Functional recovery of the post-ischemic kidney after delayed contralateral nephrectomy: Pivotal role of inflammation and miRNAs

PhD theses

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1. Introduction

Acute Kidney Injury (AKI) and chronic kidney disease (CKD) are associated with high levels of morbidity and mortality worldwide. Renal ischemia-reperfusion (IR) injury is a main cause of AKI and serious AKI is a risk factor for the development of CKD.

Under ischemic conditions, tubular epithelial cells die either by apoptosis or necrosis, leading to the release of danger-associated molecular patterns that induce proinflammatory responses, such as the generation of several proinflammatory cytokines, chemokines and the complement system. Chemokines promote the recruitment of leukocytes to the site of injury. Infiltrating neutrophil and macrophage cells may further contribute to the proinflammatory responses. If the ischemic episode is moderate, lasts only a short time, the injured kidney may regain its functional and structural integrity by adaptive repair mechanisms. If IRI is severe, persistent or recurrent, the damaged kidney may not be able to recover, and maladaptive repair mechanisms might lead to the establishment of chronic kidney disease (CKD). This process is called the AKI-to-CKD transition.

The exact pathomechanism of the AKI-to-CKD transition is largely unknown. Among the main pathological processes, infiltrating leukocytes play an important role in maintaining the proinflammatory environment. The damaged vasculature contributes to the development of hypoxia and oxidative stress. Furthermore, the transformation of resident and infiltrating fibroblasts into myofibroblasts leads to excess extracellular matrix (ECM) deposition resulting in tubulointerstitial fibrosis. Moreover, epigenetic changes occur in the injured kidney such

as the alteration of the microRNA (miRNA) expression patterns. MiRNAs regulate their targets at the post-transcriptional or translational level, hence may play a role in the AKI-to-CKD transition.

There are several animal models investigating both AKI and CKD. Regarding IR-induced AKI models, two major arms exist, the cold and warm IR. In case of warm IR, unilateral or bilateral subtypes can be distinguished. Unilateral IR enables to take into consideration the effect of the uninjured contralateral kidney that can be removed at a delayed time point. The advantage of the unilateral IR with delayed nephrectomy (Nx) method is that it allows to apply severe renal injury and to investigate the functional recovery of the post-ischemic kidney in the absence of the compensating contralateral kidney.

2. Objectives

Our main objective was to gain new, clinically relevant insights into the mechanisms of acute kidney injury and chronic kidney disease based on the model of unilateral ischemia-reperfusion with delayed contralateral nephrectomy. To achieve this goal:

1. we investigated the molecular basis of the functional recovery of the post-ischemic kidney induced by the delayed contralateral nephrectomy. In particular, we focused on inflammatory, fibrotic, hypoxic, and oxidative stress processes.
2. in addition, we aimed to determine the miRNAs that may be involved in the functional recovery of the post-ischemic kidney.

3. Methods

3.1 Animals

Male NMRI mice, weighing 20-25 g, were used in all studies. Animals were kept under standard housing conditions with free access to standard rodent chow and tap water *ad libitum*.

3.2 Unilateral ischemia-reperfusion with delayed contralateral nephrectomy

Under ketamine+xylazine anesthesia, mice were subjected either to 30 min of warm ischemia-reperfusion or to sham surgery on the left kidney. 7 days later contralateral nephrectomy or sham Nx was performed. Hence, the following groups were examined: Sham IR-Sham Nx (S-S), Sham IR-Nx (S-Nx), IR-Sham Nx (IR-S) and IR-Nx.

Six separate series of experiments were performed with a total of 134 animals. The experiments were terminated on the following days: 7, 8, 10, 14, 28 and 140.

3.3 Sample collection

Blood samples were collected on day: -1, 1, 6, 8, 10, 14 and once weekly until the termination of the study.

Kidneys were collected either at the time of nephrectomy or at the time of sacrifice. The animals were sacrificed by cervical dislocation. Blood was collected from the chest cavity after cross-section of the vena cava superior. Then, the mice were perfused with ice-cold saline solution.

Kidneys were cut into three pieces. For total RNA isolation, the upper pole was immerged in TRI Reagent® then snap frozen in liquid nitrogen. Cross-section of the kidney at the hilus level was fixed in 4% buffered formaldehyde for histological analysis. The lower part of the kidney was submerged into liquid nitrogen for protein level measurement.

All samples were kept at -80°C until further analysis.

3.4 Kidney function analysis

The functional status of the post-ischemic kidney was monitored by measuring plasma urea levels using enzymatic (UV) method.

3.5 Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

After reverse transcription of 1 μg of total RNA into cDNA using random hexamer primers and the High Capacity cDNA Reverse Transcription Kit, the following genes were measured

by quantitative PCR (qPCR): alpha-smooth muscle actin (α-SMA), collagen 1a1 (Col1a1), complement component 3 (C3), fibronectin 1 (FN1), hypoxia inducible factor 1-alpha and 2-alpha (HIF-1α and HIF-2α, respectively), interleukin-6 (IL-6), lipocalin-2 (LCN2), C-C motif chemokine 2 (CCL2), nuclear factor erythroid 2-related factor 2 (NRF2), transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α). 18S ribosomal RNA was used for normalization.

3.6 miRNA microArray

To determine which miRNAs may be involved in the functional recovery of the post-ischemic kidney, a miRNA microArray analysis was performed by Exiqon A/S. 20 samples were selected from the 8-day experiment based on kidney function and TNF-α and TGF-β expression. 6-6 samples were chosen from the IR-S and IR-Nx groups and 4-4 samples from the S-S and S-Nx groups. To validate the altered expression profile of relevant miRNAs found by the miRNA microarray, the following 13 miRNA was transcribed into cDNA using Applied Biosystems™ TaqMan™ Advanced miRNA cDNA Synthesis Kit and their expressions were measured by RT-qPCR: Mus musculus miRNA-(mmu-miR)-21a-3p, mmu-miR-21a-5p, mmu-miR-142a-3p, mmu-miR-142a-5p, mmu-miR-146a-5p, mmu-miR-199a-3p/mmu-miR-199b-3p, mmu-miR-199a-5p, mmu-miR-214-3p, mmu-miR-223-3p, mmu-miR-762, mmu-miR-2137, mmu-miR-2861, mmu-miR-3102-5p.

3.7 Histology

For all histological analysis the uninjured right kidney was used as a control. All staining was performed on paraffin embedded renal tissue samples.

Periodic acid-Schiff (PAS) staining was performed for renal tubular injury evaluation. The following pathological alterations were analyzed: 1. bush border loss, 2. tubule dilatation, 3. cast in the tubules

Masson's trichrome staining was used for the assessment of tubulointerstitial fibrosis. The extent of tubulointerstitial fibrosis was determined by scoring 0-4 per field of view.

Macrophage specific F4/80 immunostaining was performed for the analysis of macrophage infiltration. Color development was performed using diaminobenzidine Quanto Chromogen kit. F4/80-positive and the total number of pixels in each image were quantified with ImageJ software and their ratios were calculated to evaluate the extent of macrophage infiltration.

3.8 Pathway analysis

MiRNA target network analysis was performed based on the successfully validated miRNAs. MiRNA-target interaction networks were created using miRNAtarget software. The following miRNA-target interaction data sources were used: miRDB v5.0, TargetScan Mouse 7.2, miRTarBase 8.0.

3.9 Western blot analysis

To validate the functional connection between predicted miRNA and its target, the protein levels of CD2-associated protein (CD2ap) and Plexin A2 (Plxna2) were determined by Western blot analysis. Tissue samples were homogenized in lysis buffer then 20 μg protein was loaded onto 4-20% polyacrylamide gel and separated with 90V. After separation, the protein was transferred onto polyvinylidene difluoride membranes then blocked. Membranes were incubated overnight

with primary antibodies, washed, then incubated with a secondary antibody at room temperature for 2 h. After washing, the membranes were incubated with enhanced chemiluminescence reagent for 5 min to detect the corresponding bands. Total protein content was measured as loading control.

3.10 Statistics

If Bartlett's test indicated significant inhomogeneity of variances, base-10 logarithmic transformations performed before data analysis. Possible outliers were detected by the ROUT method ($p=0.01$), and these data were omitted from the analysis. Two groups were compared using unpaired nonparametric Mann-Whitney test. In case of more than two groups, one-way or two-way analysis of variance (ANOVA) were performed followed by Dunnett or Tukey's post-hoc test, respectively. For data analysis GraphPad Prism v8.0.2 was used. The level of statistical significance was set at $p<0.05$.

4. Results

4.1 Removal of the healthy kidney restores the function of the post-ischemic kidney

Plasma urea levels used as kidney function marker did not alter after severe unilateral IR in the presence of intact contralateral kidney. This observation suggests that an uninjured kidney can rapidly compensate for the loss of kidney function caused by severe unilateral IR. Furthermore, the contralateral nephrectomy on day 7 revealed, that the post-ischemic kidney hardly functioning. The plasma urea level was rapidly and markedly increased after Nx and remained elevated for several

days. However, this elevation proved to be temporary, as it showed decreased level one week after Nx, and plasma urea concentrations became comparable to those of the sham nephrectomised mice three weeks later.

A long-term study was performed to shed light on whether the Nx-induced improved kidney function is permanent or transient. No alterations in plasma urea concentrations were seen in the S-S, S-Nx and IR-S groups during the study. In the IR-Nx group, the plasma urea concentration increased sharply after Nx and then decreased to a comparable level to that of the other three groups by day 14, similarly to that seen in short-term experiments. This decrease lasted up to several weeks with constant fluctuations in a narrow range. However, from week 19, the plasma urea concentration began to increase sharply in the IR-Nx group and the study was terminated 20 weeks after IR (Figure 1).

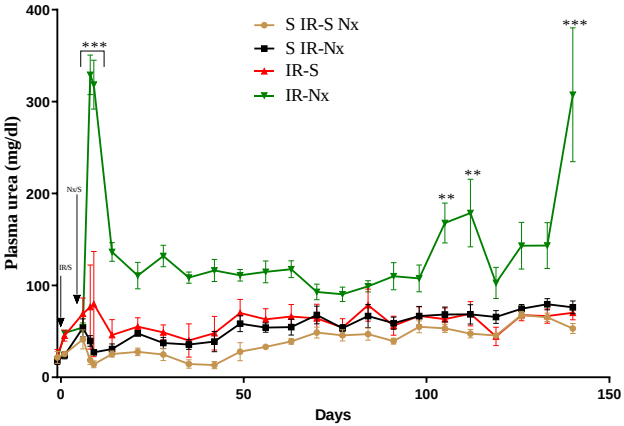

Figure 1. Kidney function changes in the long-term study. IR: ischemia-reperfusion, Nx: contralateral nephrectomy, S: sham

4.2 Contralateral nephrectomy markedly decreased the extent of tubular injury and the expression of inflammation-related genes

No histological damage was observed in the uninjured control kidneys at any time points investigated. Marked tubular injury was seen in the post-ischemic IR-S kidney already on day 8, and atrophy on day 28. Similar degree of tubular injury was found in the IR-Nx group on day 8, nevertheless, the tubular injury markedly decreased by day 28 in the post-ischemic kidney, and no further deterioration was detected on day 140 and atrophy were absent in these kidneys at any time points investigated.

Following histological analysis, we examined changes in the expression levels of main genes involved in tubular damage and inflammation-related processes.

The expression of tubular injury marker LCN2 mRNA level was markedly elevated in the post-ischemic IR-S and IR-Nx kidneys compared to the control kidney. However, LCN2 expression was downregulated in the IR-Nx group in comparison to the IR-S group by day 28 (Table 1).

The expression of the pro-inflammatory cytokine TNF-α and IL-6, the chemokine CCL2 and the complement system member C3 were markedly increased in the post-ischemic IR-S and IR-Nx kidneys in the first 4 weeks after IR. CCL2 and C3 showed the most robust elevation on day 10 (fold change 124.4±22.5 and 286.3±24.0, respectively). Upon contralateral nephrectomy, the investigated mRNAs started to downregulate in already one day after Nx the IR-Nx group compared to the IR-S group, except for IL-6, which was downregulated only on day 14 (Table 1).

To further characterize the inflammatory processes in the post-ischemic kidneys, macrophage infiltration was visualized using immunohistochemistry staining. IR induced robust macrophage infiltration into the post-ischemic IR-S and IR-Nx kidneys in comparison to the uninjured kidney. In align with the downregulation of the pro-inflammatory mRNAs, Nx markedly decreased the overall macrophage content of the post-ischemic kidneys in the IR-Nx group compared to the IR-S group form day 10.

4.3 Contralateral nephrectomy decreased the expression of hypoxia, oxidative stress and fibrosis-related genes in the post-ischemic kidney

The expression of hypoxia marker HIF-1α and -2α was markedly elevated in the post-ischemic IR-S kidney compared to the uninjured kidney. No difference was observed regarding these mRNAs in the IR-Nx group compared to the control kidney except on day 28 for HIF-2α. The oxidative stress marker NRF2 expression was markedly elevated in the post-ischemic IR-S and IR-Nx kidneys in comparison to the uninjured kidney. However, upon Nx, the mRNA level of NRF2 markedly decreased in the IR-Nx group compared to the IR-S group (Table 1).

The myofibroblast marker α-SMA mRNA level was markedly elevated in the post-ischemic IR-S and IR-Nx kidneys compared to the uninjured kidney. Upon Nx, α-SMA expression gradually decreased and reached the level of statistical significance by week 4 in the IR-Nx group in comparison to the IR-S group. The profibrotic TGF-β gene expression was markedly elevated in the post-ischemic IR-S and IR-Nx kidneys compared to the control kidney. Contralateral nephrectomy

markedly decreased the TGF-β mRNA level in the post-ischemic IR-Nx kidney in comparison to the post-ischemic IR-S kidney at all time points investigated (Table 1).

The ECM protein Col1a1 and FN1 expression markedly increased in the post-ischemic IR-S and IR-Nx group compared to the control group. However, their expression markedly decreased 3 days after Nx in the IR-Nx group compared to the IR-S, suggesting a lower level of ECM accumulation upon Nx in the post-ischemic kidney (Table 1).

Indeed, histological analysis revealed that while the uninjured control kidneys showed no fibrotic matrix deposition in the interstitium, pathological level of extracellular matrix deposition was observed from day 8 in the post-ischemic IR-S kidney. Nx resulted in lower level of ECM content in the post-ischemic IR-Nx kidney on days 14 and 28 compared to the IR-S group. Nevertheless, the matrix deposition persisted until day 140.

4.4 Ischemia-reperfusion markedly influenced the expression of several miRNAs

The performed miRNA microArray analysis by Exiqon revealed that 8 days after IR 20 miRNAs was up- and 6 was downregulated in the post-ischemic IR-S and IR-Nx kidneys more then 2-fold compared to the uninjured kidneys. However, contralateral nephrectomy moderately altered the miRNA expression profile 24 hours after Nx, no miRNA expression changed at least 2-fold in the IR-Nx group compared to the IR-S group (Figure 2).

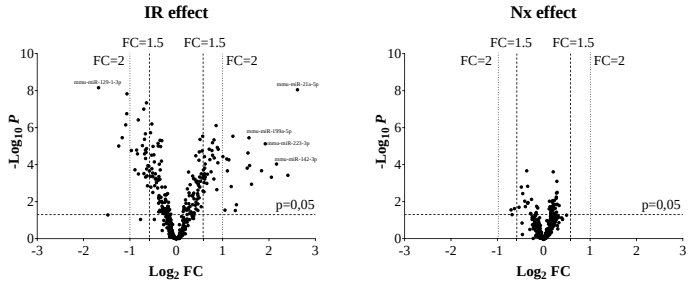

Figure 2. Results of the miRNA microArray represented as volcano plot. FC: fold change, IR: ischemia-reperfusion, Nx: contralateral nephrectomy

The expression of miRNAs, which alteration was at least 2-fold or may play a role in AKI according to the literature, were validated by RT-qPCR. In case of mmu-miR-21a-5p, -2137, -142-3p, 223-3p, -142-5p, -199a-5p, -199a-3p/-199b-3p, -214-3p, -146a-5p, the RT-qPCR results were similar to those obtained by microArray. Nevertheless, the expression profile of miR-21a-3p proved to be different in comparison to the microarray, its miRNS was significantly elevated in the IR-Nx group compared to the IR-S group.

IR markedly elevated the expression of the miR-21, -142a and -199a duplex, as well as miR-146a-5p, -214-3p and -223-3p in the post-ischemic IR-S and IR-Nx groups compared to the uninjured kidney at all time points investigated. Contralateral nephrectomy did not induced changes in the miRNA levels of the miR-21a duplex. In contrast, the miR-142a duplex was markedly reduced 3 days after Nx in the IR-Nx group compared to the IR-S group. Contralateral nephrectomy affected the two strands of mir-199a differently. MiR-199a-3p was downregulated in the IR-Nx group already on day 14 but the -5p strand decreased only on day 28 compared to the IR-S group.

Mir-146a-5p expression was markedly decreased in the post-ischemic IR-Nx kidney compared to the IR-S kidney from day 10 after Nx until day 28. Furthermore, its expression level was comparable to that of the control kidney from day 14. In case of miR-214-3p and -223-p, Nx induced their downregulation in later time points, from day 14 and only on day 14, respectively (Table 2).

4.5 MiRNA-target network analysis

MiRNA-target analysis was performed in the post-ischemic IR-S and IR-Nx kidneys separately to gain insight into the effect of IR and Nx on the miRNA-target network using all validated miRNAs. The analysis showed that several potential genes could be targeted by more than one miRNA. Four genes were predicted to be a target of four miRNAs after IR: Cd2ap, cyclin-dependent kinase 17 (Cdk17), CREB3 regulatory factor (Crebrf) and Plxna2. In response to Nx, only Plexin A2 (Plxna2) gene was regulated at least by 4 miRNAs. In case of CD2ap (expressed mainly in glomerular epithelial cells) and Plxna2 (expressed in podocytes) protein levels were measured on day 10 in order to verify the functional connection between the miRNA and its predicted target. However, none of the protein levels altered in the predicted way.

Gene	Day 8			Day 10			Day 14			Day 28		
	Ctrl vs IR-S	Ctrl vs IR-Nx	IR-S vs IR-Nx	Ctrl vs IR-S	Ctrl vs IR-Nx	IR-S vs IR-Nx	Ctrl vs IR-S	Ctrl vs IR-Nx	IR-S vs IR-Nx	Ctrl vs IR-S	Ctrl vs IR-Nx	IR-S vs IR-Nx
LCN2	↑↑	↑↑	-	↑↑	↑↑	-	↑↑	↑↑	-	↑↑	↑↑	↓↓
C3	↑↑	↑↑	↓↓	↑↑	↑↑	↓↓	↑↑	↑↑	↓↓	↑↑	↑↑	-
CCL2	↑↑	↑↑	↓↓	↑↑	↑↑	↓↓	↑↑	↑↑	↓↓	↑↑	↑↑	-
IL-6	↑	↑↑	↑↑	↑↑	↑	-	↑↑	↑↑	↓↓	↑	↑	-
TNF-α	↑↑	↑	↓	↑↑	↑	↓↓	↑↑	↑	↓↓	↑↑	↑	↓↓
HIF-1α	↑	-	-	-	-	-	-	-	-	↑	-	-
HIF-2α	-	-	-	↑	-	↓	↑	-	↓	↑	↑	-
NRF2	↑	↑	↓	↑	↑	-	↑	↑	↓	↑	↑	↓
α-SMA	↑	↑	-	↑	↑	-	↑	↑	-	↑	↑	↓
TGF-β	↑	↑	↓	↑	↑	↓	↑	↑	↓	↑	↑	↓
Col1a1	↑↑	↑↑	-	↑↑	↑↑	↓↓	↑↑	↑↑	↓↓	↑↑	↑↑	↓↓
FN1	↑↑	↑	-	↑↑	↑	↓	↑↑	↑	↓	↑↑	↑	↓↓

Table 1. Gene expression changes in the post-ischemic kidneys. Ctrl: control uninjured kidney, IR-S: severe unilateral ischemia-reperfusion without delayed contralateral nephrectomy, IR-Nx: severe unilateral ischemia-reperfusion with delayed contralateral nephrectomy. -: no statistically significant difference, ↑, ↓: Fold Change < 10, ↑↑, ↓↓: Fold Change > 10

Gene	Day 7	Day 8			Day 10			Day 14			Day 28		
	Ctrl vs IR	Ctrl vs IR-S	Ctrl vs IR-Nx	IR-S vs IR-Nx	Ctrl vs IR-S	Ctrl vs IR-Nx	IR-S vs IR-Nx	Ctrl vs IR-S	Ctrl vs IR-Nx	IR-S vs IR-Nx	Ctrl vs IR-S	Ctrl vs IR-Nx	IR-S vs IR-Nx
miR-21a-3p	↑	↑	↑↑	↑	↑	↑↑	-	↑	↑	-	↑	↑	-
miR-21a-5p	↑	↑	↑	-	↑	↑	-	↑	↑	-	↑	↑	-
miR-142a-3p	↑↑	↑	↑	-	↑↑	↑	↓	↑↑	↑	↓↓	↑↑	↑↑	↓↓
miR-142a-5p	↑	↑	↑	-	↑	↑	↓	↑	-	↓	↑↑	↑	↓
miR-146a-5p	↑	↑	↑	-	↑	↑	↓	↑	-	↓	↑	-	-
miR-199a-3p	↑	↑	↑	-	↑	↑	-	↑	↑	↓	↑	↑	↓
miR-199a-5p	↑	↑	↑	-	↑	↑	-	↑	↑	-	↑	↑	↓
miR-214-3p	↑	↑	↑	-	↑	↑	-	↑	↑	↓	↑	↑	↓
miR-223-3p	↑	↑	↑	-	↑	↑	-	↑	↑	↓	↑	↑	-

Table 2. miRNA expression changes in the post-ischemic kidneys after contralateral nephrectomy. Ctrl: control uninjured kidney, IR-S: severe unilateral ischemia-reperfusion without delayed contralateral nephrectomy, IR-Nx: severe unilateral ischemia-reperfusion with delayed contralateral nephrectomy. -: no statistically significant difference, ↑, ↓: Fold Change < 10, ↑↑, ↓↓: Fold Change > 10

5. Conclusions

In the presence of intact contralateral kidney, the post-ischemic kidney showed accelerated fibrosis and atrophy.

Our findings confirm the previous reports that the delayed contralateral nephrectomy induce the functional recovery of the post-ischemic kidney hence end-stage renal failure developed over a longer period of time.

Delayed contralateral nephrectomy induced a substantial decrease in pro-inflammatory and fibrotic processes, while hypoxia and oxidative stress reduced only in a lesser extent.

Severe unilateral ischemia-reperfusion induced the expression of numerous miRNAs in the post-ischemic kidney. Delayed contralateral nephrectomy resulted in downregulation of several miRNAs, suggesting that these miRNAs may play a role in the functional recovery of the post-ischemic kidney and/or halting its functional deterioration.

6. Publications of the author

Publications used for the thesis

Tod P, Bukosza EN, Roka B, Kaucsar T, Fintha A, Krenacs T, Szenasi G, Hamar P. (2020) Post-Ischemic Renal Fibrosis Progression Is Halted by Delayed Contralateral Nephrectomy: The Involvement of Macrophage Activation. Int J Mol Sci, 21: 3825-3842.

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Additional publications

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