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MicroRNA-466l Upregulates IL-10 Expression in TLR-Triggered Macrophages by Antagonizing RNA-Binding Protein Tristetraprolin-Mediated IL-10 mRNA Degradation

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MicroRNAs (miRNAs) are generally recognized as regulating gene expression posttranscriptionally by inhibiting translation or inducing target mRNA degradation. New mechanisms for miRNAs to regulate gene expression also still attract much attention. More and more novel miRNAs are discovered by the advanced sequencing technology, but yet their biological functions are largely unknown. Up to now, the function of miR-466l, a miRNA discovered in mouse embryonic stem cells, remains unclear. In this study, we report that miR-466l can upregulate both mRNA and protein expression of IL-10 in TLR-triggered macrophages. Furthermore, we show that miR-466l can competitively bind to the IL-10 3′ untranslated region AU-rich elements, which is a typical binding site for RNA-binding protein (RBP). Tristetraprolin is a well-known RBP, and mediates rapid degradation of IL-10 mRNA. miRNA always mediates target mRNA degradation or translation repression modestly; thus, the net effect of miR-466l’s binding to IL-10 AU-rich elements is to prevent IL-10 mRNA degradation mediated by tristetraprolin, resulting in extended 1/2 of IL-10 mRNA and elevated IL-10 expression. Thus, competitive binding with RBP to the same target mRNA and subsequent stabilization of target mRNA is an alternative mechanism for gene regulation by miRNAs. Also, a mechanism for regulation of IL-10 by miRNAs is outlined. The Journal of Immunology, 2010, 184: 000–000.

MicroRNAs (miRNAs) are emerging as important, albeit poorly characterized, regulators of many kinds of biological processes (1, 2). In the immune system, miRNAs regulate the development and function of many immune cells (3, 4). Several miRNAs play important roles in innate immunity (5, 6) and adaptive immunity (7–9). Besides, miRNAs can also contribute to lymphoproliferative diseases and autoimmunity (10). It is well accepted that miRNAs regulate gene expression posttranscriptionally by repressing translation and/or inducing target mRNA degradation. However, investigation of other new mechanisms for miRNAs to regulate gene expression attracts much attention. More and more evidence shows that posttranscriptional regulation is as important as transcriptional regulation. These two regulation mechanisms cooperate to determine the gene expression levels. It was reported that the intrinsic stability of the mRNAs of inflammatory mediators, encoded in their 3′ untranslated region (3′UTR), partially directs the sequential initiation, propagation, and resolution of inflammation (11). The AU-rich elements (ARE) located in 3′UTR of these inflammatory mediators can be regulated by both miRNAs and RNA-binding proteins (RBPs). For example, miR-16 and tristetraprolin (TTP) can cooperate to destabilize TNF-α mRNA by binding to its ARE (12). In LPS-activated macrophages, transcripts encoding cytokines, such as TNF-α, IL-1, IL-6, cyclooxygenase-2 (13), and CXCL1 (14), have ARE in their 3′UTR. TTP coordinately stabilizes these transcripts by recruiting the RNA decay machinery (15). TTP also can destabilize the anti-inflammatory cytokine IL-10 mRNA by binding to the ARE (16). It has been concluded that miRNAs mediate target mRNA degradation or translation repression modestly (17), whereas RBPs, such as TTP, can mediate rapid mRNA degradation (18, 19). Thus, whether and how miRNAs and RBPs can regulate each other and whether the target gene expression is under the control of both miRNAs and RBPs need to be further investigated.

Now, more and more new miRNAs have been identified by the advanced sequencing technology (2, 20, 21). miR-466l was identified in embryonic stem (ES) cells by using high-throughput pyrosequencing (21). However, its function remains unknown to date. Through the miRanda algorithm (22, 23), many cytokines, chemokines, and growth factors are predicted to be the targets of miR-466l. The seed sequence of miR-466l is well complementary with the AUUUA pentamer, which is a characteristic sequence of ARE. Considering that the seed sequence of miR-16 is not complementary with TNF-α ARE (12), miR-16 synergizes TTP to recognize TNF-α ARE and then mediates TNF-α mRNA degradation by a nontypical miRNA function. It is intriguing what happens when miR-466l and TTP meet at the same binding sites. We demonstrate that miR-466l can upregulate IL-10 expression in TLR-triggered macrophages both at mRNA and protein levels. We further show that miR-466l occupies the binding sites of TTP in the IL-10 3′UTR

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Abbreviations used in this paper: ActD, actinomycin D; AGO, Argonaute; ARE, AU-rich element; ES, embryonic stem; miRNA, microRNA; mTTP, murine tristetraprolin; P-bodies, processing bodies; qPCR, real-time quantitative PCR; RBP, RNA-binding protein; RIP, RNA-binding protein immunoprecipitation; RISC, RNA-induced silencing complex; siRNA, small interfering RNA; 3′UTR, 3′ untranslated region.

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ARE, thus preventing IL-10 mRNA degradation mediated by TTP and extending IL-10 mRNA t1/2.

Materials and Methods

Mice and reagents

C57BL/6 mice (5–6 wk old) were purchased from SIPPR-BK Experimental Animal (Shanghai, China). All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University (Shanghai, China). LPS, PGN, and actinomycin D (ActD) were from Sigma-Aldrich (St. Louis, MO). miR-466l mimics and control mimics were from GenePharma (Shanghai, China). TTP small interfering RNA (siRNA) and control siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA). Magenta RNA-binding protein immunoprecipitation ( RIP) RBP immunoprecipitation kit was from Millipore (Billerica, MA).

IL-10 cis elements

The IL-10 promoter was amplified from C57BL/6 mouse genome DNA (5'-GCCAACGAGGATCCCTCCCGGTTAC-3' for forward primer and 5'-TAAGACGACGACGATACACGTCG-3' for reverse primer) and subcloned into the PGL3-promoter vector (Promega, Madison, WI) to generate IL-10-pro. The IL-10 3′UTR luciferase reporter vector (nor) was constructed by amplifying the mouse IL-10 mRNA 3′UTR and cloning it into Xhol site of pGL3-promoter vector (Promega). The mutant IL-10 3′UTR (mut) was generated from the IL-10 3′UTR by mutating all of the four miR-466l potential binding sites, mut-1, mut-2, mut-3, and mut-4 were generated from IL-10 3′UTR by mutating the first, second, third, and fourth miR-466l binding site, respectively. All mutation reporter vectors were generated by changing ATTTA pentamer into ATGTA pentamer. The miR-466l with its flanking sequence was amplified from C57BL/6 mouse genome DNA (5'-GTTCATGTAGAGTGAGGTAGATC-3' for forward primer and 5'-TGTCCTGCATGACTAATCTCTAC-3' for reverse primer) and subcloned into the UBC-pRRES-EFVP vector to produce UBC-466l. All vectors were verified by sequencing.

Cell culture and transfection

HEK293T, RAW264.7, and 3LL cell lines were obtained from the American Type Culture Collection (Manassas, VA). HEK293T was cultured in DMEM (Gibco, Carlsbad, CA). Protein concentrations of the extracts were measured with a bicinchoninic acid assay (Pierce) and equalized with the extraction reagent. Equal amount of the extracts was loaded and subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted, as described previously (24). RNA immunoprecipitation experiments were performed according to the protocol provided by the kit (Millipore; catalogue 17-701).

Statistical analysis

Statistical significance was determined by Student’s t test, with values of p < 0.05 considered to be statistically significant.

Results

miR-466l is predicted to regulate gene expression of many cytokines via their ARE

The seed sequence of miR-466l is AUAAAUA, which is complementary to the typical ARE sequences. Taking advantage of the miRanda algorithm (23) and TargetScan algorithm (25), we predicted that the cytokine genes that have multiple AUUUA pentamers can be targeted by miR-466l. Among them, TNF-α, IFN-γ, IFN-β, and IL-10 are potential targets of miR-466l because they have AUUUA pentamers in the 3′UTR. For example, IL-10 has the four binding sites of miR-466l (Fig. 1).

miR-466l upregulates IL-10 expression of both mRNA and protein levels in TLR-triggered macrophages

TLR-triggered macrophages were used to study the possible role of miR-466l in the regulation of cytokine production. As shown in Fig. 2A, RAW264.7 cells transfected with miR-466l mimics expressed much higher levels of mature miR-466l. We found that, interestingly, both IL-10 mRNA and protein levels were higher in the miR-466l-overexpressed RAW264.7 cells than in mock control cells when these cells were stimulated with TLR ligands (Fig. 2B–D; Supplemental Fig. 1). The upregulation of IL-10 mRNA expression by miR-466l–expressing vector was dose dependent (Supplemental Fig. 2A). However, the protein levels of other cytokines, such as TNF-α, IFN-β, and IFN-γ, remained almost unchanged in TLR-triggered macrophages.
macrophages when transfected with miR-466l or mock control (Fig. 2E–G). In primary peritoneal macrophages, we obtained similar results (Fig. 2H). The 3LL murine lung cancer cells secrete IL-10 spontaneously and serum starvation induces 3LL cells to produce more IL-10; we took advantage of this system and found that more IL-10 was produced in the miR-466l-overexpressed 3LL cells than in mock control cells. Further experiments confirmed the upregulation of IL-10 by miR-466l in serum-starved 3LL cells (Supplemental Fig. 2B). Although miR-466l is highly expressed in ES cells, endogenous miR-466l expression is very low in macrophages (Supplemental Fig. 2D) and 3LL cells; we did not take miR-466l inhibitor to verify the effect of miR-466l on IL-10 gene expression.

**Upregulation of IL-10 production by miR-466l most likely occurs posttranscriptionally and directly**

In most cases, miRNA regulates translation repression and/or mRNA degradation of its target. It is unusual that miR-466l upregulates IL-10 production. We used the luciferase reporter systems to investigate whether miR-466l would bind to its predicted sites of IL-10 and upregulate the luciferase activity. Our data show that miR-466l did have effect on luciferase reporter vector with IL-10 3'UTR (nor), but the luciferase activity was down regulated, just as the effect of the most miRNAs on their targets (Fig. 3A). miR-466l impaired the IL-10 3'UTR reporter gene activity via the ARE, which is complementary with the miR-466l seed sequence, because miR-466l mimics could not reduce the luciferase activity of the mutant IL-10 3'UTR reporter gene (mut) (Fig. 3A). We further narrow down the critical binding site for miR-466l. We generated mut-1, mut-2, mut-3, and mut-4 by mutating four possible miR-466l binding sites,
respectively, and repeated the reporter gene experiments in RAW264.7 cells with or without LPS stimulation. Our data show that in resting RAW264.7 cells, miR-466l downregulated the wild-type IL-10 3′UTR luciferase activity, but had no effect on that of mutant IL-10 3′UTR reporter gene. Down regulation of luciferase activity is unchanged if the first, second, or fourth potential miR-466l binding sites are mutated, whereas downregulation of luciferase activity is abolished if the third binding site is mutated (Fig. 3B). In LPS-stimulated RAW264.7 cells, miR-466l upregulated the wild-type IL-10 3′UTR luciferase activity, but had no effect on that of mutant IL-10 3′UTR reporter gene. The first and fourth mutation of potential miR-466l binding sites did not block the upregulation of luciferase activity, whereas the second and third mutation abolished the upregulation of luciferase activity caused by miR-466l (Fig. 3B).

According to the above results, miR-466l decreased luciferase activity in resting RAW264.7 cells while increasing IL-10 3′UTR luciferase activity in LPS-stimulated RAW264.7 cells. The third potential binding site of miR-466l in IL-10 3′UTR may be the key position for miR-466l regulation, and the second binding site may also have some contributions.

IL-10 can be regulated both transcriptionally and posttranscriptionally. The reporter gene experiments above just indicated the posttranscriptional regulation. To address whether miR-466l could upregulate IL-10 by affecting its transcription, we constructed an IL-10 promoter luciferase reporter gene, which contains 1205 bp of IL-10 mRNA 5′UTR. The luciferase activity was much higher in the miR-466l–overexpressed RAW264.7 cells and its mock control vector (26, 27), the results of IL-10 promoter reporter gene partially suggest that the IL-10 promoter reporter is functional. However, we did not see any difference in luciferase activity between the miR-466l–overexpressed RAW264.7 cells and its mock control cells (Fig. 3C and 3D). Because most transcriptional regulator binding sites locate within the 1205 insert of the promoter reporter vector (26, 27), the results of IL-10 promoter reporter gene partially suggest that miR-466l has little effect on the IL-10 transcription.

It was shown recently that bone marrow-derived macrophages from TTP-deficient mice have longer IL-10 mRNA t1/2 than the cells from wild-type mice, indicating that IL-10 can be regulated by TTP in a posttranscriptional manner (16). It is possible that miR-466l upregulates IL-10 expression by downregulating TTP.

We examined the TTP mRNA level in LPS-stimulated RAW264.7 cells. There was no difference in TTP expression between the miR-466l–transfected cells and matched control cells (Fig. 3E), suggesting that the upregulation of IL-10 by miR-466l is not the consequence of lower TTP expression. Taken together, the results demonstrate that upregulation of IL-10 expression by miR-466l mostly likely occurs posttranscriptionally and may be directly.

miR-466l upregulates IL-10 gene expression in TLR-triggered macrophages by extending IL-10 mRNA t1/2

In TLR-triggered macrophages, the IL-10 mRNA level is upregulated in miR-466l–transfected cells compared with the mock control-transfected cells. The fold increase of upregulation was more obvious in the latter phase of LPS or PGN stimulation (Fig. 2B), indicating miR-466l may extend IL-10 mRNA t1/2. The ActD, a transcription inhibitor, was applied to block IL-10 transcription 6 h after LPS or PGN stimulation. More expression fold increase could be found in the latter phase of ActD treatment, from 2 ~ 3-fold increase to 4 ~ 5-fold increase. We took the data from three independent experiments to calculate the t1/2 of IL-10 mRNA. IL-10 mRNA level was measured by qPCR and normalized by β-actin mRNA. C and D, RAW264.7 cells were treated as described in B, and then were stimulated with LPS for indicated times; TNF-α mRNA levels (C) and IL-10 mRNA level (D) were measured by qPCR and normalized by β-actin mRNA. E and F, RAW264.7 cells were cotransfected with 5 nM indicated miRNA or siRNA. Twenty-four hours later, these cells were stimulated with LPS (100 ng/ml) for 1, 2, 4, or 8 h; IL-10 mRNA levels were measured by qPCR and normalized by β-actin mRNA (E). The culture supernatant of cells stimulated with LPS for 12 h was measured by ELISA (F). Data are shown as mean ± SEM of three independent experiments. *p < 0.01.

**FIGURE 4.** miR-466l upregulates IL-10 gene expression in TLR-triggered macrophages by extending IL-10 mRNA t1/2. RAW264.7 cells were transfected with miR-466l mimics or matched control at the final concentration of 10 nM. Twenty-four hours later, these cells were stimulated with LPS (100 ng/ml) (A) or PGN (1 μg/ml) (B) for 6 h, the culture medium was changed, and then 10 μg/ml ActD was used to treat these cells for 1, 2, or 3 h. The IL-10 mRNA were measured by qPCR and normalized by β-actin mRNA (left). The data of three independent experiments were taken to calculate the IL-10 mRNA t1/2 (right). Data are shown as mean ± SEM of three independent experiments. *p < 0.05; **p < 0.01.
mRNA in miR-466l-transfected RAW264.7 cells had a longer $t_{1/2}$, no matter if stimulated with LPS (Fig. 4A) or PGN (Fig. 4B). These data suggested that miR-466l could extend the IL-10 mRNA $t_{1/2}$, which may be the main cause of miR-466l-mediated upregulation of IL-10 expression.

miR-466l upregulates IL-10 expression in TLR-triggered macrophages by antagonizing TTP-mediated IL-10 mRNA degradation

The AUUUA pentamers located in ARE of the IL-10 3′ UTR can be bound by miR-466l, and these pentamers are also the typical binding sites of TTP. TTP can shorten the IL-10 mRNA $t_{1/2}$, but miR-466l can extend it. It has been reported that miR-16 and TTP cooperate to degrade TNF-α mRNA (12). There are also predicted models for RBP and miRNA binding to the same target (28). To determine whether miR-466l and TTP compete the same binding sites of IL-10 3′ UTR, an efficient siRNA targeting TTP was applied (Fig. 5A and 5B). In TTP-silenced RAW264.7 cells, upregulation of TNF-α is found at the early phase, whereas upregulation of IL-10 mRNA is found at late phase of LPS stimulation (Fig. 5C and 5D). The upregulation of TNF-α and IL-10 after TTP silencing is also coherent with TTP targeting TNF-α and IL-10 (16, 29).

There was no IL-10 mRNA difference between the miR-466l–transfected and its mock control-transfected RAW264.7 cells after TTP interfering, although we haven’t found miR-466l down-regulated IL-10 mRNA as we found in the IL-10 3′UTR reporter system (Fig. 5E and 5F). miR-466l cannot upregulate IL-10 gene expression after silencing TTP; therefore, we hypothesized the following: miR-466l may antagonize TTP-mediated IL-10 mRNA degradation, and thus upregulates IL-10 expression.

To test this hypothesis, we performed the RNA immunoprecipitation experiments. We used TTP Ab to isolate the RNA bound to TTP. TTP Ab precipitated large amount of IL-10 mRNA and TNF-α mRNA, whereas control IgG did not. In the immunoprecipitate, IL-10 mRNA level is lower in miR-466l–transfected cells than that of mock control-transfected cells, although the TNF-α mRNA level showed no difference between the two groups (Fig. 6A and 6B). IL-10 mRNA level was higher in miR-466l–transfected cells than that of mock control-transfected cells in the supernatant after TTP Ab immunoprecipitation, whereas the TNF-α mRNA level showed no difference between the two groups in the supernatant (Supplemental Fig. 3). It is well accepted that miRNAs mediate the RNA degradation or translation repression slightly (17), whereas the RBP, such as TTP, plays a very important role in the feedback of the inflammatory response and it degrades RNA rapidly (18, 19). The accumulation of miR-466l leads to the formation of more RNA-induced silencing complexes (RISC), allowing more ARE of IL-10 mRNA occupied by the RISC, reducing the chance of TTP-containing complexes binding to ARE. In the miR-466l–overexpressed cells, IL-10 mRNA ARE is mainly bound by miR-466l–containing RNA-induced silencing complexes; thus, degradation is lower than that in mock control cells, in which IL-10 mRNA ARE is mainly bound by TTP.

In conclusion, we demonstrate that miR-466l can upregulate IL-10 expression in TLR-triggered macrophages by antagonizing RBP TTP-mediated IL-10 mRNA degradation, and the working model we proposed is shown in Fig. 6C.

Discussion

IL-10 is one of the important regulators of the immune system that has been shown to be anti-inflammatory in many model systems (30).
It is secreted by various cell types, such as Th2 cells, regulatory T cells, B cells, macrophages, dendritic cells, mast cells, and eosinophils. It mediates lots of immunoregulatory events, such as negative regulation of maturation and activation of macrophages and dendritic cells, inhibition of T cell activation, suppression of cytokine synthesis, and Ab production (31, 32). Also, dysregulation of IL-10 leads to various immunological diseases, such as cancer, rheumatoid arthritis, asthma, infectious disorders, etc. (33). Therefore, it is essential that IL-10 expression is tightly regulated.

Regulation of IL-10 expression occurs at the transcriptional and posttranscriptional levels. Although Sp1 and Sp3 have been found to regulate transcription, it has been shown that IL-10 mRNA is constitutively transcribed in many cells (33, 34); however, the availability of its protein is significantly determined by posttranscriptional mechanisms. ARE in the 3'UTR of mouse IL-10 that leads to the degradation of its mRNA have been identified (35). miR-106a can bind the IL-10 3'UTR and regulate its expression posttranscriptionally (36). Besides, genetic variations in the IL-10 3'UTR has been shown to be associated with IL-10 levels that could lead to disease pathogenesis (37, 38). Therefore, a full understanding of the posttranscriptional regulation of IL-10 expression will be of scientific and clinical significance.

In this study, we found that miR-466l has well complementary seed sequence with the ARE motif, which is characterized by AUUUA pentamers. In TLR-triggered macrophages, miR-466l can upregulate IL-10 both at mRNA and protein levels by extending IL-10 mRNA \( t_{12} \). miR-466l decreased luciferase activity in an IL-10 3'UTR-specific manner in HEK293T and resting RAW264.7 cells while increasing IL-10 3'UTR luciferase activity in LPS-stimulated RAW264.7 cells. Based on our finding, the following mechanism was proposed: miR-466l antagonizes TTP-mediated IL-10 mRNA degradation, resulting in extended IL-10 mRNA \( t_{12} \). Upregulation of IL-10 in TLR-triggered macrophages is the net effect of the regulation by miR-466l and TTP.

RBPs and miRNAs are two key regulators in the posttranscriptional regulation of genes. They will synergize or antagonize each other. miR-16 and TTP cooperatively to degrade ARE-containing mRNA. The regulation of miR-16 in ARE-containing mRNA is sequence-specific and requires ARE-binding protein TTP. TTP does not directly bind to miR-16, but instead it binds to miR-16 through association with Ago/eif2C family members and assists its targeting to ARE (12). Because we did not detect TTP in Argonauta (AGO)2 immunoprecipitate in miR-466l-transfected macrophages (data not shown), miR-466l and miR-16 may not share the same mechanism to regulate their respective targets. miR-122 can inhibit cationic amino acid transporter 1 mRNA and reporters bearing its 3'UTR in human hepatocarcinoma cells subjected to different stress conditions. HuR, an ARE-binding protein, can stabilize its target mRNA. HuR is proved to reverse the miR-122–mediated cationic amino acid transporter 1 repression (39). This study not only determined that miRNA-mediated repression is a reversible process, but also proposed that miRNAs and RBPs may bind the same target and participate in posttranscriptional regulation together. IL-10 upregulation by miR-466l is another example of competition between miRNAs and RBPs. Our study suggested that RBP-mediated miRNA degradation can be delayed by miRNA and the net effect is the upregulation of target gene.

Vasudevan et al. (40) reported that miR-369-3 can switch target mRNA from repression to activation. As shown in the study, miR-369-3 can upregulate translation by recruiting AGO and fragile X mental retardation-related protein 1, factors associated with microribonucleoproteins. But these results cannot rule out the possibility that miR-369-3 and a RBP can antagonize each other and switch target gene on or off, the mechanism as we have proposed. RISC is a big and complicated complex. It must contain an AGO protein capable of endonucleolytic cleavage. AGO proteins bound to miRNAs, and their target mRNAs accumulate in processing bodies (P-bodies), cytoplasmic foci that are known sites of mRNA degradation (41). TTP coordinately destabilizes ARE-containing mRNA by recruiting the RNA decay machinery to the P-bodies. miRNA and TTP may locate in the same or separate P-bodies. Separate P-bodies may antagonize each other if they bind to the same or nearby target sequence. miR-466l and TTP may locate in separate P-bodies, and these two P-bodies compete for the binding to the ARE of IL-10 mRNA.

There are many ARE-containing mRNAs predicted to be the targets of miR-466l. We verified several predicted targets with the TLR-triggered macrophage system and the 3'UTR luciferase reporter gene system. Only a few of the predicted targets can be regulated by miR-466l, and IL-10 is a solid candidate in both systems. miR-466l can downregulate some predicted targets, such as TNF-\( \alpha \) in reporter gene system, but has no influence on TNF-\( \alpha \) in TLR-triggered macrophages. Whether miR-466l can regulate its predicted target in vivo may be determined by the secondary structure of the target gene mRNA.

miR-466l was discovered in ES cell by high-throughput pyrosequencing. It is relatively highly expressed in ES cells, but endogenous miR-466l expression is very low in macrophages and 3'L cells, so we did not take miR-466l inhibitor to verify the effect of miR-466l on IL-10 production. Although we have proved miR-466l can upregulate IL-10 in in TLR-triggered macrophages, the role of miR-466l in ES cells is still unknown. IL-10 is present at the maternal-fetal interface (42) and may be important for ES cell immunosuppressive properties. Placental cytrophoblasts, which are derived from ES cells, can produce the immunosuppressive cytokine IL-10 (43). Whether or not miR-466l may regulate IL-10 in these cells needs to be further investigated. In addition, the role of IL-10 regulation by miR-466l in immune homeostasis, autoimmune diseases, and even ES immune-privileged process needs to be investigated in the future with respect to the physiological and pathophysiological significance.

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Disclosures
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