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Research Article

Lentivirus-mediated bta-miR-193a Overexpression Promotes Apoptosis of MDBK Cells by Targeting BAX and Inhibits BVDV Replication

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Abstract

MicroRNAs (miRNAs) are a class of naturally occurring, short, endogenous, noncoding RNA molecules (~22 nt) involved in a wide variety of regulatory pathways, including cell growth, development, differentiation, proliferation, and apoptosis, as well as viral defense, hematopoiesis, organ formation, and metabolism. Previous studies showed that bta-miR-193a (miR-193a) was upregulated in Madin-Darby bovine kidney (MDBK) cells infected with bovine viral diarrhea virus (BVDV) strain NADL; however, the role of miR-193a in apoptosis-associated regulation remains unclear. In this study, we found that miR-193a is a novel regulator of MDBK apoptosis and that lentiviral infection exhibited a positive effect on miR-193a expression. Additionally, we observed that the miR-193a-target sequence was present in the 3′-untranslated region of B-cell lymphoma-2-associated X protein (BAX) mRNA, with miR-193a overexpression resulting in reduced BAX mRNA and protein levels. Furthermore, we observed that miR-193a promoted apoptosis and inhibited BVDV strain NADL replication according to quantitative reverse transcription polymerase chain reaction results. These findings confirmed miR-193a as a positive regulator of apoptosis and provided a theoretical basis for the important role of miRNAs in regulating BVDV replication.

Keywords: *Lentivirus, miR-193a, Apoptosis, BAX, BVDV strain NADL*

Lentivirus Aracılı bta-miR-193a Overeksprasyonu MDBK Hücrelerinin Apoptozisini Artırır ve BVDV Replikasyonunu Baskılar

Özet

MikroRNAlar (MiRNA) doğal olarak bulunan, kısa, endojen, kodlama yapmayan RNA molekülleri (~22 nt) olup, hücre büyümesi, gelişmesi, farklılaşması, çoğalması ve apoptozis gibi çok çeşitli düzenleyici yolaklarda ve ayrıca viral savunmada, hematopoieziste, organ şekillenmesinde ve metabolizmada görev yapmaktadır. Yapılan çalışmalar Bovine Viral Diare Virus (BVDV)'un NADL suşu ile enfekte Madin-Darby Bovine Böbrek (MDBK) hücrelerinde bta-miR-193a (miR-193a)'nın ekspresyonunun upregule edildiğini göstermiştir. Ancak, apoptozis ilişkili regulasyonda miR-193a'nın rolü bilinmemektedir. Bu çalışmada, miR-193a'nın MDBK apoptozisinde görev yapan bir regülatör olduğu ve lentivirus enfeksiyonunun miR-193a ekspresyonunda pozitif bir etki gösterdiği tespit edilmiştir. Ayrıca, miR-193a hedef sekansının B-hücre lenfoma-2-ilişkili X protein (BAX) mRNA'sının 3′-translasyon yapılmayan bölgesinde mevcut olduğu ve miR-193a overekspresyonunun azalmış BAX mRNA ve protein seviyesi ile ilişkili olduğu belirlendi. Kantitatif ters transkripsiyon polimeraz zincir reaksiyonu ile belirlendiği üzere miR-193a apoptozisi artırdı ve BVDV suş NADL'nin replikasyonunu inhibe etti. Elde edilen sonuçlar miR-193a'nın apoptozisin pozitif regülatörü olduğunu onaylayarak BVDV replikasyonunun düzenlenmesinde miRNA önemli rol oynadığı hakkında teorik temel oluşturmuştur.

Anahtar sözcükler: *Lentivirus, miR-193a, Apoptozis, BAX, BVDV suş NADL*

INTRODUCTION

Apoptosis plays an important role in regulating cell death and is involved in many important physiological processes, including the normal development of the immune system [1], new and old cell replacement [2], embryonic development [3], and hormone-dependent atrophy [4]. Anti-apoptotic members of the B-cell lymphoma 2 (Bcl-2) family

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include Bcl-2, Bcl-w, and Bcl-xL [5-7], whereas pro-apoptotic effectors include Bcl-2 homologous antagonist/killer (BAK) and Bcl-2-associated X protein (BAX). However, apoptosis may exhibit opposite effects on viral pathogenesis by preventing or enhancing viral transmission from infected cells [8,9]. Bovine viral diarrhea virus (BVDV) strain NADL down-regulates Bcl-2 expression by activating the endoplasmic reticulum (ER) transmembrane RNA-like endoplasmic reticulum kinase [10]. Host cells infected with cytopathic BVDV subsequently undergo unregulated apoptosis [11].

MicroRNAs (miRNAs) are endogenous, ~22-nt, small, noncoding RNAs that negatively regulate gene expression at the post-transcription level by blocking translation of or degrading target mRNAs [12-14]. miR-193a is a critical regulatory factor that targets anti-apoptotic myeloid leukemia cell sequence-1, which mediates cell proliferation and apoptosis [15-20]. Additionally, miR-193a overexpression inhibits 5-bromo-2′-deoxyuridine incorporation and induces activation of caspase-3/7, resulting in apoptotic cell death in A2780 cells [21]. Moreover, transfection of HA22T/VGH hepatocellular carcinoma cells with miR-193a results in increased apoptosis and reduced proliferation, and combined treatment with miR-193a and sorafenib results in proliferation inhibition [22]. Furthermore, ectopic expression of miR-193a leads to reduced cell proliferation, increased differentiation, and induction of apoptosis in acute myeloid leukemia blasts by targeting tyrosine-protein kinase Kit, DNA (cytosine-5)-methyltransferase 3A, cyclin D1, and mouse double minute-2 homolog [23,24].

Previous findings suggested that miR-193a is significantly upregulated in BVDV strain NADL-infected MDBK cells [25]; however, the biological roles of miR-193a in BVDV strain NADL-infected MDBK cells remain unknown. In this study, we observed that miR-193a effectively promoted apoptosis associated with BAX downregulation. These results provided a novel perspective in methods for the prevention of BVDV spreading.

MATERIAL and METHODS

Cells and Plasmids

The MDBK and HEK-293T cell lines were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and grown in Dulbecco's modified Eagle medium (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (Hyclone; GE Healthcare, Pittsburgh, PA, USA). The plasmids of the lentiviral-packing system (pLentiLox 3.7/pLL3.7, CMV-VSVG, pMDLg/pRRE, and pRSV-REV) were supplied by Dr. Bin Jia [26].

Target Prediction

Targets of miR-193a were predicted using different miRNA-target-prediction algorithms, including Microcosm Targets *(http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/genome.pl)* and TargetScan *(http://www.targetscan.org/vert_71/)*.

Lentiviral Production and Identification

To generate miR-193a-overexpressing, inhibiting, and negative-control lentiviruses, pLL3.7-pre-miR-193a, pLL3.7-pre-miR-193a IN, and pLL3.7 empty plasmids were co-transfected into HEK-293T cells using the helper plasmids (CMV-VSVG, pMDLg/pRRE, and pRSV-REV) and a high-efficiency transfection reagent (Cat. No. BW11002; Biowit, Hangzhou, China). At 48 h post-transfection, the lentiviral particles increased in number, and the viral titer was subsequently measured using the Reed-Muench method [27]. Viral titer was determined based on expression of the enhanced green fluorescent protein [28] as visualized by a fluorescence microscope (model TE2000; Nikon, Tokyo, Japan). Lentiviruses overexpressing pre-miR-193a and pre-miR-193a inhibitor were named lv-pLL3.7-pre-miR-193a and lv-pLL3.7-pre-miR-193a IN, respectively, and lentivirus packaged with pLL3.7 empty vector (negative-LV) served as a negative control. Quantitative real-time PCR (qRT-PCR) was employed to monitor the miR-193a expression at 48 h post-transfection. The primers for *Bos taurus* pre-miR-193a, pre-miR-193a inhibitor, miR-193a, and 5S rRNA (serving as an internal control) were designed using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA). The primers used are listed in *Table 1*, and qRT-PCR was performed as described in the proceeding sections.

Dual Luciferase-reporter Assay

The sequences of 3′-untranslated regions (UTRs) and corresponding BAX mutations were cloned into the *Sac*I and *Xho*I restriction sites in the dual luciferase-reporter vector pmirGLO (Promega, Madison, WI, USA). The primers for *BAX* and *GAPDH* (serving as an internal control) are listed in *Table 1*. The relative light units (firefly luciferase/Renilla luciferase) were determined using a dual luciferase-reporter assay system (E1910; Promega) in HEK-293T cells co-transfected with the indicated 3′-UTR and miRNA combinations at 48 h post-transfection as described previously [29].

qRT-PCR

Cells treated with lv-pLL3.7-pre-miR-193a, lv-pLL3.7-pre-miR-193a IN, or negative control (NC) for 48 h were harvested and subjected to total RNA extraction using a total RNA-extraction kit (TIANGEN Biotech, Beijing, China). First-strand cDNA was synthesized from 2 μg of total RNA using a reverse-transcription kit (TIANGEN Biotech) according to manufacturer instructions. The synthesized cDNA was used for qRT-PCR to analyze the mRNA levels of BAX using a LightCycler 480 (Roche, Indianapolis, IN, USA). Data were analyzed using the $2^{-\Delta\Delta Ct}$ method [30,31].

Table 1. *Primers for amplifying and real-time quantitative PCR*

Primers	Primers sequences (5′→3′)
pre-miR-193a-F	CCGTTAACGGGAGCTGAGAGCTGGGTCTTTG
pre-miR-193a-R	CCCTCGAGGGGGGCCGAGGACTGGGA
pre-miR-193a-R-IN	CCCTCGAGGGGGGCCGAGGAACTGGCCTACAAAGTCCCAGTGAACCGACACCTTCATCT
miR-193a-RT	AACTGGCCTACAAAGTCCCAGT
5S rRNA-F	GCCCGATCTCGTCTGATCT
5S rRNA-R	AGCCTACAGCACCCGGTATT
Bax 3′UTR-F	CGGAGCTCTTATGGCATTTTTCAGGGGG
Bax 3′UTR-R	CGCTCGAGCACAATTTAACTCGCCAC
Bax 3′UTR mutation-F	CGGAGCTCTTATGGCATTTTTCAGGGGG
Bax 3′UTR mutation-R	CGCTCGAGCACAATTTAACTCGCCTGACAGGCTGTGTCGGCACTGGTTACCCTCAG
GAPDH-qRT-PCR-F	GTCACCAGGGCTGCTTT
GAPDH-qRT-PCR-R	TGTGCCGTTGAACTTGC
Bax-qRT-PCR-F	CCCCGAGAGGTCTTTTTC
Bax-qRT-PCR-R	TGAGCACTCCAGCCACAA
5′UTR-qRT-PCR-F	TAAACGTGGTAACACAAGCTAGAGATA
5′UTR-qRT-PCR-R	GTCAACCCGTCAACAAGGTAAAG

Western Blot Analysis

MDBK cells were infected with lv-pLL3.7-pre-miR-193a, lv-pLL3.7-pre-miR-193a IN, or NC. At 48 h post-infection, cells were collected and treated with cell lysis buffer (Beyotime, Haimen, China). Total proteins were extracted and subjected to concentration determination using the BCA protein quantification assay kit (TIANGEN Biotech). Western blot analysis was performed as previously described [32]. For Western blots, the primary antibodies used were polyclonal anti-BAX (1:1000; ab32503; Abcam, Cambridge, MA, USA) and monoclonal anti-β-actin (1:2500; AP0060; Bioworld Technology, Louis Park, MN, USA). The horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin G (H+L; 1:5000; BS13278; Bioworld Technology) was used as the secondary antibody. Western blots were analyzed using ImageJ software (v10.2; National Institutes of Health, Bethesda, MD, USA).

Flow Cytometry Analysis

At 48 h post-infection with lv-pLL3.7-pre-miR-193a, lv-pLL3.7-pre-miR-193a IN, or NC, apoptosis was determined by flow cytometry using the BD FACSCalibur system (BD Biosciences, San Jose, CA, USA) after AnnexinV-APC/7-AAD (KGA1025; KeyGEN BioTECH, Nanjing, China) staining according to manufacturer instructions.

Detection of BVDV Replication

To determine the effects of miR-193a on BVDV NADL replication, BVDV NADL replication was measured by qRT-PCR. miR-193a mimics, miR-193 inhibitor, and NC miRNA mimics were purchased from GenePharma (Shanghai, China) and transfected into MDBK cells for 48 h, respectively. The cells were harvested after infection with BVDV strain NADL for 24 h and subjected to total RNA extraction using the Total RNA-extraction kit (TIANGEN Biotech) and reverse transcribed into cDNA. Levels of BVDV NADL mRNA and the presence of the 5′-UTR were determined by qRT-PCR.

Statistical Analysis

SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for single-factor analysis of variance statistics for the experimental data. Data are shown as the mean ± standard error. Asterisks indicate statistical significance as determined by Student's *t* test, with $P<0.05$ or $P<0.01$ indicating significance.

RESULTS

Analysis of Lentivirus-mediated miR-193a Expression

To identify the effects of pre-miR-193a-overexpressing and -inhibiting lentiviruses on miR-193a expression in MDBK cells, vectors with the targeted pre-miR-193a were designed and cloned into pLL3.7 lentiviral vectors, followed by co-transfection of the recombinant plasmids into HEK-293T cells with the helper packing plasmids. Lentiviral particles were collected at 48 h post-transfection *(Fig. 1A)*. The lentivirus was then used to infect MDBK cells, and miR-193a expression was determined by qRT-PCR. As shown in Figure 1B, miR-193a expression increased significantly following transfection with lv-pLL3.7-pre-miR-193a as compared with levels observed in cells transfected with lv-pLL3.7-pre-miR-193a IN or NC (lv- pLL3.7) at 48 h post-infection ($P<0.05$; $P<0.01$). By contrast, lv-pLL3.7-pre-miR-193a IN infection significantly reduced miR-

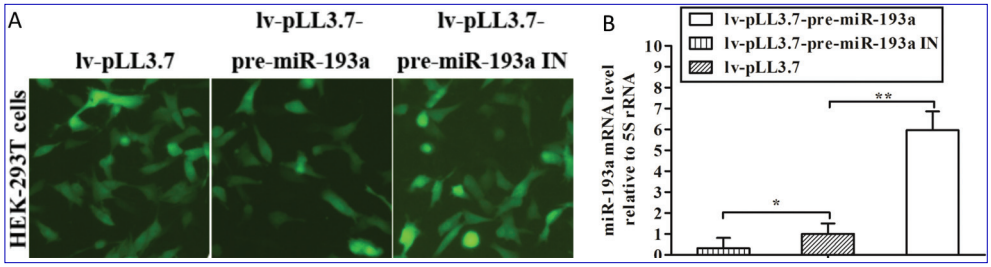

Fig 1. Analysis of lentivirus-mediated miR-193a expression (A) pLL3.7-pre-miR-193a, pLL3.7-pre-miR-193a IN, and pLL3.7 plasmids were transfected into HEK-293T cells along with helper plasmids. At 48 h post-transfection, a large number of positive cells (green fluorescence) were observed by fluorescence microscopy. (B) Analysis by qRT-PCR of miR-193a expression in MDBK cells at 48 h post-infection with lentivirus. Data showed miR-193a expression was significantly upregulated in MDBK cells infected with lv-pLL3.7-pre-miR-193a as compared with cells infected with lv-pLL3.7 at 48 h post-transfection. ** $P<0.01$. By contrast, miR-193a expression was significantly downregulated in MDBK cells treated with lv-pLL3.7-pre-miR-193a IN. $P<0.05$

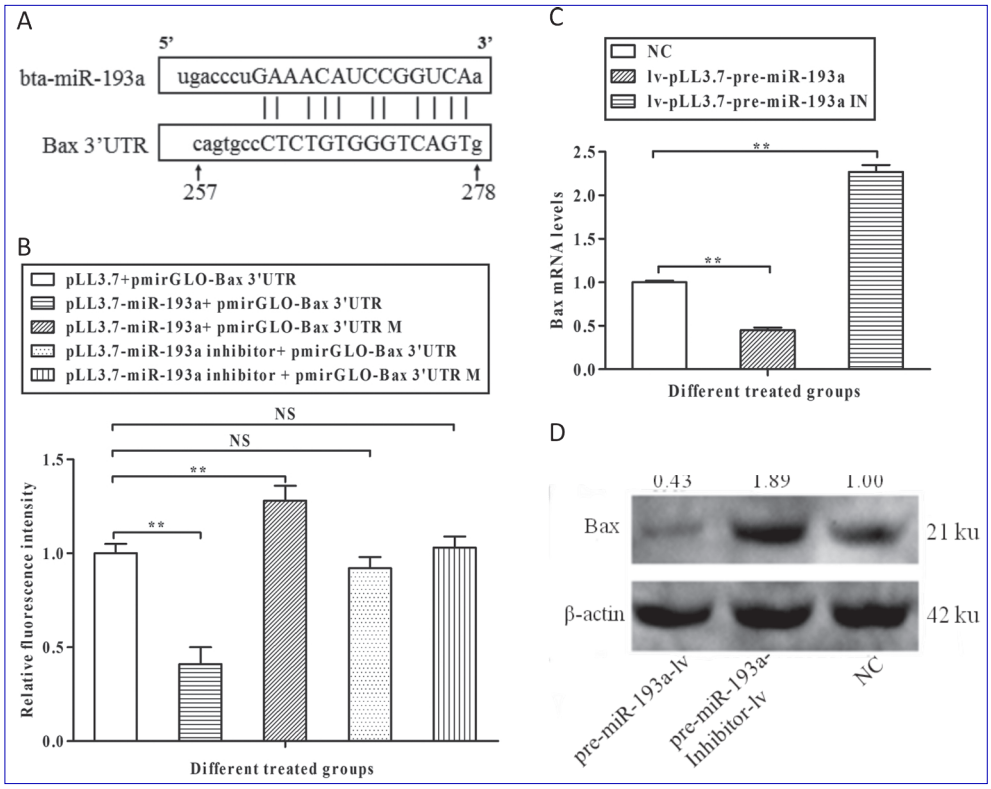

Fig 2. miR-193a targets BAX and downregulates BAX expression. (A) Schematic representation of the 3′-UTR of BAX mRNA showing the miR-193a-binding site. (B) A dual luciferase-reporter assay was performed in HEK-293T cells. Luciferase-reporter plasmids with miR-193a-target sites or corresponding mutants were co-transfected along with pre-miR-193a lentiviral vectors into HEK-293T cells. Fluorescence was visualized at 48 h post-transfection using a dual luciferase-assay system. Data represent the mean ± standard deviation *(error bars)*. ** $P<0.01$ ($n = 3$). (C) BAX mRNA levels were analyzed by qRT-PCR in cells transfected with lv-pLL3.7-pre-miR-193a, lv-pLL3.7-pre-miR-193a IN, or lv-pLL3.7. Data represent the mean ± standard deviation *(error bars)*. ** $P<0.01$ ($n = 3$). (D) Western blot analysis of BAX protein levels in the indicated MDBK cells. NC-lv represents negative-control lentivirus. Western blots were analyzed using ImageJ software ($n = 3$)

193a expression levels ($P<0.01$) *(Fig. 1B)*. These findings suggested that lentivirus-delivered miR-193a exhibited a positive effect on miR-193a expression.

miR-193a Targets the BAX mRNA 3′-UTR and Downregulates BAX Expression

The function of identifying the target sequence of miR-193a is significant to the regulation of BVDV strain NADL replication and apoptosis of infected cells. To determine whether the 3′-UTR of BAX mRNA was the functional target of miR-193a, the potential miRNA-binding site and corresponding mutations to the 3′-UTR were cloned into a dual luciferase-reporter vector (pmirGLO) *(Fig. 2A)*. The pre-miR-193a lentiviral vector and the pmirGLO-BAX 3′-UTR

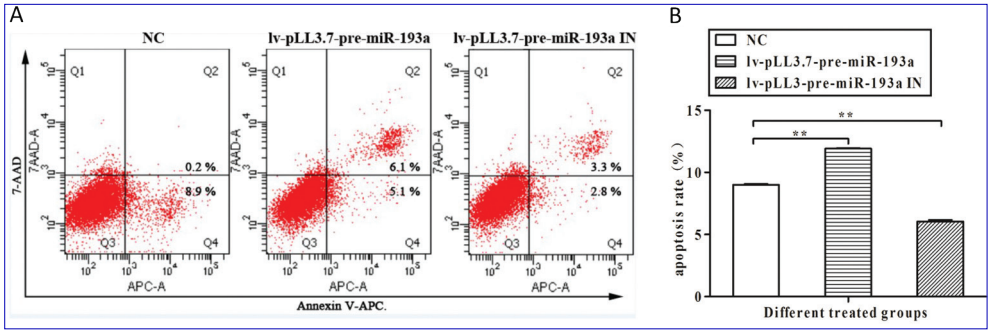

Fig 3. Infection with lv-pLL3.7-pre-miR-193a significantly promotes apoptosis in MDBK cells. (A) Representative flow cytometry images were used to assess apoptotic cells after double staining with Annexin V-APC and 7-AAD ($n = 3$). (B) Data were obtained from three independent experiments and represent the mean ± standard deviation *(error bars)*. ** $P<0.01$

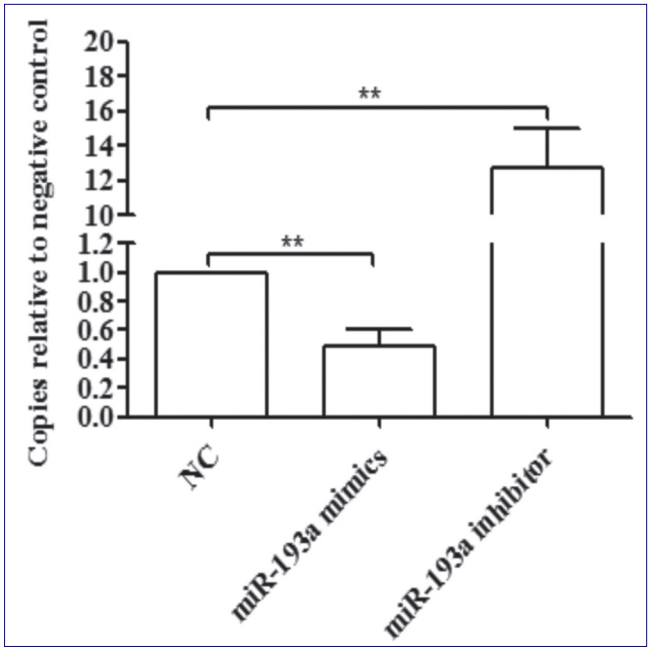

Fig 4. qRT-PCR analysis of BVDV replication in miR-193a-overexpressing and -inhibiting cells. Data represent the mean ± standard deviation *(error bars)*. ** $P<0.01$ ($n = 3$)

reporter plasmids were co-transfected into HEK-293T cells along with an internal control vector pLL3.7 + pmirGLO-BAX 3′-UTR, resulting in significant decreases in relative fluorescence activity. We observed no significant effect by the mutation on the target sequence as compared with the control vector *(Fig. 2B)*. We then determined whether miR-193a overexpression affected BAX expression according to qRT- PCR and western blot analyses. The data indicated that BAX expression was significantly inhibited by lv-pLL3.7-pre-miR-193a infection *(Fig. 2C* and *D)* ($P<0.01$). These findings showed that miR-193a directly targeted the BAX 3′-UTR and downregulated BAX expression.

miR-193a Promotes Apoptosis in MDBK Cells

To determine the relationship between miR-193a and apoptosis, we determined the effects of miR-193a overexpression on MDBK apoptosis by flow cytometry. As shown in *Fig. 3A*, the rate of apoptosis was significantly elevated in cells infected with lv-pLL3.7-pre-miR-193a as compared with the rate observed in cells infected with lv-pLL3.7-pre-miR-193a IN or lv-pLL3.7 (NC) *(Fig. 3B)*. These results suggested that miR-193a overexpression reduced BAX expression and promoted MDBK apoptosis.

Overexpression of miR-193a Reduces BVDV NADL Replication

As shown in *Fig. 4*, compared with the control group transfected with miR-193a mimics, copies of BVDV strain NADL in MDBK cells transfected with miR-193a mimics were significantly reduced ($P<0.01$). By contrast, copies of BVDV NADL were significantly increased in MDBK cells transfected with miR-193a inhibitors ($P<0.01$). These results suggested that miR-193a inhibited BVDV strain NADL replication in MDBK cells.

DISCUSSION

Previous studies reported miR-193a involvement in regulating apoptosis [21,24,33], cell proliferation [34], and differentiation [23,35]. In a previous study, we showed that BVDV NADL-infected MDBK cells exhibited upregulated levels of miR-193a. In this study, we found that miR-193a expression was regulated by miRNA-precursor-expression lentiviruses, with our data confirming that miR-193a directly targeted the 3′-UTR of BAX mRNA, thereby down-regulating BAX expression and leading to increased levels of apoptosis in MDBK cells.

BAX is a member of the Bcl-2 family and is the major pro-apoptotic protein involved in bidirectional regulation of apoptosis [36,37]. BAX overexpression results in apoptosis induction in multiple cell types and plays an important role in the neuronal cell death [38,39]. However, BAX is also a potent inhibitor of neuronal cell death in mice infected with the Sindbis virus and protects newborn mice from neuronal apoptosis [40]. According to previous studies, the Bcl-2 family, including Bcl-2, Bcl-X, and Mcl-1, are important

regulators of programmed cell death and apoptosis [41]. When intracellular Bcl-2 expression promotes apoptosis, if intracellular BAX levels are in excess, These proteins form a homologous structure resulting in a BAX-BAX dimer [42]. There are three types of apoptotic pathways related to caspases, including the mitochondrial cytochrome C pathway, the ER pathway, and the death-receptor pathway [43]. BAX is involved in the caspase-associated death-receptor-signaling pathway. The death-receptor factors fasciclin domain (Fas)1 and Fas transmit signals to the apoptosis-inducing complex, inducing free caspase-8 to form other apoptosis-inducing complexes. The BH3-interacting domain protein (Bid) is transformed into tBid when caspase-8 concentrations are too low. This is followed by activation of multi-domain BAX and BAK variants by tBid, oligomer formation on the mitochondrial outer membrane, and alterations in mitochondrial permeability and release of cytochrome C to activate caspase-9 and caspase-3 and induce apoptosis [44,45]. In a previous study, bta-miR-29b attenuates apoptosis by directly targeting caspase-7 and NAIF1 and suppresses bovine viral diarrhea virus replication in MDBK cells [32]. Here, A similar finding was that we confirmed the presence of a miR-193a-binding site in the BAX mRNA 3′-UTR according to results of a dual luciferase-reporter assay. The resulting downregulation of BAX levels promoted induction of apoptosis in lv-pLL3.7-pre-miR-193a-infected MDBK cells and inhibition of viral replication.

In conclusion, our results indicated that BAX is a critical target of miR-193a and plays a central role in the apoptosis pathway, with BAX downregulation inducing apoptosis. Moreover, miR-193a plays an important role in BVDV strain NADL replication. The mechanism associated with miR-193a-mediated apoptosis and inhibition of BVDV replication involves its interaction with mRNA of the pro-apoptotic gene BAX, induction of apoptosis, and engulfing of apoptotic debris by phagocytes for lysosomal degradation of the BVDV strain NADL virus. Our findings provided a theoretical basis for the important role of miRNA in apoptotic regulation and offer a potential target for the prevention and control of BVDV strain NADL infections.

Acknowledgments

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Conflicts of Interest Statement

The authors have declared no conflicts of interest.

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