# INHIBITION OF MEASLES VIRUS MULTIPLICATION IN CELL CULTURE BY RNA INTERFERENCE

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**Summary. –** Measles takes a toll of one million people a year worldwide, especially in developing countries. As the so far applied vaccines were not able to control this disease, novel approaches to this problem are needed. In this study, we investigated the possibility of inhibition of Measles virus (MV) infection through RNA interference (RNAi), namely the interference with the second but main receptor of MV, signaling lymphocyte activation molecule (SLAM), in an Epstein-Barr virus (EBV)-transformed marmoset B cell line B95-8. Three potential target sequences in the SLAM gene were chosen and cloned into the pSilencer 3.0-H1 vector. The vector constructs were used for transfection of B95-8 cells in which SLAM was then detected by flow cytometry, Western blot analysis and RT-PCR both at transcription and expression levels. One of the vector constructs was found to block effectively the SLAM expression as well as MV infection. These results suggest that RNAi could silence SLAM expression and inhibit the MV infection of host cells.

**Key words:** siRNAs; Measles virus; SLAM; RNAi; B95-8 cell line

## Introduction

Measles, also known as rubeola, is a common disease caused by MV (the species *Measles virus*, the genus *Morbillivirus,* the family *Paramyoviridae*). Although a live-attenuated vaccine protects against MV infection, the latter retains a high mortality worldwide, accounting for almost one million deaths per year (World Health Organization, 2000). This is partially because of inefficient immunization of young infants resulting from immaturity of their immune system and interference with transplacentally acquired maternal antibodies. Considering (i) the mortality associated with primary MV infection and secondary microbial infections due to MV-induced immunosuppression and (ii) a limited efficacy of ribavirin, the only drug approved for treatment of some paramyxovirus infections, the development of novel therapeutics that would control local outbreaks and close the immunization gap in young infants appears a priority (Plemper *et al.,* 2004).

Tatsuo *et al.* (2000) have shown that SLAM; also known as CD150, is a cellular receptor for both wild-type and vaccine strains of MV. SLAM, a 70 K molecule that mediates CD28-independent proliferation of T cells and interferon gamma (IFN-gamma) production, has been identified on human T cells, immature thymocytes, and a subset of B cells (Kruse *et al*., 2001). The human SLAM gene is located on the chromosome 1q22-q23 (Yanagi *et al*., 2002), is constitutively expressed on immature thymocytes, high memory CD45RO T cells, and a part of B cells, and is rapidly induced in all T and B cells following activation (Aversa *et al*., 1997; Cocks *et al*., 1995; Sidorenko *et al*., 1993). MV infection of lymphocytes, monocytes, macrophages, and dendritic cells could be mediated by SLAM (Yanagi *et al*., 2002). All MV strains can use SLAM as receptor, while only Edmonston and some other strains can use, besides SLAM, also CD46 as a receptor.

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**Abbreviations:** ECL = enhanced chemiluminescence; EBV = Epstein-Barr virus; APDH = glyceraldehyde-3-phosphate dehydrogenase; HIV-1 = Human immunodeficiency virus 1; MOI = multiplicity of infection; MV = Measles virus; RNAi = RNA interference; SBMA = spinobular muscular atrophy; siRNAs = small interfering RNAs; SLAM = signaling lymphocyte activation molecule

Virus in throat swabs from measles patients was titrated by plaque formation on SLAM-positive and SLAM-negative Vero cells. The results showed that most swabs produced numerous plaques on SLAM-positive cells (Ono *et al.,* 2001). It has been concluded that the great majority of MV in the bodies of measles patients uses SLAM but not CD46 as a receptor. So SLAM serves better than CD46 as a receptor. SLAM appears to be the principal receptor for MV, accounting for most of measles pathology and pathogenesis (Takeda *et al*., 2000).

RNAi is a recently discovered phenomenon that can be exploited for inhibition of gene expression. RNAi can result in specific gene silencing of homologous cellular RNA at post-transcriptional level (Fire *et al*., 1998). The precise mechanism involved is currently being elucidated in several systems and it appears that many of the steps in this process are conservative (Hammond *et al*., 2000). In brief, the RNAi pathway consists of the presentation of a 'triggering' dsRNA that is subsequently processed into 21–25 nucleotides (nt) long fragments, called small interfering RNAs (siRNAs) by an RNase III-like enzyme called Dicer (Hutvagner *et al*., 2002; Zamore *et al*., 2000). Then, siRNAs are recruited to an RNA-induced silencing complex (RISC) which in turn mediates the cleavage of the target mRNA. The latter occurs within the region of homology, directed by the original siRNAs (Elbashir *et al*., 2001). The cellular mechanism that involves the RNA-dependent RNA polymerase-mediated conversion of ssRNA into dsRNA and its amplification has been proposed (Ahlquist *et al*., 2002; Shuey *et al*., 2002). In this way siRNAs can selectively inhibit the target gene expression.

Using RNAi, a number of interesting disease-related genes have been targeted, which highlighted the therapeutical potential of this approach. A mutated RNA, associated with spinobular muscular atrophy (SBMA), was targeted in human kidney 293T cells (Caplen *et al*., 2002). Transfected cells expressing the CAG expanded androgen receptor mRNA responded to siRNA treatment by a reduction of the mutated mRNA level and, more importantly, by a rescue of the polyglutamine-induced toxicity. This example represents a proof of the principle to apply RNAi technology to diseases associated with mutated transcripts arising from one allele without affecting another (healthy) (Schütze, 2004). A specific inhibition of the oncogenic K-RAS V12 expression in human tumor cells by retroviral expressing vectors, which resulted in loss of anchorage-independent growth and tumorigenicity, indicated that viral delivery of siRNAs can be used for tumor-specific gene therapy (Brummelkamp *et al*., 2002). An effective siRNA-dependent degradation of HIV-1 ref mRNA was achieved in cells co-transfected with proviral HIV-1 DNA and siRNA-producing constructs (Lee *et al*., 2002). Moreover, human, T cells transfected with a lentiviral siRNA vector targeting the HIV-1 coreceptor CCR5 displayed a reduced CCR5 expression in HIV-1-infected cells (Qin *et al*., 2003). These findings indicate that siRNAs could be useful in antiviral strategies (Schütze, 2004).

In this study, we targeted the MV receptor SLAM gene in B95-8 cells by means of siRNAs generated from a DNA vector. We demonstrate that the siRNAs silenced effectively and specifically the SLAM expression and MV infection in B95-8 cells.

## Materials and Methods

*Vectors.* The hairpin cDNAs were generated through annealing of complementary oligonucleotides with *Bam*HI and *Hind*III sites and were inserted in the vector pSilencer 3.0-H1 (Fig. 1B), a gift from Dr. D. Guo, Wuhan University, Wuhan, P.R. China. The obtained clones were sequenced using the primers 5'-GTTTTCC CAGT CACGAC-3' and 5'-GAGTTAGCTCACTCATTAGGC-3'.

*Cell culture and transfection.* The Epstein-Barr virus-transformed marmoset B95-8 and Vero cells were cultured in RPMI 1640 medium and Dulbecco's Modified Eagle's Medium (DMEM), respectively, both supplemented with 10% of heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. B95-8 cells were seeded in 12-well plates at a density of $4\times10^5$ cells per well and cultured at 37°C in 5% $CO_2$ overnight. They were transfected at 90% cell confluence with 4 mg of DNA per well, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

*Flow cytometry.* Seventy-two hrs after transfection, the cells were incubated with an antibody to SLAM (Stratagen) at 4°C for 1 hr in 100 ml of PBS containing 0.4% BSA and 0.01 mol/l $NaN_3$. The cells were washed twice with PBS and incubated with a fluorescein isothiocyanate (FITC)-conjugated mouse anti-goat immunoglobulin (Beijing Zhong Shan Biotechnology, Co. Ltd) at 4°C for 1h in 100 ml of PBS. After two washes, the cells were fixed with 1% paraformaldehyde and analyzed in a Beckman-Coulter XL-MCL flow cytometer (Beckman Coulter, USA).

*Western blot analysis.* Seventy-two hrs post-transfection, the cells were harvested and lysed with 0.3 ml of a cold lysis buffer (20 mmol/l Tris-HCl pH 7.4, 150 mmol/l NaCl, 1mmol/l EDTA, 1% Triton X-100, 1 mmol/l $NaN_3$, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l L-glycerol phosphate, 1 mmol/l phenylmethylsulfonyl fluoride, 5 mg/ml aprotinin, and 5 mg/ml leupeptin pH 7.5). The total protein from cell lysate was separated by electrophoresis in 12% sodium dodecyl sulfate-polyacrylamide gel and electroblotted to nitrocellulose membrane (Hyclone ECI, Amersham Pharmacia Biotech). The blot was incubated with antibodies to SLAM (Stratgene) and ß-actin (Stratagene), respectively, according to the manufacturers' instructions. The signal was detected by enhanced chemiluminescence (ECL, Amersham).

*RT-PCR.* Trizol (Invitrogen) was used to extract total RNA from transfected cells following the manufacturer's protocol. The first strand cDNA synthesis was performed with the Reverse Transcription System (Promega). PCR was carried in a final volume of 20 µl with 0.8 µl of each primer (0.4 µmol/l), 3.2 µl of 25 mmol/l $MgCl_2$, 2 µl of enzyme mixture (Shanghai Casarray), 11.2 µl of $H_2O$, and 2 µl of RT product in a T1 thermocycler (Whatman Biometra, Germany). The following primers were used:

for SLAM, forward primer 5'-GATCCCAAGGGGCTCCTCTC-3' and reverse primer 5'-GCTCTCTGGAAGTGTCACAC-3'; for GAPDH, forward primer 5'-CATGGAGAAGGCTGGGGCTC-3' and reverse primer 5'-CACTGACACGTTGGCAGTGG-3'. Glyceraldehyde-3-phosphate dehy dehydrogenase (GAPDH) was used as an internal control.

After initial incubation at 95°C the mixture was subjected to 35 cycles of 95°C/10 mins (denaturation), 60°C/30 secs (annealing) and 72°C/1 min (extension) or 72°C/6 mins (final extension).

*Virus infection and titration.* B95-8 cells were infected with MV Shanghai 191vaccine strain (S191) at a multiplicity of infection of 1 72 hrs after the transfection with vectors. After 2 hrs of virus adsorption to the cells the inoculum was removed and the cells were washed with PBS. Seventy-two hrs p.i., the cells were observed by light microscopy and harvested. The virus was collected after two rounds of freeze-thawing and titrated by plaque assay in Vero cells.

## Results

*Selection of siRNA target sequences*

The SLAM mRNA (Fig. 1A) was chosen as a target for inhibition of MV infection because SLAM is a key receptor in MV infection. Beginning with the AUG start codon we scanned the SLAM mRNA for AA dinucleotides and 3'-adjacent 19 nucleotides as potential siRNA target sites. We preferably chose the sites with GC/AT ratios between 30% and 60% and eliminated all target sequences with a homology of more than 16–17 contiguous bp to other coding sequences. Based on these criteria, three specific targets were selected, namely SLAM-RNAi1 (nt 231–249), SLAM-RNAi2 (nt 372–390) and SLAM-RNAi3 (nt 637–655).

*Construction of siRNA vectors*

pSilencer3.0-H1 (pH1) was used as the vector for construction of siRNA vectors. The vector contains the H1 RNA pol III promoter and provides high levels of constitutive expression across a variety of cell types. Hairpin cDNAs were generated by annealing of the complementary synthetic oligonucleotides with *Bam*HI and *Hind*III sites and were inserted into pSilencer3.0-H1 (Fig. 1B). The insertions were checked by sequencing. The structure of hairpin ssRNAs generated from the siRNA vectors are shown in Fig. 1C.

*Silencing of SLAM expression in B95-8 cells by siRNAs*

To determine whether the siRNAs could effectively reduce the expression of SLAM protein in cultured B95-8 cells the latter were transfected with pSilencer-H1 (pH1), pH1/SLAM-RNAi1, pH1/SLAM-RNAi2 and pH1/SLAM-RNAi3 vectors, respectively. Seventy-two hrs post

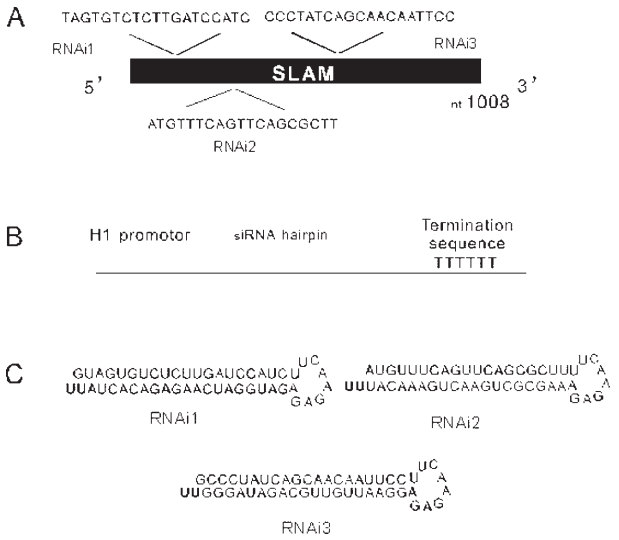


**Fig. 1**

**Structure of SLAM mRNA, siRNA vectors and siRNAs**

SLAM mRNA with siRNA target sites (A). A part of the structure vectors generating siRNAs (B). Structure of siRNAs from the vectors pH1/SALM-RNAi1, pH1/SALM-RNAi2 and pH1/SALM-RNAi3 (C).

transfection, the cells were harvested and the expression levels of surface SLAM were determined. The cells transfected with the pH1 vector were used as negative control.

Whereas pH1/SLAM-RNAi1 and pH1/SLAM-RNAi2 had only a slight effect, pH1/SLAM-RNAi3 reduced specifically the SLAM level by about 65% (Fig. 2).

In a control experiment, the SLAM expression in the pH1 vector-transfected cells was the same as that in non-transfected cells (data not shown). These experiments confirmed that they were the siRNAs that blocked the expression of the SLAM protein in B95-8 cells cells.

To further confirm the reduced level of the SLAM protein in transfected cells, Western blot analysis was performed. The results showed that whereas pH1/SLAM-RNAi1 and pH1/SLAM-RNAi2 exerted only a slight inhibitory effect (Fig. 3B and C), pH1/SLAM-RNAi3 inhibited the SLAM expression almost completely (Fig. 3D).

In a control experiment, the pH1 vector allowed a strong expression of SLAM (Fig. 3A). The ß-actin expression was used as loading control. The results of Western blot analysis confirmed those of flow cytometry, suggesting that, in general, RNAi can block the SLAM expression in cells, and, in particular, SLAM-RNAi3 was efficient in B95-8 cells.

The mode of action of RNAi resides in cleavage and degradation of the target gene mRNA that results in silencing of the target gene expression. To verify this mechanism, the SLAM mRNA level was determined by a semi-quantitative RT-PCR. The data showed that (i) all the three siRNAs

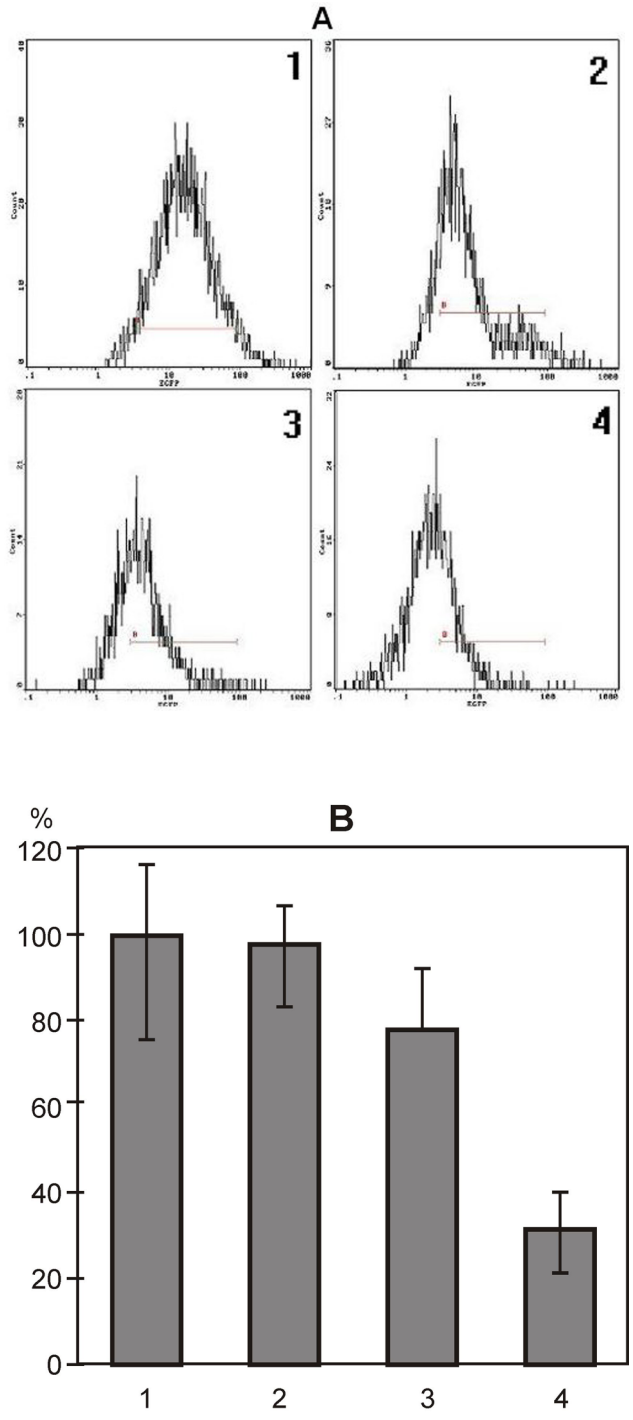


**Fig. 2**

**Flow cytometry of SLAM expression**

B95-8 cells transfected with pH1 (control, 1), pH1/SALM-RNAi1 (2), pH1/SALM-RNAi2 (3) and pH1/SALM-RNAi3 (4), respectively (A). Relative levels (%) of SLAM expression (B). Mean values from two experiments ± SE are given.

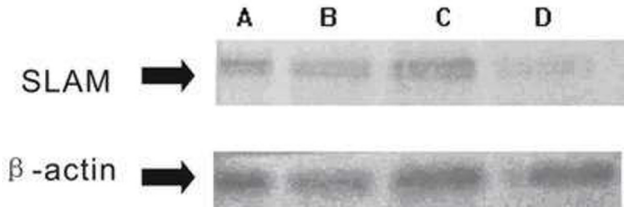


**Fig. 3**

**Western blot analysis of expression of SLAM protein**

B95-8 cells transfected with pH1 (A), pH1/SALM-RNAi1 (B), pH1/SALM-RNAi2 (C) and pH1/SALM-RNAi3 (D), respectively. ß-actin was used as internal control.

reduced the SLAM level but did not affect GAPDH mRNA and (ii) SLAM-RANi3 was most effective (Fig. 4). The data indicated that siRNAs specifically inhibited the SLAM expression through reducing the SLAM mRNA level, and thus reduced the level of SLAM in the cell membrane.

*Inhibition of MV infection by RNAi in cultured cells*

Seventy-two hrs after transfection with the RNAi vectors, B95-8 cells were infected with MV S191 vaccine strain. pH1-transfected-cells were used as control. Seventy-two hrs p.i., the cells were observed for CPE by light microscopy (Fig. 5). The control and pH1/SALM-RNAi1- and pH1/SALM-RNAi2-transfected cells displayed a typical CPE with large syncytia, which later on, expanded, detached from plate wells and died, thus resulting in the reduction of cell count. On the other hand, pH1/SALM-RNAi3 clearly blocked the CPE.

These data suggest that pH1/SALM-RNAi3 significantly reduced the level of SALM and inhibited the MV infection of B95-8 cells, apparently through blocking the entry of the virus into the cells.

To assess the effects of RNAi on MV replication, virus yields in RNAi-transfected B95-8 cells at 72 hrs p.i. with the Shanghai 191 vaccine strain of MV were determined (Fig. 6). The harvested cells were freeze-thawed twice and titrated in Vero cells. The titer in the cells transfected with

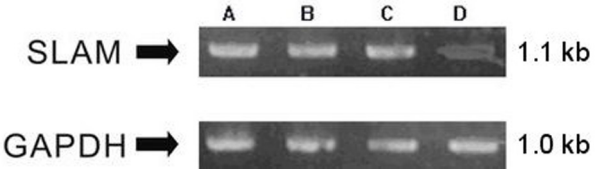


**Fig. 4**

**RT-PCR assay of SLAM mRNA accumulation**

B95-8 cells were transfected with pH1 (A), pH1/SALM-RNAi1 (B), pH1/SALM-RNAi2 (C) and pH1/SALM-RNAi3 (D), respectively. GAPDH was used as internal control.

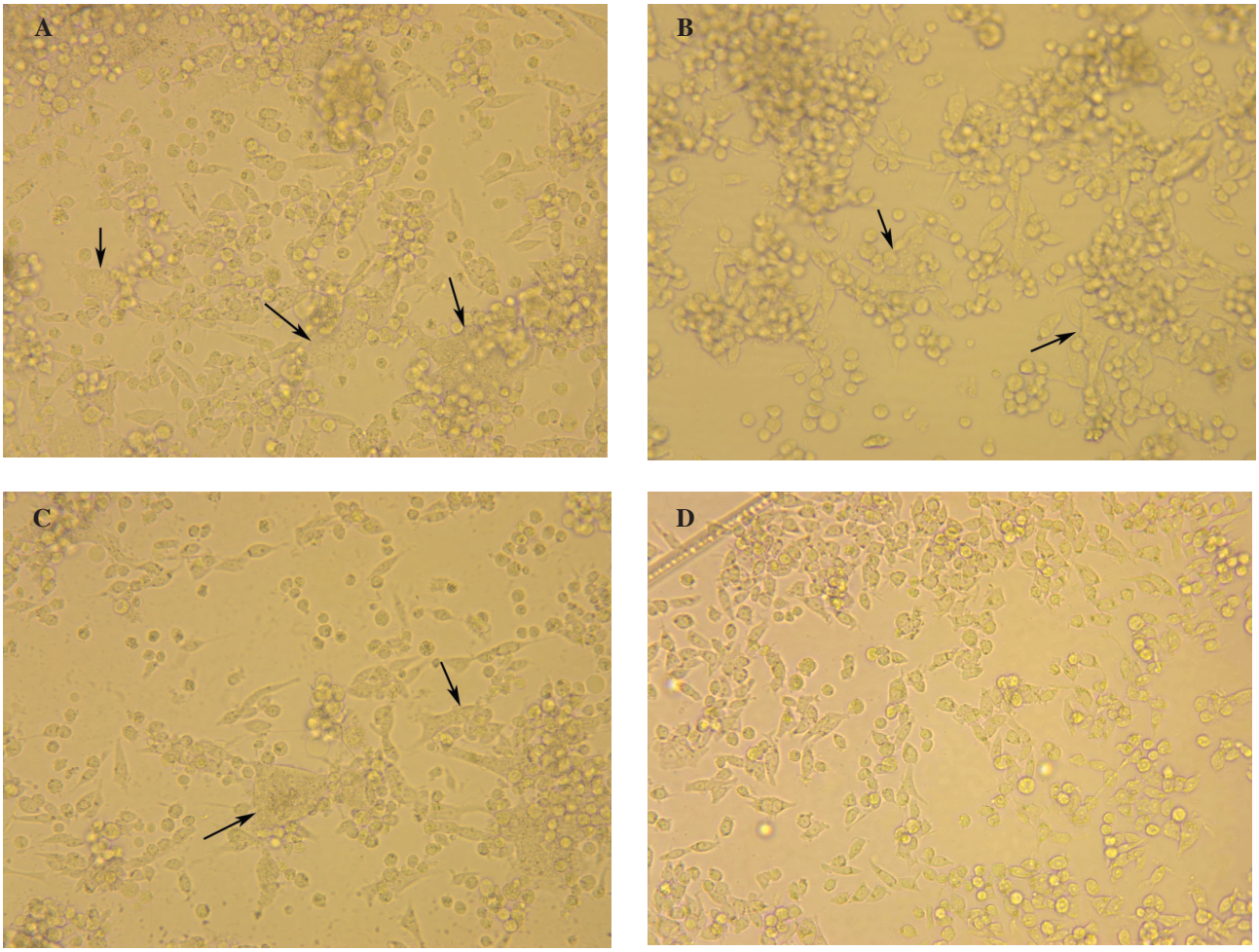


**Fig. 5**

**Morphology of RNAi-transfected B95-8 cells infected with MV**

The cells were first transfected with pH1 (A), pH1/SALM-RNAi1 (B), pH1/SALM-RNAi2 (C) and pH1/SALM-RNAi3 (D), respectively, and then infected with MV. The ells were observed under a Leica TE300 light microscope. Syncytia are marked by arrows.

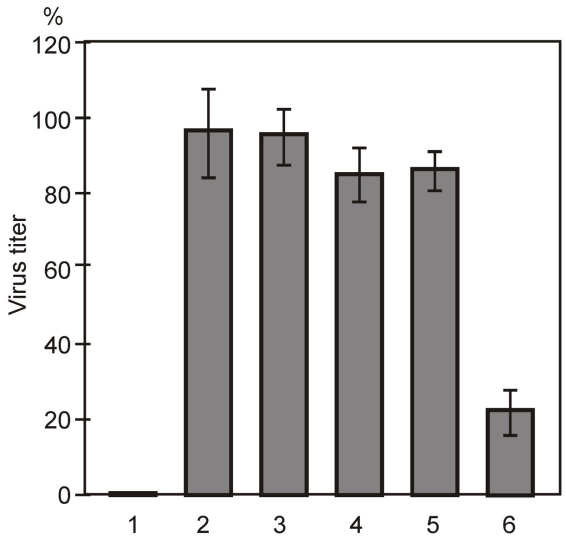


**Fig. 6**

**MV yields in RNAi-transfected B95-8 cells infected with MV**

The cells were first transfected with pH1 (2, negative control), pH1/GADPHi (3, negative control), pH1/SALM-RNAi1 (4), pH1/SALM-RNAi2 (5) and pH1/SALM-RNAi3 (6), respectively, and then infected with MV. Uninfected cells (1) served as another negative control.

pH1/SALM-RNAi3 was much lower than those in the cells transfected with pH1/GAPDHi and pH1, respectively. The titers in the cells transfected with pH1/SALM-RNAi1 or pH1/SALM-RNAi2 were only slightly lower than those in the controls. These results of CPE and titration experiments support the view that RNAi3 could reduce MV multiplication through a reduced SLAM expression.

## Discussion

RNAi is the process by which double-stranded RNA sequence-specific mRNA was degradation (Hamasaki *et al.*, 2003). RNAi is an extremely powerful research tool and clearly holds significant potential for a wide variety of gene silencing applications (David *et al*., 2002), RNAi is reported as an ideal tool to inhibit virus infection and replication in host cells. It is reported that silencing the expression of receptor protein could block the virus infection of host cells. Zhang *et al.* (2000) demonstrated silencing Fas expression could prevent liver injury by protecting hepatocytes from cytotoxicity. Novina *et al.* (2002) reported that siRNAs inhibit virus production by targeting the mRNAs for the HIV cellular receptor CD4 (Novina *et al*., 2002). To date, CD46 and SLAM were identified as cellular receptors for MV (Dorig *et al*., 1993; Naniche *et al*., 1993; Tatsuo *et al*., 2000). No MV strains have been found that cannot use SLAM as a receptor, whereas only the Edmonston and some other strains can use CD46 as receptor. Thus, the great majority of MV in the bodies of measles patients uses SLAM but not CD46 as a receptor. So in this report we chose MV receptor SLAM as the silencing target to inhibit MV infection.

In this study, we attempted to employ RNAi technology to silence SLAM expression. Because no RNAi target site of SLAM has already been identified, then we chose three different RNAi sequences to test in SLAM gene. The pH1 vector was used in the constructions of the vector-based RNAi. Initially, we showed that the RNAi3 site (637–655) selected could silence the expression of the SLAM gene in culture cells. Both flow cytometry and western blot experiments consistently suggest that the DNA vectors carrying the siRNA hairpin targeted the SLAM expression in cells. The RT-PCR of SLAM gene showed that the RNAi vector could effectively inhibit the SLAM gene mRNA accumulation. All the results above showed that RNAi3 site is a functional site of the three selected sites in the SLAM gene silencing.

Importantly, we demonstrated that RNAi inhibit SLAM protein expression in the B95-8 cells. The same cell line was used to MV infection experiment after RNAi3 inhibited SLAM expression in B95-8 cells. B95-8 is an EBV-transformed marmoset B cell line, which express a high level of SLAM (Kobune *et al*., 1990). So B95-8 cell line is sensitive to MV and is useful in studying the MV infection from clinical specimens. Through morphology observation and virus titer, pH1/SALM-RNAi3-transfected-cells exhibited little well-defined symptom of MV infection and decreased the yields of virus in the cells.

Of the three chosen target sites, only RNAi3 showed significantly effective silencing via the flow cytometry and western blot analysis, the other two RNAis just inhibited SLAM expression slightly. No obvious decrease could be observed in the following MV infection titer assay. As we know, only a small proportion of siRNAs designed for any gene was found to be effective for inducing the degradation of cognate mRNA in mammalian cells. There are many principles governing the efficacy of siRNAs. RNA-binding proteins and extensive secondary and tertiary structures with mRNA are suggested to interfere with the siRNA hybridization with their target RNA molecules (Harborth *et al*., 2003; Kretschmer-Kazemi and Sczakiel, 2003; Vickers *et al*., 2002). Maybe one of the reasons above resulted in the RNAi1 and RNAi2 failure to silence slam gene.

Taken together, we reported that the designed RNAi3 vector targeting the mRNA of MV receptor SLAM could specifically and effectively silence the receptor gene expression and inhibit MV infection in mammalian cells. Because of the important mutation between the newly discovered wild measles virus and vaccine virus (Rota *et al*., 1996), measles have broken out again in America, Europe and Asia. This study may be another choice that could be employed as a potential tool to inhibit MV infection by silencing SLAM.

The term “Antisense Therapeutics” or “Antisense Technology” encompasses several types of nucleic acids that have the ability to modulate gene expression. The aim of the Antisense strategy is simply to down-regulate the expression of disease-causing proteins by inhibiting gene expression at the level of mRNA. Recently the first antisense oligonucleotide(ODN) drug, Fomivirsen (Vitravene™) has received approval for marketing in USA (August 1998) and Europe (August 1999). This ODN is used for the treatment of cytomegalovirus retinitis infections in AIDS patients and is administered by repeated injection into the vitreous of the eye (Saghir *et al*., 2000). Can RNAi we designed be used as a form of genetic therapy for MV and associated infections? There are numerous challenges associated with converting RNAi from a laboratory technique to an antiviral therapeutic such as improvements of in vivo nucleic acid delivery technologies.

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