5\text{-}5 \times 10^3$ cells per well) were seeded on 96-well plates (Nunc Inc., Roskilde, Denmark), cultured for 24 h, and the culture medium was changed to a new medium with or without PEG-IFN-α2b (8, 32, 128, 512 or 2,048 IU/ml). After culturing for 24, 48, 72 or 96 h, the number of viable cells was measured with ImmunoMini NJ-2300 (Nalge Nunc International, Tokyo, Japan) by setting the test wavelength to 570 nm and the reference wavelength to 630 nm. To keep the optical density within linear range, all experiments were performed when the cells were in the logarithmic growth phase. The effects of IFN-α2b on the growth of VMRC-RCW cells were also examined in the same manner.

*Effects of combination therapy of PEG-IFN-α2b and 5-FU on the proliferation of RCC cell lines in vitro*. RCC cells (VMRC-RCW, 3,000 cells/well) were seeded on 96-well plates (Nunc Inc.), cultured for 24 h, and then the culture medium was changed to a new medium containing PEG-IFN-α2b alone (0 160, 317, 625, 1,250, 2,500, 5,000 or 10,000 IU/ml); 5-FU alone (0, 0.6, 1.25, 2.5, 5 or 10 μM); or both 5-FU (0, 0.6, 1.25, 2.5, 5, 10 μM) and PEG-IFN-α2b (0, 160, 317, 625, 1,250, 2,500, 5,000 or 10,000 IU/ml). After 96 h of culture, the number of viable cells was examined by MTT assay as described above.

The synergy of cooperative cytotoxicity was determined by the median-effect principle as described by Chou and Talalay (15). Data from each sample were analyzed by using CalcuSyn ver. 2 (Biosoft, Cambridge, UK).

*Morphological observation*. For morphological observation by light microscopy, 8 RCC cell lines were seeded on Lab-Tek tissue culture chamber slides (Nunc Inc.), cultured with or without PEG-IFN-α2b (1,024, 4,098 or 8,192 IU/ml) for 72 h, fixed for 30 min in Carnoy's solution, and stained with hematoxylin and eosin (H&E).

In another experiment, one RCC cell line (VMRC-RCW, 8,000 cells/chamber) was seeded on Lab-Tek tissue culture chamber slides (Nunc Inc.), cultured with PEG-IFN-α2b alone (0, 160, 317, 625, 1,250, 2,500, 5,000 IU/ml); 5-FU alone (0, 0.6, 1.25, 2.5, 5.0 μM); PEG-IFN-α2b (0, 160, 317, 625, 1,250, 2,500, 5,000 IU/ml) plus 5-FU (0, 0.6, 1.25, 2.5, 5.0 μM), or PBS, for 72 h, fixed for 30 min in Carnoy's solution and H&E stained.

*Effects of PEG-IFN-α2b and IFN-α2b on RCC cell proliferation in nude mice*. Cultured VMRC-RCW cells ($1.0 \times 10^7$ cells/mouse) were subcutaneously (s.c.) injected into the backs of 4-week-old female BALB/c athymic nude mice (n=62) (Clea Japan, Inc, Osaka, Japan). One week later when the largest diameter of the tumor reached ~10 mm (day 0), the mice were divided into 7 groups (n=8 or 9 each) in a manner to equalize the mean tumor diameter of each group. Each mouse received a subcutaneous injection of 0.1 ml of medium alone (control group), medium containing 640, 6,400, 64,000 or 640,000 IU of PEG-IFN-α2b, or medium containing 640 or 6,400 IU of IFN-α2b, twice a week for 2 consecutive weeks (on day 1, 4,

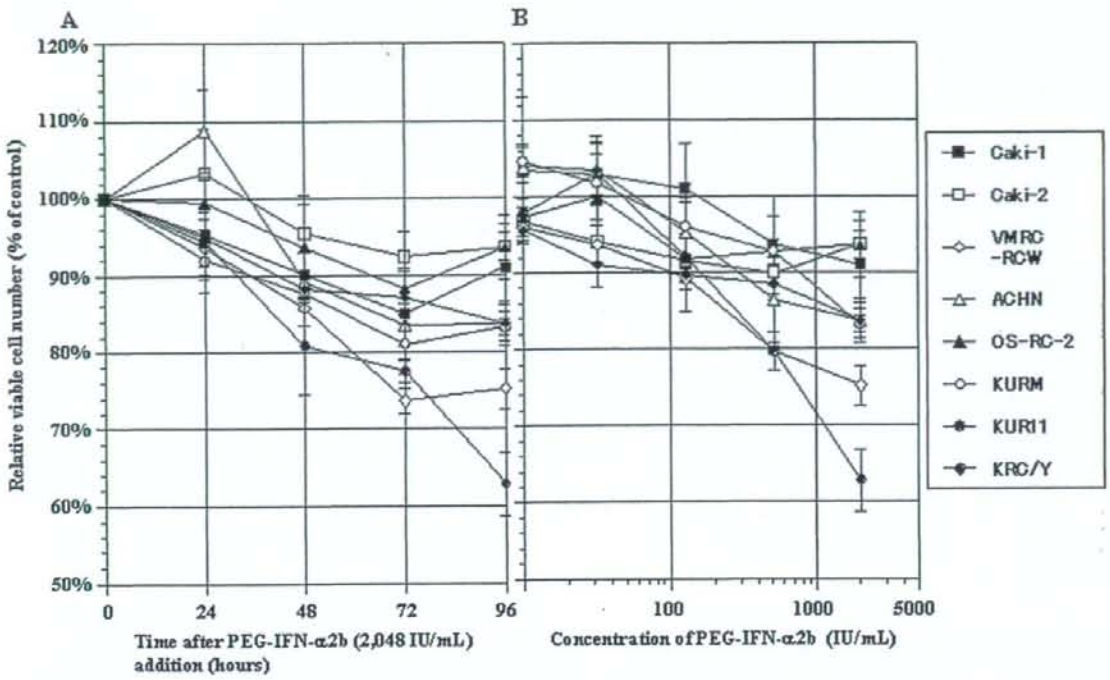


Figure 1. Anti-proliferative effects of PEG-IFN-α2b. (A) Chronological changes in relative viable cell number (% of the control) after adding 2,048 IU/ml of PEG-IFN-α2b. Growth was significantly suppressed over time in 2 cell lines (KUR11 and KRC/Y). (B) Ninety-six hours after adding 8, 32, 128, 512 or 2,048 IU/ml of PEG-IFN-α2b. Cell proliferation was suppressed in a dose-dependent manner in 6 cell lines (all but Caki-2 and OS-RC-2). Eight samples were used in each experiment. The experiment was repeated at least three times for each cell line. The values represent the average ± SE.

8 and 11). The clinical daily dose of IFN-α2b for human RCC is $600x10^4$ IU/body ($1.2x10^5$ IU/kg), and this is approximately four times the lowest dose ($3.2x10^4$ IU/kg) used in this experiment. Tumor size was measured in two directions by using calipers on the first and second days of s.c. injection (day 1 and 2) and then once every 2 days until day 14, and tumor volume ($mm^3$) was estimated by using the equation: Length x $(Width)^2$ x 0.5. Mouse body weight was measured on day 0, 8 and 14. On day 15, all mice were sacrificed and the tumors were removed.

The animals received human care according to criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985).

*Effects of combination therapy of PEG-IFN-α2b and 5-FU on RCC cell proliferation in nude mice.* VMRC-RCW cells ($7.5x10^6$ cells/mouse) were subcutaneously injected into 4-week-old female BALB/c athymic nude mice (n=58). The mice were divided into 7 groups (n=8 or 9 each) on day 7 when tumor size reached ~10 mm in diameter, and each group was assigned to one of the 7 treatments: i) PEG-IFN-α2b alone (6,400 IU); ii) IFN-α2b alone (6,400 IU); iii) low dose 5-FU alone (160 μg); iv) high dose 5-FU alone (320 μg); v) combination therapy of PEG-IFN-α2b (6,400 IU) and low dose 5-FU; vi) combination therapy of IFN-α2b (6,400 IU) and low dose 5-FU; and (vii) control.

5-FU was administered intra-abdominally every day for 2 consecutive weeks. The dose of 5-FU (160 μg/mouse, 8 mg/kg) is comparable to the clinical dose.

Tumor size measurement and IFN administration were performed in the same manner as described above. On day 15, all mice were sacrificed and each tumor was removed. After the tumor weight was measured, half of the obtained tumors were used for histological examination and the other half were used for quantitative real-time RT-PCR.

The number of cells showing characteristics of apoptosis such as cytoplasmic shrinkage, chromatin condensation and nuclear fragmentation was counted in ten 0.25 $mm^2$ areas within an H&E-stained specimen, and the average number per area was obtained. The TUNEL technique (ApopTag® Peroxidase In Situ Apoptosis Detection Kits, Chemicon International, CA, USA) was also used to detect apoptotic cells. The average number of TUNEL-positive cells per area was obtained as described above.

*Immunohistochemistry.* Double immunohistochemical staining was performed by using anti-mouse endothelial cell (anti-CD34) antibody, anti-human α smooth muscle actin (α-SMA) antibody and histofine simple stain mouse Max-Po (Rat) kits (Nichirei, Tokyo, Japan) as described elsewhere (16). We

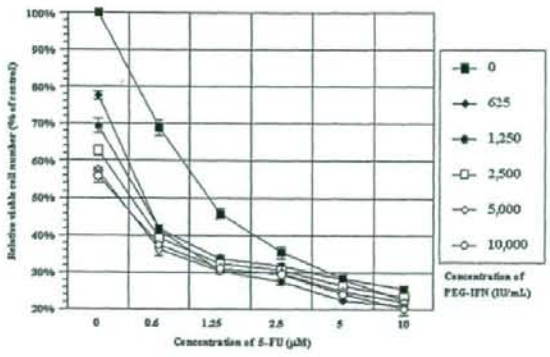


Figure 2. Anti-proliferative effects of the combination therapy of PEG-IFN-α2b and 5-FU on VMRC-RCW cells in a 96-h culture. The relative viable cell number decreased dose-dependently. Two samples were used in each experiment. The experiment was repeated three times. The values represent the average ± SE. PEG-IFN, PEG-IFN-α2b.

calculated the number of artery-like blood vessels in the entire area of each section and obtained for each the mean number per $mm^2$.

*cDNA preparation and quantitative real-time RT-PCR.* Total RNA was extracted using RNA-Bee™ (Tel-Test, Inc., TX) and reverse transcribed using Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, CA) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed with an ABI PRISM 7300 (Applied Biosystems, Foster City, CA). We examined 6 enzymes related to 5-FU metabolism, i.e., TS, TP, DPD, OPRT, UP and TK. The sequences of the primers and probes for the 6 enzymes are listed elsewhere (17). The sequences for VEGF were 5'-CCATGAACTTTCTGCTGTCTTGG-3' as the forward primer, 5'-CTGCGCTGATAGACATCCATGA-3' as the reverse primer, and 5'-TGCTCTACCTCCACCATGC CAAGT-3' as the probe. The sequences of the primers and probes for VEGFR-1, IFNAR-1, IFNAR-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems.

*Statistical analysis.* We used two-factorial ANOVA for the comparisons of tumor volume, tumor weight, number of apoptotic cells, number of artery-like blood vessels, and relative levels of mRNAs related to 5-FU metabolism.

## Results

*Effects of PEG-IFN-α2b on the proliferation of RCC cell lines in vitro.* After adding 2,048 IU/ml of PEG-IFN-α2b, the relative viable cell number of the cultured 8 cell lines was suppressed in a time-dependent manner until 72 h, but at 96 h, suppression was noted in only 2 cell lines (KRC/Y and KUR11). On the other hand, with different doses of PEG-IFN-α2b, the relative viable cell number at 96 h was suppressed in the 6 cell lines, i.e., VMRC-RCW, KRC/Y, KURM, KUR11, ACHN and Caki-1. In the 8 cell lines, $IC_{50}$ was not reached for either time- and dose-dependent suppressions, but the most sensitive case was KUR11 with the dose of 2,048 IU/ml at 96 h, i.e., the relative viable cell number was 62.7% of the control (Fig. 1).

In the VMRC-RCW cell line, the anti-tumor effects of PEG-IFN-α2b and IFN-α2b were not markedly different.

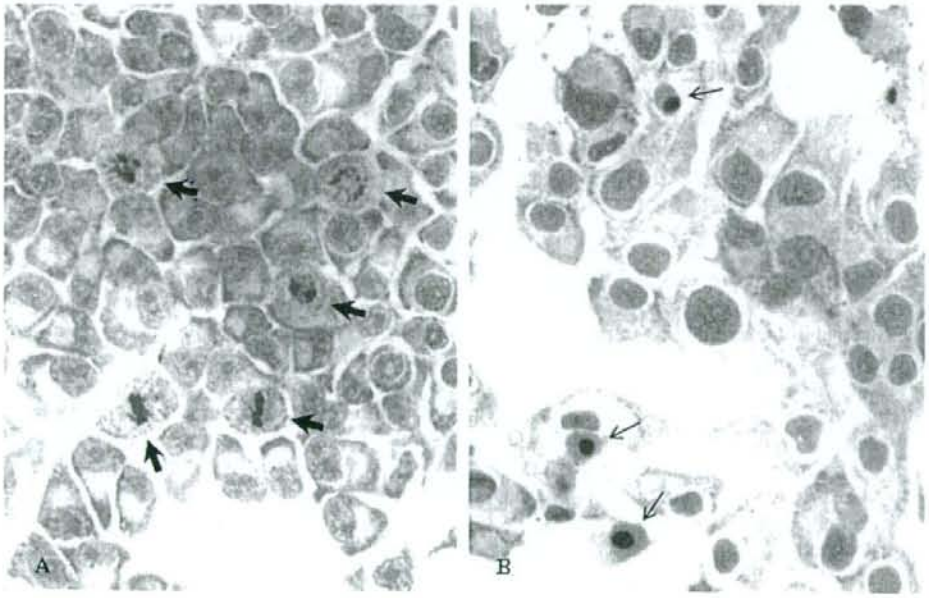


Figure 3. Photomicrograph of VMRC-RCW cells cultured for 72 h on a Lab-Tek Chamber Slide. (A) Without PEG-IFN-α2b in culture medium. Mitotic figures (thick arrows) were noted. (B) With 2,500 IU/m of PEG-IFN-α2b and 2.5 μM of 5-FU in culture medium. Apoptotic cells (thin arrows) characterized by cytoplasmic shrinkage and chromatic condensation were noted (H&E staining, x200).