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MicroRNA Profiling in Kidney Disease: Plasma versus Plasma-Derived Exosomes

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Abstract

Liquid biopsies have advanced rapidly in recent years for use in diagnostic and prognostic applications. One important aspect of this advancement is the growth in our understanding of microRNA (miRNA) biology. The measurement of miRNAs packaged within exosomes, which are constantly released into the blood stream, may reflect pathological changes within the body. The current study performed miRNA profiling using plasma and plasma-derived exosome samples from two animal models of kidney disease, the 5/6th partial nephrectomy (PNx) and two-kidney-one-clip (2K1C) models. The RT-qPCR-based profiling results revealed that the overall miRNA expression level was much higher in plasma than in plasma-derived exosomes. With 200 μ l of either plasma or exosomes derived from the same volume of plasma, 629 out of 665 total miRNAs analyzed were detectable in plasma samples from sham-operated rats, while only 403 were detectable in exosomes with a cutoff value set at 35 cycles. Moreover, the average miRNA expression level in plasma was about 16-fold higher than that in exosomes. We also found a select subset of miRNAs that were enriched within exosomes. The number of detectable miRNAs from plasma-derived exosomes was increased in rats subjected to PNx or 2K1C surgery compared to sham-operated animals. Importantly, we found that the changes of individual miRNAs measured in plasma had very poor concordance with that measured in plasma-derived exosomes in both animal models, suggesting that miRNAs in plasma and plasma-derived exosomes are differentially regulated in these disease conditions. Interestingly, PNx and 2K1C surgeries induced similar changes in miRNA expression, implying that common pathways were activated in these two disease models. Pathway analyses using DIANA-miRPath v3.0 showed that significantly changed exosomal miRNAs were associated with extracellular matrix (ECM) receptor interaction and mucin type-O-glycan synthesis pathways, which are related with tissue fibrosis and kidney injury, respectively. In conclusion, our results demonstrated that due to the differential changes in miRNAs, the measurement of exosomal miRNAs cannot be replaced by the measurement of miRNAs in plasma, or vice versa. We also showed that a set of miRNAs related with kidney injury

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and organ fibrosis were dysregulated in plasma-derived exosomes from animal models of kidney disease.

Keywords

microRNA; exosome; biomarker

Introduction

MicroRNAs (miRNAs) are non-coding RNAs, 18–25 nucleotides in length, which play critical roles in the post-transcriptional regulation of many signaling pathways and processes [1, 2]. In recent years, there has been great excitement in the use of miRNAs as circulating biomarkers of diseases, such as myocardial infarction, diabetes and cancer [3–10]. One reason for the interest in miRNAs as biomarkers is their remarkable stability in circulation [11]. Recent findings have demonstrated that miRNAs can exist within exosomes, ~30–100 nm sized vesicles, which are released into the blood stream and whose content may enter into recipient cells, remotely regulating gene expression [12–14].

Circulating miRNAs can also stably exist without the protection of vesicles. It has been ascertained that miRNAs can be carried by high density lipoprotein [15, 16] or Argonaute 2 (Ago2), a chaperone protein that is important for the function of miRNAs [17, 18]. In fact, a recent report suggests that the majority of circulating miRNAs are not found within exosomes [19]. Currently, as it relates to the study of miRNAs as biomarkers, there is no consensus on whether measuring exosomal miRNAs or total amounts of miRNAs from biological fluids is more appropriate. Some studies report only exosomal extracted miRNAs [6, 9, 10] while others reported total miRNA levels in biological fluids, irrespective of miRNA origin [3–5, 7, 8]. In fact, there is limited literature directly comparing these two methods of miRNA measurement.

To study this, we measured miRNAs in plasma and plasma-derived exosomes from Sprague-Dawley (SD) rats. We also compared the changes of miRNAs in plasma and plasma-derived exosomes using two well established models of kidney disease, namely 5/6th Partial Nephrectomy (PNx, a CKD model) and 2 Kidney 1 Clip (2K1C, a renal ischemic hypertensive model).

Materials and Methods

Animals

Male, Sprague-Dawley (SD) rats at 2 months of age were used for all studies. Studies utilizing animal experimentation described in the manuscript were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals using protocols approved by the University of Toledo College of Medicine and Life Sciences Institutional Animal Care and Use Committee.

5/6th Partial Nephrectomy (PNx) Surgery

SD rats underwent PNx surgery as detailed previously [20]. Briefly, surgery was performed on rats anesthetized with 2% isoflurane (mixed with oxygen). An incision was made in the left flank, through which arteries supplying the upper pole of the kidney were ligated with silk sutures. After ligation, the kidney was observed for a characteristic color change over approximately two-thirds of the kidney tissue, indicating successful interruption of blood flow to the discolored portion of the kidney. Once the color change was observed, the kidney was reinserted to the body cavity and the incision closed. After 1 week, the right kidney, sans adrenal gland, was removed. In sham surgeries, incisions were made as in both steps of the PNx; however, neither ligation nor removal of the opposing kidney were performed. Following euthanasia 4 weeks after surgery, whole blood was extracted and plasma was isolated. Samples were stored at -80°C until use.

Two-Kidney-One Clip (2K1C) Surgery

Male Sprague-Dawley rats underwent 2K1C surgery, a well-established method for inducing renovascular hypertension [21]. Briefly, following anesthetization with a mixture of 2% isoflurane and oxygen, a midline abdominal incision was made and 0.2 mm clip was placed around the renal artery. Sham operated animals underwent anesthesia and midline abdominal incision, but without placement of the arterial clip. 4 weeks following surgery, animals were euthanized and plasma was collected and stored as described above.

RNA isolation

One aliquot of 200 μl plasma was directly used for RNA extraction and another aliquot of 200 μl plasma from the same rat was used for exosome isolation and subsequent RNA extraction. Total RNA including small RNAs was isolated using the miRNeasy Serum/Plasma Kit (Qiagen #217184) according to manufacturer instructions. Briefly, each sample was homogenized in 1000 μl of QIAzol reagent containing phenol and guanidine thiocyanate. The lysate was then mixed with 200 μl of chloroform and centrifuged for 15 minutes at $12,000 \times g$ and 4°C . The upper aqueous phase containing RNA was then collected, thoroughly mixed with 1.5 volumes of 100% ethanol, and transferred quantitatively to a miRNeasy minElute spin column attached to a 2ml collection tube. After allowing a few minutes for equilibration and RNA binding, the column was spun, filtering out the reagents, but retaining the RNA bound to the column. Extraneous material from the column was further removed by 500 μl of the washing buffer. The column was then attached to a new collection tube, and RNA was finally eluted by adding RNase-free (14 μl) water to the column and centrifugation. Since the blood sample contained heparin during collection, heparinase treatment was applied to all samples. Heparin-containing RNA is combined with water, RNase Inhibitor (Promega), and 3 units Heparinase I (NEB). The mixture is heated for one hour at 30 degrees Celsius, followed by one minute at 99.9 degrees Celsius. The cDNA synthesis and RT-qPCR-based miRNA profiling was then performed by Qiagen Inc. using miRNA profiling plates (a total of 665 microRNA primer pairs were included in two 384-plates. The miRTC and PPC were included in each plate for reverse transcription control and positive PCR control, respectively). The RT-qPCR based miRNA profiling is

more accurate in comparison to other array methods, especially for samples with low levels of miRNAs.

Isolation of Exosomes from Plasma

Exosomes from plasma (200 μ L) were isolated using the Total Exosome Isolation Kit from Invitrogen (Cat # 4484450). Briefly, plasma was centrifuged at room temperature at $2000 \times g$ for 20 minutes. The supernatant was transferred to a new microcentrifuge tube and centrifuged at $10,000 \times g$ for 20 minutes. The supernatant was transferred to a new microcentrifuge tube. 100 μ L of PBS was added to the sample and then was mixed by vortexing. 60 μ L of the Exosome Precipitation Reagent was added and was mixed thoroughly by vortexing. The samples were incubated at room temperature for 10 minutes and then were centrifuged at $10,000 \times g$ for 5 minutes at room temperature. The supernatant was discarded and the samples were centrifuged again at $10,000 \times g$ for 30 seconds. The residual supernatant was discarded and pellets were resuspended in 200 μ L of PBS for RNA extraction and miRNA profiling.

Verification of exosome size and marker by Nanosight and Western blot

Plasma-derived exosomes were analyzed using NanoSight NS300. Exosomes were diluted in sterile PBS until they achieved 20–80 particles per frame. Samples were analyzed 5 times, for 60 seconds each. The exosome marker CD63 was probed with Western blot using anti-rat CD63 antibody from Biorad-Antibodies (Cat No: MCA4754GA). The supernatant of the last washing step was used as negative control. Briefly, following the last wash step, plasma-derived exosomal pellets were resuspended in radioimmunoprecipitation buffer (0.25% deoxycholate, 1% Nonidet P –40, 1 mM EDTA) with 1 $\times$ Halt Protease and Phosphatase inhibitor (ThermoFisher #78440). Exosomes were vortexed and then rotated at 4 $^{\circ}$ for 15 minutes. Supernatant was collected and diluted 20 $\times$ using the same radioimmunoprecipitation buffer. Following determination of protein concentration, 30 μ g of protein from exosome or supernatant was subjected to SDS-Page using a NuPage 4–12% Bis-Tris gel (Thermo-Fisher NP0322BOX). Protein was transferred to a PVDF membrane (Thermo-Fisher #88518), blocked with 5% milk and probed for CD63.

Pathway analysis

Pathway analyses based on significant miRNA changes were performed using the open-access web-server DIANA-miRPath v3.0 [22]. The target prediction of miRNAs was computed using the DIANAmicroT-CDS as previously reported [23, 24]. A conservative Fisher's exact test and the false discovery rate method were used to calculate the significantly targeted KEGG pathways, and p value threshold was set at 0.05.

Statistical Analysis

Data are presented as the mean $\pm$ SEM. miRNAs with average CT values lower than 35 were considered as detectable. Individual samples with CT values higher than 35 were set at 35 for calculation of averages. Levels of each miRNA were normalized against the global mean and delta-delta CT was used for fold change calculation between sham and PNx or 2K1C groups. Significance of miRNA change between the groups was calculated using 2-tailed

Student's t test. Lin's concordance correlation coefficient (CCC) [25] was used to evaluate the similarity of miRNA measurements produced by using plasma or plasma-derived exosomes. A descriptive scale for values of the CCC was adopted according to previous studies [26]. Values are presented as CCC with 95% confidence interval (CI).

Results

Profiling of miRNAs in plasma and plasma-derived exosomes in animal models of kidney disease

To measure the relative expression level of miRNAs in plasma and plasma-derived exosomes in animal models of kidney disease, we performed PNx and 2K1C surgery using Sprague-Dawley (SD) rats. Four weeks after surgery, blood was collected from the vena cava in heparin-containing tubes and plasma was prepared by centrifugation at 1500g for 15 min. Afterwards, one aliquot of 200 μ l plasma was directly used for RNA extraction and another aliquot of 200 μ l plasma from the same rat was used for exosome isolation and subsequent RNA extraction. Each group had samples from 3 rats. Extracted total RNAs from plasma or plasma-derived exosomes were eluted in the same volume (14 μ l) of RNAase-free water. Equal amounts of RNA from these samples were used for cDNA synthesis and RT-qPCR based miRNA profiling. The workflow of this process is illustrated in Fig. 1A. The size of the plasma-derived exosomes was measured using NanoSight. As shown in Fig. 1B, the most abundant particle size was observed at 115 nm. A small portion of particles exceeded 200 nm. We also probed for the membrane marker CD63 using Western blot and demonstrated that the isolated exosomes are CD63 positive (Fig. 1C). A total of 665 miRNAs were included in the profiling. As shown in Fig. 2A, there were 629 miRNAs detectable in plasma samples (average CT value<35) from sham-operated rats, whereas only 403 miRNAs were detectable in plasma-derived exosomes. The number of detectable miRNAs in exosomes were increased in animals subjected to PNx (510 detectable) or 2K1C (531 detectable) versus sham controls. The number of detectable miRNAs in plasma were slightly decreased in rats subjected to 2K1C surgery and had no change in PNx rats. For miRNAs detected in both plasma and exosomes, the average CT value in plasma was about 4 cycles lower than that in exosomes (29.2 ± 0.2 in plasma vs 33.4 ± 0.1 in exosomes, $p < 0.01$), indicating the average miRNA level is 16 times higher in plasma than in exosomes. We also examined the expression level of several nuclear miRNA markers (RNU6-2, SNORD61, SNORD68, SNORD72, SNORD95, and SNORD96A) in plasma and plasma-derived exosomes from sham-operated rats. As shown in Fig. 2B, these miRNAs were expressed at very low levels in exosome preparations (average CT value is 34.6 in exosome vs 27.7 in plasma), suggesting that isolated exosomes had no significant contamination from tissue or cell debris.

Despite the fact that the overall miRNA expression was significantly lower in exosomes than in plasma, exosomes were the major source for certain circulating miRNAs in sham-operated animals. A heat map was created according to the relative expression level of plasma and plasma-derived exosomes (Fig 3). Using a two sample T-test with a p-value threshold set at less than 0.01, we identified a total of 129 miRNAs in plasma-derived exosomes whose expression level were not significantly different from that in plasma.

Differential regulation of miRNA expression in plasma and plasma-derived exosomes from animal models of kidney disease

To examine how kidney disease affects miRNA expression, we analyzed the miRNA profiling data in plasma and plasma-derived exosomes from rats subjected to sham or PNx surgery. As illustrated in Fig. 4, the Volcano plot showed that in plasma, a total of 264 miRNAs were changed by at least 2 fold (106 down regulated and 158 up regulated), while in plasma-derived exosomes, a total of 163 were changed by at least 2 fold (72 down regulated and 91 up regulated). However, using two-sample T-test analysis, there were only 17 miRNAs whose change was above 2 fold and also reached statistical significance ($p < 0.05$), whereas in exosomes, 51 miRNAs met both criteria (greater than 2-fold change and $p < 0.05$). We defined miRNAs that met both criteria as “true positive change” and used Prevalence-Adjusted Bias-Adjusted Kappa (PABAK) analysis [27] to compare the agreement in miRNA regulation between plasma and plasma-derived exosomes. The result showed that there is no agreement for “true positive change” between plasma and exosome from either PNx ($\kappa = -0.03$, 95% CI $[-0.09, 0.02]$, $p = 0.27$; PABAK = 0.01) or 2K1C ($\kappa = -0.04$, 95% CI $[-0.09, 0.01]$, $p = 0.14$; PABAK = -0.19).

We further performed an agreement analysis that included all measured miRNAs using Lin's concordance correlation coefficient (CCC) method to evaluate whether miRNAs that were dysregulated in exosomes were also dysregulated in plasma. We found that there was no agreement between plasma and plasma-derived exosomes. The CCC value (ρ_c) was 0.06 $[-0.001, 0.128]$ between plasma and plasma-derived exosomes in animals subjected to PNx surgery (Fig. 5A). In 2K1C animals, the ρ_c value was $-0.05 [-0.104, 0.009]$ (Fig. 5B). These results suggest that specific miRNAs might be selectively packaged into exosomes or other carriers that are released into the blood stream during disease conditions. Interestingly, the change in miRNA expression in exosomes from PNx and 2K1C animals had a very similar pattern (Fig. 6A). The change in miRNA expression in plasma was also similar in these two animal models (Fig. 6B), implying that PNx and 2K1C may share common pathways in regulating miRNA expression and trafficking.

Pathway analysis in animal model of kidney disease based on miRNA changes in plasma or plasma-derived exosomes

To identify potential biological functions affected by changes in miRNAs in animals with kidney disease, we included miRNAs from either plasma or plasma-derived exosomes with a statistically significant, greater than 2-fold change, for pathway analyses using the DIANA-miRPath v3.0 web server. As shown in Fig. 7, we found that miRNAs significantly dysregulated in exosomes from PNx animals were associated with ECM-Receptor Interaction and Mucin Type-O-Glycan Biosynthesis pathways. The former is closely related with organ fibrosis [28], while the latter is related with kidney injuries [29]. Specifically, miRNAs such as let-7c-5p, miR-532-3p, miR-429, miR-143-3p, miR-770-5p, miR-224-5p, miR-29b-3p and let-7a-5p were identified as related to ECM-Receptor Interaction pathways. These miRNAs target genes in the collagen family as well as Itgb1 and FN1, which are known to be related with tissue fibrosis [30]. In addition, let-7c-5p, miR-129-5p, miR-448-3p, miR-30e-3p, let-7a-5p, miR-22-3p, miR-125a-5p, miR-125b-5p, miR-139-5p and miR-448-3p targets genes related to Mucin Type-O-Glycan Biosynthesis pathways,

which include genes in the GALNT family and Gnt [29]. Other associated pathways include Cancer (miR-15b-5p and miR-195-5p), N-glycan Biosynthesis (miR-125a-5p and miR-125b-5p), Fatty Acid Degradation (let-7a-5p and miR-434-3p), Calcium Reabsorption (miR-143-3p and miR-3583-5p), and Prion Diseases (miR-107-5p). Pathways regulated by miRNAs that fit the aforementioned criteria (at least 2-fold change and $p < 0.05$) in plasma samples were found to be related with Fatty Acid Degradation (miR-192-3p and let-7d-5p), Cell Adhesion Molecules (miR-190a and miR-362-3p), Biosynthesis of Unsaturated Fatty Acids (miR-362-3p), and Hematopoietic Cell Lineage pathways (miR-34b-5p and miR-540-5p) (Fig. 8).

Discussion

Exosomes were discovered in the 1980s as membrane-bound vesicles [31] and were later found to carry proteins and nucleic acids that function as important mediators of intercellular communication [32]. The existence of miRNAs in exosomes sparked great interest for their roles in intercellular epigenetic regulation, as well as using circulating miRNAs as biomarkers in liquid biopsy that reflects pathological changes in the tissues from which exosomes were released [14, 33, 34]. The translational application of using exosomal miRNAs as therapeutic mediators or biomarkers was recently challenged by the finding that miRNA copy numbers are extremely low in isolated exosomes from either cancer cell lines or blood samples from cancer patients [19].

Our current study compared miRNA profiles in plasma and plasma-derived exosomes from SD rats. RT-qPCR-based measurements demonstrated that overall miRNA expression levels in plasma-derived exosomes was about 16 times lower than that in plasma samples, which is in line with recent publications showing low abundance of miRNAs in exosome preparations [19, 35]. However, we did observe that a subset of miRNAs were relatively enriched in plasma-derived exosomes, meaning the relative expression of the particular miRNA in exosomes was close to its expression in plasma. This suggests specific and differential mechanisms in miRNA packaging, trafficking, and secretion, a concept supported by previous studies [36, 37]. We also found that overall, exosomal miRNA expression was higher in animals subjected to PNx or 2K1C surgery compared to sham-operated controls. More importantly, we found that miRNAs in plasma compared to plasma-derived exosomes were differentially regulated in these disease animal models as shown in Fig. 5. It is known that the expression profile of miRNAs in exosomes is not simply a copy of the original cells. Certain molecules such as neural sphingomyelinase 2 and Ago2 play key roles in the differential sorting of miRNAs into exosomes [33]. Our results show that specific exosomal miRNAs are significantly changed in disease conditions despite their relative low baseline levels, and equivalent changes cannot be detected in plasma samples. Two methods have been used in the literature to define a significant change in miRNA expression, one is at least 2-fold change [7], and the other one is $p < 0.05$ by Two-Sample T-Test [23]. In this study, we combined the two methods and defined significant changes as meeting both criteria (at least 2-fold change and $p < 0.05$). It was found that fewer miRNAs in plasma samples were significantly changed. There were 264 miRNAs in plasma that were altered at least 2 fold, but only 17 of them were statistically different ($p < 0.05$), whereas in exosomes samples, 51 out of 163 miRNAs met both criteria. We noticed that the amplitude of miRNA change was

much higher in plasma than in exosomes (Fig 5). However, there was also higher variability in plasma samples compared to exosomes, which resulted in statistical insignificance. This high variability is possibly due to the complexity in miRNA origination within plasma samples. In addition, since most miRNAs in exosomes are only a small subset of total plasma miRNAs, significant changes in miRNAs that can be detected in exosomes may not be detectable in plasma samples. The identity of plasma-derived exosomes was verified by NanoSight as well as by Western blotting for CD63 (Fig. 1B–C). The predominant population of particles was found near 115 nm, a size consistent with previous reports using both ultracentrifugation and commercially available exosome isolation methods [38, 39]. It was noted that there was also a small portion of particles in the preparation that exceeded 200 nm, which could be contamination from other membrane vesicles. However, given the small percentage of these particles in the preparation, this is unlikely to alter the results and conclusions of the study.

Our previous study showed that miR-29b-3p as well as a set of other anti-fibrotic miRNAs were significantly decreased in cardiac tissues from rats subjected to PNx surgery [40]. The current study found that many of these anti-fibrotic miRNAs (miR-29b-3p, miR-20a-5p, and miR-19a-3p) were also decreased in the circulating exosome samples from both PNx and 2K1C animals, indicating that changes in exosomal miRNAs may reflect pathological changes in the tissue. Interestingly, even though changes of miRNAs in exosomes are significantly different from that in plasma samples, the two kidney disease models bear a very similar pattern of miRNA changes in either exosome or plasma samples. It is known that PNx and 2K1C represent chronic kidney disease and ischemic renal hypertension models, respectively. However, PNx and 2K1C have many similarities in terms of phenotype and disease pathogenesis. They both induce renal tissue ischemia, high blood pressure, and renal and cardiac fibrosis [20, 41–43]. The current observations of high similarity of miRNA changes in blood samples from these two kidney disease models indicate that they activate common pathways that regulate the release of miRNAs into the bloodstream. It should be noted that we did not specifically measure exosomes released from the kidney. Future studies to identify candidate kidney-injury related biomarkers is warranted to test the linkage between kidney injury and changes in circulating miRNAs.

In summary, our study revealed that the differential changes of miRNAs can be detected in both exosomal and plasma samples, implying that different mechanisms are used in regulating the packaging of miRNAs to exosomes or other carriers. Our results also provide information towards the dispute in the field regarding whether exosomes or whole plasma should be used for circulating miRNA measurements. Our results suggest that the two measurements represent snapshots of different biological processes occurring during disease pathogenesis and may not be replaced by one another.

Acknowledgments

Acknowledgements and Conflict of Interest

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List of Abbreviations

miRNA microRNA

PNx	5/6 th partial nephrectomy
2K1C	two-kidney-one-clip
RT-qPCR	real time quantitative polymerase chain reaction
ECM	extracellular matrix
Ago2	Argonaute 2
SD	Sprague-Dawley
PABAK	Prevalence-Adjusted Bias-Adjusted Kappa
CCC	Lin's concordance correlation coefficient

Highlights

- Changes in miRNAs can be detected in both plasma and plasma-derived exosomes.
- Measurement of miRNAs in plasma and exosomes are not necessarily interchangeable.
- Exosomal miRNAs measurement in CKD animals suggests activation of pro-fibrotic pathways.

Fig 1. Workflow and validation of microRNA profiling in plasma and plasma-derived exosomes
A): Schematic detailing experimental procedure. **B):** Size distribution of plasma-derived exosomes isolated from sham-operated rats was analyzed using NanoSight technology. One representative sample, of three, is depicted. **C):** Representative immunoblot of CD63 from plasma-derived exosomes isolated from sham-operated rats. Relative CD63 expression was compared between the exosome pellet and the non-exosomal supernatant obtained following the exosome isolation procedure. A total of 4 independent samples were analyzed.

A **B**

Fig. 2. Differential expression of microRNAs (miRNAs) in plasma and plasma-derived exosomes
A): Plasma and plasma-derived exosomes were isolated from rats subjected to either sham, PNx, or 2K1C surgery at the end of 4th week as described in Method section. A total of 665 rat microRNAs were included in the profiling plates for measurements. Three rats were included in each group. An average CT value <35 by RT-qPCR measurement was defined as a detectable miRNA. **B):** Expression of tissue conservative miRNAs in plasma and plasma-derived exosomes.

Fig. 3. Expression level of miRNAs in plasma and plasma-derived exosomes from sham-operated rats

The heat map was generated using relative expression levels of miRNAs. Results are expressed as a ratio of individual miRNAs vs. the global mean of either plasma or plasma-derived exosomes.

Fig. 4. PNx-caused changes of miRNAs in plasma and plasma-derived exosomes

The volcano plot was generated based on the changes of each miRNA in plasma (A) or plasma-derived exosomes (B) from rats subjected to PNx or 2K1C surgery. The X-axis shows the $\log_2(\text{Fold change})$ and the Y-axis shows the $-\log_{10}(\text{p-value})$. Black circles represent individual miRNAs that had changes that were neither statistically significant nor greater than 2 fold. Gold circles represent individual miRNAs that had changes that did not reach statistical significance but were greater than 2 fold. Red circles represent individual miRNAs that had changes that did reach statistical significance but were not greater than 2 fold. Blue circles represent individual miRNAs that had changes that were both statistically significant and greater than 2 fold.

Fig. 5. Comparison of changes in exosomal and plasma miRNAs in PNx and 2K1C
Lin's Concordance Correlation Coefficient was calculated for fold regulation of individual miRNAs in A): Plasma versus exosomes in PNx animals; B): Plasma versus exosomes in 2K1C animals.

Fig. 6. Comparison of changes in miRNA in PNx and 2K1C
Scatterplots depicting fold regulation of individual miRNAs in A): PNx versus 2K1C in exosomes; B): PNx versus 2K1C in plasma.

Fig. 8. Pathway analysis based on changes of miRNAs in plasma
Plasma miRNAs from PNx rats that have at least two-fold change and $p < 0.05$ were included in the pathway analysis using DIANA-miRPath v3. 0 web server.