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Original Article

Small interfering RNA-mediated inhibition of respiratory syncytial virus infections in Hela cells

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Abstract: Respiratory syncytial virus (RSV) is one of the major causative pathogens of acute infantile respiratory infections. Until now, no drugs or vaccines have proven to be effective in the prevention and treatment of RSV infections. The purpose of the current study was to illustrate whether siRNAs could inhibit RSV infections in Hela cells via silencing endocytosis-associated genes. Custom-tailored siRNAs were used to silence mRNA expression of clathrin heavy chain, caveolin-1, and flotillin-1, respectively. Quantitative real-time PCR (qRT-PCR) was employed to test gene knockdown efficiency levels of siRNAs on targeted genes. Viral titer (quantified by Fifty Percent Tissue Culture Infective Dose ($TCID_{50}$)) and N gene expression levels (quantified by q RT-PCR) of RSV were determined to investigate the effects of siRNAs on RSV infections in Hela cells. Present results showed that mRNA expression levels of clathrin heavy chain, caveolin-1, and flotillin-1 were significantly decreased by corresponding siRNAs. Viral titer and N gene expression levels of RSV were obviously lower in all siRNA groups, compared with the control group and negative control group. The current study demonstrates that siRNAs may inhibit RSV infections in Hela cells through silencing endocytosis-related genes, including clathrin heavy chain for clathrin-mediated endocytosis, caveolin-1 for caveolae-mediated endocytosis, and flotillin-1 for clathrin/caveolae-independent endocytosis.

Keywords: Respiratory syncytial virus, endocytosis, small interfering RNA

Introduction

Respiratory syncytial virus (RSV), a widespread viral pathogen, belongs to the Pneumoviridae family. It is one of the most common viruses causing lower respiratory tract infections in infants and children. It has been estimated that 90% of children are affected by RSV within their first 2 years of life [1]. Recently, Shi et al. estimated that, in 2015, there were 33.1 million cases of RSV-associated acute lower respiratory illnesses, worldwide, resulting in approximately 3.2 million hospitalizations and 59,600 in-hospital deaths in children younger than 5 years of age [2]. Broad-spectrum antiviral drugs and symptomatic treatment are usually adopted in clinical practice for RSV infections. However, due to side effects and limited efficacy levels of these treatments, safe and effective methods for RSV infections remain a major concern.

It has been established that virus entry into host cells is a crucial step of virus infections. Like other enveloped viruses, the entry procedure of RSV involves endocytosis [3]. Normally, there are four kinds of endocytic pathways, including clathrin-mediated endocytosis, caveolae-mediated endocytosis, clathrin/caveolae-independent endocytosis, and macro-pinocytosis [4]. Clathrin-mediated endocytosis is currently the most intensely studied endocytic pathway for virus entry. Evidence has shown that viruses binding to receptors of host cells can trigger activation of clathrin-mediated endocytosis, leading to clathrin coat assembly, membrane curvature, and virus internalization [5]. Many viruses, including RSV, often exploit this pathway to gain entry into host cells [6, 7]. One previous study demonstrated that RSV infections were inhibited by inhibiting genes associated with clathrin-mediated endocytosis using small interfering RNA [8].

Unlike clathrin-mediated endocytosis, the impact of caveolae-mediated endocytosis and clathrin/caveolae-independent endocytosis on RSV infections has not been fully examined.

Table 1. Primers of siRNAs used in the present study

siRNA		Sequence
siCLTC	Forward	5'-AUCCAAUUCGAAGACCAAUTT-3'
	Reverse	5'-AUUGGUCUUCGAAUUGGAUTA-3'
siCAV1	Forward	5'-GCAAGUGUACGACGCGCACTT-3'
	Reverse	5'-GUGCGCGUCGUACACUUGCTT-3'
siFLOT1	Forward	5'-AACAGATCCAGAGGATCTCTC-3'
	Reverse	5'-CAGAUCCAGAGGAUCUCUCTT-3'

The present study aimed to silence three endocytosis-related genes, including clathrin heavy chain, caveolin-1, and flotillin-1, by small interfering RNAs (siRNAs), determining the potential roles of clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin/caveolae-independent endocytosis in RSV infections.

Materials and methods

Cells and viruses

Hela cells, a gift from the Respiratory Laboratory of the Children's Affiliated Hospital of Chongqing Medical University, were maintained in Dulbecco's Modified Eagle Medium (DMEM). They were supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% CO_2. The A2 strain of RSV, also a gift from the Respiratory Laboratory of the Children's Affiliated Hospital of Chongqing Medical University, was stored at -80°C until use.

Transfection of siRNAs

Transfection of siRNAs into Hela cells was performed, according to manufacturer instructions. Briefly, 1.5×10^3 cells/well were plated in 96-well plates and incubated overnight. After 24 hours of incubation, the cells were transfected with pre-prepared transfection compounds. Transfection compounds, formed by 30 μl DMEM medium, 1.5 μl HiPerFect transfection reagent, and 1 μl siRNA, were replaced with complete medium 48 hours after transfection. Moreover, siRNAs were targeted to the following genes: Clathrin heavy chain (CLTC, SI00299880), caveolin-1 (CAV1, SI00299642), and flotillin-1 (FLOT1, SI00300237), manufactured and validated by the Qiagen Company (Valencia, CA, USA). Positive control siRNAs (Allstars cell death control siRNA, 1027280), negative control siRNAs (Allstars Negative Control siRNA, 102798), and HiPerFect transfection reagent were also purchased from the Qiagen Company (Valencia, CA, USA). Primers used in the present study are summarized in **Table 1**.

Determination of transfection efficiency of HiPerFect transfection reagent

Hela cells were cultured using the abovementioned methods. Cultured cells were divided into 5 groups and transfected with pre-prepared compounds, including the blank control group (32.5 μl DMEM), negative control group (30 μl DMEM medium, 1.5 μl HiPerFect transfection reagent, and 1 μl negative control siRNA), positive control group (30 μl DMEM medium, 1.5 μl HiPerFect transfection reagent, and 1 μl positive control siRNA), positive siRNA only group (32.5 μl positive siRNA), and transfection reagent only group (32.5 μl HiPerFect transfection reagent). Each group was cultured at 37°C in 5% CO_2 for 48 hours, then tested for cell viability using Cell Counting Kit-8 assays (Dojindo Laboratories, Kumamoto, Japan), according to kit instructions. Additionally, 450 nm OD-values of each well were measured and used to calculate the cell viability of Hela cells. The following equation was used: Cell viability (%)=(As-Ab)/(Ac-Ab)×100%, where As represents the OD value of the experimental well, Ab represents the OD value of the blank well, and Ac represents the OD value of the control well.

Gene knockdown verification

To knockdown expression of targeted genes, cultured Hela cells were transfected with siRNAs (40 nM, 60 nM, and 100 nM) using the abovementioned methods: (1) Blank control group (32.5 μl DMEM); (2) Negative control group (30 μl DMEM medium, 1.5 μl HiPerFect transfection reagent, and 1 μl negative control siRNA); (3) siCLTC group (30 μl DMEM medium, 1.5 μl HiPerFect transfection reagent, and 1 μl siCLTC); (4) siCAV1 group (30 μl DMEM medium, 1.5 μl HiPerFect transfection reagent, and 1 μl siCAV1); and (5) siFLOT1 group (30 μl DMEM medium, 1.5 μl HiPerFect transfection reagent, and 1 μl siFLOT1). Next, mRNA expression levels of target genes were verified using quantitative real-time PCR (qRT-PCR) methods, employing the CFX96TM real-time system (Bio-Rad, USA). The following conditions were applied: Denaturation at 95°C for 5 minutes, followed

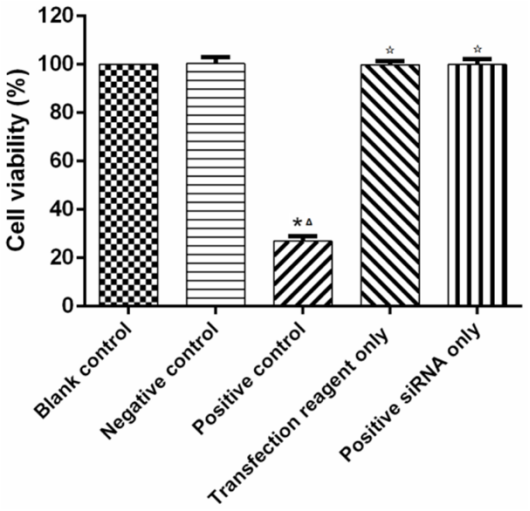

Figure 1. Cell viability determined by CCK-8 assays (n=3). * vs. the blank control group, $p<0.05$; Δ vs. the negative control group, $p<0.05$; ☆ vs. the positive control group, $p<0.05$.

by 40 cycles of 95°C for 20 seconds, 42°C for 10 seconds, and 72°C for 20 seconds. Primer sets for CLTC (QT00081977), CAV1 (QT02408112), and FLOT1 (QT00036743) were purchased from the Qiagen Company. GAPDH was used as the reference gene. Results were normalized relative to GAPDH and are represented by mean ± SD.

RSV infection and viral titer determination

Hela cells at 1.5×10^4 cells/well were seeded in 12-well plates and divided into 5 groups, including: (1) Blank control group; (2) Negative control group; (3) siCLTC group; (4) siCAV1 group; and (5) siFLOT1 group. When cell density reached 70%-80% confluence, cultured Hela cells were transfected with siRNAs using above-mentioned methods. Forty-eight hours after siRNAs transfection, the cells were incubated with RSV at a multiplicity of infection of 1 for 2 hours at 37°C in 5% CO_2. When 70%-80% cells of the blank control group became impaired, supernatants containing viruses of each group were collected. Serially-diluted-supernatant samples (from 10^{-1} to 10^{-8}) were prepared and added to Hela cells with 5 replicates for each dilution. Hela cells were then incubated for 7 days at 37°C, daily observing virus-induced cytopathic effects. $TCID_{50}$ values were calculated using the Reed-Muench method [9]. The viral titer was expressed as $log_{10}TCID_{50}$/mL.

Viral RNA quantification

N gene mRNA expression of RSV was detected, aiming to provide estimates of the "virus load" in supernatants [10]. Briefly, viral RNA was extracted from 20 μl supernatant using the Qiamp Viral RNA mini Kit (Qiagen, Hilden, Germany). N gene expression of RSV was determined with One-step qRT-PCR using the iScript™ One-Step RT-PCR Kit with the SYBR® Green kit. The following conditions were applied: Denaturation at 95°C for 3 minutes, followed by 39 cycles of 95°C for 10 seconds, 58°C for 30 seconds, and 72°C for 20 seconds. Primer sets for N gene were: Forward primer: 5'-CATCCAGCAAATACACCATCCA-3'; Reverse primer: 5'-TTCTGCACATCATAATTAGGAGTATCAA3'. GAPDH was used as the reference gene.

Statistical analysis

Data are expressed as mean ± SD and were analyzed with SPSS 19.0 software (SPSS, Chicago, IL, USA). Group comparisons were performed using one-way ANOVA. *P*-values less than 0.05 indicate statistical significance.

Results

Transfection efficiency of the HiPerFect transfection reagent

After 48 hours of incubation, massive cell death was observed under the light microscope in the positive control group. Cell viability rates of the blank control group, negative control group, positive control group, transfection reagent only group, and positive siRNA only group were 100%, 100%, 27%, 100%, and 100%, respectively (**Figure 1**). Cell viability of the positive control group was significantly lower than that in other groups, indicating that the HiPerFect transfection reagent provides good transfection efficiency in Hela cells.

Gene knockdown verification

Hela cells were transfected with abovementioned siRNAs. Gene knockdown effects of the siRNAs at the transcriptional level were evaluated 48 hours after siRNA transfection, employing qRT-PCR. **Figure 2** shows that all three siRNAs exhibited significant gene knockdown effects on targeted genes. Effects were most pronounced at 100 nM. Therefore, 100 nM siRNAs were used in the following experiments.

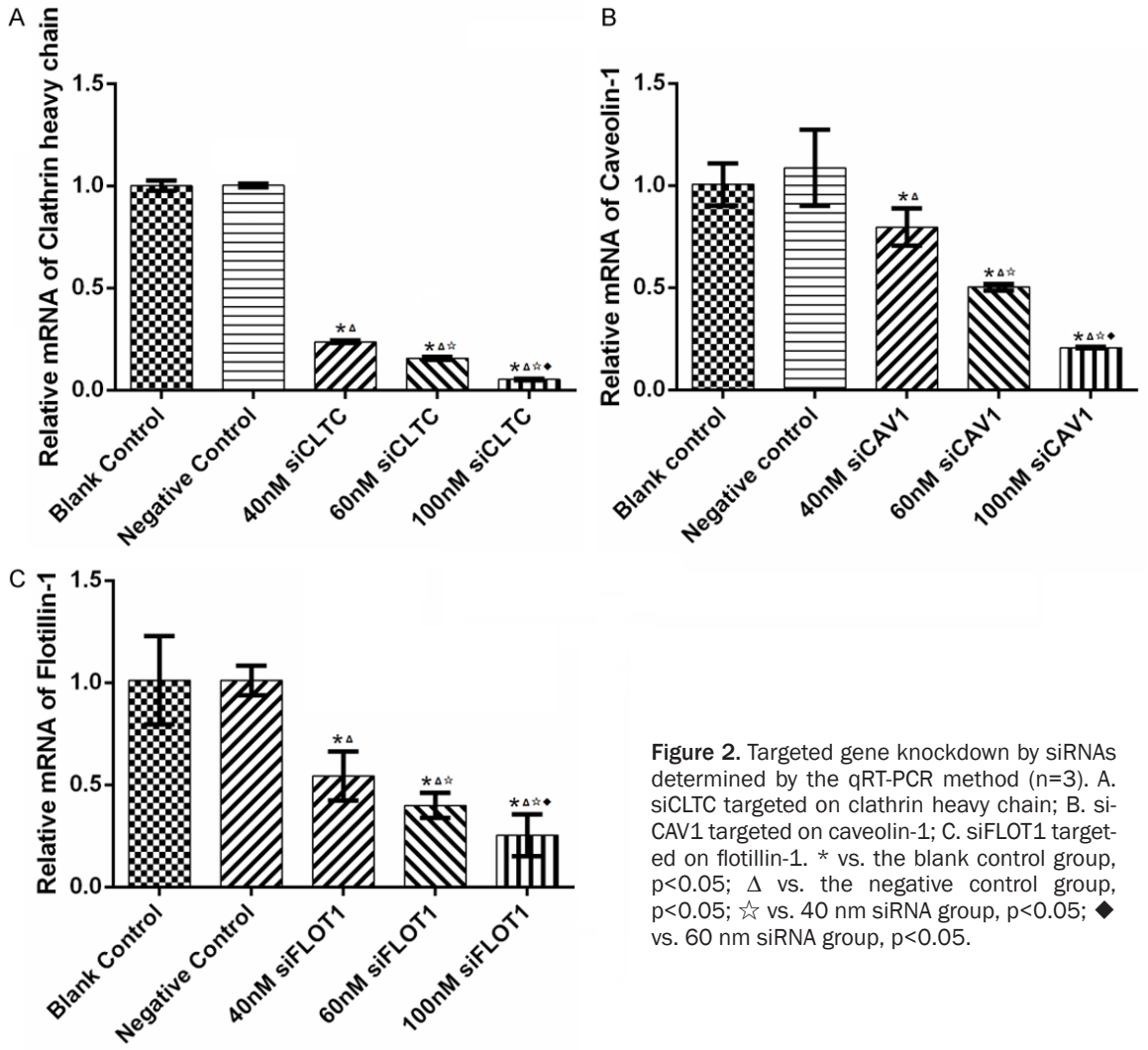

Figure 2. Targeted gene knockdown by siRNAs determined by the qRT-PCR method (n=3). A. siCLTC targeted on clathrin heavy chain; B. siCAV1 targeted on caveolin-1; C. siFLOT1 targeted on flotillin-1. * vs. the blank control group, $p<0.05$; Δ vs. the negative control group, $p<0.05$; ☆ vs. 40 nm siRNA group, $p<0.05$; ◆ vs. 60 nm siRNA group, $p<0.05$.

Inhibitory effects of siRNA on RSV infections

Viral titer, expressed as $\log_{10}TCID_{50}$/mL, was increased with time in each group, reaching a plateau after 3-5 days of incubation. At day 7, viral titer levels of the blank control group, negative control group, siCLTC group, siFLOT1 group, and siCAV group were 7.52 ± 0.31, 7.37 ± 0.34, 4.78 ± 0.09, 5.16 ± 0.44, and 5.48 ± 0.60, respectively. Viral titer levels were significantly deceased in all siRNA groups, compared with those in the blank control group and negative control group, indicating that RSV infectivity was restricted by siRNAs (**Figure 3**).

N gene mRNA expression of RSV

Compared with the blank control group, N gene mRNA expression levels of RSV were significantly decreased in the siCLTC group, siCAV1 group, and siFLOT1 group ($p<0.05$), indicating that RSV replication was restricted by siRNAs (**Figure 4**).

Discussion

RNA interference (RNAi) is a natural RNA inhibitory phenomenon that exists in most species, including humans and viruses. Moreover, siRNAs, the effector molecules of RNAi, have been widely studied in many kinds of viruses, including RSV [11], HIV [12], and HBV [13], making it a potential tool for treatment of virus infections [14]. The current study aimed to illustrate whether siRNAs could inhibit RSV infections in Hela cells via silencing endocytosis-associated genes. Results showed that siRNAs targeted on CLTC, CAV1, and FLOT1 genes could suppress

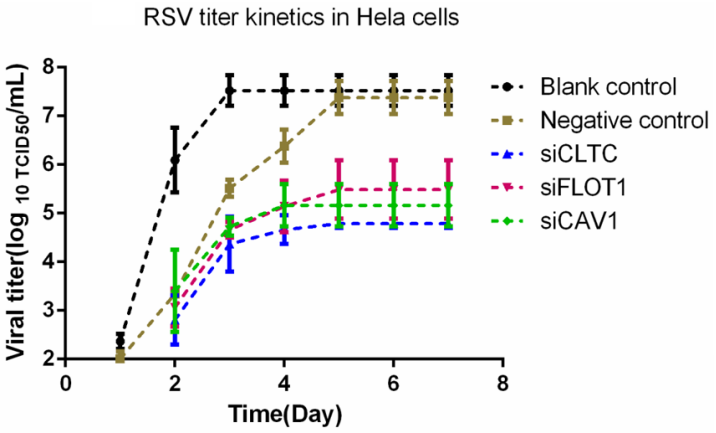

Figure 3. Viral titer determined by the Reed-Muench method.

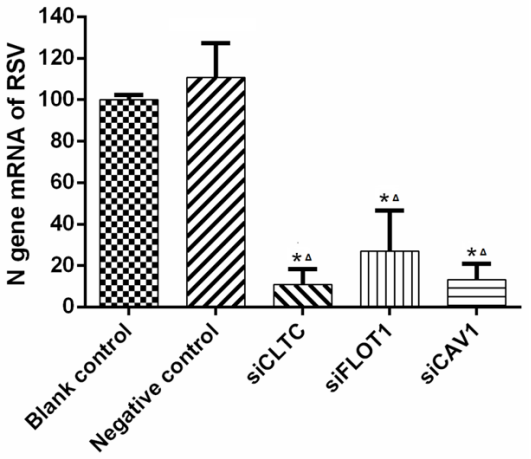

Figure 4. N gene mRNA expression of all groups determined by the qRT-PCR method (n=3). * vs. the blank control group, $p<0.05$; Δ vs. the negative control group, $p<0.05$.

viral titer and N gene expression levels of RSV in Hela cells, indicating that RSV infections were restricted by siRNAs.

Clathrin-dependent endocytosis plays an important role in RSV infections. In pre-experiment tests, the current study used chemical antagonists, such as promethazine, successfully blocking clathrin-mediated endocytosis. This inhibited RSV infections in Hela cells. However, low specificity and strong cytotoxicity levels of the chemical blockers influenced the evaluation of experimental results. Clathrin heavy chain is the major component of clathrin-dependent endocytosis. Moreover, siRNAs targeted on this gene have shown potential in treating virus infections. For example, Alhoot et al. transfected clathrin heavy chain siRNA (siCLTC) into host cells, successfully inhibiting HCV and dengue virus infections [15]. The current study used custom-tailored siCLTC, instead of chemical antagonists, aiming to investigate the roles of CLTC in RSV infections. Results showed that $log_{10}TCID_{50}$/mL and N gene mRNA expression levels of the siCLTC group were significantly lower than those of the blank control group and negative control group, indicating that siCLTC effectively inhibited RSV infections in Hela cells.

Caveolae-mediated endocytosis involves in membrane transport, signal transduction, tumor suppression, and the uptake of pathogenic viruses [16]. In mammalian cells, caveolins include 3 homologous subtypes, caveolin-1, caveolin-2, and caveolin-3. Caveolin-1 usually coexists with caveolin-2, while caveolin-3 exists only in muscle cells [3]. Although caveolin-1 is currently considered to be an important component of Caveolae and closely related to lipid domains of the cell membrane, the importance of caveolin-1 for virus infections has not yet elucidated. Few studies have focused on the relationship between caveolin-1 and virus infections. For instance, Brown et al. found that caveolin-1 may have some association with the replication of RSV [17]. Smart et al. revealed that downregulation of caveolin-1 on the cell membrane led to inhibition of HIV infections [18]. The present study tested the efficacy of siCAV1, a custom-tailored siRNA specifically targeting caveolin-1, on inhibition of RSV infections. Results showed that both $log_{10}TCID_{50}$/mL values and N gene mRNA levels of the siCAV1 group were significantly lower than those of the blank control and negative control group, indicating that siCAV1 effectively inhibited RSV infections in Hela cells.

Flotillin plays important role in cell adhesion, signal transduction, and transmembrane transport [19]. There are two homologous members of flotillin, flotillin-1 and flotillin-2. Both are associated with membrane microdomains by acylation and participate in virus endocytosis [20]. A previous study reported that flotillin-1 mediated clathrin-independent endocytosis in

mammalian cells [21]. However, little is known about the impact of flotillin-1 on RSV infections. Present data suggests that, just like siCLTC and siCAV1, siFLOT1 could also effectively inhibit RSV infections in Hela cells.

Conclusion

Present results suggest that siRNAs could inhibit RSV infections in Hela cells through silencing endocytosis-related genes, including clathrin heavy chain for clathrin-mediated endocytosis, caveolin-1 for caveolae-mediated endocytosis, and flotillin-1 for clathrin/caveolae-independent endocytosis. The present study may provide new insight for the development of anti-RSV drugs and vaccines.

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Disclosure of conflict of interest

None.

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