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Selective gene silencing of rat ATP-binding cassette G2 transporter in an *in vitro* blood–brain barrier model by short interfering RNA

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Abstract

The aim of the present study was to specifically silence the rat ATP-binding cassette transporter G2 (rABCG2) gene in brain capillary endothelial cells by transfection of short interfering RNA (siRNA). Four different siRNAs designed to target rABCG2 were each transfected into HEK293 cells with myc-tagged rABCG2 cDNA. Quantitative real-time PCR and western blot analyses revealed that three of the siRNAs were able to reduce exogenous rABCG2 mRNA and protein levels in HEK293 cells. Moreover, rABCG2-mediated mitoxantrone efflux transport was suppressed by the introduction of these three siRNAs into HEK293 cells. In contrast, the other siRNA and non-specific control siRNA did not significantly affect the mRNA expression, the protein level or the transport activity. Endogenous rABCG2 mRNA and protein expression in a conditionally immortalized rat brain capillary endothelial cell line (TR-BBB13) was suppressed by the most potent siRNA among the four siRNAs tested. Furthermore, this siRNA did not affect the mRNA levels of other ABC transporters, such as ABCB1, ABCC1 and ABCG1, and the protein level of ABCB1 in TR-BBB13 cells, suggesting that it can selectively silence rABCG2 at the blood–brain barrier. This should be a useful and novel strategy for clarifying the contribution of rABCG2 to brain-to-blood transport of substrate drugs and endogenous compounds across the blood–brain barrier.

Keywords: ABC transporter, ATP-binding cassette transporter G2, 17β-estradiol, blood–brain barrier, *in vitro* blood–brain barrier model, short interfering RNA.

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The blood–brain barrier (BBB), which is formed by the tight intercellular junctions of brain capillary endothelial cells (BCECs), strictly regulates the transfer of substances between the circulating blood and the brain (Terasaki and Hosoya 1999; Hosoya *et al.* 2002). Therefore, the molecular mechanisms of efflux transport from the brain have important implications for drug delivery and CNS homeostasis.

ABCG2 (BCRP/MXR/ABCP1) is an ATP-binding cassette (ABC) transporter localized on the luminal side of brain capillaries in humans (Cooray *et al.* 2002) and rats (Hori *et al.* 2004), and transports a diverse array of compounds out of the cells (Allen and Schinkel 2002). Therefore, ABCG2 present in BCECs may act to restrict the penetration of xenobiotics into the brain and to pump out potential toxins or metabolites from the brain. ABCG2 transports sulfated conjugates of drugs and sterols (Suzuki *et al.* 2003), whereas p-glycoprotein (P-gp), a well-characterized efflux transporter at the BBB, preferentially transports hydrophobic

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Abbreviations used: ABC, ATP-binding cassette; BBB, blood–brain barrier; BCEC, brain capillary endothelial cell; DHEAS, dehydroepiandrosterone sulfate; NC, non-specific control; PMSF, phenylmethylsulphonyl fluoride; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; siRNA, short interfering RNA; TR-BBB, conditionally immortalized brain capillary endothelial cell line.

compounds. Therefore, ABCG2 may have a distinct role in efflux transport at the BBB.

Several ABC transporters and organic anion transporters are expressed at the abluminal and/or luminal membrane of the BBB as well as ABCG2 (Gao *et al.* 1999; Virgintino *et al.* 2002; Mori *et al.* 2003). Clarifying the transport properties and the contribution of each transporter at the BBB is an important issue for understanding the physiological roles of these molecules. However, the substrate and inhibitor specificities of these transporters sometimes overlap. For example, dehydroepiandrosterone sulfate (DHEAS) is transported from brain to the circulating blood across the BBB via organic anion transporting polypeptide 2 (Asaba *et al.* 2000), while other transporters at the BBB, such as ABCG2 and ABCC4 (Zhang *et al.* 2000; Cooray *et al.* 2002; Hori *et al.* 2004), also accept DHEAS as a substrate (Suzuki *et al.* 2003; Zelcer *et al.* 2003).

Three effective inhibitors of ABCG2 have been described thus far. GF120918 was developed as a P-gp (ABCB1) inhibitor (Hyafil *et al.* 1993), but a later study found that it also inhibits ABCG2 (de Bruin *et al.* 1999). Such a dual-specificity inhibitor is unsuitable for clarifying the distinct transport activity of each transporter. Fumitoremorgin C and Ko143 are potent and selective inhibitors for ABCG2, being much less active towards P-gp and ABCCs (Rabindran *et al.* 2000; Allen *et al.* 2002). Nevertheless, the specificity of these inhibitors is concentration-dependent, and an influence of these two inhibitors on unidentified transporters at the BBB cannot be ruled out.

RNA interference is a conserved biological response to double-stranded RNA, which results in sequence-specific gene silencing (Hannon 2002). In mammalian cell cultures, double-stranded RNA-mediated interference with gene expression has also been accomplished by transfection of synthetic RNA oligonucleotides composed of 21 or 22 base pairs (short interfering RNA, siRNA; Elbashir *et al.* 2002). Sequence-specific silencing of transporter genes using siRNA should make it possible to evaluate properly the transport properties of a targeted transporter at the BBB.

Conditionally immortalized BCEC lines are useful *in vitro* BBB models which retain the *in vivo* transport properties towards various compounds (Hosoya *et al.* 2000a, 2000b; Terasaki *et al.* 2003). Endothelial cells are generally resistant to the introduction of exogenous DNA, and molecular analysis of endothelial cells has been hampered by the difficulty of transiently transfecting genes with high efficiency. Therefore, siRNA-induced specific knockdown of target transporter genes in BCECs should allow us to improve our understanding of the physiological and pharmacological functions of the efflux transport systems at the BBB.

The purpose of this study was therefore to specifically silence rABCG2 gene by the introduction of siRNA into BCECs, in order to clarify the role of ABCG2 at the BBB.

Materials and methods

Reagents

Endothelial cell growth factor (ECGF) was purchased from Boehringer Mannheim (Mannheim, Germany). Benzylpenicillin potassium and streptomycin sulfate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Non-specific Control Duplex XI (NC siRNA; Dharmacon, Lafayette, CO, USA) is claimed by the manufacturer to show no RNAi effect, and its target sequence is 5′-NNATAGATAAGCAAGCCTTAC-3′. No rat gene sequences with homology to NC siRNA were found by Blast search. β-Actin siRNA was purchased from Qiagen (Tokyo, Japan); its target sequence is 5′-AATGAAGATCAAGATCATTGC-3′. The sequence of β-actin siRNA is identical at 20 bp out of 21 bp with the corresponding sequence of rat β-actin (the underlined base in the sequence of β-actin siRNA is changed to 'C' in that of rat β-actin). All other chemicals were commercial products of analytical grade.

siRNA preparation

Four different siRNA duplexes were designed based on the coding sequence of rABCG2 cDNA (GenBank accession number AB105817). All 21-nucleotides (nt) siRNAs contained 3′-dTdT extensions and their GC contents were less than 70%. The sequences, positions and GC contents of siRNA targeting rat ABCG2 are shown in Table 1. All of the siRNA duplexes were

Table 1 Sequences of rABCG2 short interfering RNAs (siRNAs)

Number of rABCG2 siRNA	Sequences (upper, sense; lower, antisense)	Positions*/GC (%)
01	5′-CAGAGAAACAAGAACGGCCdTdT dTdTGUCUCUUUGUUCUUGCCGG-5′	95–113/52.6%
02	5′-UGUGCUAAGUUUUCAUCACdTdT dTdTACACGAUUCAAAAGUAGUG-5′	160–178/36.8%
03	5′-CCCUGACAGUGAGAGAAAAdTdT dTdTGGGACUGUCACUCUCUUUU-5′	450–468/47.4%
04	5′-GCAAACAAGACAGAAGAGCdTdT dTdTCGUUUGUUCUGUCUUCUCG-5′	998–1016/47.4%

*GenBank accession number AB105817.

chemically synthesized and HPLC-purified by Proligo (La Jolla, CA, USA).

Cell culture

HEK293 cells (American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) supplemented with 20 mM sodium bicarbonate, 100 U/mL benzylpenicillin potassium, 100 μg/mL streptomycin sulfate and 10% fetal bovine serum (Moregate, Bulimba, Australia; culture-medium A) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. TR-BBB13 cells are a conditionally immortalized BCEC cell line (Hosoya *et al.* 2000a) that has been used as an *in vitro* BBB model (Terasaki *et al.* 2003). TR-BBB13 cells were grown in culture-medium A with 15 ng/mL ECGF. The cells were maintained at 33°C, which is a permissive temperature at which temperature-sensitive SV40 large T-antigen is activated, in a humidified atomosphere of 95% air and 5% CO_2.

Transfection of siRNA into HEK293 cells or TR-BBB13 cells

HEK293 cells were plated in six-well plates at 4×10^5 cells/well, grown for 24 h then transfected with 3 μg of rABCG2 siRNA-01, rABCG2 siRNA-02, rABCG2 siRNA-03, rABCG2 siRNA-04 or NC siRNA using Lipofectamine 2000 and OPTI-MEM I reduced serum medium (Invitrogen, Carlsbad, CA, USA). In some experiments, 1 μg of myc-tagged rABCG2 cDNA (pCMV-Tag3A/rABCG2 (Hori *et al.* 2004)) or a control plasmid (pCMV-Tag3A, Stratagene, La Jolla, CA, USA) was co-transfected into HEK293 cells simultaneously with siRNA. The mRNA expression and the transport activity were examined at 48 h after the transfection. The protein expression was examined at 24, 48 and 72 h after the transfection.

For quantitative real-time PCR analysis, TR-BBB13 cells were plated in six-well plates at 4×10^5 cells/well, grown for 24 h at 33°C then transfected with 4 μg of rABCG2 siRNA-03, β-actin siRNA or NC siRNA using Lipofectamine 2000 and OPTI-MEM I reduced serum medium (Invitrogen). At 24 h after siRNA transfection, TR-BBB13 cells were treated with or without 100 nM 17β-estradiol. Culture was continued for a further 24 h at 33°C. For western blot analysis, TR-BBB13 cells were plated in six-well plates at 4×10^5 cells/well, grown for 24 h at 33°C then transfected with 4 μg of rABCG2 siRNA-03 or NC siRNA using Lipofectamine 2000 and OPTI-MEM I reduced serum medium (Invitrogen). The protein expression was examined at 36 h after the transfection.

Quantitative real-time PCR analysis

Total RNA was extracted from HEK293 cells or TR-BBB13 cells with an RNeasy kit (Qiagen) according to the manufacturer's protocol. RNA integrity was checked by electrophoresis on an agarose gel. Single-stranded cDNA was prepared from 1 μg of total RNA by RT (ReverTraAce, Toyobo, Osaka, Japan) using oligo dT primer. Quantitative real-time PCR analysis was performed using an ABI PRISM 7700 sequence detector system (PE Applied Biosystems, Foster City, CA, USA) with 2 × SYBR Green PCR Master Mix (PE Applied Biosystems) according to the manufacturer's protocol. To quantify the amount of specific mRNA in the samples, standards for each run were prepared using pGEM-T Easy Vector containing ABCG2, ABCB1, ABCC1, ABCG1, β-actin or GAPDH (dilution ranging from 0.1 fg/μL to 1 ng/μL). The standard curves of each gene were obtained by linear regression between the logarithm of the standards of each gene and the corresponding threshold cycle (Ct) values. The Ct value indicates the cycle number at which the reaction begins to be exponential. All the plots showed high linearity, and the Ct values of all samples were within the range of the standard plots. The ABCG2, ABCB1, ABCC1 or ABCG1 mRNA levels were normalized relative to the β-actin mRNA level. The β-actin mRNA level was normalized relative to the GAPDH mRNA level. In Fig. 1, each rABCG2 mRNA level is indicated as a percentage of the mean of those in HEK293 cells co-transfected with NC siRNA and rABCG2 cDNA ($n = 3$; open column, NC+). In Fig. 4, each mRNA level is indicated as a percentage of the mean of mRNA levels in TR-BBB13 cells treated with non-siRNA (–) and 17β-estradiol (E2; $n = 3$; the leftmost column). The control lacking the RT enzyme was assayed in parallel to monitor any possible genomic contamination. The PCR was run for 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min after pre-incubation at 95°C for 10 min, using specific primers. The sequences of primers were as follows: sense primer 5′-CAATGGGATCATGAAACC-TG-3′, antisense primer 5′-GAGGCTGATGAATGGAGAA-3′ for ABCG2; sense primer 5′-ACAGAAACAGAGGATCGC-3′ and antisense primer 5′-CGTCTTGATCATGTGGCC-3′ for ABCB1/mdr1a; sense primer 5′-CTGGCTTGGTGTGAACTGAT-3′ and antisense primer 5′-AGGCTCTGGCTTGGCTCTAT-3′ for ABCC1; sense primer 5′-TGCCCGCCGGGTTGAAACTGTTC-3′ and antisense primer 5′-ACTGTCTGCATTGCGTTGCATTGC-3′ for ABCG1; sense primer 5′-TTTGAGACCTTCAACACCCC-3′ and

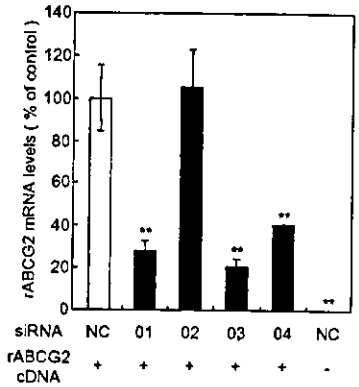

Fig. 1 Effects of rABCG2 siRNAs on the exogenous rABCG2 mRNA level in HEK293 cells co-transfected with myc-tagged rABCG2 cDNA. HEK293 cells were transfected with siRNAs (rABCG2 siRNA-01, rABCG2 siRNA-02, rABCG2 siRNA-03 and rABCG2 siRNA-04 (01, 02, 03 and 04) or non-specific control (NC) siRNA) with (+) or without (–) co-transfection of myc-tagged rABCG2 cDNA. At 48 h after transfection, the cells were collected for quantitative real-time PCR analysis. The sequences of rABCG2 siRNAs are shown in Table 1. Each column represents the mean ± SEM ($n = 3$). The rABCG2 mRNA level was normalized relative to the β-actin mRNA level. Each rABCG2 mRNA level is shown as percentage of the mean of the rABCG2 mRNA level in the NC siRNA-treated HEK293 cells cotransfected with myc-tagged rABCG2 cDNA (NC+). $^{**}p < 0.01$, significantly different from the NC+.

antisense primer 5′-ATAGCTCTTCTCCAGGGAGG-3′ for β-actin; sense primer 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ and antisense primer 5′-TCCTTGGAGGCCATGTAGGCCAT-3′ for GAPDH.

Western blot analysis

HEK293 cells were lysed with lysis buffer containing 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 4% CHAPS, 1 mM phenylmethylsulfonyl fluoride, and a protease-inhibitor cocktail (Sigma Chemical Co., St Louis, MO, USA). The lysate was centrifuged at 15 000 *g* for 30 min and the supernatants were collected. TR-BBB13 cells were homogenized by mean of the nitrogen cavitation technique (800 psi, 15 min, 4°C) in buffer containing 10 mM HEPES-NaOH (pH 7.4), 250 mM sucrose, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF). The homogenized samples were centrifuged at 10 000 *g* for 10 min and the supernatants were collected. These supernatants were centrifuged at 100 000 *g* for 1 h, and a crude membrane fraction was obtained from the pellets. The pellets were suspended in lysis buffer. The protein concentration of samples was measured by the Bradford method using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA, USA). Protein samples (HEK293 cells, 12 μg; TR-BBB13 cells, 40 μg (for rABCG2) or 20 μg (for Na^+,K^+-ATPase and ABCB1) per lane) were resolved by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE; Bio-Rad) and subsequently electrotransferred to nitrocellulose membranes. Membranes were treated with blocking buffer (4% skimmed milk in 25 mM Tris–HCl (pH 8.0), 125 mM NaCl, 0.1% Tween-20 for 2 h at 20°C and incubated with anti-c-myc antibody (0.1 μg/mL; Bethyl Laboratories Inc., Montgomery, TX, USA), anti-β-actin antibody (1 : 2000; Sigma), anti-Na^+,K^+-ATPase antibody (0.1 μg/mL; Upstate Biotechnology, Lake Placid, NY, USA), anti-ABCB1 antibody (C219) (1 : 100; Signet, Dedham, MA, USA), or anti-ABCG2 antibody (G2-Ab1) (1.0 μg/mL) (Hori *et al.* 2004) as the primary antibody at 4°C for 16 h after blocking. The membranes were washed three times with blocking buffer and incubated with horseradish peroxidase-conjugated second antibody. The bands were visualized with an enhanced chemiluminescence kit (SuperSignal; Pierce, Rockford, IL, USA). The relative densities of the bands were measured using NIH image software (National Institutes of Health, Bethesda, MD, USA).

Transport assay

For transport studies, HEK293 cells were incubated for 1 h at 37°C in a medium containing 20 μM mitoxantrone. The cells were then washed in ice-cold phosphate-buffered saline and placed on ice until measurement. Relative cellular accumulation of mitoxantrone was determined by flow cytometry with a 635 nm red diode laser and 661 nm bandpass filter (FACs Calibur, BD Biosciences, Lexington, KY, USA). A total of 20 000 events were collected. Debris was eliminated by gating on forward versus side scatter. The mean channel number for each histogram was used as a measure of drug fluorescence for calculation.

Data analysis

Unless otherwise indicated, all data represent the mean ± SEM. An unpaired, two-tailed Student's *t*-test was used to determine the significance of differences between two group means. One-way ANOVA followed by the modified Fisher's least-squares difference method was used to assess the statistical significance of differences among means of more than two groups.

Results

Silencing of exogenous rABCG2 gene in HEK293 cells

To determine the effects of four different siRNAs (rABCG2 siRNA-01, rABCG2 siRNA-02, rABCG2 siRNA-03 and rABCG2 siRNA-04; Table 1) on rABCG2 gene expression, quantitative real-time PCR analysis was performed using HEK293 cells co-transfected with myc-tagged rABCG2 cDNA. After treatment with rABCG2 siRNA-01, rABCG2 siRNA-03 or rABCG2 siRNA-04 for 48 h, the rABCG2 mRNA levels were suppressed in rABCG2-transfected HEK293 cells by 71.8%, 78.8% or 54.7%, respectively (01+, 03+ and 04+, Fig. 1), compared with those in cells treated with non-specific control (NC) siRNA (NC+, Fig. 1). In contrast, treatment with rABCG2 siRNA-02 had no significant effect on the rABCG2 mRNA level (02+, Fig. 1).

Effects of siRNAs on rABCG2 protein level in HEK293 cells

To clarify whether rABCG2 protein was reduced concomitantly with the suppression of rABCG2 mRNA, the level of exogenous rABCG2 protein was examined by western blot analysis. The protein was detected using anti-c-myc antibody, as rABCG2 protein was fused with the myc epitope. Myc tagged-rABCG2 proteins were detected at 80 kDa in HEK293 cells co-transfected with NC siRNA and myc-tagged rABCG2 cDNA (NC+, Fig. 2a), while no band was detected in HEK293 cells co-transfected with NC siRNA and the vector alone (i.e. without the myc-tagged rABCG2 cDNA insert) (NC–, Fig. 2a). rABCG2 siRNA-01, rABCG2 siRNA-03 and rABCG2 siRNA-04 each reduced the level of rABCG2 protein in HEK293 cells co-transfected with myc-tagged rABCG2 cDNA (01+, 03+ and 04+, Fig. 2a). rABCG2 siRNA-03 was the most effective (03+, Fig. 2a), and it reduced the relative density of the bands by 99.7 ± 0.1% (mean ± SEM; $n = 3$) compared with NC siRNA. In contrast, the rABCG2 protein level was not affected by rABCG2 siRNA-02 (02+, Fig. 2a). The level of β-actin protein was unchanged by any of the rABCG2 siRNAs (Fig. 2a). As shown in Figs 2(b and c), western blot analysis at 24 h and 72 h after transfection clearly demonstrated that co-transfection of rABCG2 siRNA-03, but not NC siRNA, significantly reduced the level of rABCG2 protein.

Effects of rABCG2 siRNAs on mitoxantrone efflux transport in rABCG2 cDNA-transfected HEK293 cells

Mean fluorescence intensity of mitoxantrone was significantly reduced in HEK293 cells following co-transfection with NC siRNA and myc-tagged rABCG2 cDNA (NC+, Fig. 3a) compared with NC siRNA alone (NC–, Fig. 3a). The proportion of transiently rABCG2 cDNA-transfected cells was 25.7 ± 0.4% (mean ± SEM; $n = 3$; gated area, Fig. 3b). rABCG2 siRNA-01, rABCG2 siRNA-03 and rABCG2 siRNA-04 significantly increased the mean fluorescence intensity of mitoxantrone (01+, 03+ and 04+, Fig. 3a), and indeed, rABCG2 siRNA-03 completely reversed the reduction of the mitoxantrone level. Representative histogram and dot plots showed that the population of rABCG2-transfected cells almost completely

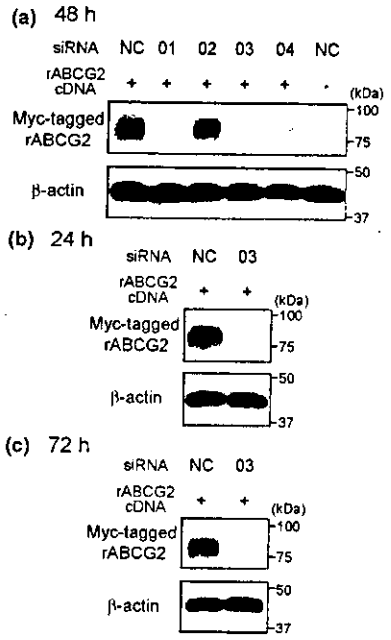

Fig. 2 Effects of rABCG2 siRNAs on exogenous rABCG2 protein in HEK293 cells co-transfected with myc-tagged rABCG2 cDNA. HEK293 cells were transfected with siRNAs (rABCG2 siRNA-01, rABCG2 siRNA-02, rABCG2 siRNA-03 and rABCG2 siRNA-04 (01, 02, 03 and 04) or non-specific control (NC) siRNA) with (+) or without (-) co-transfection of myc-tagged rABCG2 cDNA. The sequence of rABCG2 siRNAs are shown in Table 1. At 48 h (a), 24 h (b) or 72 h (c) after transfection, the cells were collected for western blot analysis using anti-c-myc and anti-β-actin antibodies. Typical results from repeated experiments are shown.

overlapped with that of non-transfected cells (03+, Fig. 3b). In contrast, rABCG2 siRNA-02 had no significant effect on the mean fluorescence intensity of mitoxantrone in HEK293 cells (02+, Fig. 3a).

Selective inhibition of endogenous rABCG2 gene in a conditionally immortalized BCEC line (TR-BBB13) by siRNA

rABCG2 siRNA-03, which is the most potent siRNA for attenuating rABCG2 function, was used to suppress endogenous rABCG2 expression in TR-BBB13 cells. At 24 h after siRNA transfection, TR-BBB13 cells were treated with 17β-estradiol, which has been reported to induce ABCG2 mRNA expression in cancer cells (Ee *et al.* 2004), or not treated. In the absence of 17β-estradiol, the rABCG2 mRNA level was reduced by 42.2% by transfection of rABCG2 siRNA-03 into TR-BBB13 cells (G2-03), whereas transfection of NC siRNA had no effect (NC)[E_2(−), Fig. 4a]. The rABCG2 mRNA level was significantly induced in non-siRNA-transfected TR-BBB13 cells following treatment with 17β-estradiol (open columns, Fig. 4a). The rABCG2 mRNA level was reduced by 75.7% by transfection of rABCG2 siRNA-03 into TR-BBB13 cells in the presence of 17β-estradiol (G2-03)[E_2(+), Fig. 4a]. In contrast, the transfection of NC siRNA did not affect the rABCG2 mRNA level in TR-BBB13 cells (NC)[E_2(+), Fig. 4a]. Treatment with siRNA targeted to β-actin decreased the β-actin mRNA level by 57.9 ± 2.2% (mean ± SEM; n = 3) in TR-BBB13 cells, supporting the view that siRNA was successfully transfected into TR-BBB13 cells.

To confirm the selectivity of the inhibitory effects of siRNA, the expression levels of other ABC transporters expressed in BCECs were examined. rABCG2 siRNA-03 did not significantly affect the ABCB1, ABCC1 and ABCG1 mRNA levels in TR-BBB13 cells in either the presence or absence of 17β-estradiol (Figs 4b–d). Following the 17β-estradiol treatment, the ABCB1 mRNA level was increased in TR-BBB13 cells (Fig. 4b), whereas the ABCC1 mRNA level showed a tendency to decrease (Fig. 4c), and the ABCG1 mRNA level was unchanged (Fig. 4d).

Suppression of endogenous rABCG2 protein expression in TR-BBB13 cells by siRNA

The rABCG2 protein expression was suppressed by transfection of rABCG2 siRNA-03 into TR-BBB13 cells (G2-03) compared with untransfected (-) and NC siRNA-transfected (NC) TR-BBB13 cells (Fig. 5a, upper panel). The expression of ABCB1 protein and Na^+,K^+-ATPase protein, used as a standard, was not changed by any of the treatment conditions (Fig. 5a, middle and lower panel, respectively). As shown in Fig. 5(b), the density ratio of rABCG2 to Na^+,K^+-ATPase density was significantly decreased by 62.1% by transfection of rABCG2 siRNA-03 into TR-BBB13 cells (G2-03) compared with untransfected TR-BBB13 cells (-).

Discussion

The present study demonstrated that introduction of any of three rABCG2 siRNAs efficiently decreased the expression of rABCG2 and suppressed the apparent efflux function of mitoxantrone, a substrate drug of rABCG2. Moreover, rABCG2 siRNA selectively suppressed the mRNA and protein expression of rABCG2 in a conditionally immortalized brain capillary endothelial cell line (TR-BBB), an *in vitro* BBB model.

Three of the siRNAs designed to target the rABCG2 gene induced sequence-specific suppression of the expression and function of the rABCG2 transporter (Figs 1–3). None of the siRNAs affected the β-actin protein levels (Fig. 2). This is the first evidence that rABCG2 function can be suppressed by siRNA-induced RNA interference. The differences in efficacy among these three siRNAs could be due to altered ability to silence the rABCG2 gene rather than altered transfection efficiency, because rABCG2 siRNA was present in about 900-fold molar excess over rABCG2-expression plasmid (the amount/length of the rABCG2 siRNA and the plasmid was 3 μg/21 bp and 1 μg/about 6300 bp, respectively). The protein expression and the transport activity of rABCG2 were completely suppressed at 48 h after rABCG2 siRNA-03 transfection (Figs 2 and 3), while reduction of the mRNA expression was around 80% (Fig. 1). This apparent difference may be because the protein level was below the detection threshold of western blot analysis, and below the level required for exerting its function. The expression of

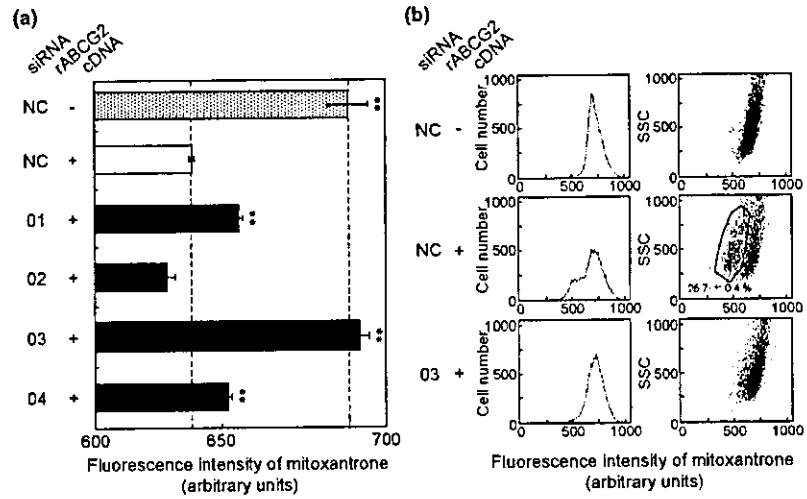

Fig. 3 Effects of rABCG2 siRNAs on mitoxantrone efflux transport in HEK293 cells co-transfected with myc-tagged rABCG2 cDNA. (a) HEK293 cells were transfected with siRNAs (rABCG2 siRNA-01, rABCG2 siRNA-02, rABCG2 siRNA-03 and rABCG2 siRNA-04 (01, 02, 03 and 04) or non-specific control (NC) siRNA) with (+) or without (–) cotransfection of myc-tagged rABCG2 cDNA. The sequences of rABCG2 siRNAs are shown in Table 1. At 48 h after transfection, the cells were incubated with 20 μM mitoxantrone for 1 h at 37°C. Mitoxantrone fluorescence in arbitrary units was determined by flow cytometry with a 635 nm red diode laser and 661 nm bandpass filter. Each column represents the mean ± SEM ($n = 3$). $^{**}p < 0.01$, significantly different from the NC siRNA-treated HEK293 cells co-transfected with myc-tagged rABCG2 cDNA (NC +). (b) Representative histogram plot and dot plot of HEK293 cells transfected with siRNAs [NC siRNA or rABCG2 siRNA-03 (03)] with (+) or without (–) myc-tagged rABCG2 cDNA, showing the mitoxantrone fluorescence vs. cell number and side scattered light (SSC), respectively. The gated cell population (solid line) identified the mitoxantrone-effluxing cells. The number shown is the proportion of total rABCG2-transfected cells contained in the gated cell population (mean ± SEM; $n = 3$).

exogenous rABCG2 protein was also completely suppressed at 24 h and 72 h after rABCG2 siRNA-03 transfection (Fig. 2), indicating that this siRNA remains effective at least from 24 h to 72 h. The sequence of rABCG2 siRNA-03 is 100% identical with the corresponding sequence of mouse ABCG2 (GenBank accession number NM011920). Therefore, this siRNA could be also effective for suppressing the function of mouse ABCG2.

The sequence locations of the effective rABCG2 siRNA-01, rABCG2 siRNA-03 and rABCG2 siRNA-04 (Table 1) were not limited to within 100-nucleotides downstream from the first ATG in contrast to the previous siRNA design (Elbashir *et al.* 2002). This result is in agreement with the recent report indicating that the major determinant of siRNA activity is the target sequence itself, rather than its location (Yoshinari *et al.* 2004). Recently, eight criteria for rational siRNA design for RNA interference were proposed (Reynolds *et al.* 2004). Indeed, the most effective rABCG2 siRNA (rABCG2 siRNA-03) satisfied as many as six of the criteria. For instance, this siRNA has moderate to low G/C content (30–52%), low internal stability of the sense 3′-end (at least three A/U bases at 15–19 nt) and a lack of internal repeats. Moreover, rABCG2 siRNA-03 has 'A' and 'U' at positions 19 and 10, respectively. It has been reported that these sequence-related criteria had a strong impact on improved selection of highly potent siRNAs (the increase in the probability of selecting siRNAs which induce more than 95% gene silencing was 7.2% and 12.8% for A19 and U10, respectively; Reynolds *et al.* 2004).

The present study has demonstrated that the delivery of siRNA suppresses rABCG2 mRNA and protein expression in TR-BBB13 cells (Figs 4a and 5), which are an *in vitro* BBB model expressing functional rABCG2 (Hori *et al.* 2004). There have been reports that the protein and function of targeted transporters were suppressed concomitantly with silencing of the corresponding genes (Wu *et al.* 2003; Nabokina *et al.* 2004; Said *et al.* 2004). Indeed, the endogenous rABCG2 protein level was suppressed in TR-BBB13 cells concomitantly with its gene silencing. rABCG2 siRNA-03 presumably suppresses transport activity of endogenous rABCG2 in TR-BBB13 cells by the reduction of rABCG2 protein level. The rABCG2 siRNA suppressed the induction of the rABCG2 mRNA level by 17β-estradiol to the same level as in untreated cells (Fig. 4a). This result suggests that this siRNA was efficiently delivered into TR-BBB13 cells and blocked the induced gene expression of rABCG2. Further study using labeled siRNA would be useful for distinguishing the transfection efficiency of siRNA from the efficacy of siRNA on endogenous rABCG2.

The rABCG2 mRNA level increased in TR-BBB13 cells following treatment with 100 nM 17β-estradiol (Fig. 4). Estrogen is thought to reach a maximum concentration of

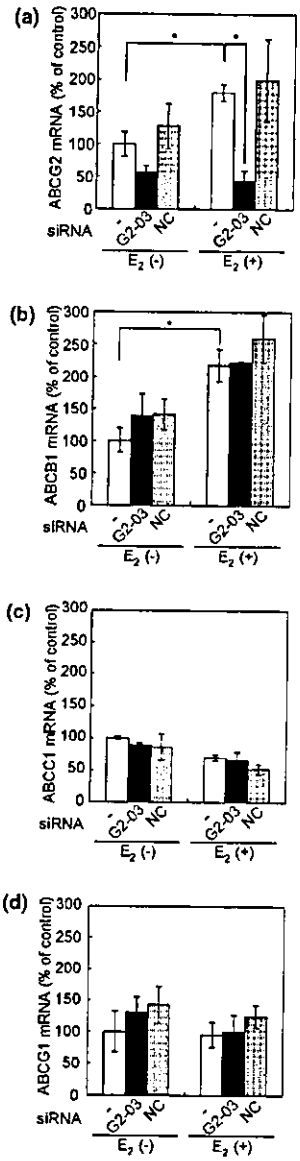

Fig. 4 Selective gene silencing of rABCG2 in TR-BBB13 cells by siRNA.TR-BBB13 cells were transfected with rABCG2 siRNA-03 (G2-03, ■) or non-specific control siRNA (NC, ▣), or untransfected (–, □). After 24 h transfection of siRNAs, the culture medium was changed to that with (+) or without (–) 17β-estradiol (E_2), and culture was continued for another 24 h. The ABCG2 (a), ABCB1 (b), ABCC1 (c), and ABCG1 (d) mRNA levels were determined by quantitative real-time PCR analysis. Each column represents the mean ± SEM ($n = 3$). Each mRNA level was normalized relative to the β-actin mRNA level. Each mRNA level is shown as percentage of the mean of the mRNA levels in TR-BBB13 cells treated with non-siRNA (–) and 17β-estradiol (E_2) (the leftmost column). $^*p < 0.05$, significant difference.

150 nM during the third trimester of pregnancy (Clarke *et al.* 2001). Under such conditions, there is possibility that brain-to-blood transport activity via ABCG2 would be induced.

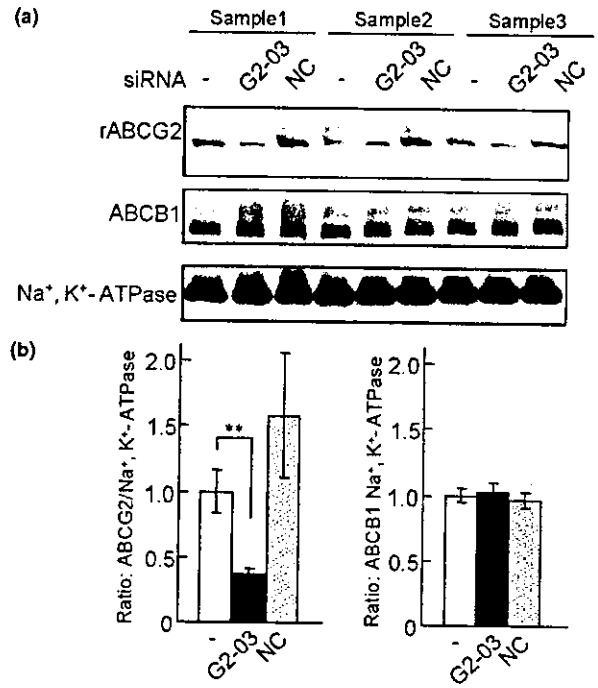

Fig. 5 Selective suppression of rABCG2 protein expression in TR-BBB13 cells by siRNA.TR-BBB13 cells were transfected with rABCG2 siRNA-03 (G2-03) or non-specific control siRNA (NC), or untransfected (–). After 36 h transfection of siRNAs, the cells were collected for western blot analysis using anti-ABCG2, anti-ABCB1 and anti-Na^+,K^+-ATPase antibodies.(a) Results from three independent western blot analyses (samples 1–3) are shown. (b) The ratio of ABCG2 (left panel) or ABCB1 (right panel) densities to Na^+,K^+-ATPase density. Each column represents the mean ± SEM ($n = 3$). $^{**}p < 0.01$, significantly different from untransfected cells (–).

17β-Estradiol also regulates the expression of ABCB1 and ABCC1 mRNAs in TR-BBB13 cells, suggesting that these ABC transporter-mediated transport systems may be affected by exposure to 17β-estradiol. Recently, it has been reported that the promoter region of human ABCG2 gene contains a novel and functional estrogen response element (ERE) which has 83.3% (10 aa/12 aa) homology with a classical consensus ERE (Ee *et al.* 2004). A search of the rat genome sequence revealed that the first intron of rABCG2 also has a sequence which shows 83.3% (10 aa/12 aa) homology with the classical consensus ERE. It has been reported that 17β-estradiol enhances the ABCG2 mRNA expression in estrogen receptor (ER)-positive human cancer cell lines (Ee *et al.* 2004), and that BCECs express multiple subtypes of ER-α (Stirone *et al.* 2003). Investigation of the sensitivity of the ERE-like sequence should provide a better understanding of the mechanism of rABCG2 induction by 17β-estradiol treatment.

Introduction of siRNA into TR-BBB13 cells would be a promising approach to clarify the specific role of each transporter at the BBB because the cells retain the *in vivo*

transport properties towards various compounds (Terasaki *et al.* 2003). The efficiency of a DNA vector-based transfection using cationic liposomes was less than 5% in TR-BBB13 cells (unpublished data). The present study suggests that oligonucleotide (siRNA)-based transfection is far more effective than DNA vector-based transfection in the case of TR-BBB13 cells. ABCG2 confers multidrug resistance upon cancer cells, so ABCG2 siRNA-induced RNA interference may also be useful for overcoming drug resistance.

The luminal localization of ABCG2 at the BBB has been clearly demonstrated in humans (Cooray *et al.* 2002) and rats (Hori *et al.* 2004). However, the functional contribution of ABCG2 at the BBB *in vivo* remains unclear (Allen and Schinkel 2002). Following rABCG2 siRNA-03 transfection into TR-BBB13 cells, the mRNA level of ABCG1, which has sequence homology with ABCG2, was unchanged (Fig. 4d), and those of ABCB1 and ABCC1, which can transport some ABCG2 substrates, were unaffected for at least 48 h after the siRNA transfection (Figs 4b.c). These data suggest that the silencing effect of the siRNA is specific for the ABCG2 gene in this *in vitro* BBB model. Because the rABCG2 siRNA selectively suppressed rABCG2 mRNA and protein, the siRNA study should allow us to clarify the contribution of the transporter to the BBB efflux transport. Indeed, it has recently been reported that siRNA designed to distinguish thiamine transporter subtypes induced subtype-specific gene silencing in Caco-2 cells, and that the functional contribution of the subtypes to thiamine uptake in the cells was clearly demonstrated by using the siRNA (Said *et al.* 2004). Such a sequence-specific silencing by siRNA may be a promising way to achieve a deeper understanding of the physiological and pharmacological roles of rABCG2 at the BBB. Regarding transporter gene knockdown at the BBB, the siRNA transfection into BCECs should be more specific than suppression by inhibitors, and easier to carry out as compared with the development of knockout mice. Moreover, the siRNA technique would be useful for silencing plural transporter genes because mixtures of siRNAs can be delivered.

In conclusion, the present study has demonstrated that delivery of siRNA into this *in vitro* BBB model specifically reduced endogenous rABCG2 protein level as well as its mRNA level. Application of the siRNA technique to BBB research should increase our understanding of ABCG2 role at the BBB.

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RNAiを用いたC型肝炎の遺伝子治療

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C型慢性肝炎は日本全国で200万人が罹患しており，インターフェロンによる治療の有効性は不十分で新たな治療法が必要である．近年，ターゲット遺伝子の発現を配列特異的に抑制できるsiRNAを，抗ウイルス剤として応用する研究が急速に進んでいる．5′-UTR IRESや非構造タンパク質領域をターゲットにデザインされたsiRNAは高率にHCV RNA配列を切断して，HCVレプリコンレベルではHCVゲノムの増殖を抑制する．その有効性は従来の核酸試薬であるアンチセンスやリボザイムをはるかにしのぐ．さらに，これをウイルスベクターに導入することにより臨床応用への可能性が追究されている．

Key words hepatitis C, HCV, siRNA, IRES, adenovirus vector

肝細胞癌による死者は年間3万人を超え，この死亡者数は癌のなかで男性では第3位，女性では第4位を占め，年々増加傾向にある．その最大の原因はC型慢性肝炎であり，肝細胞癌患者の約75％はC型肝炎ウイルス（HCV）抗体陽性である．C型慢性肝炎は日本全国で200万人が罹患しており，年間で約30万人がインターフェロン（IFN）による治療を受けているがその効果は遺伝子型により異なり，20万人は治癒しない．しかもIFN治療は発熱，頭痛の副反応は必発で，重篤な精神症状，甲状腺異常などの副作用もあり，6か月に及ぶIFN治療の身体的，経済的負担は大きい．近年開発されたリバビリンの併用療法や，新しいウイルスタンパク質合成阻害薬も大幅な治療効果の改善には至っていない．

RNAレベルで配列特異的にその発現を抑制することのできるshort interfering RNA（siRNA）は，本来細胞のもつウイルスに対する防御機構とも考えられ，ウイルスゲノムはsiRNAのよいターゲットとされ[1]，新しい治療薬になる可能性が期待されている．ここではC型肝炎に対するsiRNA治療戦略の現状と展望を概説したい．

○C型肝炎ウイルス（HCV）

*HCV*遺伝子は9,600塩基から成るプラス一本鎖RNAで，5′と3′非翻訳領域（UTR）にはさまれたORF（open reading frame）から成る．5′側の341塩基のUTRは複雑なRNA構造のIRES（internal ribosome entry site）（一部コアタンパク質コード領域に及ぶ）を含み，HCV RNAはキャップ非依存的にこの5′-IRESにより翻訳される．3′-UTRには

Gene therapy of hepatitis C virus with RNAi

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よこた・たかのり　1990年東京医科歯科大学大学院修了（医学博士）．98年米国バーナム研究所，99年米国バック神経変性疾患研究所留学を経て，2000年より現所属．専門は分子生物学と遺伝子治療学．現在の主な研究テーマはアルツハイマー病など神経疾患の病態解明と遺伝子治療．学問のための研究ではなく，実際に疾患を分子レベルで治療するつもりで研究を進めたい．

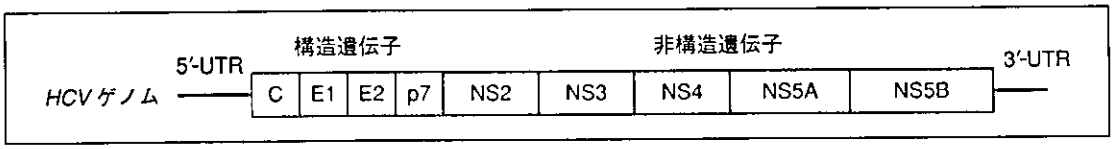

図1　HCVの遺伝子構造

表1　HCVに対するsiRNAの報告

報告者	標的	siRNA	抑制効果(倍)
Seo MY et al[3]	5'-UTR	合成siRNA	6.7
Randal G et al[4]	NS3, NS5B	合成siRNA	8.8
Kapadia SB et al[5]	NA4B	合成siRNA	50
Wilson JA et al[6]	NS3, NS5B	合成siRNA	10.4
		発現DNAベクター	4
Yokota T et al[7]	5'-UTR	合成siRNA	33.3
		発現DNAベクター	5.3
		アデノウイスルベクター	＞1,000

poly(U)配列と98塩基から成る3'X領域が存在している．ORFは5'側からC，E1，E2，p7の構造タンパク質，NS2，NS3，NS4，NS5A，NS5Bの非構造タンパク質を含む3,010のアミノ酸から成る1本の大きなポリプロテインをコードしている(図1)．

HCVは一本鎖RNAウイルスであるがゆえ，プルーフリーディング機能がなく，ウイルス複製時に特にORF領域においてRNAポリメラーゼの読み違いによる変異を起こしやすい（一般にRNAポリメラーゼのエラーの頻度は～10^{-4} base/site/replicationと予想されている）．*HCV*遺伝子が同定されて以来，さまざまな遺伝子型が報告されてきたが，現在では分子進化学的に遺伝的に距離をもつ6つの遺伝子型に分類・整理されている．また，同一個体内においても遺伝子配列の異なったウイルス集団が存在してquasispeciesと呼ばれている[2]．

○siRNAターゲットサイトの選択

2003年にHCVレプリコンを用いたHCVに有効なsiRNAが相次いで報告された（表1）[3～7]．quasispeciesの問題から，もしsiRNAにその配列上ターゲットサイトとのミスマッチ変異が生じた場合，特にその変異部位が19塩基のうち5'側から9～13塩基目付近であると，たとえ1塩基のミスマッチでも大きく切断効率を下げる場合のあることが知られている[8,9]．そこで筆者らは，HCVの遺伝子型にかかわらず92～98％配列が保存されている5'-UTR IRES[10]にsiRNAのターゲットを絞ってデザインした（図2）．まず，5'-UTR IRESのRNAの二次構造[11]から一本鎖のループの部分を選び，(AA/CA/GA)N_{19}の配列で，後のU6プロモーターを用いたベクター化のためにN_{19}がGに始まりCに終わるなど[12]，いくつかの有効なsiRNA選択の配列のルールからターゲットの部位を選んだ．

○培養細胞によるHCV増殖モデル

HCVは通常の培養細胞には感染せず，感染培養細胞モデルがないことがHCV研究の大きな妨げとなっていたが，1999年にBartenschlagerらによりヒト肝細胞癌株Huh-7細胞を用いてHCVの自己増殖を可能にしたHCVレプリコンが報告された[13]．これは*HCV*ゲノムの構造タンパク質をコードする部分をネオマイシン耐性遺伝子に置換した構造で，ヒト肝細胞癌株Huh-7細胞に導入して，レポーター遺伝子としてルシフェラーゼ遺伝子を融合させて，*HCV*遺伝子複製効率をルシフェラーゼ活性によって簡便に評価できるようにした安定発現細胞系である（図3）．これによって，抗HCV薬の培養細胞での評価が可能となった．

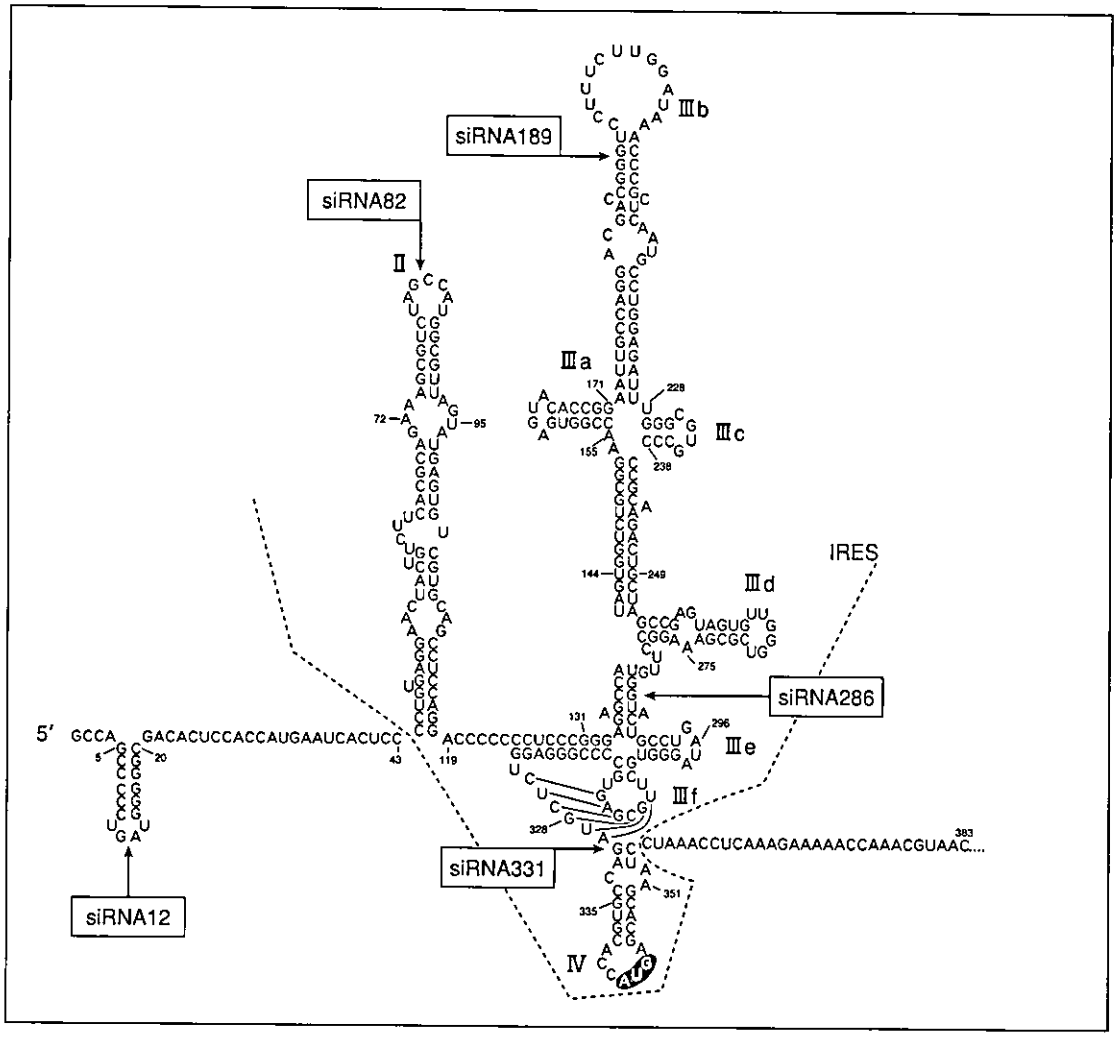

図2　HCV 5′-UTR IRES の RNA の二次構造と siRNA のターゲット部位
（二次構造：Brown EA, et al : *Nucletic Acids Res* 20, 5041-5, 1992[11] より）

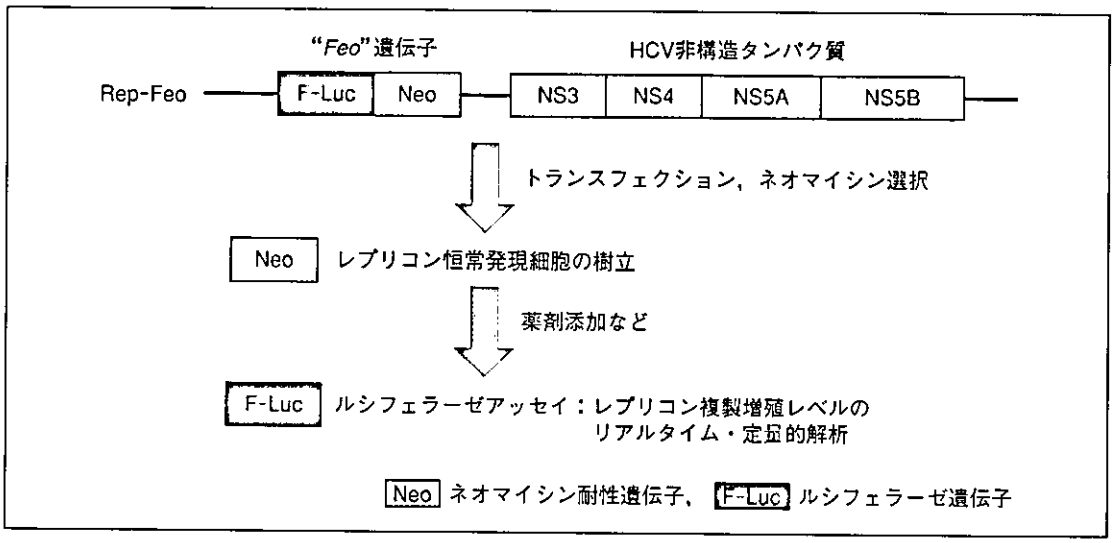

図3　HCV レプリコン（Rep-Feo）の構造

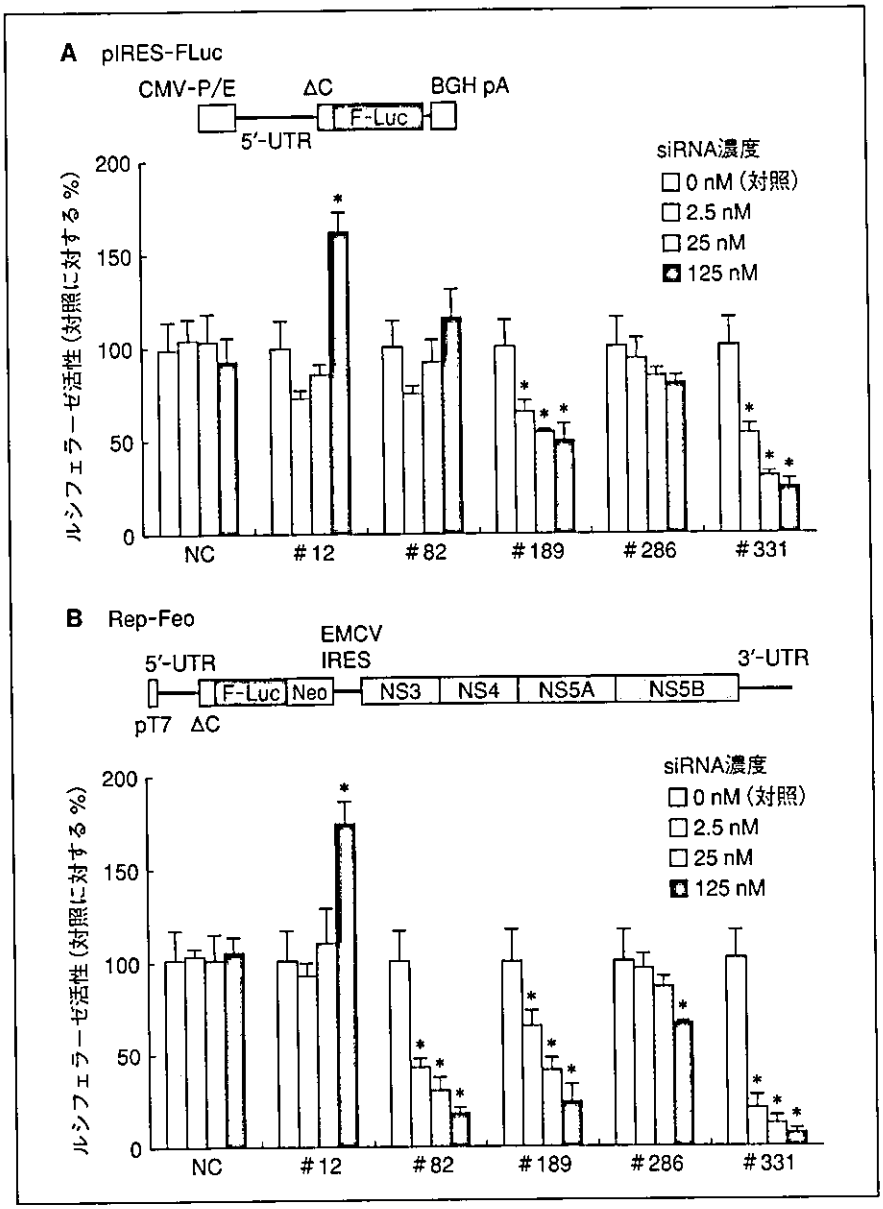

図4　合成siRNAの5′-IRESベクター（pIRES-FLuc）（A）とHCVレプリコン（Rep-Feo）（B）におけるルシフェラーゼ活性抑制効果

*：$p<0.05$, NC：ネガティブコントロール
（Yokota T. et al：*EMBO Rep* 4, 602-8, 2003[7] より）

○HCVに対するsiRNAの効果

図4に筆者らが5′-UTR IRESに対してデザインしたsiRNAの効果を示す．5′-IRESベクター（pIRES-FLuc）においてもHCVレプリコン（Rep-Feo）においてもsiRNA331が最も有効にHCV RNAの増殖発現を抑制した．HCVレプリコンにおいて，対照に比較して125 nMのsiRNA濃度では97％のルシフェラーゼ活性の抑制が達せられ，2.5 nMの非常に低濃度siRNAでも約80％の抑制がみられた．この結果はHCVレプリコンRNAのノーザンブロットや非構造タンパク質のウェスタンブロットでも確かめられた（図5）．5′-IRESベクターよりHCVレプリコンでの抑制率が高かったのは，このsiRNAが複製の鋳型を減らすということだけでなく，5′-IRESを切断することがRNAの転写活性そのものを低下させ，さらに複製に必要なRNAポリメラーゼなど非構造タンパク質の翻訳を抑えることにより，相乗的にHCV RNAの増殖を抑制したものと考えている．HCV 5′-UTRを標的としたこのsiRNAは，従来の機能性核酸試薬であるアンチセンスオリゴDNA[14]，リボザイム[15]の効果と比較しても圧倒的に低濃度でより高い抑制活性を示した（図6）．

一方，siRNA12では意外な結果が得られた．

siRNA12を作用させた結果，HCVの翻訳や増殖が逆に増加したのである．従来の欠失変異株の実験から5′-UTR IRESのヘリックス1はIRESの翻訳機能に抑制的に働いているとの報告があり[16]，それに合致するものであるが，siRNAを非翻訳領域に働かせると翻訳を促進してしまう場合があることは，遺伝子発現抑制を意図した遺伝子治療をデザインする際に注意すべきことと考えられた．

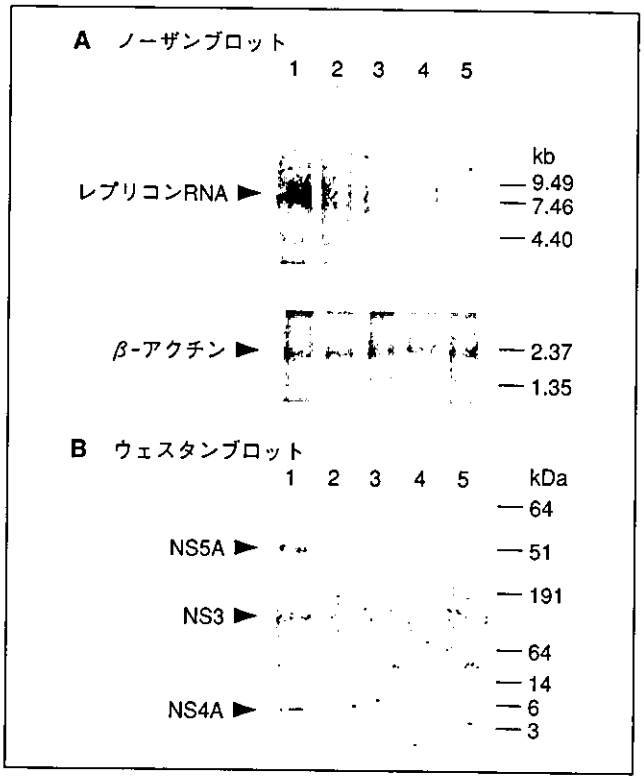

図5 siRNA331によるHCVレプリコンRNA，非構造タンパク質の発現抑制効果
A：レプリコン配列全長に対するプローブを用いたノーザンブロット．
B：NS5Bに対するプローブや抗体を用いたウェスタンブロット．
1：mock，2：siRNA331 2.5 nM，3：siRNA331 25 nM，4：siRNA331 125 nM，5：トランスフェクションなし
(Yokota T, et al : *EMBO Rep* 4, 602-8, 2003[7] より)

○HCVに対するsiRNA発現ベクターの効果

siRNAをウイルスベクターで細胞内に発現させるためにはsiRNA発現DNAベクターの作製が必要である．筆者らは，RNAポリメラーゼⅢ（Pol Ⅲ）系のプロモーターであるヒトU6プロモーターの下流にタンデムタイプ[12]とステムループタイプ[17]の2つのsiRNA発現ベクター配列を挿入して（図7）その効果を検討した．タンデムタイプでは2つのU6プロモーターからセンス鎖とアンチセンス鎖に相当するRNAが別々に転写され，細胞内でハイブリダイズしてsiRNAを産生する．一方，ステムループタイプでは，センス鎖とアンチセンス鎖がループを介してつながるヘアピンRNAが転写され，細胞質でDicerによってプロセシングされてsiRNAになる．このプロセシングを有効にするため，センス鎖とアンチセンス鎖はsiRNAの19 塩基より少し長目で，プロテインキナーゼR（PKR）に認識されない21塩基と27塩基の2種類を作製して効果を比較した．効

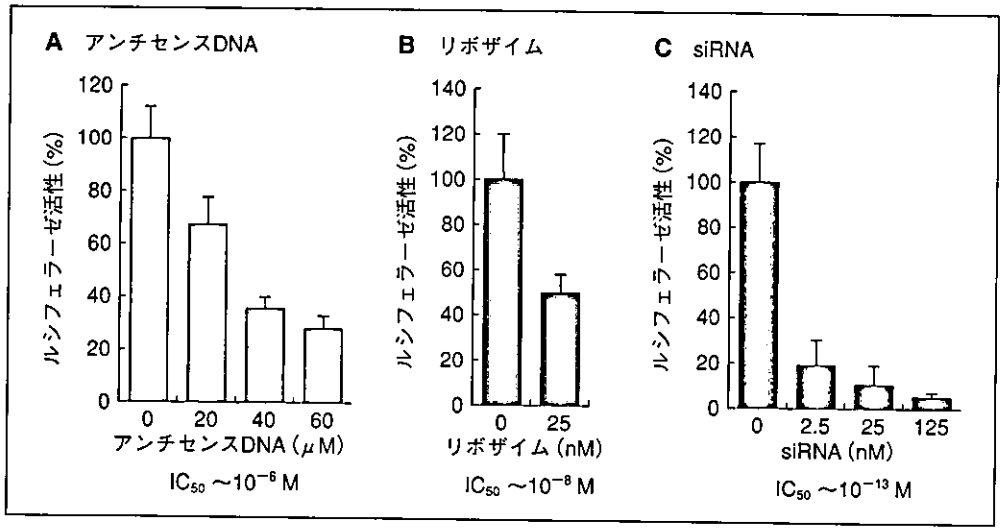

図6 HCV 5′-UTRを標的としたアンチセンスDNA，リボザイム，およびsiRNAの細胞内での効果の比較
(A：Wakita T, et al : *J Biol Chem* 269, 14205-10, 1994[14] より；B：Sakamoto N, et al : *J Clin Invest* 98, 2720-8, 1996[15] より；C：Yokota T, et al : *EMBO Rep* 4, 602-8, 2003[7] より)

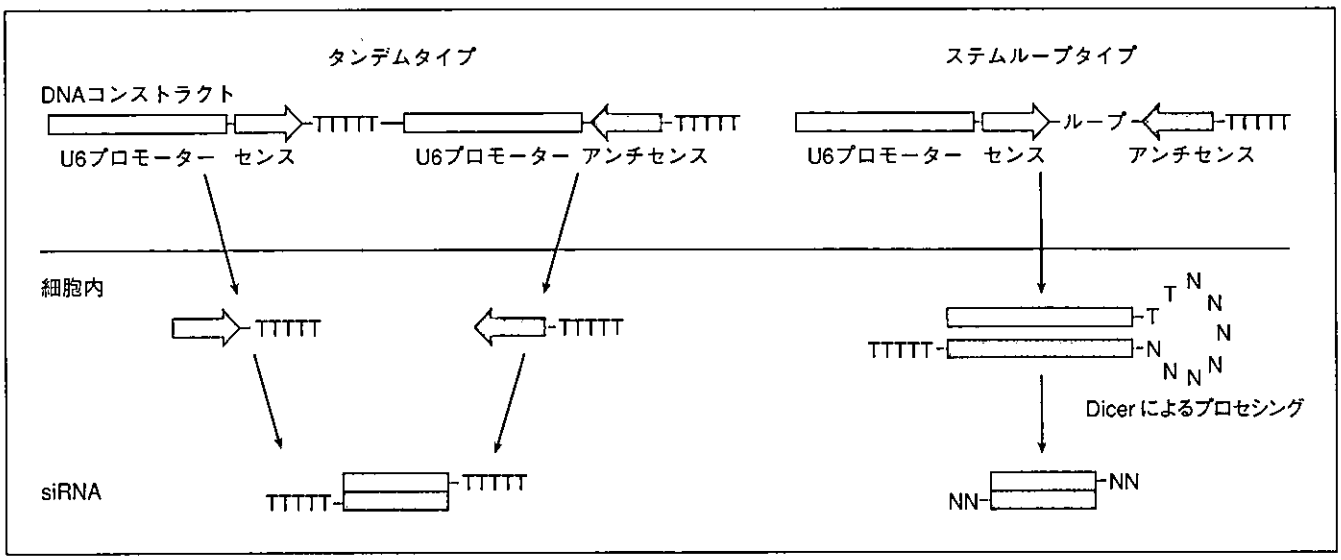

図7　siRNA発現DNAベクターの構造と細胞内でのプロセシング

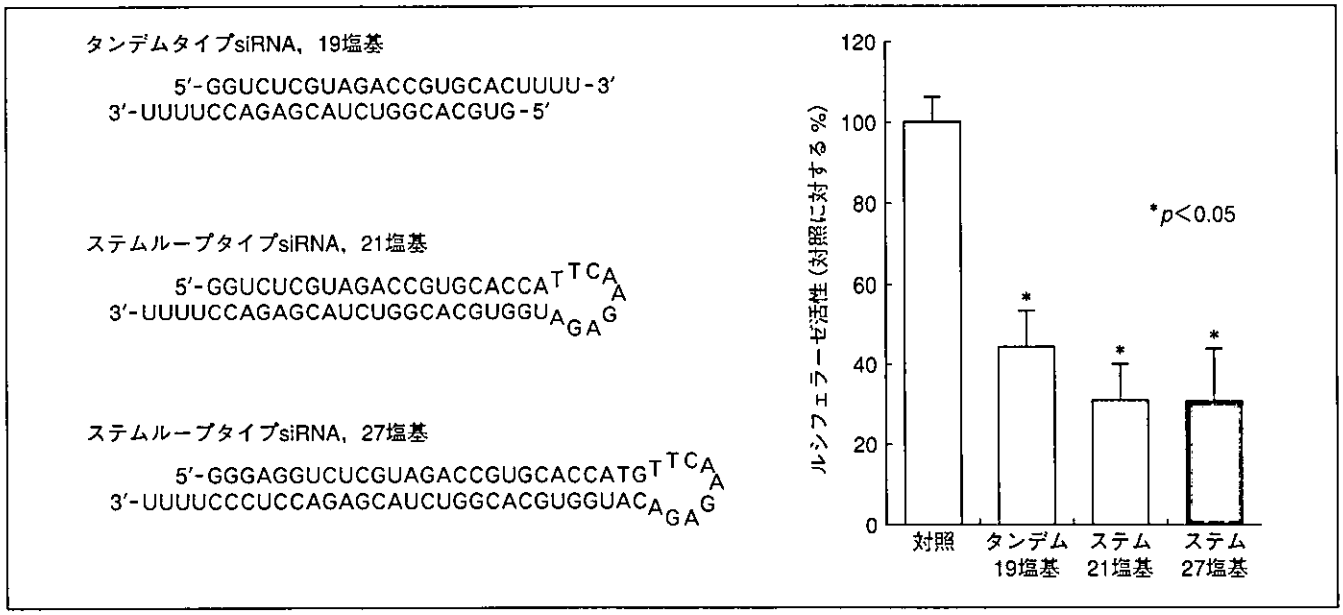

図8　siRNA発現プラスミドDNAの細胞内導入によるHCV増殖抑制効果

果は，タンデムタイプよりステムループタイプのほうがより低濃度で作用して最大効果も優れていた．ステムループタイプの鎖長は21塩基と27塩基の間では大きな差はなかった（図8）．これは，ステムループタイプのほうがセンス鎖とアンチセンス鎖がシスにあるため，より効率よくアニールできて二本鎖になることで細胞内で安定になるためと考えられる．さらに，ステムループタイプでは複製に際してセンスやアンチセンス配列に高頻度に変異が入ることが知られており，それを防ぐため複数のミスマッチ変異を導入した（未発表）．これによって，HCVゲノム切断効率は下がらず，より安定なsiRNA発現ベクターが完成した．

筆者らの作製したこれらのsiRNA，siRNA発現ベクターは，いずれも同時期に発表された欧米の報告に比較して高いHCV増殖抑制効果を示した（表1）．

○siRNAの肝細胞へのデリバリーと *in vivo* での抑制効果

McCaffrey ら[18]は，マウスの尾静脈から10 μgのNS5Bに対する合成siRNAやsiRNA発現ベクターを体重の5～10％の大量のリン酸緩衝生理食塩水（PBS）を用いて5～7秒という短時間で注入するハイドロダイナミクス導入法によって，*in vivo* の肝細胞にsiRNAの導入に成功した．さらに最近，このハイドロダイナミクス法で導入されたFasに対する合成siRNA〔2′-bis (acetoxyethoxy) -methyl etherで化学的に修飾したsiRNAでその安定性の上昇を図っている〕により，マウスに誘発された劇症肝炎の死亡率を低下させたことが報告された[19]．このハイドロダイナミクス導入法をそのまま臨床応用することは到底できないが，siRNAが*in vivo* の肝細胞で有効に作用することを示した重要な報告である．また，この報告でFasの発現レベルが20日後には正常に戻っている点は，長期のsiRNAによる副作用が不明である現在ではかえって臨床応用によいかも知れない．

一方，もう少し長期の抑制効果にはウイルスベクターが必要となる．siRNA発現ベクターコンストラクトをアデノウイルス[20]やレンチウイルス[21]，レトロウイルス[22]などのウイルスベクターに組み込んで作製したsiRNA発現ウイルスベクターを用いて，*in vivo* 細胞へのsiRNA導入の報告が次々となされている．筆者らも，上記siRNA発現ベクターをアデノウイルスに組み込んでHCVレプリコン培養細胞株でHCVの増殖をほぼ完全に抑制できるきわめて有効なsiRNA発現アデノウイルスベクターの作製（表1）に成功しており，臨床応用の可能性に期待している（未発表）．

HCVは宿主としてヒトとチンパンジーしか感染しないとされ，臨床応用には感染実験を可能とする小動物の開発が必要である．最近，新世界ザル，小型原猿類の一種であるタマリン[23]やツパイ[24]において，HCVあるいはHCVに近縁なウイルスであるGBV-Bを感染させることにより急性肝炎を起こすことが可能となった．これらはC型肝炎の疾患動物モデルとなりうる可能性がある．肝臓はウイルスベクターやハイドロダイナミクス導入法などを用いることによりsiRNAがアクセスしやすい点から，siRNAによる臨床応用のターゲットとして最もよい臓器の一つと考えられ，今後の発展に大いに期待がもたれる．

この研究は東京医科歯科大学消化器内科 坂本直哉先生，榎本信幸先生，東京大学工学部化学生命工学 宮岸真先生，多比良和誠先生との共同研究であり深謝する．また，図表の一部は坂本直哉先生の協力を得た．

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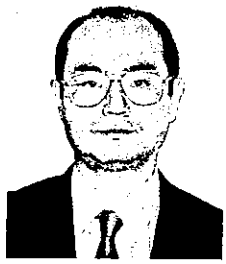
RNAiを用いたウイルス性疾患の遺伝子治療

Gene therapy of virus disease with RNAi



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◎今日までのウイルス増殖抑制の方法はワクチンかウイルス蛋白やウイルス特異酵素をターゲットとした創薬であった．近年，開発された強力な遺伝子発現抑制法であるsmall interfering RNA(siRNA)がウイルス核酸をターゲットとした新しい抗ウイルス薬としてその可能性が期待されている．すでに，AIDSウイルス，C型・B型肝炎ウイルス，SARSウイルスなどではそれぞれに特異的なsiRNAが作製されて*in vitro*ではこれらの有効性が確認されている．今回，C型肝炎ウイルスに対するsiRNAの具体例を示し，siRNAのウイルス性疾患への臨床応用の展望について述べたい．

Key word：C型肝炎，HIV，siRNA，B型肝炎，アデノウイルスベクター

今日までのウイルス増殖抑制の方法はワクチンかウイルス蛋白やウイルス特異酵素をターゲットとした創薬であった．ここ数年，ウイルスゲノム複製にかかわる転写，翻訳を核酸レベルで直接抑制しようという試みが，アンチセンスオリゴマー，ライボザイム，DNAエンザイムなどで行われていたが，十分な抑制効果は得られなかった．最近，新しい遺伝子発現抑制(gene silencing)として，これらをはるかに凌ぐ効果をもつRNAiが注目されている．

長い二本鎖RNAiによって誘導される遺伝子発現抑制であるRNAi現象は，植物から昆虫，哺乳動物に至るまで広く保存して観察され，元来真核細胞に備わった抗ウイルス機構として知られていた．しかし，哺乳動物における二本鎖RNAの導入はPKRや2'5'oligosynthetaseの活性化による非特異的な翻訳抑制やRNAの分解が生じ，宿主の細胞が死んでしまうため，分子生物学的手法としても遺伝子治療の方法としてもRNAiの利用の大きな妨げになっていた．しかし，近年，RNAi機構の中間産物であるsmall interfering RNA(siRNA)を用いることによってこれらの副反応が回避され，siRNAの配列に特異的な遺伝子発現抑制が可能となった(詳細は他稿を参照)．

細胞内においてsiRNAと内因性のゲノムから転写されたRNAとが会合(切断)する場と，外因性のウイルスの遺伝子(RNA)と会合(切断)する場が同じかどうかはわかっておらず，siRNAが内因性のゲノムの発現抑制と同様の効率でウイルスRNAを切断できるかどうかは完全には証明されていない．しかし，siRNAの機能は核酸配列によって規定されており，内因性の遺伝子であろうと，ウイルスのように外因性の遺伝子であろうとあらゆる遺伝子の発現にその作製は可能であり，多くのウイルスはその複製から成熟の過程において，siRNAが細胞質において裸のウイルスRNAに結合できる場面は多くあることが予想され，siRNAがウイルス疾患の治療薬として有効である可能性が高い[1]．

siRNAの発見以来，すぐにいくつかのウイルスにおいて細胞内でのウイルス遺伝子の切断やウイルス遺伝子複製モデルでにsiRNAが有効である

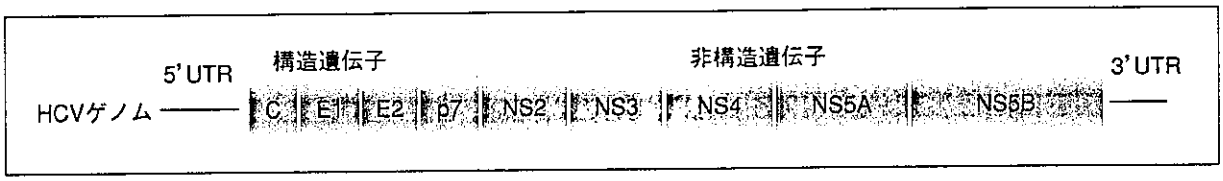

図 1　HCV の遺伝子構造

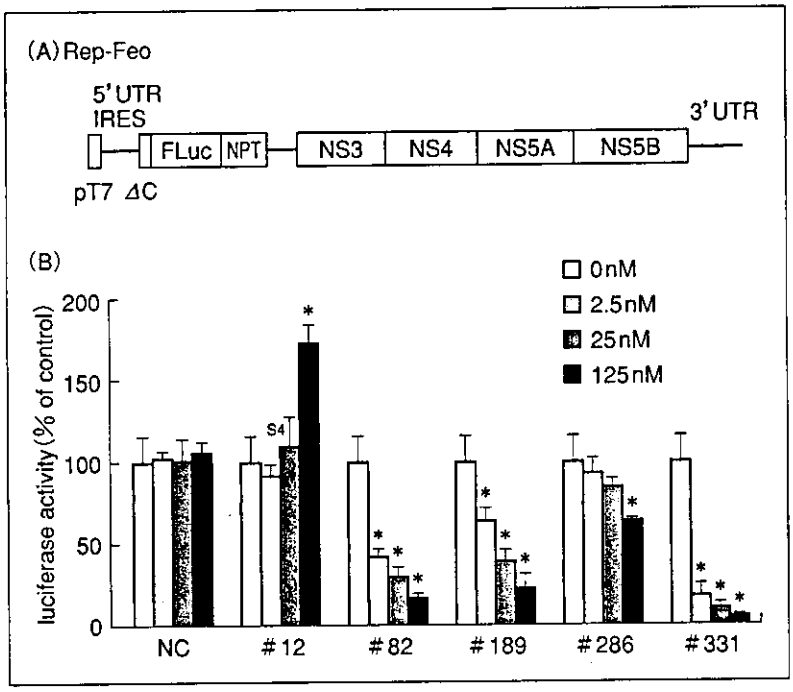

図 2

A：HCV レプリコン(Rep-Feo)の構造．Fluc：ルシフェラーゼ遺伝子，NPT：ネオマイシン耐性遺伝子，C：コア蛋白遺伝子．

B：合成 siRNA の HCV レプリコン(Rep-Feo)(B)へのルシフェラーゼ活性抑制効果(文献[4]から転載)．

との報告があいついでいる．現在まで，AIDS ウイルス(HIV)[2,3]，C 型[4-7]・B 型[8,9]肝炎ウイルス，SARS ウイルス[10]を含むいくつかのウイルスで有効な siRNA の報告がある．ここでは，著者らが作製した C 型肝炎ウイルス(HCV)に対する siRNA について[4]紹介する．

C 型肝炎ウイルス(HCV)

HCV 遺伝子は 9600 塩基からなるプラス一本鎖 RNA で，5'と 3'非翻訳領域(UTR)に挟まれた ORF からなる．5'側の 341 塩基の UTR は複雑な RNA 構造の IRES(internal ribosome entry site)(一部コア蛋白コード領域に及ぶ)を含み，HCV RNA はキャップ非依存的にこの 5'IRES により翻訳される．3'UTR にはポリ U 配列と 98 塩基からなる 3'X 領域が存在している．ORF は 5'から C，E1，E2，p7 の構造蛋白，NS2，NS3，NS4A，NS5A，NS5B の非構造蛋白を含む 3010 のアミノ酸からなる 1 本の大きなポリプロテインをコードしている(図 1)．

HCV は一本鎖 RNA ウイルスであるがゆえ，プルーフリーディング機能がなく，ウイルス複製時にとくに ORF 領域において RNA ポリメラーゼの読み違いによる変異を起こしやすい．HCV 遺伝子が同定されて以来，さまざまな遺伝子型が報告されてきたが，現在では分子進化学的に遺伝的に距離をもつ 6 つの遺伝子型に分類，整理されている．また，同一個体内においても遺伝子配列の異なったウイルス集団が存在して quasispecies とよばれている[11]．