Determining the Role of Tubulointerstitial Nephritis Antigen-Like 1 in Renal Ischemic Reperfusion Injury

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A Thesis in the Field of Biotechnology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

March 2018

Abstract

Acute kidney injury (AKI) is a broad term that applies to a wide range of pathological etiologies characterized by a sudden increase in serum creatinine, a hallmark of malfunctioning kidneys. Ongoing efforts to elucidate the pathophysiology of AKI has shed light on certain proteins that may be involved during the kidney injury and repair process. One protein of interest is tubulointerstitial nephritis antigen like-1 (TINAGL1). In mice that has been subjected to renal ischemic reperfusion injury (IRI) surgery to induce AKI, mRNA transcripts of Tinagl1 has been found to be significantly increased shortly after AKI. We hypothesize that the upregulation of Tinagl1 plays an important role in mitigating the damage done by AKI and serve as an essential component during the process of repairing kidney tubules. In this study, we generated a mouse with Tinagl1 knockout and evaluated its susceptibility to renal tubular damage following IRI surgery. Results show that mice with knockout of Tinagl1 have far greater renal tubular damage compared to wild type littermates following kidney injury. Furthermore, we are able to demonstrate Tinagl1 is capable of potentiating Wnt signaling. Thus, our findings suggest Tinagl1 is a significant factor in mitigating kidney injury and can modulate the Wnt signaling pathway.

Dedication

This effort is dedicated to my parents and my brothers, who have always been supportive of my academic pursuits.

Acknowledgements

I would like to thank Dr. Andrew McMahon for his support and letting me pursue an independent research project in his laboratory for the fulfillment of the ALM degree requirements. This would not have been made possible without his mentorship and training to perform high-caliber science with cutting-edge technology. I would also like to thank the members of the McMahon lab who have taken the time out of their experiments and busy lives to help me with the project.

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Chapter I

Introduction

General function of the kidney is to act as a filtration system for blood circulating the body as well as regulating acid-base and fluid balance. The kidneys receive 20% of the cardiac output and filters approximately 120 to 150 quarts of blood, producing one to two quarts of urine. This section will review the physiology of the kidney and its pathological outcomes upon injury followed by current therapeutic options available for those suffering from kidney injury. Furthermore, this section will introduce Wnt signaling and how it can be an exciting area for the research into kidney injury and repair through the protein Tinagl1.

Physiology of the Kidney

Each kidney is composed of roughly one million smaller units called nephrons, which are the functional units of the kidney. The nephron can be separated into two parts: the glomerulus and the tubule. The glomerulus acts as a filter to prevents blood cells and large molecules such as proteins from passing to the tubules, whereas the tubules act to balance electrolytes. Blood enters the kidney through branching renal arteries that terminate in afferent arterioles in the glomerulus, forming a network of capillaries

responsible for glomerular filtration. The efferent arterioles in the glomeruli located within the outer and mid-cortex of the kidney leads to the network of capillaries along the proximal and distal convoluted tubules. The efferent arterioles in the glomeruli located near the inner or juxtamedullary glomeruli, on the other hand, form the vasa recta that runs parallel to the loop of henle. These distinct parts of the kidney correlate to specific functions; blood flow in the outer cortex is primarily glomerular filtration whereas other cortical areas are associated with nutrient delivery to tissues and return reabsorbed solutes and waters to the systemic circulation. In doing so, the kidneys are able to regulate blood homeostasis by maintaining a stable balance of electrolytes including sodium, potassium, and phosphate. A summary of substances balanced by the kidneys to maintain homeostasis and the description of each substance is listed in Table I.

Table 1. Substances regulated by the kidneys for balance of extracellular homeostasis.

Substance	**Description**
Glucose	Almost complete reabsorption in the proximal tubule by sodium-glucose transport proteins and the GLUT glucose transporter.
Peptides	Almost complete reabsorption in the proximal tubule.
Urea	Partial reabsorption in proximal tubules, secreted in the loop of henle, and final reabsorption in the collecting duct. Urea is important for the regulation of osmolality.
Sodium	Reabsorption occurs throughout the kidney with majority (65%) occurring at the proximal tubule, 25% at the loop of henle, 5% in the distal tubules, and the remaining 5% in the collecting duct.
Chloride	Reabsorption process is similar to sodium.
Water	Aquaporin water channels allow osmosis to occur as water follows solutes in reabsorption at the proximal tubules, loop of henle, and collecting duct.
Bicarbonate	Major reabsorption occurs at proximal tubule with remainder of the reabsorption occurring at the loop of henle and collecting duct. Bicarbonate acts as the buffer system in the blood and helps maintain acid-base balance.
Protons	Secreted by the collecting duct by a proton pump to form a proton gradient.
Potassium	Reabsorption occurs in the proximal tubule and loop of henle. Collecting duct can also cause reabsorption or secretion depending on dietary needs.
Calcium	Most reabsorption occurs in proximal tubules, loop of henle, and distal tubules.
Magnesium	Most reabsorption occurs in proximal tubules, loop of henle, and distal tubules, but competes with calcium.
Phosphate	Majority of reabsorption (85%) occurs in proximal tubules.
Carboxylate	Complete reabsorption in the proximal tubules by carboxylate transporters.

Note: This table lists the known substances being regulated in the kidney.

In addition to maintaining extracellular homeostasis, the kidney also produces and secretes hormones. Erythopoietin is necessary for the regulation of red blood cell production in the bone marrow. Prostaglandins are also produced as well as calcitriol that is essential for the production of vitamin D. Of particular importance in regulating blood pressure is the secretion of renin as it is involved with the renin-angiotensin-aldosterone system. The renin-angiotensin-aldosterone system contributes to the regulation of fluid balance while also regulating blood pressure through specialized cells in the kidneys called the juxtaglomerular cells. Juxtaglomerular cells convert prorenin present in the blood into renin that then secretes directly into the blood circulation. The renin is responsible for converting angiotensinogen produced in the liver to angiotensin I, which is then subsequently converted to angiotensin II by angiotensin-converting enzyme in the lungs. Angiotensin II is a potent vasoconstrictor that narrows blood and in doing so, increases the blood pressure. Furthermore, Angiotensin II stimulates the secretion of aldosterone from the adrenal cortex, which then acts back on the renal tubules to increase reabsorption of water and sodium while excreting potassium to increase the volume of extracellular fluid in the body, thereby increasing blood pressure.

Acute Kidney Injury

Given the multiple essential functions of the kidney, it is clear that damage to the organ can cause severe consequences. Acute kidney injury (AKI) is an abrupt decrease in kidney function, precisely defined as within 48 hours, based on serum creatinine and

other specific laboratory and clinical values used to guide diagnosis (Mehta *et al.*, 2007). AKI can be caused by a variety of factors and in most cases, the causes are actually multi-factorial. Such factors involve numerous cell types within the kidney in addition to changes to systemic circulation. The first factor we will review is hemodynamic instability. Patients with septic shock often results in decreased urine along with decreased creatinine clearance, which is indicative of kidney damage (Martin *et al.*, 1990).

Another factor that can cause AKI is the cessation of blood flow to the kidney followed by restoration and reoxygenation of tubular cells- a process referred to as ischemic reperfusion injury (IRI). IRI can be caused by infarction, sepsis, and organ transplantation, causing oxidative stress among other cellular insult including inflammatory responses of reactive oxygen species (ROS), cytokines, chemokines, and leukocyte activation that further exacerbate tissue damage (Sharfuddin *et al.*, 2011). The diminished renal physiological function results in poor prognosis with high morbidity and mortality, with mortality rates commonly exceeding 50% in patients admitted to the intensive care unit. Furthermore, survivors of AKI are consequently at a significantly increased risk of developing chronic kidney disease (CKD) and ultimately progress to end-stage renal disease (ESRD) (Chertow et al., 2005) (Coca et al., 2012). The severity of CKD and ESRD calls for renal replacement therapies including hemodialysis and kidney transplantation.

Current Treatment for Acute Kidney Injury

Following AKI, the kidney has an extensive innate repair mechanism in attempt to re-establish its tubular structure and maintain proper function. Lineage tracing experiments have demonstrated surviving epithelial cells within the kidney tubules are capable of reconstituting the entire epithelium post-IRI, suggesting the unlikeliness of extrarenal cells entering the tubule and differentiating into epithelial cells during the repair process (Humphreys et al., 2008). However, this process remains poorly understood as the complexity and extent of renal damage extends beyond epithelial cells and include the damage to renal vasculature. In this context, recent evidence suggest extrarenal endothelial cells are capable of infiltrating the renal vasculature and partially regenerate the vascular structure to help mediate the repair process.

In response to the ongoing concern of AKI, current research aims to better understand the pathophysiology of AKI to help ameliorate damage or prevent its onset following kidney insult. Recent studies have demonstrated benefits of various chemical compounds that target certain areas of the known pathophysiological pathway. Among such therapeutic compounds include doxycycline to reduce the level of pro-inflammatory cytokines, leptin to decrease tumor necrosis factor alpha that is involved in regulating immune cells, and the use of levosimendan and ascorbic acid as antioxidative agents to relieve oxidative stress (Kucuk et al., 2009). However, these compounds remain insufficient or inefficient in treating AKI and patients continue to be at risk for renal failure. Ongoing investigation into the kidney's molecular and cellular responses to AKI

reveal novel factors and pathways utilized by the kidney to ameliorate damage. Once such factors are better understood, they can then be manipulated to treat or prevent AKI.

Translating Ribosome Affinity Purification

The kidney can be broken down to various types of tissues with different cell types. This cellular diversity in an organ creates a challenge when attempting to profile mRNA expression for each cell differs in gene expression. Prior methods include microdissections or cell sorting to isolate specific cell populations for mRNA profiling. However, such methods require steps that may affect cellular integrity, including the need to fixate the tissues or inducing cellular stress with single-cell suspensions. To overcome this challenge, researchers at Rockefeller University established a method to profile translated mRNA in a specific cell population. This methodology, called translating ribosome affinity purification (TRAP), enables researcher to break down the cellular diversity by combining cell type-specific expression with affinity purification of translating ribosomes (Heiman et al., 2014).

The McMahon laboratory has applied this method toward profiling mRNA expression in various cell types within the kidney during acute kidney injury (Liu et al., 2014). L10a ribosomal protein subunit was tagged with EGFP under CRE recombinase-dependent activation. Thus, TRAP of mRNA populations can then be used on CRE-expressing cells. This mouse line is then combined with cell type-specific CRE-driver lines to specifically profile mRNA from cell types of interest. These mice were then

subjected to IRI and profiled in comparison to wild type. In doing so, we can identify cell-type specific changes in mRNA after acute kidney injury.

Tubulointerstitial Nephritis Antigen-like 1

Tubulointerstitial Nephritis Antigen-like 1 (TINAGL1), also known as tubulointerstitial nephritis antigen related protein (TIN-ag-RP), Lipocalin7 (LCN7), and adrenocortical zonation factor 1 (AZ-1), is a 52.4 kD protein located in the extracellular matrix with a 467-amino acid sequence. It was first discovered and identified from a search for novel cathepsin-related proteins in the interest of identifying thiol-dependent cathepsins, which are cysteine proteases essential to cellular processes including terminal protein breakdown in lysosomes, bone resorption, MHC-II class-mediated antigen presentation, and activation of zymogens (Bromme et al., 2000). Structurally, TINAGL1 consists of two major homology domains: a cysteine rich EGF-like domain and a cathepsin B-like domain that is proteolytically inactive due to a serine mutation of the critical cysteine residue in the highly conserved catalytic triad (cysteine, histidine, and asparagine) present in cathepsin B. TINAGL1 was found to be localized in vesicular compartments and secreted from the cell, initially suggesting its potential involvement in the endosomal trafficking pathway. It is also a member of the lipocalin family of small extracellular proteins, some of which are capable of binding to a range of small hydrophobic molecules, enabling the transport of insoluble ligands of major signaling pathways such as Wnt (Flower et al., 2000) (Ganfornina et al, 2000).

Recently, the *Drosophila melanogaster* ortholog of TINAGL1 known as Secreted Wg-interacting molecule (Swim) has been found to play an essential role in Wingless (Wg) signaling, which is orthologous to Wnt signaling in mammals (Mulligan et al., 2012). Swim binds to Wg in a lipid-dependent manner and with nanomolar affinity to facilitate long-range Wg diffusion through the extracellular matrix. This suggests that in mammalian systems, TINAGL1 could bind to Wnt proteins and facilitate its diffusion to act on Wnt receptors at a distance. Wnt proteins are hydrophobic proteins that are largely insoluble, thus its ability to act on cells at a distance remains unclear and can be elucidated by TINAGL1.

Previous studies have demonstrated TINAGL1 to be predominantly localized to vascular tissues of different organ types including vascular smooth muscle, lung capillary endothelium, vascular basement membranes of adrenocortical sinusoidal capillaries, and the glomerular basement membranes (Wex et al., 2001). Further studies in zebrafish suggest TINAGL1 is a positive regulator of angiogenesis that mediates endothelial cell adhesion and invasion, angiogenic cell sprouting, and more importantly it promotes TGF-β signaling (Brown et al., 2010). However, the physiological role and function of TINAGL1 remains largely unexplored in normal kidney function.

Tubulointerstitial Nephritis Antigen

TINAGL1 and Tubulointerstitial Nephritis Antigen (TINAG) are 46% identical in structure and therefore the function and characterization of TINAG is important to

determine potential functional redundancy. TINAG has been demonstrated to be a regulator of tubulogenesis during mammalian kidney development. Given that ischemic AKI primarily damages the tubular epithelium of the nephron, a protein regulating tubulogenesis could be of interest in repairing the kidney tubules (Bonventre and Yang, 2011). Furthermore, mutant mice lacking TINAG are more susceptible to cisplatin-induced injury with increased tubular cell damage. The tubular cell damage was partially reversed in cells that were maintained on TINAG substratum, demonstrating the importance of this extracellular matrix protein in tubular repair. Previous studies have also shown that treatment with TINAG antisense oligonucleotide and anti-TINAG antibody induced dysmorphogenesis of the embryonic metanephroi, malformation of the S-shaped body, and decrease in tubular population during kidney development (Kanwar et al., 1999).

Wnt Signaling Pathway

Wnt proteins are a family of secreted lipid-modified signaling proteins consisting of 19 members in both humans and mice, though Wnt expression differs between the two. In mammals, Wnt signaling appear to occur predominantly between cells that are close to each other rather than at a distance (Clevers et al., 2012). The Wnt signaling pathway has been identified to play important roles for cellular interactions during embryonic development, where it controls patterning, specification, proliferation, and migration (Gilbert, 2010).

The Wnt signaling pathway can be categorized into two distinct pathways; a canonical and non-canonical pathway depending on the involvement of β-catenin. In the non-canonical Wnt signaling pathway, Wnt can be involved with a calcium pathway or an alternative planar cell polarity pathway. In the interest and relevance of our research project, the focus will be on the canonical Wnt pathway involving β-catenin. In the canonical pathway, extracellular Wnt proteins bind to a heterodimeric receptor complex on the membrane consisting of a low-density lipoprotein-related protein-5 or -6 (LRP5/6) and a seven-transmembrane Frizzled (Fz) receptor. Under normal circumstances without Wnt interaction to the cell surface receptors, a "destruction complex" is present in the cytoplasm. This complex is composed of protein Axin interacting with the tumor suppressor protein APC, Dishevelled, casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). CK1 and GSK3 is able to phosphorylate β-catenin, followed by its ubiqutinatation by E3 ubiqutin ligase, which ultimately results in proteasomal degradation of β-catenin.

When Wnt proteins bind with LRP5/6 and Fz receptors on the cell surface, LRP5/6 is phosphorylated by Axin that has been recruited to the cytoplasmic tail of the heterodimeric receptor, enabling LRP5/6 to interact with the destruction complex. This causes a conformational change that leads to the destabilization of Axin, resulting in the dissociation of the destruction complex and thus preventing the phosphorylation and degradation of β-catenin. Without the degradation of β-catenin, it accumulates in the cytoplasm and consequently translocate to the nucleus where it can bind to and activate T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors, causing the induction of target genes. β-catenin can also act beyond TCF/LEF and

become involved with other signaling pathways in different cell types, such as the phosphoinositide 3-kinase (PI3K) pathway in epithelial cells (Pece et al., 1999).

Wnt Signaling in Kidney Injury and Repair

In kidney injury and repair, there are evidence indicating the importance of Wnt in mitigating the injury process and maintaining proper repair (Kawakami et al., 2013). The importance of Wnt signaling pathway in the kidney was first observed during kidney development, where Wnt9b and Wnt4 activated induction signals that stimulate uncommitted mesenchymal cells to differentiate into epithelial cells that form the nephrons of the kidney (Schmidt-Ott et al, 2008). In an uninjured kidney, the Wnt pathway is active in cells of the papillary region of the kidney where there is a constant cell stress and relatively high cell turnover due to low oxygen tension (Lin et al., 2010). Upon AKI, the Wnt pathway becomes widely expressed. When mice with reduced numbers of Wnt co-receptors LRP5 and LRP6 are subjected to IRI, the severity of AKI becomes more extensive and repair is hindered with the formation of cysts in the tubules. Frizzled 4 (Fz4) is a major receptor in the kidney epithelium for Wnt and upon its deletion, a similar effect to decreased LRP5/6 is observed, supporting the importance of Wnt signaling in AKI.

One Wnt of particular importance in AKI is Wnt4, where it has been found to be expressed in proximal tubules in rat kidney after IRI (Terada et al., 2003). Its expression pattern is related to the expression patterns of cell cycle progression markers such as

cyclin D1 and A, suggesting Wnt signals may contribute to the proliferation of renal tubule cells recovering from injury. Additionally, the Wnt pathway results in the accumulation of β-catenin and specific ablation of β-catenin in renal epithelial aggravates AKI (Zhou et al., 2012). There are several studies that suggests Wnt may be deleterious to the kidney and aggravates or even initiate the injury process. Among such studies include the role of Wnt signaling in promoting renal interstitial fibrosis, promoting podocyte dysfunction, albuminuria, and proteinuria (He *et al.*, 2009, Dai *et al.*, 2009, and He *et al.*, 2011). Given these mixed studies of whether Wnt is beneficial or deleterious to the kidney in the event of AKI, further studies into its role and involvement is necessary. Therefore, the investigation into Tinagl1 will serve as a useful tool to help elucidate the effects of Wnt on AKI.

Chapter II

Materials and Methods

This section outlines the experimental methods and techniques used to investigate the effects of Tinagl1 on acute kidney injury induced in mice. Briefly, this includes the use of translating ribosome affinity purification for the discovery of Tinagl1 and its relationship to AKI, the design and production of the Tinagl1 knockout mice, and the surgeries that were performed. All experiments involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Southern California Keck School of Medicine.

Translating Ribosome Affinity Purification

Mice were generated with *Rosa26-EGFP-L10a* allele using recombination-mediated cassette exchange in embryonic stem (ES) cells of C57BL6/J; 129/Sv mice (Figure 1) (Tsanov et al., 2012). A *Rosa26* vector containing F3- and FRT-flanked ATG-F3-Puro-pA cassette with a PGK promoter that drives the expression of FLPo recombinase was used to target F1 cells and generate a parental ES clone. A neomycin resistance gene (Neo) without the ATG start codon was inserted by a CAGGS promoter and a loxP-flanked triple SV40 polyA signal along with EGFP-L10a cassette. To block

influences on the promoter activity, three copies of the 400-bp region of β-globin insulator from chicken were inserted upstream of the promoter. The successful combination of the cassette (CAGGS-loxP-2xpA-loxP-EGFP-L10a-pA) followed by Neo expression from the endogenous Rosa 26 promoter was confirmed by PCR and expression examined by immunostaining for GFP. Four cell type-specific *Cre* lines were used to drive EGFP-L10a expression: *Six2-L10a* (renal tubules), *Foxd1-L10a* (interstitial cells), *Cdh5-L10a* (endothelial cells), and *Lyz2-L10a* (macrophages).

Twenty-four hours after ischemic reperfusion injury surgery (described in more detail in a subsequent section), mice were anesthetized followed by perfusion with pre-chilled phosphate-buffered saline (PBS). Kidneys were then removed and rapidly minced on ice following 4 ml of chilled polysome extraction buffer for homogenization. Anti-GFP antibody (monoclonal, from Memorial Sloan-Kettering Cancer Center, New York, New York, USA) were coupled to Dynabeads and 200 µl was then added to approximately 1 ml of kidney extract. After 30 minutes of incubation at 4ºC with agitation, beads were washed with wash buffer and resuspended in lysis buffer followed by RNA isolation. DNaseI digestion (QIAGEN RNAeasy Mini Kit) was used to remove contaminating DNA. 200 ng of purified RNA was then used to generate cDNA for microarray analysis.

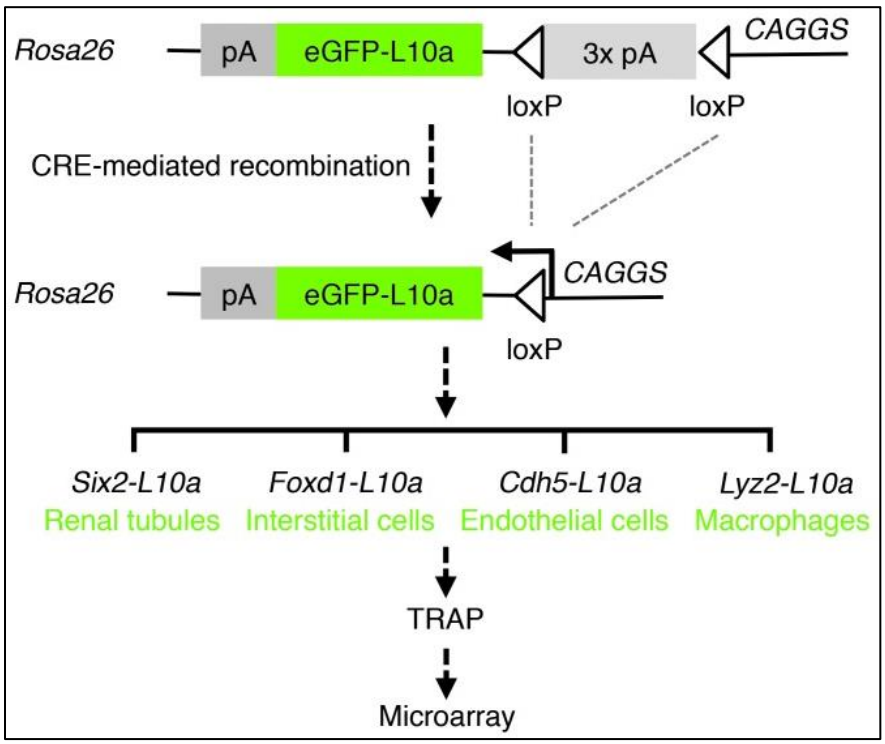

Figure I. Translating Ribosome Affinity Purification. TRAP analysis for various kidney cell types (*Liu et al.*). *Rosa26-EGFP-L10a* allele with a CAGGS promoter expressed with cell-specific *Cre* lines to obtain mRNA from certain cell types of interest.

Ischemic Reperfusion Injury (IRI) Surgery

To induce ischemic acute renal failure, 6 to 12-week old male mice were used and protocols for survival surgery were approved by IACUC. Mice were anesthetized with intraperitoneal injection of 105 mg ketamine/kg and 10 mg xylazine/kg. Body temperature was closely monitored and maintained at 37ºC throughout the procedure. Sterile equipment was used to make flank incisions to expose the kidneys followed by

clamping of renal pedicles for 28 minutes using microaneurysm clips (Roboz Surgical Instrument Co.). Restoration of blood flow and coloration of the kidney were carefully monitored following the removal of microanuerysm clips. Mice that are sham-operated followed the same procedure minus clamping of the renal pedicles. All animals were then closely monitored for four hours post-surgery for proper recovery and carefully evaluated for signs of lethargy or moribund.

Generating Tinagl1 Knockout Mice

Specific embryonic stem cell gene targeting of Tinagl1 and the generation of chimeric knockout mice was done by the Genome Modification Facility at Harvard University. Briefly, the Tinagl1 gene was knocked out through homologous recombination of a knockout-first allele (Figure 2), followed by electroporation of ES cells, drug-selection, colony picking, and expansion of the clones for transplantation into a surrogate mother to produce chimeric progenies. The chimeric mice were then sent to the housing facilities at USC, where they were carefully observed under quarantine by animal technicians and veterinarians according to protocol. After clearance of quarantine, the Tinagl1 knockout chimeric mice was mated to wild type C57BL/6 mice to determine germline transmission and produce heterozygous progenies carrying both the Tinagl1 knockout allele and wildtype allele. The heterozygous male and female progenies were then mated together to produce homozygous knockout mice.

To confirm the heterozygosity and homozygosity of the mice, genotyping was performed with a PCR assay. Primers were designed to flank a 34-base pair synthetic loxP site on the allele specific to the knockout mice on the tm1a background (Figure 2). Gel electrophoresis was performed following PCR amplification of the synthetic loxP site. In mice with a knockout allele, a size fragment of 34 bp larger than a wild type band was observed at the same region without the synthetic loxP site. Furthermore, RNA was isolated from the kidneys of the knockout, heterozygous, and wildtype mice followed by RNA sequencing performed at the University of Southern California's Epigenome Center.

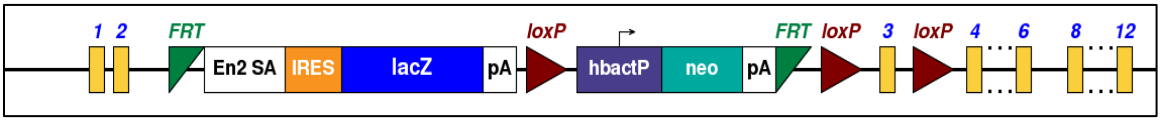

Figure 2. Schematic representation of knockout-first allele. LacZ gene is expressed followed by a poly-A, creating knockout/reporter mice. Essential exon of Tinagl1 is flanked with synthetic loxP sites to make a conditional knockout after FLP-FRT recombination if there is embryonic lethality.

Immunofluorescence Imaging and Analysis

Mice that underwent IRI surgery in addition to sham controlled mice were anesthetized and intra-cardiac perfusion was done with chilled 4% paraformaldehyde. Kidneys were then prepared for cryosectioning by being embedded in Optical Cutting Temperature (OCT) compound and frozen into cryoblocks using 100% ethanol in dry ice. 6 μm cryosections were made on glass slides and washed in 0.1% PBST, blocked in 5% donkey serum, and incubated overnight with primary antibodies specific for kidney injury molecule-1 (KIM1) at 1:1000 dilution (goat, R&D Systems #AF1817) or fluorescein labeled Lotus Tetragonolobus Lectin (LTL, Vector Labs #FL-1321) at 4ºC. KIM1 is an established biomarker for kidney injury and LTL is a marker for the renal proximal tubule where kidney injury has been shown to occur. Following overnight incubation, sections were then incubated with a fluorescently labeled Alexa Fluor 555 secondary antibody against goat (Invitrogen) at room temperature for one hour. Sections were then stained with Hoechst 33342 (Invitrogen) to stain nuclei and mounted with Immu-Mount (Fisher Scientific). Images were acquired using confocal microscopy (Carl Zeiss LSM 780).

Luciferase Reporter for Wnt Signaling

To determine the contributions of Wnt signaling pathway to the actions of Tinagl1, an *in vitro* assay will be used to assess Wnt activity. Super 8x TOPFlash plasmid (M50 Super 8x TOPFlash, plasmid #12456, addgene) were used as a beta-catenin reporter to serve as a surrogate method of quantifying the extent of Wnt signaling, which is upstream of beta-catenin and TCF/LEF in the signaling pathway (Figure 3). In brief, the TCF/LEF sites are upstream of a luciferase reporter so upon activation of TCF/LEF by beta-catenin, luciferase will also be activated. M51 Super 8x FOPFlash is used as a control with a mutated TCF/LEF binding site (Figure 4). Plasmids were then transfected into HEK293T cells with FuGENE 6 transfection reagent (Promega) to successfully culture cells that are capable of determining the extent of Wnt signaling. Transfection is optimized using 2 μg of DNA per well at various ratios of FuGENE 6 transfection reagent to DNA from 3 μL, 6 μL, 8 μL, and 12 μL. Once added, regents were gently mixed and the FuGENE 6 Transfection Reagent with DNA complex is incubated at room temperature for 15 minutes. Cells were cultured with Dulbecco's modified eagle medium (DMEM, ThermoFisher, #11320082) supplemented with glutamine and penicillin/streptomycin. In a 96 well plate, 100 μL of cells in the growth medium were plated and 5 μL of the FuGENE 6 transfection reagent/DNA complex was added. Gentle mixing by pipetting was done for 10-30 seconds and placed back into the incubator for 24 hours. Transfection efficiency was then assayed using the reporter system.

A Myc-DDK-tagged mouse Tinagl1 clone (Origene, #MR219690) was transfected into HEK293T cells and cell media was collected after 24 hours of incubation with the premise that Tinagl1 is a secreted protein and will be present in the media. The collected media was then serially diluted in fresh media and administered in equal amounts to Super TOPFlash HEK cells. A constant dose of 100 ng of Wnt3a, a widely used activator of the beta-catenin Wnt signaling pathway, were then administered to the cells and luciferase expression was measured 4 hours later.

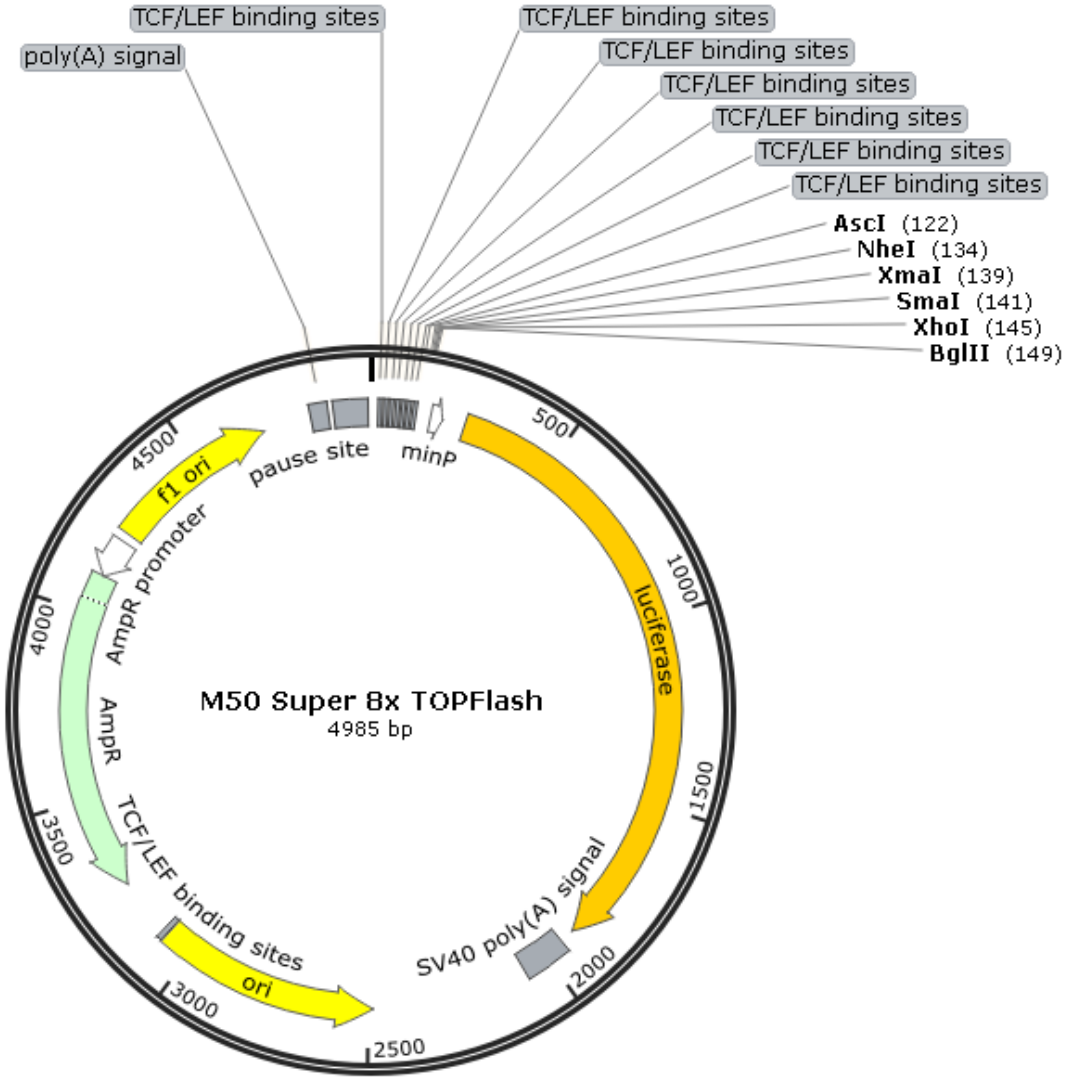

Figure 3. Schematic representation of M50 Super 8x TOPFlash plasmid. This plasmid will be used as a reporter for *in vitro* assay for Wnt signaling.

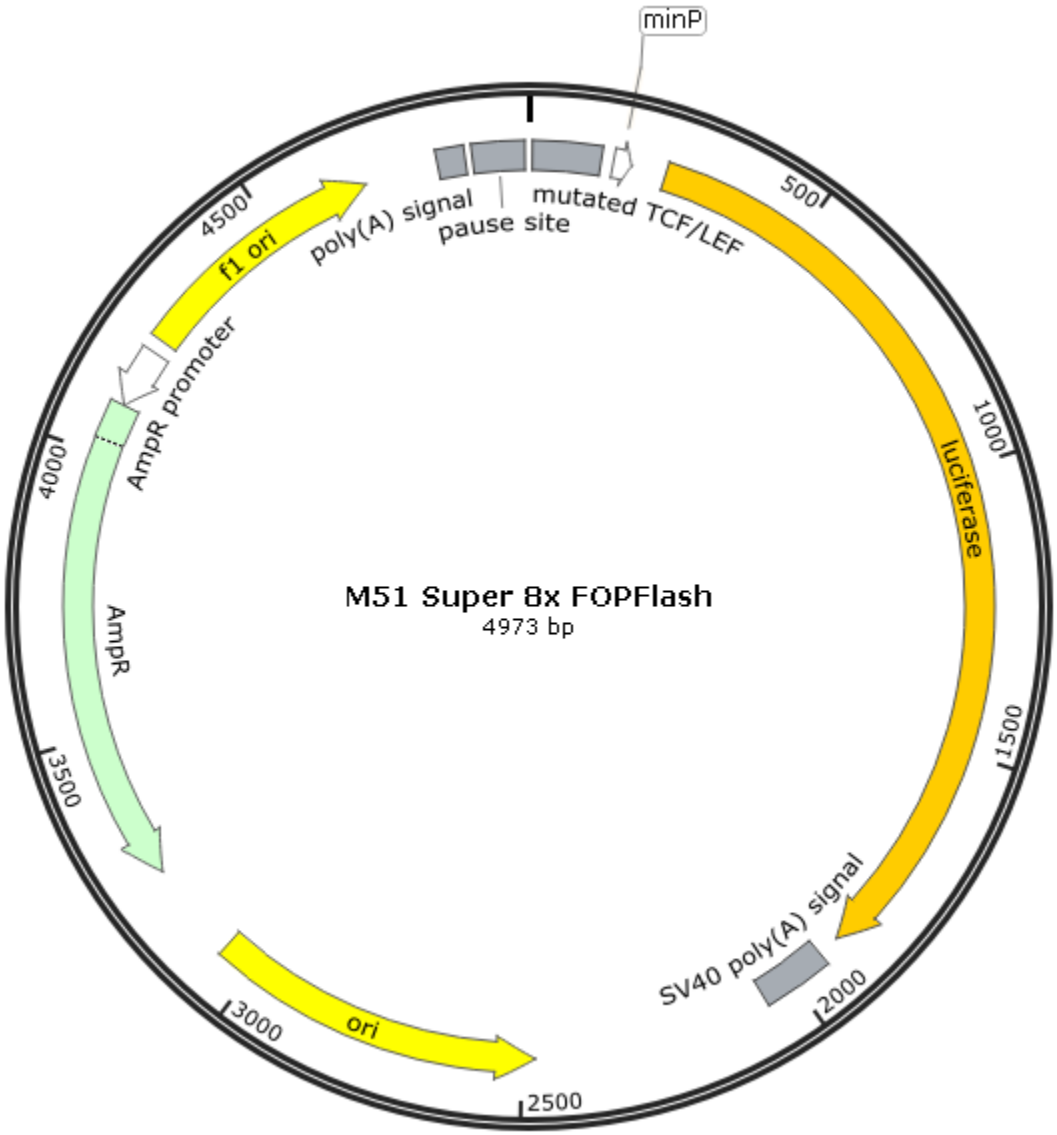

Figure 4. Schematic representation of M51 Super 8x FOPFlash plasmid. This plasmid has mutated TCF/LEF binding sites to serve as a negative control for in vitro assays of Wnt signaling.

Chapter III

Results

This section will describe the findings of the experiment, including the discovery of Tinagl1 from TRAP, successfully generating homozygous knockout of Tinagl1 in mice, inducing acute kidney injury, immunostaining for kidney injury, and results from the *in vitro* Wnt signaling assay.

Translating Ribosome Affinity Purification

A mouse line that enables the identification of translational profiles in specific cell types through TRAP was successfully generated by the McMahon lab. EGFP-L10a mouse line was generated and crossed to four different Cre strains that enable the expression of EGFP-L10a in four cellular components of the kidney with no major overlaps among the compartments. Tissue-specific expression of EGFP-L10a was verified upon Cre activation. Six2-Tet-GFP-Cre was exclusive to nephron progenitors (Kobayashi *et al.*, 2008). Foxd1-GFP-Cre was specific to interstitial cell lineages including mesangial and nonglomerular pericyes (Humphreys et al., 2010). Cdh50-Cre is in the vascular endothelium (Alva et al., 2006). Lyz2-Cre specifically labeled cells of the

myeloid lineage, particularly monocytes, macrophages, granulocytes, and dendritic cells (Clausen et al., 1999).

Following the crosses of EGFP-L10a to the four different Cre strains, TRAP RNA was prepared from the kidneys of 6 to 12-week old male mice in each line and analyzed by microarray. The number of cells labeled was proportional to the TRAP RNA yield: 10 ng/kidney for non-transgenic mice as a control, 30 ng/kidney for L10a kidneys in absence of CRE as a second control, 4 μg for Six2-L10a, 1.5 μg for Foxd1-L10a, 250 ng for Cdh5-L10a, and 200 ng for Lyz2-L10a. It was observed that TRAP RNA from L10a kidneys without CRE had approximately 3-fold greater yield compared to non-transgenic controls, suggesting a potential CRE-independent background activity of L10a.

From the TRAP RNA, 1,545 genes specific to the nephron were identified with 555 genes specific to interstitial cells, 384 specific to endothelial cells, 907 specific to myeloid lineages. To determine the specificity of these genes to its cellular compartments, Gene Ontology (GO) enrichment analysis was done and a clear relationship was established to match cell populations within each TRAP RNA sample; the nephron matched with transport function, interstitial cells matched with cell adhesion, endothelial cells matched with vasculature, and macrophage-enriched myeloid populations matched with the immune response. This provides us with the conclusion that the TRAP RNA from the mouse models created are able to accurately and robustly identify cell-specific RNA signatures, which can now be applied in the context of kidney injury.

Surgical Renal Ischemic Reperfusion Injury for TRAP Analysis

Mice generated for trap were each successfully operated on to induce renal ischemic reperfusion injury. A 28-minute bilateral clamping of the renal vasculature at 6 to 12-week old males significantly increased serum creatinine levels 24 hours after the surgery as expected, which indicated successful induction of acute kidney injury. Animals operated under sham conditions did not show similar signs of increased serum creatinine and also had absent signs of AKI compared to their renal IRI-induced counterparts. Signs of AKI include renal tubular necrosis, sloughing of cells in the outer medullary region, and evidence of casts within the tubules. Furthermore, diagnostic markers of AKI (Kim-1 and NGAL) were evaluated with RNA in situ hybridization analysis that confirmed IRI in the experimental group and absent from sham-surgery controls. It is also important to note that cellular pattern of Cre-L10a expression was not altered by IRI.

Following IRI surgery to induce AKI and obtaining microarray data, principle component analysis (PCA) was performed to determine similarities and differences in gene expression from the microarray. PCA was applied to data from mice without surgery, sham-operated, and IRI induced mice for all four Cre-L10a TRAP data. Biological replicates were clustered indicating a high degree of similarity. Additionally, clustering of from the two control groups were distinct from the IRI data.

TRAP Reveals Changes in Tinagl1 After Kidney Injury

From the TRAP data, Tinagl1 was identified as a gene of interest in the role of kidney injury and/or repair. mRNA transcripts of Tinagl1 were significantly increased in all kidney cell types that were measured 24 hours after acute ischemic reperfusion injury (Figure 5). Endothelial cells (indicated by *Cdh5*) had the greatest expression of Tinagl1 in both controls and after IRI. However, the most significant change was found in renal tubular cells (indicated by *Six2*) with nearly a 3-fold increase. Other cell types show nearly a 2-fold increase after acute IRI.

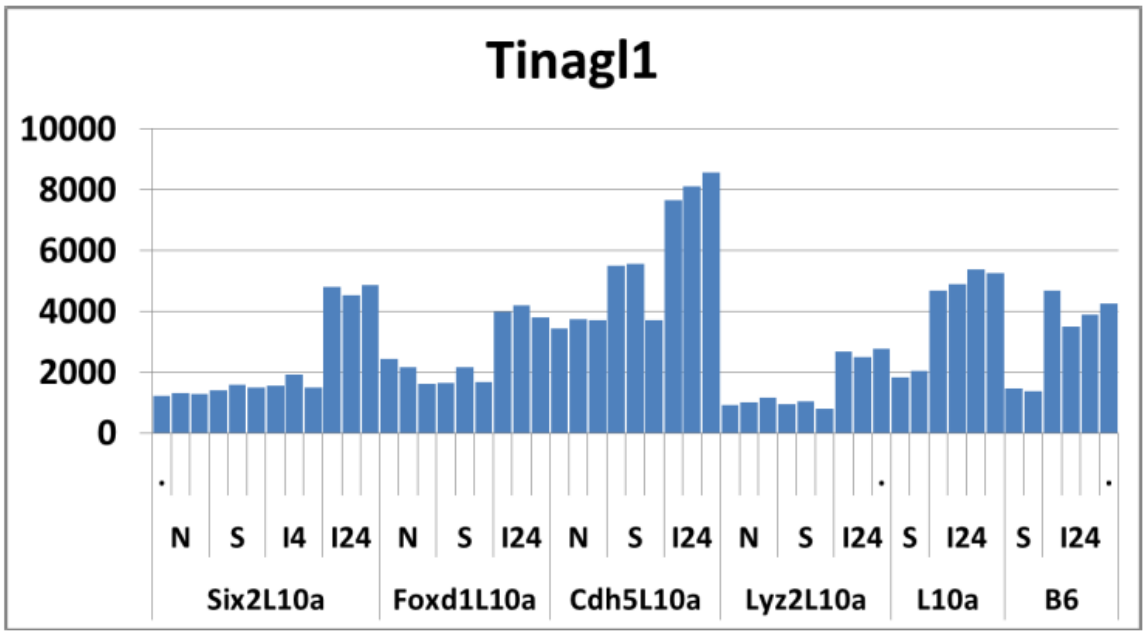

Figure 5. Tinagl1 expression in various cell types after IRI. TRAP of renal tubular cells (Six2), interstitial cells (Foxd1), endothelial cells (Cdh5), and macrophages (Lyz2) 24 hours after IRI compared to sham.

Minimal change in Tinagl1 mRNA transcript compared to sham-operated mice were observed two and four hours after acute IRI (Figure 6). However, a significant increase in Tinagl1 occurred 24 hours following acute ischemic reperfusion injury in mice compared to sham controls. Tinagl1 mRNA steadily decreases starting at 48-hours until it reaches back to normal levels 28 days after IRI.

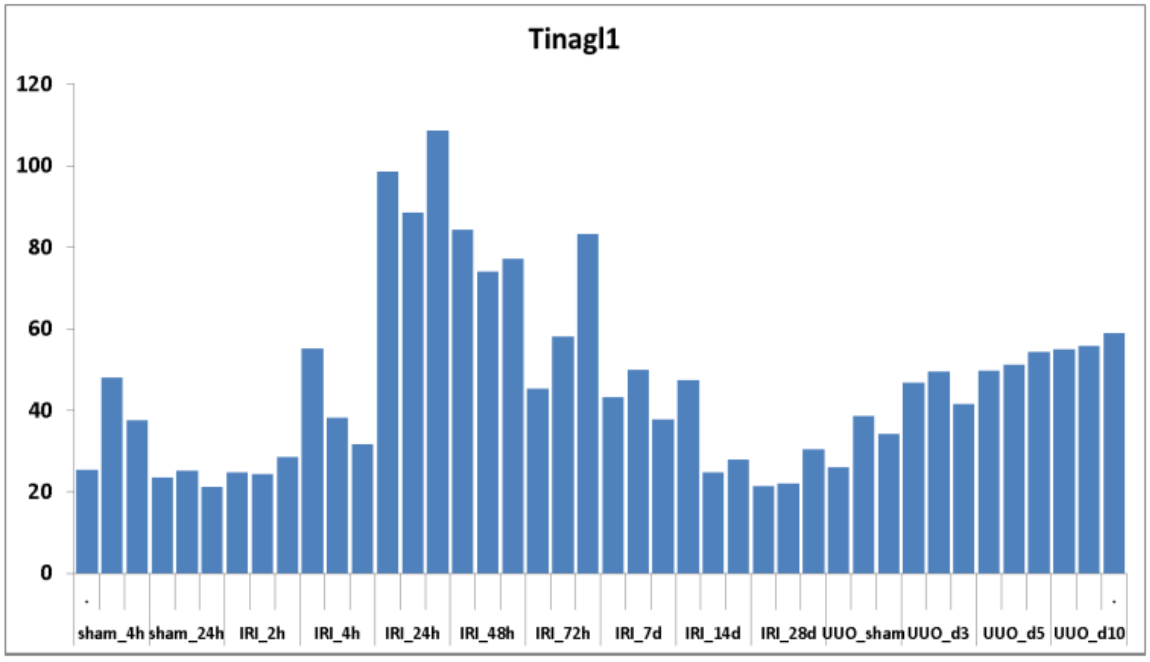

Figure 6. Tinagl1 expression over time after IRI and UUO. TRAP performed on mice with acute IRI and followed at various timepoints (n=3 for each timepoint). Sham and unilateral ureteral obstruction (UUO) were done for comparison to IRI.

Generating Tinagl1 Knockout Mice

The knockout-first allele (refer to Figure 2) was successfully inserted by the Genome Modification Facility at Harvard University. Briefly, electroporation of ES cells followed by drug-selection to select colony, which were then expanded for transplantation into a surrogate mother to produce chimeric progenies. These chimeric founder mice were then sent to the University of Southern California where it underwent quarantine and monitored by animal technicians and veterinarians according to protocol. Following clearance of quarantine, the chimeric founders were crossed with wild type C57BL/6 mice to generate heterozygotes. Genotyping was performed by PCR amplification with primers flanking the loxP sequence, thereby providing a larger band in mice with the loxP allele. Results provided the bands of expected size and also validated germline transmission of the knockout-first allele (Figure 7). Male and female heterozygote mice were then mated with each other to produce wild type, heterozygous, and homozygous knockouts at close to the mendelian ratio of 1:2:1, respectively. Importantly, homozygous knockout of the allele was shown to be not embryonic lethal and mice are viable with no noticeable difference in survivability. The appearance of homozygous knockout mice for Tinagl1 were also indistinguishable from wild type littermates. To validate the knockout, RNA sequencing was done on kidney tissue from these mice and results confirmed the absence of Tinagl1 RNA.

Figure 7. Genotyping of Tinagl1 Knockout. Heterozygous x heterozygous matings were genotyped. Lanes with two bands are indicative of a mouse with heterozygous expression of the allele. Top band indicates wild type expression of the allele in the mouse whereas bottom band indicates homozygous expression of the allele.

Tinagl1 Knockout Exacerbates Proximal Tubular Damage after AKI

Tinagl1 knockout mice and wild type littermates were subjected to acute IRI surgery as previously described. After 48 hours post-surgery, kidneys from the mice were harvested and 6 μm frozen sections were cut on its sagittal plane onto microscope slides using the cryostat. Frozen sections were maintained at -80ºC until staining. Optical cutting temperature medium was removed from the slides by washing with phosphate buffered saline (PBS) and blocking solution was placed for one hour followed by applying the primary antibodies overnight. Kidney Injury Molecule-1 (KIM-1) was used as a biomarker to assess the extent of renal tubular damage and co-stained with lotus tetragonolobus lectin (LTL) to indicate proximal tubules. Secondary antibody was applied after overnight incubation and images were acquired. In wild type mice, kidney damage was localized to the outer cortex with some tubules injured, but majority of tubules were undamaged based on the pattern of KIM1 staining (Figure 8). These results were expected and consistent with the pattern of tubular damage following IRI surgery. In Tinagl1 knockout mice, the extent of tubular damage in the cortex was extensive. KIM1 is expressed in nearly all proximal tubules in Tinagl1 KO mice (Figure 9).

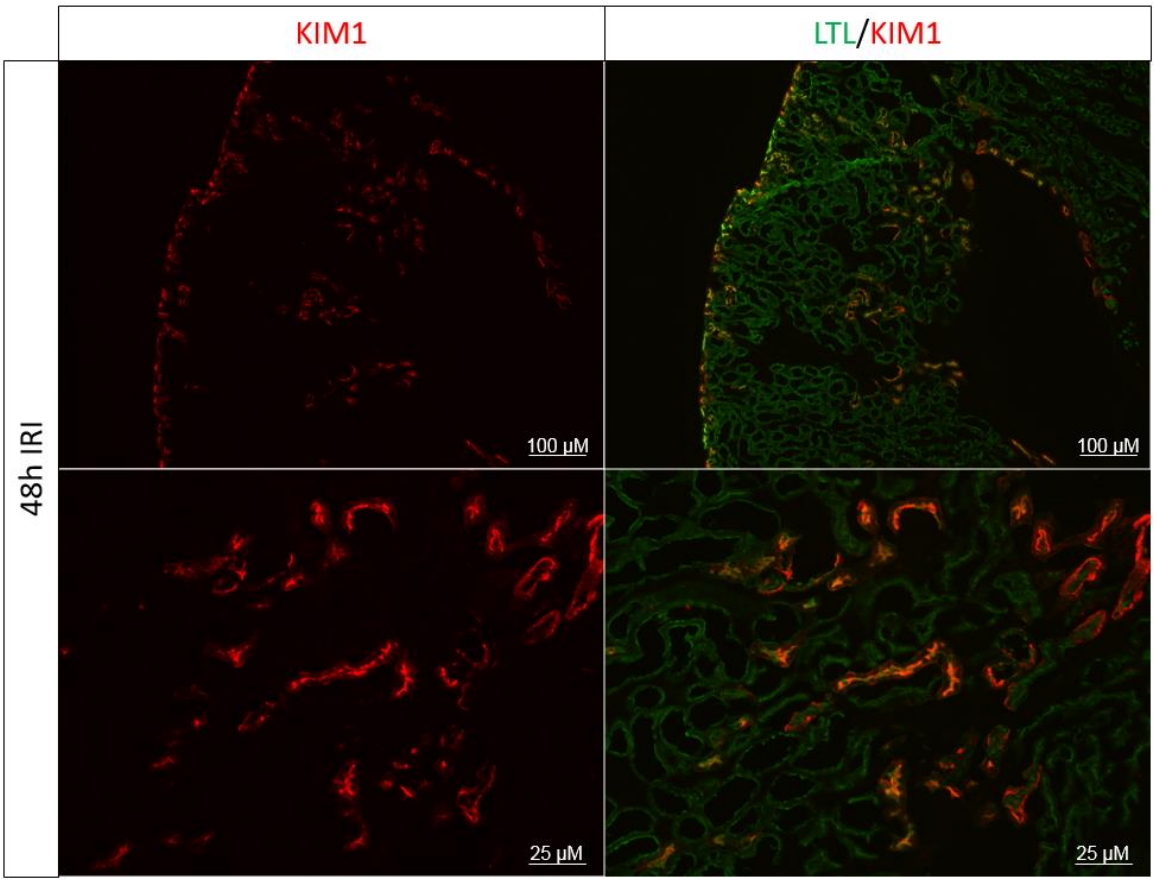

Figure 8. Immunofluorescent imaging of wild type mice 48 hours after acute IRI surgery. Some staining of KIM1 is noticeable in the outer cortex, indicating some proximal tubular damage.

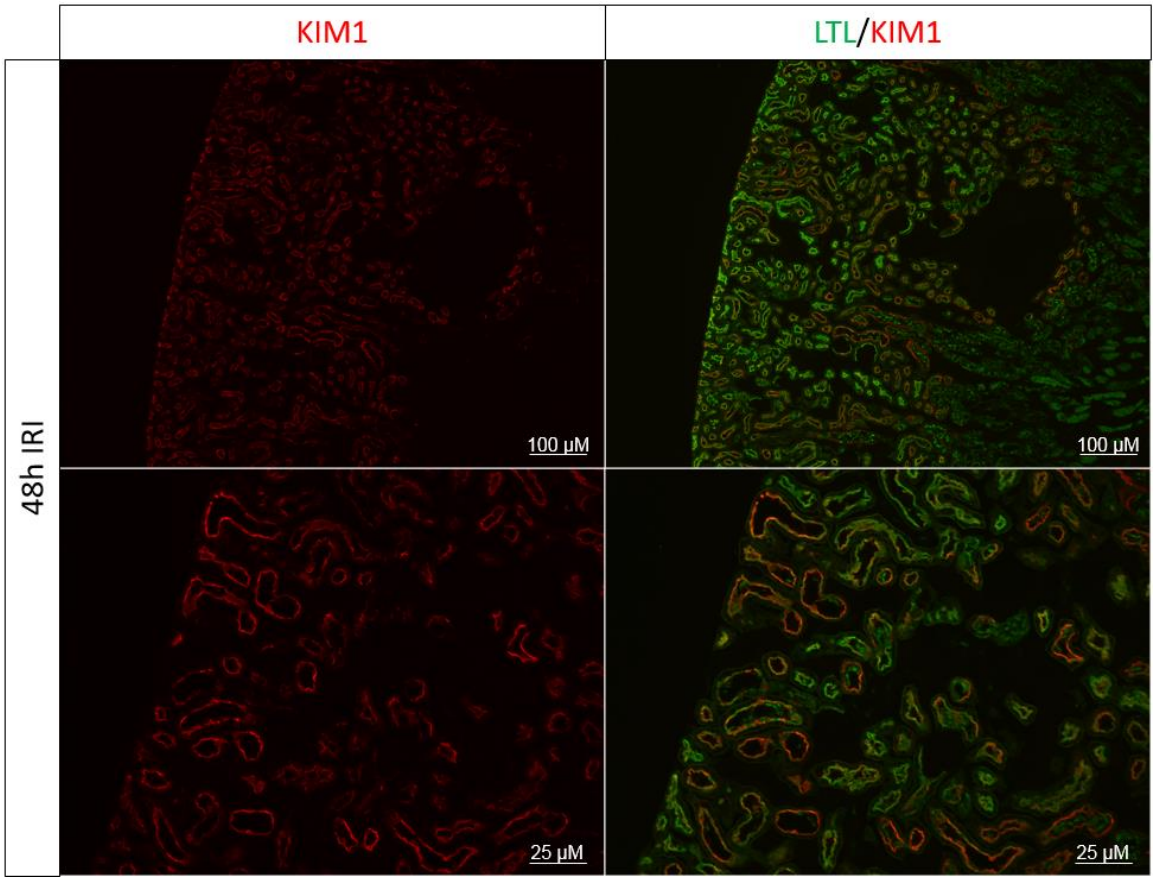

Figure 9. Immunofluorescent imaging of Tinagl1 KO mice 48 hours after acute IRI surgery. Expression of KIM1 was expressed throughout the outer cortex of the kidney, indicating widespread and extensive damage to proximal tubules.

Tinagl1 Promotes Wnt Signaling

Tinagl1 clone (Origene) was successfully transfected into HEK293T cells using FuGene 6 transfection reagents (Promega) and incubated for 24 hours. Cell media was then collected. Super 8x TOPFlash was also successfully transfected into HEK293T cells to serve as a Wnt reporter. Cell media from Tinagl1 transfected cells were serially diluted and administered to HEK293T cells transfected with Super 8x TOPFlash. 100 ng of Wnt3a was then added into each mixture and incubated for four hours. Analysis of luciferase expression showed drastic changes to Wnt activity corresponding to amount of Tinagl1 applied to the reporter cells (Table 1). Media collected from mock-transfected HEK293T cells that were applied to SuperTOPFlash cells were used as a transfection control and reported luciferase expression of 507.94. With relative luciferase activity expressed in ratio to control, 507.94 is set to a ratio of 1. A secondary control with just fresh media had similar amounts as the transfection control with a relative luciferase activity of 0.87. When undiluted media from Tinagl1-transfected HEK293 cells were applied to SuperTOPFlash cells, a substantial 1630-fold increase in Wnt activity is shown through the luciferase reporter. At a 1:1 dilution, the relative luciferase activity is still 120-times greater than the control. Even at 1:4 and 1:8 dilutions, the relative luciferase activity was still shown to be substantially increased at 8.2x and 3.3x, respectively. This dose dependence of Tinagl1 on the luciferase activity is a strong indicator that Tinagl1 influences Wnt activity.

Table 2. Relative Luciferase activity from Tinagl1 on Wnt Activity.

	Control	**Undiluted**	**1:1**	**1:4**	**1:8**	**Fresh Media**
Relative Luciferase activity (ratio to control)	1	1630.4	120.9	8.2	3.3	0.87

Note: Results of luciferase reporter in SuperTOPFlash cells with Tinagl1 administered into the media.

Chapter IV

Discussion

The aim of the study was to investigate Tinagl1 in the context of acute kidney injury. TRAP analysis has demonstrated that the expression of Tinagl1 is significantly increased following IRI surgery, an established method of inducing acute kidney injury in mice. The significant increase found in various cell types of the kidney suggests the possibility that Tinagl1 has an important role in restoring kidney structure and/or function following kidney injury. It is interesting to note that the greatest change in Tinagl1 occurs in the renal tubules, where cellular damage from acute kidney injury is most prominent. However, the most abundant expression of Tinagl1 is found in the endothelial cells, suggesting it plays a significant role in angiogenesis and restoration of renal vasculature. This is consistent with previous studies that show Tinagl1 is predominantly expressed in vascular smooth muscle cells and may be contributing to angiogenesis (Brown *et al.*, 2010). The relationship of Tinagl1 to the renal vasculature plays an important role in understanding microvasculature renal blood flow, which may correlate with glomerular perfusion due to changes that may occur on the afferent and efferent arterioles (Abuelo, 2007). Injury from various states of diseases are prevented by an adequate amount of macrovascular renal perfusion pressure (Bagshaw *et al*., 2008, Sun *et al.*, 2015, and Walsh *et al.*, 2013). Beyond the microvasculature, the microvascular tone of the afferent and efferent arterioles can change and be manipulated in diseased states. A

tubuloglomerular feedback from the juxtaglomerular apparatus to the afferent arteriolar tone exists through several molecules including angiotensin II, which also affects efferent tone (Schnermann, 2015). Consequentially, glomerular perfusion pressure can be negatively affected as a result of changes to the vasculature that ultimately leads to kidney injury (Whiting et al., 2017 and Schlondorff, 1993). Given the potential role and effects of Tinagl1 on the renal vasculature, it can play an important role in a variety of diseases as well as normal physiology.

However, it is important to note that renal perfusion is important for the mechanism of kidney injury, but injury can also occur without hypotension with glomerular hypoperfusion leading to tubular damage (Sutton *et al.*, 2002). Similarly, the tubular damage can also be a result of oxidative stress and inflammation with proposed mechanisms for its pathophysiology (Wang et al., 2012 and Ko et al., 2011). An example is the increased adherence of leukocytes to transmigrate toward the renal interstitium due to the upregulation of adhesion molecules from activated endothelial cells. The leukocytes that leave the peritubular capillaries are near the tubular epithelial cells and can be a direct cause of tubular cell injury (Verma *et al.*, 2015). The results from our TRAP data supports the role of Tinagl1 in macrophages, but further work needs to be done to investigate whether there are changes to oxidative stress related to Tinagl1.

Evaluation of Tinagl1 expression following IRI shows minimal increase at two and four hours post-surgery. The increase occurs starting at 12 hours and peaks at nearly 24 hours. Due to this delayed response, we suspect that Tinagl1 was upregulated as part of the repair process and not a mere byproduct or a pathological outcome of acute kidney injury. Furthermore, the upregulation of Tinagl1 was also found in kidneys with induced

unilateral ureteral obstruction, a severe form of injury that induces significant pressure and damage throughout the kidney. This reduces the likelihood that the upregulation is due to the surgery itself or the hypoxic environment induced by IRI.

We have also successfully created mice with Tinagl1 knockout. These mice are not embryonic lethal and thus far, we have not noticed any premature deaths. If Tinagl1 does play a role in angiogenesis we can conclude that it is not an essential factor throughout the developmental stages and into adulthood. Further study needs to be done to closely examine any changes in development that may not be phenotypically apparent. The study of its developmental effects, if any, can shed insight on the topic of whether kidney repair and regeneration is a molecular recapitulation of developmental processes (Little *et al.*, 2017). If Tinagl1 is upregulated in repair but there are no signs of its significance in development, then it is likely that the protein is specific to kidney repair and suggest a distinct repair pathway than recapitulating developmental processes.

IRI surgeries done on Tinagl1 knockout mice shows a substantial difference in kidney damage compared to their wild type littermates. This provides further evidence of Tinagl1 playing an important role in mitigating kidney damage in the event of acute injury. However, the mechanism on how it is able to reduce the extent of kidney damage remains largely unknown. It is worthwhile to note that Tinagl1 is expressed in all cell types we looked at, including macrophages where a significant increase is also found after IRI. The role of Tinagl1 in macrophages is not yet known and could also be responsible for mitigating damage following kidney injury. Additional work and characterization needs to be done with our Tinagl1 knockout mice after IRI including the evaluation of differences in inflammation to help elucidate the role of Tinagl1 and

macrophages. Furthermore, better characterization of kidney damage including serum creatinine and urine analysis would provide additional information on whether Tinagl1 is truly capable of mitigating damage aside from what was found through immunofluorescence staining.

Of particular interest and relevance to our work is the question of whether Wnt signaling pathway is beneficial or deleterious to the kidney following injury. Experiments have provided evidence that Wnt signaling can significantly contribute to atrophy and fibrosis, but others have shown that the pathway can also foster regenerative processes in acute tubular injury. For example, Dickkopf3 (DKK3) has been demonstrated as an agonist for Wnt signaling in chronic kidney disease and fosters fibrotic inflammation in the tubulointerstitial compartment (Grone *et al.*, 2017). Atrophy of the tubular epithelium and interstitial fibrosis with chronic tubulointerstitial damage are prominent in chronic kidney disease and serves as a predictor for its progression.

There is evidence to believe Tinagl1 is able to potentiate Wnt signaling based on previous work done on its drosophila homolog. It has been suggested that Tinagl1 can bind to Wnt and help solubilize it to potentiate its effects over a greater area. Our results with SuperTOPFlash cells supports such possibility of agonizing the effects of Wnt. Given that Tinagl1 knockout demonstrates a more severe injury to the proximal tubules following IRI surgery, our evidence suggests that Wnt plays an important role in preventing the injury of the proximal tubules following acute injury. However, the mechanism and role of Wnt in kidney repair and regeneration is also not fully understood. Future directions to this project will aim to address key questions as it relates to not only kidney injury, but also Wnt signaling in general. With Tinagl1 knockout mice,

it could serve as a useful tool to investigate the role of Wnt signaling in kidney damage and repair.

Ultimately, it would be beneficial to determine if there are therapeutic effects to administering Tinagl1 as a preventative treatment. Instead of mice with Tinagl1 knockout, an overexpression of Tinagl1 would be informative to determine if such mice would be more resistant to acute kidney injury. Alternatively, and ambitiously, Tinagl1 could perhaps be solubilized and administered to mice following IRI surgery to determine if it has potent therapeutic effects. However, there remains significant limitations due to its unknown effects in other organs and tissues that also needs further research and evaluations before considering its use as a therapeutic agent. Nonetheless, our findings show a significant role of Tinagl1 in responding to kidney injury that requires further investigations to better elucidate mechanisms of kidney repair and improve the lives of patients with acute kidney injury and subsequently chronic kidney disease.

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