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Generation of microRNA-30e-producing recombinant viral hemorrhagic septicemia virus (VHSV) and its effect on in vitro immune responses

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ABSTRACT

MicroRNAs (miRNAs) are non-coding small RNAs involved in the regulation of gene expression. In the present study, we firstly reported the use of a fish RNA virus, viral hemorrhagic septicemia virus (VHSV), as a delivery vehicle of a miRNA-30e, and the effect of miR-30e produced by the recombinant VHSV on the immune responses of Epithelioma papulosum cyprini (EPC) cells was investigated. The expression of functional miR-30e using a CMV promoter-driven vector was verified by the significantly lower eGFP expression in cells transfected with a vector containing miR-30e sponge sequence than that in cells transfected with a control vector that had mutated miR-30e sponge sequence. Furthermore, the down-regulation of reporter gene containing 3′-UTR of NF-κb inhibitor α-like protein B (NFκbiαb) by miR-30e was demonstrated, suggesting that miR-30e overexpression can increase immune responses related to NF-κB activation through inhibition of IκB. A miR-30e-expressing recombinant VHSV (rVHSV-A-miR30e) that had primary microRNA-30e sequence between N and P genes was rescued using the reverse genetic method, and the successful expression of miR-30e in the cells infected with rVHSV-A-miR30e was demonstrated using Northern blot and qRT-PCR. Cells infected with rVHSV-A-miR30e showed the increase of NF-κB activation and type I interferon induced genes expression, suggesting that rVHSV-A-miR30e can produce functional miR-30e in fish cells, and VHSV can be used as a vehicle to deliver functional microRNAs in fish.

1. Introduction

MicroRNAs (miRNAs) are non-coding small RNAs (−22 nucleotides in length) in eukaryotic cells or in some viruses, and participate in the regulation of gene expression through binding to complementary nucleotides sequences located usually in the 3′ untranslated region (UTR) of mRNAs [1]. Canonical primary miRNAs in the nucleus consist of a stem-loop region (about 80 bp) and flanking regions, and are cleaved by Drosha guided by DGCR8, resulting hair-pin structured precursor miRNAs that are transported to cytoplasm by exportin-5. In the cytoplasm, the loop region of a precursor miRNA is cleaved by Dicer, and one strand of the double-stranded RNA (mature miRNA) forms an RNA induced silencing complex (RISC). As one miRNA can have multiple target genes and one gene can be a target for multiple miRNAs, the wide range of pathways in cells is regulated by miRNAs, and more than 2/3 of genes in human are known to be regulated by miRNAs [2–4].

Members in the miRNA-30 family including miR-30a, miR-30b, miR-30c, miR-30d and miR-30e share the similar seed sequence. However, as each miRNA-30 family member has different nucleotide sequence at flanking regions and localizes at different chromosomes, each member is often differentially regulated, which allows miRNA-30 family members to participate in more diverse biological processes [5,6]. To date, almost all researches on the role of miRNA-30 have been conducted in mammals, while the role of miRNA-30 in fish cells has been poorly investigated. As far as we know, there have been two papers on the role of miRNA-30 family in fish; one is on the regulatory role of miRNA-30a in the muscle development of zebrafish [7], and the other is on the role of miR-30c in the salt tolerance of tilapia [8].

Among miRNA-30 family members, recently, the regulatory role of miR-30e in the progression of diverse cancers in mammals has been reported [9–11], and one of the way to regulate cancers by miR-30e was the activation of NF-κB through the suppression of IκBα [12.13]. In relation to viral infections, the upregulation of miRNA-30e by various viral infections has been reported in mammals [14–17]. Especially, Zhu et al. [15] reported the overexpression of miR-30e induced anti-Dengue virus responses through the NF-κB activation-mediated interferon-ß induction. On the other hand, there has been no available information on the immunological role of miR-30e in fish cells.

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The artificial expression of miRNAs is usually accomplished through plasmid-based expression vectors or mimics. Recently, there are reports on the application of recombinant viruses as a vehicle for miRNAs delivery, and most of the reports have focused on the availability of DNA viruses. RNA viruses were not into consideration due to the concerns of viral genome degradation by miRNAs produced by itself. However, recently, the possibility of various RNA viruses as a delivery vehicle for miRNAs has been reported [18–23]. In fish, there is little information on the availability of fish viruses, even in DNA viruses, for the delivery of miRNAs. In the present study, we firstly reported the use of a fish RNA virus, viral hemorrhagic septicemia virus (VHSV), as a delivery vehicle of a miRNA, and the effect of miR-30e produced by the recombinant VHSV on the immune responses of Epithelioma papulosum cyprini (EPC) cells was investigated.

2. Materials and methods

2.1. Cells and virus

EPC cells (ATCC CRL-2872), zebrafish embryonic cell line (ZF4) (ATCC CRL-2050), and baby hamster kidney (BHK-21) cells (KCLB-10010) were grown in Leibovitz medium (L-15, Sigma), Dulbecco's modified Eagle's medium/nutrient mixture F-12 ham (DMEM/F12, Sigma), and minimum essential medium (MEM, Thermo Fisher), respectively. Each medium was supplemented with penicillin-streptomycin solution (100 U/ml penicillin 100 μg/ml streptomycin, Welgene) and 10% fetal bovine serum (FBS, Welgene). The wild-type VHSV (VHSV KJ2008) [24] and recombinant VHSVs were propagated in monolayer of EPC cells at 15 °C in the presence of 2% FBS. When the cytopathic effect (CPE) was broadly observed, medium was frozen and melted, then, centrifuged at 8000 rpm. Supernatant was filtered with 0.45 μm syringe filter (Advantec) and kept at −80 °C until use.

2.2. Construction of vectors for primary miRNA-30e expression and miR-30e sponge

To clone the primary miRNA-30e sequence from the genomic DNA of ZF4 cells, the region containing 150 bp flanking region at each 5′ and 3′ end was amplified by PCR using a primer pair in Table 1, and the amplified product was cloned into pGEM-T easy vector (Promega). The primary miRNA-30e sequence was inserted downstream of the CMV promoter of pFC vector (System Biosciences) after digestion of the vectors with XhoI and XbaI. To know whether the functional miR-30e could be produced by the primary miRNA-30e expressing cassette, fragments with the sponge sequence for miR-30e or with several nucleotides-mutated sponge sequence were multimerized (4 repetitive sequences) by the annealing of oligonucleotides fragments, and were cloned into pGEM-T easy vectors. To use enhanced green fluorescent protein (eGFP) as a reporter protein, eGFP ORF was inserted into pcDNA3.1 (Invitrogen) (pcDAN3.1-eGFP), then, each sponge and mutated sponge sequence (restricted with NotI and ApaI) was inserted downstream of the eGFP ORF. The eGFP-sponge cassette and the eGFP-mutated sponge cassette were inserted into the primary miRNA-30e expressing vector using restriction enzymes (KpnI and EcoRI), and were designated as pmiR30e-eGFP-sponge and pmiR30e-eGFP-mutated sponge, respectively.

To analyze the effect of the interaction between miR-30e and sponge on eGFP reporter expression, EPC cells were transfected with each vector using FuGENE HD (Promega), and the mean of fluorescence intensity (MFI) value was measured by flow cytometry (BD FACSVerse) at 48 h post-transfection.

2.3. Construction of primary miRNA-30e expressing vector and quantitation of miR-30e

To construct a primary miRNA-30e expressing vector with an eGFP cassette as a reporter, the pmiR30e-eGFP-sponge vector and pcDNA3.1-eGFP vector were digested with KpnI and EcoRI, then, the fragment of eGFP cassette was inserted into the pmiR30e-eGFP-sponge vector (designated as pmiR-30e-eGFP). The construction of its control vector was performed by the removal of the stem-loop region of primary miRNA-30e using Overlap cloner kit (Elpisbio, Korea) and primers (Table 1), and designated as pmiR-30e-eGFP-Con.

EPC cells were transfected with the vectors using FuGENE HD (Promega), and at 48 h post-transfection, 2×10^5 of eGFP positive cells were sorted among the transfected cells by FACS Aria III (BD Biosciences). Small RNA was isolated from the eGFP-positive cells using Hybrid-R™ miRNA kit (GeneAll, Korea) following manufacturer's instructions. HB miR Multi Assay Kit™ system II (HeimBiotek, Korea) was used to synthesize cDNA and to quantify miR-30e. Amplification of small nucleolar U6 used as a reference gene and miR-30e was done using specific primers provided by HeimBiotek. The miRNA quantitative RT-PCR was performed with LightCycler (Roche), and relative quantification of miR-30e level was analyzed through the comparative threshold method ($2^{-\Delta\Delta Ct}$).

2.4. Construction of miR-30e vectors targeting the 3′-UTR of NF-κb inhibitor α-like protein B

The prediction of target genes for miR-30e was performed using a miRNA target prediction tool, miRanda [25], from the assembled expressed tag sequences (EST) of fathead minnow (*Pimephales promelas*) retrieved from NCBI database, and NF-κb inhibitor α-like protein B (NFκbiαb) was predicted as one of the target genes of miR-30e. To know whether miR-30e inhibits NFκbiαb expression in EPC cells, the 3′-UTR of NFκbiαb was amplified by PCR, and cloned into Topo cloning vector (Elpisbio, Korea). A reporter vector containing the primary miRNA-30e expression cassette and the eGFP reporter cassette with 3′-UTR of NFκbiαb (pmiR30e-NFκbiαb) was constructed through the assembling of three fragments (fragment #1, #2, #3) using Overlap cloner kit (Elpisbio) and primer sets (Table 1). The fragment #1 having PGK promoter and eGFP ORF was amplified from the pmirGLO-Bmpr2-Δ17 vector (Addgene plasmid # 49380) [26] that was modified by the replacing of luciferase ORF with eGFP ORF. The fragment #2 corresponding to 3′-UTR of NFκbiαb was amplified from the cloned Topo vector, and the fragment #3 (the primary miRNA-30e cassette) was amplified from pmiR30e-eGFP-sponge vector. Each amplified fragment was treated with DpnI to remove methylated DNA template, then, assembled according to the manufacturer's instruction. A control vector (pmiR30e-NFκbiαb-Con) was constructed by the deletion of the stem-loop region of primary miRNA-30e and the assembling of fragments using Overlap cloner. EPC cells were transfected with the constructed vectors, and the reporter mean fluorescence intensity (MFI) was analyzed by Flow cytometry (FACSVerse, BD Biosciences) at 48 h post-transfection.

In addition, to further verify NFκbiαb as a target gene of miR-30e, dual luciferase assay was performed. The pmirGLO-Bmpr2-Δ17 vector was used as a dual luciferase reporter vector, and the 3′-UTR of NFκbiαb was inserted into the down-stream of the firefly luciferase gene using SalI and XhoI restriction enzymes, and designated as pmirGLO-NFκbiαb 3UTR. A control vector (pmirGLO-NFκbiαb 3UTR SDM) was constructed by the site directed mutagenesis (SDM) using primers in Table 1. Due to the low transfection efficiency of fish cell lines, BHK-21 cell line was used for the assay. BHK-21 cells at a density of 6×10^3 cells/well were seeded into 96-well plates and were cultured for 24 h. The FuGENE HD transfection reagent (1 μl) was added on 100 μl MEM medium, 100 ng of pmirGLO-NFκbiαb 3UTR vector, and 200 nM of miR-30e mimics (Bioneer, Korea) were added on 100 μl MEM medium. Each mixture was incubated for 5 min at RT and mixed well by tapping, then, incubated for 15 min at RT. The transfection mixture was added to the cells drop-wise. The day after transfection, media changed to 10% FBS MEM culture medium. As a control, pmirGLO-NFκbiαb

Table 1
Primers used in this study.

Primers	Sequence (5′-3′)	
For Cloning of primary miRNA		
miR30e-XhoI-F	ATACTCGAGACAGCCATGCCATAGTTTTAGG	
miR30e-XbaI-BglII-R	AGCTCTAGAAGATCTAGTTCATCATATGACCAGTGAC	
For miR-30e sponge synthesis		
NotI_antisense_F	GCGGCCGCgctgcaaacatccgactgaaagTAGCTAAgct	
XhoI_antisense_R	CTCGAGctttcagtcggatgtttgcagcTTAGCTA	
XbaI_antisense_F	TCTAGAgctgcaaacatccgactgaaagTAGCTAAgct	
ApaI_antisense_R	GGGCCCctttcagtcggatgtttgcagcTTAGCTA	
For miR-30e mutated sponge synthesis		
miR30e_AS_mutation_F1	GCGGCCGCgctggaatcaacctacagatagTAGCTAAgct	
miR30e_AS_mutation_R1	CTCGAGctatctgtaggttgattccagcTTAGCTA	
miR30e_AS_mutation_F2	TCTAGAgctggaatcaacctacagatagTAGCTAAgct	
miR30e_AS_mutation_R2	GGGCCCctatctgtaggttgattccagcTTAGCTA	
For Cloning of NFκbiαb 3′UTR in EPC cell		
EPC_iKB_3UTR_F	GGAAAGAAGGACTAGAAGGACTTCTGTTGAATG	
EPC_iKB_3UTR_R	CATGCACTTGATTTCCAAGGAAGGACAC	
For Construction of pmiR30e-NF κbiαb vector		
Fragments #1	Vector_FOR	GGAAATGTGCGCGGAACCC
	eGFP_pmiR_OC_R	AATTCAACTTGTTTATCATGCACTTGATTTCCAAGGA
Fragments #2	EPC_iKB_3UTR_OC_F	GCCGCTTCGAGCAGACGAAAGAAGGACTAGAAGGACTTCTGTTG
	EPC_iKB_3UTR_OC_R	AATTCAACTTGTTTATCATGCACTTGATTTCCAAGGA
Fragments #3	CMV_OC_F	ATAAACAAGTTGAATTCCATAGCCCATATATGGAGTTCCGCG
	SV40_Pmirglo_OC_R	GTTCCGCGCACATTTCCGCGTTAAGATACATTGATGAGTTTGGAC
For Construction of pmirGLO – NFkbiab 3′UTR SDM vector		
EPC_ikb_3UTR_SDM_F	CATGTAAATGTGTATATAGAAGgTcGcAgCGTAACGCATTTTAAATTA	
EPC_ikb_3UTR_SDM_R	TAATTTAAAATGCGTTACGcTgCgAcCTTCTATATACACATTTACATG	
For deletion of miR-30e stem-loop		
Fragments #1	Vector.FOR	GGAAATGTGCGCGGAACCC
	miR30e_universal_R	AGCCCGTACTGCCAGCTGT
Fragments #2	miR30e_deletion_F	TGGCAGTACGGGCTAAGCCAACTGCTGTTACTCTCG
	AmpR-pr-F	GGGGTTCCGCGCACATTT
For Construction of pVHSV-A-eGFP		
EGFP-NdeI-R	CATATGATGGTGAGCAAGGGCGAGG	
EGFP-SalI-R	GTCGACTTACTTGTACAGCTCGTCCATGCCG	
For Construction of pVHSV-A-miR30e		
Dre_miR30e_OC_F	AAGACAAACACTGAGATCATATGTACAGCCATGCCATAGTTTTAGG	
Dre_miR30e_OC_R	GACGGAAAGGCTTGGTCGACAGTTCATCATATGACCAGTG	
For real-time RT-PCR		
EPC 18S	F	AATGTCTGCCCTATCAACTTTC
	R	TGGATGTGGTAGCCGTTTC
EPC Mx1	F	TGGAGGAACCTGCCTTAAATAC
	R	GTCTTTGCTGTTGTCAGAAGATTAG
EPC ISG15	F	TGATGCAAATGAGACCGTAGAT
	R	CAGTTGTCTGCCGTTGTAAATC
EPC IFN-α	F	CGCTCAGGCATGGGAGCTG
	R	GAGGACGTGGCATTGCTTGC

(Underline: restriction enzyme site, Lowercase: sponge and sponge mutation sequence).

3UTR SDM vector was transfected as the same protocols. At 48 h post-transfection, firefly and *Renilla* luciferase activity assay was conducted using Dual-Glo Luciferase Assay kit (Promega) according to the manufacture's protocol with Victor X3 plate reader (PerkinElmer). The activity was measured 5 times with 1 min interval. The mean of the relative luciferase ratio (firefly luciferase/*Renilla* luciferase) of the control was set to one.

2.5. Generation of miR-30e expressing recombinant VHSV (rVHSV-A-miR30e)

A previously constructed vector (pVHSV-A-IFNγ) [27] that harboring the ORF of olive flounder IFN-γ between N and P genes was used to construct a vector for recombinant VHSV that expressing primary miRNA-30e. The pVHSV-A-IFNγ vector was digested with NdeI and SalI restriction enzymes, then, assembled with a PCR amplicon of precursor miRNA-30e using Overlap cloner, and designated as pVHSV-A-miR30e. As a control vector, the IFNγ ORF in pVHSV-A-IFNγ vector was replaced with eGFP ORF using restriction enzymes (NdeI and SalI), and designated as pVHSV-A-eGFP. EPC cells expressing T7 RNA polymerase were seeded on 35 mm dish about 80% confluence and transfected with a mixture of pVHSV-A-miR30e or pVHSV-A-eGFP (2 μg) and previously constructed helper vectors, pCMV-N (500 ng), pCMV-P (300 ng), and pCMV-L (200 ng) using Fugene HD transfection reagent according to manufacturer's protocol. When extensive cytopathic effect (CPE) was observed, the cells were freeze-thawing and centrifuged at 8000 rpm for 10 min at room temperature (RT). The resulting supernatant was filtered with 0.45 μm syringe filter and passaged on EPC cells that cultured on T25 flask at 15 °C. At 5–7 d post-inoculation, the supernatant was collected and stored at −70 °C before being used.

To check the titers of each viral stock (rVHSV-A-miR30e and rVHSV-A-eGPF), EPC cells (2×10^6 cells/35 mm dish) were infected for 2 h at 15 °C with each serially diluted virus stock (10^{-3} to 10^{-5}). After the removal of the media, cells were overlaid with plaque media (0.8% Agarose, 2% FBS, 100x antibiotics) and incubate on 15 °C until viral plaque distinguishable. The fixing and staining was performed with 10% formalin and 10% crystal violet for 2 h each, then, rinsed with distilled water and plaque-forming units (PFU) were counted. To compare the growth of each recombinant virus, EPC cell (2×10^6 cells/35 mm dish) were inoculated with each recombinant virus (rVHSV, rVHSV-A-eGFP, rVHSV-A-miR30e) at MOI 0.001 at 15 °C. Each supernatant was collected at 1, 3, 5 days post-infection and filtered through

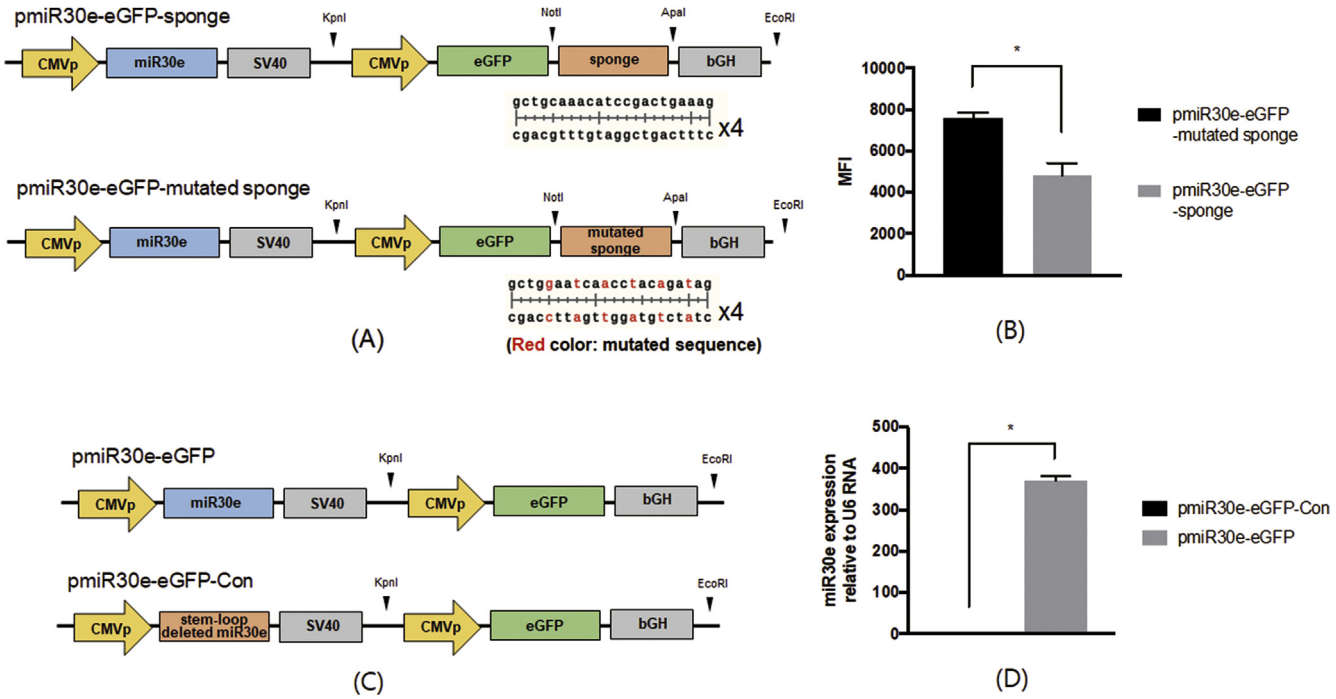

Fig. 1. (A) Vector maps showing a primary miRNA-30e (miR30e)-expressing cassette and an eGFP-miR-30e sponge cassette (pmiR30e-eGFP-sponge) or an eGFP-miR-30e mutated sponge cassette (pmiR30e-eGFP-mutated sponge). CMVp, cytomegalovirus immediate early promoter; SV40, simian virus 40 poly A signal; bGH, bovine growth hormone poly A signal. (B) The mean of fluorescence intensity (MFI) value of cells transfected with pmiR30e-eGFP-sponge vector or pmiR30e-eGFP-mutated sponge vector. (C) Vector maps showing a primary miRNA-30e-expressing vector (pmiR30e-eGFP) and a control vector that contains the stem-loop-deleted primary miRNA-30e (pmiR30e-eGFP-Con). (D) The expressed amount of miR-30e from cells transfected with pmiR30e-eGFP-sponge vector or pmiR30e-eGFP-mutated sponge vector. The miR-30e expression level was analyzed by quantitative RT-PCR using U6 RNA as a control. The asterisk on the bar in (B) and (D) represents statistically significant at $p < 0.05$.

0.45 μm syringe filter then measure the viral titers by the plaque assay.

2.6. Verification of miR-30e production by rVHSV-A-miR30e using Northern blot and qRT-PCR

To verify the expression of miR-30e by rVHSV-A-miR30e using Northern blot, EPC cells were infected with the recombinant viruses (rVHSV-A-eGFP, rVHSV-A-miR30e) with a multiplicity of infection (MOI) 0.05, and total RNA was extracted by Hybrid-R miRNA purification kit (GeneAll) at 48 h post-infection. Extracted total RNA was measured by BioSpectrometer (Eppendorf) and 30 μg of the RNA was mixed with 2x RNA loading dye (NEB) then loaded on 15% TBE urea polyacrylamide gel at 200 V 1 h. The gel transfer to the Hybond XL membrane was done using the Mini Trans-Blot (BioRad) in 0.5x TBE at 0.4 A for 1 h. The membrane was UV cross-linked at 1200 μ joules for 20 min and dried at 50 °C for 30 min. The cross-linked membrane was pre-hybridized for 3 h at 42 °C, then, hybridization was performed overnight with appropriate probe. For U6 RNA, a biotin-labeled probe (5′-ATG AGGAACGCTTCACGAATT-Biotin-3′) was synthesized from Macrogen (Korea). For miR30e, oligonucleotide (5′-GCTGCAAACATC CGACTGAAAG-3′) was tailed with DIG-dUTP and dATP using DIG Oligonucleotide Tailing kit (Roche). The hybridized membrane was rinsed with washing buffer 1 (2xSSC, 0.1% SDS) and washing buffer 2 (0.1xSSC, 0.1% SDS) for two times. The Dig labeled probes were detected using anti-digoxigenin-AP and visualized with the chemiluminescence substrate CDP-Star (Roche). The Biotin labeled probes were detected using streptavidin-HRP and visualized with Cheminate-HRP Picodetect (Applichem). The membranes were captured using an LAS 4000 (Fujifilm).

To analyze the expression of miR-30e by rVHSV-A-miR30e using qRT-PCR, 500 ng total RNA extracted from the above was used for the synthesis of miRNA cDNA using HB II RT reaction kit according to manufacturer's instruction. Synthesized miRNA cDNA was used for quantitative analysis by LightCycler (Roche) using HB miR Multi Assay Kit system II and calculate the expression level of miR-30e relative to RNA U6 using $2^{-\Delta\Delta Ct}$ method.

2.7. Effect of rVHSV-A-miR30e infection on NF-κB activity and the expression of IFN-α, Mx1, and ISG15 in EPC cells

To measure NF-κB activity, EPC cells were transfected with pNFκB-MetLuc2-Reporter plasmid (Clontech) using FuGENE HD according to the manufacture's instructions, and selected using G418 (400 μg/ml, Sigma) [28]. Cells (2×10^6 cells/35 mm dish) containing NF-κB reporter vector were infected with recombinant VHSVs (rVHSV-Wild, rVHSV-A-eGFP, and rVHSV-A-miR30e) with MOI 0.2. At 24 h post-infection, 50 μl of the culture media was added into 96 well plates, and analyzed luciferase activity using Ready-To-Glow secreted luciferase reporter assay kit (Clontech). The signal of luciferase activity was measured in a Victor3 multilabel plate reader (PerkinElmer). The experiment was performed in triplicate.

To analyze the expression of genes related to type I interferon responses (Mx1, ISG15, and interferon-α), EPC cells (2×10^6 cells/35 mm dish) were infected with recombinant viruses (rVHSV-wild, rVHSV-A-eGFP, rVHSV-A-miR30e) with MOI 0.001, then, at 24 and 48 h post-infection, total RNA was extracted using Hybird-R Kit (Geneall). The 1 μg of the total RNA was used to synthesize first-strand cDNA using M-MLV Reverse Transcriptase (Elpisbio). Synthesized cDNA was analyzed with LightCycler (Roche) using SYBR Green reagent (Enzynomics) and each primer set (Table 1). To verify the reproducibility of genes expression, one more experiment with the same scheme was performed.

2.8. Statistical analysis

Statistical significance was analyzed using SPSS for Windows (Chicago, IL, USA). Data were analyzed by using one-way ANOVA

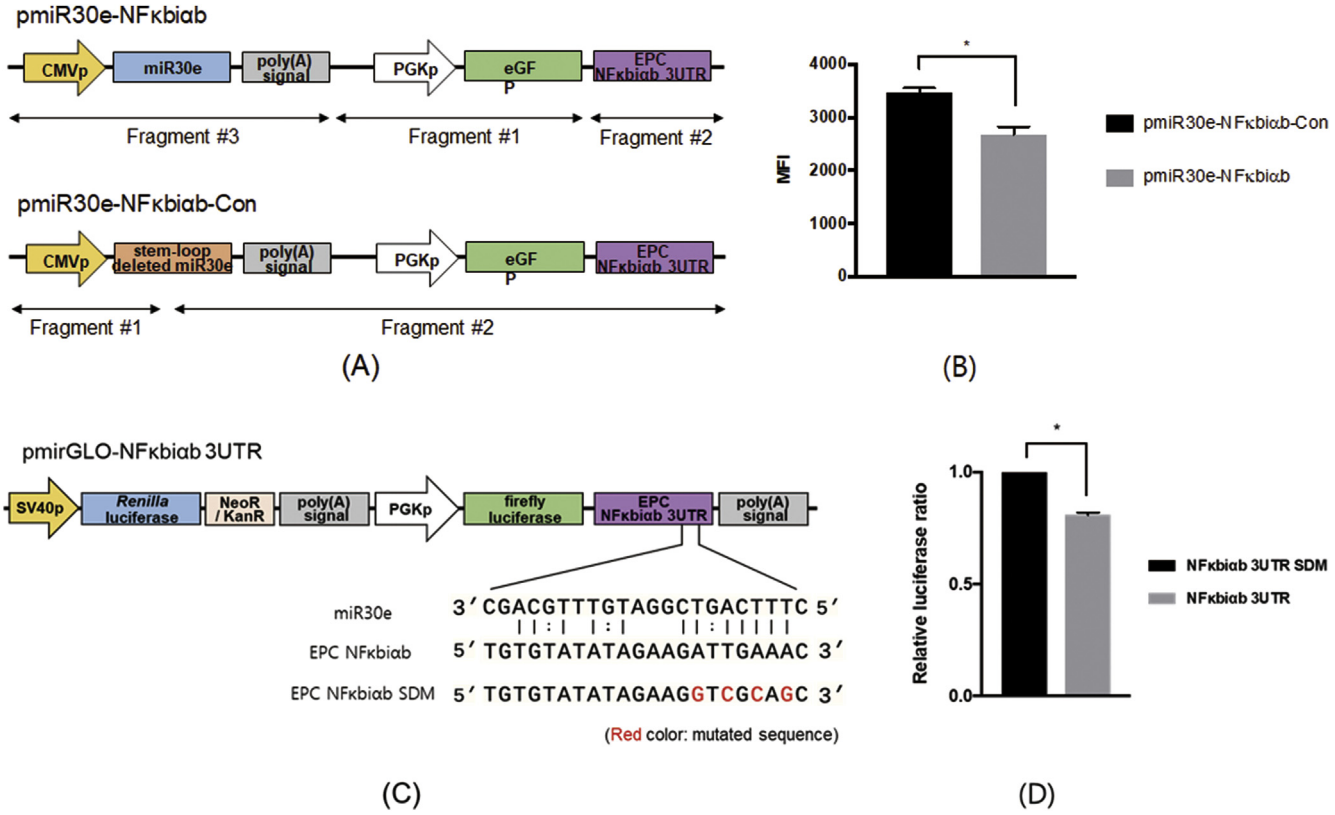

Fig. 2. (A) A vector with an expressing cassette for primary miRNA-30e (miR30e) and a reporter cassette for eGFP harboring NFκbiαb 3′UTR (pmiR30e-NFκbiαb) for the verification of miR-30e targeting to NFκbiαb. A control vector (pmiR30e-NFκbiαb-Con) contains stem-loop deleted primary miRNA-30e sequence. (B) The mean of fluorescence intensity (MFI) value of cells transfected with pmiR30e-NFκbiαb vector or pmiR30e-NFκbiαb-Con vector. (C) Diagram of the firefly and *Renilla* luciferase reporter plasmid containing either NFκbiαb 3′UTR sequence or mutated NFκbiαb 3′UTR sequence (red letters) behind the firefly luciferase ORF. (D) The value of the relative luciferase ratio (firefly luciferase versus *Renilla* luciferase activity) in cells simultaneously transfected with miR-30e mimics and each vector. The asterisk on the bar in (B) and (D) represents statistically significant at $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

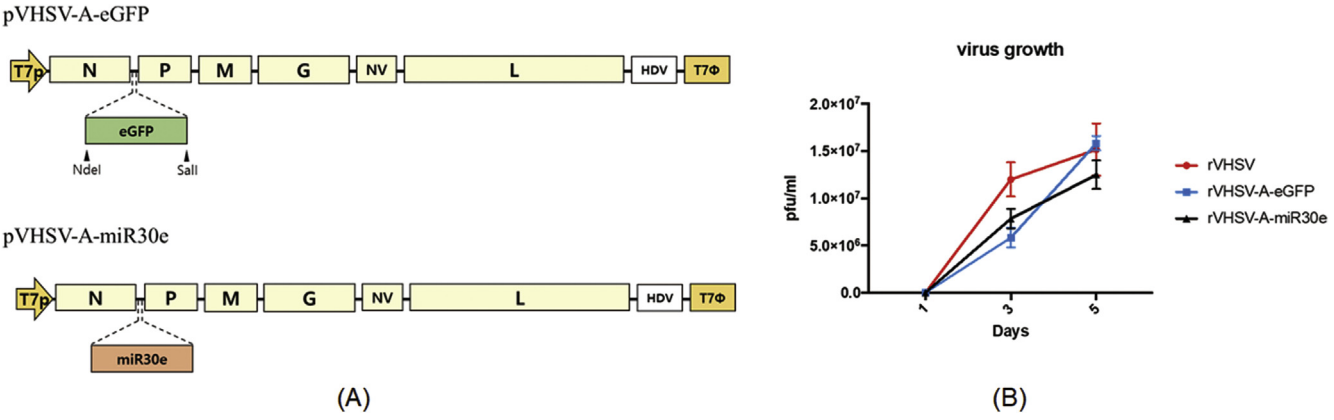

Fig. 3. (A) The schematic representation of vectors for the generation of an eGFP-expressing VHSV (rVHSV-A-eGFP) and a primary miRNA-30e-expressing VHSV (rVHSV-A-miR30e). The eGFP sequence or primary miR30e sequence was inserted between N gene and P gene using restriction enzymes. T7p, T7 RNA polymerase promoter; N, nucleoprotein, P, phosphoprotein; M, matrix protein; G, glycoprotein; NV, non-virion protein; L, RNA-dependent RNA polymerase; HDV, hepatitis delta virus ribozyme; T7Φ, T7 RNA polymerase terminator. (B) The growth of each recombinant virus (rVHSV, rVHSV-A-eGFP, and rVHSV-A-miR30e) in EPC cells. At 1, 3, 5 days post-infection, each supernatant was collected and the viral titers were measured by the plaque assay.

followed by Tukey HSD post-hoc test or Student's t-test, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Verification of functional miR-30e production by primary miRNA-30e expressing vector

To verify the production of functional miR-30e by the present CMV promoter-driven miR-30e vector, EPC cells were transfected with

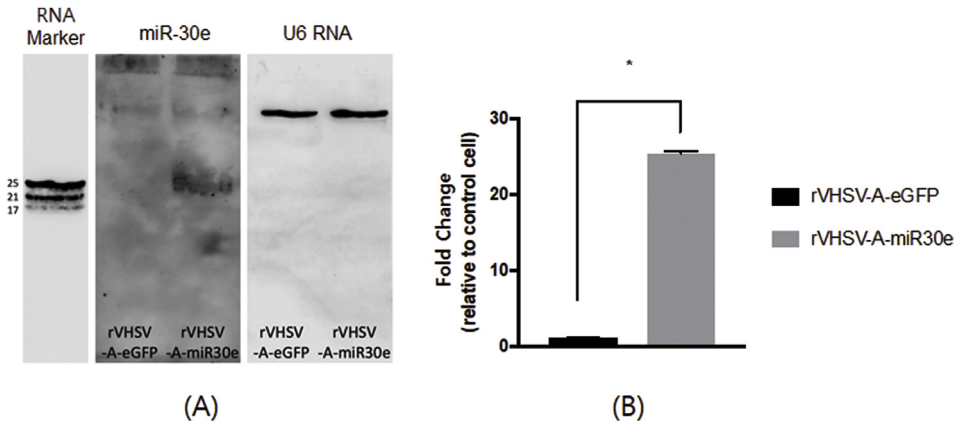

Fig. 4. The verification of miRNA-30e production from cells infected with rVHSV-A-miR30e by Northern blot (A) and quantitative RT-PCR (B). Cells infected with rVHSV-A-eGFP were used as control. The asterisk on the bar represents statistically significant at $p < 0.05$.

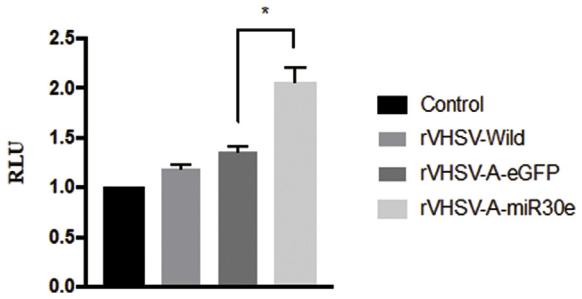

Fig. 5. Effect of rVHSV-A-miR30e infection on NF-κB activity. EPC cells containing the NF-κB-MetLuc2 reporter vector were infected with recombinant VHSVs (rVHSV-Wild, rVHSV-A-eGFP, or rVHSV-A-miR30e) and the relative luminescence units (RLU) were measured by analyzing luciferase activity. Cells with the reporter vector but not infected were used for control. The asterisk on the bar represents statistically significant at $p < 0.05$.

pmiR30e-eGFP-sponge vector or pmiR30e-eGFP-mutated sponge vector (Fig. 1A), and compared the fluorescent levels (Fig. 1B). The cells transfected with the vector containing miR-30e sponge sequence (pmiR30e-eGFP-sponge) showed significantly lower eGFP expression than cells transfected with the control vector that had mutated miR-30e sponge sequence (pmiR30e-eGFP-mutated sponge) (Fig. 1B).

To analyze the amount of miR-30e production by a CMV promoter-driven vector, EPC cells were transfected with pmiR-30e-eGFP vector or pmiR-30e-eGFP-Con vector in which the stem-loop region of primary miRNA-30e was deleted (Fig. 1C). As the transfection efficiency of EPC cells was low, cells expressing fluorescence were collected after transfection using cell sorter, and the amount of miR-30e was evaluated using qRT-PCR (Fig. 1D). The overexpression of miR-30e in EPC cells transfected with a primary miRNA-30e-expressing vector compared to cells transfected with the control vector was confirmed by qRT-PCR (Fig. 1D).

3.2. NFκbiαb as a target of miR-30e

Using a miRNA target gene prediction tool (miRanda), NFκbiαb was predicted as a target gene of miR-30e. To verify whether NFκbiαb gene of EPC cells is a target for miR-30e, EPC cells were transfected with pmiR30e-NFκbiαb vector that containing the 3′-UTR of NFκbiαb behind the reporter gene (eGFP) ORF or a control vector in which the stem-loop region of primary miRNA-30e was deleted (pmiR30e-NFκbiαb-Con) (Fig. 2A). At 48 h post-transfection, the MFI level of EPC cells transfected with the vector containing a primary miRNA-30e expression cassette was significantly lower than that of cells transfected with the vector containing a stem-loop region-deleted primary miRNA-30e expression cassette (Fig. 2B).

To further confirm whether NFκbiαb gene is a target of miR-30e, a dual luciferase reporter assay was performed. BHK-21 cells were simultaneously transfected with miR-30e mimics and either pmirGLO-NFκbiαb 3UTR vector or pmirGLO-NFκbiαb 3UTR SDM vector that contained mutated NFκbiαb 3′ UTR in the region binding to seed sequence of miR-30e (Fig. 2C). At 48 h post-transfection, the relative luciferase ratio in cells transfected with pmirGLO-NFκbiαb 3UTR vector was significantly lower than that of cells transfected with pmirGLO-NFκbiαb 3UTR SDM vector (Fig. 2D).

3.3. Generation of miR-30e expressing recombinant VHSV

Recombinant VHSVs harboring primary miRNA-30e sequence or eGFP sequence (control) between N and P gene (Fig. 3A) was successfully generated by reverse genetic method. The growth of rVHSV-A-miR30e was not significantly different from that of rVHSV-A-eGFP (Fig. 3B).

To verify the production of miR-30e by rVHSV-A-miR30e using Northern blot, EPC cells were infected with rVHSV-A-miR30e or rVHSV-A-eGFP. A band corresponding to the size of mature miR-30e was detected cells infected with rVHSV-A-miR30e (Fig. 4A), on the other hand, no corresponding band was detected from cells infected with rVHSV-A-eGFP. The evaluation of miR-30e production using qRT-PCR, also, showed significantly higher miR-30e level in cells infected with rVHSV-A-miR30e than cells infected with rVHSV-A-eGFP (Fig. 4B).

3.4. Functional analysis of rVHSV-A-miR30e in vitro

The NF-κB activity of EPC cells infected with rVHSV-A-mir30e was significantly higher than cells infected with rVHSV-wild or rVHSV-A-eGFP (Fig. 5).

The expression of type I interferon related genes (Mx1, ISG15, and Interferon α) was significantly higher at both 24 h and 48 h after rVHSV-A-mir30e infection compared with rVHSV-A-eGFP or rVHSV-wild infection (Fig. 6).

4. Discussion

Although microRNAs play important roles in the regulation of genes expression, the regulatory effects can be variable according to the ratio between the amount of a microRNA and target genes transcript. The additional artificial supply of a microRNA can break the natural balance, which can more evidently show the effects of a microRNA. Therefore, artificial microRNA expression systems can be harnessed to modify certain characteristics of cells for the purpose of a research. In

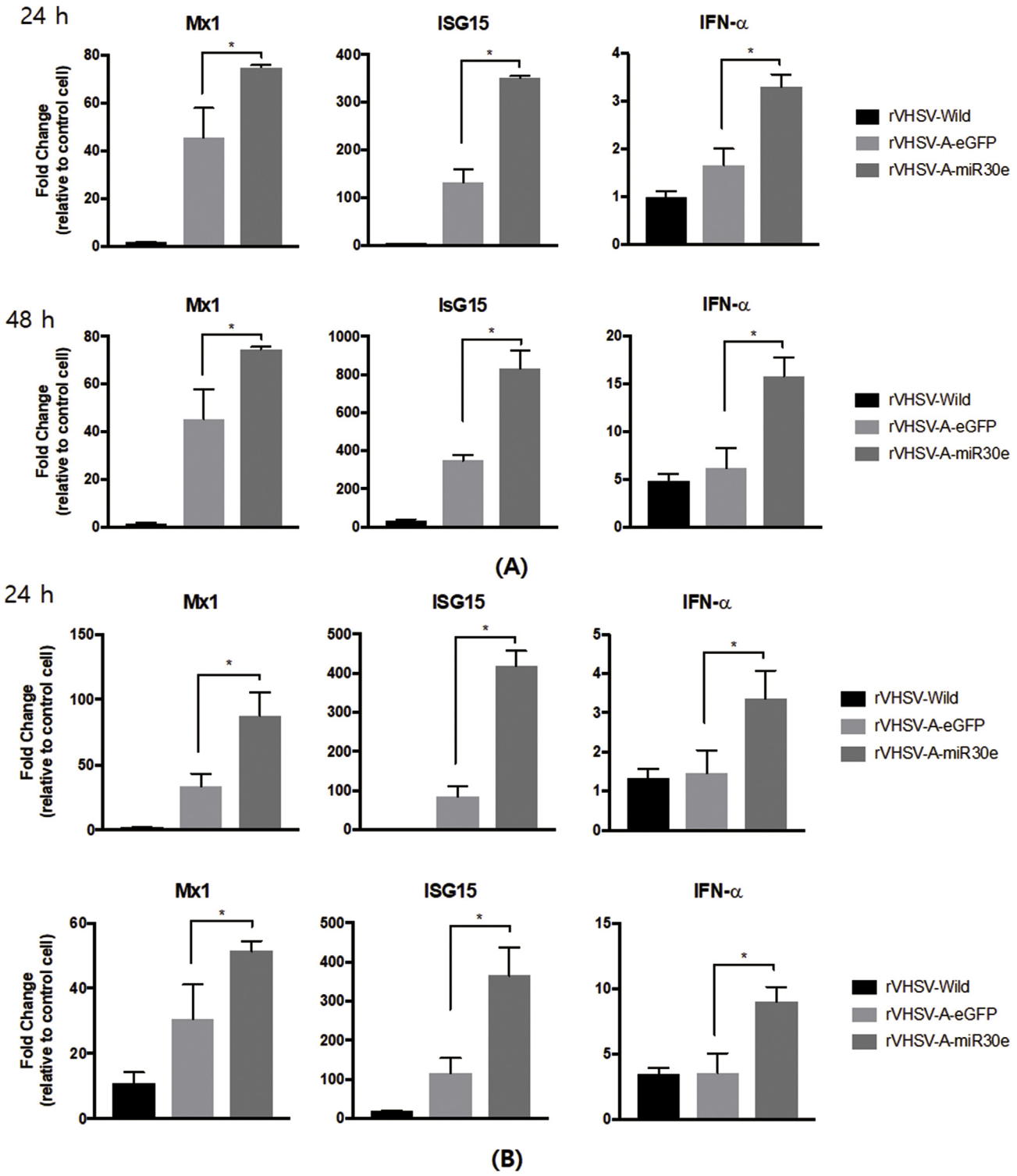

Fig. 6. (A) The expression of genes related to type I interferon – Mx1, ISG15, and interferon-α (IFNA) - in EPC cells infected with recombinant VHSVs (rVHSV-Wild, rVHSV-A-eGFP, or rVHSV-A-miR30e). Cells were infected with each recombinant VHSV at MOI 0.001 and the expression of genes was analyzed at 24 and 48 h post-infection. (B) To confirm the reproducibility of the quantitative RT-PCR results, an experiment with the same scheme of (A) was performed. The asterisks on the bars represent statistically significant at $p < 0.05$.

the present study, we constructed a vector that had a CMV promoter-driven primary microRNA-30e, and the high expression of miR-30e and its inhibitory effect on reporter gene expression in EPC cells were verified by the miR-30e-specific qPCR and a vector containing a miR-30e sponge cassette, suggesting that the overexpression of miR-30e would significantly (or abnormally) down-regulate the expression of its target genes. In this study, through a search for miR-30e target genes, a binding site for miR-30e was found from the 3′-UTR region of NFκbiαb in EPC cells, and the down-regulation of reporter gene containing NFκbiαb 3′-UTR by miR-30e was demonstrated. NF-κB is a transcript factor and involved in diverse immune responses. In the cytoplasm, NF-κB exists as an inactivated form by being bound to IκBs, and the

detachment of IκBs activates NF-κB to move into the nucleus. The activation of NF-κB by the overexpression of miR-30e has been reported in mammalian cells, in which miR-30e repressed the translation of IκBα [13,15]. Therefore, the present results suggest that fish miR-30e can also decrease IκB expression, and miR-30e overexpression can increase immune responses related to NF-κB activation.

Delivery of microRNAs using recombinant viruses can be a way to overcome the low transfection efficiency of cells and to modulate cell immune responses. As the processing of primary microRNAs into pre-microRNAs is occurred in the nucleus by Drosha and DGCR8, most of recombinant viruses for microRNAs delivery have been DNA viruses, or RNA viruses with a DNA intermediate such as Retrovirus and Lentivirus, or nucleic RNA viruses such as influenza viruses [19]. Although cytoplasmic RNA viruses do not enter the nucleus, the production of microRNAs in mammalian cells using a recombinant cytoplasmic RNA virus has been reported [18]. Especially, Langlois et al. [21] reported the production of miR-124 using a recombinant VSV (a member of Rhabdoviridae) that contained the primary microRNA-124 sequence between G and L genes. Similarly, in the present study, we inserted primary microRNA-30e sequence between N and P genes of VHSV genome, and demonstrated the successful expression of miR-30e by the recombinant VHSV (rVHSV-A-miR30e) using Northern blot and qRT-PCR. Although mechanisms associated with the cytoplasmic primary microRNA processing are not clearly elucidated, recently, Shapiro et al. [29] reported the relocalization of Drosha to the cytoplasm following virus infection and the production of mature miRNAs from virus-derived cytoplasmic primary microRNAs. Therefore, as VHSV RNA cannot enter into nucleus, the present results suggest that Drosha and other primary microRNA processing factors in the nucleus might be translocated to the cytoplasm.

In our previous study, the expression of miR-30e in EPC cells was decreased by VHSV infection [30], and, in this study, the expression level of miR-30e in cells infected with rVHSV-A-eGFP was low. Therefore, the production of miR-30e by rVHSV-A-miR30e infection can bring artificial effects in EPC cells. In the present study, cells infected with rVHSV-A-miR30e showed the higher increase of NF-κB activation and type I interferon induced genes expression than cells infected with rVHSV-A-eGFP, suggesting that rVHSV-A-miR30e can produce functional miR-30e in fish cells, and VHSV can be used as a vehicle to deliver microRNAs in fish. We are now performing experiments to solve the problem related to the cytotoxicity of VHSV in infected cells for the development of a safe and effective delivery of functional microRNAs using recombinant VHSVs in fish.

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