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Enhanced inflammatory damage by microRNA-136 targeting Klotho expression in HK-2 cells by modulating JAK/STAT pathway

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MiR-136 acts as a tumor suppressor by promoting cell apoptosis and downregulating Bcl-2 in glioma cells. Hence, an attempt has been made to evaluate the role of miR-136 in regulation of inflammatory damage in HK-2 cells. HK-2 cells were cultured and assessed for viability. The cells were then transfected with miR-136 mimic, si- miR-136, si-Klotho, and NC. Dual luciferase test was performed to confirm the target of miR-136 which was assumed to be Klotho. Cell viability, apoptosis, expressions of inflammatory cytokines like TNF-α, IL-1β, IL-6 and IL-8 were assessed in HK-2 cells with overexpressing miR-136 or with knocked down miR-136 activities, following exposure to LPS. LPS induced inflammatory damage decreased cell viability, induced cell apoptosis, and increased the expression of different inflammatory cytokines. It was found that LPS decreased the expression of miR-136. Over-expression of miR-136 inhibited cell viability, enhanced apoptosis, and increased expression of inflammatory cytokines while knockdown of miR-136 showed opposite results with p-values < 0.05. MiR-136 negatively regulated the expression of Klotho with p-value < 0.05. Over-expression of miR-136 inhibited the expression of Klotho and activated JAK/STAT and mTOR signaling pathways and vice versa. Hence, it can be concluded that miR-136 enhances inflammatory damage probably by targeting klotho as has been observed in luciferase assay by inactivation of JAK/STAT and mTOR signaling pathways.

1. Introduction

Chronic kidney disease (CKD), one of the widespread global health conditions, has affected more than 20 million people in the United States alone (2010). Despite considerable progress in laboratory-based and epidemiological research, the underlying risk factors, and pathophysiologic pathways involved in CKD progression is yet to established. A better understanding of CKD and outcomes associated with it may be achieved by elucidating the genetic contributions to the processes responsible for progressive renal injury ultimately leading to end-stage renal disease (Bowden 2003). Kidneys play a pivotal role in various basic physiological functions including blood pressure control, salt and water homeostasis, blood cell production, acid-base balance, and calcium homeostasis. Hence, any kind of renal dysfunction can result from or lead to a variety of pathologies. As per the National Center for Health Statistics, more than 3.9 million adults have been diagnosed with kidney disease in the United States, a number that amounts to approximately 2% of the adult population (Schiller et al. 2012). Mortality due to renal diseases is eighth leading cause of death and hypertensive renal disease is not far behind so as to rank it the 13th most abundant cause of mortality (Murphy et al. 2012). Renal diseases are classified as acute or chronic, depending upon the onset of the disease.

Abbreviations:

mTOR- Mammalian target of rapamycin; LPS- Lipopolysaccharide; JAK/STAT- Janus kinase/ Signal Transducer and Activator of Transcription; GFR- Glomerular filtration rate; qRT-PCR- Quantitative real time-polymerase chain reaction

Acute kidney disease is usually associated with bacterial infection, sepsis or ischemia-reperfusion injury, whereas chronic kidney disease (CKD) is usually the result of diabetic complications, hypertension, obesity, and autoimmunity. The factors that promote renal disease can be quite different; however, acute kidney disease can lead to chronic kidney disease, and, if left untreated, both can lead to end-stage renal disease (ESRD). Equally important factors are presence of inflammation and immune system, common manifestations for both acute and chronic kidney diseases. Cytokines and inflammatory mediators such as TNF-α and ILs have been shown to influence sodium excretion, renal blood flow, and GFR (Harris and Neilson 2006; Noronha et al. 2002). In a report, TNF-α decreased renal blood flow and GFR and has been shown to induce natriuresis in mice (Shahid et al. 2008). The reduction in renal blood flow was found to be consistent with a study conducted in rabbits (Girardin et al. 1994). In a number of studies, IL-1 and IL-6 were demonstrated to dilate skeletal muscle arterioles, basilar arteries, and coronary arteries (Kenny et al. 1990; Minghini et al. 1998; Osuka et al. 1997). IL-1 increased sodium excretion, but somehow the response was not associated with increased renal blood flow or GFR (Cannon 1988; Kohan et al. 1989). Thus, IL-1 was found to dilate peripheral arteries but not renal arterioles and increase sodium excretion by directly acting on renal epithelial cells. The aforementioned studies demonstrated that cytokines and inflammatory mediators can alter renal blood flow and GFR. A kidney protein, Klotho (α-Klotho), is linked to ageing, phosphate metabolism, and inflammation. Klotho deficiency is associated with premature ageing in mice (Kuro-O et al. 1997). It is a single-pass transmembrane protein of 135 kDa, which downregulates inflammation (Hu et al. 2012).

MicroRNA is a class of small non-coding RNA approximately 20-22 nucleotides in length and plays an important role in regulating gene expression by directly binding to the 3' UTR of target mRNAs (Bartel 2004). The microRNAs bind to the 3' untranslated region (UTR) of mRNA, thereby leading to inhibition of translation of mRNA and finally facilitating its degradation. A review by He et al. (2004), reviewed that there are a number of studies where miRNAs play pivotal roles in diverse biological processes (He and Hannon 2004). MiR-136 is considered as a tumor suppressor because literature survey reveals that it promotes cell apoptosis by downregulating Bcl-2 in glioma cells. Hence, an attempt has been made in this study to evaluate the role of miR-136 in the regulation of inflammatory damage in HK-2 cells along with exploration of the possible underlying mechanisms.

2. Investigations and results

2.1. Effect of LPS-induced inflammatory damage in HK-2 cells on cell viability and cell apoptosis

Cell viability of LPS-induced inflammatory damaged HK-2 cells decreased significantly in a dose-dependent manner. It was found to be less than 50%, approximately 50% and almost 100% at 10 µg/ml, 5 µg/ml, and 1 µg/ml of LPS when compared to the control cells where cell viability was 100% (p-value< 0.05) (Fig. 1a). The findings for cell viability correlated well with apoptosis which increased significantly in a dose-dependent manner with increasing concentration of LPS when compared with the control cells with p-values < 0.05 (Fig. 1b). Furthermore, expression of inflammatory cytokines like TNF-α, IL-1β, IL-6, and IL-8 when

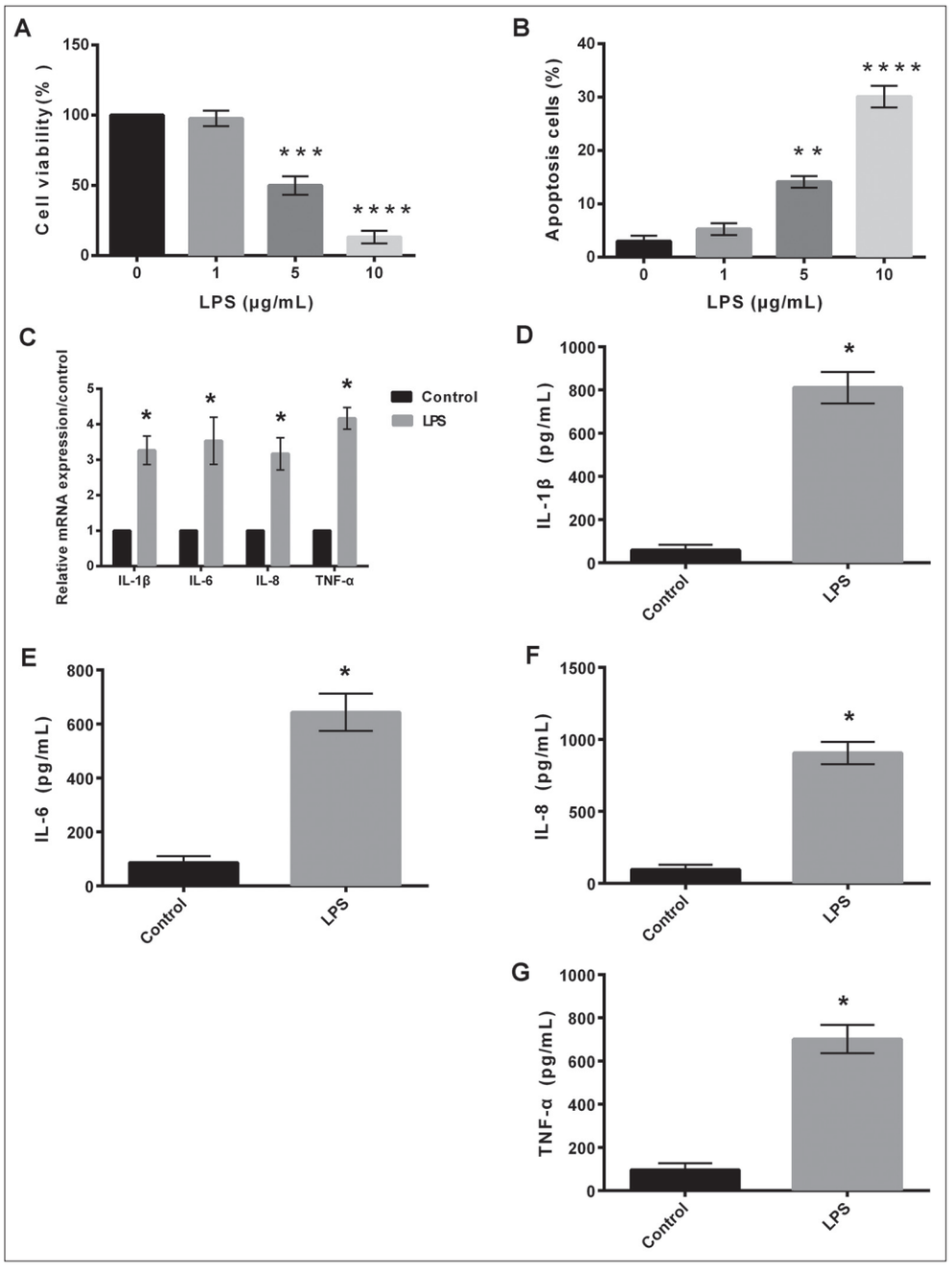

Fig. 1: LPS induced inflammatory damage in HK-2 cells. *, p<0.05

assessed by ELISA, revealed a significant increase in LPS challenged HK-cells compared to their expression in control cells (p-value< 0.05) (Fig. 1c-g).

2.2. Expression of miR-136 in LPS treated HK-2 cells

There was a significant decrease in miR-136 expression in LPS challenged HK-2 cells compared to the control cells (p-value< 0.05) (Fig. 2).

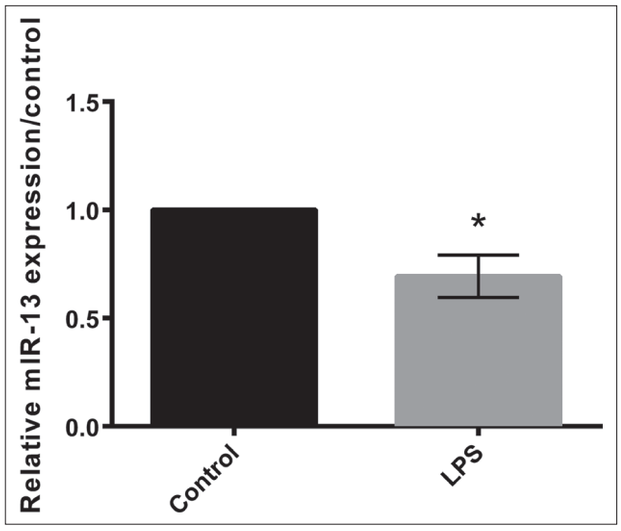

Fig. 2: LPS decreased the expression of miR-136. *, p<0.05

2.3. Expression of miR-136 after transfection

The expression of miR-136 was found to be either over-expressed (in miR-136 mimic group) or suppressed (in si-miR-136 group) in HK-2 cells relative to control or scramble cells (Fig. 3) (p-value< 0.05).

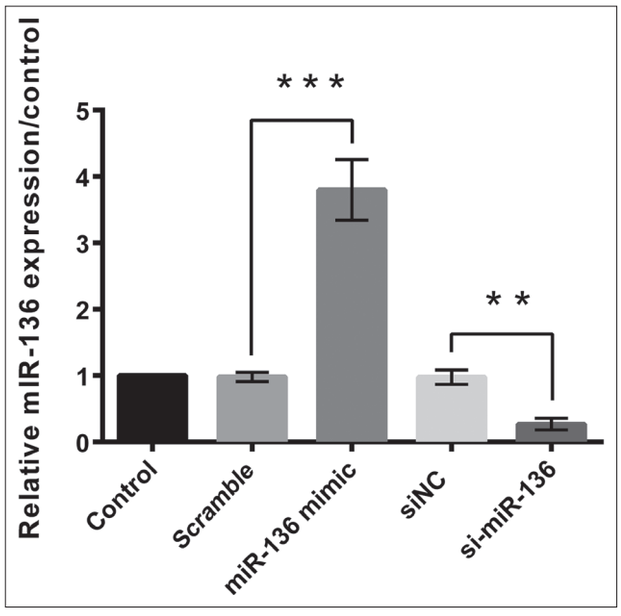

Fig. 3: The expression of miR-136 was overexpressed or suppressed in miR-136-mimic group and si-miR-136 group of HK-2 cells. *, p<0.05

2.4. Effects of over-expression of miR-136

Over-expression of miR-136 inhibited cell viability, while knockdown of miR-136 showed opposite results (Fig. 4a) (p-value< 0.05). Similarly, over-expression of miR-136 enhanced cell apoptosis, while knockdown of miR-136 showed opposite results (Fig. 4b) (p-value< 0.05). Over-expression of miR-136 led to significant increase in expression of inflammatory cytokines, while knockdown of miR-136 showed opposite results (Fig. 4c-g) (p-value< 0.05).

2.5. MiR-136 targeted klotho gene

HK-2 cells transfected with miR-136 negatively regulated the expression of klotho mRNA (Fig 5a, b) (p-value< 0.05). This was further confirmed by luciferase assay where miR-136 binding elements down-regulated the expression of klotho promoter significantly compared to the expression of U6 (Fig 5c) (p-value< 0.05 and p-value < 0.01).

2.6. Effects of knock-down of miR-136

Knockdown of miR-136 promoted cell viability by up-regulation of klotho (Fig 6a) (p-value< 0.05). Similarly, knockdown of miR-136 inhibited cell apoptosis by up-regulation of klotho (Fig 6b) (p-value< 0.05 and p-value < 0.01). Hence, as expected knockdown of miR-136 decreased the expression of inflammatory factors by up-regulation of klotho (Fig 6c-g) (p-value< 0.01). Interestingly, it was also observed that a silenced klotho gene led to a significant decrease in cell viability (p-value < 0.05), increased cell apoptosis (p-value < 0.001) and an increase in pro-inflammatory cytokine IL-1β, IL-6, IL-8 and TNF-α levels (p-values < 0.05 and p-values < 0.001).

2.7. Effect of miR-136 on JAK/STAT and mTOR signaling pathways

Over-expression of miR-136 inhibited the expression of klotho and activated JAK/STAT and mTOR signaling pathways, whereas knockdown of miR-136 promoted expression of Klotho and inactivated JAK/STAT and mTOR signaling pathways (Fig 7).

3. Discussion

In the present study, we explored the functional role of miR-136 in renal tubular inflammatory injury, along with the probable underlying mechanism. The results showed that the expression of miR-136 was decreased in LPS-induced inflammatory injury in HK-2 cells, indicating that miR-136 might be involved in the regulation pathway. A similar observation was found in a study by Chen et al. (2014), where miR-136 was highly downregulated in LPS stimulated cord blood cells. It has also been observed in this study that over-expression of miR-136 led to an increase in pro-inflammatory cytokines assessed which included TNF-α, IL-1β, IL-6, and IL-8, thus suggesting that miR-136 might promote inflammation. Klotho is an anti-inflammatory modulator in kidney, depletion of which in a mouse model was shown to increase inflammation in a mouse model (Hamano 2015). In our study we also observed that HK-2 cells transfected with knocked-down miR-136, decreased the expression of inflammatory factors which was again found to be associated with upregulation of the klotho gene, thereby alleviating inflammatory injury.

Apart from the above findings, we also found that over-expression of miR-136 further aggravated the injury. Moreover, results from the dual luciferase assay showed that Klotho was a target of miR-136, and miR-136 negatively regulated the expression of Klotho. Results also demonstrated that miR-136 regulated cell inflammatory injury by downregulation of Klotho. The results of western blot showed that Klotho alleviated the LPS-induced injury by inactivation of the JAK/STAT and mTOR signaling pathways which contradicts the finding as reported by Hamano (2015), where klotho activated mTOR pathway (Zhao et al. 2011). Considering the aforementioned facts we can conclude that miR-136 enhances

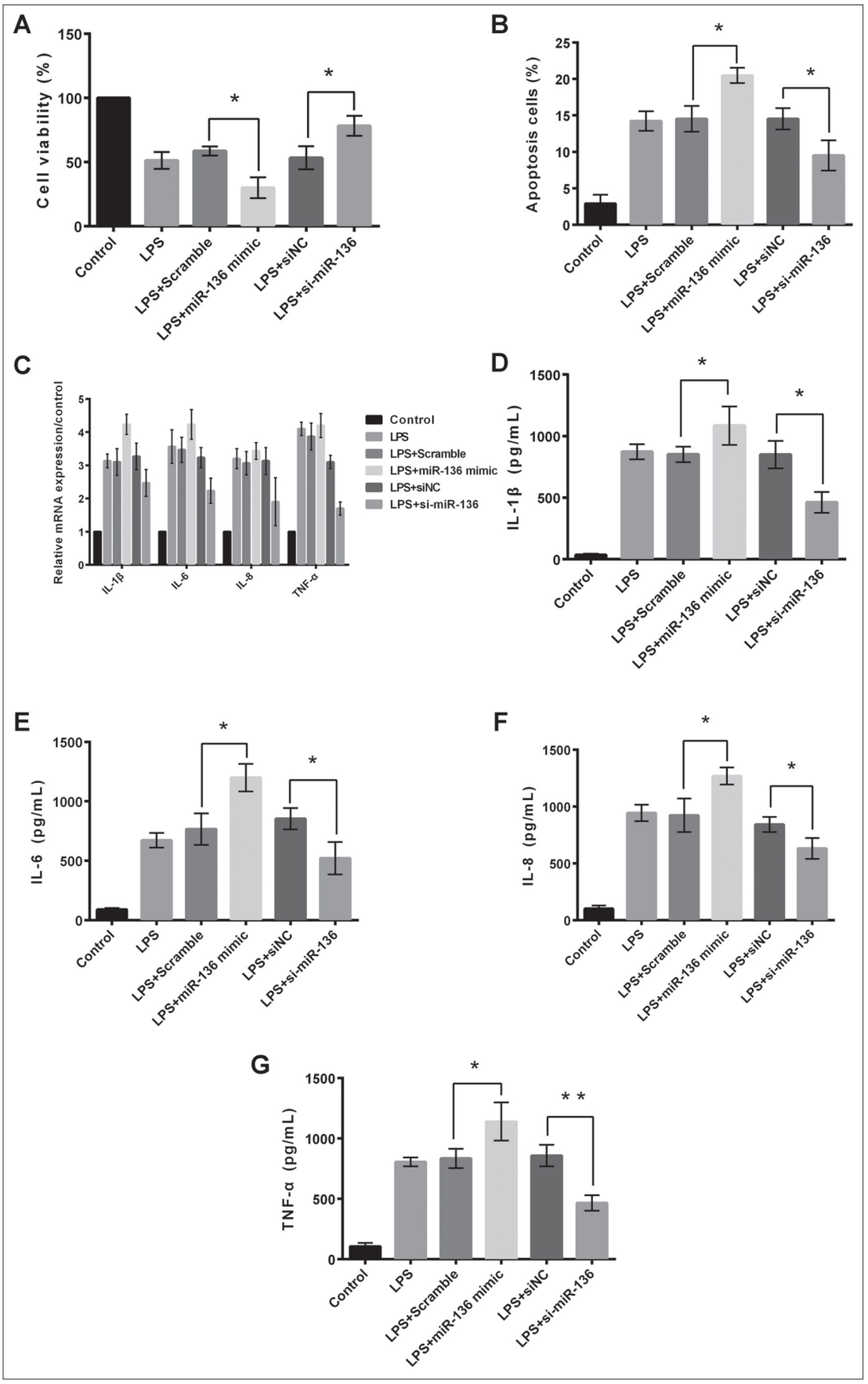

Fig. 4: Effects of over-expression of miR-136 on cell viability, apoptosis, and inflammatory cytokines. *, $p<0.05$

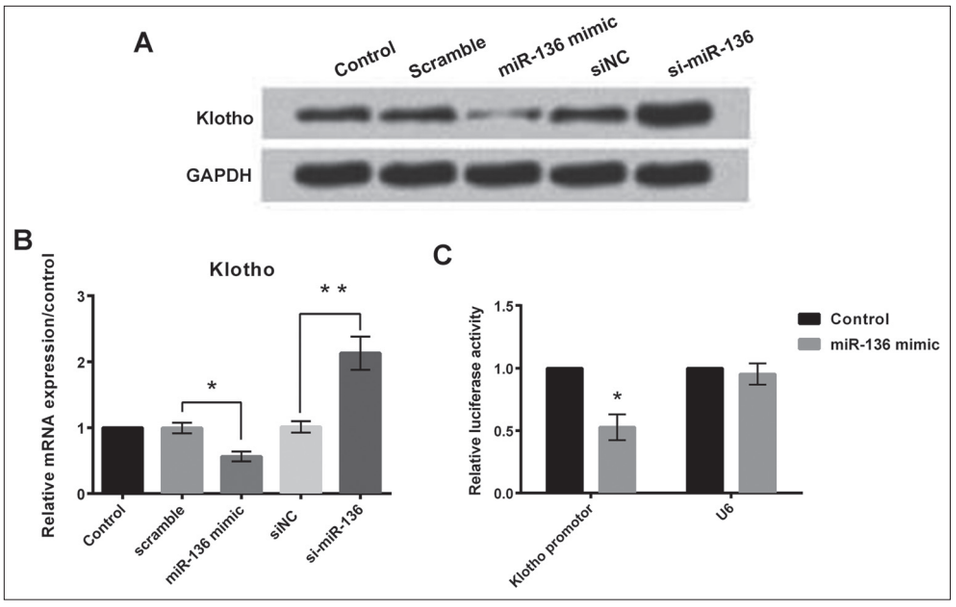

Fig. 5: Klotho a target of miR-136 which down-regulates the expression. *, p<0.05

inflammatory damage probably by targeting klotho as has been observed in the luciferase assay by inactivation of JAK/STAT and mTOR signaling pathways.

4. Experimental

4.1. Cell culture and treatment

HK-2 cells were obtained from Riken cell bank (Ibaraki, Japan). Cells were cultured in a culture medium; RPMI- 1,640 (Wako, Osaka, Japan) supplemented with 10% heat- inactivated fetal bovine serum (FBS), penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. MH7A cells were treated by LPS in a series of concentration for 5 h.

4.2. CCK-8 assay

Cells were seeded in 96-well plate with 5000 cells/well, cell proliferation was assessed by a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). Briefly, after stimulation, the CCK-8 solution was added to the culture medium, and the cultures were incubated for 1 hour at 37 °C in humidified 95% air and 5% CO_2. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

4.3. Apoptosis assay

Flow cytometry analysis was performed to identify and quantify the apoptotic cells by using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). The PC-12 cells (100,000 cells/well) were seeded in a 6 well-plate. Treated cells were washed twice with cold PBS and resuspended in buffer. The adherent and floating cells were combined and treated according to the manufacturer's instruction and measured with flow cytometer (Beckman Coulter, USA) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

4.4. ELISA

Culture supernatant was collected from 24-well plates and concentrations of inflammatory cytokines measured by enzyme-linked immunosorbent assay (ELISA) using protocols supplied by the manufacturer (R&D Systems, Abingdon, UK) and normalized to cell protein concentrations.

4.5. miRNAs transfection

miR-136 mimic, si- miR-136 and si-Klotho, and the NC controls were synthesized by GenePharma Co. (Shanghai, China). Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol.

4.6. Qualitatitive real time-PCR (qRT-PCR)

Total RNA was extracted from cells and tissues using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay of miR-136 and U6 (Applied Biosystems, Foster City, CA, USA) were used for testing the expression levels of miR-136 in cells.

4.7. Dual luciferase activity assay

The 3'UTR target site was generated by PCR and the luciferase reporter constructs with the klotho 3'UTR carrying a putative miR-136-binding site was transfected into pMiR-report vector were amplified by PCR. Cells were co-transfected with the reporter construct, control vector and miR-136 or scramble using Lipofectamine 3000 (Life Technologies, USA). Reporter assays were done using the dual-luciferase assay system (Promega) following to the manufacturer's information (Zhou et al. 2016).

4.8. Western Blot

The protein used for western blotting was extracted using RIA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. GAPDH antibody was purchased from Sigma. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibody was incubated with the membrane at 4 °C overnight, followed by washing and incubation with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the Polyvinylidene Difluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 µl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

4.9. Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean±SD. Statistical analyses were performed using SPSS 19.0 statistical software. The P-values were calculated using a one-way analysis of variance (ANOVA). P-values of <0.05 were considered to indicate a statistically significant result.

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Conflict of interest: There is no conflict of interests.

References

Bartel DP (2004) MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116:281-297.

Bowden DW (2003) Genetics of kidney disease. Kidney Int Suppl 63: S8–S12.

Cannon JG (1988) Interleukin-1 induces natriuresis in conscious rats: role of renal prostaglandins. Kidney Int 33: 1059-1065.

Centers for Disease Control and Prevention (2010) National Chronic Kidney Disease Fact Sheet: general information and national estimates on chronic kidney disease in the United States. Atlanta, GA: U.S., Department of Health and Human Services, Centers for Disease Control and Prevention.

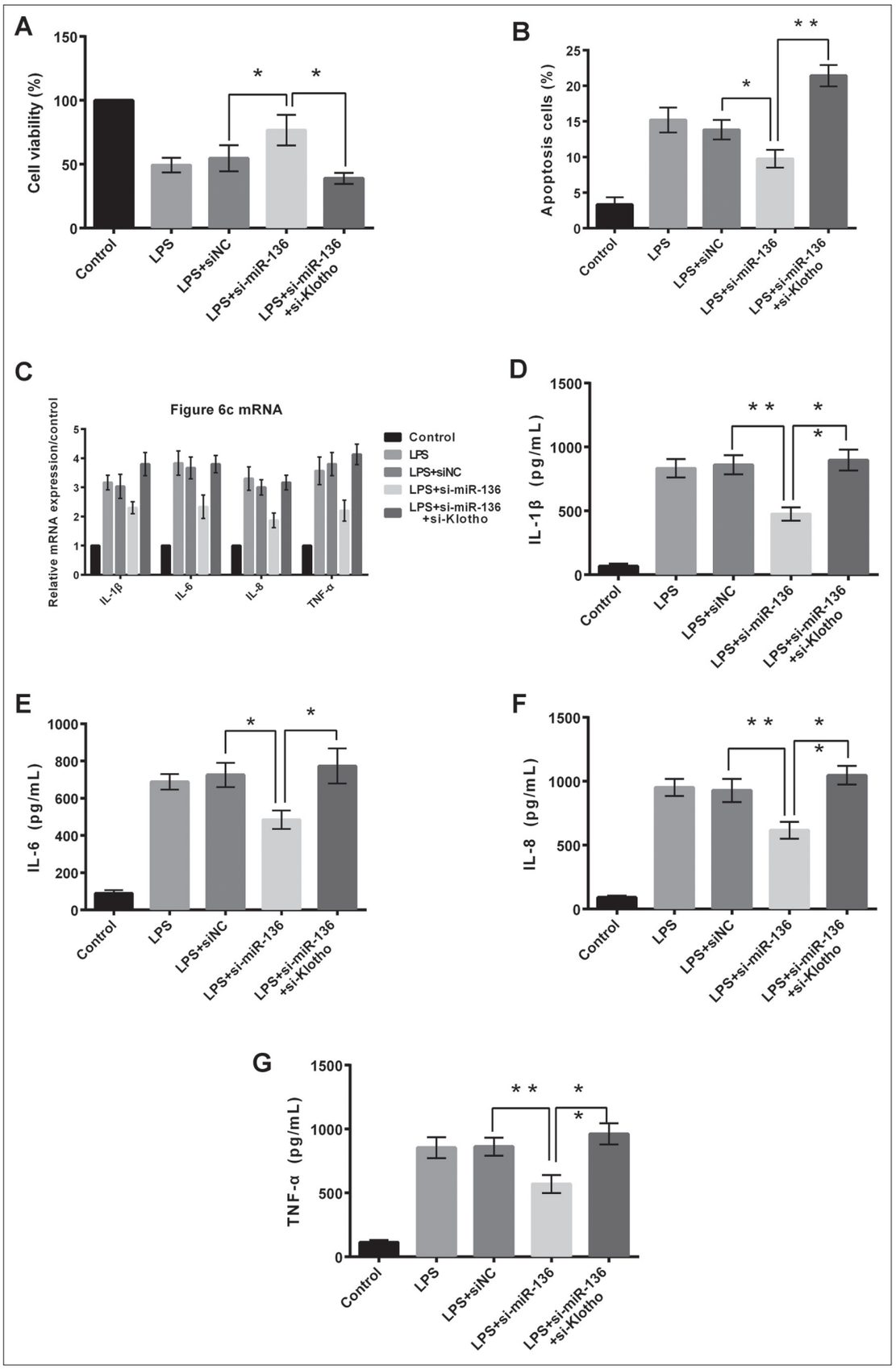

Fig. 6: MiR-136 knock-down alleviated inflammatory damage. *, $p<0.05$

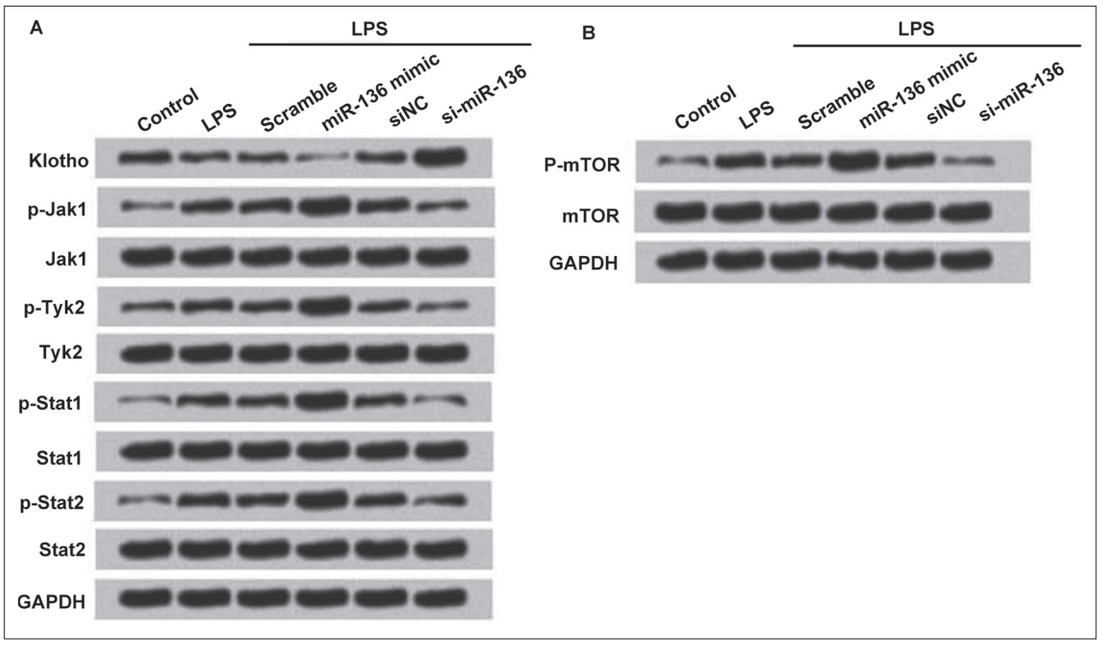

Fig. 7: MiR-136 regulates the expression of Klotho by incativating JAK/STAT pathway.

Chen J, Liu Z, Yang Y (2014) In vitro screening of LPS-induced miRNAs in leukocytes derived from cord blood and their possible roles in regulating TLR signals. Pediatr Res 75: 595-602.

Girardin E, Grau GE, Paunier L, Le CC (1994) Early hemodynamic and renal effects of tumor necrosis factor alpha: role of thromboxane. Circulat Shock 42: 20-26.

Hamano T (2015) Klotho upregulation by rapamycin protects against vascular disease in CKD. Kidney Int 88: 660-662.

Harris RC, Neilson EG (2006) Toward a unified theory of renal progression. Medicine 57: 365-380.

He L, Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. Nature Rev Genet 5: 522-531.

Hu MC, Kuroo M, Moe OW (2012) The emerging role of Klotho in clinical nephrology. Nephrol Dial Transplant 27: 2650-2657.

Kenny D, Mccarthykenny G, Pelc LR, Cheung HS, Brooks HL, Warltier DC (1990) Vasodilator actions of interleukin-1 in the canine coronary circulation. Basic Res Cardiol 85: 279-284.

Kohan DE, Merli CA, Simon EE (1989) Micropuncture localization of the natriuretic effect of interleukin 1. Am J Physiol 256: 810-813.

Kuro-O M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurabayashi M, Kaname T, Kume E (1997) Mutation of the mouse klotho gene leads to a syndrome resembling ageing. Nature 390: 45-51.

Minghini A, Britt LD, Hill MA (1998) Interleukin-1 and interleukin-6 mediated skeletal muscle arteriolar vasodilation: in vitro versus in vivo studies. Shock 9: 210-215.

Murphy SL, Xu JQ, Kochanek KD (2012) Deaths: Preliminary Data for 2010. Nat Vital Stat Rep 60: 1-51.

Noronha IL, Fujihara CK, Zatz R (2002) The inflammatory component in progressive renal disease—are interventions possible? Nephrol Dial Transplant 17: 363-368.

Osuka K, Suzuki Y, Watanabe Y, Dogan A, Takayasu M, Shibuya M, Yoshida J (1997) Vasodilator effects on canine basilar artery induced by intracisternal interleukin-1 beta. J Cerebral Blood Flow Metabol 17: 1337-1345.

Schiller JS, Lucas JW, Ward BW, Peregoy JA (2012) Summary health statistics for U.S. adults: National Health Interview Survey, 2010. Vital and Health Statistics. Series 10, Data from the National Health Survey: 1-207.

Shahid M, Francis J, Majid DS (2008) Tumor necrosis factor-alpha induces renal vasoconstriction as well as natriuresis in mice. Am J Physiol Renal Physiol 295: F789-F796.

Zhao Y, Banerjee S, Dey N, Lejeune WS, Sarkar PS, Brobey R, Rosenblatt KP, Tilton RG, Choudhary S (2011) Klotho depletion contributes to increased inflammation in kidney of the db/db mouse model of diabetes via RelA (serine)536 phosphorylation. Diabetes 60: 1907-1916.

Zhou F, Li Y, Hao Z, Liu X, Chen L, Cao Y, Liang Z, Yuan F, Liu J, Wang J (2016) MicroRNA-300 inhibited glioblastoma progression through ROCK1. Oncotarget 7: 36529-36538.