

MicroRNA-326 Upregulates B Cell Activity and Autoantibody Production in Lupus Disease of MRL/lpr Mice

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B cells are recognized as key participants in various autoimmune diseases, including systemic lupus erythematosus (SLE). Although sets of transcription factors and cytokines are known to regulate B cell differentiation, the roles of microRNAs are poorly understood. Our previous study proved that microRNA-326 (miR-326) was markedly upregulated in SLE patients; however, the biological function of miR-326 during SLE pathogenesis remained unknown. In this study, we found that miR-326 overexpression in MRL/lpr mice led to B cell hyperactivity and severe SLE. Moreover, E26 transformation-specific-1 (Ets-1), a negative regulator of B cell differentiation, was identified as a target of miR-326. Therefore, a novel mechanism has been found in which the elevated miR-326 in B cells of SLE promotes plasmablast development and antibody production through downregulation of Ets-1.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex inflammatory autoimmune disease. One of the hallmarks of this disease is the production of antinuclear autoantibodies by uncontrolled over-activated B cells.¹ These autoantibodies can form immune complex deposition in different tissues and organs, resulting in chronic inflammation and tissue damage. Renal involvement (lupus nephritis [LN]) is a severe consequence of SLE and an important cause of morbidity and mortality in SLE.²

Autoreactive B cells have an important role in SLE by producing autoantibodies, presenting autoantigens to T cells, and secreting proinflammatory cytokines.³ Increased plasmablast numbers correlate well with the titers of double-stranded DNA (dsDNA)-specific antibodies and disease flares in patients with SLE.⁴ A substantial increase in plasmablast numbers can be observed around 7 days after secondary immunization.⁵ The transcription factor E26 transformation-specific-1 (Ets-1) is a negative regulator of B cell differentiation, and Ets-1 deficiency can drive terminal differentiation of B cells into immunoglobulin M (IgM)- and immunoglobulin G (IgG)-secreting plasma cells.⁶ Despite the lines of evidence described above, the upstream mechanisms of Ets-1 controlling B cells in SLE were not completely understood.

MicroRNAs (miRNAs) represent a large family of endogenous noncoding small RNAs that negatively regulate gene expression at the post-transcriptional level. A growing body of literature has revealed essential roles of miRNAs in the regulation, development, and function of the immune response, and they could potentially serve as disease biomarkers and therapeutic targets.⁷ miRNAs are critical in maintaining immune tolerance, and Dicer, one of the RNase III family enzymes, plays an important role in the biogenesis of miRNAs. Regulatory T cell (Treg)-specific Dicer ablation leads to fatal autoimmune disease.⁸ Mice lacking Dicer in B cells spontaneously developed lupus-like disease, characterized by high titers of serum autoantibodies and renal immune complex deposition.⁹ Changes in miRNA expression profiles have been identified in various autoimmune diseases, including multiple sclerosis (MS), rheumatoid arthritis (RA), and SLE. It has been reported that miR-326 correlates well with disease severity and interleukin-17 (IL-17) production, suggesting a key role for miR-326 in Th17 cell function.¹⁰ However, the role of miRNA-326 in B cells tolerance remains unexplored.

In the present study, we investigated the role of miR-326 in SLE B cell differentiation and found that silence or overexpression of miR-326 alleviated or aggravated SLE, respectively. In addition, Ets-1, a negative regulator of B cell differentiation, was confirmed as a target of miR-326. Our data suggest that miR-326 is a B cell-associated miRNA that contributes to the pathogenesis of SLE.

RESULTS

miR-326 Regulates B Cell Differentiation

To identify whether miR-326 regulates B cell differentiation, we performed an *in vivo* injection of lentivirus (LV)-326 or LV-sponge to

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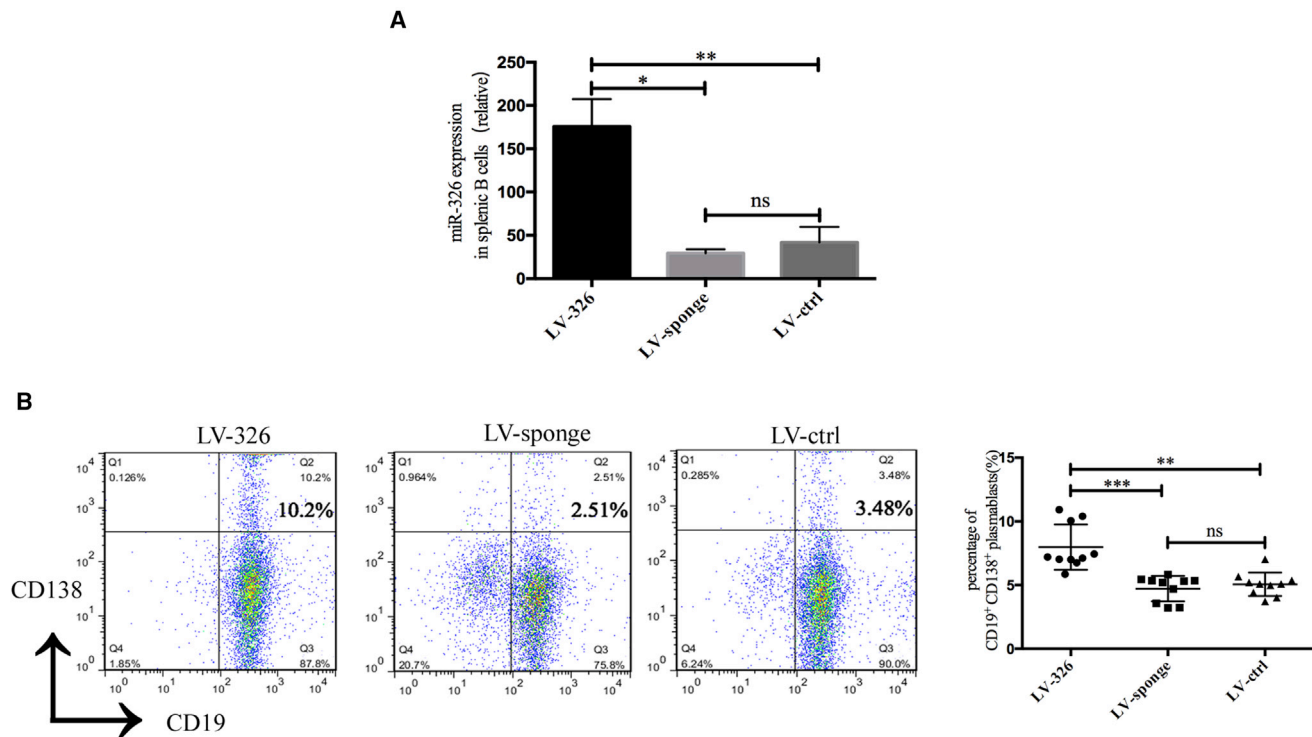


Figure 1. miR-326 Regulates B Cells Differentiation

Splenic CD19⁺ B cells were isolated from LV-326, LV-sponge, and LV-ctrl mice. (A) Real-time PCR analysis of levels of miR-326 in CD19⁺ B cells among the three groups on day 7 after lentivirus injection. (B) Flow cytometry analysis of the percentage of CD19⁺CD138⁺ plasmablasts in splenic CD19⁺ B cells among the three groups on day 7 after lentivirus injection. Data are presented as mean \pm SEM. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

increase or silence miR-326 expression in MRL/lpr mice, respectively (Figure 1A). Mouse B lymphocytes (CD19⁺ cells) from the spleen were purified, and we analyzed the percentage of plasmablasts (CD19⁺ CD138⁺ cells) in B lymphocytes. As shown in Figure 1B, LV-326-injected mice had a higher frequency of plasmablasts in splenic CD19⁺ B cells compared with LV-sponge-injected mice and LV-control (ctrl)-injected mice. No significant difference in miR-326 expression and frequency of plasmablasts between LV-ctrl-injected mice and non-LV-injected mice was observed.

miR-326 Enhances the Levels of Anti-dsDNA in Serum

To further investigate the contribution of miR-326 to B cell differentiation in MRL/lpr mice, serum titers of IgG anti-dsDNA autoantibody were quantified by ELISA. As shown in Figures 2A and 2B, increasing or silencing the expression of miR-326 significantly upregulated or downregulated the serum levels of IgG anti-dsDNA autoantibody, respectively. Compared with LV-sponge-injected and LV-ctrl-injected mice, LV-326-injected mice had higher titers of IgG anti-dsDNA 7 days after lentivirus injection (Figure 2D), and LV-sponge-injected mice had lower titers of IgG anti-dsDNA autoantibody, but with no statistical significance. Furthermore, the serum levels of IgG anti-dsDNA autoantibody were positively associated with the percentage of CD19⁺CD138⁺ plasmablasts ($r = 0.2527$) (Figure 2E).

miR-326 Regulates the Expression of B Cell Cytokines in Serum

The serum levels of IL-6 and IL-10 were significantly increased after injection of LV-326 (Figures 3A and 3B), whereas tumor necrosis factor (TNF) levels were not changed (Figure 3C). Moreover, 7 days after injection, the levels of IL-6 were significantly higher in LV-326-injected mice compared with LV-sponge-injected mice and LV-ctrl-injected mice (Figure 3D). However, there were no significant differences in serum levels of IL-10 and TNF among the three groups (Figure 3E and 3F).

miR-326 Promotes B Cell Hyperactivity via Ets-1

We investigated whether Ets-1, a critical gene for terminal differentiation of B cells into Ig-secreting plasma cells, is also targeted by miR-326 in SLE. *Ets-1* mRNA in CD19⁺ B cells was tested by real-time PCR analysis. LV-326-injected mice showed lower levels of *Ets-1* mRNA in CD19⁺ B cells, and LV-sponge-injected mice displayed higher levels of *Ets-1* mRNA in CD19⁺ B cells, both compared with LV-ctrl-injected mice (Figure 4A). In addition, the levels of *Ets-1* mRNA were negatively correlated with the expression of miR-326 in CD19⁺ B cells ($r = 0.5951$) (Figure 4B) and the percentage of plasmablasts ($r = 0.5132$) (Figure 4C). Higher Ets-1 expression in the kidneys of LV-sponge-injected mice compared with LV-326-injected mice and LV-ctrl-injected mice was also found (Figure 4D).

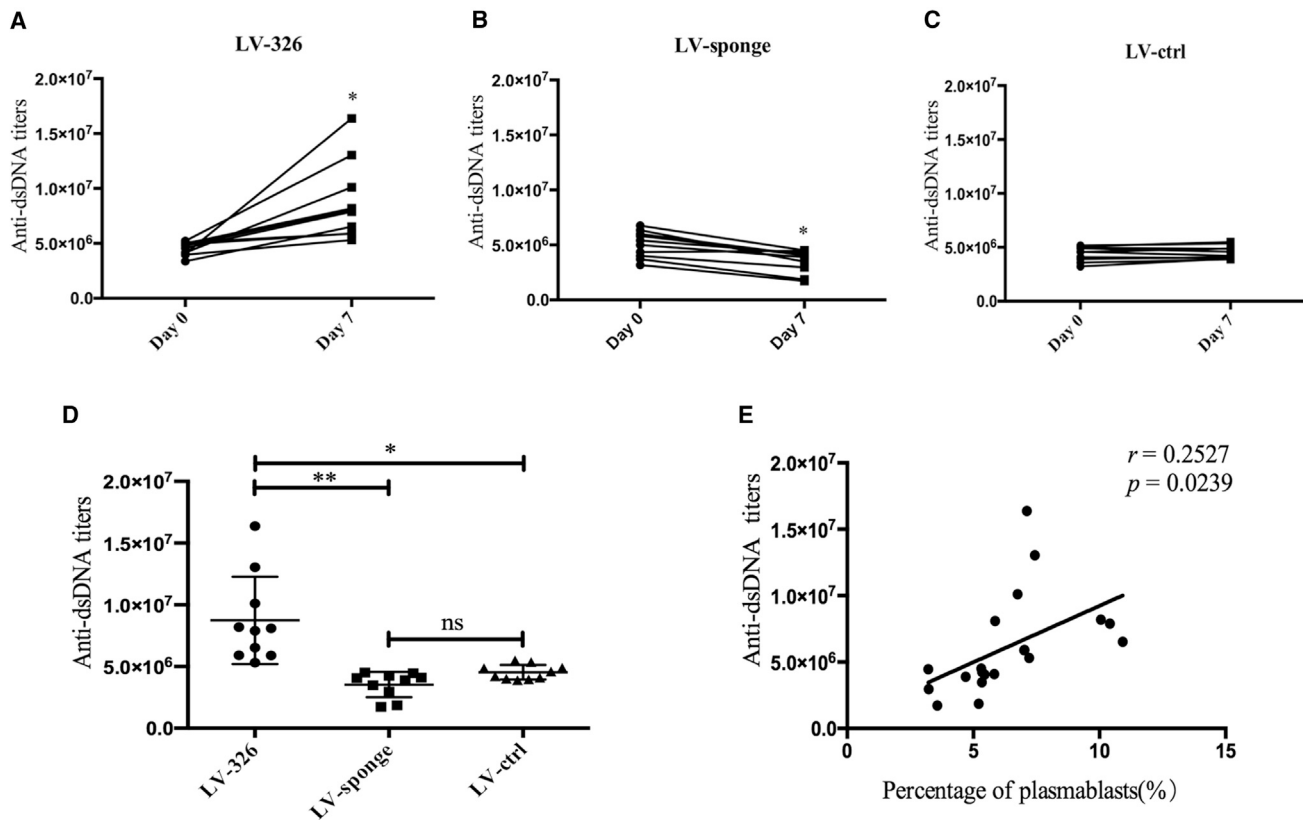


Figure 2. miR-326 Enhances the Serum Levels of Anti-dsDNA in MRL/lpr Mice

Serum was collected on day 0 and day 7 from LV-326, LV-sponge, and LV-ctrl mice. (A–C) ELISA analysis of the changes of serum titers of IgG anti-dsDNA autoantibody between day 0 and day 7 in LV-326(A), LV-sponge (B), and LV-ctrl (C). (D) ELISA analysis of serum titers of IgG anti-dsDNA autoantibody among the three groups on day 7 after lentivirus injection. (E) The serum levels of IgG anti-dsDNA autoantibody were positively correlated with the percentage of CD19⁺CD138⁺ plasmablasts ($r = 0.2527$). Data were determined using GraphPad Prism 6. Data are presented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

miR-326 Helps Immune Complex Deposits in the Kidneys

To assess whether B cells changes in our mice with lentivirus injection correlated with the Ig deposition in kidneys, we performed immunohistochemistry (IHC) in LV-326-injected mice, LV-ctrl-injected mice, and LV-sponge-injected mice. LV-326 injection increased the deposition of IgG, IgG1, and IgM in the mouse glomerulus (Figures 5A, 5B, and 5D), whereas IgG2 had no significant change (Figure 5C).

miR-326 Promotes the Development of LN

MRL/lpr mice often succumb to renal failure associated with the active inflammation and immune complex deposition in the kidneys. To investigate the effect of miR-326 in mouse kidney damage, miR-326 expression in the kidneys was tested by real-time PCR analysis. Data showed that the miR-326 levels in kidney tissue of LV-326-injected mice were higher than in LV-sponge-injected mice and LV-ctrl-injected mice (Figure 6A). H&E staining results showed that, compared with LV-ctrl-injected mice, LV-sponge-injected mice had lower mononuclear cell infiltration and LV-326-injected mice had more mononuclear cell infiltration (Figure 6B). Glomerular damage

was assessed by histological examination of periodic acid-Schiff (PAS) staining. LV-326-injected mice showed significant tissue injury in terms of intracapillary and extracapillary proliferative and necrotizing (crescentic) glomerulonephritis as well as tubulointerstitial inflammation (Figure 6C).

DISCUSSION

One of the characteristics of SLE is that B cell hyperactivity lead to the production of various autoantibodies.¹¹ The abnormal expression of miRNAs plays an important role in the pathogenesis of SLE.⁷ Recently, many studies of the expression of autoimmune-associated miRNAs have reported significant differences between SLE patients and healthy controls, which provided available information for understanding SLE pathogenesis and for promoting the development of biomarkers for diagnosis and new targets for treatment.¹² The underlying mechanisms of specific miRNA-mediated regulation of B lymphocyte development in SLE has been investigated; for example, decreased miR-1246 expression through the AKT-P53 signaling pathway and the downstream effect on the expression of EBF1, leading to further activation of B cells in SLE.¹³ In the present study, we

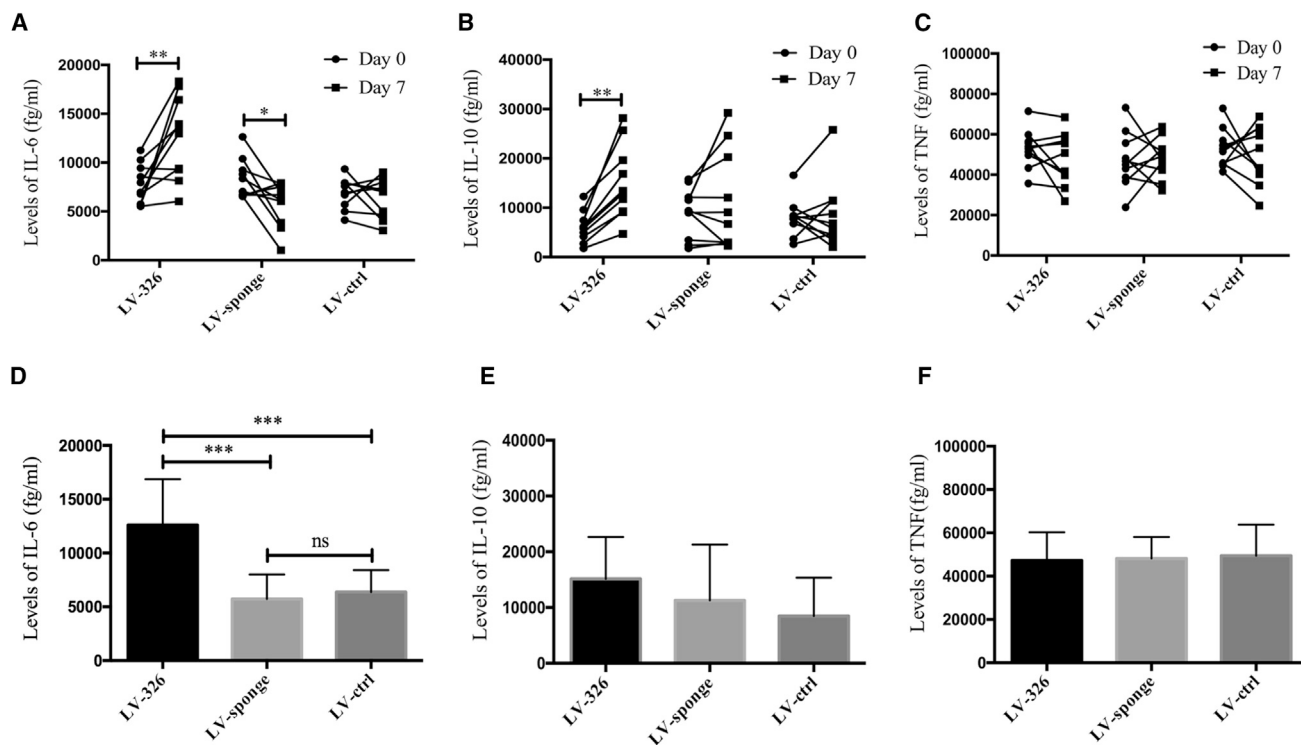


Figure 3. miR-326 Regulates the Expression of B Cell Cytokines in Serum

Serum was collected on day 0 and day 7 from LV-326, LV-sponge, and LV-ctrl mice. (A–C) CBA analysis of the changes in serum levels of IL-6 (A), IL-10 (B), and TNF (C) between day 0 and day 7. (D–F) CBA analysis of serum levels of IL-6 (D), IL-10 (E), and TNF (F) among the three groups on day 7 after lentivirus injection. Data are presented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

found, for the first time, that miR-326 is an effective regulator of B cell differentiation and autoantibody production in SLE.

miR-326, an autoimmunity-associated miRNA, has been found to be significantly increased in patients with MS and is a presumed diagnostic biomarker for the disease.¹⁰ However, the role of miR-326 in SLE has been poorly studied. Our previous study has demonstrated that the levels of miR-326 in SLE patients were increased,¹⁴ but its molecular mechanisms remain to be further studied. Here we demonstrated that overexpression of miR-326 in B cells enhanced the responsiveness of B cells through downregulating the expression of Ets-1.

Ets-1 plays a crucial role in the development of B cells and is highly expressed in normal B lymphocytes.^{15,16} Downregulation of Ets-1 in B cells can drive terminal differentiation of B cells into Ig-secreting plasma cells.¹⁷ Bories et al.¹⁸ reported that mice with a targeted mutation in the Ets-1 gene develop B cell terminal differentiation into IgM-secreting plasma cells, but the underlying mechanisms by which Ets-1 regulates B cell differentiation in SLE remained unclear. In recent years, several studies have drawn a connection between Ets-1 and autoimmune diseases.^{19–21} Ets-1 mRNA levels in peripheral blood mononuclear cells (PBMCs) of SLE patients were lower compared with healthy controls.²² Du et al.¹⁰ showed that miR-326

could suppress the expression of Ets-1 protein encoded by a wild-type Ets-1 3' UTR in a dose-dependent way. Additionally, miR-326 also regulated the expression of endogenous Ets-1 in CD4⁺ T cells.⁶ These data indicated that miR-326 specifically target Ets-1. In the current study, we identified that miR-326 could directly inhibit the expression of Ets-1 in B cells and that the level of Ets-1 was negatively associated with the percentage of plasmablasts, which suggested that the increased miR-326 could promote B cell differentiation via Ets-1. B cells lacking Ets-1 are generally hyperresponsive in terms of autoantibody production and development of Ig-secreting plasma cells.²³ Ets-1-deficient mice could develop lupus-like disease, characterized by high titers of IgM and IgG autoantibodies, including dsDNA.¹⁷ Here miR-326 overexpression mice exhibited higher titers of IgG anti-dsDNA autoantibody and more IgG, IgG1, and IgM deposition, suggesting that miR-326 is a positive regulator in facilitating autoantibody production, which might be a partial reason for B cell hyperactivity in the pathogenesis of SLE.

Cytokines play a key role in SLE as effectors. Elevated serum IL-6 levels are observed before the first positive autoantibodies in preclinical SLE, suggesting that IL-6 signals play an important role in maintaining B cell tolerance.²⁴ Arkatkar et al.²⁵ found that B cell-derived IL-6 is a costimulatory signal necessary for spontaneous germinal center (GC) formation, leading to B cell hyperactivity and systemic

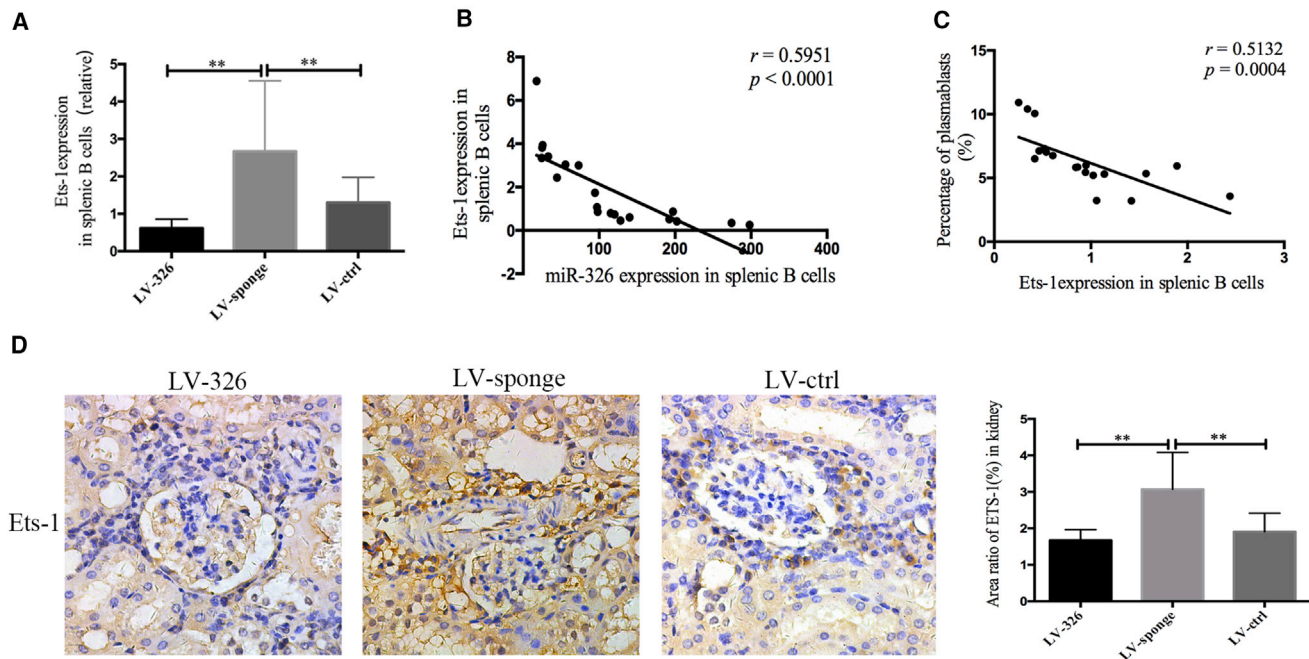


Figure 4. miR-326 Promotes B Cell Hyperactivity via Ets-1

(A) Real-time PCR analysis of CD19⁺ B cell mRNA levels of Ets-1 among the three groups on day 7 after lentivirus injection. (B) The levels of Ets-1 mRNA were negatively correlated with the expression of miR-326 for mice with lentivirus administration ($r = 0.5951$). Data were determined using GraphPad Prism 6. (C) The levels of Ets-1 mRNA were negatively correlated with the percentage of plasmablasts in CD19⁺ B cells for mice with lentivirus administration ($r = 0.5132$). Data were determined using GraphPad Prism 6. (D) Representative photomicrographs of immunostaining for Ets-1 in kidney tissue sections taken from LV-326, LV-sponge, and LV-ctrl mice. Expression of Ets-1 as shown in the level of brown color (dark to light) in pictures and the levels Ets-1 were semiquantitatively scored as described in [Materials and Methods](#) on the basis of immunohistochemical results. Data are presented as mean \pm SEM. ** $p < 0.01$.

autoimmunity. SLE patients were found to have significant higher serum levels of IL-10.²⁶ IL-10 plays a key role in the pathogenesis of SLE, including regulation of B cell differentiation and autoantibody production, which may be the reason for B cell hyperactivity in SLE.²⁷ Autocrine and paracrine effects of IL-10 signaling may also result in B cell hyperactivity.²⁸ In the current study, overexpression of miR-326 upregulated the expression of serum IL-6 and IL-10; it is further confirmed that miR-326 has the effect of promoting B cell activities.

LN is a highly complex autoimmune disease caused by SLE that contributes to the major cause of morbidity and mortality in SLE,²⁹ although the pathogenesis of LN remains incompletely understood. Animal model experiments confirmed that glomerulonephritis is initiated by renal deposition or formation of immune complexes (ICs), resulting in the activation of complement and Fc γ receptors (Fc γ R).²⁹ In fact, renal immune deposits can be found in all patients with SLE. Here we identified that miR-326 overexpression mice developed severe glomerulonephritis with massive immune complex deposits and glomerular damage, which implies that upregulation of miR-326 aggravates renal injury in the lupus mouse and might be an important regulator of LN.

These data provide new insights into the relevance of miR-326 to SLE. However, the regulation of expression of miR-326 is not revealed. The

molecular mechanisms of transcription and maturation of miR-326 in B cells need to be determined in the future.

In conclusion, miR-326 has been found, for the first time, to contribute to B cell hyperactivity and autoantibody production by inhibiting Ets-1 expression in SLE. Overexpression of miR-326 promotes the secretion of inflammatory cytokines and progression of renal pathology in SLE. miR-326 plays an important role in SLE and could be used to regulate B cell differentiation into autoantibody-secreting cells in SLE.

MATERIALS AND METHODS

Mice

Thirty 8-week-old female MRL/lpr mice were purchased from the SLAC Laboratory (Shanghai, China) and subsequently bred and maintained under standard laboratory conditions at the Animal Care Commission of Anhui Provincial Hospital. The MRL/lpr mouse was chosen as the model mouse for this study because it develops high titers of dsDNA antibodies and immune complex deposits in the kidneys at 12–16 weeks of age.³⁰ The protocols for the animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC). All mice were maintained under specific pathogen-free conditions.

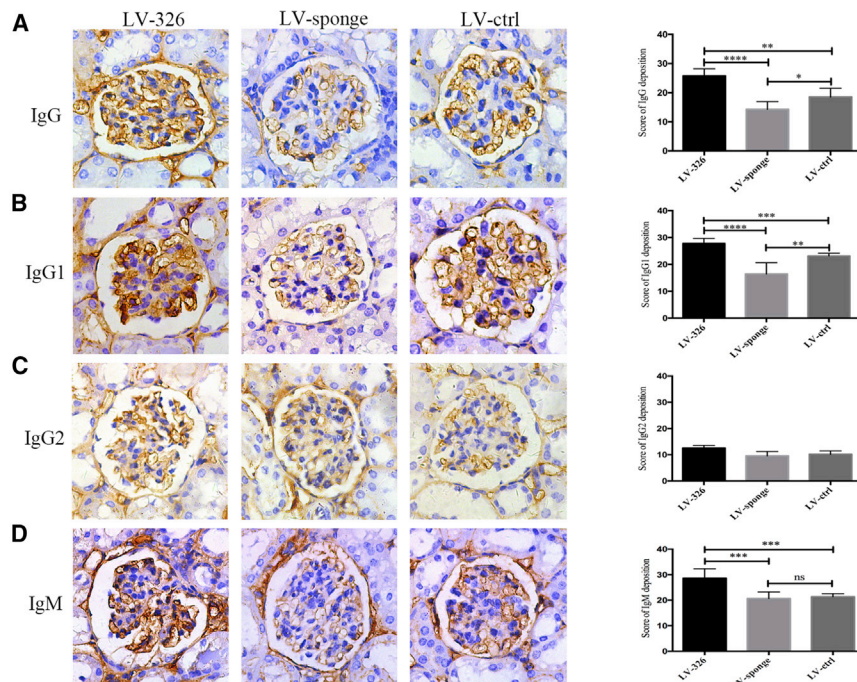


Figure 5. miR-326 Helps Immune Complex Deposits in the Kidneys

(A–D) Representative photomicrographs of immunostaining for IgG (A), IgG1 (B), IgG2 (C), and IgM (D) in kidney tissue sections taken from LV-326, LV-sponge, and LV-ctrl mice. Deposition of the Igs is shown by the levels of brown color (dark to light) in the pictures, and deposition volume was scored as described in [Materials and Methods](#) on the basis of immunohistochemical results. Data are presented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Changing miR-326 Expression via Lentivirus Injection

Mice were divided into 3 groups of 10 mice each at the age of 14 weeks and injected with approximately 2×10^7 transforming units of recombinant lentivirus (GeneChem, China) into the tail vein of the mice, encoding, respectively, pre-miR-326 (LV-326), an miR-326-specific inhibitor (LV-sponge), and LV-ctrl. No difference in mouse basal weight among the groups was observed. Mice were sacrificed after 7 days. LV-326 improves miR-326 transcript amounts without replication, and LV-sponge is a miR-326-specific decoy target that can suppress endogenous miR-326 activity without transcription.³¹

Blood Collection

Blood samples were collected from the corneal vein on the day before lentivirus injection (day 0) and on day 7 after injection (day 7) using a capillary blood collection tube and placed in a 1.5-mL microcentrifuge tube. Samples were allowed to clot by standing for 30 min at room temperature and then centrifuged at 10,000 rpm for 10 min. The serum was decanted into fresh tubes and stored at -80°C for long-term storage.

B Cell Isolation

CD19⁺ B lymphocytes from spleen were isolated using Mouse B Lymphocyte Enrichment Set-DM (BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

Flow Cytometry

B cells isolated from mouse spleens were stained with phycoerythrin-conjugated anti-mouse CD138(281-2) (BD Biosciences, San Jose, CA). Samples were acquired with a Calibur (BD Biosciences) flow cytometer, and the data analysis was performed with FlowJo software version 7.6 (Tree Star, Ashland, OR, USA).

ELISA

The levels of dsDNA antibodies in serum were quantified using the Varelisa dsDNA antibody ELISA kit (Phadia, Uppsala, Sweden) according to the manufacturer's protocols. The absorbance was read at 450 nm using a microplate reader.

Cytometric Bead Array

Serum B cell cytokine levels were measured by cytometric bead array (CBA; BD Biosciences, San Jose, CA). The CBA was simultaneously performed with specific antibodies for IL-6,

IL-10, and TNF according to the manufacturer's protocols. Samples were analyzed with a BD Canto2 (BD Biosciences, San Jose, CA) flow cytometer, and data analysis was performed with FCAP Array 2.0 software (Soft Flow, St. Louis Park, MN).

Real-Time qPCR

Total RNA was extracted from splenic B cells using TRIzol reagent (Sigma) according to the manufacturer's protocol. The expression levels of miR-326 were validated using the miScript primer assay (QIAGEN, Hilden, Germany) and miScript SYBR Green PCR kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocols. The specific primer (miR-326-5p: MniRQP1229) and housekeeping gene U6 (MniRQP9002) used in qRT-PCR were purchased from GeneCopoeia (USA). The expression levels of Ets-1 were validated using the QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) and QuantiTect SYBR Green RT-PCR Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The primers and β -actin were designed and synthesized by Shanghai Shenggong Biotechnology (Shanghai, China). All cDNA samples were amplified in the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, California, USA). The $\Delta\Delta\text{Ct}$ value was calculated by subtracting the Ct value for U6 from the Ct value for the gene of interest.

Morphologic Examinations

The kidneys of mice were isolated, fixed with 4% paraformaldehyde, and embedded in paraffin wax. Sections were cut at 2 μm , and deparaffinized tissue sections were used for histological examination. H&E-stained kidney sections were evaluated by ImageJ software. PAS-stained kidney sections were evaluated, and glomerular damage

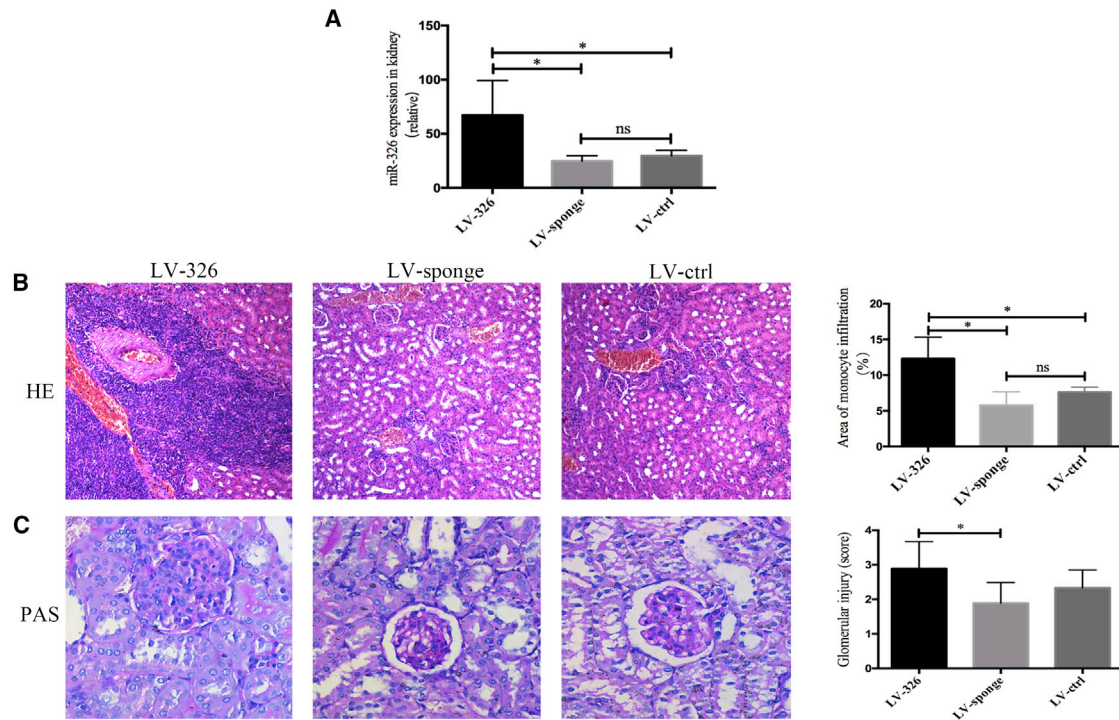


Figure 6. miR-326 Promotes the Development of Lupus Nephritis

(A) Real-time PCR analysis of renal miR-326 levels among three groups on day 7 after lentivirus injection. (B) Histopathology revealed by H&E staining of kidney tissue sections from LV-326, LV-sponge, and LV-ctrl mice. The infiltration area of mononuclear cells was evaluated by ImageJ. (C) Glomerular histology revealed by PAS staining of kidney tissue sections from LV-326, LV-sponge, and LV-ctrl mice. Glomerular injury was semiquantitatively scored as described in [Materials and Methods](#) on the basis of immunohistochemical results. Data are presented as mean \pm SEM. * $p < 0.05$.

was scored on a scale of 0–4 as follows:³² 0 = normal, 1 = mild intracapillary proliferation, 2 = moderate intracapillary proliferation, 3 = strong intracapillary proliferation or moderate intracapillary proliferation and extracapillary proliferation, 4 = strong intracapillary proliferation. Antibodies against mouse IgG (Jackson ImmunoResearch Laboratories, Suffolk, UK) (dilution 1:500), mouse IgM (Jackson ImmunoResearch Laboratories, Suffolk, UK) (dilution 1:1,000), mouse IgG1 (Abcam, Cambridge, UK) (dilution 1:500), and mouse IgG2 (Abcam, Cambridge, UK) (dilution 1:500) were used for the assessment of glomerular deposits and scored as follows:³² 0 = no deposition, 1 = mild deposition, 2 = moderate deposition, 3 = strong deposition. Ets-1 expression in renal interstitial was also evaluated using an antibody against mouse Ets-1 (Novus Biologicals, Littleton, CO, USA) (dilution 1:100) and visualized with enhanced chemiluminescence and quantified by densitometry (ImageJ, NIH).³³ The slides were visualized and photographed with a light microscope (Leica DM4000, Solms, Germany).

Statistical Analysis

Data are presented as mean \pm SEM. To determine statistical significance among multiple comparisons, we used a one-way ANOVA test followed by a *post hoc* analysis (Newman-Keuls test). Differences between two individual experimental groups were compared by a two-tailed t test. $p < 0.05$ was considered statistically significant.

AUTHOR CONTRIBUTIONS

Conceptualization, J.-H.T. and N.X.; Formal Analysis, Y.X., X.F., and L.J.; Investigation, Y.X., X.F., and X.-J.D.; Writing – Original Draft, Y.X.; Writing – Review & Editing, Y.-P.W., J.-H.T., and X.-P.L.; Funding Acquisition, X.-P.L.; Supervision, X.-P.L. and X.-M.L.

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