miR-183 potentially inhibits NF-κB1 expression by directly targeting its 3’-untranslated region

Fushen Sha1,2, Shuangxing Wu1,2, Hui Zhang1,2*, and Xuemin Guo1,2,3*

1Zhongshan School of Medicine, Institute of Human Virology, Sun Yat-Sen University, Guangzhou 510080, China
2Key Laboratory of Tropical Disease Control, Sun Yat-Sen University, Ministry of Education, Guangzhou 510080, China
3Department of Biochemistry, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou 510080, China
*Correspondence address. Tel: +86-20-87330101; Fax: +86-20-87332588; E-mail: xguo2005@yahoo.com (X.G.); zhangh92@mail.sysu.edu.cn (H.Z.)

Nuclear factor-κB (NF-κB) is an important transcription factor. While the NF-κB signaling pathway is modulated by many microRNAs (miRNAs), very few have been reported to target NF-κB1 gene directly. In this study, we used multiple miRNA target prediction programs to predict miRNAs with putative NF-κB1 3’-untranslated region (UTR) binding sites. miR-183 was strongly implicated and experimentally validated by reporter assays. The results showed a reduced expression of the NF-κB1 3’UTR containing luciferase vector by ~30%, which was comparable to the reduction by miR-9 (the only known miRNA targeting the NF-κB1 3’UTR). Mutagenesis of the miR-183 seed region binding sequence in the NF-κB1 3’UTR abolished the inhibitory effect of miR-183, as noted by the NF-κB1 3’UTR-containing reporter. Moreover, similar to miR-9, miR-183 could down-regulate the expression of the reporter driven by NF-κB promoter to some degree, suggesting that miR-183 might negatively regulate the endogenous NF-κB1. Overall, our data provide computational and experimental evidence that NF-κB1 is a potential target of miR-183.

Keywords NF-κB1; microRNA; computational prediction; reporter assay

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Introduction

Activation of nuclear factor-κB (NF-κB) signaling pathway is a ubiquitous stress response to a wide variety of stimuli, resulting in the expression of a vast array of NF-κB-dependent genes [1,2]. NF-κB family, an important transcription factor, comprises five related members, RelA (p65), RelB, c-Rel, and the precursor proteins NF-κB1 (p105) and NF-κB2 (p100), which are processed into p50 and p52, respectively. These proteins play distinct gene regulatory roles by forming different homodimers and heterodimers, with p50-RelA heterodimer being the most abundant form [3]. In most cases, NF-κB is retained in the cytoplasm by binding to inhibitors of κB (IκB). Once a cell receives stimuli, NF-κB signal transduction is triggered, resulting in activation of IκB kinase (IKK) complex and subsequent degradation of IκB. These events allow translocation of the released NF-κB proteins into nucleus and transcription activation of a set of genes containing κB sites [3,4]. More than 150 NF-κB-responsive genes have been identified in many types of cells responding to different stimuli [2,5], implying a required rapid signal termination to maintain cell homeostasis. It is very clear that the NF-κB pathway is tightly controlled by both positive and negative feedback regulations to generate coordinated signals essential for cell viability [6]. The NF-κB pathway, in particular, is under the tight control of negative autoregulatory feedback loops at multiple levels, such as the NF-κB–IκB loop affecting the duration of NF-κB binding in the nucleus, and the loop of NF-κB and tumor necrosis factor-receptor (TNFR)-associated factor (TRAF)-associated factor (TRAF) affecting TNFR activity [5,7]. Despite 28 years of research centered on NF-κB since its discovery, additional regulators that either activate or repress NF-κB or its target genes remain to be identified and elucidated.

MicroRNAs (miRNAs) are emerging as a type of new and important regulators of NF-κB pathway, which are evolutionarily conserved 20–23 nucleotides non-coding RNAs that regulate gene expression at post-transcriptional level primarily by binding to mRNA 3’-untranslated region (UTR) [8,9]. Many miRNAs have been shown to attenuate NF-κB signaling by targeting different NF-κB-activating proteins [10]. For examples, miR-199a inhibits NF-κB signaling by targeting IκKB in ovarian carcinoma cells [11]; miR-223, miR-15, and miR-16 down-modulate the signaling by targeting IKKa during the monocyte-macrophage differentiation [12]. Currently, MiR-9 is the only known miRNA that directly targets the NF-κB1 3’UTR in monocyte, whose expression leads to the reduction of NF-κB1/p105 protein level.
miR-183 targeting NF-κB1 3’UTR

Given that a gene may be targeted by multiple miRNAs [14], and that NF-κB is the central orchestrator of both inflammation and immune response [15], we speculated that NF-κB expression might be co-operatively regulated by multiple or a cluster of miRNAs. Here, we screened the NF-κB1 3’UTR sequence for possible miRNA binding sites using a collection of online microRNA target prediction programs, and identified miR-183 as a potential regulator of NF-κB1 by targeting its 3’UTR.

Materials and Methods

microRNA prediction
Six online microRNA and microRNA target prediction programs were used to screen for microRNAs with potential binding sites in the 3’UTR of NF-κB1, including RegRNA (http://regrna2.mbc.nctu.edu.tw/), miRANAda (http://www.microrna.org/microrna/home.do), TargetScan (http://www.targetscan.org/), EIMMo (http://www.mirz.unibas.ch/EIMMo2/), microRNAMAP (http://mirnamap.mbc.nctu.edu.tw/), and PicTar (http://pictar.mdc-berlin.de/). The predicted micorRNAs with a high integrated ranking were chosen for further experimental analysis.

Plasmid construction
Total RNAs were extracted from HEK293T cells and reverse-transcribed using oligo(dT) primers. The resulting first-strand cDNA was used as template for the amplification of NF-κB1 3’UTR by polymerase chain reaction (PCR) with the primers 5’-CACCTCGAGCTGTGCAACATTCCCA-3’ and 5’-ACGCAGCCCTGAGTCTCAATTTGCT-3’ (restriction sites underlined). The amplicon was digested with XhoI and NotI and inserted downstream of the Renilla luciferase (R-luc) coding region in the psiCHECK2 vector (Promega, Madison, WI), with the resulting plasmid named luc-3’UTRNF-κB1-WT. The co-expressed firefly luciferase (F-luc) driven by the HSV-TK promoter in the psiCHECK2 vector was served as an intraplasmid transfection normalization reporter. To map the sequences responsive to miR-183, NF-κB1 3’UTR, from the 5’-end to the 3’-end, was divided into five overlapping fragments via PCR with luc-3’UTRNF-κB1-WT as the template, the primers 5’-CACCTCGAGCTGTGCAACATTCCCA-3’ and 5’-ACGCAGCCCTGAGTCTCAATTTGCT-3’ for D1 (1–170 bp), 5’-CACCTCGAGCTGTGCAACATTCCCA-3’ and 5’-ACGCAGCCCTGAGTCTCAATTTGCT-3’ for D2 (121–310 bp), 5’-CACCTCGAGCTGTGCAACATTCCCA-3’ and 5’-ACGCAGCCCTGAGTCTCAATTTGCT-3’ for D3 (241–410 bp), 5’-CACCTCGAGCTGTGCAACATTCCCA-3’ and 5’-ACGCAGCCCTGAGTCTCAATTTGCT-3’ for D4 (361–550 bp), 5’-CACCTCGAGCTGTGCAACATTCCCA-3’ and 5’-ACGCAGCCCTGAGTCTCAATTTGCT-3’ for D5 (481–709 bp). All of the upstream primers contained XhoI site (underlined) and downstream primers contained NotI site (underlined). After digestion, the fragments were inserted into the XhoI and NotI sites of psiCHECK 2 vector, and the resulting plasmids were named luc-3’UTRNF-κB1-D1, luc-3’UTRNF-κB1-D2, luc-3’UTRNF-κB1-D3, luc-3’UTRNF-κB1-D4, and luc-3’UTRNF-κB1-D5. The putative miR-183-binding site at the 3’ UTR of NF-κB1 was mutated by site-directed mutagenesis with luc-3’UTRNF-κB1-WT as the template and the oligonucleotides 5’-TGAGAGTTCATGCATGTGGGATTTAAAA AAAAGGCATA-3’ and 5’-TATGCCTTTTTTTTTAAAAACC ACATCAAGTGACTCTCA-3’ (bases introducing the mutations into the predicted seed sequence for miR-183 are italicized and underlined) as the primers, and the resulting plasmid was designated as luc-3’UTRNF-κB1-mut.

Transfection and luciferase reporter assay
HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Grand Island, USA) supplemented with 10% fetal bovine serum at 37°C. Before transfection, HEK293T cells were seeded into 24-well plates and grown to 80% confluence. The resulting NF-κB1 3’UTR-containing plasmids (200 ng) were then transfected either alone or with 6 pmol synthesized microRNA mimics (Ribobio, Guangzhou, China) using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instruction. A reporter vector containing five copies of NF-κB response elements that drive transcription of firefly luciferase (pGL4.32, Promega) was used to detect the effect of miR-183 on NF-κB1 expression. The synthetic miRNA mimics (50 pmol) were co-transfected with 500 ng pGL4.32 together with 50 ng pRL-TK (a control vector expressing Renilla luciferase, Promega) into HEK293T cells using Lipofectamine 2000 according to the manufacturer’s instruction, followed by 10 ng/ml TNF-α treatment to induce the activation of NF-κB. Cell lysates were prepared using passive lysis buffer (Promega) at 48 h post-transfection and the luciferase activity was measured using the dual-luciferase assay kit (Promega) according to the manufacturer’s instruction.

Statistical analysis
Data were expressed as mean ± standard deviation (SD). Statistical significance was determined using the Student’s t-test, and a value of P < 0.05 was considered statistically significant.

Results
Prediction of miRNAs targeting the NF-κB1 3’UTR
In silico analysis using six different miRNA prediction programs, miRANAda, TargetScan, EIMMo, microRNAMAP, RegRNA, and PicTar, revealed a large number of miRNAs with putative NF-κB1 3’UTR binding sites. The probability

of these predicted miRNAs was ranked based on their occurrence frequency and ranking in different algorithm programs, and the top 20 of them are listed in Table 1. miR-9 ranked first and was the only miRNA predicted by all above six programs simultaneously, and the sole miRNA predicted by PicTar.

The NF-κB1 3’UTR was targeted by miR-183

To experimentally verify whether the predicted miRNAs have regulatory roles on NF-κB1, a reporter assay system was generated with the NF-κB1 3’UTR fused downstream of R-luc coding sequence (Fig. 1A). HEK293T cells were transfected with the resulting reporter construct alone or with the respective highly ranked miRNA mimic (Table 1), including miR-9, -183, -16, -195, -129, -15a, and -15b, in which, miR-9 was used as a positive control [13]. Compared with the relative luciferase signal of the cells transfected with the reporter alone, the addition of miR-9 and miR-183 inhibited the reporter expression by $\approx 22\%$ and $\approx 32\%$, respectively (Fig. 1B). In contrast, the other five miRNAs changed little or up-regulated the reporter expression to some degree. These results indicated that miR-183 could down-regulate the expression of NF-κB1 3’UTR-containing reporter, suggesting that miR-183 might target NF-κB1 3’UTR directly, similar to miR-9.

### Table 1. Ranking of top 20 miRNAs targeting NF-κB1 predicted by *in silico* analysis

<table>
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<tr>
<th>Integrated rank herein</th>
<th>Name of miRNA</th>
<th>Ranking$^a$</th>
<th>RegRNA</th>
<th>miRANDA</th>
<th>TargetScan</th>
<th>EIMMo</th>
<th>microRNAMAP</th>
<th>PicTar</th>
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NA: not available.

$^a$Total miRNAs predicted by an algorithm were sorted based on their scores from high to low. A smaller rank number indicated a higher probability.

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Figure 1. The effect of miR-183 on the expression of NF-κB1 3’UTR-containing reporter

(A) The reporter system was constructed by inserting the 3’UTR of NF-κB1 into the 3’-terminus of *Reinilla* luciferase coding sequence (CDS), driven by the SV40 promoter. The resulting construct was designated as Luc-3’UTRNF-κB1-WT. (B) Luc-3’UTRNF-κB1-WT was transfected into HEK293T cells alone or with different miRNA mimics. The activities of dual reporters, R-luc and F-luc, were measured 48 h post-transfection. The ratio of R-luc to F-luc was set as 1 in the cells transfected the reporter alone (marked with –). Results are expressed as mean ± SD of three independent experiments. *$P < 0.05$. 

A possible interaction of miR-183 with NF-κB1 3’UTR was further estimated by mapping the interaction site. All the used online programs, except for PicTar, pointed to the same
miR-183 targeting NF-κB1 3’UTR

Figure 2. miR-183 interacted with the predicted binding sequences in the 3’UTR of NF-κB1 (A) The 3’UTR sequence of NF-κB1 was divided into five overlapped fragments and cloned downstream of Renilla luciferase in the psiCHECK2 vector. The numbers indicate the positions of the ends of the fragments in the NF-κB1 3’UTR. The predicted miR-183 binding site is labeled with a black rectangle. The resulting constructs were transfected into HEK293T cells alone (marked with -) or with miR-183 mimics with luciferase activities measured at 48 h post-transfection. (B) The putative interacting sequences between miR-183 and NF-κB1 3’UTR are shown. The NF-κB1 3’UTR mutation shown in bold abolished the complementarity with miR-183 seed region and was cloned into the same reporter vector as the NF-κB1 3’UTR. The predicted miR-183 binding site is shown in black. These findings indicated that miR-183 could inhibit the transcription activity mediated by NF-κB, suggesting a reduction in active NF-κB protein level.

Figure 3. The effect of miR-183 on the reporter driven by NF-κB promoter (A) The reporter vector pGL4.32 contains five NF-κB responsive element copies (NF-κB-RE) in the promoter region of firefly luciferase (F-luc) gene. (B) The pGL4.32 vector was transfected into HEK293T cells along with a control vector, pRL-TK, and the mimics of miR-183, or miR-9 or miR-ctrl. TNF-α was added to a final concentration of 10 ng/ml immediately after the transfection to stimulate F-luc expression. The luciferase activity was measured at 48 h post-transfection and the ratio of F-luc to R-luc was calculated. The ratio in the cells transfected with the miR-ctrl was set as 1. **P < 0.01.

miR-183 down-regulated the expression of the reporter driven by NF-κB promoter

The effect of miR-183 on endogenous NF-κB1 protein was estimated utilizing a reporter system driven by NF-κB promoter, pGL4.32 (Fig. 3A). The mimics of control miRNA (miR-ctrl), miR-183, and miR-9 were individually transfected into HEK293T cells along with pGL4.32 and pRL-TK, followed by TNF-α treatment to induce the strong expression of F-luc driven by NF-κB promoter. Similar to miR-9, miR-183 inhibited the luciferase activity relative to the miR-ctrl (Fig. 3B). These findings indicated that miR-183 could inhibit the transcription activity mediated by NF-κB, suggesting a reduction in active NF-κB protein level.

Discussion

As the response activated by NF-κB signaling is so strong, multiple negative regulatory mechanisms have evolved to attenuate NF-κB pathway by affecting the expression, localization, or modification of NF-κB or NF-κB-activating proteins at different levels [5,7]. In this study, we provided computational and experimental evidence for the identification of miR-183 as a new regulator of NF-κB signaling. Our data showed that miR-183 could inhibit the expression of NF-κB1 3’UTR-containing reporter via interaction with the predicted binding site and down-regulate the reporter expression driven by NF-κB promoter (Figs. 2 and 3), thus strongly suggesting
that miR-183 targets the NF-κB1 3’UTR directly. Nevertheless, western blot analysis of endogenous NF-κB1/p105/p50 showed unaltered protein levels after miR-183 mimic treatments (Supplementary Fig. S1). Similarly, no change was observed in cells transfected with the positive control miR-9 (Supplementary Fig. S1). There are several possible explanations for this inconsistency, including but not limited to the limitations of exogenous nucleotides transfection efficiency (~72%), determined by flow cytometry assay of 5-FAM fluorescence-labeled negative control oligo transfection to HEK293T cells using Lipofactamine 2000), the intrinsic weak NF-κB1-knockdown efficiency of miR-183 and miR-9 mimics, the cell type-specific biological properties, and the complicated regulation mechanisms of the NF-κB pathway and potential crosstalk. Therefore, more experiments are required to further confirm that NF-κB1 is directly targeted by miR-183, including the antagonistic effect of miR-183 antisense oligonucleotides on miR-183, the expression and tissue distribution of endogenous miR-183, the effect of miR-183 on the expression of NF-κB-responsive genes, etc.

A collection of miRNA prediction algorithms is currently available that weight different factors, such as seed region pairing, evolutionary conservation, and thermodynamic stability of the mRNA/miRNA duplex, thus generating a diverse range of predictions [16]. While it is hard to determine which algorithm is the most reliable or accurate, both TargetScan and PicTar provide high sensitivity and specificity and have produced experimentally verifiable data sets [17]. Here, we used six different online programs to predict the miRNAs targeting NF-κB1 3’UTR and generated an integrated ranking to these miRNAs by compiling their occurrence number and ranking in each program. Consistent with the positive experimental results previously [13] and herein, miR-9 and miR-183 were assigned an integrated ranking of No. 1 and 2, respectively, at targeting NF-κB1. It is worth mentioning that miR-129 was not predicted by PicTar or microRNAmap, but received a relatively high probability score in the other four programs, with ranking No. 1 in miRanda, No. 2 in TargetScan, No. 3 in EIMMo, and No. 6 in RegRNA (Table 1). Nevertheless, miR-129 did not exhibit an inhibiting effect on NF-κB1 3’UTR following the reporter assay (Fig. 1B), which is more reflective of the low integrated ranking assigned herein of No. 12. These findings further support the enhanced accuracy of an integrated analytical approach that provides more reliable predictions and ultimately increases the positive rate of experimental confirmations.

Negative auto-regulatory feedback control is an important feature of NF-κB signaling, which allows for transient NF-κB activation, avoids undesirable prolonged inflammatory responses, and finally restores cell homeostasis [5,7]. Notably, a couple of NF-κB-dependent miRNAs have been identified to participate in negative autoregulatory feedback control of NF-κB activation, which provides a new insight into the NF-κB signaling regulation. These miRNAs include miR-146, which targets IRAK-1 and TRAF6 and thereby down-regulates the MyD88-dependent NF-κB response [18]; miR-9, which targets NF-κB1 directly and prevents the LPS-induced excessive activation in monocytes [13]. To estimate whether transcription of miR-183 is NF-κB-dependent, the Genomatix MatInspector software, a renowned software tool for finding promoter and transcription factor binding sites [19], was used to search for possible NF-κB binding sites in the region around the predicted miR-183 transcription start site (TSS) (~5.4 kb upstream of mature miR-183 sequence) [20]. Two c-Rel binding sites were predicted, one located at ~400 bp downstream of TSS with 95.1% matrix similarity and the other at ~2.1 kb upstream of TSS with 97.5% matrix similarity (Supplementary Fig. S2). This in silico analysis suggests that miR-183 transcription might be NF-κB-responsive, and that miR-183 mediated NF-κB regulation might form a negative autoregulatory feedback loop. Considering that c-Rel expression is primarily high in B and T cells, we speculated that, unlike other NF-κB proteins that are ubiquitously expressed [21], miR-183 might exert cell-specific autoregulation on NF-κB signaling. However, this speculation is yet to be experimentally verified, which will also be our next research goal.

Supplementary Data

Supplementary Data are available at ABBS online.

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References