miR-204 regulates epithelial-mesenchymal transition by targeting SP1 in the tubular epithelial cells after acute kidney injury induced by ischemia-reperfusion

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Abstract. Acute kidney injury (AKI) is a disease where kidney function is lost almost instantaneously; it can develop very rapidly over few hours to maximum of few days. Despite the advent of technology, the clinical management against this disease is very poor, and most of the time it is life-threatening. AKI has been actively regulated by extracellular matrix proteins (ECM), however, its underlying mechanism of regulation during AKI progression is very poorly understood. In this study, we explored the integrated network of mRNA and microRNAs (miRNAs) that maintains the progression of ECM after induction of AKI by lethal ischemia. To identify key regulators of ECM, we screened large number of transcriptomes using laser capture microdissection (LCM) technique in addition to microarray and RT-qPCR. Our result clearly showed that 9 miRNAs including miR-21, miR-483, miR-5115, miR-204e, miR-128, miR-181c, miR-203, miR-204 and miR-204c were highly regulated, out of which miR-204 expression change (decrease) was most drastic during ischemia/reperfusion. Detail mechanistic study utilizing combined experimental and computational approach revealed that TGF-β signaling pathway was potentially modulated by deregulated miRNA-204 through SP1, where the TGF-β signaling pathway plays a vital role in ECM regulation. Apart from targeting SP1 and antagonizing epithelial-mesenchymal transition (EMT) signaling our result also showed that miR-204 protects interstitial tissue of renal tubules from chronic fibrotic change. Altogether our study provides sufficient details of how miRNA mediated ECM regulation occur during AKI, which can be effectively utilized in future for better AKI management and diagnosis.

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Introduction

Acute kidney injury (AKI) is defined as a set of clinical-based syndromes characterized by rapid deterioration of glomerular filtration rate (1). The pathogenesis rate of AKI is approximately 30% in intensive care unit (ICU) patients with complications of clinical nephrology with mortality rate of up to 50% (2,3). Apart from being a strong independent risk factor in developing progressive chronic kidney disease (CKD) and end-stage renal disease (ESRD), AKI also determines non-renal outcomes, for instance as seen in cardiovascular disease (4-8). One of the primary causes of AKI is ischemia-reperfusion (I/R) injury of the kidney which occurs by means of severely restricting blood supply to the kidney, particularly towards renal medullar. At this point, hypotension and hypoxia-based injuries are more prone on epithelial cells located at the S3 segment of the outer medullary of proximal tubule (9). In animal models of I/R-induced AKI, reperfusion causes cellular inflammation, oxidative stress, endothelial dysfunction and necro-apoptotic processes, paradoxically exacerbating cell damage (10). Furthermore, the kidney tubular epithelial cells are paramount in tubulointerstitial fibrosis, as a result of oxidative stress and excessive protein exposure. Subsequent overexpression and accumulation of extracellular matrix proteins (ECM) mainly accounts for it (11). With growing body of evidence, it was further established that variety of forms of AKI are associated with CKD (12). In fact, up to 50% of clinically defined AKI cases displayed certain degree of fibrosis.

Chronic AKI can be pathologically defined as extensive collection of ECM in glomeruli and tubulointerstitial that serves as the major cause of renal failure worldwide. ECM is actively involved in cellular signaling, and thus plays an essential role in renal damage and repair during I/R by modulating scaring, inflammatory processes, proliferation and transdifferentiation of fibroblasts. Due to the fact that I/R-induced

AKI involves disruption of certain signal activation and transduction pathways, it is vital for us to fully understand the mechanism especially in terms of working and signaling molecules (13,14). At the moment, a suitable solution for this disorder has not yet been made available.

Epithelial-mesenchymal transition (EMT) is a complex reprogramming process that provides epithelial cells with a mesenchymal phenotype. EMT takes place via numerous different pathways and plays an essential role during organogenesis, tissue repair and fibrosis, in addition to tumor invasion and metastasis (15). Studies have shown that the EMT can take place in adult kidney during chronic injury, which plays very important roles in renal fibrosis (16,17). Foremost causes for the AKI are ischemia, hypoxia and nephrotoxicity. The mechanisms involved in kidney injury and repair are complex. The kidney is particularly susceptible to ischemia and toxins, resulting in vasoconstriction, endothelial damage, and activation of inflammatory processes. This susceptibility arises in part from the vascular-tubular relationships in the outer medulla of the kidney, where the partial pressure of oxygen is low, even at baseline, making them more vulnerable to a decreased renal blood flow (18). A general strategy for treating renal fibrosis is focusing on the signal pathways that causes tubular EMT, or even on myofibroblasts transformation (19).

Recently, another group of powerful regulators which are given high regards from disease initiation and/or progression viewpoint are the microRNAs (miRNAs). They are essentially small noncoding RNA transcripts with approximate length of 22 nucleotides. Their high complementary sequences make them bind to 3'-untranslated region of mRNA targets and subsequently lead to repression of protein synthesis (20). Another unique feature of these regulators are the fact they could modify the expression of multiple genes at any point of time and this serves as a major regulator of whole disease-specific signaling cascades and pathways instead of single gene. This feature specifically highlights the immense potential of miRNAs. As such, any alterations in the levels of miRNAs could possibly be the underlying mechanism of dysregulated expression of protein which also includes kidney disease progression. They are even being studied as internal biomarkers for polycystic disease (21), renal cancer (22) and fibrosis (23). Regardless, only few studies were made available in describing the role played by miRNAs in chronic AKI induced by I/R. In addition, the alteration of miRNA dysregulation and mRNA expression especially in chronic AKI induced by I/R are not fully elucidated. Despite this, the presence of miRNAs and corresponding functions were studied in a limited extent especially pertaining to chronic AKI induced by I/R. Same pattern holds true regarding network integrating dysregulation of miRNA as well as changed mRNA expression which takes place in chronic AKI by I/R.

So far, most published miRNA expression studies utilized whole kidney tissues in I/R-induced AKI mouse without separating the tubular cells that have a central role in tubulointerstitial fibrosis after AKI, following excessive protein exposure. Kidney medulla and cortex are extensively described as having differential patterns of kidney-related miRNAs (24). The expression profile of miRNA specific to kidney samples/specimens is always challenged with heterogenicity of cells which are reflected in the analysis that most of time drowns the specific markers of renal tubular epithelia.

Our work attempted to understand the network properties in regulating both mRNAs and miRNAs that maintain the progression of ECM after AKI induced by lethal ischemia (45-min, unilateral) and following reperfusion injury. This study focussed on elucidating groups of miRNAs that have direct relations with ECM after lethal ischemia and following reperfusion injury. Here the combination of few methods which include RT-qPCR, microarray and laser capture microdissection (LCM) to screen transcription molecule patterns originating from concentrated cell groups harvested from murine kidney with I/R-induced AKI, as an effort to describe networking pathways related to pathologically-defined fibrotic issues. By using the array technology we obtained a thorough selection of miRNAs related to tubular epithelial cells those captured by LCM and the differential profiles of mRNAs in the kidney tissues, as verified by qPCR with subsequent analysis of bioinformatics.

Materials and methods

Animal studies. C57BL/6J male mice were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (licensed no.: SCXK Hu 2007-0005) and were taken care as per the conditions described by Guide for Care and Use of Laboratory Animals. All mice were kept in animal housing facility under temperature of 21±1˚C with 50-80% relative humidity with 12-h light-dark cycle every alternate day except during surgery. They were acclimatized for 2 weeks prior to experiments and their food was commercial rodent chow with water supply *ad libitum*. Ethical approval was obtained from Xinhua Hospital Animal Care and Use Committees.

Renal ischemia-reperfusion injury. A total of 40 C57BL/6J mice (weight range: 20-24 g) were subjected to renal unilateral I/R injury for 45 min as previously described (25). Among them, 8 mice underwent sham surgery. In brief, all the experiments were performed at room temperature (24±0.5˚C), as described by the standard operating procedures. By using heating pad (HK-3, DOPS) the intra-abdominal temperature was kept within acceptable range. The animals were anesthetized via intraperitoneal (i.p.) injection of 45 mg/kg pentobarbital. Lethal (45 min) ischemia injury was obtained via clamping of left renal artery. Preparation for sham-operated control mice were carried out with no clamping on the renal pedicle.

Sacrifice and organ collection. Animals were sacrificed upon completion of the experimental treatment by adopting cervical dislocation method at 3, 24, 72 and 128 h following reperfusion. Sham mice were sacrificed by cervical dislocation method 24 h later (n=8/time point). Both kidneys were taken out upon deliberate trans-cardiac perfusion with 20 ml pre-cooled (4˚C) physiological salt solution. Both kidneys from randomly chosen mice undergoing I/R were excised and stored accordingly for further use in microarray analysis of microRNA. Other renal tissue samples were kept at -80˚C via snap-frozen method in liquid nitrogen.

Histology of renal tissue injury and immunohistochemistry. Fixed renal tissue samples at 4% buffered formaldehyde were dehydrated and embedded in paraffin wax (FFPE) for histology and immunohistochemistry experiments. They were morphologically evaluated by using tissue microarray (TMA) as previously described (26).

Subsequent immuno-staining required TMA sections to be incubated in 1% BSA in TBS (pH 7.4) buffer for 15 min, followed by primary antibody [rabbit anti-mouse α-SMA immunoglobulin-G (IgG) (1:100; R&D Systems] for 16 h and in secondary antibody [goat anti-rabbit IgG EnVision-peroxidase polymer kit (Dako)] for 40 min, all at room temperature. Immunostained slides were then digitalized using a Pannoramic Scan, and the results were analyzed via the Pannoramic Viewer software (3DHISTECH).

Laser capture microdissection. The kidney samples were subjected to LCM with the Leica LMD7000 System as suggested by the manufacturer. In brief, serial cut sections of 10 μm were mounted on polyethelene membrane slides (Leica Membrane Slides), and were fixed in 100% ice-cold ethanol for 15 min and kept for approximately 2 h at -80˚C. The slides were then washed with DEPC treated water and stained with hematoxylin solution (MHS128; Sigma-Aldrich, St. Louis, MO, USA) for one min. Then, the slides were dehydrated for 204 sec with 100% ethanol and xylene for 5 min and later air-dried. The slides were positioned onto Leica LMD7000 Laser Microdissection Instrument (Leica LMD7000, DE). As described by manufacturer's recommended protocol, kidney tubular epithelial cell populations (~$2x10^5$) were selected and captured using UV laser cutting. Immediately, LCM cap was placed in a micro-centrifuge tube that contained 400 μl lysis/binding buffer (Ambion, Austin, TX, USA), and stored for RNA isolation at -80˚C.

Total RNA isolation and quality control. Total RNA was extracted from tissue sections by means of mirVana™ RNA Isolation kit (Applied Biosystems, Foster City, CA, USA) as per provided protocol. Quantification of RNA was performed with NanoDrop ND-2000 (Thermo Scientific) and its integrity was evaluated via Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The quality was determined by RNA integrity number (RIN). On a scale of 1-10, a RIN score was generated for each sample. The quality is considered to be excellent for RIN ≥7-10, good for RIN ≥5 and poor for RIN <5.

Genome-wide miRNA profiling analysis. Agilent Mouse miRNA V21.0 (8x60 K) microarrays (Agilent Technologies) were utilized to match the expression profiles of LCM selected epithelial cells taken from 6 kidney tissues (n=3 left kidney at 24 h following reperfusion, n=3 right kidney of the same mouse at 24 h following reperfusion). The microarray consists of probes with 1881 mouse mature miRNAs from Sanger miRBase V21.0. Cy3 incorporation was performed on a total RNA of 100 ng from each of the kidney samples. Scanning of microarray slides were by the Agilent Scanner G2505C (Agilent Technologies). The subsequent processing of labeling and hybridization were accomplished at Shanghai Oebiotech Company as per the protocols in the Agilent miRNA system. In brief, total RNA were dephosphorylated, denatured and then labeled with Cy3. After purification, RNAs were hybridized onto the microarray. Upon washing, the arrays tentatively were scanned with the Agilent Scanner G2505C (Agilent Technologies). Next, Gene-spring software (version 12.5; Agilent Technologies) was engaged to complete the primary analysis with the raw data. Initially, the raw data were normalized with quantile algorithm. In conditions where the probes detects at least 1 condition out of 2 conditions have flags and were chosen for further data analysis. miRNAs expressed differentially were then recognized through fold change and P-value was generated using t-test. The threshold for up- and down-regulated genes was set at a fold change of ≥2.0 and a P-value ≤0.05, respectively. Target genes of differentially expressed miRNAs were the intersection predicted with three databases (TargetScan, microRNAorg, Pita). GO analysis and KEGG analysis were functional to govern the parts of these interested target genes played in GO terms or the pathways. Expression pattern of miRNAs among samples was distinguished by means of Hierarchical Clustering.

MicroRNA-mRNA co-expression network analysis. Putative functions mediated by mRNA targets were identified by the miRNA of interest. We first predicted expression-based mRNA targets of miRNA. Differentially expressed miRNA-related target genes of interest were the intersection predicted with three databases (TargetScan, microRNAorg and Pita). GO and KEGG analysis were performed to detect the function of these target genes played in GO terms or the pathways. Predicted mRNAs were validated by microarray profiling of differential mRNA expression in kidney tissues, and mRNA targets that showed consistent expression patterns were chosen for further study. Predicted mRNAs were validated by microarray profiling of differential mRNA expression in kidney tissues, and mRNA targets that show consistent expression patterns were chosen for further study.

RT-qPCR analysis of miRNAs and mRNA. Total RNA of 2 μg was reverse transcribed with Bulge-Loop miRNA-specific reverse transcription-primers (RiboBio, Guangzhou, China). Real-time PCR reactions were carried out with Platinum SYBR Green qPCR SuperMix-UDG chemicals (Invitrogen, Carlsbad, CA, USA) and Bulge-Loop primers (RiboBio) on PRISM 7900HT (Applied Biosystems, Carlsbad, CA, USA) with small nuclear RNA U6 as the normalization control. Initially, a total of 5 ng RNA samples were reverse-transcribed (RT) to complementary DNAs (cDNAs) using a miRNA-specific, stem-loop RT primer (for miR-21, miR-483, miR-5115, miR-30e, miR-128, miR-181c, miR-203, miR-204, miR-30c, the primers are shown in Table I) as described by the manufacturer. The PCR end products were augmented from the cDNA samples using the TaqMan Small RNA Assay along with the TaqMan Universal PCR Master Mix2.

Western blotting. The expression of TGF-β, SP1, E-cadherin, vimentin, α-SMA and Collagen I were detected by means of standard Western blotting. Cells were lysed in RIPA buffer and 20 μg of proteins were separated on 8-10% SDS/PAGE gel before transferring onto PVDF membranes (Millipore, Billerica,

Table I. The primers for AKI mice.

miRNA	Primer	Sequence (5'-3')
mmu-mir-204	RNA	UUCCCUUUGUCAUCCUAUGCCU
MIMAT0000237	RT stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGCAT
	Forward	GCGCTTCCCTTTGTCATCCT
	Reverse	CAGTGCAGGGTCCGAGGTA
mmu-mir-203	RNA	AGUGGUUCUUGACAGUUCAACA
MIMAT0004547	RT stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGTTGA
	Forward	GCGCAGTGGTTCTTGACAGT
	Reverse	CAGTGCAGGGTCCGAGGTA
mmu-mir-181c	RNA	AACAUUCAACCUGUCGGUGAGU
MIMAT0000674	RT stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCAC
	Forward	GCGCAACATTCAACCTGTCG
	Reverse	CAGTGCAGGGTCCGAGGTA
mmu-mir-128	RNA	CGGGGCCGUAGCACUGUCUGA
MIMAT0016982	RT stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGAC
	Forward	GCGCCGGGGCCGTAGCACT
	Reverse	CAGTGCAGGGTCCGAGGTA
mmu-mir-30c	RNA	UGUAAACAUCCUACACUCUCAGC
MIMAT0000514	RT stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCTGAG
	Forward	GCGCTGTAAACATCCTACACT
	Reverse	CAGTGCAGGGTCCGAGGTA
mmu-mir-30e	RNA	UGUAAACAUCCUUGACUGGAAG
MIMAT0000248	RT stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTTCCA
	Forward	GCGCTGTAAACATCCTTGAC
	Reverse	CAGTGCAGGGTCCGAGGTA
mmu-U6	RT	GGGCCATGCTAAATCTTCTC
NR_003027	Forward	ATGGGTCGAAGTCGTAGCC
	Reverse	TTCTCGGCGTCTTCTTTCTCG

MA, USA). Upon incubation with necessary antibodies, the membranes were then visualized using the ECL system.

Cell culture and cell transfection. HK2 cells were cultured in DMEM supplemented with 100 mg/ml of streptomycin, 100 U/ml penicillin and 10% fetal bovine serum (FBS). They were cultivated at 37˚C in a humidified incubator of 5% CO_2. They were seeded in 24-well plates and incubated overnight, before transiently being transfected with Lipofectamine 2000 (Invitrogen) reverse transfection protocol as per manufacturer's protocol. miR-204 inhibitor (10 nM; both Qiagen), acted as negative control, unspecific AllStars negative control RNA from Qiagen or LNA control from Exiqon was used and all the primers are shown in Table II.

Luciferase reporter assay. HK2 cells were seeded on 24-well plates and co-transfected with 100 ng per well of the resulting luciferase UTR-report vector, 20 ng per well of miR-204 precursor molecules or control precursor (Applied Biosystems) and 2 ng per well of pRLCMV vector (internal control, Promega) using Lipofectamine 2000 (Invitrogen) as per manufacturer's protocol. The cells were lysed after 24 h and their corresponding luciferase activity was assessed with the DualLuciferase Assay Reporter System (Promega).

Statistical analysis. Statistical analysis was accomplished with SigmaStat software (Jandel Scientific Software, San Rafael, CA, USA). One-way analysis of variance, followed by the Student-Newman-Keuls was performed for comparison between groups. $P<0.05$ was considered statistically significant in all cases.

Results

I/R injury results in significant renal tubule interstitial tissue chronic fibrotic changes. In order to characterize whether I/R injury could cause renal tubule interstitial tissue chronic fibrotic changes, we employed a well-designed mouse settings of 45 min unilateral renal I/R injury. The murine models were sacrificed at 24 h, 72 h and 7 days upon reperfusion exhibited renal tubular cell damage evaluated by H&E (Fig. 1A). Extensive tubule-interstitial damage was noted especially in

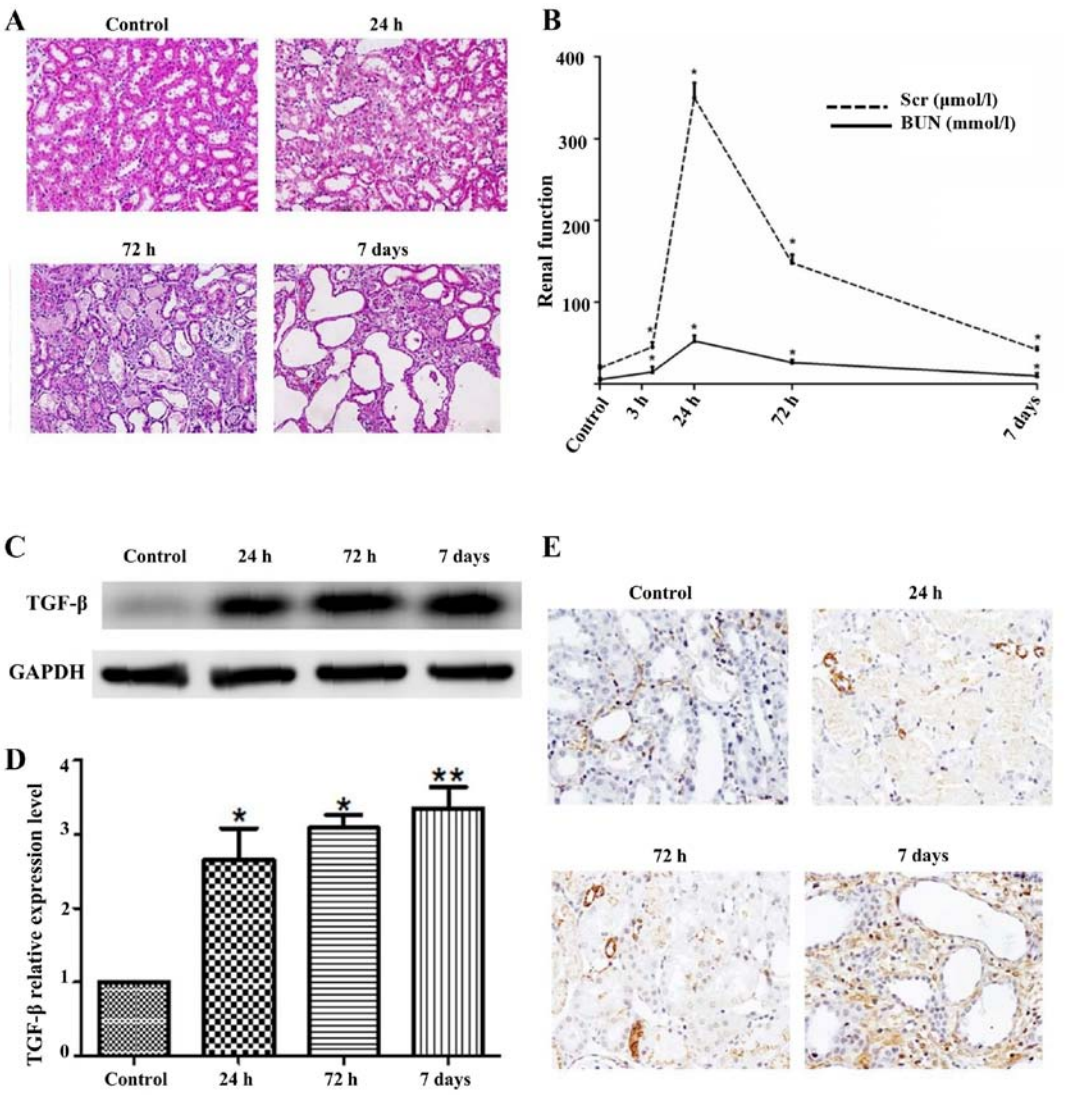

Figure 1. I/R injury results in significant renal tubule interstitial tissue chronic fibrotic changes. (A) H&E of mouse kidney tissue after I/R 24 h, 72 h, 7 days, control with sham operation; (B) Renal function after I/R 3 h, 24 h, 72 h, 7 days, control with sham operation; (C) western blotting of TGF-β after I/R, control with sham operation; (D) the relative expression levels of TGF-β; (E) IHC of α-SMA after I/R, control with sham operation. Data are shown as the mean ± SD, *P<0.05 vs. control, **P<0.01 vs. control. Student's t-test was performed to evaluate the statistical difference.

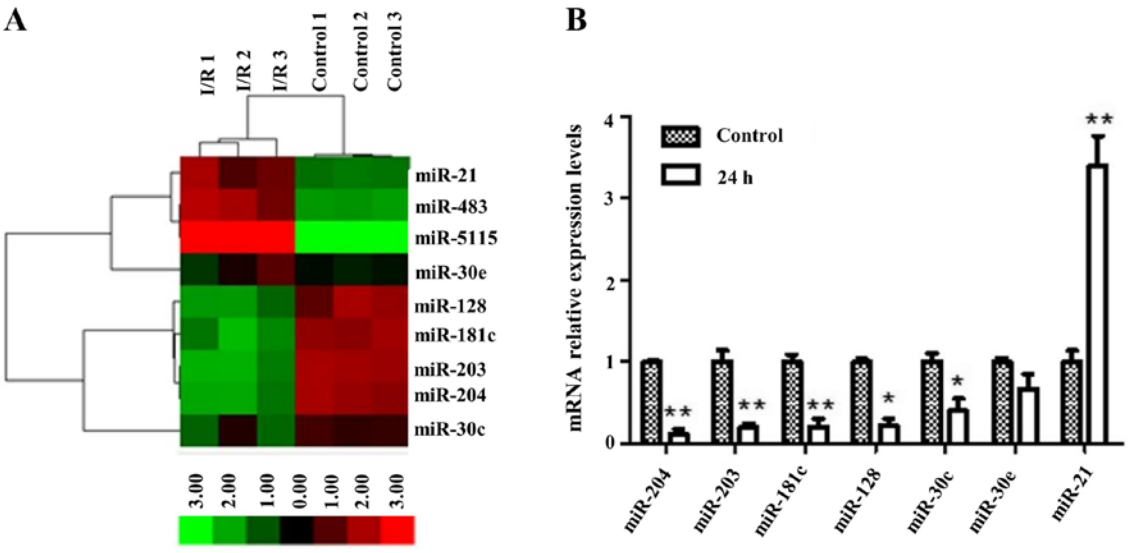

Figure 2. I/R Injury changed the expression of miRNAs in the kidney tissue. (A) Heath map of the differential expression of miRNAs; (B) relative expression levels of miRNAs; data are shown as mean ± SD, *P<0.05 vs. control, **P<0.01 vs. control. Student's t-test was performed to evaluate the statistical difference.

Table II. The primers for HK-2.

miRNA	Primer	Sequence (5'-3')
hsa-mir-204	RNA	UUCCCUUUGUCAUCCUAUGCCU
MIMAT0000265	RT stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGCAT
	Forward	GCGCTTCCCTTTGTCATCCT
	Reverse	CAGTGCAGGGTCCGAGGTA
SP1	Forward	TGGCAGCAGTACCAATGGC
NM_001251825	Reverse	CCAGGTAGTCCTGTCAGAACTT
GAPDH	Forward	GGAGCGAGATCCCTCCAAAAT
NM_001256799	Reverse	GGCTGTTGTCATACTTCTCATGG
α-SMA	Forward	GTGTTGCCCCTGAAGAGCAT
NM_001613	Reverse	GCTGGGACATTGAAAGTCTCA
Collagen I	Forward	GAGGGCCAAGACGAAGACATC
NM_000088	Reverse	CAGATCACGTCATCGCACAAC
E-cadherin	Forward	CGAGAGCTACACGTTCACGG
NM_004360	Reverse	GGGTGTCGAGGGAAAAATAGG
Vimentin	Forward	GCCCTAGACGAACTGGGTC
NM_001017921	Reverse	GGCTGCAACTGCCTAATGAG

S3 segments located at tubules of proximal origin and also in the corticomedullary as well as outer medulla junctions. In addition, events of necrosis and degenerative shifts were profound in individual tubules with lumen closure via cellular debris and desquamated cells. Mild infiltration of chronic inflammation was seen after 24 h of post-treatment where less quantity of necrotic cells were noted in the tubules, especially in their lumens.

In addition, renal function indicated that Scr level was induced at 24 h, but gradually reduced after 24 h, while the level of BUN changed slightly with time (Fig. 1B). Western blot results revealed that the level of TGF-β gradually increased in time dependent manner (Fig. 1C). Moreover, the mRNA expression of TGF-β was seen to be consistent with the WB outcome (Fig. 1D). Immunohistochemical staining of alpha smooth muscle actin (α-SMA), was an important marker of renal tubule interstitial tissue chronic fibrotic change, and showed stronger staining for α-SMA at 7 days than the control (Fig. 1E). Above all, these data demonstrated that I/R injury could cause significant renal tubule interstitial tissue chronic fibrotic changes.

I/R injury induces distinct downregulation of miR-204 in the kidney tissue. To explore the molecular mechanism how I/R injury altered the miRNA expression, we assessed the miRNA profile by means of a novel micro-bead based technology Agilent Mouse miRNA Microarray Scanner formats (8x60 K) which contain probes for 1881 mouse mature miRNAs from the Sanger miRBase V21.0. Upon excluding miRNAs that have minute fluorescent indication (<100 MFI), a total of 9 miRNAs (namely miR-483, miR-21, miR-5115, miR-30e, miR-128, miR-181c, miR-203, miR-204, miR-30c) of the tubule epithelial cell population were selected and captured by LCM. These miRNAs had significantly different expression relative to that of right kidney without damage by I/R. These studies revealed that the miR-204 levels were decreasing in I/R injury group as compared to that of control group. Profound changes in levels of miRNA upon I/R injury in all groups are depicted in Fig. 2A.

Validation of array data was performed by means of Real-time quantitative PCR (RT-qPCR) to detect levels of miR-204 when injury occurs. Fig. 2B shows 5- to 6-fold lower expression of miR-204 under I/R-caused damage as compared to control group. The analysis of Gene Ontology was performed to elucidate distinct biological processes and molecular functions in relation with I/R injury groups and control groups.

The most enriched term for each group can be seen in Fig. 3A and B listing all significant GO categories with a $P<0.05$. Noteworthy, we observed the GOs which showed target gene of downregulation. miRNAs were potentially relevant in intracellular signal transduction, positively modulated cAMP-dependent protein kinase activity, small GTPase mediated signal generation, augmentation of ERK1 and ERK2 cascade and canonical Wnt signaling pathway. Enriched GO terms associated with target gene of upregulated miRNAs were involved in the functions of cell fate commitment, Notch signaling pathway, augmentation of cytotoxicity due to natural killer cells, attenuation of cell differentiation and augmentation of smooth muscle cell apoptotic process. In Fig. 3C and D, the most enriched pathway for each group is shown. Pathway analysis showed that target gene of downregulation miRNAs were implicated in the following pathways: Signaling pathways regulating stem cell pluripotency, TNF signaling pathways, Rap1 signaling pathways, Ras signaling pathways, Hippo signaling pathways, ErbB signaling pathways and PI3K-Akt signaling pathways.

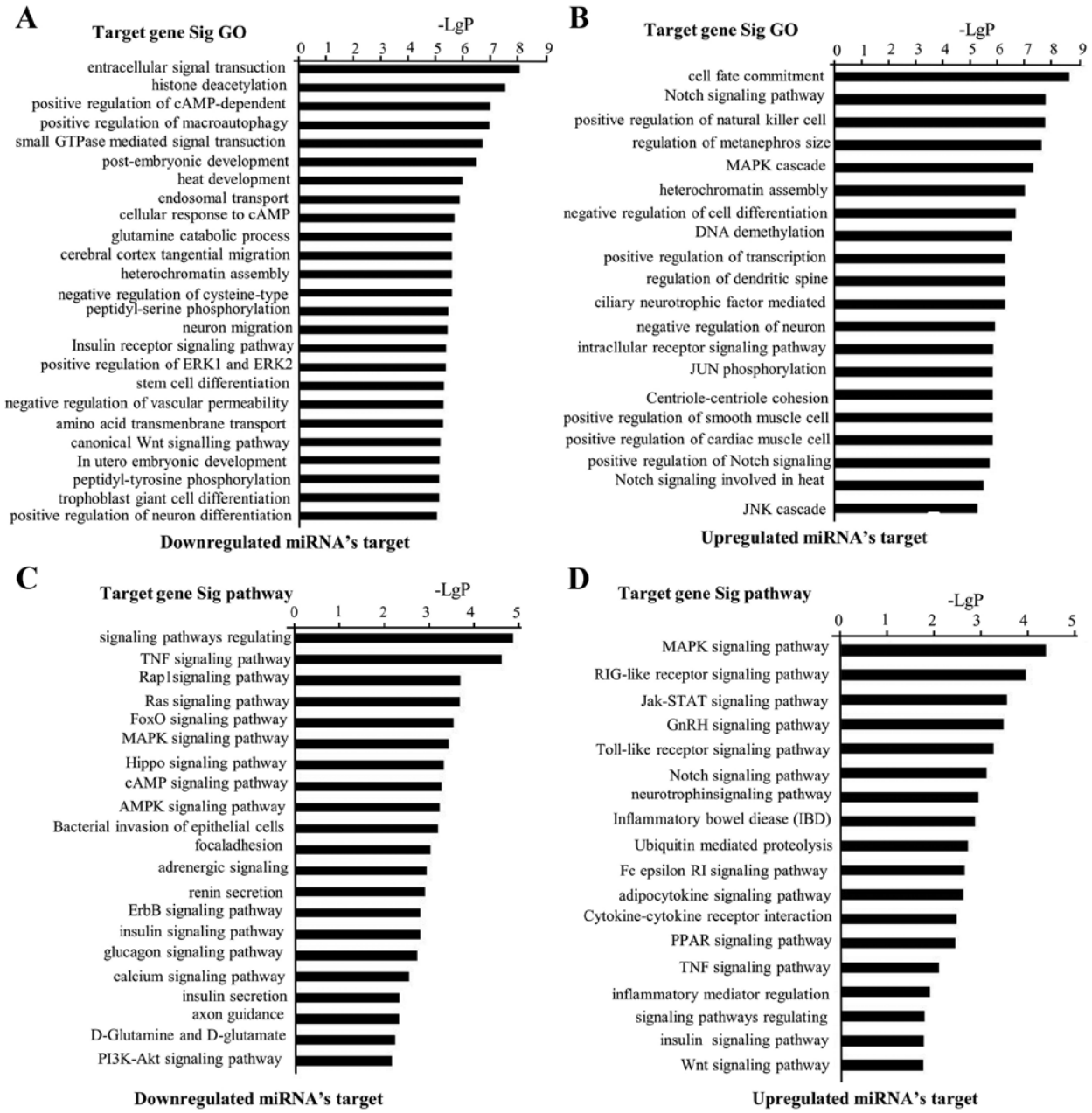

Figure 3. I/R Injury changed the GO and pathways of differential expression miRNA target genes in the kidney tissue. (A) The downregulated miRNA target gene sig GO; (B) the upregulated miRNA target gene sig GO; (C) the downregulated miRNA target gene sig pathway; (D) the upregulated miRNA target genes sig pathway.

Moreover, analysis of pathways demonstrated that target gene of upregulation miRNAs were implicated in following pathways: MAPK signaling pathways, RIG-1-like receptor signaling pathways, Jak-STAT signaling pathways, Notch signaling pathways, Cytokine-cytokine receptor interactions, PPAR signaling pathways, TNF signaling pathways and Wnt signaling pathways.

miR-204 expression was downregulated while SP1 expression was upregulated by I/R injury. To search for potential protein-coding RNAs and miRNAs involved in kidney from I/R-induced AKI mice, we globally analyzed the protein-coding RNA and miRNA expression profiles of normal group and I/R injury group. Co-expression networks of protein-coding RNAs and miRNAs were constructed (Fig. 3A). This is due to co-expression of modules that may correspond to functional and biological pathways and that of protein-coding RNAs can also be searched from RefSeq in National Center for Biotechnology Information (NCBI), we concentrated further on the co-expression models that have high proportion of protein coding RNAs in the CRC co-expression networks. miR-204 in CRC was identified via such modality. In this co-expression network, miR-204 is connected to 13 protein-coding transcription molecules which are highly relevant for genes involving I/R by modulating scaring, inflammatory processes, proliferation and transdifferentiation of fibroblasts (Fig. 4A). The expression pattern of miR-204 in Fig. 3A was validated using RT-qPCR analysis and the expression of miR-204 was suppressed at 24 h, 72 h and 7 days (Fig. 4B). Correspondingly, SP1 protein and mRNA level was induced

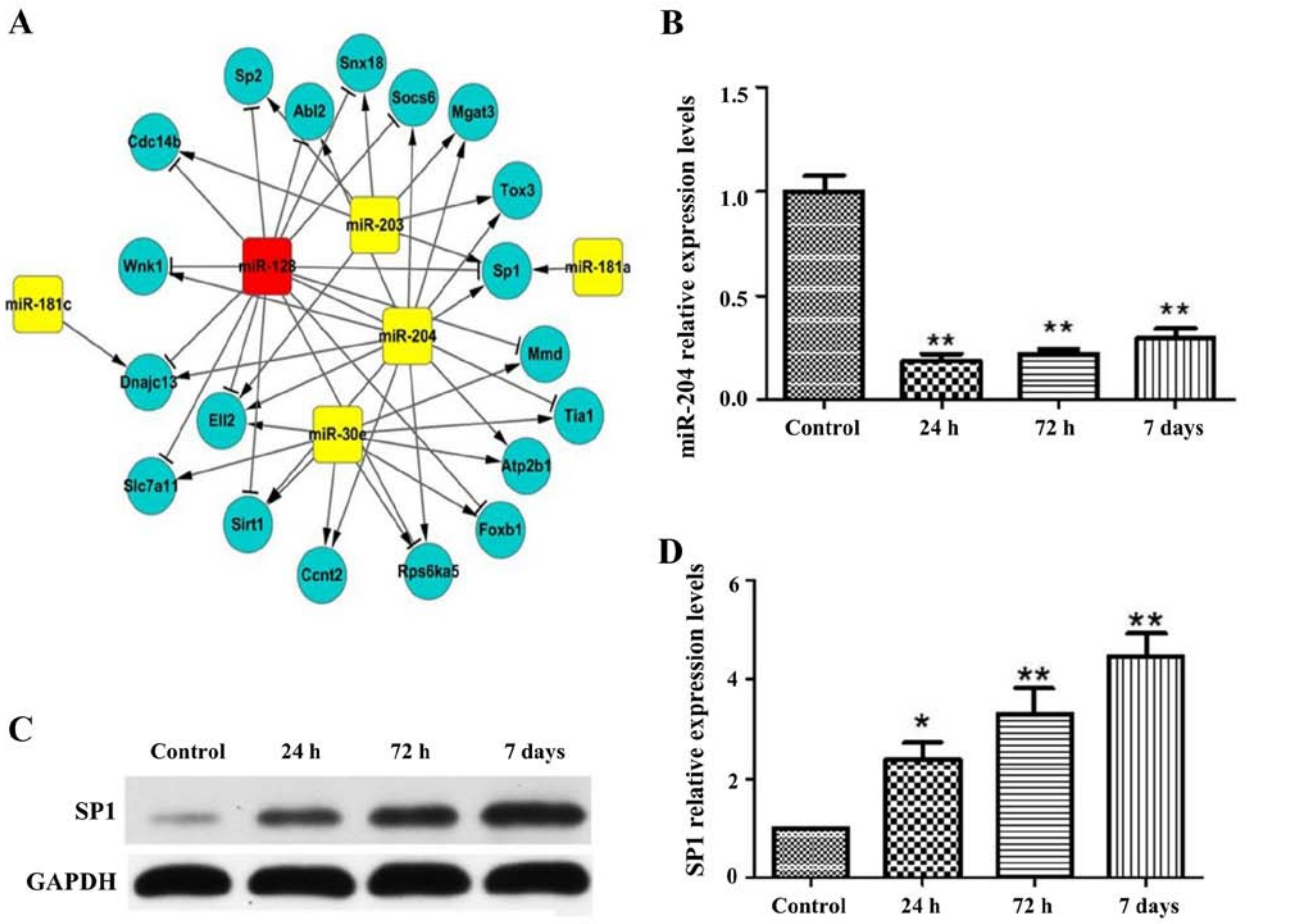

Figure 4. miR-204 expression is downregulated while SP1 expression is upregulated by I/R injury. (A) The network of miRNA and target gene; (B) relative expression levels of miR-204; (C) the western blot of SP1; (D) relative expression levels of SP1 protein. The data are shown as the mean ± SD, *P<0.05 vs. control, **P<0.01 vs. control. Student's t-test was performed to evaluate the statistical difference.

at I/R injury 24 h, 72 h and 7 days with western blot and RT-qPCR analysis (Fig. 4C and D).

miR-204 suppresses hypoxia-induced SP1/EMT signaling pathway by directly targeting SP1. We hypothesized that miRNA/mRNA network would operate I/R injury under hypoxia. The expression of miR-204 was markedly decreased by hypoxia while SP1 mRNA level was induced significantly under hypoxia (Fig. 5A). Furthermore, the increased levels of SP1 induced by hypoxia could be partially reversed by overexpression of miR-204 with miR-204 mimic (Fig. 5A). The mRNA expression of vimentin, α-SMA and Collagen I, acting as mesenchymal cell markers, were induced under hypoxic condition (Fig. 5A). As expected, the interruption approach using miR-204 mimic also reversed the hypoxia-induced vimentin, α-SMA and Collagen I mRNA level (Fig. 5A). E-cadherin, as expression of epithelial cell markers, displayed the opposite expression patterns to mesenchymal cell markers (Fig. 5A). Consistent with RT-qPCR data, western blot also proved that hypoxia-induced SP1 protein level could be partially reversed by overexpression of miR-204 (Fig. 5B and C). Similarly, hypoxia promoted the expression levels of vimentin, α-SMA and Collagen, which could be partly terminated by miR-204 (Fig. 5B and C).

Using TargetScan software, SP1 gene was further validated as candidate target of miR-204 since it carries corresponding miR-204 target sites in the 3'-UTR. As a way to verify this phenomenon, a luciferase-based reporter vector containing oligo-nucleotides that fully complements the 3'-UTR of wild-type SP1, or its relevant mutants was constructed. Using HK2 cell lines, non-functional control miR-NC RNAs or pre-hsa-miR-204 RNAs co-transfection were performed. It was further noted that wild-type SP1 3'-UTR and miR-204 target sequences had distinct reduction in its relative luciferase activity, especially upon presence of miR-204 and not the mutant (Fig. 5D). Additionally, miR-204 inhibitor could also induce the relative luciferase activity of SP1 3'-UTR but not mutant sequence. Taken together, the data from luciferase reporter assay presented that SP1 is both a direct and an exact target of miR-204.

Discussion

Numerous studies have depicted that simultaneous clamping especially with non-traumatic microaneurysm clamps, of the renal pedicle of both kidneys could induce acute kidney injury (AKI) (5). In our current study, we first globally screened microRNAs whose levels are affected during AKI using a microarray; then we validated these screened-out targets by qPCR on a one-by-one basis. Our goal is to identify specific microRNA candidates that might play essential roles in AKI so that we can further study in detail these validated

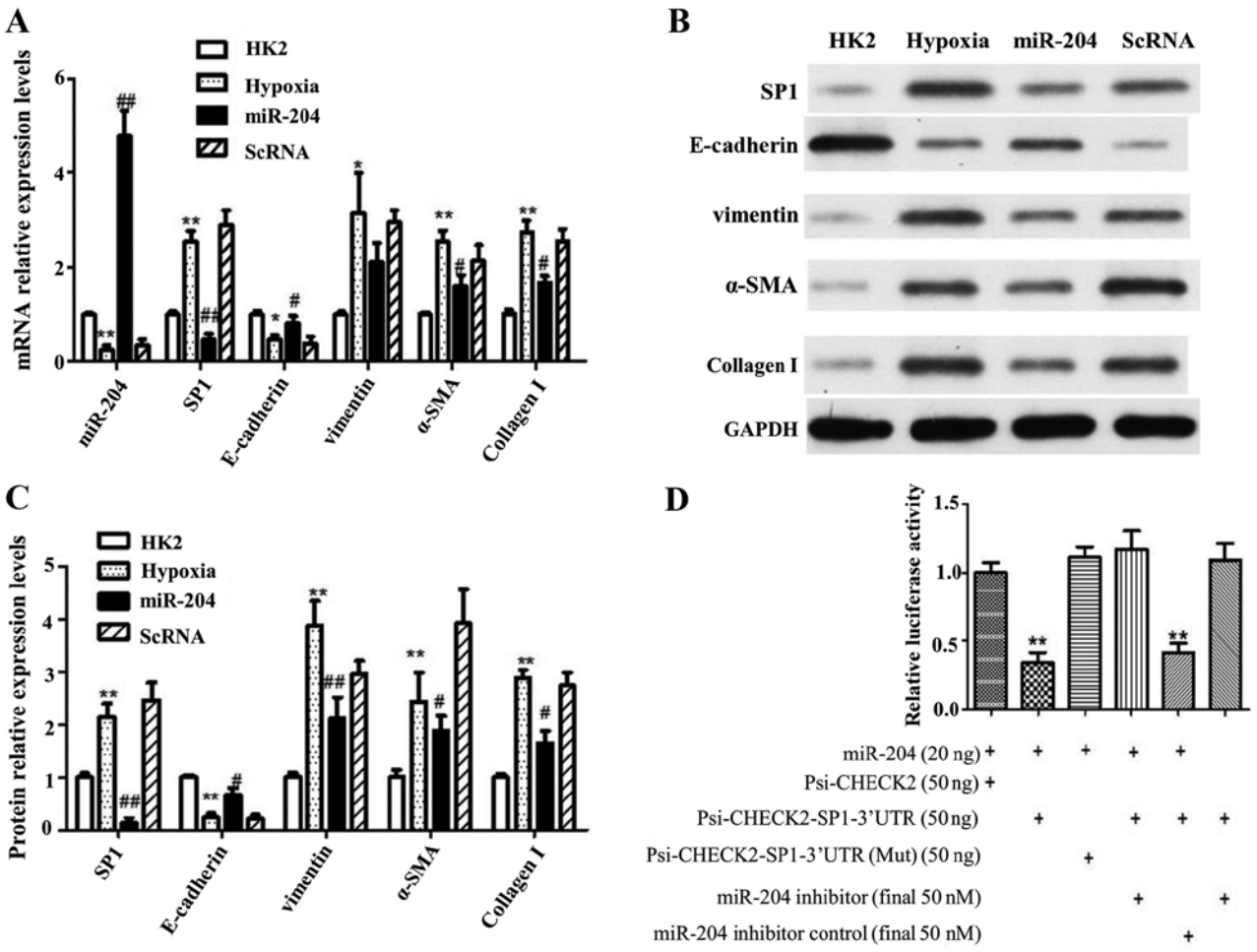

Figure 5. miR-204 suppresses hypoxia-induced SP1/EMT signaling pathway by directly targeting SP1. (A) relative expression levels of mRNA, as a control, HK2 was normally treated, hypoxia was treated with hypoxia in HK2 cells as I/R model, miR-204 was treated with both hypoxia and overexpression miR-204, and ScRNA was treated with both hypoxia and random sequence; (B) western blotting of EMT proteins and SP1; (C) the relative expression levels of western blotting; (D) luciferase activity decreased significantly in HEK293 cells co-transfected with miR-204 wild-type 3'UTR of SP1, but mutant of 3'UTR of SP1 did not. Data are shown as the mean ± SD, *P<0.05 vs. control, **P<0.01 vs. control, $^{\#}$P<0.05 vs. model, $^{\#\#}$P<0.01 vs. model. Student's t-test was performed to evaluate the statistical difference.

microRNA molecules in cell culture to animal models of AKI. It is a known observation, which we also have taken notice of, that there are fluctuations of even base-line RNA levels among individual mouse; and considering also the high-throughput nature of microarrays, we aimed to minimize RNA level fluctuations in each individual mouse, therefore, in our study, AKI were induced instead by unilaterally clamping using non-traumatic microaneurysm clamps on the renal pedicle of the left kidney and paired comparison were done on microRNA levels between the left and right kidneys within the same mouse. Reflecting on our interpretations of opposing expression levels in both left and right kidney tissue, it is plausible to roughly assess the different levels of miRNAs playing a crucial role on the mechanism of extracellular matrix proteins (ECM) through the progress of kidney injury and tissue repair process following I/R.

Laser capture microdissection (LCM) has been proven useful in separating cell populations from tissue sections and the standard genomic methods have served as an added tool and further improved the analysis of complex tissues. The limitation, however, is that only small amount of material with compromised quality could be recovered from LCM for genomic usage. To overcome this issue, tissue preparation protocol was optimized and specific miRNA expression profile approach was performed. Furthermore, miRNAs are highly stable and difficult to degrade in instances such as in kidney tissues and clinical plasma (27). Several studies have reported that differences in miRNA expression did not overlap each other in the post-I/R tissues (28). As a result, we attempted extra work to detect I/R injury-altered miRNAs.

The goal of this study was to describe the expression patterns of miRNAs in kidney tissues upon induction with I/R. It was obviously noted that miR-21, miR-483, miR-5115, miR-204e, miR-128, miR-181c, miR-203, miR-204, miR-204c were highly regulated as compared to sham. This was indeed a brand new protocol in identifying 29 core mRNA target genes related to miR-204 ECM upon kidney injury. miRNA expression over extended period of time is crucial to detect key miRNA that are relevant to different levels of kidney injury which include repair and damage processes (29). Our study demonstrated that renal tubules interstitial tissue chronic fibrotic changes were significant after I/R injury at 24 h. We shifted our focus of analysis on 24 h as the time point encircling miRNA microarray for the reason that we presented a

complete outline for prediction of the relationship between the progression of the chronic fibrotic changes of renal tubule interstitial tissue and miRNAs, and in detail, the molecule mechanisms of inhibition and interruption of chronic AKI.

Our results demonstrated that the miR-204 expression was increased during the recovery and maintenance phase in I/R-induced AKI models. It is known that miR-204 belongs to miR-204/211 family, which includes miR-204 and miR-211. All of them have similar sequence in their 5' terminus known as 'seed sequence'. This miRNA is profound in kidney and play vital roles for podocyte homeostasis and pro-nephros development. It has been previously shown that hepatic miR-204 was spotted in CCl4-induced liver fibrosis. The downregulation of miR-204 was described to stimulate epithelial-mesenchymal transition (EMT) of epithelial thyroid cells especially in anaplastic thyroid pancreatitis. In this study, a drastic decline of miR-204 expression was noted especially in fibrotic kidney.

Moreover, Jiang *et al* (30) reported that miR-204 was downregulated by TGF-β1 in NRK-52E cells though there was partial blockage by Smad7 transfection. As such, implication by Smad signaling activation has direct effect on downregulation of the miR-204. Taken together, Jiang *et al* (30) results indicate that the miR-204 downregulation could be a vital facilitator for tubular-cell extracellular matrix production induced by TGF-β1 in renal fibrosis in a mouse model. This study however depicted that level of miR-204 corresponds to severity of AKI though the correlation is not yet fully understood as it can also indicate protective behaviour. As previously described (6), miR-204 had control over apoptosis and necrosis of renal tubular epithelial cells as well as promoting cell proliferation. As such, clearly this molecule has a huge role to play as kidney protector in AKI. In addition, release of miR-204 also takes place in response to inflammation, stress and also protects the kidneys from delayed ischemia preconditioning.

It should be noted that the whole body responds to I/R injury and microRNA circulation could perhaps be initiated in another place. Even if injured kidneys are speculated to be the primary source of microRNAs in circulation, an extensive amount of work are required to recognize the exact cell type which produce them. In our study, our data concluded that miR-204 is a specific I/R induced chronic AKI suppressor gene, thereby antagonizing the SP1/EMT-signaling pathway.

Less information is available regarding the integration of miRNA dysregulation networks which are prevalent in chronic AKI. In that sense, perhaps an integrative network consisting of both mRNA and miRNAs my hold the clue to detect key factors of chronic AKI induced by I/R. Our current work identified an integrative regulatory network of transcript levels and altered miRNAs that govern chronic AKI induced by I/R. The current study was designed to be a pilot with limitation in terms of mechanistic experiments that would give us a clearer data regarding microRNAs individually. As such, we truly consider that profiling of microRNAs systematically is essential to study their molecular properties. To conclude, this study provided distinct indication that miR-204 are linked with hazard of chronic AKI by I/R. Studies using large cohort would be paramount to ascertain the molecular mechanisms involved in pathophysiology of chronic AKI. Accurate measures to detect and classify the miRNAs with respect to AKI would help in terms of healing mediations that can be harnessed, in near future.

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