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Research Note

siRNAs to Knockdown Antiviral Chemokine-related Genes in FRhK-4 Cells

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ABSTRACT

The objective of this study was to generate small interfering RNA (siRNA) to knockdown antiviral chemokine-related genes in fetal rhesus monkey kidney (FRhK-4) cells. We generated siRNA duplexes to suppress antiviral chemokines like *CXCL10* and *CCL4* in FRhK-4 cells by downregulating interferon regulatory factor (*IRF*) *3* and *IRF7*. Three siRNA duplexes (si-F-IRF3-1, si-F-IRF3-2, and si-F-IRF3-3) targeting *IRF3*, and one siRNA duplex (si-F-IRF7) targeting *IRF7* were generated. A nontarget siRNA duplex was used as the negative control. The nontarget or target siRNA duplexes (si-F-IRF3-1, si-F-IRF3-2, si-F-IRF3-3, and si-F-IRF7) were transfected into FRhK-4 cells using transfection reagents, and they were then incubated at 37°C for 6 h with 5% CO_2. After 6 h, the medium was removed, and fresh medium was added to each cell, and they were then incubated at 37°C for 48 h with 5% CO_2. The transfected FRhK-4 cells were infected with hepatitis A virus (HAV) HM-175/18f (viral titer: 10^5 PFU/mL) and incubated at 37°C for 3 h with 5% CO_2 for HAV propagation. The expression levels of chemokines, including *CXCL10* and *CCL4*, under the regulation of *IRF3* and *IRF7* in the transfected FRhK-4 cells were measured using quantitative real-time polymerase chain reaction after 3 h of HAV infection. The results indicated that *CXCL10* and *CCL4* expression levels were decreased in FRhK-4 cells transfected with si-F-IRF3-1, si-F-IRF3-3, or si-F-IRF7 ($p < 0.05$) compared to those in the negative control. These results indicate that si-F-IRF3-1 and si-F-IRF3-3, and si-F-IRF7 successfully knocked down *IRF3* and *IRF7* in FRhK-4 cells, respectively and suppressed antiviral chemokines. These siRNAs could be used to suppress antiviral chemokines in FRhK-4 cells.

Hepatitis A virus (HAV) is mainly transmitted via the fecal-oral route, either by person-to-person contact or through contaminated water and foods, particularly through salads, fruit, and shellfish (Acheson & Fiore, 2004; Hollinger & Emerson, 2001). HAV, the prototype of the *Hepatovirus* genus within the family Picornaviridae (Fauquet & Stanley, 2005), replicates in the liver and can cause acute hepatitis after a relatively long incubation period of approximately 28 d. Recently, the incidence of HAV infection has increased in developed countries. Although the fatality rate of HAV infections is lower than that of norovirus infections, HAV outbreaks tend to be more severe (Lee & Yoon, 2021; Wheeler et al., 2005; Webb et al., 2020; World Health Organization, 2015). For instance, in Pennsylvania, USA, HAV-contaminated green onions caused three deaths among 601 cases in 2003 (Wheeler et al., 2005). In South Korea, 17,598 hepatitis A cases were reported in 2019, presenting an increase of 722% from that in 2018 (Korea Centers for Disease Control & Prevention, 2020). Therefore, methods are needed for HAV detection and culture; however, only a few mammalian cells are available for this purpose. Although the FRhK-4 cell line derived from rhesus monkey is frequently used for this purpose, it has limitations because it expresses antiviral chemokines.

Induction of interferons (IFNs), which is crucial for innate immunity against viral infection, is mediated by the binding of viral nucleic acids to receptors on the cell surface and to cytosolic pattern recognition receptors. In the classical pathway, the early phase of IFN induction, following viral RNA binding to toll-like receptor 3, retinoic–acid-inducible protein-1, and melanoma differentiation-associated gene 5, leads to the activation of *IRF3*, which translocates to the nucleus and binds to specific promoter elements of *IFN-β*, initiating its transcription (Debing et al., 2014; Feng et al., 2014; Kato et al., 2006). *IRF7* is expressed predominantly in the lymph myeloid tissue and is transcriptionally induced by viral infection, type I IFNs, and pathogen-associated molecular patterns, including dsRNA and lipopolysaccharide (Honda et al., 2006). Nine IRF family members have been identified in mammals (Honda et al., 2006), and *IRF3* and *IRF7* have been found to play essential roles in controlling the IFN response during

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viral infection by producing chemokines, including CC motif ligand 4 (*CCL4*) and CXC motif ligand 10 (*CXCL10*) (Huang et al., 2020).

RNA interference (RNAi) is an endogenous posttranscriptional regulatory process that involves small regulatory RNAs, including small interfering RNAs (siRNAs) or microRNAs (miRNAs), which silence target mRNAs in a sequence-specific manner (Lunavat et al., 2016). RNAi is mainly used in gene therapy and for the identification of genes and signaling-associated biological pathways (Chen et al., 2014; Chou et al., 2013; Peng et al., 2015; Zhu et al., 2014).

The objective of this study was to generate siRNAs to suppress the antiviral chemokines (*CCL4* and *CXCL10*) in FRhK-4 cells by downregulating *IRF3* and *IRF7*.

Materials and methods

Cell line and virus culture conditions. Fetal rhesus monkey kidney (FRhK-4, ATCC CRL-1688) cells were maintained in Dulbecco's modified Eagle medium (DMEM; WelGENE) supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific) and 1% penicillin-streptomycin (PS; GenDEPOT) at 37°C under 5% CO_2. FRhK-4 cells were infected with cytopathic HM-175/18f strain of HAV (ATCC VR-1402), and then incubated at 37°C for 5 days under 5% CO_2. The infected FRhK-4 cells were then incubated at 37°C for 5 min under 5% CO_2 and frozen at −20°C for 10 min. This procedure was repeated thrice to lyse the FRhK-4 cells. The resulting lysates were centrifuged at 1,952 × *g* at 25°C for 10 min. The supernatant was then transferred to a sterile CryoTube (Thermo Fisher Scientific) and stored at −80°C until used.

siRNA generation and siRNA transfection. siRNA (si-F-IRF3-1, si-F-IRF3-2, si-F-IRF3-3, and si-F-IRF7) duplexes targeting *IRF3* and *IRF7* in FRhK-4 cells were generated with an online program provided by Dharmacon Inc. The stock solution of the siRNA duplex was prepared according to the manufacturer's instructions (stock concentration, 100 μM) (Bioneer). The siRNA duplex was transfected into FRhK-4 cells using Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions. A nontarget siRNA duplex (Bioneer) was used as a negative control. One day before transfection, FRhK-4 cells (3.0×10^5 cells/mL) were cultured in 6-well plates (SPL Life Sciences) and grown to 50–60% confluence. The growth medium was removed, and fresh medium (500 μL) without serum was added to each cell. The target or nontarget siRNA duplexes with the diluted Lipofectamine™ RNAiMAX transfection reagent was added to each cell and mixed gently by manually rocking the plate back and forth; the cells were then incubated at 37°C for 6 h under 5% CO_2. After incubation for 6 h, the growth medium was then removed, and fresh medium containing serum was added to each cell, and the cells transfected with the target or nontarget siRNA duplexes were incubated at 37°C for 48 h with 5% CO_2. Subsequently, the transfected FRhK-4 cells were infected with HAV strain HM-175/18f (viral titer: 10^5 PFU/mL) at 37°C for 1 h with 5% CO_2. The uninvaded HAV was removed, and Eagle's Minimum Essential Medium (ATCC) supplemented with 2% FBS and 2.5 μg/mL amphotericin B (Gibco) was added to the infected FRhK-4 cells, and the cells were then incubated at 37°C for 3 h with 5% CO_2 for HAV propagation. After 3 h, the expression levels of chemokines, including *CXCL10* and *CCL4*, which are regulated by *IRF3* and *IRF7* were measured in FRhK-4 cells transfected with target or nontarget siRNA duplexes.

mRNA extraction and complementary DNA (cDNA) synthesis. FRhK-4 cells transfected with target or nontarget siRNA duplexes were subjected to mRNA extraction using TRIzol™ Reagent (Invitrogen) according to the manufacturer's instructions with slight modifications. Briefly, TRIzol™ Reagent (400 μL) was added to FRhK-4 cells transfected with target or nontarget siRNA duplexes to disrupt the cell membrane, and were then incubated at 25℃ for 5 min. The cell lysate (400 μL) was then transferred to a microcentrifuge tube. Chloroform (80 μL; Samchun Chemical Co.) was added to the microcentrifuge tube containing the cell lysate and then mixed by pipetting. The mixture was incubated at 25℃ for 3 min, followed by centrifugation at 12,000 × *g* and 4°C for 15 min. The aqueous phase containing RNA was transferred to a new microcentrifuge tube. Isopropanol (200 μL) (Samchun Chemical Co.) was added to the microcentrifuge tube and mixed by pipetting. The mixture was incubated at 4°C for 10 min, followed by centrifugation at 12,000 × *g* at 4°C for 10 min; the supernatants were then discarded. Ethanol (400 μL) (Duksan) was added to the pellet in a microcentrifuge tube and mixed by pipetting. The mixture was centrifuged at 7,500 × *g* at 4°C for 10 min, and supernatant were then discarded. The pellet was air dried at 25℃ for 10 min. RNase-free water (30 μL) was added to the pellet and mixed by pipetting. The dissolved RNA was incubated at 60°C for 15 min in a dry bath incubator (Allsheng Instruments). cDNA was synthesized from mRNA using the Quantitect Reverse Transcription Kit (Qiagen), as described below. Genomic DNA was eliminated by adding gDNA Wipeout Buffer (2 μL) (Qiagen) and incubating at 42°C for 2 min. Quantiscript reverse transcriptase (1 μL) (Qiagen), Quantiscript RT Buffer (4 μL) (Qiagen), and RT Primer Mix (1 μL) (Qiagen) were added to the microcentrifuge tube and then mixed by pipetting on ice. The mixture was incubated at 42°C for 15 min and then heated at 95°C for 3 min to inactivate the Quantiscript Reverse Transcriptase (Qiagen).

Measurement of chemokine gene expression using quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR was performed using the Rotor-Gene SYBR Green PCR kit (Qiagen) to measure the chemokine gene expression levels. The cDNA template (1 μL), forward and reverse primers (2.5 μL), RNase-free water (6.5 μL) (Qiagen), and SYBR Green PCR master mix (12.5 μL) (Qiagen) were added to a microcentrifuge tube, and they were then mixed by pipetting. The qRT-PCR was performed on the Rotor-Gene Q (Qiagen) by incubation at 95°C for 5 min and amplified for 40 PCR cycles of 95°C for 5 s and 55°C for 10 s. The primer sequences used for amplification are listed in Table 1. Cycle threshold (CT) values, determined using Rotor-Gene Q series software (Stratagene), were used to evaluate the relative expression of target mRNA. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Statistical analysis. All experiments were performed in triplicate and the data were analyzed using the general linear model procedure of the SAS® program version 9.3 (SAS Institute Inc.). Least-square

Table 1
Sequences of primers used to amplify *GAPDH*, *CXCL10*, and *CCL4* gene via quantitative real-time polymerase chain reaction

Cell	Gene	Primer sequences (5'→3')		Reference
FRhK-4	*GAPDH*	Forward	GAA ATC CCA TCA CCA TCT TCC AGG	(Ahn et al., 2008)
		Reverse	GAG CCC CAG CCT TCT CCA TG	
	CXCL10	Forward	TCA GCA CCA TGA ATC AAA CTG	(Basu et al., 2002)
		Reverse	CCT CTG GTT TTA AGG AGA TCT	
	CCL4	Forward	ACC ATG AAG CTC TGC GTG ACT G	
		Reverse	AGC TCA GTT CAG TTC CAG GT	

means among groups were compared using a pairwise t test at $\alpha = 0.05$.

Results and discussion

Three siRNA duplexes (si-F-IRF3-1, si-F-IRF3-2, and si-F-IRF3-3) targeting *IRF3* and one siRNA duplex (si-F-IRF7) targeting *IRF7* in FRhK-4 cells were successfully generated. The si-F-IRF3-1 sense sequence was 5′ - GUA AAU AGC AGG AGA UAG A = UU - 3′; the si-F-IRF3-1 anti-sense sequence was 5′ - UCU AUC UCC UGC UAU UUA C = UU - 3′, the si-F-IRF3-2 sense sequence was 5′ - CUG UAU AAC CCA AGG CAA A = UU - 3′, the si-F-IRF3-2 anti-sense sequence was 5′ - UUU GCC UUG GGU UAU ACA G = UU - 3′, the si-F-IRF3-3 sense sequence was 5′- GGA AGG AGG AGG AAG UAU A = UU - 3′, the si-F-IRF3-3 anti-sense sequence was 5′- UAU ACU UCC UCC UCC UUC C = UU - 3′, the si-F-IRF7 sense sequence was 5′- GCA CCU GGA CGG ACA CUU A = UU - 3′, and the si-F-IRF7 anti-sense sequence was 5′- UAA GUG UCC GUC CAG GUG C = UU - 3′. In a study by Ma et al. (2021); *CCL4* expression was found to be regulated in an *IRF3*-

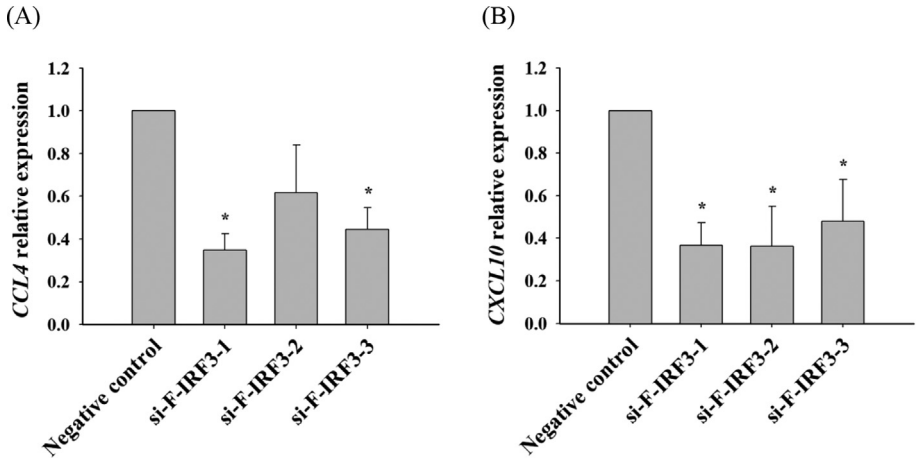

Figure 1. Relative gene expression levels of *CCL4* (A) and *CXCL10* (B) in FRhK-4 cells transfected with siRNA duplexes targeting *IRF3* after 3 h of HAV infection. Negative control: FRhK-4 cells transfected with nontarget siRNA; si-F-IRF3-1: FRhK-4 cells transfected with si-F-IRF3-1; si-F-IRF3-2: FRhK-4 cells transfected with si-F-IRF3-2; si-F-IRF3-3: FRhK-4 cells transfected with si-F-IRF3-3. *Statistical significance was determined by comparing the tested sample with the negative control. Data are shown as the mean ± standard deviation.

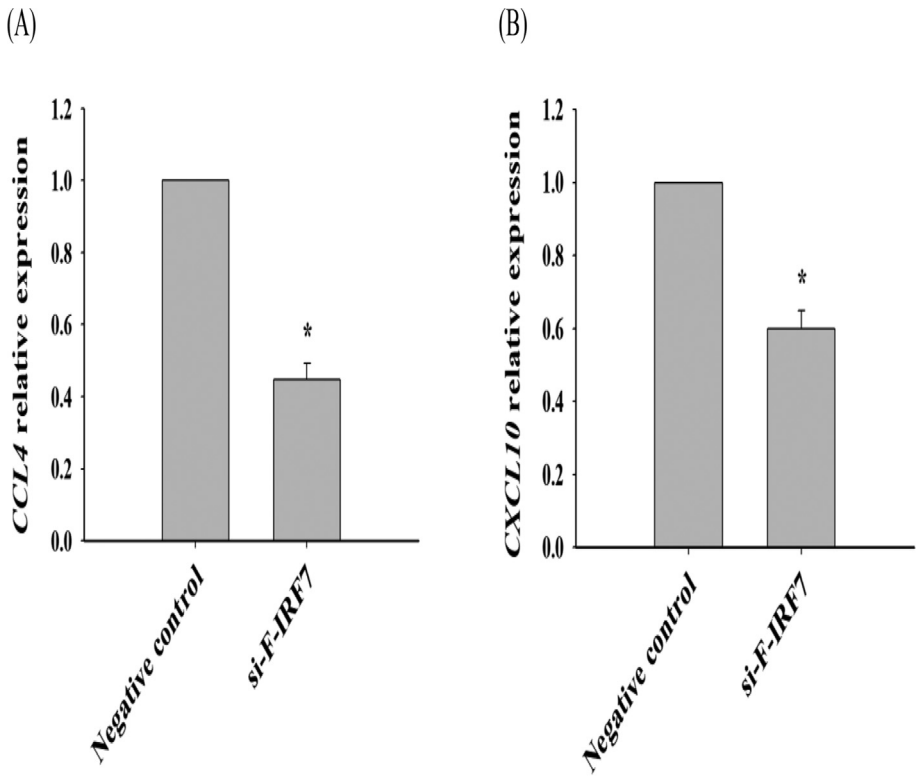

Figure 2. Relative gene expression levels of *CCL4* (A) and *CXCL10* (B) in FRhK-4 cells transfected with an siRNA duplex targeting *IRF7* after 3 h of HAV infection. Negative control: FRhK-4 cells transfected with nontarget siRNA; si-F-IRF7: FRhK-4 cells transfected with si-F-IRF7. *Statistical significance was determined by comparing the tested sample with the negative control. Data are shown as mean ± standard deviation.

dependent manner. *CCL4* promotes T-cell chemotaxis from the bloodstream to the infected area, increases IL-17 production, and regulates the migration of monocytes related to antiviral immune responses (Ma et al., 2021; Maurer & Von Stebut, 2004). In this study, *CCL4* expression levels in FRhK-4 cells transfected with si-F-IRF3-1 or si-F-IRF3-3 were lower ($p < 0.05$) than those in the negative control (Fig. 1A). In the early stage of the immune response to HAV infection, *CXCL10*, which is regulated in an *IRF3*-dependent manner, is also increased (Lanford et al., 2011). *CXCL10* expression levels in FRhK-4 cells transfected with si-F-IRF3-1, si-F-IRF3-2, or si-F-IRF3-3 were lower ($p < 0.05$) than those in the negative control (Fig. 1B). Further, *CCL4* and *CXCL10* expression levels in FRhK-4 cells transfected with si-F-IRF7 were lower ($p < 0.05$) than those in the negative control (Fig. 2A and 2B). Downregulation of chemokines *CCL4* and *CXCL10* could be a limiting factor for antiviral immune responses and promote viral infection. Similarly, Kim et al. (2022) reported that Vero cell line deficient in single *IFN*-γ (*IFNG*) and *IFNG* receptor 1 shows increased susceptibility to HSV-1. Our results indicated that the expression of antiviral factors, *CXCL10* and *CCL4*, were significantly lower in FRhK-4 cells treated with the siRNAs si-F-IRF3-1, si-F-IRF3-3, and si-F-IRF7. In conclusion, the siRNAs such as si-F-IRF3-1 and si-F-IRF3-3 targeting for *IRF3*, and si-F-IRF7 targeting for *IRF7* could be used for development of the mammalian cells for HAV detection and culture, because the siRNAs can suppress antiviral chemokines in the FRhK-4 cells.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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