

Dysregulated microRNAs in amyotrophic lateral sclerosis microglia modulate genes linked to neuroinflammation

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MicroRNAs (miRNAs) regulate gene expression at post-transcriptional level and are key modulators of immune system, whose dysfunction contributes to the progression of neuroinflammatory diseases such as amyotrophic lateral sclerosis (ALS), the most widespread motor neuron disorder. ALS is a non-cell-autonomous disease targeting motor neurons and neighboring glia, with microgliosis directly contributing to neurodegeneration. As limited information exists on miRNAs dysregulations in ALS, we examined this topic in primary microglia from superoxide dismutase 1-G93A mouse model. We compared miRNAs transcriptional profiling of non-transgenic and ALS microglia in resting conditions and after inflammatory activation by P2X7 receptor agonist. We identified upregulation of selected immune-enriched miRNAs, recognizing miR-22, miR-155, miR-125b and miR-146b among the most highly modulated. We proved that miR-365 and miR-125b interfere, respectively, with the interleukin-6 and STAT3 pathway determining increased tumor necrosis factor alpha (TNF α) transcription. As TNF α directly upregulated miR-125b, and inhibitors of miR-365/miR-125b reduced TNF α transcription, we recognized the induction of miR-365 and miR-125b as a vicious gateway culminating in abnormal TNF α release. These results strengthen the impact of miRNAs in modulating inflammatory genes linked to ALS and identify specific miRNAs as pathogenetic mechanisms in the disease.

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Subject Category: Neuroscience

Amyotrophic lateral sclerosis (ALS) is a widespread motor neuron disorder causing injury and death of lower and upper motor neurons.¹ Identification of superoxide dismutase 1 (SOD1) mutations accounting for about 20% of familial ALS² allowed the production of transgenic mice overexpressing mutated *SOD1* genes, with the SOD1-G93A strain being the most exploited. These mice develop loss of motor neurons together with symptoms resembling human ALS.³ Moreover, clinical and electrophysiological data show that human SOD1-G93A phenotype resembles sporadic ALS in terms of disease progression, thus identifying the SOD1-G93A model as appropriate to investigate the molecular mechanisms of the sporadic disease.⁴

Mutated SOD1-mediated toxicity derives from both motor neurons and neighboring glia, with microgliosis highly contributing to neurodegeneration,^{5–8} although far little attention has been paid to the study of how mutated SOD1 affects microglia features in particular.^{9,10} Multiple mechanisms control the proper levels of protein expression during inflammation and, among these, microRNAs (miRNAs).¹¹ These small, non-coding RNAs are important regulators of protein synthesis under rapid environmental changes such as receptor activation.¹² Apart from their recognized role in cellular specification and physiopathological mechanisms, the

recent discovery of circulating miRNAs also suggests that they provide novel means for paracrine and systemic communication.^{13,14} Current findings demonstrate a correlation between miRNAs expression and microglia activation.¹⁵ For instance, mutations of TDP43, a gene involved in miRNAs biogenesis, were lately found correlated to ALS.¹⁶ Moreover, dysregulation of miRNAs in the best model for miRNAs ablation, the Dicer knockout mice, causes spinal motor neuron disease.¹⁷ Finally, lack of miR-206 accelerates disease progression in ALS mouse.¹⁸ All these findings strengthen the role of miRNAs in ALS pathology.

Microglia activation can occur through different means, among which released tumor necrosis factor alpha (TNF α) and extracellular ATP binding to ionotropic purinergic P2X7r.¹⁹ In CNS, P2X7r is abundantly expressed in microglia and involved in various pathologies.²⁰ In ALS, P2X7r is found upregulated in microglia of human spinal cord and in sections from mutated SOD1 rats.^{21,22} In a previous work, we showed that ALS microglia exhibit P2X7r-dependent enhancement of TNF α , COX-2, CD68, iNOS, NOX and pERK proteins, together with toxic effects exerted on neuronal cells.^{23,24} However, despite a hypothesized role of P2X7r in ALS pathology, the full signaling and pathways involved are still unknown.

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Abbreviations: ALS, amyotrophic lateral sclerosis; nt, non-transgenic; SOD1, superoxide dismutase; miRNA, microRNA; TNF α , tumor necrosis factor alpha; QPCR, quantitative RT-PCR; IL-6, interleukin-6; UTR, untranslated region; (BzATP), 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate; NF- κ B, nuclear factor kappa B
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The aim of this work was to define the miRNAs' signature of microglia from non-transgenic (nt) and SOD1-G93A mice in both resting and P2X7r-activated conditions. We detected a specific subset of dysregulated inflammatory miRNAs that might contribute to ALS alterations. Our results strengthen the impact that miRNAs might have on post-transcriptional modulation of genes linked to inflammation and particularly to ALS.

Results

Profiling of miRNA transcriptome of brain microglia from nt and SOD1-G93A mice. To examine the transcriptional profile and downstream regulation of individual miRNAs likely relevant to the ALS SOD1-G93A microglia phenotype, as appropriate model system we adopted primary microglia purified from newborn cerebral cortex of nt and SOD1-G93A mice. Of the total 627 mouse miRNAs included on the microarray chip employed, only a subset of about 130 miRNAs showed significant hybridization signal and this occurred in both nt and ALS microglia. We defined this novel subset as the miRNAs transcriptome of the brain microglia. As microglia share properties with both immune and nervous system cells (being the immune-competent cells of the brain), we compared the microglia miRNA transcriptome with the complete mouse miRNA profile of Landgraf *et al.*²⁵ (Supplementary Figure 1). The majority of miRNAs that we found significantly expressed in the brain microglia cultures were also present in the whole immune system and brain.²⁵ Of these, ~60% were immune system enriched, ~35% were shared by immune system and brain transcriptome, only ~5% belonged exclusively to the brain (Figure 1a).

The transcriptional profile of the miRNA transcriptome of the brain microglia was then tested for differential expression in nt and ALS microglia. We found that the overexpression of human SOD1-G93A transgene increased the overall transcriptional profile of microglia (Figure 1b), with 78 different miRNAs being significantly upregulated (listed in Supplementary Figure 1C). We then adopted two selection criteria for further validation and investigation: (1) statistical significance; (2) well-known implication in immune system regulation. By this way, we came up with six promising candidates (miR-155, miR-146b, miR-22, miR-365, miR-125b and miR-214) that were confirmed to be upregulated by quantitative real-time (RT)-PCR (QPCR) (Figure 1c), in line with the microarray results (Figure 1d).

IL-6-negative regulation by miR-365 in ALS microglia.

The putative binding site of miR-365 to the interleukin (IL)-6 3'-untranslated region (UTR) is broadly, although not strictly, conserved among vertebrates and was previously validated only in human.²⁶ As miRNA/mRNA interactions are very stringently dependent on sequence composition, we first validated by *in vitro* assay whether mmu-miR-365 binds to the 3'-UTR of mouse IL-6 causing translational inhibition. We constructed two plasmids encoding a renilla luciferase transcript with either wild-type or mutant IL-6 3'-UTR (Figure 2a), which were co-transfected with a miR-30-based lentiviral vector driving mature miR-365 overexpression (Figure 2b). We found that miR-365 inhibited the expression of the transcript containing wild-type IL-6 3'-UTR but not mutant IL-6 3'-UTR (Figure 2c), thus demonstrating a specific inhibitory effect of miR-365 on IL-6 3'-UTR, through direct interaction.

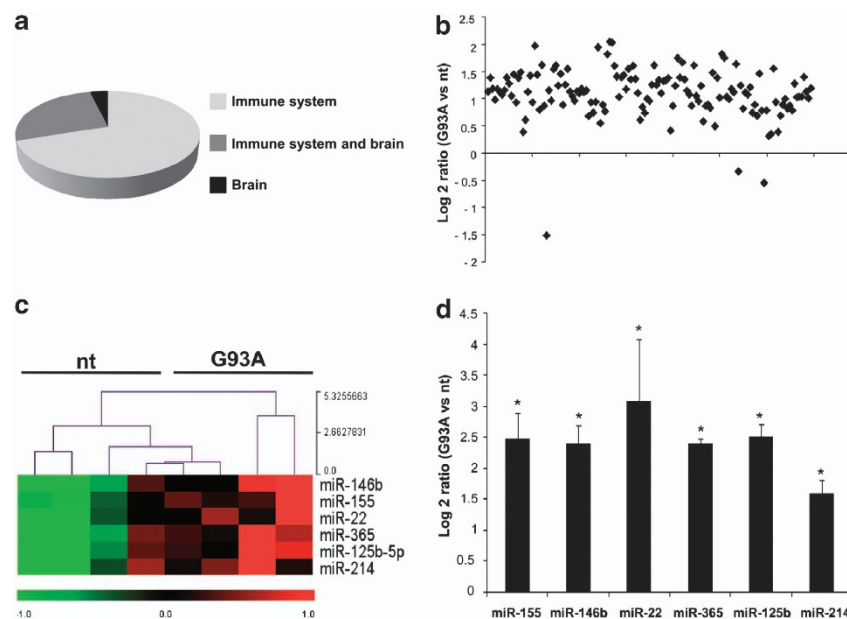


Figure 1 MiRNA profiling of microglia from nt and SOD1-G93A mice. (a) Immune system and brain-related miRNAs expressed in microglia on the basis of Landgraf *et al.*²⁵ (b) Overall statistics of down- and upregulated miRNA genes in SOD1-G93A microglia. Log₂ ratios (SOD1-G93A/nt) are shown. Only miRNAs with a mean expression value > 20.0 in linear scale were selected in order to avoid values too close to noise level. (c) Heat maps of six differentially expressed miRNAs in nt and SOD1-G93A microglia. Red to green indicates high to low expression ($P \leq 0.05$). (d) QPCR validation of the miRNA microarray data of the six differentially expressed miRNAs represented as log₂ $\Delta\Delta\text{CT}$

As target regulation by miRNAs is under the influence of the specific intracellular milieu of each cell type, *in vitro* miRNA/mRNA interactions had to be necessarily validated in our microglia culture system. In order to prove miR-365 as a negative regulator of IL-6 in microglia, we overexpressed the mature sequence of miR-365 by lentiviral transduction. Western blot analysis of IL-6 protein performed at 96 h post transduction showed that exogenous miR-365 repressed IL-6 production in microglia (Figure 2d). Given that IL-6 was found

to be a target of miR-365 (Figures 2c and d), and that miR-365 was found augmented in ALS (Figures 1c and d), we directly measured the content of IL-6 in ALS microglia, by semi-quantitative RT-PCR, western blotting and ELISA. A significant reduction of IL-6 occurred at both mRNA, total and secreted protein levels in SOD1-G93A microglia (Figures 3a–c), thus establishing a novel inverse correlation between IL-6 and miR-365 levels.

STAT3-negative regulation by miR-125b in ALS microglia.

STAT3 is the major downstream effector of IL-6 in murine brain²⁷ and mouse STAT3 3'-UTR contains two conserved binding sites for miR-125b, which we found upregulated in ALS microglia (Figures 1c and d). Predictive hybridization between miR-125b and STAT3 3'-UTR by RNA hybrid²⁸ shows very low minimum free energy with two binding sites (Figure 4a), suggesting STAT3 as a candidate target of miR-125b. Moreover, STAT3 3'-UTR is a validated target of miR-125b *in vitro* and in a mouse granulocytic cell line.²⁹ With the necessity to prove this same interaction in microglia,

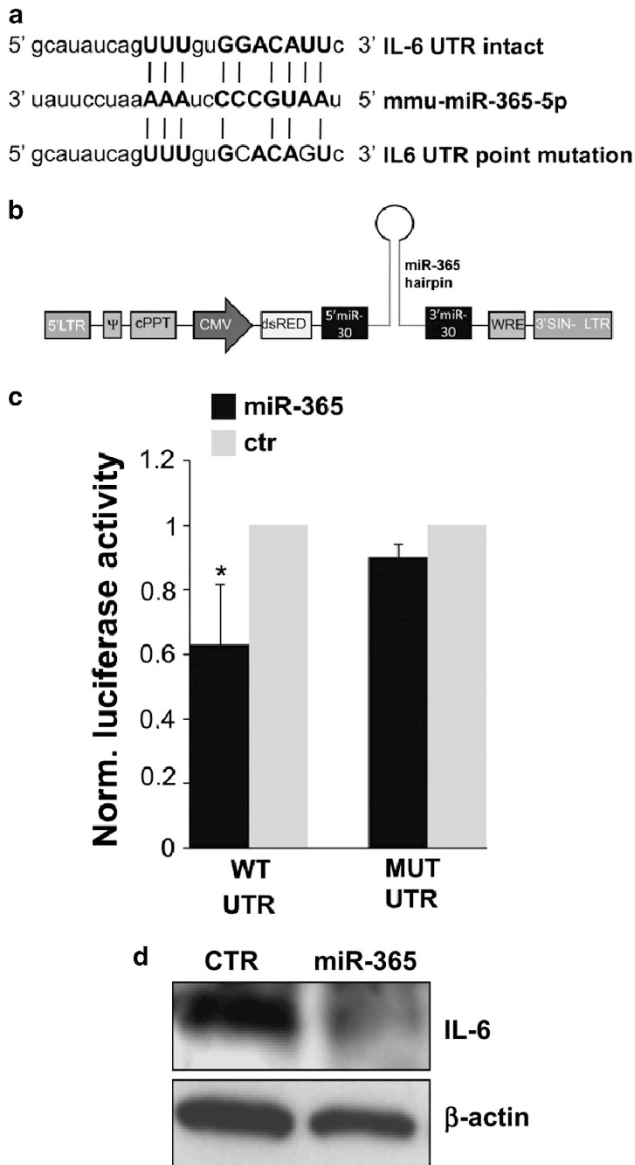


Figure 2 Validation of mouse IL-6 as direct miR-365 target. (a) Alignment of miR-365 and its target sites in intact or mutated IL-6 3'-UTR. (b) Schematic representation of pprime-dsRed-miR-365 construct. (c) Normalized luciferase activities of IL-6 3'-UTR renilla luciferase reporter plasmid, and IL-6 3'-UTR-mutant renilla luciferase reporter plasmid, 48 h after co-transfection together with pprime-miR-365 or empty vector in HEK293 cells. (d) Western blotting with anti-IL-6 antibody of total lysates from empty vector and pprime-miR-365-infected microglial cells, at 96 hours post virus transduction. β-actin was used for protein normalization

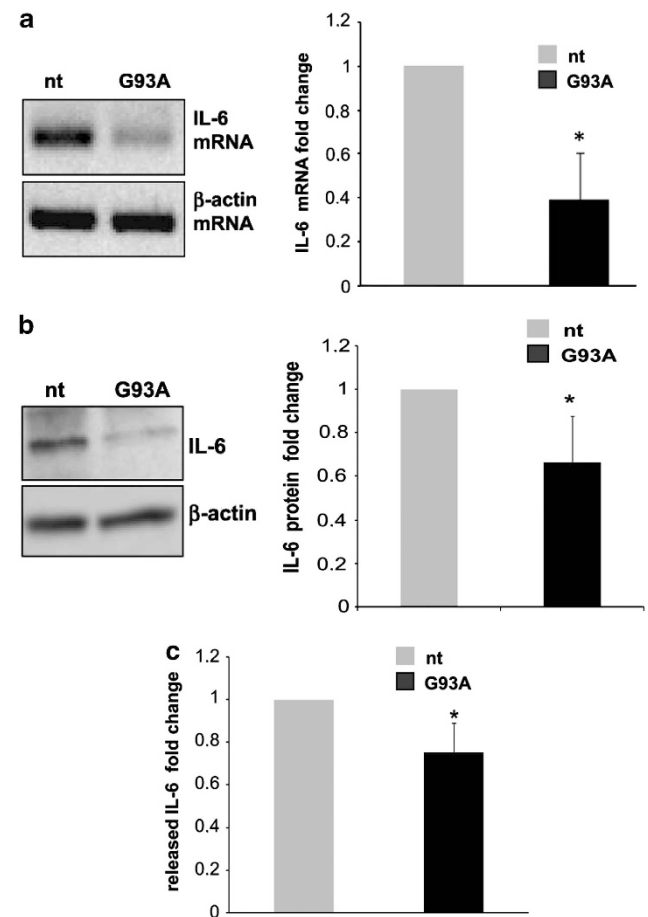


Figure 3 IL-6 downregulation in SOD1-G93A mouse microglia. (a) Semi-quantitative RT-PCR using specific primers for IL-6 and β-actin mRNAs, on total RNA from nt and SOD1-G93A microglia. β-actin was used for normalization. (b) Western blotting with anti-IL-6 antibody on total lysates from nt and SOD1-G93A microglia. β-actin was used for protein normalization. (c) IL-6 levels in the culture media of microglia from nt and G93A mice, after 6 h incubation in fresh media, as assessed by ELISA

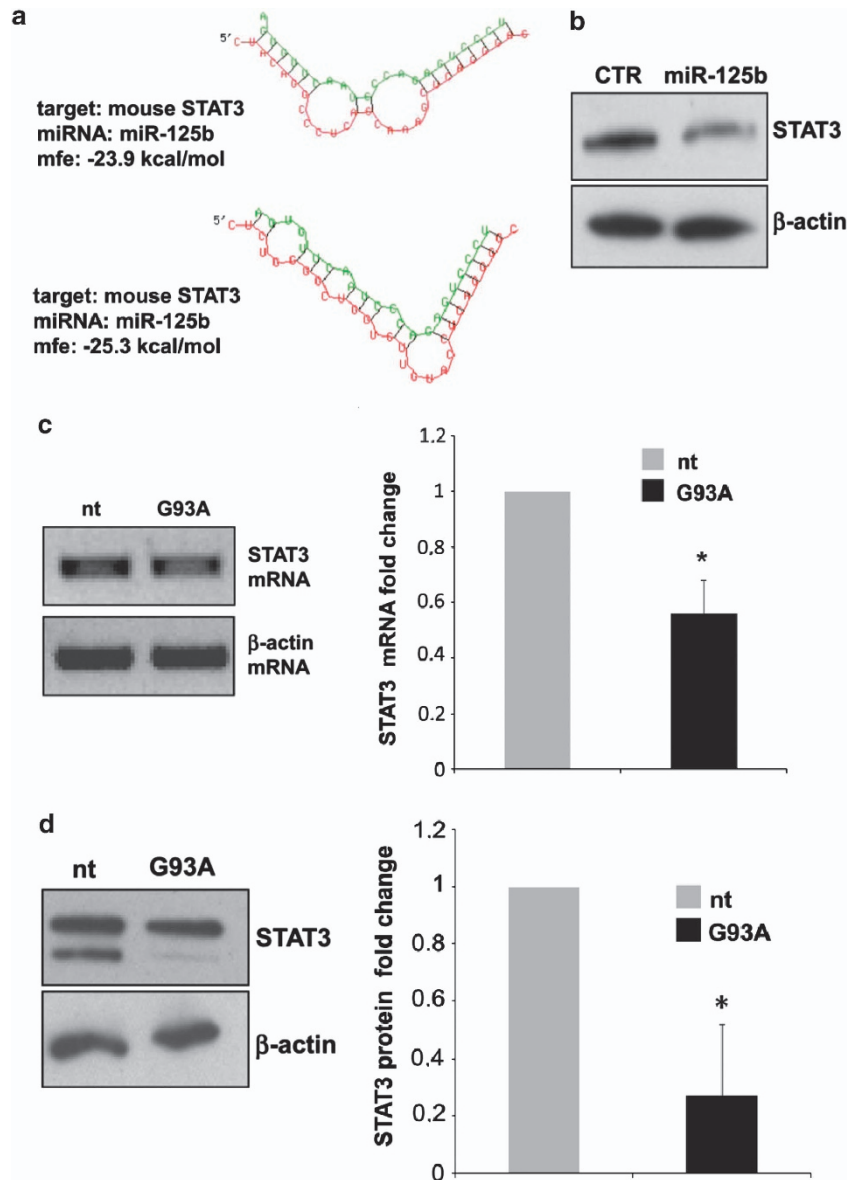


Figure 4 MiR-125b-mediated STAT3 downregulation in SOD1-G93A mouse microglia. (a) The two minimum free energy duplexes of miR-125b-5p and 3'-UTR of mouse STAT3 (NM_011486.4) as predicted by RNA hybrid. (b) Western blotting with anti-STAT3 antibody of total lysates from empty vector and pprime-miR-125b infected microglia at 96 hours post virus transduction. β -actin was used for protein normalization. (c) Semiquantitative RT-PCR using specific primers for STAT3 and β -actin mRNAs of total RNA from nt and SOD1-G93A microglia. β -actin was used for normalization. (d) Western blotting with anti-STAT3 antibody of total lysates from nt and SOD1-G93A microglia. β -actin was used for protein normalization

we transduced nt cells with a lentiviral system driving the overexpression of mature miR-125b and performed western blot analysis at 96 h post transduction. We demonstrated that exogenous miR-125b reduces STAT3 protein (Figure 4b). Having demonstrated that STAT3 is a target of miR-125b (Figure 4b), and that miR-125b is increased in ALS (Figures 1c and d), we then directly measured the content of STAT3 in ALS microglia, by semiquantitative RT-PCR and western blotting. The reduction of STAT3 that we demonstrated in SOD1-G93A microglia at both mRNA and total protein levels (Figures 4c and d) thus underlines a novel inverse correlation between STAT3 and miR-125b levels.

TNF α regulation by miR-365 and miR-125b. Elevated production of TNF α is a common feature of several inflammatory diseases including ALS. We demonstrated here that TNF α mRNA increases about 40% in SOD1-G93A microglia (Figure 5a), thus confirming previous data on protein level.²³ As the IL-6/STAT3 pathway is known to downregulate TNF α synthesis³⁰ only in monocytes after LPS stimulation and similar data are not available in microglial cells, in order to validate the hypothesis that miR-365 and miR-125b regulate TNF α through the IL-6/STAT3 pathway, we transfected SOD1-G93A microglia with specific miR-365/miR-125b inhibitors and demonstrated an approximately

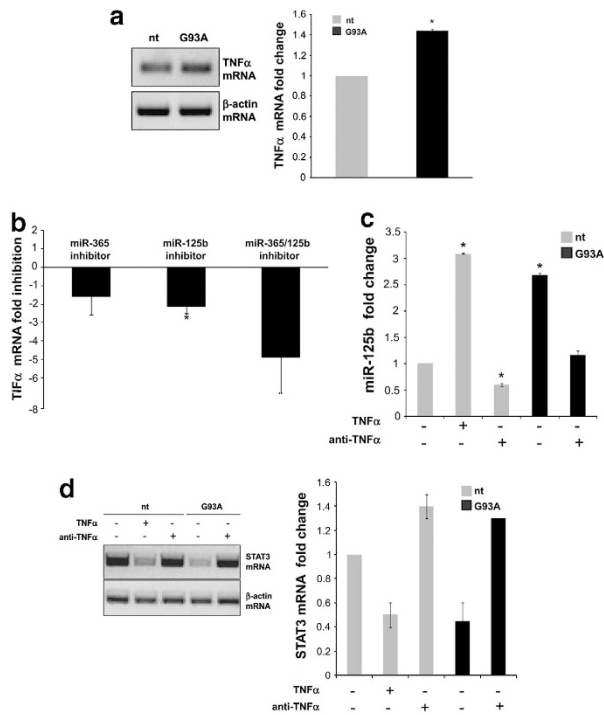


Figure 5 TNF α -mediated miR-125b upregulation and STAT3 repression in microglia. (a) Semiquantitative RT-PCR performed using specific primers for mouse TNF α and β -actin mRNAs on nt and SOD1-G93A microglia total RNA. β -actin was used for normalization. (b) QPCR quantification of TNF α mRNA was performed at 48 h after transfection with the specified miRNA inhibitors. (c) QPCR quantification of miR-125b and (d) semiquantitative RT-PCR of STAT3 mRNA in nt and SOD1-G93A microglia upon 24 h of TNF α (10 ng/ml) or anti-TNF α (0.5 ng/ml) treatment

fivefold downregulation of TNF α mRNA by QPCR (Figure 5b). Most importantly, the single transfection of miR-125b inhibitor directly targeting STAT3 3'-UTR, was able *per se* to produce a significant inhibition of TNF α mRNA (Figure 5b).

TNF α regulation of STAT3 via miR-125b in microglia. TNF α is known to regulate miR-125b expression in monocytes and in a macrophage cell line after LPS stimulation,^{31,32} whereas this mechanism is not yet proven in microglia. We thus investigated whether exogenous TNF α could directly control miR-125b production in ALS microglia. We stimulated nt cells with TNF α (10 ng/ml) for 24 h and by QPCR we found that miR-125b was overexpressed to levels comparable to ALS microglia (Figure 5c). The treatment with anti-TNF α moreover restored miR-125b to basal levels in SOD1-G93A microglia and even more in nt cells. To further prove that STAT3 is a target of miR-125b, we performed semiquantitative RT-PCR on STAT3 mRNA of nt and SOD1-G93A microglia exposed to TNF α or anti-TNF α treatments. We demonstrated a previously unknown TNF α -dependent inverse correlation occurring between miR-125b and STAT3 (Figure 5d).

P2X7r-driven miRNA expression profile of brain microglia from wild-type mice. To identify those miRNAs whose expression is regulated by activation of microglia for instance through P2X7r, a miRNA microarray analysis was carried out

using RNA isolated from nt and SOD1-G93A microglia upon 2 h from activation with the P2X7r agonist 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate (BzATP, 100 μ M). As one of the recognized effects of BzATP on ALS microglia is the induction of TNF α content,²³ we evaluated the miRNA expression profile in microglia at the peak of BzATP-mediated TNF α mRNA induction. This occurred at 2 h, in both nt and SOD1-G93A microglia (Supplementary Figure 2). Under these conditions, most of microglia-expressed miRNAs were found upregulated in nt cells (Figure 6a), thus showing a similar effect to that obtained by overexpression of SOD1-G93A in microglia. Among the P2X7r-induced miRNAs, 22 resulted more significantly upregulated (Figure 6b) and miR-155, miR-22 upregulation was then confirmed by both microarray and QPCR (Figure 6c). Induction of miR-146b and miR-125b was instead proved to be statistically significant only by QPCR analysis (Figure 6c).

P2X7r-driven miRNAs regulation in microglia from ALS mice. As previous results from our lab^{23,24} demonstrated that the inflammatory effect of BzATP in microglia is enhanced in SOD1-G93A when compared with nt cells, we investigated this effect also regarding miRNAs expression. QPCR was performed to assess the levels of miR-125b, miR-146b, miR-155, and miR-22 in SOD1-G93A microglia upon 2 h of treatment with 100 M BzATP. Also in this case, we observed an increase of miR-125b, in addition to miR-146b and miR-155 in both nt and ALS microglia (Figure 6c). Surprisingly, miR-22 expression was significantly downregulated only in SOD1-G93A microglia upon BzATP treatment.

Discussion

The responses of brain-resident macrophages, microglia, rely on dynamic, specific and tightly regulated gene expression changes ensuring the exact quality and quantity of inflammatory mediators in a time-dependent manner. Dysregulation of this system, leading to uncontrolled immune cell activation, is associated with progression of neurodegenerative diseases.³³ A prominent part in orchestrating microglia activation is reserved to protein transcription factors that induce a battery of genes responsible for switching on/off the inflammatory action. However, transcriptional regulation fails to explain all the complex timing and cell specificity of gene expression, thus suggesting the existence of additional layers of molecular control. MiRNAs operating as fine tuners of post-transcriptional events mediate neuronal gene expression³⁴ and are presently emerging as key factors also in microglia reactivity.¹⁵ As a consequence, miRNA dysregulation becomes instrumental for understanding many neuro-inflammatory diseases, including ALS.³⁵ In the present study, we examined the role of miRNAs in brain microglia from healthy mouse and carried out a comparative analysis with the SOD1-G93A ALS microglia. We performed miRNAs expression profiling by using the standardized microarray approach that is considered the most accurate to this purpose.³⁶ We established that brain microglia maintain their immune system signature despite their brain location, as shown by the expression of mostly immune-enriched miRNAs. This

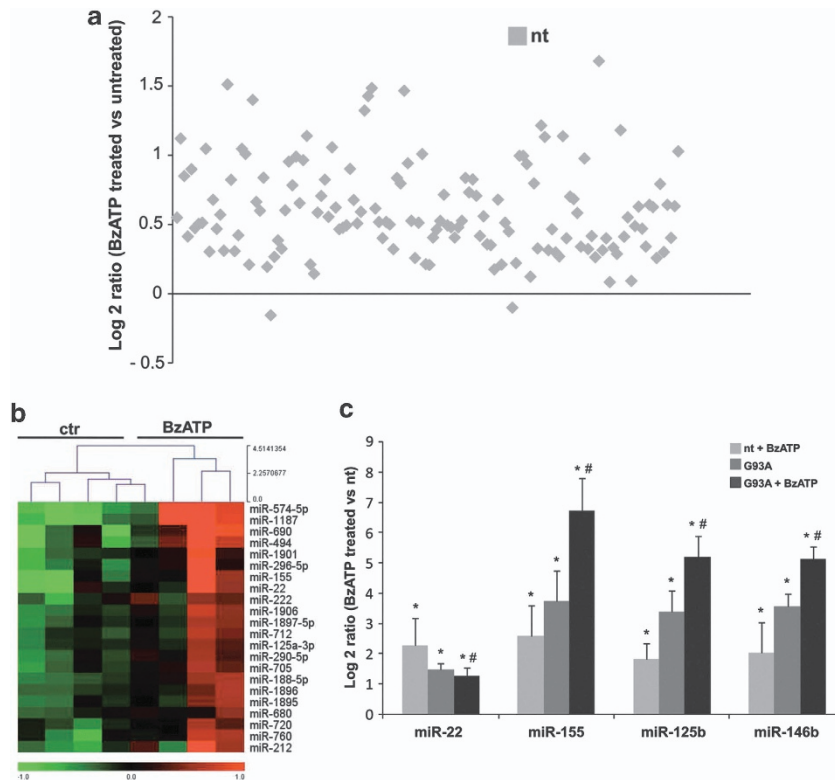


Figure 6 P2X7r-mediated miRNAs regulation in nt and ALS microglia. (a) Overall statistics of down- and upregulated miRNA genes in BzATP-treated nt microglia. Log₂ ratios (BzATP-treated nt/untreated nt) are shown. (b) Heat maps of 22 differentially expressed miRNAs in BzATP-treated nt microglia. Red to green indicates high to low expression ($P \leq 0.05$). (c) QPCR data of four differentially expressed miRNAs in BzATP-treated and untreated nt and SOD1-G93A microglia. Data are expressed as log₂ $\Delta\Delta$ CT with respect to untreated nt. * $P \leq 0.05$ with respect to untreated nt; # $P \leq 0.05$ with respect to BzATP-treated nt

corroborates the similarity between microglia and circulating macrophages previously shown by mRNA profiling.³⁷ In addition, we found that overexpressed mutated SOD1 is able *per se* to significantly upregulate a huge proportion of the brain-resident microglia miRNAs. Our findings in brain microglia further extend those reported by Butovsky *et al.*³⁸ using CD39-selected microglia from spinal cord and corroborate the importance of miRNAs dysregulation in SOD1-G93A ALS microglia. Despite the intrinsic CNS site-specific difference of microglia,³⁹ several miRNAs such as miR-146b, miR-29b, let-7a/b, miR-27b, miR-21, miR-210 and miR-155 were similarly found upregulated. Among those miRNAs that we validated, miR-125b, miR-146b and miR-155 are typical components of the innate immune system,⁴⁰ with miR-125b overexpression being also responsible for direct macrophage activation.⁴¹ Moreover, upregulation of miR-125b causing neuroinflammatory detrimental downregulation of the immune system repressor CFH⁴² was also demonstrated in Alzheimer's disease brain tissue, as well as in primary neuronal-glia cells during ROS-generating oxidative stress.⁴³ For this reason, we hypothesized that miR-125b overexpression might represent a cross-road among different neuroinflammatory conditions. As both Alzheimer's disease⁴⁴ and ALS^{45,46} share the pathological activation of cytokine-mediated nuclear factor κ B (NF- κ B) signaling, and miR-146, miR-155, miR-214 and miR-365 all converge in NF- κ B-mediated immune cell regulation,^{26,47} it will be interesting to verify

whether dysregulation of these specific miRNAs might be instrumental to abnormal NF- κ B activation in ALS.

Although neuroinflammation is a fundamental component of ALS progression, with microglia having a key causative role in the process, whether the expression of inflammatory cytokine pathway is directly regulated by miRNAs in ALS microglia has not been previously determined.

A pathological hallmark characterizing ALS is an abnormal production and release of inflammatory cytokines such as TNF α and IL-6.^{48–50} Although studies performed on total extract of mouse spinal cord found that both IL-6 and TNF α levels are increased in SOD1-G93A model,^{51,52} IL-6 results instead downregulated in isolated spinal cord microglia from asymptomatic phase to end stage of the disease,⁴⁸ suggesting that the release of IL-6 *in vivo* is not cell autonomous. Consistently with these last findings, we demonstrated a significant IL-6 downregulation in ALS brain microglia cultures.

Being known that IL-6 is a validated target of miR-365 only in human system,²⁶ with our work we demonstrated that miR-365/IL-6 interaction is conserved also in mouse, despite a single mismatch in the seed region. The presence of functional noncanonical sites with a single mismatch in the seed region is for instance reported also in the majority of miR-155 targets.⁵³ In support of the miR-365/IL-6 targeting, we next demonstrated that IL-6 protein content is inhibited by

exogenous miR-365 in primary microglia. As we also proved here that IL-6 mRNA and protein are constitutively down-regulated in ALS microglia, we established for the first time that upregulation of miR-365 is at least in part responsible for IL-6 dysregulation in ALS.

STAT3 is known to be the first downstream effector of IL-6 and, moreover, a validated target of miR-125b in both human⁵⁴ and mouse.²⁹ Here we demonstrated that also STAT3 total protein is decreased in primary microglia in the presence of exogenous miR-125b. Moreover, we found that STAT3 mRNA and total protein are decreased in ALS microglia, suggesting that STAT3 downregulation is directly controlled by miR-125b overexpression.

It is well established that IL-6 can mediate pro-inflammatory, but also anti-inflammatory signals,^{55,56} with transcriptional TNF α reduction^{30,57} as a likely readout of protective pathways, and upregulation of TNF α mostly associated with the detrimental roles of microglia.⁵⁸ The present finding of upregulated TNF α transcription in ALS microglia thus confirms our previous studies at protein level²³ and above all suggests that miR-365 and miR-125b upregulations might directly prevent the anti-inflammatory function of IL-6/STAT3. This is furthermore supported by the suppressing role exerted by inhibitors of miR-365/125b on TNF α mRNA expression that we demonstrated for the first time in SOD1-G93A ALS microglia. In this way, we established a direct causative mechanism involving mutated SOD1 expression and miRNAs dysregulation and giving rise to abnormal cytokine production, a phenomenon typically corresponding to detrimental M1 microglia phenotype (Figure 7).

One of the most widespread microglia activator, extracellular ATP binding to purinergic P2 receptors, is known to markedly increase in the nervous system in response to ischemia, trauma and several neuroinflammatory insults comprising ALS.⁵⁹ Among P2 receptors, the ionotropic P2X7r subtype mainly expressed in the CNS by microglia exerts several pro-inflammatory functions. In ALS patients, as well as SOD1-G93A models, increased immunoreactivity for P2X7r has been extensively documented in the spinal cord and brain microglia.²⁰ Moreover, we demonstrated that the pro-inflammatory action of microglial P2X7r is enhanced in the SOD1-G93A model and in turn drives increased neurotoxicity.^{23,24} Furthermore, a tuned balance of P2X7r activation was proven to be important for disease progression, as shown after ablation of P2X7r in the SOD1-G93A mouse model.⁶⁰ As several studies have identified a prominent and complex role of miRNAs as key modulators of signal propagation as well as pathological conditions, we decided to verify whether miRNAs might indeed constitute one way through which P2X7r activation participates to ALS pathogenesis and, in particular, to the inflammatory circuit described above. With our work, we proved that extracellular BzATP binding to P2X7r upregulates miRNAs transcriptome that was found similarly upregulated in ALS microglia. This finding highlights miRNAs as crossover in the signal transduction mechanisms of inflammation mediated by both P2X7r and ALS. Most importantly, as BzATP hyperactivates miR-155, miR-125b and miR-146b that are already upregulated in SOD1-G93A microglia, we suggest that these specific miRNAs might constitute an important mechanism by which P2X7r activation exacerbates the

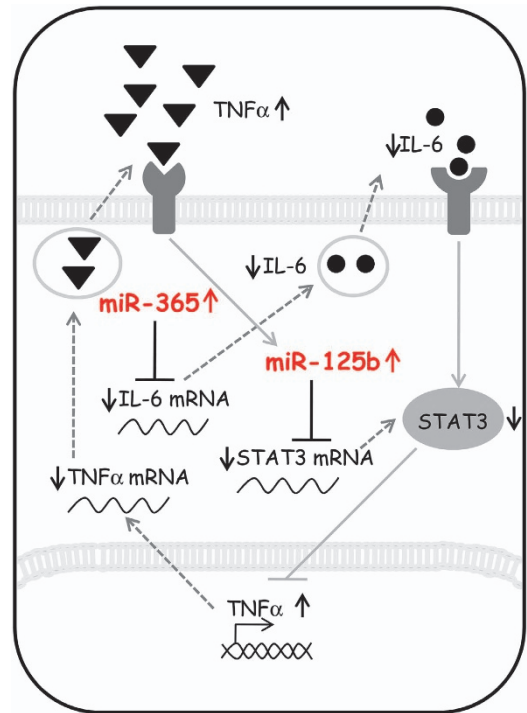


Figure 7 Proposed interplay of miR-365 and miR-125b in IL-6-STAT3 pathway and TNF α production. Mir-365 and miR-125b are overexpressed in SOD1-G93A microglia and cooperate to repress IL-6-STAT3 pathway by direct targeting. The repression of this pathway increases the activation of TNF α gene transcription. TNF α produced and released in medium is able to upregulate miR-125b, thereby enhancing its own transcription

detrimental phenotype of ALS microglia. By acting as genetic switchers or fine tuners, in our opinion miRNAs might become stringent modulators of the huge variety of cellular responses carried out by different inflammatory stimuli under both physiological and pathological conditions.

In conclusion, by comparative screening of miRNAs in resting and activated SOD1-G93A microglia, our work has identified selected miRNAs to be used as novel tools for further dissecting and controlling neuroinflammatory mechanisms. In particular, we established a pathogenic mechanism in ALS microglia that correlates the induction of miR-365 and miR-125b to IL6/STAT3 downregulation and consequent enhancement of TNF α production, all responsible for switching microglia toward a detrimental phenotype. Moreover, our results disclosing that purinergic stimulation *per se* can regulate miRNAs in brain microglia will encourage the exploitation of purinergic-regulated miRNAs in the context of ALS and additional neuroinflammatory diseases.

Materials and Methods

Primary microglia cell cultures. Mixed glial cultures from the brain cortex were prepared as previously described.²³ Briefly, neonatal SOD1-G93A and nt littermate mice were killed and, after removing the meninges, cortices were minced and digested with 0.01% trypsin and 10 μ g/ml DNaseI. After dissociation and passage through 70 μ m filters, cells were resuspended in DMEM/F-12 media with GlutaMAX (Gibco, Life Technologies, Paisley, UK) plus 10% fetal bovine serum (FBS), 100 units/ml gentamicin and 100 μ g/ml streptomycin/penicillin at a density of

62 500 cells/cm². After ~15 days, a mild trypsinization (0.08% in DMEM/F-12 without FBS) was performed for 40 min at 37 °C to remove non-microglial cells. The resultant adherent microglial cells (pure >98%) were washed twice with DMEM/F-12 and kept in 50% mixed glial cells conditioned medium at 37 °C in a 5% CO₂ and 95% air atmosphere for 48 h until used.

Microarray. For the miRNA profiling by Agilent Platform (Agilent Technologies, Milan, Italy), total RNA (100 ng) was used. The samples were labeled using the Agilent miRNA Complete Labelling and Hyb Kit (5190-0456) according to Agilent's procedure (G4170-90011, version 2.4, September 2011). The cyanine-3-labeled miRNAs were hybridized on Agilent Mouse miRNA Microarrays 8 × 15K V2 (G4472B) containing 627 mouse miRNAs and 39 mouse viral miRNAs. Hybridizations were performed at 55 °C for 20 h in a rotating oven. Microarrays were then washed with Agilent Gene Expression Buffer 1 for 5 min at room temperature and Agilent Gene Expression Wash Buffer 2 for 5 min at 37 °C. A final treatment with acetonitrile for 1 min at RT was performed. Post-hybridization image acquisition was accomplished using the Agilent scanner G2564B. Data extraction from the images was accomplished by Agilent Feature Extraction ver 10.7 software, using the standard Agilent one-color miRNA expression extraction protocol. Data analyses were performed using Agilent GeneSpringGX, Multi-ExperimentViewer for Clustering (TIGR), Microsoft Excel (Microsoft Inc., Redmond, WA, USA) and R Bioconductor (Seattle, WA, USA).

Target prediction of ALS-dysregulated miRNAs. MiRNA targets were predicted by combinatorial utilization of three different web-based prediction algorithms: TargetScan (<http://www.targetscan.org/>), miRanda (<http://www.micromi.org>) and RNA hybrid.²⁸ We also collected the experimentally *in vitro*-confirmed targets and inflammatory-related miRNAs by literature curation, and combined them with the above results.

Quantitative RT-PCR. Total RNA including small RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's instruction and was checked with the Nanodrop 100 System and the Agilent 2100 bioanalyzer. QPCR for miRNAs quantification was conducted using miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) following reverse transcription using miScript Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. All miRNA primers were purchased from Qiagen and the relative expressions were calculated using the comparative CT method with mouse U6 small nuclear RNA as the normalizing control. QPCR for TNF α quantification was performed using SYBR Green select (Applied Biosystem, Milan, Italy) following reverse transcription using Enhanced Avian RT kit (Sigma-Aldrich, Milan, Italy). Primers were as follows: TNF α forward 5'-CTGTAGCCACGTCGTAGC-3'; TNF α reverse 5'-TTGAGATCCATGCCGTTG-3'; GAPDH forward 5'-CATGGCCTTCGGTTCCTCA-3'; GAPDH reverse 5'-CCTGCTTACCACCTTCTTGAT-3'.

Semiquantitative RT-PCR. Primary microglial cells were lysed with TRIzol (Invitrogen) and total RNA was extracted following the manufacturer's instructions. After DNase treatment (Qiagen), equal amount of total RNA (1 μ g) was subjected to retro-transcription by enhanced avian RT-PCR kit (Sigma-Aldrich) and 50 ng of each cDNA were amplified with either mouse IL-6-specific primers (F: 5'-CCAGAAACCGCTATGAAGTTC-3'; R: 5'-CCATTGCACAACCTTTTCTCAT-3') or mouse STAT3-specific primers (F: 5'-CAGAAAGTGCCCTCAAGGGCG-3'; R: 5'-CGTTGTTAGACTCCTCCATGTTCC-3'). The number of cycles was fixed to 30, after verifying that under these conditions the DNA polymerase (Sigma-Aldrich) presented a linear activity in amplifying cDNAs. Moreover, to verify that the same quantity of DNA was used in each reaction, actin primers (F: 5'-ATCCTGTGGCATCCATGAAAAC-3', R: 5'-AACGCAGCTCAGTAACAGTC-3') were inserted in each PCR for 18 cycles. Amplification products (15 μ l of 50) were electrophoresed on 2% agarose gel containing ethidium bromide (1 μ g/ml, Sigma-Aldrich), photographed under UV light and quantified using Kodak Image Station 440CF (Eastman Kodak, Rochester, NY, USA), with Molecular Imaging Software 4.0.1 (Eastman Kodak).

Antibodies. IL-6 rabbit polyclonal antibody (1:500) was obtained from Millipore (Merck Millipore, Merck KGaA, Darmstadt, Germany); STAT3 rabbit polyclonal antibody (1:1000) from Cell Signalling Technology Inc. (Beverly, MA, USA); and β -actin mouse antibody (1:2500) from Sigma-Aldrich. HRP-linked anti-rabbit and anti-mouse antibodies were obtained from Cell Signalling Technology Inc.

Protein extraction, SDS-polyacrylamide gel and western blotting.

In order to isolate total protein extracts, cells in serum-free medium were collected with ice-cold RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) and added with protease inhibitor cocktail (Sigma-Aldrich). Lysates were kept for 30 min on ice and then centrifuged for 10 min at 14 000 × g at 4 °C. Supernatants were collected and assayed for protein quantification by the BCA method (Thermo Fischer Scientific, Rockford, IL, USA). Analysis of protein components was performed by polyacrylamide gel separation (Bio-Rad, Laboratories, Milan, Italy) and transfer onto nitrocellulose membranes (Amersham Biosciences, Cologno Monzese, Italy). After saturation, blots were probed overnight at 4 °C, with the specified antibody, and finally incubated for 1 h with HRP-conjugated secondary antibodies and detected on X-ray film (Aurogene, Rome, Italy) using ECL Advance western blotting detection kit (Amersham Biosciences). Quantifications were performed using Kodak Image Station.

ELISA. Microglia were incubated for 6 h in fresh media without serum, and mouse IL-6 expression was measured in the supernatants using a commercial mouse IL-6 ELISA kit (Invitrogen), according to the manufacturer's instructions.

MiR-365 and miR-125b constructs, transfection and virus transduction.

To generate pPRIME-dsRed-miR-125b and pPRIME-dsRed-miR-365, their respective hairpins were PCR amplified (F: 5'-GAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG-3'; R: 5'-CTAAAGTAGCCCCTTGAATCCGAGGCGAGTAGGCA-3') from the following templates: miR-125b 5'-TGCTGTTGACAGTGAGCGTCCCTGAGACCCTAAGTGTGATAGTGA AGCCACAGATGTAACCGGTTAGGCTCTTGGGAGCTTGCCTACTGCTCCGGA-3'; miR-365 5'-TGCTGTTGACAGTGAGCGATGAGGACTTTGGGGCAGATGTGTAGTGAAGCCACAGATGTACATAATGCCCTAAAAATCCTTATTGCTCTTGC TGCTACTGCTCCGGA-3' and cloned into the *Xho*I and *Eco*RI sites of the plasmid pPRIME-CMV-dsRED. All pPRIME-dsRed constructs were verified by automated sequencing and then purified and co-transfected together with packaging vectors into HEK-293FT cells. Supernatants were collected after 48 and 72 h, and viral particles were concentrated by ultracentrifugation for 2 h at 26,000 r.p.m. (Ultraclear Tubes, SW28 rotor, and Optima L-100 XP Ultracentrifuge; Beckman Coulter, Milan, Italy) and recovered by suspension in HBSS (Sigma-Aldrich). Titers of viral particles ranged between 10⁶ and 10⁷ TU/ml. Viral particles and polybrene (8 μ g/ml) were then added to isolated primary microglia. Lentiviral particles at a multiplicity of infection of 30 and 8 μ g/ml polybrene (Sigma-Aldrich) were added to the culture. Supernatant was removed 5 h after infection and replaced with DMEM-F-12 medium containing 10% FBS. In all the experiments, the efficiency of microglia transduction was at least 90%, as determined by counting the number of microglia expressing the dsRed molecule and counter-staining nuclei with Hoechst 33258 (1 μ g/ml for 5 min) by means of a fluorescent microscope. All experiments were performed at 96 h post infection.

Cell transfection. Primary microglia (5 × 10⁵ per well) were plated for 48 h and transfection of 20 nM miRIDIAN Hairpin inhibitors (Dharmacon Products, Thermo Fisher Scientific) was performed with lipofectamine 2000 (Invitrogen) according to manufacturers' instructions. We used a negative control inhibitor based on *Caenorhabditis elegans* miRNAs not found in humans (miRIDIAN miRNA hairpin inhibitor negative control).

Luciferase assay. The 3'-UTR of the mouse IL-6 was PCR amplified with *Xba*I-flanked primers (F: 5'-GCGCGctctagaTAGTGCGTTATGCCCTAAGCA-3'; R: 5'-GCGCGctctagaGTTTGAAGACAGTCTAAACAT-3'). The PCR products were purified, digested and cloned downstream of the luciferase-coding region in the pRL-TK vector (Promega, Milan, Italy). pRL-TK containing mutant IL-6 UTR was generated by PCR site-directed mutagenesis (F: 5'-GCATATCAGTTTGTGcACAgTCCACTGTGGTCAAG-3'; R: 5'-CTGACCACAGTGAGGAcTGTgCACAAA CTGATATGC-3') using wild-type IL-6 UTR plasmid as a template, followed by digestion with *Dpn*I. Human HEK293T cell line was co-transfected with respective luciferase constructs and pprime-miR-365 or empty vector along with firefly luciferase expression plasmid (PGL3) as a transfection control using Lipofectamine (Invitrogen). After 48 h, cells were lysed and analyzed for relative luciferase activity using the Dual Luciferase Assay Kit (Promega). Results are representative of three independent experiments.

Statistical analysis. Data are presented as mean ± S.E.M. Normality of data was assessed by the Shapiro-Wilk test. Statistical differences were verified by

Student's unpaired two-tailed *t*-test or nonparametric Wilcoxon's Mann–Whitney test using MedCalc (Medcalc Software, Mariakerke, Belgium). **P* < 0.05 was considered significant.

Conflict of Interest

The authors declare no conflict of interest.

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