INHIBITION OF UL54 AND UL97 GENES OF HUMAN CYTOMEGALOVIRUS BY RNA INTERFERENCE

M.-C. SHIN[1], S.-K. HONG[1], J.-S. YOON[1], S.-S. PARK[1], S.-G. LEE[1], D.-G. LEE[2], W.-S. MIN[2], W.-S. SHIN[2], S.-Y. PAIK[1*]

[1]Department of Microbiology, College of Medicine, and [2]Department of Internal Medicine, The Catholic Hemopoietic Stem Cell Transplantation Center, The Catholic University of Korea, Seoul, 137-701, South Korea

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Summary. – Short interfering RNAs (siRNAs), namely siUL54-1 and siU54-2 targeting UL54 (DNA polymerase) gene, and siUL97-1 and siUL97-2 targeting UL97 (phosphotransferase) gene, were used to inhibit respective genes of Human cytomegalovirus (HCMV) and consequently the virus infection process in human foreskin fibroblast (HFF) cultures. The virus infection was monitored by cell morphology (CPE), levels of UL83 and IE86 mRNAs, and virus antigen. The results showed that siUL97-2 remarkably inhibited viral CPE while other siRNAs were less inhibitory. The siRNAs reduced the levels of UL83 mRNA but not that of IE86 mRNA; again, siUL97-2 was most inhibitory. Particularly, siUL97-2 reduced the UL83 mRNA level 14, 19, 203, and 37 times at 24, 48, 72, and 96 hrs post infection (p.i.), respectively. When tested for the effect on viral antigen by immunofluorescent assay (IFA), UL97-2 exerted a marked inhibition. These results demonstrate the effectiveness of siRNAs against experimental HCMV infection and indicate their therapeutic potential.

Key words: Human cytomegalovirus; immunofluorescence assay; RNA interference; UL54; UL97; RT-PCR; real-time PCR

Introduction

HCMV is ubiquitous in humans worldwide and infects more than 50% of population as demonstrated at least serologically. Acute HCMV infection in normal immunocompetent persons has an asymptomatic latent period. However, this virus is a significant opportunistic pathogen in immunocompromised patients and the reactivation of a latent HCMV infection may have fatal outcome. For example, HCMV pneumonia is the most severe and fatal syndrome in post-bone marrow transplants (BMT) with suppressed cellular immunity (Landolfo *et al*., 2003). Moreover, solid organ transplants with HCMV pneumonia in the late post-transplant period are prone to severe infections with bacteria, fungi, and protozoa as well as to a decline or loss of function of the transplanted organ.

Despite these clinically significant points, there are no effective treatments of HCMV infections. Even though ganciclovir (GCV), foscarnet (PFA), and cidofovir (CDV) are currently used as therapeutics of HCMV infections, they are also administered as means of antiviral prophylaxis in increasing extent. Moreover, the advent of HCMV strains resistant to these medications has further limited their efficacy (Henry *et al*., 2001).

There is an urgent need for effective and safe therapeutics of HCMV infections. RNA interference

*Corresponding author. E-mail: paik@catholic.ac.kr; fax: +822-5356473.

Abbreviations: BMT = bone marrow transplantant; CDV = cidofovir; EGFP = enhanced green fluorescent protein; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; GCV = ganciclovir; HBV = Hepatitis B virus; HCMV = Human cytomegalovirus; HCV = Hepatitis C virus; HFF = human foreskin fibroblasts; HIV-1 = Human immunodeficiency virus 1; IE86 = immediate early protein 86; IFA = immunofluorescence assay; MOI = multiplicity of infection; p.i. = post infection; PFA = foscarnet; pp65 = phosphoprotein 65; RNAi = RNA interference; siRNA = small interfering RNA

(RNAi) in eukaryotes is a natural and evolutionary conserved process of post-transcriptional gene silencing by RNA-mediated RNA degradation in a sequence-specific manner. siRNAs have been developed as strong inhibitors of gene expression.

A siRNA, which is a 21–23 bp double-stranded RNA (dsRNA) that is complementary to a part of the target gene, induces sequence-specific degradation of mRNA of the target gene through the following mechanism: (i) the siRNA interacts with a multicomponent nuclease to form an RNA-induced silencing complex (RISC); (ii) the siRNA in the RISC directs the complex to the target mRNA through sequence complementarity; (iii) RNA polymerization begins from the siRNA to form a dsRNA; (iv) the dsRNA is cleaved into siRNAs which then initiate another round of RNA cleavage (Zhou *et al.,* 2004).

Since a dsRNA had been first reported to silence specific genes of *Caenorhabditis elegans* (Fire *et al.,* 1998), many reports on RNAi in animal cells appeared (Hammond *et al*., 2000; Zamore *et al*., 2000; Bernstein *et al*., 2001; Elbashir *et al*., 2001). RNAi has become a potential tool of inhibition of expression of specific genes and, consequently, of the cure of cancer and dominantly inherited genetic disorders (Martinez *et al*., 2002; Shuey *et al*., 2002).

Recently, also the inhibition of various animal viruses with siRNAs has been reported: Human immunodeficiency virus 1 (HIV-1) (Lahourari *et al*., 2006; Kenichi and Takashi, 2006; Hamamoto *et al*., 2006), Hepatitis C virus (HCV) (Hamazaki *et al*., 2006; Korf *et al*., 2005; Wilson and Richardson, 2005), influenza viruses (de Jonge *et al*., 2006; Zhou *et al*., 2004), polioviruses (Gitlin *et al*., 2005), and tumors associated with papillomaviruses (Jiang and Milner, 2002; Kapadia *et al*., 2003; McCaffrey *et al*., 2003; Seo *et al*., 2003; Shlomai *et al*., 2003).

There has been made so far only a single study on inhibition of HCMV with siRNAs (Wiebusch *et al.,* 2004) in which siRNAs targeting UL54 gene were examined. Its results showed an inhibition of virus yield, IE1 protein level, viral DNA synthesis, and UL54 mRNA level. In this paper we attempted to extend the analysis of the effect of siRNAs on HCMV by including siRNAs targeting besides UL54 also UL97 (phosphotransferase) gene involved in viral tegument formation, and by monitoring also viral CPE, viral antigen detectable by IFA, and the levels of UL83 and IE86 mRNAs.

Materials and Methods

Cells and virus. HFF cell line (ATCC CRL-1635) was maintained in DMEM supplemented with 10% of FBS at 37°C in 5% CO_2. HCMV AD169 strain (ATCC VR-538) used in this study was stored at -70°C.

siRNAs. siUL54-1 (5'-AAGGUGUAUAUUGC GGGUUCG-3', nt 79,209–79,189) and siUL54-2 (5'-AACUACGAGGUAGCCGAAGAU-3', nt 77,113–77,133) targeting UL54 (DNA polymerase) gene, and siUL-97-1 (5'-AAGAUUUGUUAUGCCGUGGAC-3', nt 141,450–141,470) and siUL97-2 (5'-AAUCACCAGUGUCGUGUAUGC-3', nt 141,822–141,842) targeting UL97 (phosphotransferase) gene were based on the sequence of HCMV AD169 strain (GenBank Acc. No. X17403). In order to ensure their specificity, they were subjected to a BLAST search against human EST library. No homology with eukaryotic DNA polymerase, phosphotransferase or other genes was found. A non-silencing fluorescein-labeled control siRNA (Roche, USA) was used as a negative control.

siRNAs assay. HFF cells (8 x 10^4cell/ml) in 12-well plates were transfected with 20 nmol/l each of the siRNAs using the HiPerFect Transfection Reagent (Qiagen, USA) according to the manufacturer's instructions. A non-silencing fluorescein-labeled siRNA (siNSF) was used as a negative control. A siRNA targeted against CDC2 was employed as a positive control. The positive control siRNA indicated a ~95% transfection efficiency (data not shown). After 24 hrs, the cells were infected with the virus at a multiplicity of infection (MOI) of 1.

RT-PCR. Total RNA was extracted using the Trizol reagent (Gibco, USA) and subjected to RT-PCR. The RT step was carried out using a reverse transcription system (Roche, USA) according to the manufacturer's instructions. The reaction with an oligo-dT primer ran at 42°C for 1 hr and at 90°C for 7 mins in a thermocycler (Perkin-Elmer, USA). The obtained cDNA was subjected to the PCR step with the primers 5'-GCACACCCAACGTGCAGACT CGGC-3' (forward, nt 170,324–170, 346) and 5'-TGG CTGCCTCGATG GCCAGGCTC-3' (reverse, nt 170,989–170,966) for IE86 gene, and 5'-CACCTGTCACCGCTG CTATATTTGC-3' (forward, nt 119,809–119,833) and 5'-CACCACGCAGCGGCCCTTGATGTTT-3' (reverse, nt 120,208–120,184) for UL83 gene (Detrick *et al*. 2001).

The amplification consisted of initial incubation at 95°C for 105 secs and 35 cycles of 95°C/15 secs, 60°C/30 secs and 72°C/2 mins. The PCR products were analyzed by agarose gel electrophoresis in a standard manner.

Real-time PCR. cDNA for real-time PCR was synthesized using the DyNamo™ SYBR Green 2-step qRT-PCR Kit (Finnzymes, Finland) according to the manufacturer's instructions. The reaction consisted of 25°C/10 mins, 37°C/30 mins, and 85°C/5 mins. Real-time PCR was carried out using the MiniOpticon Real-Time PCR System and the MJ Mini Thermal Cycler (Bio-Rad Laboratories, USA). The following primers for assaying UL83 mRNA encoding pp65 were employed: CMV 028 (5'-AAAGAGCCCGACGTCTACTACACGT-3', forward, nt 866–890) and CMV 029 (5'-CCAGGTACACC

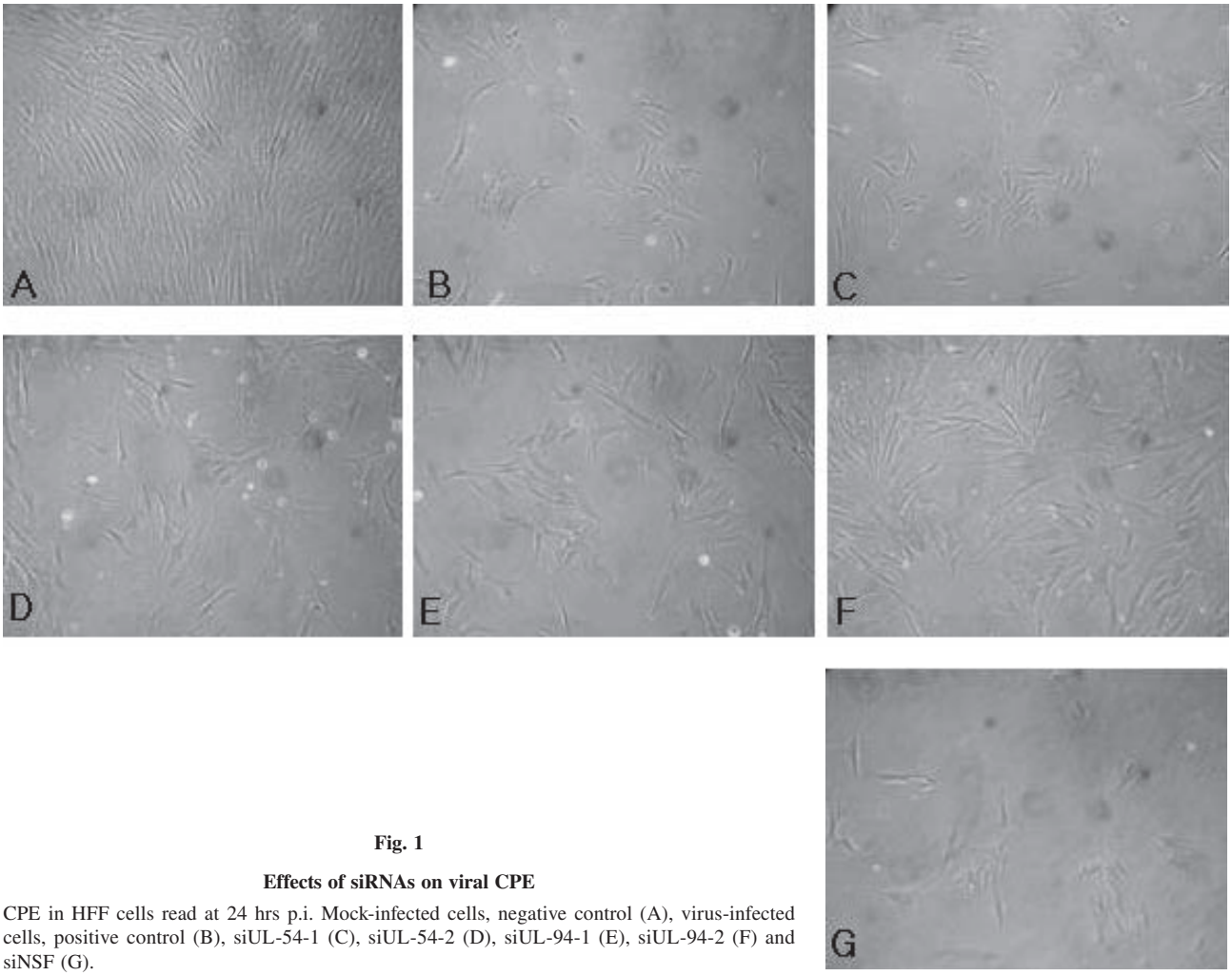

Fig. 1

Effects of siRNAs on viral CPE

CPE in HFF cells read at 24 hrs p.i. Mock-infected cells, negative control (A), virus-infected cells, positive control (B), siUL-54-1 (C), siUL-54-2 (D), siUL-94-1 (E), siUL-94-2 (F) and siNSF (G).

TTGACGTACTGGTC-3', reverse, nt 1,046–1,021). The primers were designed on the basis of known sequence (GenBank Acc. No. NC_001347) (Zaia *et al*., 1990). The relative quantification of pp65 expression was determined by comparing the amount of pp65 transcript with that of a housekeeping, ß-actin gene using the Opticon Monitor 3.1.32 program (Bio-Rad Laboratories). Melting curve analysis was performed for each reaction to ensure the specificity of the reaction. Absolute quantification of the pp65 transcript was done using a standard curve, which was generated from serial dilutions of the annealed oligomer that was used to construct the pGEM-T Easy vector (Promega) containing one copy of the UL83 gene encoding the pp65 target sequence (159 bp). HFF cells were transfected with siRNA and infected with HCMV 24 hrs later as above. The infected cells were harvested at 24, 48, 72, and 96 hrs p.i., their Rna was isolated and subjected to relative PCR for UL83 mRNA.The reaction ran in triplicate and three independent experiments were made. Quantification of UL83 mRNA was done using a standard curve determining the dependence of log of number of UL83 mRNA copies on Ct.

IFA. The cells were harvested from each well, pelleted at 1,200 rpm for 4 mins, washed with PBS, resuspended in 200 µl of PBS, and smeared onto Teflon-coated slides. These were dried at room temperature and the cells were fixed with absolute methanol at -20°C for 10 mins, rehydrated in PBS for 5 mins, and incubated with a monoclonal antibody (MAb) to HCMV (the DO-7 clone, DAKO, USA) at 37°C for 1 hr in a humidified chamber. The cells were then washed 3 times with PBS and incubated with a FITC-conjugated goat anti-mouse IgG for 45 mins. Coverslips were mounted on the glass slides and examined using fluorescence microscopy (Axiotech 100, Zeiss Jena, Germany).

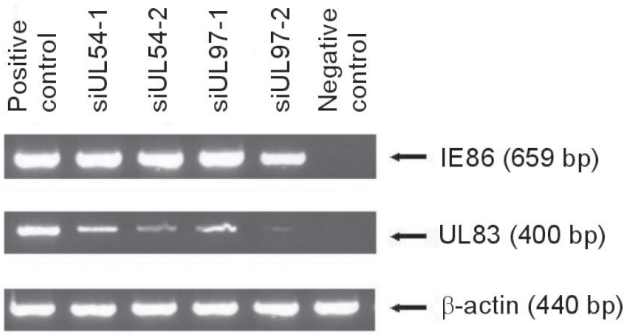

Fig. 2

Effects of siRNAs on the levels of IE86 and UL83 mRNAs

RT-PCR, agarose gel electrophoresis. ß-actin mRNA served as internal standard.

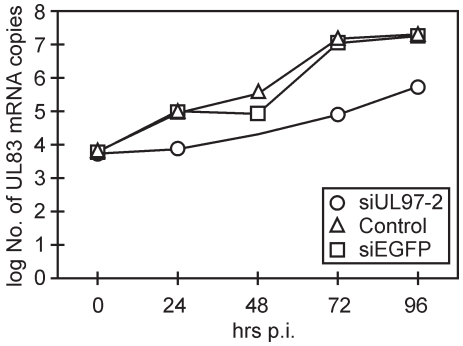

Fig. 3

Reduction of UL83 mRNA level by siUL97-2

Real-time PCR. HCMV UL83 mRNA level in the cells transfected with siUL97-2 and siEGFP, respectively, and in non-transfected cells (control).

Results

Effect of siRNAs on viral CPE in HFF cells

Inhibition of the virus infection with the siRNAs was assessed by transfecting HFF cells with 20 nmol/l siUL54-1, siUL54-2, siUL97-1, and siUL97-2, respectively, infecting them 24 later with the virus, and reading CPE at 24 hrs p.i. CPE was significantly reduced in the infected cells transfected with siRNAs, most markedly with si97-2 (Fig. 1C-F). There was an evident CPE in the infected cells and in the infected cells trasnfected with siNSF (Fig. 1B and 1G) but none in the mock-infected cells (Fig. 1A).

Effects of siRNAs on the levels of IE86 and UL83 mRNAs as determined by RT-PCR

In order to test the effects of the siRNAs on selected viral mRNAs the levels of IE86 and UL83 mRNAs in HFF cells transfected with 20 nmol/l siUL54-1, siUL54-2, siUL97-1, and siUL97-2, respectively, and infected 24 later with the virus were determined at 24 hrs p.i. by RT-PCR. The results

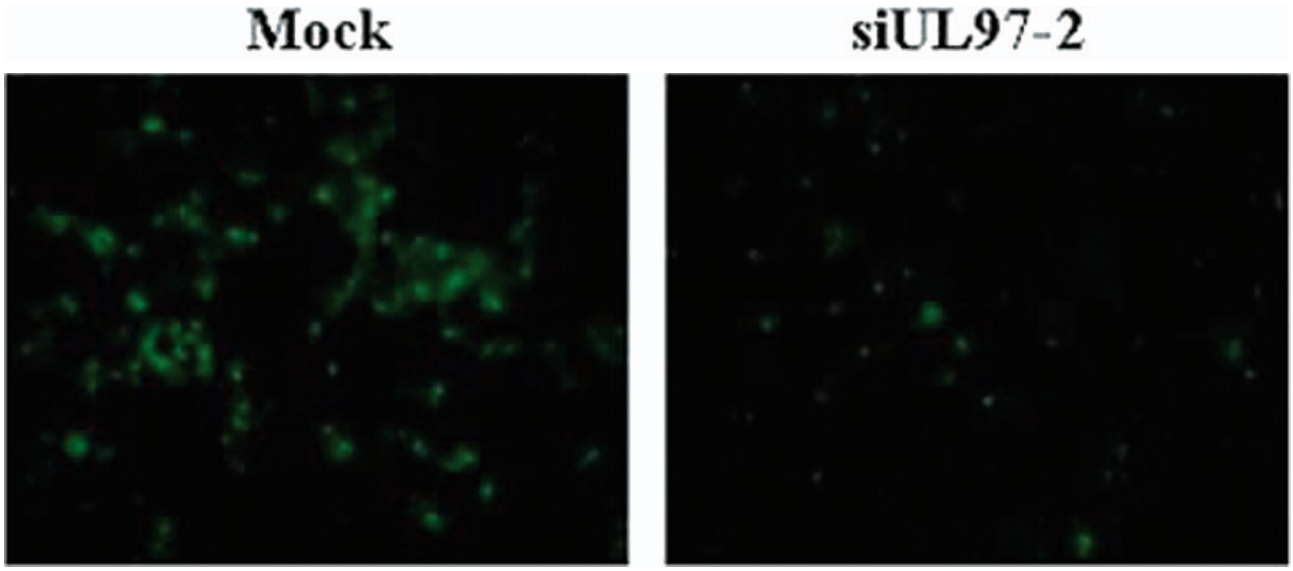

Fig. 4

Effect of siUL97-2 on viral antigen expression as determined by IFA

IFA performed at 24 hrs p.i.

showed that all four siRNAs reduced the level of UL83 mRNA but not that of IE86 mRNA (Fig. 2). Among the siRNAs siUL97-2 was most inhibitory.

Effect of siUL97-2 on UL83 mRNA level as determined by real-time PCR

The reduction of UL83 mRNA level with siUL97-2 demonstrated by RT-PCR was examined using real time PCR. The HFF cells transfected with siUL97-2 and infected 24 hrs later with the virus were examined for UL83 mRNA at 24, 48, 72, and 96 hrs p.i. by real-time PCR. The results showed that the UL83 mRNA levels were reduced at the above time intervals 14, 19, 203, and 37 times, respectively (Fig. 3).

Effect of siUL97-2 on viral antigen expression in cells as determined by IFA

The inhibitory effect of siUL97-2, the strongest inhibitor of the siRNAs tested, at the viral protein level was determined by IFA that demonstrated in cells the viral protein recognized by the given MAb. The experiment with HFF cells was set up in the same manner as above. The result showed that siUL97-2 reduced markedly the specific fluorescence of infected cells (Fig. 4).

Discussion

The results on RNAi with HCMV described in this study cannot be directly compared with those of Wiebusch *et al*. (2004) because, in spite of common virus, cells and some siRNAs (siUL54-1 and siUL54-2), they concerned different parameters of viral infection. Whereas these authors followed virus yield, IE1 protein, viral DNA synthesis, and UL54 mRNA level and found their inhibition, we concentrated on CPE, viral antigen, and levels of UL83 and UE86 mRNAs. Our study brought a few interesting findings: (i) an siRNA targeting UL97 gene (siUL97-2) was more inhibitory than the siRNAs targeting UL54 gene towards viral CPE and UL83 mRNA, (ii) siUL97-2 reduced the level of UL83 mRNA but not that of IE86 mRNA.

The reason why the effects of siRNA targetingUL54 and UL97 genes were assayed on different genes (UL83 and IE86) was the fact that these genes produce proteins (pp65 and IE86) which are characteristic markers of HCMV infection and UL83 mRNA can be well quantitated by RT-PCR or real-time PCR.

In conclusion, the siRNAs applied to HCMV might have a potential of novel therapeutics. This highly specific approach has an advantage over conventional antiviral drugs such as GCV, PFA or CDV on account of their toxic side effects and emergence of drug-resistant viral strains.

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References

Bernstein E, Caudy AA, Hammond SM, Hannon, GJ (2001): Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366.

de Jonge J, Holtrop M, Wilschut J, Huckriede A (2006): Reconstituted influenza virus envelopes as an efficient carrier system for cellular delivery of small-interfering RNAs. *Gene Ther.* **13**, 400–401.

Elbashir SM, Harborth J, Lendeckel W Yalcin A, Weber K, Tuschl T (2001): Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE Mello CC (1998): Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806–811.

Gitlin L, Stone JK, Andino R (2005): Poliovirus escape from RNA interference: short interfering RNA-target recognition and implications for therapeutic approaches. *J. Virol.* **79**, 1027–1035.

Hamamoto S, Nishitsuji H, Amagasa T, Kannagi M, Masuda T (2006): Identification of a novel human immunodeficiency virus type 1 integrase interactor, gemin2, that facilitates efficient viral cDNA synthesis in vivo. *J. Virol.* **80**, 5670–5677.

Hamazaki H, Ujino S, Miyano-Kurosaki N, Shimotohno K, and Takaku H (2006): Inhibition of hepatitis C virus RNA replication by short hairpin RNA synthesized by T7 RNA polymerase in hepatitis C virus subgenomic replicons. *Biochem. Biophys. Res. Commun*. **343**, 988–994.

Hammond SM, Bernstein E, Beach D Hannon GJ (2000): An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* **404**, 293–296.

Henry SP, Miner RC, Drew WL, Fitchett J, York-Dekalco, DC, Rapp LM, Levin AA (2001): Antiviral activity and ocular kinetics of antisense oligonucleotides designed to inhibit CMV replication. *Invest. Ophthalmol. Vis. Sci.* **42**, 2646–2651.

Jiang M, Milner J (2002): Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. *Oncogene* **21**, 6041–6048.

Kapadia SB, Brideau-Andersen A, Chisari FV (2003): Interference of hepatitis C virus RNA replication by short interfering RNAs. *Proc. Natl. Acad. Sci. USA* **100**, 2014–2018.

Kenichi I, Takashi OT (2006): Transcriptional repression of human immunodeficiency virus type 1 by AP-4. *J. Biol. Chem*. **281**, 12495–12505.

Korf M, Jarczak D, Beger C, Manns MP, Kruger M (2005): Inhibition of hepatitis C virus translation and subgenomic replication by siRNAs directed against highly conserved

HCV sequence and cellular HCV cofactors. *J. Hepatol.* **43**, 225–234.

Lahouari A, Desclaux M, Faucon-Biguet N, Mallet J, Vogel R (2006): Control of small inhibitory RNA levels and RNA interference by doxycycline induced activation of a minimal RNA polymerase III promoter. *Nucleic Acids Res*. **34**, e37.

Landolfo S, Gariglio M, Gribaudo G, Lembo D (2003): The human cytomegalovirus. *Pharmacol. Ther.* **98**, 269–297.

Martinez J, Patkaniowska A, UrlaubH, Luhrmann R, Tuschl T (2002): Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **110**, 563–574.

McCaffrey AP, Nakai H, Pandey K, Huang Z, Salazar FH, Xu H, Wieland SF, Marion PL Kay MA (2003): Inhibition of hepatitis B virus in mice by RNA interference. *Nat. Biotechnol. Nat. Biotechnol.* **21**, 639–644.

Seo MY, Abrignani S, Houghton M, Han JH (2003): Small interfering RNA-mediated inhibition of hepatitis C virus replication in the human hepatoma cell line Huh-7. *J. Virol.* **77**, 810–812.

Shlomai A Shaul Y (2003): Inhibition of hepatitis B virus expression and replication by RNA interference. *Hepatology* **37**, 764–770.

Shuey DJ, McCallus DE, Giordano T (2002): RNAi: gene-silencing in therapeutic intervention. *Drug Discov. Today* **7**, 1040–1046.

Wiebusch L, Truss M, Hagemeier C (2004): Inhibition of human cytomegalovirus replication small interfering RNAs. *J. Gen. Virol*. **85**, 179–184.

Wilson JA, Richardson CD (2005): Hepatitis C virus replicons escape RNA interference induced by a short interfering RNA directed against the NS5b coding region. *J. Virol*. **79**, 7050–7058.

Zaia JA, Gallenz-Hawkins G, Churchill MA, Morton-Blackshere A, Pande H, Adler SP, Schmidt GM, Forman SJ (1990): Comparative analysis of human cytomegalovirus ?-sequence in multiple clinical isolates by using polymerase chain reaction and restriction fragment length polymorphism assays. *J. Clin. Microbiol*. **28**, 2602–2607.

Zamore PO, Tuschl T, Sharp PA Bartel DP (2000): RNAi: Double-stranded RNA directs the ATP-dependent cleavage of Mrna at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33.

Zhou Y, Chan JH, Chan AY, Chak RK, Wong EY, Chye ML, Peiris JS, Poon LL, Lam E (2004): Transgenic plant-derived siRNAs can suppress propagation of influenza virus in mammalian cells. *FEBS Lett.* **577**, 345–350.