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Journal of Cellular Biochemistry 117:84–93 (2016)

Journal of Cellular Biochemistry

Simian Immunodeficiency Virus Impacts MicroRNA-16 Mediated Post-Transcriptional Regulation of mu Opioid Receptor in CEM ×174 Cells

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ABSTRACT

Although the mechanism which regulates transcription in the 5′-UTR of the mu opioid receptor gene (OPRM1) in lymphocytes has been well-studied, a question remains as to whether there is post-transcriptional regulation of OPRM1 gene in lymphocytes. In this study, the authors describe both the role played by miRNAs and the impact of SIVmac239 infection on post-transcriptional regulation of OPRM1 gene in CEM ×174 cells. Our results show that miR-16 is able to bind the target site in the range of 8699–8719 nt from the stop codon in MOR-1 mRNA 3′-UTR and suppress the expression of OPRM1 gene. Mutation of this target site reduces the effect of miR-16. Morphine (1 μM) inhibits the expression of miR-16, and this effect is reversed by the antagonist naloxone. Thus, morphine may up-regulate receptor level by both stimulating OPRM1 gene transcription and stabilizing its mRNA. SIVmac239 infection results in an apparent elevation of miR-16 and gradual reduction of OPRM1 gene expression. The inverse correlation of elevated miR-16 and reduced OPRM1 gene expression under viral loading confirmed the effect of SIVmac239 on post-transcriptional regulation of OPRM1 gene in lymphocytes. The authors conclude that miR-16 is a primary factor in post-transcriptional regulation of OPRM1 gene. SIVmac239 upregulates miR-16 levels and consequently suppresses OPRM1 gene expression. This finding will be helpful for full understanding of the regulatory mechanism of OPRM1 gene in lymphocytes, as well as the synergistic mechanism of HIV infection and morphine addiction in the pathogenesis of AIDS. J. Cell. Biochem. 117: 84–93, 2016.

KEY WORDS: MICRORNAs; MU OPIOID RECEPTOR; LYMPHOCYTES; SIMIAN IMMUNODEFICIENCY VIRUS; POST-TRANSCRIPTION

Although transcriptional regulation of the mu opioid receptor (MOR) has been well demonstrated in neurons, it is definitely different in lymphocytes. For instance, it has reported [Liang et al., 1995; Choe et al., 1998; Xu and Carr, 2000; Choi et al., 2005] that nerve cell lines showed multiple transcription initiation sites. In comparison, our previous work [Wei et al., 2005] showed that lymphocytes have only one transcription initiation site which is located -110 bp from start codon. Unlike neurons, transcriptional efficiency of OPRM1 gene in lymphocytes is at relative low level that leads to difficult detection [Wick et al., 1996]. However, on the other hand, the differential expression of opioid receptor genes in various cells explains diversity in regulating developmental and tissue-specific gene expression [Pan, 2005].

It has been well known that human MOR mediates a variety of physiological and pharmacological effects, while morphine administration in many tissues. It is particularly important that MOR as the primary receptor is responsible not only for opioid tolerance but also for aberrant immune response. It has been documented [Zhang et al., 2012] that the activation of MOR is subjected to influence of microenvironment in lymphocytes. The mechanism underlying expression of OPRM1 gene in lymphocytes [Li et al., 2008] involves functional interaction of transcription factors in the crucial step of transcription initiation.

The signal pathway involving transcription of OPRM1 gene under the influence of morphine treatment has been investigated in lymphocytes, and results have shown that morphine promotes

Conflict Of Interest: The authors declare no conflict of interest.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 81072710, 81370522; Grant sponsor: Beijing Natural Science Foundation; Grant number: 7101006; Grant sponsor: Guangdong Province Natural Science Fund Project; Grant number: S2012010010732; Grant sponsor: Shenzhen Research Fund; Grant number: JCYJ20120616153140835.

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Manuscript Received: 18 March 2015; Manuscript Accepted: 4 June 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 6 June 2015

DOI 10.1002/jcb.25251 • © 2015 Wiley Periodicals, Inc.

phosphorylation of PI3K and AKT, which result in interruption of the interaction of PTEN and p53. At the same time, levels of E2F1, which is a downstream effector of AKT, and the interaction of E2F1 with YY1 are elevated, which leads to initiation of OPRM1 gene transcription. Therefore, the results from the study [Liu et al., 2010] showed that PI3K/AKT signaling is involved in initiation of the OPRM1 gene transcription under morphine stimuli. Infection of lymphocytes with simian immunodeficiency virus (SIVmac239) [Liu et al., 2009] may result in silencing of OPRM1 gene expression in view of methylation of the promoter region which leads to failure of the interaction of Sp1 with YY1 and their binding to the elements.

Although the regulatory mechanism in the 5′-UTR has been well investigated in lymphocytes, the role of 3′-UTR in expression of OPRM1 gene is as yet unexplored. MicroRNAs (miRNAs) are small non-coding RNAs which regulates gene expression by binding mainly to 3′-untranslated region (3′-UTR) of target mRNAs. miRNAs have enriched expressions in the nervous system. There have been plenty of evidences [Kosik, 2006] which showed that miRNAs play fundamental role in neuronal development, plasticity, metabolism, and apoptosis. It has been reported that let-7 [He et al., 2010] and miR-23b [Wu et al., 2008; Wu et al., 2009; He et al., 2010] can regulate the expression of OPRM1 gene and play an integral role in opioid tolerance in the nervous system. However, the roles played by miRNAs in OPRM1 gene expression in lymphocytes, as well as its correlation with disease, have not yet been reported though its role in B cell function has been reviewed [Juan et al., 2013].

Whether miRNAs mediate post-transcriptional regulation of OPRM1 gene in lymphocytes, and the affect of SIVmac239 infection on OPRM1 gene are of particular interest in this study, as HIV-1 replication and susceptibility of the host to HIV infection are directly relevant to morphine and miRNA in immune cells as reported previously [Li et al., 2003; Wang et al., 2011; Hwang et al., 2012; Purohit et al., 2012]. It is hoped that clarification of the mechanism of post-transcriptional regulation of OPRM1 gene in lymphocytes will provide further insights for understanding alterations of the immune system in morphine tolerance, dependence, and addiction, particularly in cases of drug abuse associated with disease, such as AIDS.

MATERIAL AND METHODS

CELL CULTURE AND SIVmac239 INFECTION

The CEM ×174 cell line is a human lymphocytic cell line which is highly susceptible to SIVmac239 infection and routinely utilized investigation of the propagation of SIVmac239. CEM ×174 cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum at 37 °C in a humidified atmosphere with 5% CO_2. In the SIVmac239-infected group, CEM ×174 cells at a density of 3×10^5 cells/ml in 25 cm^2 flasks were treated with a 1/10 volume of SIVmac239 supernatant of free-cells (1×10^3 $TCID_{50}$/ml). Heat-inactivated SIVmac239 was used as the control.

PREDICTION OF miRNAS AND TARGET SITES IN THE 3′-UTR OF MOR-1 mRNA

UCSC (http://genome.ucsc.edu/) and Genebank were used to obtain OPRM1 3′-UTR sequence. Websites PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html), RegRNA (http://regrna.mbc.nctu.edu.tw/html/tutorial.html), RNA22 (http://cbcsrv.watson.ibm.com/rna22.html) [Ritchie et al., 2009], and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) [Rehmsmeier et al., 2004] were used to predict miRNAs targeting the 3′-UTR of MOR-1 mRNA and putative target sites for miR-16.

MORPHINE TREATMENT AND DETECTION OF miR-16

To examine the effects of different concentrations of morphine on miR-16, cells (2×10^5 cells/ml) were added to six well plates and treated with different concentrations of morphine chloride (0.01–100 μM). After 4 h incubation, cells were pelleted and total cellular RNA was extracted from cells with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. In the evaluation of different incubation times under stimulation with morphine, morphine at a concentration of 1 μM was added into the cultures and cells were harvested at various time intervals from 0 to 24 h. Effect of morphine (1 μM) on primary transcripts (pri-miRNA) and precursor miRNA (pre-miRNA), as well as the mature form were observed with qRT-PCR. In the naloxone opiate receptor antagonist blocking assay, the CEM ×174 cells were pre-incubated with 1 μM naloxone for 30 min, and subsequently treated with 1 μM of morphine for 4 h. Routine RNA extraction was then performed. Detection of mature miR-16 was carried out by quantitative real-time PCR after stem-loop reverse transcription (stem-loop qRT-PCR) with primers as listed in Table I. U6 was used as reference control for mature miR-16, and β-actin was used as control for pri/pre-miR-16-1/2.

PLASMID CONSTRUCTS AND SITE-DIRECTED MUTAGENESIS

Based on prediction of potential miRNAs targeting the 3′-UTR of MOR mRNA, genome sequences expressing the precursors of miRNAs, namely, pre-miR-16-1, pre-miR-1301, and pre-miR-215 were amplified and inserted into the BamHI/XbaI restriction sites of pcDNA3.1(+) vector to generate corresponding constructs. Pre-miR-211 was a gift from Dr. Mao, Department of Biochemistry and Molecular Biology, Peking University Health Science Center. All constructs were verified by DNA sequencing. Primers used for all constructs were also listed in Table I.

Genomic DNA from CEM ×174 cells was used as a template for PCR to obtain 10110 bp (DNA sequence from 1–10110) and 3538 bp (DNA sequence from 10095–13633) fragments of OPRM1 gene 3′-UTR, which were inserted into the SpeI /PmeI sites of the pMIR-REPORT containing the firefly luciferase reporter gene to generate pMU1 and pMU2. pMU1 and pMU2 contain 6 and 1 putative miR-16 binding sites, respectively. Six miR-16 binding sites in pMU1 were further constructed to generate pMU-A, pMU-B, pMU-C, pMU-D, pMU-E, and pMU-F.

The mutated construct pMU-Fm (miR-16 target site mutated) was generated by mutating the seed match sequence (5′-TGTTGCTG-3′) of the miR-16 target site in pMU-F to 5′-TATGGATG-3′ by site-directed mutagenesis. The pMU-Fd (miR-16 target site deleted) was constructed to delete the match sequence (5′-TGTTGCTG-3′) with overlapping PCR. MiR-16 mimics and inhibitors were purchased from Shanghai Genephama, Inc (Suzhou, China).

TABLE I. Sequences of Oligonucleotides Used for Constructs of MOR-1 3′-UTR and Pre-miRNAs, Stem-Loop RT, qRT-PCR, and miRNA Assays

	Oligonucleotides sequences	Product size (bp)
Primers for constructs of MOR-1 3′-UTR		
pMU1	Sense: 5′-GGACTAGTCAGGGTCTCATGCCATTC-3′	10110
	Antisense: 5′-GGGTTTAAACGAGCTAGAAGAAACAATACTAGT-3′	
pMU2	Sense: 5′-CGACGCGTATTGTTTCTTCTAGC-3′	3538
	Antisense: 5′-GGGTTTAAACTGTATCAGTTTTAGGTTTTATTTATC-3′	
pMU-A	Sense: 5′-GGACTAGTCTCAGCTCAGAATCCTTATG-3′	444
	Antisense: 5′-CCAAGCTTGGAGAGCTTCTAGGCAT-3′	
pMU-B	Sense: 5′-GGACTAGTCTGGCAATTACCAAGATAAGAT-3′	434
	Antisense: 5′-CCAAGCTTCAGTGGTTAGAGTTGCAGTG-3′	
pMU-C	Sense: 5′-GGACTAGTCTGTGTTCTAAGCGCTTCTG-3′	493
	Antisense: 5′-CGACGCGTCTGCTCCGTGGGAACTTG-3′	
pMU-D	Sense: 5′-GGACTAGTGTACTTCATATGCTCGAAACTAAAG-3′	453
	Antisense: 5′-CGACGCGTGTATTGCTCCCACAGGACAGAAG-3′	
pMU-E	Sense: 5′-GGACTAGTATCTGGTTGTGGTGGCAATG-3′	428
	Antisense: 5′-CCAAGCTTGACTAGCTGGGATTACAGGC-3′	
pMU-F	Sense: 5′-GGACTAGTCCTACCATAGTGATACATGTGG-3′	392
	Antisense: 5′-CCAAGCTTGTCAAGTCATGAGCACAGGTAC-3′	
pMU-Fm	Sense: 5′-GGTTTTGTTTGGGTTTTTTATGGATGTTGCTTGTTTATTTGTTTG-3′	
	Antisense: 5′-CAAACAAATAAACAAGCAACATCCATAAAAAACCCAAACAAAACC-3′	
pMU-Fd	Sense: 5′-CTTAGGGGGATGCTTGTTTATTTGT-3′	
	Antisense: 5′-TAAACAAGCATCCCCCTAAGCCCCT-3′	
Primers for plasmid constructs of pre-miRNAs		
miR-16-1	Sense: 5′-GGGGTACCAAACTTGATGGCA-3′	580
	Antisense: 5′-CGCGGATCCTACTTAAAATCTCCTT-3′	
miR-23b	Sense: 5′-CGCGGATCCGCGTATGGTCATCTCTGGAGTC-3′	446
	Antisense: 5′-GCTCTAGAGCATCAGCTAAGCTCTGCAC-3′	
miR-215	Sense: 5′-CGCGGATCCGCGTGTGGTCACTTGGACTC-3′	444
	Antisense: 5′-GCTCTAGAGCTACATAGGTTGACTGGTCAG-3′	
miR-1301	Sense: 5′-CGCGGATCCGCGGTGGTTCACTGATAGCCTAG-3′	400
	Antisense: 5′-GCTCTAGAGCCCAGGGGTCATTGAGTGTTC-3′	
Synthesized miRNAs		
miRNA mimics N.C	Sense: 5′-UUCUCCGAACGUGUCACGUTT-3′	
	Antisense: 5′-ACGUGACACGUUCGGAGAATT-3′	
hsa-miR-16-5p mimic	Sense: 5′-UAGCAGCACGUAAAUAUUGGCG-3′	
	Antisense: 5′-CCAAUAUUUACGUGCUGCUAUU-3′	
miRNA inhibitor NC	Sense: 5′-UUGUACUACACAAAAGUACUG-3′	
hsa-miR-16-5p inhibitor	Sense: 5′-CGCCAAUAUUUACGUGCUGCUA-3′	
Primers for RT-qPCR of mRNAs		
MOR-1	Sense: 5′-TGACTCAACTGGATGGGCTAAGG-3′	279
	Antisense: 5′-GCAAGGAAAGAGGGATGAAGGA-3′	
β-actin	Sense: 5′-CCAACCGCGAGAAGATGA-3′	97
	Antisense: 5′-CCAGAGGCGTACAGGGATAG-3′	
Primers for RT-qPCR of primary and precursor miR-16		
Pri-miR-16-1	Sense: 5′-GCAATTACAGTATTTTAAGAGATGAT-3′	169
	Antisense: 5'-CATACTCTACAGTTGTGTTTTAATGT-3′	
Pre-miR-16-1	Sense: 5′-GCAGCACGTAAATATTGGCGT-3′ 61	
	Antisense: 5′-CAGCAGCACAGTTAATACTGGAGA-3′	
Pri-miR-16-2	Sense: 5′-GTGTGACAGGGATACAGCAAC-3′ 184	
	Antisense: 5′-GTGCTTAGGTAAATCAAACACCAAG-3′	
Pre-miR-16-2	Sense: 5′-GCAGCACGTAAATATTGGCGT-3′ 61	
	Antisense: 5′-AAGCAGCACAGTAATATTGGTGTT-3′	
Stem-loop primers for reverse transcription of mature miRNAs		
miR-16	Sense: 5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCGCCAAT-3′	
miR-15b	Sense: 5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTGTAAACC-3′	
miR-23b	Sense: 5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGGTAATCC-3′	
miR-215	Sense: 5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGTCTGTCA-3′	
U6	Sense: 5′-CGCTTCACGAATTTGCGTGTCAT-3′	
Primers for quantitative real-time PCR of miRNAs		
miR-16	Sense: 5′-CTTCCAGGTAGCAGCACGTAAATA-3′	80
miR-15b	Sense: 5′-CTTCCAGGTAGCAGCACATCAT-3′	78
miR-23b	Sense: 5′-CTTCCAGGATCACATTGCCAGGG-3′	79
miR-215	Sense: 5′-CTTCCAGGATGACCTATGAATTG-3′	79
universe	Antisense: 5′-TGCGTGTCGTGGAGTC-3′	
U6	Sense: 5′-GCTTCGGCAGCACATATACTAAAAT-3′	89
	Antisense: 5′-CGCTTCACGAATTTGCGTGTCAT-3′	

ELECTROPORATION

CEM ×174 cells were transfected with constructs of pre-miRNA and wild-type/mutated fragments of OPRM1 gene 3′-UTR or miRNA mimic/inhibitor (synthesized by Shanghai GenePharma Co., China) using the ECM 830 square wave electroporation system. Electroporation settings were adjusted to single pulse, 210 v and 30 ms. Immediately prior to transfection, the CEM ×174 cells were harvested in logarithmic growth phase by low speed centrifugation and resuspended in complete growth medium at a density of 1×10^7 cells/ml. Aliquots of cells (100 μl) were briefly preincubated with 1.6 pmol vector plasmids, 100 pmol miRNA mimic, negative control, or 200 pmol miRNA inhibitor individually, which were no more than 20 μl in total. The cell mixture was then transferred to a BTX 4 mm gap cuvette which was placed in the BTX chamber to conduct electroporation. After electroporation, cells were promptly and gently transferred into pre-warmed complete growth medium

and allowed to recover for 12–48 h prior to analysis. Supplementary Figure S1 showed the transfection efficiency under such condition (Supplementary Figure S1).

QRT-PCR, REAL-TIME QUANTITATIVE PCR (QPCR), AND WESTERN BLOTTING

Total RNA was isolated using TRIzol reagent (invitrogen) for both mRNA and miRNA analyses. Reverse transcription was routinely performed according to the manufacturer's instructions. In brief, 2 μg mRNA was used as template for reverse transcription in 20 μl system. 1/10 of the reverse transcription product was used for real-time PCR with control Cq(quantitative cycle) value of 29. Reverse transcription for miRNAs was carried out with stem-loop primers. Relative levels of MOR-1 mRNA were examined using SYBR green qPCR (Vazyme Biotech, Inc, Nanjing, China) and were normalized to levels of β-actin mRNA. Extra primers were designed for amplifying fragment from the end of CDS to the back half of 3′-UTR in order to ensure that MOR mRNA detected in fact have the 3′-UTR sequences targeted by miR-16 (Supplementary Table S1). Supplementary Figure S2 showed the fragments amplified with these primers. For analysis of miR-16 expression, qPCR analyses were conducted with TransStart Green qPCR SuperMix (TransGene Biotech, Inc, Beijing, China) and were normalized to the expression of U6. Primers used in qRT-PCR are listed in Table I. Relative expression was calculated using the $2^{-\triangle\triangle CT}$ method. The specificity of qRT-PCR primers was determined using a melting curve after amplification to show that only a single species of qPCR product was amplified from the reaction. The qPCR and qRT-PCR experiments were repeated at least three times. The relative concentration of MOR-1 mRNA is presented as mean fold-change of samples to control. Western blot was carried out for detection of MOR-1 expression as described previously [Liu et al., 2009]. Primary antibodies against MOR-1 and GADPH were purchased from (Santa Cruz Biotechnology, Santa Cruz, CA).

LUCIFERASE REPORTER ASSAY

Luciferase assays were routinely performed [Wei et al., 2005]. The pRL-SV40 vector (Promega, Madison, WI) containing Renilla luciferase was cotransfected as a reference control. CEM ×174 cells were transfected with all constructs and subjected to a dual luciferase reporter assay 48 h after transfection with electroporation. Firefly luciferase activity was normalized to renilla luciferase activity. The original pMIR-REPORT vector served as a negative control.

STATISTICAL ANALYSIS

Statistical analysis was carried out using SPSS version 13.0, and significance was determined with the two-tailed student's *t*-test. All data are represented as mean ± S.D and $P < 0.05$ is considered statistically significant.

RESULTS

miR-16 INHIBITS EXPRESSION OF OPRM1 GENE

To determine the potential role of miRNAs in regulation of OPRM1 gene expression, four constructs expressing different miRNAs (miR-1301, miR-16, miR-211, and miR-215) were transfected into CEM ×174 cells. Potential binding sites predicted by different websites for these four miRNAs were listed in Supplementary Table S2. Of these four miRNAs, only miR-16 reduced apparently content of MOR protein as shown in Figure 1A. Some miRNAs, such as let-7 and miR-23b, exhibited inhibitory role in neuron did not apparently change the level of MOR-1, indicating the difference between neuron and immune cells (Supplementary Figure S3). This effect was most obvious at 36 and 48 h after transfection with miR-16 mimic (Fig. 1B). The effect of miR-16 on OPRM1 gene expression was further validated by using miR-16 inhibitor, which showed that inhibitor was able to up-regulate OPRM1 gene expression (Fig. 1B). Specificity of inhibitor was verified by observation of reduction of miR-23b following transfection with inhibitor for miR-23b (Supplementary Figure S4). As expected, transfection with miR-16 mimic was able to suppress the expression of MOR-1 mRNA, which was reversed by transfection with miR-16 inhibitor (Fig. 1C). MiRNA-16 inhibitor was able to reduce level of endogenous miR-16 (Fig. 1D). The specificity of inhibitor for miR-16 was further confirmed by observation of the effect of inhibitor on various miRNAs (Fig. 1E).

miR-16 INHIBITS MOR-1 mRNA 3′-UTR ACTIVITY BY BINDING TO ITS TARGET SEQUENCE

To determine whether miR-16 suppresses OPRM1 through specific binding to the putative 3′-UTR target sites, CEM ×174 cells were cotransfected with miR-16 mimic and two different fragments of OPRM1 3′-UTR, namely, pMU1 (containing six potential miR-16 target sites designated as A–F) and pMU2 (containing one potential miR-16 target site) (Fig. 2A). The numbers in parentheses indicate nucleotide sites downstream from OPRM1 stop codon. Co-transfection of miR-16 mimic with pMU1 caused a ∼40% decrease of luciferase activity compared with the control as shown in Figure 2B. The activity of pMU2 was not affected by miR-16 mimic. Co-transfection with miR-16 inhibitor led to an apparent increment in luciferase activity of pMU1, while the activity of pMU2 was unchanged (Fig. 2C). As there are six binding sites for miR-16 in pMU1, we generated six constructs corresponding to these individual sites designated as pMU-A, pMU-B, pMU-C, pMU-D, pMU-E, and pMU-F. Co-transfection of certain individual constructs with miR-16 inhibitor lead to significant increases in the luciferase activity of pMU-F (Fig. 2D). The results indicated that the site F may be the target site of miR-16. The pMU-F containing target site in OPRM1 3′-UTR was conserved in different species (Fig. 2E). Furthermore, the authors mutated or deleted the target sequence of miR-16 in pMU-F to generate pMU-Fm or pMU-Fd (Fig. 2E). Of three constructs, only luciferase activity of pMU-F was significantly declined after cotransfection with mimic (upper panel of Fig. 2F). Mutation or deletion of target sequence resulted in significant reverse of the repressive effect of miR-16. Furthermore, co-transfection of miR-16 inhibitor with pMU-F leads to significant increase in the luciferase activity (lower panel of Fig. 2F). However, the increase of luciferase activity was not observed in pMU-Fm or pMU-Fd group. These results suggest a sequence-specific interaction between miR-16 and its binding site in MOR-1 mRNA 3′-UTR.

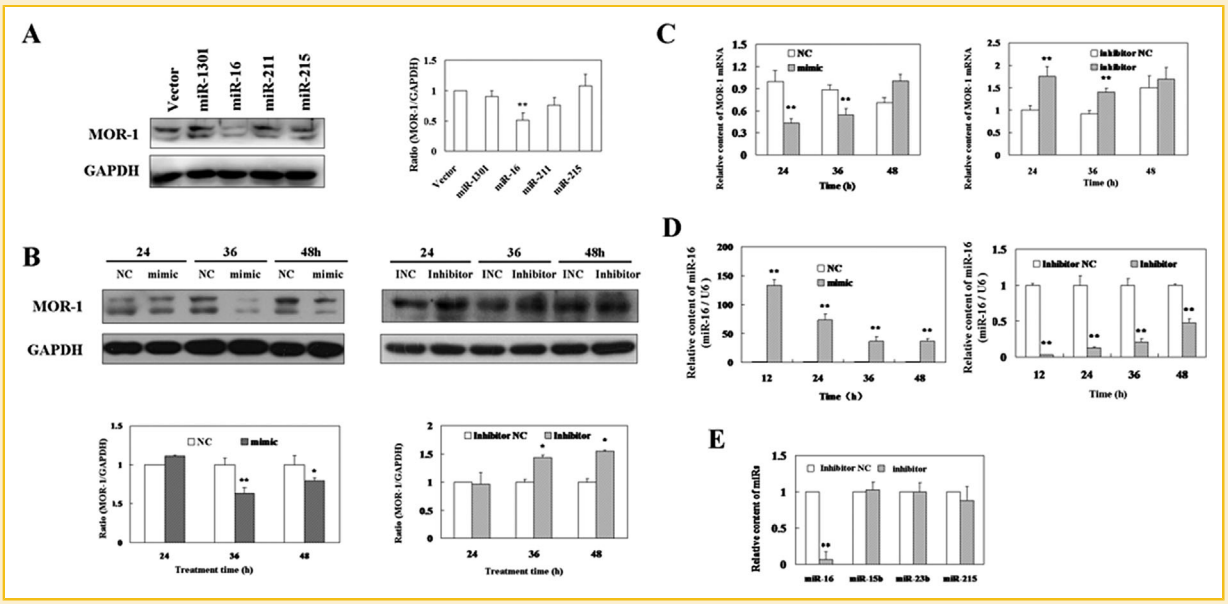

Fig. 1. Effect of miR-16 on expression of MOR-1 in CEM ×174 cells evaluated by western blotting or qRT-PCR. (A) Effect of transfection of constructs expressing various pre-miRNAs on expression of MOR-1. Left panel of image is the densitometric analysis. (B) Effect of miR-16 mimic or inhibitor on MOR-1 protein and (C) MOR-1 mRNA 24, 36, or 48 h after transfection. (D) The effect of hsa-miR-16-5p mimic (100 pmol/1 × 10^6cells) or inhibitor (200 pmol/1 × 10^6cells) on miR-16 levels at 12, 24, 36, or 48 h after transfection. NC: negative control for miR-16 mimic. Inhibitor NC or INC: negative control for inhibitor. The panels below images are the densitometric analysis. These experiments were repeated at least three times. The each image is representative of three independent experiments. Data represent mean ± s.d. of three samples. $^{**}P < 0.01$ as compared with controls.

THE ROLE OF MORPHINE ON EXPRESSION OF miR-16 AND OPRM1 GENE

To verify the impact of morphine on expression of endogenous miR-16, CEM ×174 cells were treated with various concentrations of morphine (0.01–100 μM). The results showed that morphine apparently inhibits expression of miR-16 in the concentration range (Fig. 3A). The morphine (1 μM) had no effect on expression of miR-23b, rather than that in neuron, indicating that the effect of morphine is different in immune cells (Supplementary Figure S5). The inhibitory role of morphine was observed at treatment time 4 h and lasted up to 20 h in the presence of morphine (1 μM), which has proved to be an optimal dose as reported in our previous experiments [Hao et al., 2003] (Fig. 3B).

To identify whether morphine treatment affects the expression of miR-16 at the transcriptional level, the authors examined the expression of primary transcripts (pri-miRNA) and precursor miRNA (pre-miRNA), as well as the mature form by qRT-PCR. As shown in Fig. 3C, morphine (1 μM) exposure may result in obvious suppression of pri/pre-miR-16-1 (transcribed with miR-15a from Chromosome 13) and pri/pre-miR-16-2 (transcribed with miR-15b from Chromosome 3), suggesting a transcriptional regulation of these two clusters by morphine (Fig. 3C).

Morphine-induced suppression of miR-16 expression was reversed by the opiate receptor antagonist naloxone, indicating that the classical and specific mu receptor reaction was involved (Fig. 3D). As expected, morphine treatment (1 μM) may lead to an obvious elevation of OPRM1 gene expression (Fig. 3E). It thus appears that the role of morphine in maintenance of OPRM1 gene expression is achieved by both stimulating transcription of OPRM1 gene and stabilizing its mRNA.

SIVmac239 UPREGULATES miR-16

To determine whether SIVmac239 infection affects expression of OPRM1 gene by post-transcriptional regulation, the authors sampled SIVmac239-infected CEM ×174 cells to analyze for miR-16 and OPRM1 genes. After treatment with SIVmac239 for 24 h, formation of syncytia was observed in CEM ×174 cells (Fig. 4A). SIVmac239 infection led to significant elevation of miR-16 (Fig. 4B), and at the same time led to gradual reduction of OPRM1 gene expression at the mRNA (Fig. 4C) and protein levels (Fig. 4D). The heat-inactivated SIVmac239 (hSIV) as control did not change OPRM1 expression. The role of SIV on miR-16 was rather specific, since no effect on miR-23b was observed after SIV loading (Supplementary Figure S6). It is noted that inhibitory role of SIVmac239 to OPRM1 gene was properly reversed by miR-16 inhibitor, indicating that inhibitory role of SIVmac239 to expression of OPRM1 gene was at least partly eliminated owing to miR-16 inhibition and consequently decreased degradation of MOR-1 mRNA (Fig. 4E). The inverse correlation of elevated miR-16 and reduced OPRM1 gene under viral loading implied strongly the effect of SIVmac239 on post-transcriptional regulation of OPRM1 gene in CEM ×174 cells.

DISCUSSION

OPRM1 gene is expressed constitutively and at high levels in defined neurons. These receptors are normally expressed in lymphocytes but at lower levels. This is of particular interest as MOR is known to mediate a variety of the physiologic and pharmacologic effects of morphine in lymphocytes via regulatory mechanisms which differ

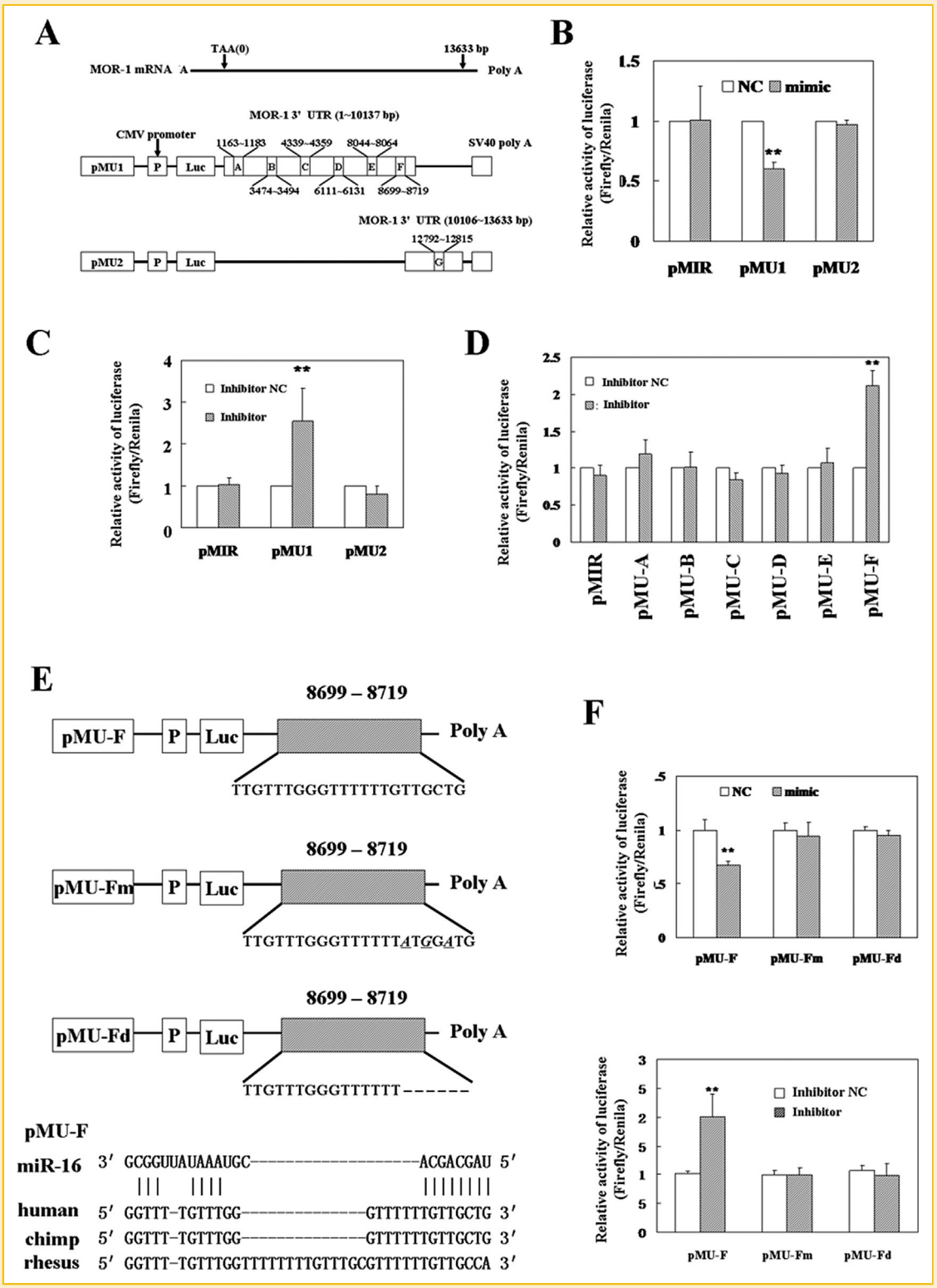

Fig. 2. Determination of miR-16 binding sites in the 3′-UTR of MOR-1 mRNA in CEM ×174 cells. (A) Schematic shows potential binding sites of miR-16 in pMU1 and pMU2 constructs. Luciferase activity after transfection of pMU1 or pMU2 constructs and miR-16 mimic (B) or inhibitor (C). (D) Luciferase activity after transfection of constructs expressing different binding sites (pMU-A, pMU-B, pMU-C, pMU-D, pMU-E, or pMU-F) of miR-16 in the 3′-UTR of MOR-1 mRNA and inhibitor. (E) A schematic plot of the luciferase constructs pMU-F, pMU-Fm, and pMU-Fd (upper panel). Homology analysis of miR-16 in human, chimp, and rhesus (lower panel). Italic and underlined letters in schematic for pMU-Fm represent mutated nucleotides. Dashes in pMU-Fd represent deleted nucleotides. (F) Luciferase activity after co-transfection of pMU-F, pMU-Fm, or pMU-Fd and mimic (upper panel) or after co-transfection of inhibitor and pMU-F, pMU-Fm, or pMU-Fd (lower panel). These experiments were repeated at least three times. Data represent mean ± s.d. of three samples. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with controls.

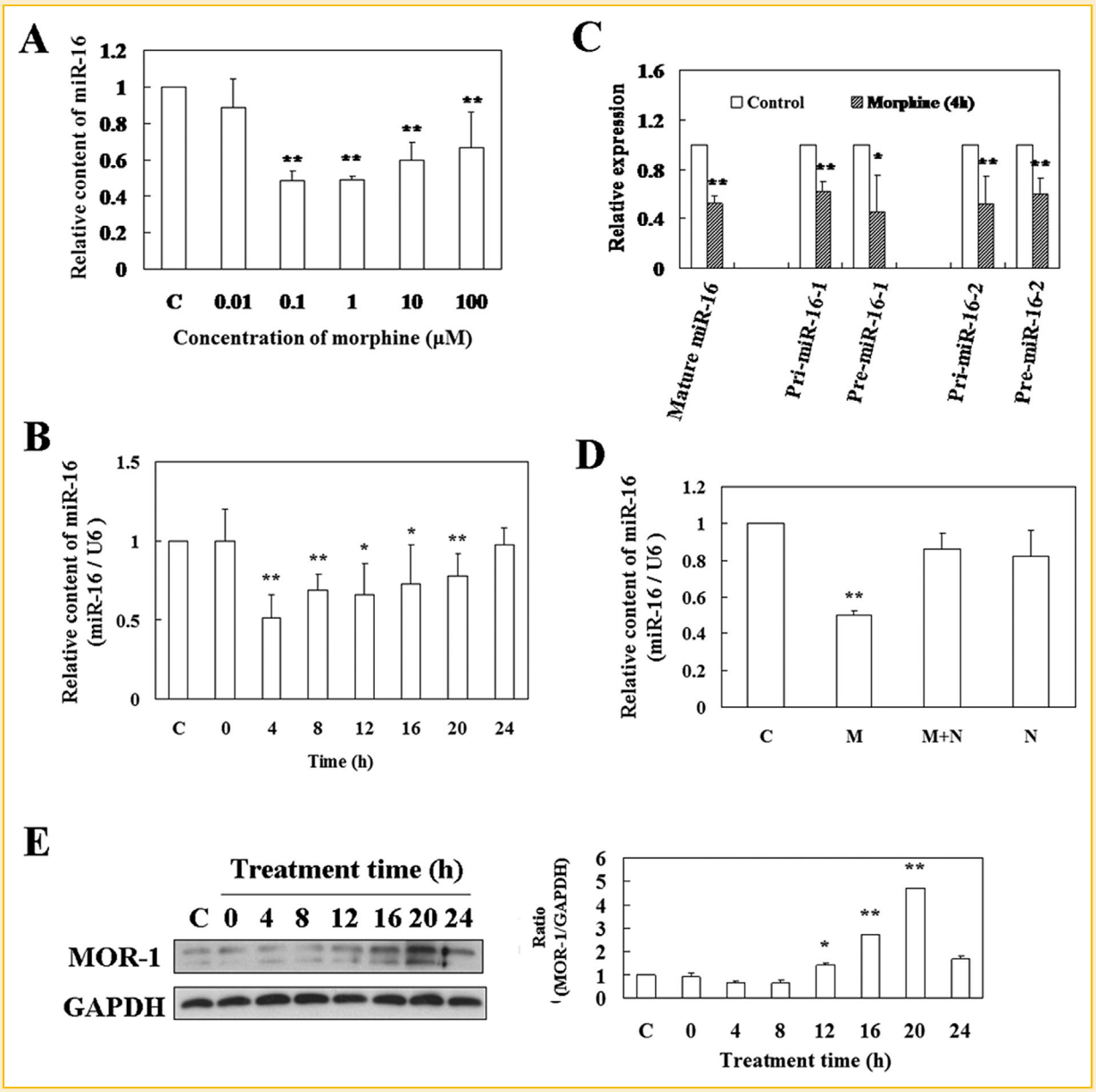

Fig. 3. Effects of morphine on miR-16 expression in CEM ×174 cells. (A) Effects of different concentrations of morphine (0.01–100 μM) on expression of miR-16 at treatment time 4 h. (B) Effect of morphine (1 μM) on expression of miR-16 at different time points. (C) Effects of morphine (1 μM) on pri-, pre-, and mature miR-16. (D) Blockade of the effect of morphine by naloxone on expression of miR-16. These experiments were repeated at least three times. M: 1 μM morphine treated group; N: 1 μM naloxone treated group; MN: morphine and naloxone treated group. (E) Western blotting analysis of the effect of morphine treatment on expression of OPRM1 gene. The image is representative of three independent experiments. The panels next to images are the densitometric analysis. Data represent mean ± s.d. of three samples. $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with controls.

from those in neural cells. Changes in expression of OPRM1 gene in lymphocytes which result from opioid abuse have been shown to be of importance in the pathologic progression of immune disorders such as AIDS [Xu et al., 2004].

Over the past decade, there has been a substantial increase in our understanding of the mechanism underlying transcriptional initiation of OPRM1 gene in lymphocytes [Wei et al., 2005; Li et al., 2008; Liu et al., 2009]. However, all evidence for understanding of this mechanism in lymphocytes was garnered through study of the role of the 5′ regulatory region of OPRM1 gene. Post-transcriptional regulation of OPRM1 gene in neurons has been studied. It has been shown that a number of miRNAs are involved in regulation of OPRM1 gene expression. For example, let-7 miR was identified as a mediator for the translocation and sequestering of OPRM1 gene mRNA to P-bodies, leading to translation repression. These results [He et al., 2010] suggest that let-7 plays an integral role in opioid tolerance. In other studies [Wu et al., 2008, 2009], it was found that miR-23b interacts with MOR 3′-UTR via a K box motif in SH-SY5Y and mouse P19 cells. However,

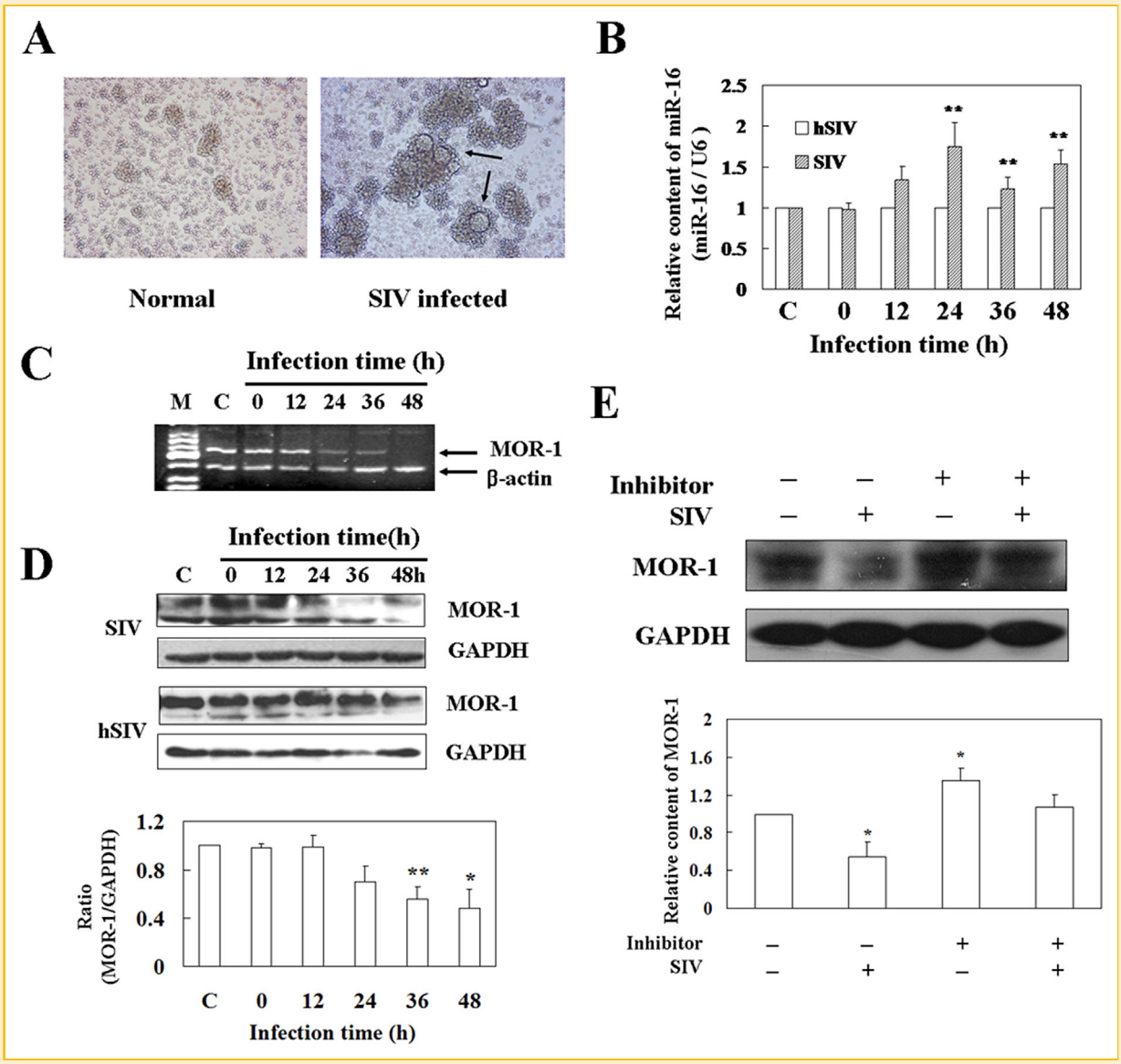

Fig. 4. The effects of SIVmac239 infection on expression of OPRM1 gene and miR-16 in CEM ×174 cells. (A) Cells were observed under the microscope after 48 h incubation with SIVmac239. Arrows indicate syncytia formation. (B) Effects of SIVmac239 infection on miR-16 expression at different time points. C: control; SIV: SIVmac239-infected cells; hSIV: cells treated with heat-inactivated SIVmac239. (C) Effects of SIVmac239 infection on expression of OPRM1 gene at different time points analyzed by qRT-PCR, and (D) western blotting. (E) Western blotting analysis of the effect of SIVmac239 infection associated with transfection of miR-16 inhibitor on OPRM1 gene expression. The panels below images are the densitometric analysis. The each image is representative of three independent experiments.

transfection with miR-23b or let-7 did not change the expression of OPRM1 gene, indicating the difference between neuron and immune cells. A recent study [Ni et al., 2013] showed that the miR-134 expression level is inversely related to OPRM1 gene expression. OPRM1 gene expression in SH-SY5Y cells is up-regulated after inhibition of miR-134, indicating that OPRM1 is a target of miR-134. Study [Zheng et al., 2012] showed that morphine is able to stimulate change in microRNA expression which may be relevant to OPRM1 gene transcription and opioid tolerance. Although previous study [Dave and Khalili, 2010] showed that morphine treatment led to slight increase in human monocyte-derived macrophages, binding of opioids to opioid receptors have discrepant impact in different immune cells [Martin-Kleiner et al., 2006].

With increasing understanding of OPRM1 gene post-transcriptional regulation in the nervous system, the mechanism underlying analogous regulation in lymphocytes seems insufficiently understood and remains to be worked out as it may be completely different from that in neurons. With respect to characteristics of OPRM1 gene expression in human lymphocytes, particularly during the course of viral infection, the regulatory detail will be one of the least well-understood aspects of this mechanism. The data the authors present here imply that miR-16 is the primary miRNA in post-transcriptional regulation of OPRM1 gene in

lymphocytes which is distinctly different from the nervous system. MiR-16 achieves its elaborate regulation of OPRM1 gene expression by binding to one of its elements in 3′-UTR region, and this was confirmed by mutation of bases in the seed sequence. It appears that morphine promotes expression of OPRM1 gene and suppresses the expression of miR-16, and as a consequence, the level of MOR-1 is maintained.

AIDS has been considered to be the major cause of mortality among users of injected drugs. Dysfunction and eventual death of lymphocytes under the influence of viral loading is a hallmark of this disease′s pathology. Studies [Kumar et al., 2004; Hauser et al., 2005; Hauser et al., 2006] showed that opioids such as morphine, which are significant predisposing risk factors, can exacerbate the immunopathogenesis of HIV infection. To clarify the role of opiate exposure in the progression of HIV-1/AIDS, researchers [Chuang et al., 2005] have used nonhuman primate models of morphine and simian immunodeficiency virus (SIV) infection. Studies performed in the rhesus macaque model demonstrated that SIVmac239 infection may influence the expression of OPRM1 gene and contribute to lymphocyte dysfunction. Previous study [Liu et al., 2009] demonstrated that SIVmac239 infection may result in aberrant methylation of OPRM1 gene promoter which leads to failure of binding of transcription factors to their elements, and consequently leads to silencing of the gene. Apart from the 5′-UTR OPRM1 gene regulation, post-transcriptional regulation is also an important factor which leads to repression of the gene. Using SIV as a model of HIV, the relationship of miRNAs with AIDS was evaluated [Witwer et al., 2011; Winkler et al., 2012; Sisk et al., 2013]. However, few were involved in miR-16. The data in the current study show that miR-16 is up-regulated following SIVmac239 loading, which apparently accelerates degradation of MOR-1 mRNA. These findings will be helpful in understanding the synergistic role of HIV infection and morphine addiction in the pathogenesis of AIDS.

Previous work [Hao et al., 2003] has shown that morphine alleviates the damage of lymphocytes by SIV infection. This is particularly true in early stages of SIVmac239 infection. It was interesting to note that morphine exposure in early stages of immunodeficiency virus infection may properly improve cell survival [Xu et al., 2004]. Studies [Donahoe and Vlahov, 1998] by others using SIV to investigate the effect of morphine on susceptibility of rhesus macaques have suggested that morphine has a protective role against virus infection. AIDS progression in opiate-dependent monkeys was reported to be slower than expected. Previous studies [Roy et al., 1997; Yossuck et al., 2008] showed that reduced levels of OPRM1 gene expression due to SIV infection may relieve morphine stimuli resulted cellular impairment.

The present results together with the previous work on regulation of the 5′-UTR showed that down regulation of OPRM1 gene expression through synergism of transcription and post-transcriptional gene regulation during the course of viral infection will lead to incapacity of lymphocytes to morphine stimuli and eventually to alleviated death of virus-infected cells. Nevertheless, short-term exposure of SIV-infected lymphocytes to morphine may only temporarily alleviate the progression of SIV-induced apoptosis, as the longer survival of infected cells will favor the replication and propagation of viral particles, leading eventually to the acceleration of cell death [Chuang et al., 2005]. The change in OPRM1 gene expression is, therefore, still a factor contributing to accelerated pathologic progression of AIDS. This result further clarifies the mechanism of OPRM1 gene expression in lymphocytes. The impact of the interaction of SIV infection and morphine on cellular function has recently been addressed [Perez-Casanova et al., 2007; Perez-Casanova et al., 2008; Banerjee et al., 2011].

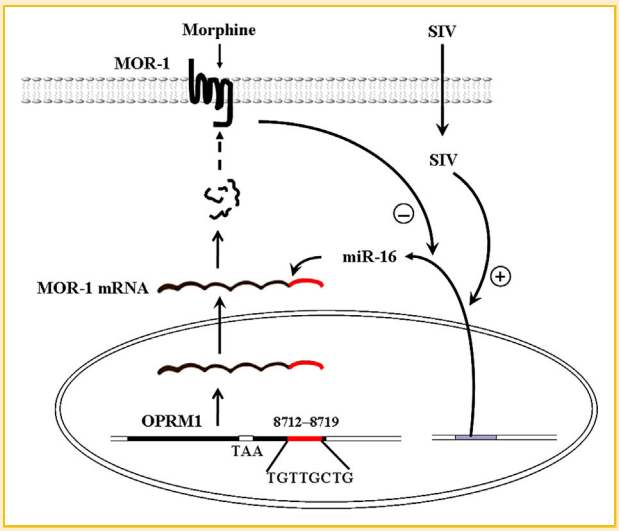

Fig. 5. Schematic model of the possible role of miR-16 in post-transcription regulation of the OPRM1 gene of lymphocytes.

In conclusion, our data address the role of miR-16 in post-transcriptional regulatory mechanisms which is schematically outlined in Figure 5. SIV infection upregulates expression of miR-16 and as a result further inhibits expression of OPRM1 gene, which is one cause leading to repression of OPRM1 gene in SIV-infected lymphocytes. The reduced ability of lymphocytes to response to morphine due to silencing of OPRM1 gene during viral loading will temporarily favor survival of infected cells and in turn cause braking of AIDS progression. This analysis of this mechanism may provide new insights for understanding of the effect of opioid addiction on AIDS progression.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No. 81072710 and 81370522) and the Beijing Natural Science Foundation (No. 7101006), the Guangdong Province Natural Science Fund Project (No. S2012010010732), and the Shenzhen Research Fund (No. JCYJ20120616153140835).

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