

Inhibition of microRNA-30a prevents puromycin aminonucleoside-induced podocytic apoptosis by upregulating the glucocorticoid receptor α

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Abstract. It is well-established that steroid-resistant nephrotic syndrome commonly involves podocytic injury, however, the underlying mechanism remains to be elucidated. Previous studies have demonstrated that glucocorticoids enhance podocytic recovery predominantly through the functional isoform, glucocorticoid receptor (GR) α . Our previous study demonstrated the reduced expression of GR α in podocytes from patients with steroid-resistant nephrotic syndrome compared with those with steroid-sensitive syndromes, which suggested that microRNAs (miRNAs) may be a potential therapeutic target in the modulation of steroid sensitivity. The present study screened miRNAs in murine injured podocytes by microarray and identified 10 miRNAs significantly upregulated, including miR-30a, miR-30d, miR-100, miR181c, miR-5099, miR-3535, miR-140-3p, miR-148-3p and miR-103-3p. Bioinformatic target prediction indicated that the GR was a candidate target gene of miR-30a. The data also indicated that miR-30a negatively regulated GR α in normal and injured podocytes induced by puromycin aminonucleoside (PAN). In addition, the inhibition of miR-30a prevented podocytic apoptosis induced by PAN. However, luciferase reporter assay data suggested an indirect effect on the transcriptional activity of GR α . The present study indicated that silencing of miR-30a may improve steroid sensitivity in injured podocytes, although the mechanism cannot be explained by conventional means and remains to be elucidated.

Introduction

Podocytes are critical in maintaining the integrity of the glomerular filtration barrier. Acquired proteinuric glomerulopathies, including minimal change disease and focal segmental glomerulosclerosis, commonly exhibit abnormalities in podocytic phenotypes, including effacement and apoptosis (1,2). Glucocorticoids, which are the primary therapy for the treatment of proteinuric glomerulopathies, exert profound effects through their interaction with specific glucocorticoid receptors (GRs) (3). GRs are abundantly expressed in human and mouse podocytes (4,5), and exist in different isoforms as a result of alternative splicing. GR α is the functionally active isoform located in the cytoplasm. Glucocorticoids are reported to induce recovery in podocyte injuries predominantly via two GR α -mediated mechanisms. Genetic regulation occurs following the binding of glucocorticoids at low doses (0.5 mg/kg/day), resulting in the activation and translocation of GR α into the nucleus. Subsequently, activated GR α binds at the glucocorticoid response elements within the genome to mediate the direct regulation of target genes leading to anti-inflammatory and immunosuppressive effects (5). The alternative mechanism involves the direct effect of glucocorticoids on podocyte-expressed GR α (4), which reduces podocytic apoptosis and promotes actin aggregation (6). Therefore, GR α regulation is a crucial determinant of steroid efficacy.

Our previous study demonstrated a reduced expression of GR α in podocytes from patients with steroid-resistant idiopathic nephrotic syndrome compared with steroid-sensitive patients (7). Therefore, upregulation of the GR α may be a promising target for the improvement of steroid sensitivity. miRNAs are post-transcriptional regulators of gene expression, which bind to complementary sequences in the target mRNA to induce gene repression (8,9). Previous data indicates that GR α -specific miRNAs represent potential therapeutic targets in T-cell acute lymphoblastic leukemia and multiple myeloma (10,11). However, the regulation of GR α by miRNAs in podocytes remains to be determined. In the present study, the miRNA expression profile in normal murine podocytes and injured podocytes induced by puromycin aminonucleoside

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(PAN) was compared, and demonstrated the existence of an association between miR-30a and the expression of GR, based on bioinformatic analysis. Subsequently, the present study observed that miR-30a downregulated GR α in injured podocytes. Furthermore, inhibition of miR-30a reduced podocytic apoptosis by upregulating GR α . These findings indicated that inhibition of miR-30a may reduce podocytic apoptosis via a GR α -mediated mechanism.

Materials and methods

miRNA microarray. miRNA microarray analysis was performed using miRNA microarrays (RiboBio Co., Ltd., Guangzhou, China), according to the manufacturer's instructions. Briefly, 600 ng total RNA was isolated from differentiated murine podocytes and injured podocytes induced by PAN, and was dephosphorylated with a calf intestine alkaline phosphatase treatment for 30 min at 37°C prior to labeling. The samples were diluted with dimethylsulfoxide, denatured for 10 min at 100°C and were subsequently labeled with pCp-Cy3 (Biorbyt, Cambridge, UK) in T4 RNA ligation buffer (BestBio, Shanghai, China). The labeled RNA was hybridized using 2X hybridization buffer (Agilent Technologies, Palo Alto, CA, USA), washed with 6X SSC (Solarbio Science & Technology Co., Ltd., Beijing, China)/0.005% Triton X-102 (DOW, Midland, MI, USA), stained with horseradish peroxidase-streptavidin (RiboBio Co., Ltd.) and scanned with an Agilent 2200 Bioanalyzer (Agilent Technologies). Microarray data analysis was performed using an Illumina HiSeqTM2500 (Illumina Inc., San Diego, CA, USA).

Prediction of murine GR targeting miRNAs. The following target prediction algorithms were used to predict murine GR targeting miRNAs: Pictar (<http://pictar.mdc-berlin.de/>), miRanda (<http://www.microrna.org/>) and TargetScan/TargetScanS (<http://www.targetscan.org/>). The highest threshold value was used for the prediction using miRanda.

Podocytes culture. The conditionally immortalized mouse MPC5 podocyte cell line was cultured, as previously described (6). Briefly, undifferentiated podocytes were grown at 33°C with a relative humidity of 100% and 5% CO₂-containing atmosphere in RPMI-1640 medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies, Grand Island, NY, USA), 100 U/ml penicillin, 100 μ g/ml streptomycin (Proteintech Group, Inc., Chicago, IL, USA) and 10 U/ml mouse interferon- γ (IFN- γ ; Sigma Chemical Co., St. Louis, MO, USA). Podocyte differentiation was induced by culturing at 37°C for 10-14 days in the presence of rat tail collagen type I (Sigma Chemical Co.) without IFN- γ .

Podocyte treatment. Podocyte injury was induced *in vitro* with PAN (Sigma Chemical Co.) using the following procedure: Differentiated podocytes (day 12) were incubated in RPMI-1640 medium, supplemented with charcoal-stripped FBS for 24 h. Drug treatments were performed 24 h following transfection. The podocytes were exposed to PAN at a concentration of 45 ng/ml and were incubated for 48 h. Podocyte recovery was induced using dexamethasone (Dex; Sigma

Chemical Co.). The cells were pretreated for 1 h with the GR agonist, Dex (1 μ M) prior to PAN treatment. Correspondingly, the cells were pretreated with the GR antagonist, RU486 (20 μ M; Sigma Chemical Co.), for 30 min prior Dex treatment.

Transfection. Using the methods described by the relevant manufacturers, the podocytes (day 12) were transiently transfected with 50 nM synthetic miR-30a mimics, 100 nM inhibitor and the corresponding scrambled control oligonucleotides (RiboBio Co., Ltd.). At 48 h post-transfection, the cells were harvested and prepared for analysis.

Total RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). On the 12th day of podocyte differentiation (day 12), the total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. The RNA concentration and purity was assessed by measuring the absorbance at 260 and 280 nm using a Nanodrop spectrophotometer 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

The primers for assay of miR-30a were purchased from ABI (Applied Biosystems, Foster City, CA, USA). RT reactions, containing the total RNA (5 μ l), 50 nM stem-loop RT primer (3 μ l), 10X RT buffer (1.5 μ l), 100 mM dNTPs (0.15 μ l), multiScribe reverse transcriptase (1 μ l) and RNase inhibitor (0.19 μ l), were performed using a GeneAmp PCR system 9700 (Applied Biosystems) for 30 min at 16°C, 30 min at 42°C and 5 min at 85°C. PCR amplification was performed using a standard TaqMan PCR kit (Applied Biosystems) with the RT product (1.33 μ l), 2X TaqMan Universal PCR Master mix (14.5 μ l), 20X TaqMan MicroRNA assay (1.5 μ l). The PCR amplification conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The intra-experimental variation was assessed based on the average of the mean $\Delta\Delta$ Ct values following normalization against U6. All samples were assessed in triplicate.

The gene expression levels were analyzed by RT-qPCR on a LightCycler (Roche, Mannheim, Germany) using the following primers (Takara Bio, Inc., Otsu, Japan): GR α , sense: 5'-AAAGAGCTAGGAAAAGCCATTGTC-3' and antisense: 5'-TCAGCTAACATCTCTGGGAATTCA-3'; GAPDH, sense: 5'-GAAGGTGAAGGTCGGAGT-3' and antisense: 5'-GAA GATGGTGATGGGATTTTC-3'. The total RNA (2 μ l) was used for RT using the SYBR[®] PrimerScriptTM RT-PCR kit (Takara Bio, Inc.), according to the manufacturer's instructions. The resulting data were analyzed using the comparative $\Delta\Delta$ C_t method for relative gene expression quantification against GAPDH (12).

Analysis of the expression of GR α by western blotting. The protein concentration of the cell lysates (100 μ g) was determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). MPC5 cells grown in six-pore plates (Costar Corning, Corning, NY, USA) were washed twice with PBS, then treated with RIPA cell lysis solution (Solarbio Science & Technology Co., Ltd.) on ice for 30 min, then centrifuged at 12,000 x g for 30 min at 4°C to attain the supernatant. The protein samples were heated to 100°C for 5 min prior to 20 μ l aliquots being separated by SDS-PAGE (Beijing Solarbio Science & Technology Co.,

Ltd.) and transferred onto polyvinylidene membranes (Beijing Solarbio Science & Technology Co., Ltd.). The membranes were incubated with a polyclonal rabbit-anti-mouse GR α primary antibody (cat. no. sc-37642; 1:250; Santa Cruz Biotechnology, Inc., CA, USA) in 10 ml dilution buffer (1X Tris-buffered saline, 0.1% Tween-20 and 5% BSA), with agitation overnight at 4°C. The membranes were subsequently incubated with the appropriate horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (cat. no. zf-0311; 1:2,000; Zhongshan Biotechnology Co., Ltd., Beijing, China) in 10 ml dilution buffer with agitation for 1 h at 25°C. The expression of GR α was normalized against GAPDH, as an internal control. The bands were detected using an enhanced chemiluminescence kit (Amersham, Pittsburgh, PA, USA) and the protein expression was quantified using Labworks™ Image Acquisition and Analysis software version 4.6 (UVP LLC, Upland, CA, USA).

Flow cytometric analysis of podocyte apoptosis. The apoptosis rate in the transfected podocytes exposed to Dex and PAN was assessed using a fluorescein isothiocyanate (FITC)-conjugated annexin V apoptosis detection kit (Molecular Probes, Eugene, Oregon, USA). Briefly, the podocytes were gently trypsinized and pelleted by centrifugation at 900 x g for 5 min at room temperature. The pellet was washed with phosphate-buffered saline (PBS) and resuspended in binding buffer. The cells were subsequently stained with FITC-annexin V and propidium iodide (PI) for 5 min on ice in the dark. The stained cell data was acquired on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed using CellQuest Pro software 5.1 (BD Biosciences, San Jose, CA, USA). At least 1x10⁶/ml cells were assessed per sample and the apoptotic cells were defined as annexin V positive and PI negative.

Podocyte viability assays. Podocytes transfected with the miR-30a mimics and inhibitor were pretreated with Dex followed by PAN. The viability was determined by fluorescence microscopy (BX51TF; Olympus, Tokyo, Japan) following staining with an EdU incorporation kit (RiboBio Co. Ltd.), according to the manufacturer's instructions.

Cloning of pmiR-RB-REPORT™ construct. Prediction of the 3'-untranslated region (UTR) and coding sequence (CDS) was performed with miRanda v1.0b and RNA22, respectively. The GR α 3'-UTR (1-2021 bp) fragment, bearing the predicted miR-30a binding site, was isolated from murine cDNA by PCR. The PCR product was isolated by agarose gel electrophoresis, purified and cloned into the pmiR-RB-REPORT™ vector (RiboBio Co., Ltd.). The resulting construct was designated 3'-UTR (1-2021 bp) WT. The predicted miR-30a binding site (GTTTGTG) was mutated (CTATCTC), inserted into another vector and designated 3'-UTR (1-2021 bp) MT. The GR α 3'-UTR (2020-3953 bp) and CDS fragments were cloned into another two vectors, and were designated 3'-UTR (2020-3953 bp) WT and CDS WT, respectively. The primer sequences (RiboBio Co., Ltd.) were as follows: 3'-UTR (1-2021 bp) WT and 3'-UTR (1-2021 bp) MT, sense: 5'-CCGCTCGAGCTGCCTTACTAAGAAAGGCTGCCTTAAAG-3' and antisense: 5'-GAATGCGGCCGCAGCCTCTGTTTCTGTTAACC-3'; 3'-UTR (2020-3953 bp) WT, sense: 5'-CCGCTCGAGACAAGAGTCCATTTCCAA

GTAAG-3' and antisense: 5'-GAATGCGGCCGCAGT CACATAATGAAAAAGCAG-3'; CDS WT, sense: 5'-CCGCTCGAGATGGACTCCAAAGAATCCTTAGC-3' and antisense: 5'-GAATGCGGCCGCCTCATTCTGTATGAAACAGAAGC-3'. The plasmid constructs were confirmed by automated DNA sequencing.

Luciferase assays. NIH3T3 murine fibroblast cells were seeded into a 96-well plate (4,000 cells/well) and were co-transfected with a reporter construct (100 ng/ml) and synthetic miR-30a (50 nM), according to the manufacturer's instructions (RiboBio Co. Ltd.). At 24 h post-transfection, the cells were harvested in 100 μ l Passive Lysis Buffer (Promega, Madison, WI, USA) and the luciferase activity was measured in the lysates (35 μ l) using a luciferase reporter assay kit (Promega).

Statistical analysis. SPSS version 18.0 (IBM SPSS, Chicago, IL, USA) was used for statistical analyses. For microarray analysis, the raw expression signals were log transformed, normalized and filtered, according to the median corrected signal of all the miRNAs with an intensity >100. The miRNAs displaying differential expression between differentiated murine podocytes and injured podocytes induced by PAN were detected using a two-sample t-test. The data are presented as the mean \pm standard deviation and interpreted by analysis of variance with Tukey's test. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of differentially expressed miRNA in differentiated murine podocytes and injured podocytes induced by PAN. Of the 1,908 murine miRNAs represented on the microarrays, 187 were expressed in differentiated podocytes and 172 in injured podocytes induced by PAN. A total of 19 miRNAs were identified to be significantly upregulated and 35 were significantly downregulated (Table I). Following the application of a fold change threshold >2 (false discovery rate <0.01), 10 miRNAs, including miR-30a, miR-27a-3p, miR-30d, miR-100, miR181c, miR-5099, miR-3535, miR-140-3p, miR-148-3p and miR-103-3p were significantly upregulated.

GR is a putative target of miR-30a in murine podocytes. To identify miRNAs with the potential to modify GR activity by binding to the 3'-UTR in the 10 significantly upregulated miRNAs, an *in silico* search was performed using Pictar, miRanda and TargetScan/TargetScanS databases. Based on the maximum likelihood of binding conservation and accessibility, all three databases predicted that GR was a putative target of miR-30a in the mouse.

miR-30a negatively regulates GR α in normal and injured podocytes induced by PAN. Based on the results of the bioinformatics investigations, the expression levels of GR α and miR-30a were analyzed in undifferentiated and differentiated podocytes by RT-qPCR. While the expression of miR-30a increased, the expression of GR α decreased during the process of podocytic maturation (Fig. 1A and B). Subsequently, it was revealed that transfection of undifferentiated and differentiated podocytes with the miR-30a mimics caused a reduction in the mRNA

Table I. Identification of differentially expressed miRNA in differentiated murine podocytes and injured podocytes induced by PAN.

miRNA ID	Differentiated podocytes	Podocytes induced by PAN	P-value
mmu-miR-30a-5p	8928.94	33333.19	<0.01
mmu-miR-27a-3p	5597.11	18809.07	<0.01
mmu-miR-30d-5p	4084.54	10617.27	<0.01
mmu-miR-100-5p	576.49	2146.32	<0.01
mmu-miR-140-3p	472.84	1605.43	<0.01
mmu-miR-103-3p	343.27	3171.62	<0.01
mmu-miR-148a-3p	228.49	1193.20	<0.01
mmu-miR-181c-5p	23.17	2069.71	<0.01
mmu-miR-5099	0.00	28.84	<0.01
mmu-miR-3535	0.00	12.11	<0.01
mmu-miR-10b-5p	9607.71	23206.82	<0.05
mmu-miR-10a-5p	8993.11	22036.14	<0.05
mmu-miR-106b-5p	359.89	881.80	<0.05
mmu-miR-28a-3p	89.02	252.70	<0.05
mmu-miR-542-3p	31.25	98.28	<0.05
mmu-miR-195a-5p	3.81	22.69	<0.05
mmu-miR-429-3p	3.35	20.99	<0.05
mmu-miR-6236	0.00	7.00	<0.05
mmu-miR-26a-5p	5669.51	376.40	<0.01
mmu-miR-107-3p	1315.63	81.05	<0.01
mmu-miR-378d	639.75	0.00	<0.01
mmu-miR-128-3p	574.66	72.69	<0.01
mmu-miR-181b-5p	478.94	215.67	<0.01
mmu-miR-425-5p	458.21	126.43	<0.01
mmu-miR-22-3p	408.67	95.38	<0.01
mmu-miR-191-5p	403.18	144.69	<0.01
mmu-miR-3074-2-3p	323.61	142.13	<0.01
mmu-miR-29b-3p	172.70	77.29	<0.01
mmu-miR-423-3p	124.54	58.70	<0.01
mmu-miR-615-3p	115.54	39.59	<0.01
mmu-miR-16-1-3p	87.19	11.77	<0.01
mmu-miR-25-5p	74.39	7.85	<0.01
mmu-miR-362-5p	64.94	2.39	<0.01
mmu-miR-671-5p	51.52	5.29	<0.01
mmu-miR-450a-5p	33.69	16.72	<0.01
mmu-miR-296-3p	29.57	13.31	<0.01
mmu-miR-24-3p	26.98	0.00	<0.01
mmu-miR-340-3p	23.78	5.12	<0.01
mmu-miR-24-2-5p	17.83	6.48	<0.01
mmu-miR-30c-2-3p	14.94	5.97	<0.01
mmu-miR-152-5p	14.63	3.92	<0.01
mmu-let-7d-3p	14.48	5.46	<0.01
mmu-miR-350-5p	14.33	3.58	<0.01
mmu-miR-126a-3p	10.21	3.58	<0.01
mmu-miR-199b-3p	10.21	0.00	<0.01
mmu-miR-466e-3p	10.06	0.00	<0.01
mmu-miR-132-5p	6.25	0.00	<0.01
mmu-miR-301a-3p	14.18	7.00	<0.05
mmu-miR-1306-3p	7.77	2.05	<0.05
mmu-miR-182-3p	6.25	2.05	<0.05
mmu-miR-181b-1-3p	6.10	0.00	<0.05
mmu-miR-3102-3p.2-3p	5.79	0.00	<0.05
mmu-miR-1964-3p	5.03	0.00	<0.05

PAN, puromycin aminonucleoside; miRNA, microRNA; miR, microRNA.

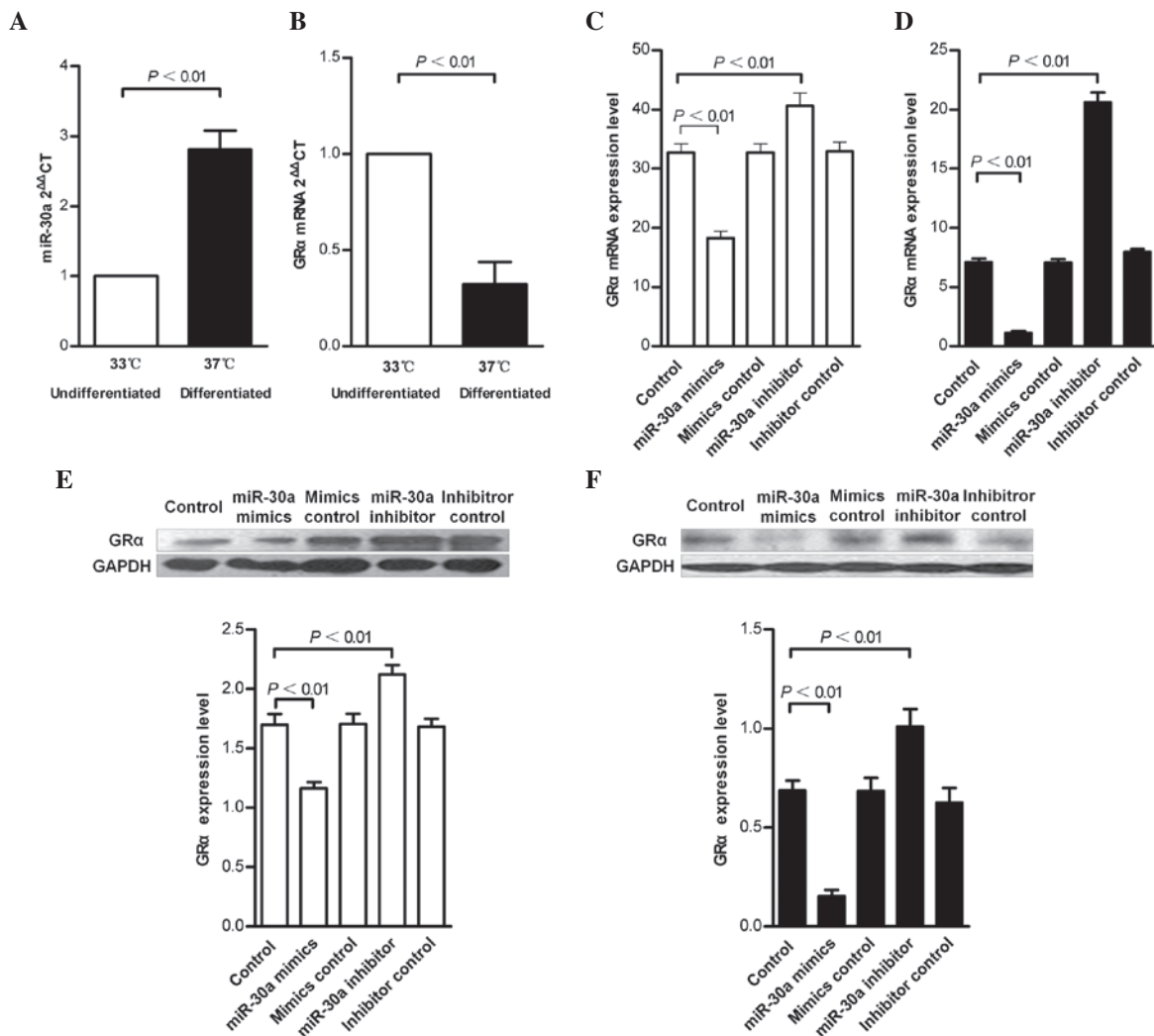


Figure 1. miR-30a negatively regulates GR α in undifferentiated and differentiated murine podocytes. RT-qPCR analysis of the mRNA expression levels of (A) miR-30a and (B) GR α in undifferentiated and differentiated podocytes. RT-qPCR analysis of the mRNA expression of GR α in podocytes transfected with miR-30a mimics or inhibitor in (C) undifferentiated and (D) differentiated podocytes. Western blot analysis of the protein expression of GR α in podocytes transfected with miR-30a mimics or inhibitor in (E) undifferentiated and (F) differentiated podocytes. GAPDH was used as a loading control. The data are expressed as the mean \pm standard deviation of three independent experiments. GR, glucocorticoid receptor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR, microRNA.

and protein expression levels of GR α (25-60 and 25-40%, respectively). By contrast, treatment with the miR-30a inhibitor resulted in increased mRNA and protein expression levels of GR α (25-35 and 10-25%, respectively; Fig. 2C-F).

An *in vitro* model of podocyte injury induced by PAN was established to further confirm the effect of miR-30a on GR α . Compared with the normal group, the mRNA expression of GR α decreased in injured podocytes and the expression of miR-30a increased, which confirmed the microarray results (Fig. 2A and B). An identical pattern was observed in response to PAN-injury following miR-30a mimic or inhibitor transfection of the podocytes. Transfection with the miR-30a mimics caused a 25% reduction in the mRNA and protein expression levels of GR α , while miR-30a inhibitor treatment resulted in a 25% increase in GR α RNA, and a 10% increase in GR α protein (Fig. 2C-E).

Inhibition of miR-30a prevents PAN-induced podocyte apoptosis. Apoptosis is the predominant characteristic of

PAN-induced podocytic injury. Flow cytometric analysis revealed that podocyte apoptosis increased following PAN treatment and was ameliorated by Dex (P<0.05). However, this amelioration was inhibited by the GR inhibitor, RU486 (P>0.05). Transfection of PAN and Dex pretreated podocytes with miR-30a mimics or the miR-30a inhibitor resulted in increased and decreased levels of apoptosis, respectively (P<0.05; Fig. 3A and B).

Furthermore, EdU incorporation analysis of the injured podocytes demonstrated that the proliferation rate was increased by Dex and that this increase was inhibited by RU486 (P<0.05). Transfection of PAN and Dex pretreated podocytes with miR-30a mimics or the miR-30a inhibitor resulted in decreased (P<0.05) and increased proliferation, respectively (Fig. 3C).

Expression of GR α is downregulated by miR-30a interference, however, the 3'-UTR and CDS of GR α are indirect miR-30a targets. To further confirm the association between miR-30a

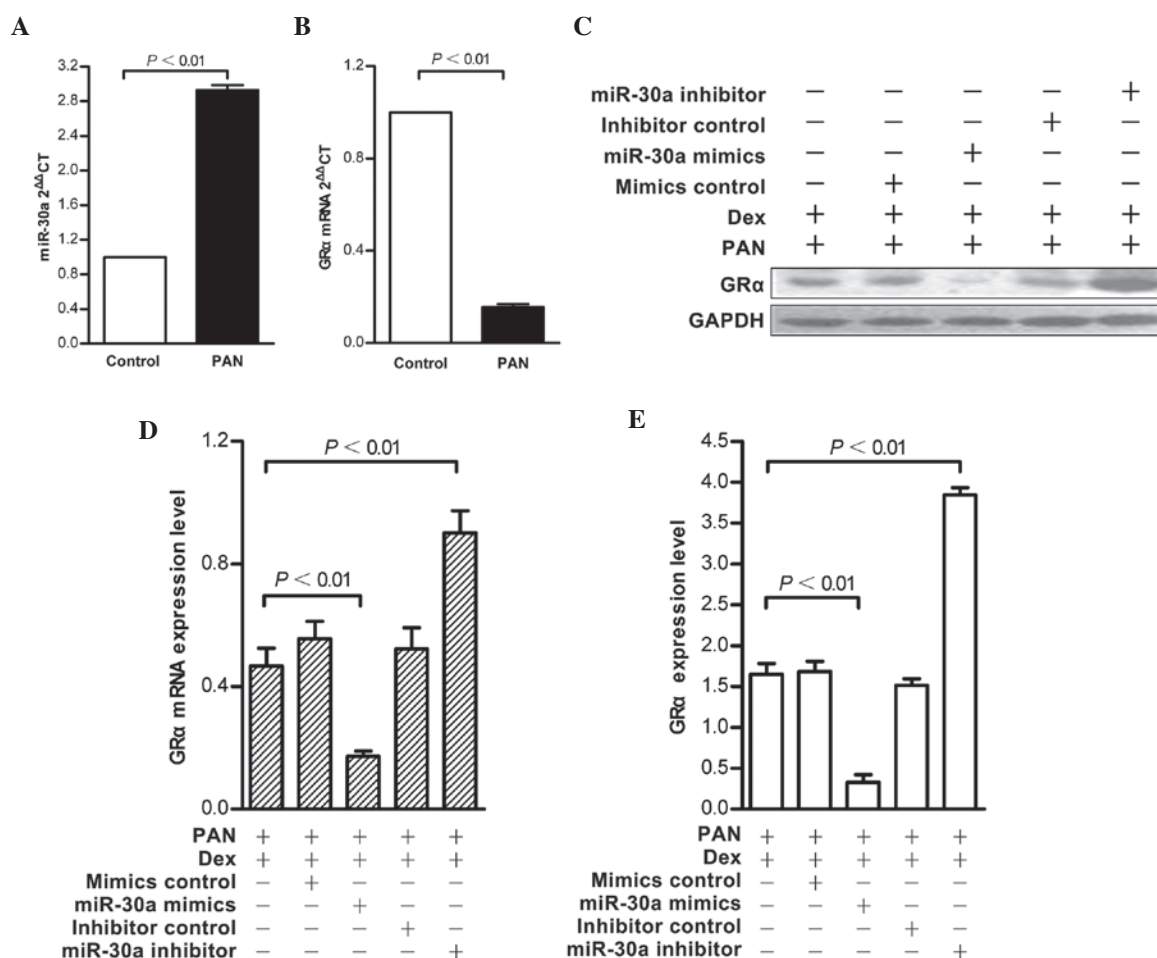


Figure 2. miR-30a negatively regulates GR α in PAN-injured podocytes. RT-qPCR analysis of the mRNA expression levels of (A) miR-30a and (B) GR α in PAN-injured podocytes. (C) RT-qPCR analysis of the mRNA expression of GR α in podocytes transfected with miR-30a mimics and inhibitor prior to PAN-mediated injury. (D and E) Western blot analysis of the protein expression of GR α in podocytes transfected with miR-30a mimics or inhibitor prior to PAN-mediated injury. GAPDH was used as a loading control. The data are expressed as the mean \pm standard deviation of three independent experiments. GR, glucocorticoid receptor; PAN, puromycin aminonucleoside; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR, microRNA; Dex, dexamethasone.

and GR α , the targeted binding site was examined using a dual-luciferase reporter system. Firstly, the consensus sequences for miR-30a in the most common targeted binding site, the GR α 3'-UTR, were predicted. The full-length GR α 3'-UTR (3,953 bp) was revealed to contain six predicted binding sites for miR-30a: 330-351, 741-761, 824-842, 941-969, 1605-1629 and 2108-2137 bp (Fig. 4A). The site located at 941-969 bp was identified as the most likely binding site, according to the continuity of seed sequences, the lowest free energy and conservation between species. To confirm the predicted consensus sequences and to determine whether these miR-30a seed sequences directly contributed to the negative regulation of GR α expression, substitution mutants (at positions 963, 965, 967 and 969) of the GR α 3'-UTR fragment were cloned into vectors containing the WT miR-30a targeting sequences. Furthermore, analysis of other possible candidates, including the GR α -5'-UTR and CDS, indicated that nucleotides 66-71 bp of the CDS region is also a predicted miR-30a binding consensus sequence (Fig. 4B). However, co-transfection of murine cells with 50 nM miR-30a mimics revealed no change in luciferase activity of the vectors, therefore, demonstrating that miR-30a binds to either the 3'-UTR or CDS of GR α (Fig. 4C).

Discussion

GR α is a well-known receptor isoform, which mediates glucocorticoid functions. Investigation of the miRNA-regulation of GR α in podocytes may shed new light on the mechanisms underlying steroid-resistance in podocyte-associated nephrology. By comparing a microarray miRNA expression profile in normal and injured murine podocytes, the present study identified 10 candidate miRNAs significantly upregulated in injured podocytes, along with the downregulation of their potential target gene, GR α .

Since a certain regularity exists in the interaction of miRNAs and target genes, computational algorithms, which are fundamental to our understanding of the role of miRNAs in gene regulation, have proved to be highly effective in predicting candidate miRNAs, which can target an mRNA of interest (13,14). However, this predicted correlation requires biological confirmation in the context of several influencing factors (15). The three most common bioinformatics target prediction tools were used in the present study and all indicated that GR was putative target gene of miR-30a. However, the interaction between miR-30a and the GR α isoform of GR

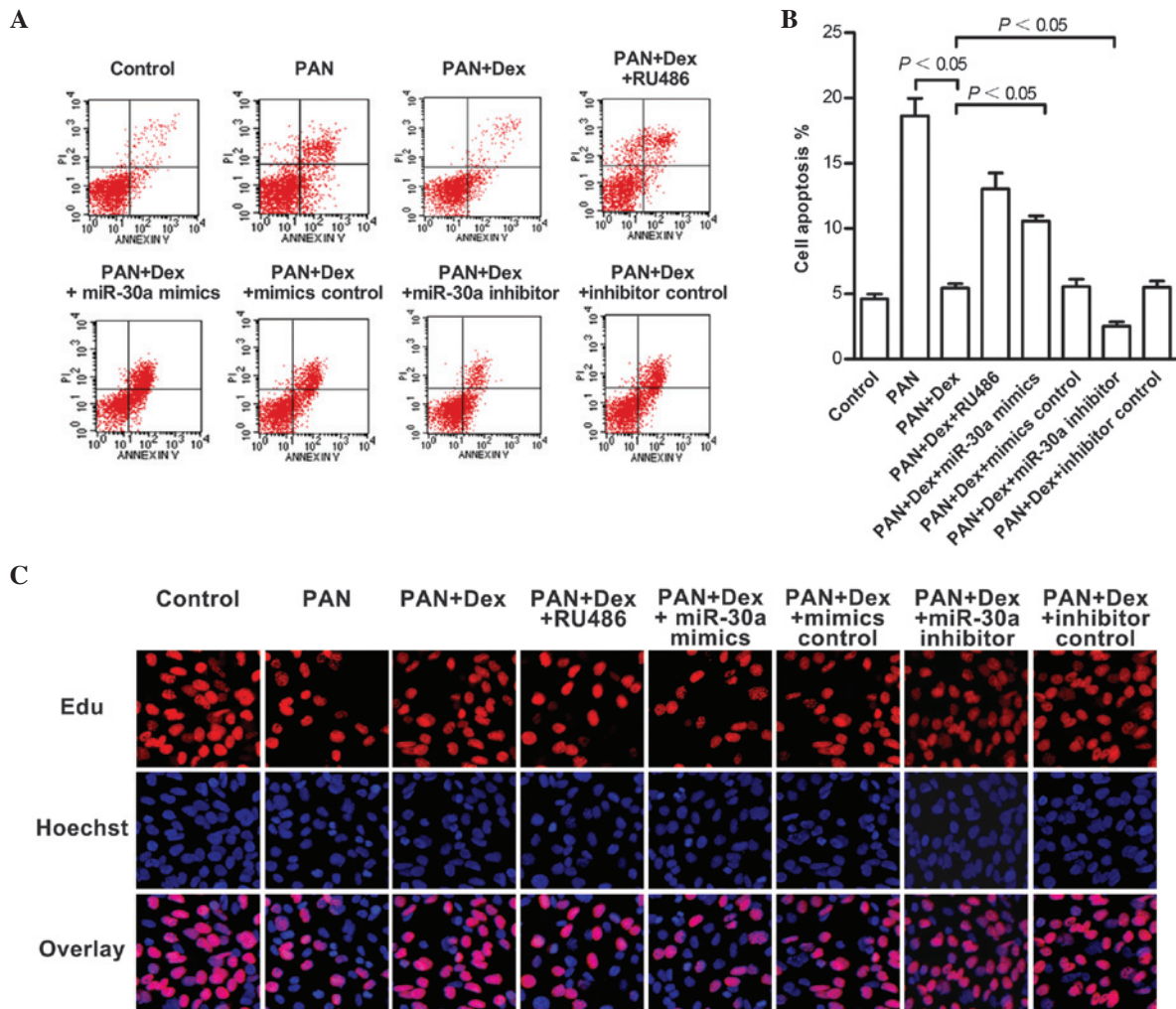


Figure 3. Inhibition of miR-30a prevents PAN-induced podocytic apoptosis. (A and B) Flow cytometric analysis of the apoptotic cell death marker, annexin V, in PAN-injured podocytes following recovery induced by Dex combined with GR antagonist, RU486, and following transfection with miR-30a mimics and the inhibitor. The data are expressed as the mean \pm standard deviation of three independent experiments. (C) EdU incorporation was used to observe cell proliferation. Dex, dexamethasone; GR, glucorticoid receptor; PAN, puromycin aminonucleoside; miR, microRNA.

remains to be elucidated experimentally. As classical negative regulators in mammals, miRNAs were initially hypothesized to mediate the repression of target proteins at the translational level (16). Later, high-throughput studies confirmed that miRNA-mediated changes occur at the mRNA and protein levels, and are predominantly mediated by target mRNA destabilization (17,18). In the present study, it was identified that the marked reduction in the mRNA expression of GR α mediated by miR-30a was in contrast to the more moderate influence on the corresponding protein, indicating that miR-30a regulates GR α expression at the translational and transcriptional levels.

The podocyte is a terminally differentiated cell with limited proliferative capacity and podocyte number is a hallmark of progressive glomerulosclerosis (19,20). Therefore, podocytic apoptosis is a critical determinant of the progression of glomerulosclerosis and protection against podocytic apoptosis is a vital means of slowing down glomerulosclerosis (21). Additionally, previous studies have suggested that miR-30a is involved in kidney development, including podocyte differentiation (22-24). Therefore, the present study aimed to determine whether miR-30a was involved in podocytic apoptosis. Besides the conventional anti-inflammatory or

immunosuppressive actions, Dex causes modest upregulation and increased nuclear localization of GR in podocytes, and also enhances and accelerates podocytic maturation. PAN treatment causes podocytic apoptosis, resulting in the development of massive proteinuria resembling human podocytopathies. This method has provided insights into the mechanisms of podocyte-induced injury *in vitro*. Glucocorticoids can protect and enhance recovery from PAN-induced injury in cultured murine podocytes via actin filament stabilization (7). The protective effects of Dex on PAN-induced podocyte apoptosis are associated with decreased expression of p53, increased expression of Bcl-x1, inhibition of apoptosis-inducing factor translocation and ERK phosphorylation (25-27). This also remains controversial as certain previous studies have demonstrated that Dex significantly enhances podocyte number owing to increased survival of individual cells as opposed to a reduction of apoptosis (4). To confirm the microarray results, quantitative detection of miR-30a and the expression was higher in the injured podocytes induced by PAN, confirming the microarray results. However, Wu *et al* (28) previously documented that the expression of the miR-30 family in human

computational target prediction programs were identified to achieve precision reaching ~93% (35,36). Accordingly, false positives obtained through *in silico* prediction may account for the negative result observed in the luciferase reporter assay in the present study. However, the correlation between miR-30a and GR α at the functional level was demonstrated in this study. A similar effect was reported by Vreugdenhil *et al* (37) and revealed that miR-18 was predicted to bind GR mRNA *in silico* and that miR-18 overexpression reduced GR protein levels. However, miR-18 was unable to bind to the predicted seed region in the GR 3'-UTR. It was suggested that the predicted miRNA binding sites were unavailable for miRNA binding due to differences in the tertiary RNA folding of the target gene sequence in the predicted and *in vivo* situations (37). Therefore, these data fail to completely preclude a potential miR-30a effect on the gene expression of GR α . The functions of miRNAs are particularly complicated. While a single miRNA is thought to target numerous genes, multiple miRNAs are co-expressed and are likely to mediate coordinated regulation of identical mRNAs (28,38), resulting in corresponding multiplex associations. Furthermore, synergy and antagonism also exist in the interaction of miRNAs and represent an additional layer of diversity to the function of miRNAs (39). The mechanisms by which miRNAs mediate gene regulation are still open to discussion, and further investigations are required to improve our understanding of the function of each miRNA. Only in this way will the network of interactions, which exist between miRNAs and target genes, be better elucidated.

The major finding of the present study was that silencing of miR-30a may have a potential effect on improving steroid sensitivity in murine podocyte injury. However, although miR-30a downregulates GR α at the functional level, the mechanism requires further investigation.

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