MicroRNA-150 deletion in mice protects kidney from myocardial infarction-induced acute kidney injury

Punithavathi Ranganathan, Calpurnia Jayakumar, Yaoping Tang, Kyoung-mi Park, Jian-peng Teoh, Huabo Su, Jie Li, Il-man Kim, and Ganesan Ramesh

Department of Medicine and Vascular Biology Center, Georgia Regents University, Augusta, Georgia

Submitted 19 February 2015; accepted in final form 18 June 2015

MicroRNA-150 deletion in mice protects kidney from myocardial infarction-induced acute kidney injury. Am J Physiol Renal Physiol 309: F551–F558, 2015. First published June 24, 2015; doi:10.1152/ajprenal.00076.2015.—Despite greater understanding of acute kidney injury (AKI) in animal models, many of the preclinical studies are not translatable. Most of the data were derived from a bilateral renal pedicle clamping model with warm ischemia. However, ischemic injury of the kidney in humans is distinctly different and does not involve clamping of renal vessel. Permanent ligation of the left anterior descending coronary artery model was used to test the role of microRNA (miR)-150 in AKI. Myocardial infarction in this model causes AKI which is similar to human cardiac bypass surgery. Moreover, the time course of serum creatinine and biomarker elevation were also similar to human ischemic injury. Deletion of miR-150 suppressed AKI which was associated with suppression of inflammation and interstitial cell apoptosis. Immunofluorescence staining with endothelial marker and marker of apoptosis suggested that dying cells are mostly endothelial cells with minimal epithelial cell apoptosis in this model. Interestingly, deletion of miR-150 also suppressed interstitial fibrosis. Consistent with protection, miR-150 deletion causes induction of its target gene insulin-like growth factor-1 receptor (IGF-1R) and overexpression of miR-150 in endothelial cells downregulated IGF-1R, suggesting miR-150 may mediate its detrimental effects through suppression of IGF-1R pathways.

ACUTE KIDNEY INJURY (AKI) due to ischemia is a serious and frequent problem in hospitalized patients and patients who undergo surgery (7, 16, 30). Currently, there are no therapies available to treat or prevent the development of AKI. Moreover, therapies developed based on the currently existing renal ischemia-reperfusion injury were not translatable. This is mainly due to the inadequacy of currently used animal models of bilateral renal pedicle clamping in mimicking hypoxic renal injury in humans during bypass surgery or artificial ventilation. Except during partial nephrectomy, all other forms of AKI in humans develop without complete stoppage of blood supply as opposed to the mouse model of bilateral clamping. Therefore, underlying mechanisms and cell types that may be affected could be quite different. In addition, the definition of 50% increase of creatinine over baseline (2-fold increase) for diagnosis of AKI (26, 31) as opposed to 10-fold increase in the mouse model with significant necrosis in the tubules suggests extreme difference in the severity. Induction of myocardial infarction (MI) through ligation of left coronary artery with artificial ventilation of lung in the mouse is a much closer and ideal model that is closely related to human cardiopulmonary bypass surgery-induced AKI in humans. Due to its time-consuming nature and complicated surgical protocol requiring much needed surgical skills, it is not practiced as a routine model for inducing AKI in the mouse. With the help of a cardiac surgeon, we performed this model to determine the underlying mechanism of MI-induced AKI and role of miRNA-150 in AKI.

MicroRNAs constitute a class of noncoding RNAs that play key roles in the regulation of gene expression. Acting at the posttranscriptional level, these fascinating molecules may fine-tune the expression of as much as 60% of all mammalian protein-encoding genes. Recent studies have shown that microRNAs (miRs) are involved in the pathogenesis of chronic kidney disease and renal fibrosis (6, 17, 21). MiR-150 is shown to mediate its profibrotic effects through suppression of SOCS1 in tubular epithelium and mesangial cells (37). However, its role in AKI is unknown. Our results demonstrate for the first time that miR-150 deletion in mice protects the kidney against MI-induced AKI as well as bilateral renal ischemia-reperfusion injury.

MATERIALS AND METHODS

Mouse strains. MiR-150 knockout mice and their wild-type (WT; C57BL/6J) counterparts were purchased from Jackson Laboratories. The Institutional Animal Care and Use Committee of the Georgia Regents University approved all of the protocols and procedures for using animals (approval number BR10-10-369).

Mouse model of MI-induced AKI. Neck area and the left side of the ribcage were shaved and disinfected using 80% ethanol. The mouse was placed on its back and a facemask was placed over its nose and mouth to keep up the anesthesia, isoflurane. Unconsciousness of the mouse was confirmed by pinching the toe. Midline cervical incision was performed by separating the skin, muscle, and tissue covering the trachea under the dissection microscope. A small hole was cut in the exposed trachea between two cartridge rings below the glottis to insert the endotracheal tube and then a tube was inserted. The thoracic movement was checked to make sure that both lungs were well-ventilated. The respiration rate was ~110 breaths/min, with an inspiratory pressure of 17 to 18 cm H2O. The mouse was turned carefully to its right side, facing its left side. Left-sided thoracotomy was performed between the third and the fourth rib, and the tissue and muscle were dissected carefully using a cauter to prevent bleeding. The thorax was opened carefully and the heart was located without touching the lung with any sharp object. After that, part of the pericardial sac that is covering the heart was removed. The left anterior descending artery (LAD) was identified which is located between the pulmonary artery and the left auricle. With the use of an 8-0 Prolene suture (Ethicon, Norderstedt, Germany), the LAD was ligated in the proximal region with one single suture. A chest tube

Address for reprint requests and other correspondence: G. Ramesh, Dept. of Medicine and Vascular Biology Center, CB-3702, Georgia Regents Univ., 1459 Laney Walker Blvd., Augusta, GA 30912 (e-mail: gramesh@gru.edu).
Regained consciousness.

Intraperitoneally. The mice were kept in a warm incubator until they wounds were closed and mice were given 1 ml of warm saline procedure except the renal pedicles were not clamped. Surgical control, sham-operated animals were subjected to the same surgical protocol. As a confirm visually upon release of the clamps. As a knockout and their WT counterparts were anesthetized with pentobarbital sodium (50 mg/kg body wt ip) and were placed on a heating pad to maintain body temperature at 37°C. Both renal pedicles were identified through dorsal incisions and clamped for a period of 26 min.

Reperfusion was confirmed visually upon release of the clamps. As a control, sham-operated animals were subjected to the same surgical procedure except the renal pedicles were not clamped. Surgical wounds were closed and mice were given 1 ml of warm saline intraperitoneally. The mice were kept in a warm incubator until they regained consciousness.

Renal ischemia reperfusion. Eight- to nine-week-old miR-150 knockout and their WT counterparts were anesthetized with pentobarbital sodium (50 mg/kg body wt ip) and were placed on a heating pad to maintain body temperature at 37°C. Both renal pedicles were identified through dorsal incisions and clamped for a period of 26 min. Reperfusion was confirmed visually upon release of the clamps. As a control, sham-operated animals were subjected to the same surgical procedure except the renal pedicles were not clamped. Surgical wounds were closed and mice were given 1 ml of warm saline intraperitoneally. The mice were kept in a warm incubator until they regained consciousness.

Renal function. Renal function was assessed by measurements of serum creatinine (DZ072B, Diazyme Laboratories, Poway, CA) (28, 32).

TACS TdT in situ apoptosis detection. To identify apoptotic cells, tissue sections were stained using TACS TdT in situ Apoptosis Detection kit (R&D Systems) according to the manufacturer’s instructions. Briefly, tissue sections were deparaffinized, hydrated, and washed with PBS. Sections were digested with proteinase K for 15 min at 24°C. Slides were then washed and endogenous peroxidase activity was quenched with 3% H2O2 in methanol. Slides were washed and incubated with TdT labeling reaction mix at 37°C for 1 h and then with streptavidin-horseradish peroxidase. Color was developed using TACS blue label substrate solution. Slides were washed, counterstained, and mounted with Permount. Sections were photographed and labeled cells were counted and quantified.

Quantitation of mRNA by real-time RT-PCR. Real-time RT-PCR was performed in an Applied Biosystems 7700 Sequence Detection System (Foster City, CA). Total RNA (1.5 μg) was reverse transcribed in a reaction volume of 20 μl using Omniscript RT kit and random primers. The product was diluted to a volume of 150 μl and 6-μl aliquots were used as templates for amplification using the SYBR Green PCR amplification reagent (Qiagen) and gene-specific primers. The primer sets used were as follows: mouse TNF-α (forward: GCATGATCCGACGTGGAA; reverse: AGATCCATGCGACGTGGAA).

Fig. 1. microRNA (miR)-150 deletion protects the kidney against myocardial infarction (MI)-induced acute kidney injury (AKI). A: description of MI surgery protocol. B: time course of serum creatinine rise after sham and MI surgery in wild-type (WT) mice. *P < 0.001 vs. corresponding time point in sham; n = 4–6. C: serum creatinine at 48 h after sham and MI surgery in WT and miR-150 knockout (KO) animals. *P < 0.05 vs. other groups; n = 4–6. D: RT-PCR analysis of inflammatory cytokine and kidney injury markers in WT and miR-150 KO animals. *P < 0.05 vs. other groups; n = 4. E: periodic acid Schiff (PAS)-stained section from sham and MI-operated WT and miR-150 KO animals showing no visible structural changes in the kidney with MI.
CGTTG GCCAG), MCP-1 (forward: ATGCAGGTCCCTGTCATG; reverse: GCTTGAGGTGGTTGTGGA), ICAM-1 (forward: AGATTCACTACGGTGCTG; reverse: CTTCAGAGGCAGGAAACAGG), KIM-1 (forward: ATGAATCAGATTCAAGTCTTC; reverse: TCTGGTTTGTGAGTCCATGTG), and NGAL (forward: CCCCATCTGTGCTCACTGTC; reverse: TTTTCTGGACCGCATTG). The amount of cDNA was normalized to the β-actin signal amplified in a separate reaction (forward primer: AGAGGGAAATCGTGCGTGAC; reverse: CAATAGTGATGACCTGGCCGT).

Histology and immunostaining. Kidney tissue was fixed in buffered 10% formalin for 12 h and then embedded in paraffin wax. For assessment of injury, 5-μm sections were stained with periodic acid Schiff (PAS) followed by hematoxylin. To quantify leukocyte infiltration, sections were stained with rat anti-mouse neutrophil antibody (Abcam, Cambridge, MA; 1:200 dilution) followed by goat anti-rat biotin conjugate. Color was developed after incubation with ABC reagent (Vector Lab). Stained sections were photographed and five ×40 fields of neutrophils were examined for quantification of leukocytes. To determine whether TdT-mediated dUTP nick end labeling (TUNEL)-positive cells are endothelial cells, after staining the section for FITC-based TUNEL assay (R&D Systems), sections were counterstained with CD31 antibody (Abcam) followed by Cy5-conjugated secondary antibody. Stained sections were photographed using an Olympus inverted microscope with color CCD camera.

Cell culture and transfection. Immortalized mouse cardiac endothelial cell line was purchased from Cedarlane and maintained according to the company’s recommendation. For gain-of-function studies, we transfected cytomegalovirus expression plasmids for miR-150 (Origene, SC400788) or miR-150 mimics (Life Technologies, MC10070). All in vitro assays were performed 60–72 h after transfection when overexpression is maximum. Overexpression was confirmed by RT-PCR and showed over 20,000-fold more miR-150 than control transfected cells.

Statistical methods. All assays were performed in duplicate or triplicate. The data are reported as means ± SE. Statistical significance was assessed by an unpaired, two-tailed Student’s t-test for single comparison or ANOVA for multiple comparisons. P < 0.05 is considered significant.

RESULTS

MI-induced AKI in mice follows a similar time course of human cardiopulmonary bypass surgery. Immediately after the MI surgical procedure (Fig. 1A), kidney function was monitored by measuring serum creatinine. Sham-operated WT mice with just ventilation showed a small but insignificant increase in serum creatinine. However, WT mice subjected to left coronary artery ligation with ventilation developed a significant rise in serum creatinine on day 1 and peaked at day 2 (Fig. 1B). In the subsequent days, serum creatinine started falling to reach sham-operated animal levels. The time course and serum creatinine rise were very similar to previously reported pediatric human cardiopulmonary bypass surgery (20, 23).

Interestingly, we carried a similar procedure in miR-150 knockout mice and they are protected against MI-induced kidney dysfunction as seen by reduced serum creatinine rise at
Fig. 3. Quantification of apoptosis after sham and MI surgery in WT and miR-150 KO animals. A: quantification of TdT-mediated dUTP nick end labeling (TUNEL)-positive cells in the kidney cortex. B: quantification of TUNEL-positive cells in the kidney medulla. *P < 0.001 vs. sham. #P < 0.001 vs. WT MI; n = 4–6. C: representative images showing TUNEL staining in WT and miR-150 KO mouse kidney.

Fig. 4. Colocalization of CD31 (endothelial marker) with TUNEL-positive cells in the WT kidney. A: CD31 staining shown in red. B: TUNEL-positive cells shown in green. C: nuclear staining shown in blue. D: overlay of 3 images. Yellow arrow indicates colocalization of CD31 cells and TUNEL-positive nuclei.
Kidney dysfunction in WT mice was associated with a significant increase in inflammatory cytokines such as TNF-α, IL-6, and MCP-1 as well as kidney injury markers such as NGAL and KIM-1 (Fig. 1D). These changes were minimal in miR-150 knockout mice. Interestingly, PAS-stained sections did not show any visible structural alteration such as cast formation and brush-border damage in both WT and miR-150 knockout mice (Fig. 1E).

Despite increased inflammatory cytokine expression, there was no neutrophil infiltration seen in the kidney after MI (not shown). In addition, macrophage/monocyte infiltration was not increased after MI in the WT and miR-150 knockout animal kidney (Fig. 2) in both the cortex and medulla.

**MI with ventilation induced a prominent peritubular interstitial cell apoptosis.** To determine whether kidney dysfunction was associated with tubular epithelial cell apoptosis similar to the bilateral renal pedicle clamping model, a kidney section was stained with TUNEL staining to determine apoptosis. TUNEL staining showed a large increase in apoptotic positive cells in WT. Surprisingly, very little apoptosis was found in the tubular epithelium of the cortex. A large number of interstitial cells were positive for TUNEL in WT mice subjected to MI and maximum apoptosis was found in the inner medulla (Fig. 3, A–C). Moreover, many cells were positive for TUNEL within the vessels. miR-150 knockout mice showed a significant reduction in MI-induced apoptosis of interstitial cells (Fig. 3, A–C).

To determine whether apoptotic interstitial cells are endothelial cells, immunofluorescence double staining was done for TUNEL and endothelial cell marker CD31. As shown in Fig. 4, apoptotic cells are indeed endothelial cells. However, we also saw few cells that were negative for endothelial cell marker. The identity of these CD31-negative cells is unknown.

**Mild interstitial fibrosis seen at 8 wk was suppressed in miR-150 knockout mice.** Previous studies have shown that miR-150 blockade suppressed interstitial fibrosis in a model of lupus nephritis (37). However, it was not clear whether such phenomenon is also seen in other models of interstitial fibrosis. To determine the effect of miR-150 deletion on AKI-induced fibrosis in vivo, kidney tissue sections were stained with Trichrome. As shown in Fig. 5, mild interstitial fibrosis was seen in WT mice that were subjected to MI which was completely suppressed in miR-150 knockout mice. Consistent with trichrome staining, a modest but significant increase in collagen I and connective tissue growth factor mRNA was seen in WT kidney after MI that was suppressed in the miR-150 knockout animal kidney with MI.

**miR-150 knockout mice are resistant to bilateral renal ischemia-reperfusion injury model as well.** To determine whether miR-150 knockout mice are also resistant to bilateral renal clamping model of ischemia-reperfusion injury, the renal pedicle was clamped for 26 min and then the kidney function and inflammatory responses were measured at 24 h. As shown in Fig. 6, WT mice developed severe renal injury as shown by increased serum creatinine over sham-operated animals. However, miR-150 knockout mice showed significantly lower levels of serum creatinine compared with WT mice that were subjected to ischemia-reperfusion injury. Consistent with improved renal function, the biomarker expressions of KIM-1 and NGAL were significantly reduced in miR-150 knockout mice compared with WT mice after ischemia-reperfusion injury. In addition, the expressions of inflammatory cytokines were also reduced in the kidney of miR-150 knockout compared with WT mice.

**miR-150 represses IGF-1R expression in the kidney.** IGF-1 pathways play a critical protective role in vascular damage and kidney reperfusion injury (8, 13, 14, 22). Recent studies showed that miR-150 targets insulin-like growth factor-1 receptor (IGF-1R) expression in pancreatic cancer cells through c-myc transcription factor thereby inducing apoptosis (11). Therefore, we determined whether miR-150 induces apoptosis through downregulation of IGF-1R expression. As shown in Fig. 7A, in response to reperfusion injury the expression of miR-150 was downregulated but the downregulation was not complete. However, complete deletion of miR-150 in mice causes upregulation of IGF-1R expression in the kidney (Fig. 7, B and C). Interestingly, when we overexpressed miRNA in endothelial cells, IGF-1R and c-myc expression were downregulated, suggesting that IGF-1R is a target for miR-150 that may be regulated indirectly through c-myc downregulation.
DISCUSSION

For more than two decades, bilateral renal pedicle clamping model (warm ischemia reperfusion) was used to study human ischemic kidney disease (24, 34). Simplicity and uniform kidney injury response lead to widespread use of this model among renal researchers. Although a large amount of knowledge was generated from this model, whether it is an appropriate reflection of human ischemic kidney disease remains a big question. Except during partial nephrectomy, renal pedicle clamping was not employed in any of the ishemic kidney diseases in humans. Rather, reduced renal blood flow or reduced oxygenation of the kidney is the common underlying cause of ischemic injury in humans. This is mainly due to ventilation and/or in combination with surgery. Difficulties of translating preclinical mouse studies for therapies in humans suggest that the model system used may not be appropriate. To find an optimal model that has a similar course of kidney pathogenesis, we employed the MI model in combination with ventilation similar to cardiopulmonary bypass surgery in humans.

The creatinine rise and time course are very similar to that as seen after cardiopulmonary bypass surgery (20). Moreover, the severity of AKI is also similar to human AKI. Consistent with human studies (12, 20), both NGAL and KIM-1 expression increased significantly in the kidney. These data suggest that the model system we chose is close to human ischemic kidney disease.

Tubular necrosis and epithelial cell apoptosis were shown to be prominent features of ischemia-reperfusion injury of the kidney using the bilateral renal pedicle clamping model (4, 5, 25, 33). This was attributed to the sensitivity of S3 segment of the proximal tubular epithelium unable to adopt anaerobic metabolism. Although in vitro studies did not support this notion (27), it is still widely believed in the field. In contrast to this, the currently employed model does not induce a large amount of apoptosis in S3 segment of the nephron, rather it induced apoptosis in the circulating and interstitial cells. Our double staining with endothelial cell marker CD31 suggests that these apoptotic cells are indeed endothelial cells. In addition, we also see CD31-negative interstitial cells that are positive for TUNEL. The identities of these interstitial cells are not clear.

MiR-150 deletion suppressed both MI-induced AKI as well as renal clamping-induced AKI despite differences in their primary injury mechanism. The best understood role of miR-150 in the hematopoietic system is its regulation of the transcription factor Myb during B cell development. It is becoming clear, however, that miR-150 has additional relevant targets that affect growth, maturation, and the immune response in both B and T lymphocytes and that downregulation of miR-150 in lymphatic tissue results in unregulated proliferation that contributes to tumorigenesis (1, 3, 36). Overexpression of miR-150 induced apoptosis of pancreatic cancer by targeting IGF-1R (11), suggesting the proapoptotic role of this miR-150. MiR-150 induced apoptosis of pancreatic cancer by targeting IGF-1R (11), suggesting the proapoptotic role of this miR-150.
critical role in endothelial cell survival, proliferation, and regeneration (8, 14). Consistent with this view, our data show that IGF-1R is upregulated in the kidney of miR-150 knockout mice, whereas overexpression of miR-150 downregulated IGF-1R in endothelial cells. Moreover, administration of recombinant IGF-1 suppressed AKI and accelerated recovery of the kidney from AKI in the animal model (10, 19), suggesting IGF-1 pathways are protective in the kidney. Therefore, it is possible that deletion of miR-150 could abolish ischemia-induced changes in IGF-1R expression and enhance endothelial cell survival. Recent studies also show that miR-150 plays a critical role in kidney fibrosis through suppression of SOCS1 (37). Consistent with this report, our results also show that miR-150 deletion suppressed MI-induced interstitial fibrosis at 8 wk after surgery. It is interesting to note that miR-150 exhibits tissue specificity. Recent studies showed that miR-150 knockout exacerbated MI-induced cardiac dysfunction and fibrosis (29), whereas the same mice show protection in the kidney against MI-induced AKI as well as kidney fibrosis (37). A similar tissue-specific difference in microRNA activity was seen for miR-214 as well (2, 9, 18). To determine whether hypoperfusion due to decreased fractional shortening may contribute to observed sensitivity of the kidney in different groups, we could not find any relation with decrease in fractional shortening and development of AKI (data not shown), suggesting that an additional mechanism may exist for protective effects in miR-150 knockout mice.

In conclusion, our studies demonstrate that MI-induced AKI shows a distinct pattern of interstitial cell apoptosis and serum creatinine rise similar to human cardiac bypass surgery. Deletion of miR-150 suppressed interstitial cell apoptosis and these interstitial cells are mostly endothelial cells and miR-150 represses IGF-1R expression in endothelial cells. Our studies suggest that inhibition of miR-150 may be useful for treatment of MI-induced AKI in humans.

GRANTS
This work was supported by American Heart Association (AHA) Greater Southeast Affiliate Postdoctoral Fellowship 13POST16840074 to P. Ranganathan, National Institutes of Health (NIH) R01 DK083379 to G. Ramesh, AHA...
REFERENCES


DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: P.R., C.J., Y.T., K.-m.P., J.-p.T., and G.R. performed experiments; P.R., Y.T., K.-m.P., J.-p.T., H.S., J.L., I.-m.K., and G.R. analyzed data; P.R., K.-m.P., J.-p.T., H.S., I.-m.K., and G.R. interpreted results of experiments; P.R. prepared figures; P.R. and C.J. drafted manuscript; P.R., Y.T., K.-m.P., J.-p.T., H.S., J.L., I.-m.K., and G.R. approved final version of manuscript; I.-m.K. and G.R. conception and design of research.

REFERENCES