An Omics Approach to Identify Model-agnostic Disease-driving Nodes in AKI: Implications for Drug Development

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ABSTRACT

Acute Kidney Injury (AKI) is the abrupt loss in kidney function caused by either injury or impairment. To classify the severity of cases of AKI, Acute Tubular Necrosis (ATN) scoring is utilized. Clinical treatments of AKI are often punctuated with failures. Lack of robust translational success can at least in part be explained by the fact that model systems may not fully recapitulate human AKI. It is therefore important to develop strategies that are governed by the same pathway in both human and animal models to properly treat AKI. This study examines the expression of Rho-associated protein kinase (ROCK) in AKI in mice induced by mercury chloride, folic acid, and domoic acid and correlates expression with ATN scoring.

Prepared hematoxylin-eosin-stained kidney slides were obtained from mice treated with mercury chloride, folic acid or domoic acid and examined by microscopy. Tissues were categorized by the size of their urinary casts and scores were designed to mirror ATN scoring. Analysis of ROCK2 expression by RT-PCR showed that there was significantly increased in expression in all models when compared to the control (Sham). The highest level of ROCK2 expression can be found in the HgCl2 model of AKI. Moreover, renal ROCK2 expression level exhibited a significant and direct correlation with ATN scores.

This is the first report of increased renal ROCK2 expression in multiple models of AKI accompanied by ATN. These data suggest that ROCK2 is a model agnostic injury driving node in AKI and a potential therapeutic target for treatment.

INTRODUCTION

Acute Kidney Injury (AKI) is defined as the abrupt loss in kidney function caused by either injury or impairment. An increase in serum creatinine levels, which indicate that improper or impaired functioning of the kidneys, is an identifier of Acute Kidney Injury (Ostermann and Joannidis, 2016). AKI can be caused by decreased blood flow which can be caused by overuse of pain medication, heart failure, severe allergy or major surgery. It can also be caused by direct damage to the kidneys which could be provoked by multiple myeloma (cancer of the plasma cells), sepsis (bacteria infects bloodstream) and interstitial nephritis (allergic reactions to certain drugs). A third cause associated with AKI can be the blockage of urinary tract triggered by kidney stones, blood clots in the urinary tract and bladder, prostate cancer or cervical cancer (Makris and Spanou, 2016).

In order to identify the severity of cases of AKI, Acute Tubular Necrosis (ATN) scoring is utilized. AKI could either be categorized into pre-renal AKI or ATN. In order to identify AKI as ATN, a biopsy is necessary. Within kidneys that have been diagnosed with AKI, there is a formation of urinary casts, specifically granular casts, and hyaline casts (Perazella *et al*, 2008). Granular casts are indicators of kidney disease while hyaline casts are formed in the absence of tubular lumen (Higuchi *et al,* 2019).

There is also an increase in Renal Tubular Epithelial Cells (RTEC) of kidneys diagnosed with AKI. Although all of these symptoms have been identified in AKI, there has been no specific number of casts necessary for a certain kidney to have a certain ATN score (Perazella *et al*, 2008).

Acute Kidney Injury has been reported in 8 to 16% of hospitalized patients. There are approximately 13.3 million cases of AKI each year ("International Society of Nephrology", 2019). Over the past 25 years, the instances of non-dialysis-requiring AKI have increased in many countries such as the United States, Canada, and the United Kingdom. Although the number of episodes of AKI has increased, the mortality rate has decreased. This is due to the fact that there is an increase in the awareness of AKI. Dialysis-requiring AKI has also increased in the past 25 years whether it be caused by general hospital admissions, surgeries (specifically cardiac), coronary interventions, infectious disease, sepsis or strokes (Sawhney and Fraser, 2017). AKI has a death rate of about 20% of hospital admissions and that percentage increases to 50% in Intensive Care Units (Luo *et al*, 2017). The significant presence of AKI in the hospital can also lead to financial burdens on the patients with nearly $7500 of excess hospital costs (Chertow *et al*, 2005).

AKI can be induced by mercury chloride, folic acid, and domoic acid. Mercury is a toxic heavy metal that comes in both organic and inorganic forms. Mercury toxicity significantly affects the kidneys and can be caused by fish consumption or dental amalgam (Dhanapriya *et al*, 2016). Many times within Asian communities, mercury chloride is seen as an at-home remedy. Folic Acid is a synthetic dietary supplement. It is often present in artificially enriched foods and pharmaceutical vitamins. Many times, vitamins containing folic acid are recommended to pregnant women (Greenberg *et al*, 2011). Domoic acid is naturally produced in phytoplankton and accumulates in seafood during harmful algae blooms (Ferriss *et al*, 2017).

There have been lots of disparities surrounding AKI and its diagnosis. The most common definition used for diagnosis includes an increase of at least 44.2 μmol/L of serum creatinine or

urine output less than 400-500 ml per day are also considered indicators (Gameiro *et al,* 2018). However, a worldwide classification of AKI was not created until 2004. **R**isk, **I**njury, **F**ailure, **L**oss of kidney function and **E**nd-stage kidney disease or RIFLE was created by a consortium of nephrologists and intensivists to establish a more organized system of classification for AKI. This classification takes into account the criteria of clinical applicability, sensitivity, and specificity (Biesen *et al*, 2006). The RIFLE classification system would use serum creatinine and urine output as determinants of the severity of AKI. The severity can be classified into three groups: risk, injury, and failure. RIFLE has become an important tool in diagnosing AKI in intensive care units (ICU) and predicting patient outcomes (Lopes and Jorge, 2013).

However, RIFLE does have its flaws. First, the baseline serum creatinine levels necessary to define AKI are not well understood throughout medical practice. The Modification of Diet in Renal Disease (MDRD) equation is used to identify the baseline equation (Swart *et al*, 2010). RIFLE also does not consider the causation of AKI in patients which can affect the quality of the treatment for the patients (Lopes and Jorge, 2013).

The usage of an animal model is crucial in biological research and medicine. Animal models can be used to identify certain diseases. Relationships between humans and other animals, specifically mammals, can facilitate understanding of the human body. Most vaccines created that have been successfully used on humans were developed using an animal model. Basic science and medical research is possible because of these models. In fact, 90% of the drugs used to treat animals are exactly identical or very similar to the drugs that are used on humans (Barré-Sinoussi and Montagutelli, 2015).

Mus musculus (the house mouse) has historically been used as a model for investigating human biology and the effect of certain diseases (Perlman, 2016). Specifically in AKI, the mouse model can be used to investigate the effect of AKI in various organs. *Mus musculus* can be used to test potential therapies and identify physiological mechanisms in relation to AKI. In mice with AKI, relationships between organs such as the heart, liver, brain, and lung can be identified (Grams and Rabb, 2012).

Rho-associated protein kinase (ROCK) is a protein kinase necessary for cell adhesion, migration, proliferation, cytokine activation, inflammatory cell migration, smooth muscle cell contraction and cell cycle regulation (Hartmann et al, 2015). Inhibition of the ROCK pathway has been shown to have anti-inflammatory, anti-apoptosis and antioxidant effects (Amano et al, 2010). Treatments with Fasudil-HCl (HA-1077), a Rho-kinase inhibitor, have been shown to protect mice from significant impairments in renal structure and suppressed renal inflammation during oxidative stress. There are two isoforms of ROCK that have significant impacts on renal function. ROCK 1 has been shown to be necessary for chemotactic leukocyte migration into the cell while ROCK 2 is shown to be responsible for the regulation of inflammation and fibrosis within kidneys (Wang *et al*, 2008).

Clinical trials in AKI are punctuated with failures. Lack of robust translational success can at least in part be explained by the fact model systems might not fully recapitulate human AKI. ATN, the hallmark pathological feature of AKI, might not necessarily be governed by the same pathway in animal models in comparison to human subjects. *My hypothesis is that a model-agnostic injury-driving network is more than likely to play a role in human tissue injury.*

Identification of such an injury-driving network might spur the development of drugs that neutralize those networks not merely in animal models but more importantly in human subjects.

APPROACH

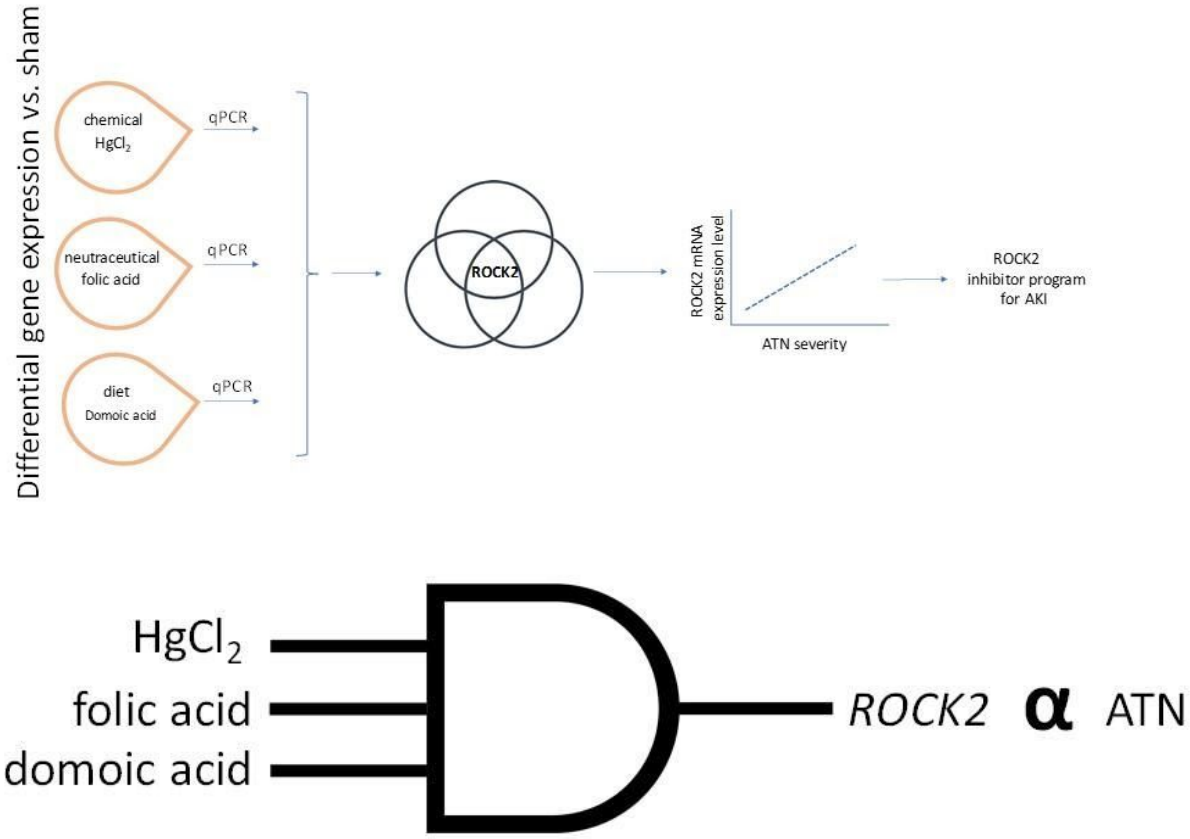

Figure 1. Functional Transcriptome Mapping. Top. Renal *ROCK2* expression will be queried across 3 distinct models of AKI. Expression level will be correlated against ATN (blinded observer). *Iff* (if and only if) renal *ROCK2* expression is increased across all 3 models and titrates ATN, then it will be deemed a functional injury-driving node in AKI and submitted as a target of interest for drug development. Bottom. Boolean expression of the above.

I employed a disruptive and potentially highly impactful approach termed inter-model functional transcriptomic analysis to identify whether renal ROCK2 is an injury-driving node in AKI (Figure 1).

METHODOLOGY

Prepared hematoxylin-eosin-stained kidney slides were obtained from mice treated $HgCl_2$ (7 mg/kg, IP, n= 9), folic acid (300 mg/kg, IP, n=4) or domoic acid (2.5 mg/kg, IP, n =3) (**IACUC #)**. These slides were prepared and provided by mentor for analysis. These slides were examined using a compound light microscope and sorted into five categories based on the size of their urinary casts, seen as hyaline and granular casts in AKI. The largest-sized casts received a score of 5 and the smallest received a score of 0 or 1. These scores were designed to mirror Acute Tubular Necrosis (ATN) Scores (Perezella et al, 2008). After the slides were sorted by microscopy, the sizes of the casts measured using ImageJ to ensure that there was statistical significance between a score of 4 from lower scores. A *t*-test was used to determine the significance between the measured values of the Sham and AKI groups. A Pearson Product Moment Correlation was used to determine the strength of the association between the genes queried and the increase in Bowman's Space. A p-value ≤ 0.05 was considered to be statistically significant

Upon determining the presence of AKI, RNA was obtained from frozen tissue (IACUC #: 2016-004). RNA was previously isolated by mentor and used for other purposes than this study. Renal homogenates (n=8/group) were submitted to qPCR for the evaluation of *ROCK2* expression. A sham group (n=6) served as control.

Quantitative Polymerase Chain Reaction (qPCR) is an amplification of DNA to determine the relative concentration of a known template (Kubista et al, 2006). Specifically, qPCR uses fluorescent probes to quantify the levels of DNA produced. The template DNA used can be genomic DNA or cDNA. To perform a qRT-PCR, it is necessary to obtain RNA from

tissues. RNA is then converted into cDNA using the enzyme reverse transcriptase. The cDNA can then be amplified using gene specific primers and quantified by qPCR.

In order to convert RNA into cDNA, high capacity cDNA Reverse Transcription Kit (Qiagen, Cat #: 204054) was used. First, the concentrations of the RNA were tested using a nanodrop.

Sample	Concentration	A260/A280
1	279.8 ng	2.17
2	63.1 ng	2.21
5	58.4 ng	2.64
7	234.3 ng	2.17
11	445.4 ng	2.19
21	226.5 ng	2.16
22	343.6 ng	2.19
23	457.8 ng	2.18

Next, using the concentrations of the RNA, the amount of water and RNA required to normalize the RNA samples was calculated. All samples must be normalized to the same concentration and the same total amount of RNA and water.

Sample	Concentration	Concentration Desired	RNA (to be added)	Water (to be added)
1	279.8 ng	500 ng	1.68 µl	8.32 µl
2	63.1 ng	500 ng	7.9 µl	2.1 µl
5	58.4 ng	500 ng	8.56 µl	1.44 µl
7	234.3 ng	500 ng	2.06 µl	7.94 µl

11	445.4 ng	500 ng	1.12 µl	8.88 µl
21	226.5 ng	500 ng	2.4 µl	7.6 µl
22	343.6 ng	500 ng	1.4 µl	8.6 µl
23	457.8 ng	500 ng	1.14 µl	8.85 µl

A Master Mix was created from the cDNA Reverse Transcription Kit and each component was multiplied by the number of samples (extra was made in order to calculate for human error).

Reagent	**Amount (µl)**	**Amount for 10 samples**
10x RT Buffer	2.0 µl	20 µl
25x DNTP Mix	0.8 µl	8 µl
10x Random Primers	2.0 µl	20 µl
Multiscribe Reverse Transcriptase	1.0 µl	10 µl
Nucleus-Free Water	4.2 µl	42 µl
Total:	10 µl	100 µl

The corresponding amounts of RNA and water were then added to each PCR tube. Water and RNA were added, and a 10 µl of the Master Mix was added to each tube and mixed by pipetting. The PCR tubes were then sealed and briefly centrifuged placed into the thermocycler. The RT-conditions were:

Setting	**Step 1**	**Step 2**	**Step 3**	**Step 4**
Temperature	25℃	37℃	85℃	4℃
Time	10 mins.	120 mins.	5 mins.	Forever

After the cDNA was synthesized, Power-Up SYBR Green Master Mix, Micro- Amp Endura Plate Optical 96-well Clear Reaction Plate, PCR Plate Covers, RNase-Free Water, Primers and cDNA are all used to prepare wells for qPCR. First, use primers to make a stock solution.

Reagent	Amount (Large Stock)	Amount (Small Stock)
Forward Primer (100UM)	75 µl	7.5 µl
Reverse Primer (100UM)	75 µl	7.5 µl
Water	1350 µl	135 µl

Then, a layout of the PCR plate was created in which it was ensured that there was room for each sample to run in triplicates. Primers and SYBR Green Master Mix was also created by multiplying each component by the number of wells (always add 5 for standard error).

Reagent	Amount (µl)	Amount for 28 wells
SYBR Green	5 µl	140 µl
Primer	1 µl	28 µl
Water	3 µl	84 µl
Total	9 µl	243 µl

A Master Mix was made for each primer being tested. Then 9 µl of the master mix was pipetted into the corresponding wells. After that, 1µl of cDNA of a certain sample was pipetted in. Once completed, the plate was vortexed and briefly centrifuged. The primers used for ROCK 2 were Forward: TGGCCCAGTTTGCATCTTTC and Reverse: AGCAAGTTGTGTTCCCAACC; for PPIA the primers were Forward: AAGTTTTCTGCTGTCTTTGG and Reverse: GTGTTCTTCGACATCACG.

RESULTS

Analysis of the prepared slides showed that when compared to the sham cohort, all models of AKI demonstrated ATN albeit to varying degrees (Figure 2). The domoic acid model was accompanied by mild nephron dropout and folic acid demonstrating increased nephron loss. The $HgCl_2$ model had kidneys with the most severe ATN accompanied by inflammation.

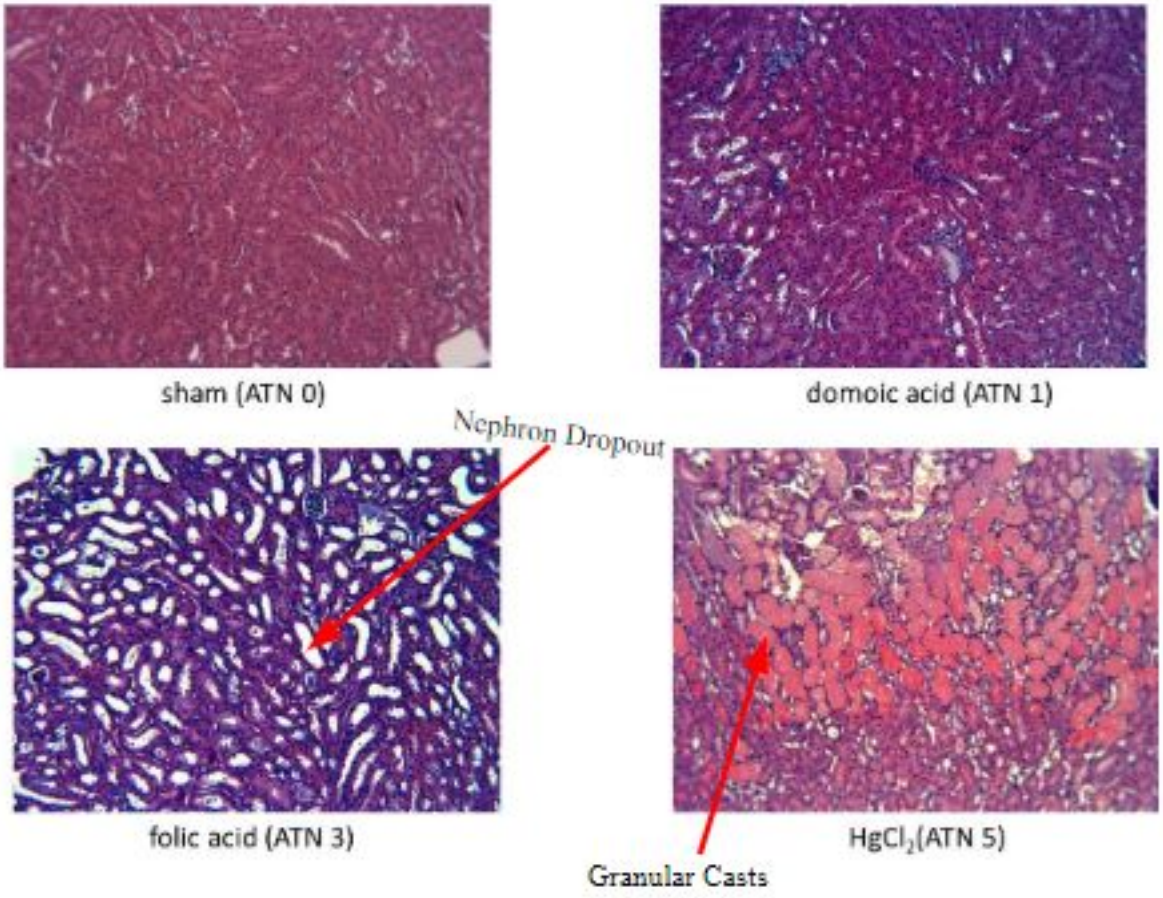

Figure 2. Models of AKI. Representative H&E-stained images (10X) of kidneys from sham, domoic acid, folic acid and $HgCl_2$ models of AKI demonstrating varying amounts of ATN (0-5 scale, blinded observer).

To correlate the physiological observation (ATN scoring) with the molecular expression of ROCK2, RT-PCR was performed. As seen in Table 1 and Figure 3, Renal *ROCK2* expression was increased significantly (Figure 3) in all models when compared to the control (sham). The highest level of ROCK2 expression can be found in the $HgCl_2$ model of AKI.

Group	*ROCK2* expression level	ATN (0-5)
sham	1	0
sham	1	0
sham	1	0
sham	1	0
sham	1	0
sham	1	0
$HgCl_2$	15	5
$HgCl_2$	13.6	5
$HgCl_2$	14	5
$HgCl_2$	12.1	5
$HgCl_2$	9	5
$HgCl_2$	10.7	5
$HgCl_2$	12.5	5
$HgCl_2$	13	4
$HgCl_2$	10.7	4
folic	1.3	1
folic	1.8	3
folic	1.4	1
folic	1.55	1
Domoic	3.3	2
Domoic	3.3	2
Domoic	2.2	1

Table 1. Renal *ROCK2* expression and ATN scores for each kidney across all models tested.

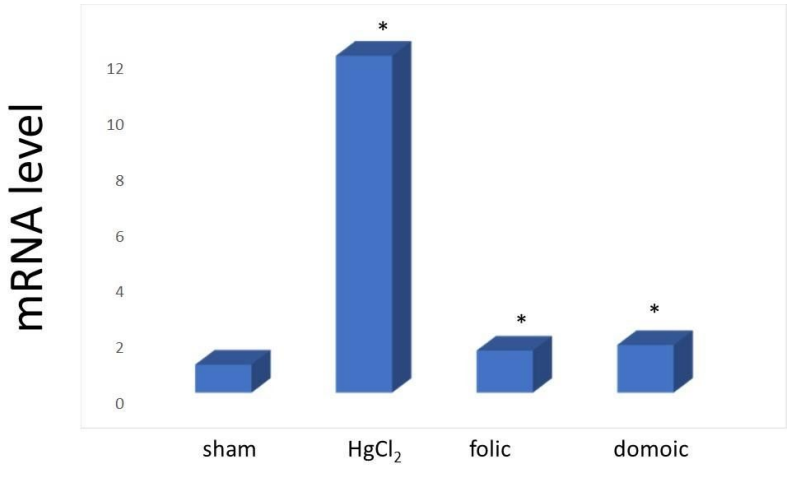

Figure 3. Renal *ROCK2* Expression Levels. Compared to the sham cohort, renal *rock2* expression levels were increased in each model of AKI.

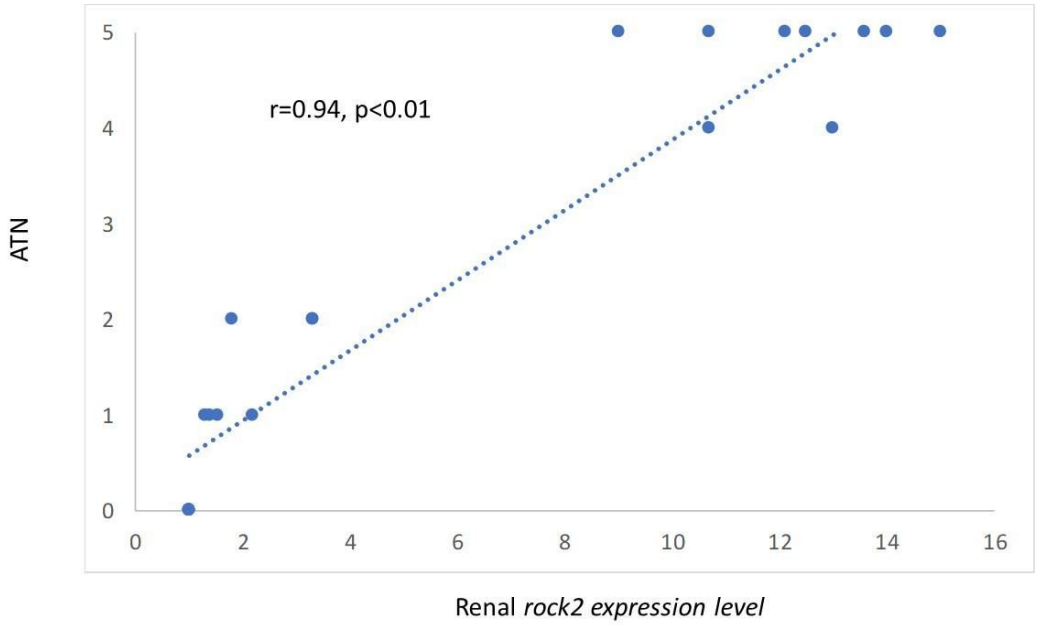

Figure 4. Renal *ROCK2* Expression Levels and ATN.

Renal ROCK2 expression level exhibited a significant and direct correlation with ATN scores (Figure 4).

DISCUSSION

This is the first report of increased renal *ROCK2* expression in multiple models of AKI accompanied by ATN. Expression levels of renal *ROCK2* mRNA exhibited a direct correlation with ATN. These data suggest that *ROCK2* is a model agnostic injury driving node in AKI.

AKI remains a challenge for both the nephrologist and intensivist as 3-5% of hospitalized patients and up to 30% of patients in intensive care units develop AKI. Morbidity, need for renal replacement therapy and mortality increase with the severity of AKI. Acute tubular necrosis (ATN) is the hallmark pathological feature in AKI that then triggers renal dysfunction. To date,

there is no approved therapy for AKI other than hydration (dilution is the solution) and renal replacement therapy. Clinical trials in this space are punctuated with failures as a number of drugs failed to mitigate renal injury. It is important therefore to develop clinic therapeutics that can mitigate AKI and attenuate morbidity and mortality (Wang et al, 2017). There is evidence that inhibition of renal Rho kinase (ROCK) attenuates AKI, and that the administration of ROCK inhibitors mitigates AKI (Prakash et al, 2008). Although inhibition of both ROCK1 and ROCK2, attenuates AKI, a ROCK2-selective inhibitor is clinically preferred as it is not associated with severe hypotension and risk of syncope associated with ROCK1 inhibition (Kentrup et al, 2011).

Three etiologically distinct but clinically relevant models of AKI were investigated herein. These models comprised of chemical injury, nutraceutical-induced injury, and diet-induced injury. In each of these 3 models, ATN was evident to varying degrees. Each of these models exhibited a significant increase in renal *ROCK2* expression suggesting that this gene's upregulation is model agnostic. Secondly, the expression of renal *ROCK2* correlated with ATN. These data suggest that *ROCK2* is a functional injury driver in AKI. These findings also suggest that ROCK2 may be a disease driver in human AKI.

FUTURE STUDIES

Future studies would involve testing various types of drugs specifically targeting the ROCK2 signaling pathway and determining their effects in treating AKI. If preclinical proof-of-concept studies with such a drug are successful, it will trigger safety and efficacy studies with a ROCK2 inhibitor in AKI patients. Results from this study have the potential to

leverage the platform of Precision Medicine for a number of indications as a ROCK2 inhibitor would find use in tissue/disease agnostic basket trials involving subsets of patients whose disease (lung, kidney, etc.) is being driven by *ROCK2*.

Nevertheless, this study exhibits some limitations. For one, this study only considers murine models; it would be advantageous to study other animal models as *ROCK2* may only be prevalent in mice. *ROCK2* may only be the tip of the iceberg, suggesting other possible injury drivers that were not tested in this study. Finally, it remains to be investigated whether the ROCK2 enzyme actually plays a functional role in human AKI which can only be confirmed in protocol-approved human kidney biopsies correlated with ATN scores.

CONCLUSIONS

AKI can be induced by various models (chemical injury, nutraceutical-induced injury and diet-induced injury). The degree or severity of the ATN scoring is affected by the model used as a HgCl2 (chemical injury) produced the most severe ATN in contrast to domoic acid and folic acid. The expression levels of renal *ROCK2* mRNA exhibited a direct correlation with ATN. These data suggest that *ROCK2* is a model agnostic injury driving node in AKI.

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