miR-155* mediates suppressive effect of PTEN 3′-untranslated region on AP-1/NF-κB pathway in HTR-8/SVneo cells

P. Xue a,1, M. Zheng a,1, Z. Diao b, L. Shen c, M. Liu a, P. Gong d, H. Sun b, Y. Hu a,∗

a Drum Tower Clinical Medical College, Nanjing Medical University, Nanjing 210029, China
b Department of Obstetrics and Gynecology, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing 210008, China
c Nanjing University Medical School, Nanjing 210093, China
d Nanjing University of Traditional Chinese Medicine, Nanjing 210023, China

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ABSTRACT
Among miRNAs, miR-155 is a known regulator of immune system. Accumulating studies have revealed the connections between miR-155 and activator protein 1 (AP-1)/nuclear factor (NF)-κB. However, miR-155*, a miR-155 paralog, has so far been less studied. Here we demonstrated that miR-155*, induced by lipopolysaccharide (LPS) in an AP-1/NF-κB dependent manner, played a positive feedback role in AP-1/NF-κB pathway via targeting interleukin-1 receptor-associated kinase M (IRAKM) and NF-κB inhibitor interacting Ras-like 1 (NKIRAS1) in trophoblasts. Our study further proved that miR-155*-targeted PTEN 3′-untranslated region (3′UTR) increased IRAKM and NKIRAS1 expression by competing for miR-155* binding, thereby suppressing AP-1/NF-κB activation induced by LPS.

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1. Introduction
Appropriate regulation of innate immune system at the maternal–fetal interface is crucial for the success of pregnancy [1,2]. Toll-like receptor 4 (TLR4), a receptor known to mediate immune response in trophoblasts at the maternal–fetal interface, discerns lipopolysaccharide (LPS) from gram-negative bacteria and initiates the activation of intracellular negative regulators, such as interleukin-1 receptor-associated kinase M (IRAKM) and NF-κB inhibitor interacting Ras-like 1 (NKIRAS1), are activated to maintain fine-tuned balance of TLR4 signaling [3–5]. Disorders of TLR4 signaling in trophoblasts may result in some adverse pregnancy outcomes including spontaneous abortion, preterm labor and preeclampsia [1,2].

miRNAs are a class of small RNAs that post-transcriptionally repress gene expression by partially complementing with the 3′-untranslated region (3′UTR) of target mRNAs. miRNAs are initially transcribed as primary miRNA transcripts (pri-miRNAs) that are processed to precursor miRNA hairpins (pre-miRNAs). Then pre-miRNAs are transported to the cytoplasm, where they are cleaved from the stem loop by Dicer to form mature miRNA/miRNA* duplex.

Following processing, miRNAs are assembled into the RNA-induced silencing complex for target recognition [6].

Among miRNAs, miR-155 is a known regulator of immune system [7,8]. Several known target genes of miR-155, such as c-Jun, IKK ε, and TAB2, are associated with TLR4-mediated immune response [9–11]. Previous studies have revealed that miR-155 is up-regulated via the LPS/AP-1/NF-κB pathway in trophoblasts and contributes to preeclampsia by down-regulating cysteine-rich 61 and cyclin D1 [12–14]. However, little is known about the possible function and regulation of miR-155* in trophoblasts.

Phosphatase and tensin homolog (PTEN), a critical tumor suppressor, normally functions to antagonize phosphoinositide-3-kinase (PI3K)/Akt signaling, thereby regulating cell survival, metabolism, mobility and immune response [15]. Recent studies reveal that miRNAs mediate the crosstalk between PTEN and multiple RNA transcripts within competitive endogenous RNA (ceRNA) network [16–18]. Each miRNA may repress up to tens or hundreds of targets whereas individual RNA transcripts often harbor numerous miRNA binding sites for multiple distinct miRNAs [19]. Recently, the ceRNA network has been identified in which multiple RNA transcripts can communicate with each other by competing for the common miRNAs [20]. Furthermore, the ceRNA mechanism explains the regulatory function of 3′UTRs [20–22]. However, no data to date are available on the miRNAs-mediated function of PTEN 3′UTR.

According to bioinformatics analysis that six bases in the 3′UTR of PTEN are complementary to miR-155* seed sequence, we speculated...
that miR-155* may mediate the regulatory function of PTEN 3′UTR in trophoblasts. In this study, we proved that LPS-induced miR-155* acted as a positive feedback regulator of AP-1/NF-κB pathway by targeting IRAKM and NKIRAS1, and further demonstrated that exogenous PTEN 3′UTR resulted in higher expression of IRAKM and NKIRAS1 by competing for miR-155* binding, and subsequently played a suppressive role in AP-1/NF-κB pathway.

2. Materials and methods

2.1. Cell culture

Immunofloralized first-trimester trophoblast cell line (HTR-8/SVneo) was kindly provided by Dr. Graham from Queen’s University, Canada. The cells were grown in RPMI 1640 (HyClone, South Logan, USA), supplemented with 10% fetal bovine serum (HyClone), 100 U/ml penicillin and 100 μg/ml streptomycin (HyClone), at 37 °C in a humidified 5% CO2 incubator.

HTR-8/SVneo cells were incubated in the presence of Escherichia coli LPS (100 ng/ml for 0–48 h or 0–200 ng/ml for 6 h; Sigma–Aldrich, St. Louis, USA) to detect the expression of miR-155* following LPS treatment. Additionally, the cells were cultured respectively with pyrimidine dithiosemicarbazone (PDTC, an inhibitor of NF-κB) (Sigma), SP600125 (an inhibitor of c-Jun N-terminal kinase (JNK); Sigma), and the both for 1 h before LPS stimulation (100 ng/ml for 6 h). Then the cells were collected to investigate the relationship between signal pathways and the expression of miR-155*.

2.2. Plasmid construction

2.2.1. The 3′UTR recombinant plasmids pEGFP-PTEN-3′UTR and pEGFP-NKIRAS1-3′UTR, and mutant 3′UTR recombinant plasmids pEGFP-mut-PTEN-3′UTR and pEGFP-mut-NKIRAS1-3′UTR

The base pair primers with a Nhe I restriction site (bold), encompassing the miR-155* binding site (underlined) in the 3′UTR of PTEN (nt 2523–2580, accession no. NM_000314.4) (AGCTTAGCAGTACATGCTATGGCAGTTGTCG) and NKIRAS1 (nt 2080–2138, accession no. NM_001142523.1) (AGCTTAGCTTAATGCTAATATGTAGGAGCTAGC) were synthesized by Shenggong (Shanghai, China). The primers used for plasmid construction are shown in supplemental Table 1. Data were analyzed by using the 2−ΔΔCT method [23].

2.2.2. The recombinant vector pGFP-miR-155* recombinant plasmid pGFP-miR-155* and sponge miR-155* recombinant plasmid pEGFP-sponge-miR-155*

The recombinant vector pGFP-miR-155* was constructed from pEGFP-PTEN-3′UTR, which was digested with Nhe I to remove the fragment containing GFP and PTEN 3′UTR and then ligated to form the recombinant vector pGFP-PTEN-3′UTR with a mutated segment at miR-155 seed site (bold) (GGCTTTAGCTAATGCTAATATGTAGGAGCTAGC) and NKIRAS1 3′ UTR (underlined) (AGCTTAGCAGTACATGCTATGGCAGTTGTCG). The recombinant vector was transfected into HTR-8/SVneo cells in 12-well plates separately with one of the plasmids containing pGFP-155*, pGFP-PTEN-NKIRAS1-3′UTR, and pGFP-sponge-miR-155* recombinant plasmid pEGFP-sponge-miR-155*.

2.3. Luciferase reporter assays

HTR-8/SVneo cells in 12-well plates were separately transfected with one of the plasmids containing pGFP-155*, pGFP-PTEN-NKIRAS1-3′UTR, and pGFP-sponge-miR-155* recombinant plasmid pEGFP-sponge-miR-155*.

2.3.1. Involvement of AP-1/NF-κB pathway in LPS-induced miR-155* expression

It has been found that the promoter region of pri-miR-155 contains putative AP-1- and NF-κB-binding sites and demonstrated that both the AP-1 and NF-κB pathways induce pri-miR-155 production [11,12,24]. In our previous study, trophoblastic miR-155 expression was found to be induced by LPS via the AP-1/NF-κB pathway [12]. Additionally, miR-155 and its star-form partner miR-155* are processed from the same precursor (pre-miR-155) [11]. To investigate the expression of miR-155* following LPS stimulation in trophoblast, we treated HTR-8/SVneo cells with 100 ng/ml LPS for various periods of time or various concentrations of LPS for 6 h, and detected the expression of miR-155*. As shown in Fig. 1A, miR-155* levels induced by LPS (100 ng/ml) reached peak levels at 6 h and then decreased. For concentrations of LPS between 50 and 200 ng/ml, significant increases of miR-155* levels were noted and the peak increase occurred at 100 ng/ml LPS (Fig. 1B). When HTR-8/SVneo cells were pre-treated with PDTC (50 μM) or SP600125 (20 μM), respectively, or both PDTC and SP600125 for 1 h, miR-155* expression was significantly down-regulated in the presence of LPS (100 ng/ml) (Fig. 1C). These findings suggest that miR-155* is induced by LPS through the AP-1/NF-κB pathway in trophoblasts.
3.2. IRAKM and NKIRAS1 are targeted by PTEN-targeting miR-155*

Analysis with Targetscan Version 5.2 showed that miR-155* targeted PTEN, IRAKM [11] and NKIRAS1 (Fig. 2). To determine whether miR-155* may directly target PTEN 3′UTR, we constructed GFP-tagged plasmids with wild-type and mutant PTEN 3′UTR. Both the wild-type and the mutant plasmids were introduced into HTR-8/SVneo cells, respectively. Compared to that in the cells transfected with pΔGFP, GFP expression of the wild-type, but not the mutant, was significantly reduced in the cells transfected with miR-155* (Fig. 3A). Furthermore, GFP-positive cells of the wild-type were markedly decreased, but not the mutant, in the miR-155*–transfected cells (Fig. 3A). The same strategy has been employed in studying miR-155* targeting in NKIRAS1 3′UTR. GFP expression and GFP-positive cells of the wild-type NKIRAS1 3′UTR were significantly reduced following transfection with miR-155*, whereas the mutant was unaffected by transfection with miR-155* (Fig. 3B).

As shown in Fig. 3C, transfection of HTR-8/SVneo cells with miR-155* resulted in decreased PTEN, IRAKM and NKIRAS1 protein levels; however, restoration of their protein levels was observed in the sponge miR-155*–transfected cells (Fig. 3D). Additionally, transfection of HTR-8/SVneo cells with miR-155* significantly decreased PTEN, IRAKM and NKIRAS1 mRNA levels (Fig. 3C), whereas sponge miR-155* expression contributed to the restoration of their mRNA levels (Fig. 3D). These results indicate that PTEN, IRAKM [11] and NKIRAS1 are targets of miR-155*.

3.3. Coding-independent regulation of IRAKM and NKIRAS1 by PTEN 3′UTR

The ceRNA mechanism suggests that 3′UTRs regulate gene expression by acting as decoys for the common miRNAs [20]. It may be supposed that PTEN 3′UTR expression should incur increased translation of IRAKM and NKIRAS1. As shown in Fig. 4A, we indeed observed markedly elevated expression of IRAKM and NKIRAS1 proteins in the PTEN 3′UTR-transfected HTR-8/SVneo cells, compared to the pEGFP-C1–transfected cells. However, in the cells transfected with mutant PTEN 3′UTR, IRAKM and NKIRAS1 protein levels were not obviously affected (Fig. 4B).

To further ascertain whether this observed effect was a consequence of PTEN 3′UTR expression regulating endogenous IRAKM and NKIRAS1 expression by competing for miR-155* binding, HTR-8/SVneo cells were transfected with PTEN 3′UTR, together with either IRAKM or NKIRAS1 luciferase reporter vectors (pGL3-promoter-IRAKM-3′UTR or pGL3-promoter-NKIRAS1-3′UTR) for a series of luciferase reporter assays. PTEN 3′UTR was found to possess the up-regulated activities on both IRAKM and NKIRAS1 3′UTR linked to luciferase reporter vectors, which showed higher luciferase activities (Fig. 4C and D). However, when the miR-155* binding sites were mutated, there was a restoration in luciferase activities (Fig. 4C and D). These results indicate that miR-155* binding site is essential for the regulation of IRAKM and NKIRAS1 expression by PTEN 3′UTR.

3.4. PTEN 3′UTR has a suppressive activity in AP-1/NF-κB pathway

To determine whether PTEN 3′UTR may regulate AP-1/NF-κB pathway, we compared the luciferase activities of AP-1 and NF-κB in the cells transfected with wild-type and mutant PTEN 3′UTR respectively. As shown in Fig. 5A, after PTEN 3′UTR was introduced into the cells treated with 100 ng/ml LPS, luciferase activities of AP-1 were significantly reduced, compared to that in the cells transfected with pEGFP-C1. Meanwhile, the mutant PTEN 3′UTR, which contains a mutated segment at miR-155* binding site, did not inhibit AP-1 activation induced by LPS (Fig. 5A). Similarly, luciferase activities of NF-κB after PTEN 3′UTR transfection in the cells treated with 100 ng/ml LPS were markedly decreased, but not the mutant, compared to that in the cells transfected with pEGFP-C1 (Fig. 5B). These results suggest that PTEN could exert in part its suppressive activity in AP-1/NF-κB pathway through its 3′UTR with miR-155* binding site.

Fig. 1. Induction of miR-155* transcription via LPS/AP-1/NF-κB pathway. Expression of miR-155* following LPS (100 ng/ml) stimulation for the indicated time (A), or the indicated concentrations of LPS stimulation for 6 h (B), or LPS (100 ng/ml) stimulation for 6 h after pre-treatment with PDTC (50 μM) and/or SP600125 (20 μM) (C). miR-155* levels were measured by qPCR and normalized to U6. Results are representative of at least three independent experiments. Error bars = SD. *Means p < 0.05. **Indicates p < 0.01.

Fig. 2. Prediction of miR-155* targets. Predicted miR-155* binding site in 3′UTRs of PTEN, IRAKM and NKIRAS1 mRNAs was determined with Targetscan version 5.2. Mutated nucleotides are indicated in underlined bold type.
Fig. 3. PTEN, IRAKM and NKIRAS1 are targeted by miR-155*. (A) Effects of miR-155* on PTEN 3'UTR. HTR-8/SVneo cells were transfected with 1.6 μg of either pΔGFP-miR-155* or pΔGFP, together with 0.4 μg of either pEGFP-PTEN-3'UTR or pEGFP-mut-PTEN-3'UTR for 12 h. GFP-positive cells were observed by fluorescence microscopy (left) and whole-cell extracts were subjected to western blot analysis to detect GFP (right). β-actin is shown to confirm equal loading. (B) Effects of miR-155* on NKIRAS1 3'UTR. As described in A, HTR-8/SVneo cells were transfected with 1.6 μg of either pΔGFP-miR-155* or pΔGFP, together with 0.4 μg of either pEGFP-NKIRAS1-3'UTR or pEGFP-mut-NKIRAS1-3'UTR for 12 h. (C–D) Effects of miR-155* (C) or sponge miR-155* (D) on PTEN, IRAKM and NKIRAS1. HTR-8/SVneo cells were transfected with 1.6 μg of either pΔGFP-miR-155* (C), or pEGFP-sponge-miR-155* (D), and/or treated with LPS (100 ng/ml) for 12 h. qPCR was performed to detect PTEN, IRAKM and NKIRAS1 mRNA expression and normalized to 18S rRNA (left). Western blot analysis was used to detect PTEN, IRAKM and NKIRAS1 protein expression (right). β-actin is shown to ensure equal loading in all lanes. The data are representative of at least three independent experiments. Error bars = SD. *Means p < 0.05.
found that NKIRAS1, a negative regulator of NF-κB resulted in down-regulation of IRAKM (Fig. 3C). We also demonstrated that transfection of HTR-8/SVneo cells with miR-155* in trophoblasts using HTR-8/SVneo, 155* in trophoblasts remains undetermined. In this study, we explored the role of miR-155* in trophoblasts using HTR-8/SVneo, and NF-κB activation induced by LPS (Fig. 5A and B). Meanwhile, IRAKM and NKIRAS1 protein levels were up-regulated in the transfected cells (Fig. 5C). Both of AP-1 and NF-κB are well-documented transcription factors. AP-1 and NF-κB, can also be regulated by oxidative stress and some other stimuli including TNF-α and IL-1 [25]. Reportedly, AP-1 and NF-κB play a critical role in immune response during pregnancy, excessive or inappropriate activation of AP-1 and NF-κB may result in pathological outcomes [1,2,12]. A number of the AP-1 transcription factors of extravillous trophoblasts are abundant in the tissue samples from normal pregnancy and several trophoblast diseases [26,27]. Furthermore, significantly higher JunB, FosB, NF-κB expression have been detected in human preeclamptic placentas [12]. Therefore, attempts of modifying AP-1/NF-κB activation in trophoblasts may improve adverse pregnancy outcomes in the future.

PTEN, a critical antagonist of PI3K/Akt pathway, blocks the activation of Akt and its downstream targets, thereby regulating TLR4-mediated immune system. Paradoxically, PTEN appears to up-regulate the activation of mitogen-activated protein kinase pathways (p38 and JNK) and the downstream target AP-1 in some cases [28,29], but not in others [15,30]. Likewise, PTEN has been shown to promote NF-κB activation in some cell systems [29] and suppress NF-κB in others [31,32]. The opposing effects of PTEN may be due to the activation of downstream effectors, of which there are many. In this study, PTEN was identified to be one of miR-155* targets, indicating that miR-155* may mediate the functional effect of PTEN on AP-1/NF-κB pathway in trophoblasts. We revealed that transfection of HTR-8/SVneo cells with PTEN 3'UTR blocked the AP-1/NF-κB activation induced by LPS (Fig. 5A and B). Meanwhile, IRAKM and NKIRAS1 protein levels were up-regulated in the transfected cells (Fig. 4A). Luciferase assays further validated that PTEN 3'UTR enhanced IRAKM and NKIRAS1 expression by competing for miR-155* binding (Fig. 4C and D). Taken together, we provide a new mechanism underlying the miR-155*-mediated regulation of AP-1/ NF-κB pathway by PTEN 3'UTR.

Although accumulating studies have shown the connections between PTEN and AP-1/NF-κB pathway, less is known about the regulation of AP-1/NF-κB pathway by PTEN 3'UTR. In this study, PTEN 3'UTR was found to increase the expression of IRAKM and NF-κB in HTR-8/SVneo cells (Fig. 5C), suggesting that PTEN 3'UTR may mediate the functional effect of PTEN on AP-1/NF-κB pathway in trophoblasts.
NKIRAS1 by competing for miR-155* binding, thereby blocking AP-1/NF-κB activation (Fig. 5C). Our results are in consistent with other works in which exogenous 3′UTRs exert biological activities by competing endogenous miRNAs [21,22]. The ceRNA mechanism explains the regulatory function of exogenous 3′UTRs, and suggests that multiple RNA transcripts, including messenger RNAs, transcribed pseudogenes and long noncoding RNAs, compete with each other for the common miRNAs, generating a large-scale regulatory network across transcriptome [20]. Therefore, while analyzing the role of PTEN in trophoblasts, we may need to consider not only the proteins but also the remaining non-coding transcripts.

In summary, while AP-1/NF-κB pathway is a key determinant of immune response during pregnancy, excessive or inappropriate activation of this pathway may result in pathological outcomes. As a result, our study promotes to be developed as some approaches to keep tight control of the AP-1/NF-κB pathway during pregnancy. We propose that LPS-induced miR-155* plays a positive feedback role in AP-1/NF-κB pathway via inhibiting IRAKM and NKIRAS1 expression, whereas exogenous PTEN 3′UTR can block AP-1/NF-κB activation via competing for miR-155* binding (Fig. 5C).

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2013.04.015.

**References**


