Cadherin-11 mediates kidney injury through alpha-1 antitrypsin

By

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DEDICATION

For my parents, Kirk and Donna Huffstater,

who believed in me and taught me to believe in myself;

And for Monica Wright,

whose friendship and support got me through it.

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LIST OF COMMON ABBREVIATIONS

Abbreviations and Key Terms	Definition
AA	Aristolochic acid
AAN	Aristolochic acid nephropathy
AngII	Angiotensin II
CDH11	Cadherin-11
Col1α1	Collagen 1α1
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
IgG2a	Isotype control to SYN0012
IL	Interleukin
KIM-1	Kidney injury marker 1
LTL	Lotus tetragonolobus lectin
MI	Myocardial infarction
PT	Proximal tubule
PTEC	Proximal tubule epithelial cell
PSR	Picrosirius red
qPCR	Quantitative polymerase chain reaction
SYN0012	CDH11 blocking antibody
TGFβ	Transforming growth factor β
TNF-α	Tumor necrosis factor α
Unx	Uninephrectomy
UUO	Unilateral ureteral obstruction

Chapter 1

Introduction and Motivation

1.1 Renal Disease Burden

Both chronic kidney disease (CKD) and acute kidney injury (AKI) are increasing in prevalence among adults in the United States. AKI is associated with extended hospital stays, increased mortality and morbidity, and is an independent risk factor for the development of CKD and end stage renal disease (ESRD), and can exacerbate pre-existing CKD.[1–4] CKD is likewise a risk factor for AKI and has become a global health crisis, affecting 15% of adults in the United States.[5–7] The two most common causes of CKD are diabetes and hypertension; 1 in 3 people with diabetes and 1 in 5 with hypertension will develop CKD.[6] The CDC estimates that nearly half of all 30-year-olds will develop CKD in their lifetime, 80% of these will develop stage 3 to 5 CKD, and 11% of those who reach stage 3 will end up with kidney failure.[8,9] Additionally, CKD greatly raises the risk of cardiovascular disease and stroke, the leading two causes of death worldwide.[10] Treatment of CKD is also a massive economic burden – while CKD and end stage renal disease (ESRD) patients make up just 13% of the Medicare population, their treatment constitutes 23% of the total cost of Medicare, over $114 billion.[9,10] These numbers will only increase as the population ages and there remain no curative or preventative treatments for AKI, AKI progression to CKD, or CKD. The approved pharmacological treatments for these conditions function only to slow the progression of the disease – by managing blood pressure, blocking the renin-angiotensin system, and normalizing blood sugar – or manage the symptoms of declining renal function. New therapies to treat and prevent, rather than simply manage, AKI and CKD are sorely needed.

1.2 Kidney Physiology

One challenge to the development of treatments for renal injuries is the complexity of the kidneys and the major roles they play in regulating the body. The function of the kidneys is simply described as maintenance of homeostasis. However, “maintaining homeostasis” requires balancing of many complex physiological properties, including blood pressure, body temperature, and composition and volume of the blood, as well as clearance of toxic substances.[11] These functions are carried out by over 20 specialized cell types, many of which make up the functional unit of the kidney, or the nephron. Each kidney contains approximately one million nephrons. The nephron is composed of several functional units: the glomerulus, which is responsible for filtration of blood; the proximal tubules, responsible for crucial reabsorption and secretion of vital or toxic substances; the loop of Henle, which concentrates the filtrate to prevent loss of water and form urine; the distal tubules, which also performs some reabsorption and secretion; and the collecting duct, which collects urine for excretion. The kidneys perform this “cleaning” process on approximately 180 liters of blood per day, which corresponds to all blood in the body flows getting “cleaned” approximately 60 times per day. Without these clearance and balancing processes, other tissues, including nerves, muscles, and cardiac tissue, cannot function properly because their microenvironment and metabolic requirements would be completely disrupted.

1.3 Chronic and Acute Kidney Injury

As mentioned above, CKD and AKI represent a significant disease burden within the United States. There are many significant risk factors for CKD, from diabetes to cardiovascular diseases to AKI. Regardless of the initial cause of CKD, fibrosis, tubular atrophy, and interstitial inflammation are the common pathological features resulting in CKD progression.[12] Clinically, CKD is defined as a reduction of kidney function for a period longer than 3 months. It is usually

diagnosed by sustained measurement of increased proteinuria or decreased glomerular filtration rate (GFR) and patients are stratified into 5 different stages of disease depending on the extent of renal function loss.[13,14] However, one of the significant problems associated with treatment of CKD is that it is a silent disease, meaning patients often don't come in for examination until the disease progressed to an advanced state. AKI is clinically defined by a rapid reduction in renal function, most often diagnosed by spikes in serum creatinine or blood urea nitrogen (BUN), compounds that are largely excreted by healthy kidneys. While AKI can be caused by many things, one of the most significant risk factors for AKI is admission to a hospital, particularly if the patient is critically ill. AKI occurs in approximately 2% of hospital patients, in 60% of ICU patients over the course of their stay, and with even further increased risk if the patient is in sepsis.[15,16] These statistics are staggering, particularly when the fact that there are extremely limited treatment options is considered. Treatment for AKI generally involves administration of drugs to help maintain homeostasis to act as a crutch for kidney function to allow for renal recovery.

Most causes of AKI occur in the hospital.[17] In the ICU, the most common cause of AKI is sepsis, whereby the cells of the kidney are essentially poisoned by bacterial toxins in the blood. In most other cases, AKI is caused by ischemia as a result of reduced blood flow to the functional cells of the kidney. As the most metabolically active cells in the kidney, the proximal tubule cells are also the most vulnerable to ischemic injury.[18,19] Additionally, proximal tubule death in AKI has been shown to be a major predictor of CKD and ESRD.[20–22] Following an acute injury, the injured epithelial cells secrete both paracrine and autocrine signaling factors to induce proliferation, recruit immune cells such as macrophages, and activate fibroblasts. In a moderate injury, such signaling processes result in a coordinated wound recovery response and the damaged tubules are restored to their healthy morphology and function (**Figure 1.1**). However,

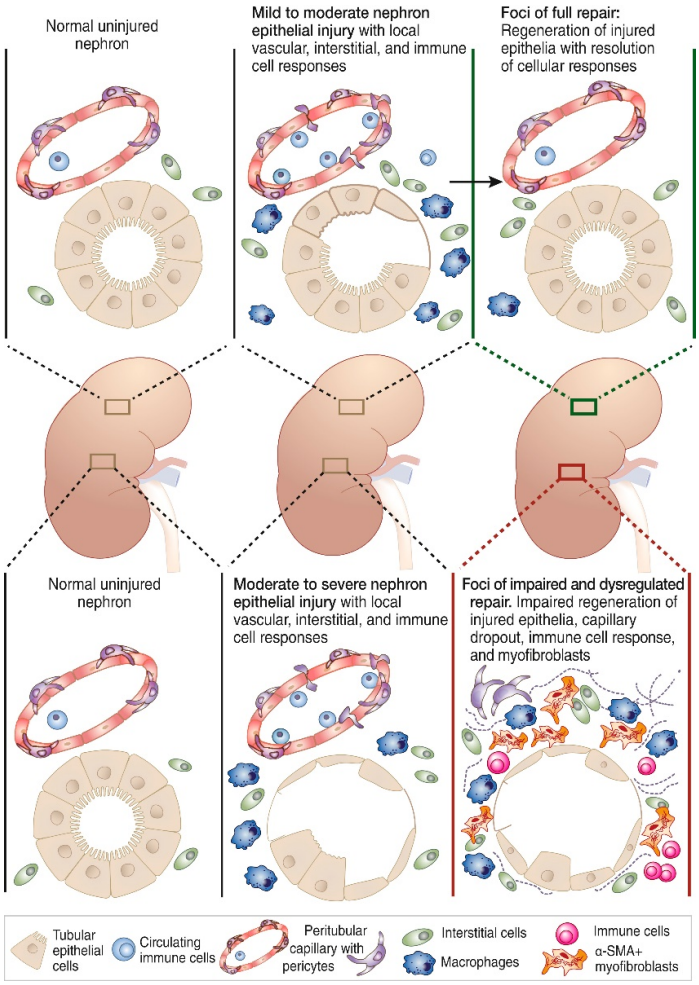

Figure 1.1 Renal response to injury following an AKI
Following an acute injury, the damaged tubular epithelial cells initiate wound healing responses. If the wound is too severe, these responses become dysregulated and lead to inappropriate recruitment of immune cells and activation of fibroblasts. This results in further injury to the tubular epithelium and prevents recovery. Adapted from Kumar, 2018.

if the injury is severe or sustained, this repair response becomes dysregulated and can contribute to further injury and lead to inflammation and fibrosis.

As is shown in **Figure 1.1**, renal fibrosis can be generally described as the result of an inappropriate wound healing response following an injury to kidney epithelium. Fibrosis is the pathological accumulation of extracellular matrix (ECM) components, such as collagen and fibronectin. This excessive ECM secretion is produced primarily by myofibroblasts, an activated, highly secretory form of fibroblast or pericyte characterized by expression of smooth muscle α-actin (αSMA) and vimentin.[23–25] Both myofibroblasts and infiltrating immune cells play an

important role in establishing and perpetuating the fibrogenic environment of the chronically injured kidney.[24,26] This mechanism becomes self-sustaining, as the injured epithelium secretes pro-fibrotic and pro-inflammatory cytokines, such as transforming growth factor β1 (TGF-β1) and interleukin-6 (IL-6), respectively (**Figure 1.2**).[23,26,27] The recruited immune cells also secrete TGF-β1 and continue to recruit other inflammatory cells, perpetuating the inflammation and

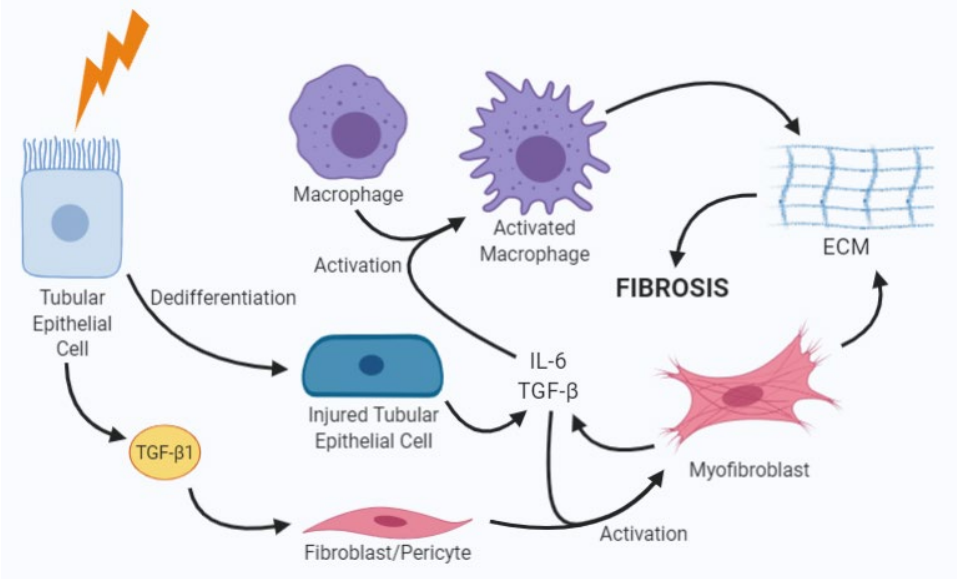

Figure 1.2 Fibrosis results from inappropriate wound healing response following injury to epithelium.
Following injury to tubular epithelial cells, activation of myofibroblasts and immune cells contribute to a fibrogenic environment, in which significant secretion of ECM and infiltration of immune cells exacerbate the injurious state of the epithelial cells. *This figure was created with Biorender.com.*

myofibroblast activation. This positive feedback between inflammation and buildup of ECM leads to further tubular atrophy, hypoxia, and vascular rarefaction, all of which contribute to progressive loss of nephrons and renal function.[23,24,28]

1.4 Cadherin-11

Cadherins are transmembrane proteins important for cell-cell adhesion junctions, which are partially responsible for maintaining tissue structure. As a group, these proteins generally have three functional domains: extracellular, transmembrane, and intracellular. The extracellular domain typically forms homophilic bonds with the extracellular domain of another cadherin on a

neighboring cell. The transmembrane domain anchors the extracellular domain to the cell and the intracellular domain. The intracellular domain forms complexes with proteins such as β-catenin, p120-catenin, and α-catenin. Binding with these proteins provides connections to the cytoskeleton, allowing cadherins to participate in signaling through both traditional mechanisms and force transduction.[29] Major shifts in cell phenotypes are observed with expression of different cadherins, which can lead to various pathological processes – this has resulted in significant studies on the role of cadherins in disease.[30–36]

Cadherin-11 (CDH11), also known as OB-cadherin, is notable within this protein family because its homotypic bond is twice as strong as that formed between other cadherins, and cadherin switching to CDH11 from other cadherins has been implicated in disease.[37–39] Like many other cadherins, CDH11 is involved in many signaling pathways, including those of TGF-β, Wnt, and ERK, all of which are known to have significant effects on cell survival, proliferation, and differentiation.[40,41] Several studies have implicated CDH11 in fibrotic diseases, such as rheumatoid arthritis, pulmonary fibrosis, dermal fibrosis, and heart valve disease, and have shown that inhibition of CDH11 significantly improves outcomes in mouse models of these conditions.[38,42–48] CDH11 has been shown to modulate ECM production and induce fibroblasts to produce IL-6, and it can both increase and be increased by expression of TGF-β1.[38,43,47–49] While many tissues express CDH11, the functional role of CDH11 in fibrosis seems to be mediated primarily by fibroblasts and immune cells.[44,45,47,48,50] However, this role has not been definitively identified, particularly in the context of renal injury and fibrosis. Recent studies have shown increased CDH11 expression both in mouse models of renal fibrosis and human biopsies and urine, highlighting CDH11 as a potential biomarker for CKD.[51] This evidence, combined with efficacy of CDH11 inhibition in other fibrotic diseases provides strong support for the study of CDH11 as a drug target for treatment of CKD.

1.5 Dissertation overview

My doctoral work has investigated the role of CDH11 in kidney injury. I first identified which cellular population was responsible for CDH11 expression in the kidney after injury. The second aim was to evaluate the effects of CDH11 inhibition using genetic knockout mice and pharmacological inhibition with a CDH11 blocking antibody. Lastly, my work identified genetic alterations associated with CDH11 inhibition in kidney injury and identified a novel relationship between CDH11 and alpha-1 antitrypsin (AAT).

In this dissertation, I present a background of kidney injury, CDH11, and the effects of CDH11 inhibition in other fibrotic diseases. Subsequently, I present a summary of my doctoral research into the benefits of CDH11 inhibition in three distinct murine models of kidney injury, using both genetic and pharmacologic means of inhibiting CDH11. Inhibition of CDH11 was beneficial in improving renal function, reducing kidney injury, and reducing fibrosis and inflammatory signaling. Next, I present my results on genetic alterations associated with CDH11 inhibition and describe the relationship we identified between CDH11 and AAT. RNA sequencing and *in vitro* assays were implemented to identify changes in genetic expression and determine whether CDH11 regulates expression of AAT. Finally, I discuss the impact of this work and potential future directions the research could be taken.

Chapter 2

Wnt/β-catenin in Acute Kidney Injury and Progression to Chronic Kidney Disease

Text for Chapter 2 was adapted from **Huffstater T**, Merryman WD, and Gewin LS. *Wnt/β-catenin in Acute Kidney Injury and Progression to Chronic Kidney Disease*, in Seminars in Nephrology, Joseph V. Bonventre, Editor. Elsevier, PA.

2.1 Introduction

Acute kidney injury (AKI) is increasing in incidence and is associated with longer hospitalizations and reduced survival[1]. AKI is also an independent predictor for the development of chronic kidney disease (CKD), end-stage renal disease (ESRD), and a risk factor for the progression of pre-existing CKD[2–4]. According to the United States Renal Data System, CKD affects 14.8% of Americans, greatly raises the risk of cardiovascular disease and stroke, and places a huge economic burden on the healthcare system once it progresses to ESRD[52]. Thus, treatment of AKI and the prevention of AKI progression to CKD would have an enormous benefit on morbidity, mortality, and the cost of healthcare. Unfortunately, there are no drugs that can either prevent AKI or prevent the progression of AKI to CKD.

AKI injures many cells but primarily targets the tubule epithelium given this compartment's high energetic demands. After injury, the interplay of injured tubules with inflammatory cells, the microvasculature, and fibroblasts is important in the response to AKI and its transition to CKD. Growth factors mediate many of the cellular responses, both reparative and injurious, following AKI. Many of these growth factors, such as Wnt/β-catenin, play a critical role in renal development, have little activity in the uninjured adult kidney, but become re-

expressed after injury. This review focuses on the role of Wnt/β-catenin in AKI and AKI to CKD and its potential as a therapeutic target.

2.1.1 Wnt/β-catenin Signaling

The protein β-catenin has dual functions, serving as both a structural protein and a transcription factor, and the specific role depends upon its cellular location and the presence of Wnt ligands. Membrane-bound β-catenin plays a structural role as part of the adherens junction complex that, together with β-catenin and cadherins, mediate cell-cell interactions. In the uninjured kidney, cytosolic β-catenin is usually targeted for degradation by the destruction complex consisting of axin, adenomatous polyposis coli (APC), glycogen synthase kinase-3β (GSK-3β), and casein kinase 1[53]. β-catenin is phosphorylated by GSK-3β, which then targets it for ubiquitination and proteasomal degradation[54]. However, ligands from the Wnt family (Wnt is a portmanteau named for the Drosophila homologue Wingless and the Int-1 integration site in murine breast tumors) can rescue cytosolic β-catenin from degradation (Figure 1). There are 19 mammalian Wnt ligands, and these bind to a frizzled (Fzd)/low density lipoprotein receptor-related protein (LRP) receptor complex. This binding recruits the protein Dishevelled to facilitate removal of the destruction complex away from β-catenin, thus allowing β-catenin to accumulate in the nucleus and act as a transcription factor to affect gene expression. It is important to note that Wnt ligands can also affect proximal/distal epithelial polarity, or planar cell polarity, but this signaling occurs independent of the β-catenin-dependent (i.e. canonical Wnt signaling) pathway. This review focuses specifically on the canonical Wnt/β-catenin signaling pathway in the context of renal injury and repair.

Once liberated from the destruction complex, β-catenin accumulates in the nucleus and interacts with other transcription factors in the T cell factor/lymphoid enhancer factor (TCF/LEF) family (Figure 1). TCF/LEF transcription factors act as transcriptional repressors while bound to Groucho in the absence of β-catenin. Nuclear β-catenin displaces Groucho and leads to activation of transcriptional responses[55]. One classic transcriptional target of β-catenin/TCF/LEF is axin2

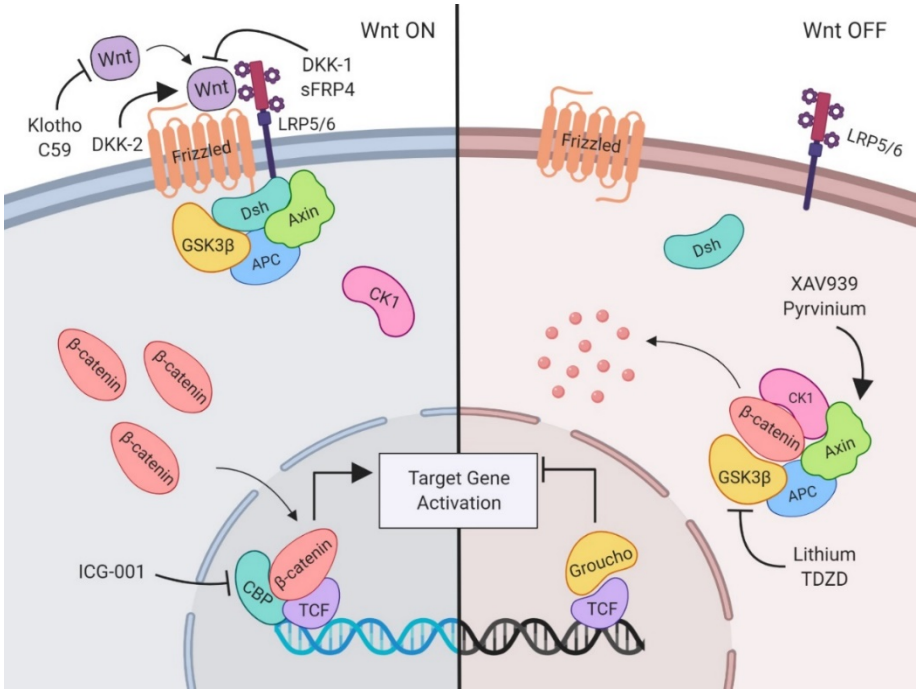

Figure 2.1 Canonical Wnt/β-catenin signaling pathway and pharmacologic pathway mediators.

Wnt ligands bind to Frizzled and LRP5/6 leading to Dsh recruitment of the β-catenin destruction complex, including GSK3-β, Axin, and APC. This prevents the degradation of β-catenin, allowing for cytosolic accumulation and nuclear translocation. In the nucleus, β-catenin and CBP form a complex to displace Groucho and bind to TCF, activating transcription of TCF family target genes. Early pathway inhibitors include C59 which prevents secretion of Wnt ligands; Klotho binds to and sequesters Wnt ligands[86]; DKK-1 binds to LRP5/6 to prevent Wnt binding; sFRP4 binds Wnt and Frizzled to disrupt signaling; and DKK-2 binds to LRP5/6 to enhance binding of Wnt. Several pharmacologic agents alter the activity of components of the destruction complex, including lithium and TDZD-8, which inhibit the activity of GSK3-β and GSK-3, respectively, and prevents β-catenin degradation. Pyrvinium prevents Axin degradation and XAV939, a tankyrase inhibitor, stabilizes cytosolic Axin with both compounds leading to enhanced β-catenin degradation[87, 88]. ICG-001 binds to CBP and reduces β-catenin interactions with TCF, thus blocking β-catenin/TCF-dependent target gene expression. *This figure was created with Biorender.com.*

which serves as a feedback repressor given that axin is a component of the destruction complex[56]. Axin2 transcripts are frequently used as a measure of β-catenin/TCF/LEF activity. Indeed, some reporter mice for β-catenin activity utilize the axin2 promoter to express LacZ[57,58]. The downstream transcriptional responses of β-catenin/TCF/LEF are important in modulating cell

proliferation and differentiation, key biological responses in injury. However, the exact target genes affected vary depending upon the target cell type.

The microenvironment is also critical in determining the targets downstream of β-catenin. During oxidative stress, nuclear β-catenin has been shown to preferentially bind FoxO transcription factors which outcompete TCF/LEF binding[59]. FoxO/β-catenin signaling promotes different transcriptional responses including cell cycle arrest, antioxidant production, and cell survival, all biological functions associated with the FoxO family. Investigators have shown that this switch from TCF/LEF- to FoxO-mediated β-catenin-dependent transcription is relevant to bone formation and liver metabolism[60–62]. However, the role of β-catenin/FoxO-dependent signaling in vivo has not been well investigated in the context of kidney injury with the notable exception of T cell differentiation as will be discussed later. A recent publication shows a protective effect of FoxO1 in the context of TGF-β treatment in murine tubular epithelial cells in vitro, and future studies should explore β-catenin/FoxO interactions in vivo[63].

2.1.2 Wnt/β-catenin and Renal Development

An extensive discussion of Wnt/β-catenin in renal development is beyond the scope of this manuscript, and interested readers are directed to prior reviews[64,65]. Wnt is one of many growth factors involved in development (e.g. Notch, hedgehog) that subsequently become re-expressed after renal injury[66]. An understanding of their biological actions in development may provide insights into their function in the injured kidney. The developing kidney requires finely controlled cellular communication between the epithelial cells of the invading ureteric bud (UB) and the undifferentiated metanephric mesenchyme (MM). The UB rapidly branches and forms the ureter, papilla, and collecting ducts, while the MM condenses around the invading UB to form the remaining nephron (glomerulus, proximal tubule down to collecting duct). Expression of Wnt ligands as well as a TCF/LEF reporter (i.e. β-catenin) localizes Wnt/β-catenin activity to the

distal UB tips and surrounding MM[67], suggesting that Wnt/β-catenin signaling plays an important role in this epithelial/mesenchymal crosstalk.

Murine genetic studies indicate that Wnt/β-catenin signaling controls renal tubular differentiation. Deleting β-catenin in the UB using the homeobox B7 (Hoxb7)-Cre caused a spectrum of malformed kidneys from renal agenesis to cystic/dysplastic kidneys with the severity depending upon the degree of β-catenin suppression[68,69]. Cells lacking β-catenin had premature expression of differentiated collecting ducts as noted by aquaporin-3 and zona occludens-1α expression. In a model in which β-catenin was stabilized (i.e. activated) in the UB, aquaporin-3 expression was reduced[69]. These data suggest that UB-derived β-catenin signaling maintains epithelial cells in a de-differentiated precursor state. In the MM, β-catenin signaling is necessary for the formation of tubules as deletion of β-catenin in the MM (Six2-Cre) led to fewer nephrons and these remaining nephrons retained β-catenin expression[70]. This persistent β-catenin was thought to be in the earliest formed renal vesicles prior to induction of Cre recombinase[70]. Conversely, overexpression of β-catenin in the MM caused ectopic tubule induction without proper epithelialization as defined by lack of differentiation and mesenchymal-to-epithelial transition[64,70]. In both the UB and MM, suppression and over-activation of Wnt/β-catenin signaling led to aberrant renal development. Thus, there may be a critical dose-dependent effect as well as spatial and temporal control of this pathway to ensure proper tubule development and differentiation. It is likely that these variables are also important in determining the role of Wnt/β-catenin in the injured kidney.

2.2 Wnt/β-catenin and Acute Kidney Injury

Wnt/β-catenin signaling is expressed at low levels in the uninjured adult kidney, primarily in the papilla, but this pathway is upregulated in rodent models of acute and chronic renal injury[58,71,72]. Wnts and β-catenin activity have been localized to the proximal tubule after the

ischemia-reperfusion injury (IRI) model[58,73], a common rodent model of AKI involving temporary clamping of the renal pedicle. This signaling pathway is also present in the interstitium after IRI and unilateral ureteral obstruction (UUO), the classical chronic injury model of tubulointerstitial fibrosis[74]. There is also evidence that the Wnt/β-catenin pathway is altered in human kidney disease. Transcriptomic data from human kidneys showed increased Wnt signaling in diabetic nephropathy[75]. In addition, renal biopsy tissue from proteinuric patients revealed alterations in Wnt/β-catenin signaling, particularly in the proximal tubules[76]. More recently, urinary levels of Dickkopf-3 (Dkk3), a modulator of Wnt/β-catenin signaling, predicted preoperatively the risk of AKI after cardiac surgery and was a biomarker for renal decline in patients with CKD[77,78]. Although these data support a link between Wnt/β-catenin signaling and kidney injury, the critical question is whether Wnt/β-catenin signaling promotes repair and recovery or exacerbates the response to injury.

2.2.1 The role of Wnt/β-catenin in Acute Kidney Injury

AKI is a rapid decline in renal function caused by many different injuries such as drugs, ischemia from cardiopulmonary bypass, sepsis, and toxins. The initial target of AKI is thought to be the renal tubules, particularly the proximal tubule and thick ascending limb, both of which are very metabolically active segments with reduced oxygen tensions[79]. Injured tubular epithelial cells undergo cell death through a variety of processes (e.g. apoptosis, necrosis, necroptosis), and even viable cells may slough off the tubular basement membrane due to alterations in integrin expression. The surrounding viable cells then de-differentiate, migrate to cover the denuded basement membrane, proliferate to replace the lost tubule cells, and ultimately re-differentiate to restore tubular function[80]. Although the tubular epithelial cells play a key role in AKI, several other cells types (e.g. inflammatory cells, microvasculature, pericytes/fibroblasts) also modulate the response to AKI and influence AKI progression to CKD.

Abundant data suggest that Wnt/β-catenin signaling is protective in the context of AKI, and the beneficial effects are likely mediated by the proximal tubule. Studies that systemically block the activity of GSK-3β, the kinase that targets β-catenin for degradation, improved renal recovery after AKI induced by mercuric chloride, cisplatin, or IRI[81,82]. Genetic deletion of GSK-3β in proximal tubules reduced apoptosis and mortality after mercuric chloride-induced AKI, suggesting that GSK-3β activity specifically within the proximal tubule plays an important role in the outcome of AKI[81]. One study used lithium, a known inhibitor of GSK-3β, and showed histologic improvement in proximal tubules after injury by the cisplatin or IRI models[82]. One cautionary note regarding these studies is that GSK-3β can affect signaling proteins other than β-catenin, so the beneficial effects in the proximal tubules might not be solely due to augmented β-catenin signaling. Addressing this concern, tubule-specific β-catenin deletion (using the Ksp-Cre) worsened the response to two different AKI rodent models: IRI and folic acid nephropathy indicating that β-catenin per se plays a protective role in proximal tubules[83]. β-catenin deletion also can affect the adherens junctions, though the authors showed that β-catenin (aka plakoglobulin) compensated at the adherens junctions in these conditional knockout mice. However, it is possible that subtle differences in cell/cell interactions also contributed to the phenotype. Another study found that Wnt7b produced by macrophages had a protective effect on kidney tubular epithelial cells after IRI[58]. This same group demonstrated that Dickkopf-2 (Dkk2), a soluble protein that binds to LRP5/6 on the cell surface and enhances canonical Wnt signaling, reduced the number of TUNEL-positive apoptotic tubule cells after injury. Thus, both genetic and pharmacologic approaches show that Wnt/β-catenin signaling is protective to renal tubules in AKI (Table 2-1).

Table 2-1 Pharmacologic and genetic mediators of Wnt/β-catenin signaling in acute kidney injury.

Pharmacologic	Mechanism	Effect	Reference
DKK-2	Binds to LRP5/6 to enhance binding of Wnt and pathway activation	Reduced number of apoptotic tubular epithelial cells in the IRI model of AKI	[58]
Lithium	Inhibits GSK3β activity, preventing β-catenin degradation	Improved renal morphology, especially in proximal tubules, in cisplatin and IRI models of AKI	[81]
TDZD-8	Specifically inhibits GSK3β activity, preventing β-catenin degradation	Reduced tubular epithelial cell damage and death in the IRI model of AKI	[84]
Genetic	**Mechanism**	**Effect**	**Reference**
γ-GT-Cre GSK3β Deletion	GSK3β ablated specifically in proximal tubular epithelium	Reduced apoptosis and mortality in toxin induced and IRI models of AKI	[80]
Ksp-Cre β-catenin Deletion	β-catenin selectively deleted in tubular epithelium	More severe injury and worse mortality in IRI and folic acid models of AKI	[82]
Gli1-Cre β-catenin Deletion	β-catenin selectively deleted in Gli1+ fibroblasts	Attenuated inflammation and tubular injury in IRI model of AKI	[85]

2.2.2 Mechanisms Whereby Wnt/β-catenin is Protective in AKI

There may be several mechanisms by which Wnt/β-catenin signaling protects renal tubules including modulation of apoptosis and survival pathways (Figure 2). Mice containing a tubule-specific deletion of β-catenin sustained greater tubular apoptosis after IRI or folic acid administration[83]. This increased apoptosis was associated with greater expression of Bcl-2 associated X protein (Bax), a pro-apoptotic protein in the Bcl-2 family that induces mitochondrial injury. In addition, the conditional knockout mice had greater expression of p53, a protein that may promote apoptosis through upregulation of Bax[86], and reduced phosphorylated protein kinase B (Akt), which can suppress Bax expression, after folic acid injection[83]. Consistent with these in vivo findings, β-catenin induced tubular cells' Akt phosphorylation and reduction of Bax

activation, mitochondrial membrane injury, and apoptosis following ATP depletion *in vitro*[84]. In addition, the GSK-3 inhibitor TDZD-8 reduced tubule damage in rats after IRI associated with decreased Bax and cell death[87]. In addition to suppressing pro-apoptotic proteins, Wnt/β-catenin signaling has also been shown to increase expression of survivin, a member of the inhibitor of apoptosis protein (IAP) family[88]. In the IRI model, levels of survivin were decreased in two separate mouse models with reduced β-catenin signaling[83,89]. Thus, Wnt/β-catenin signaling likely protects tubular epithelial cells in AKI by modulating the expression of pro/anti-apoptotic proteins to favor tubule survival.

Wnt/β-catenin signaling may also improve repair and regeneration after AKI by promoting proliferation of the surviving renal tubule cells. One of β-catenin's downstream target genes is cyclin D1 which binds with cyclin dependent kinases (CDK) 4 and 6 to promote cell

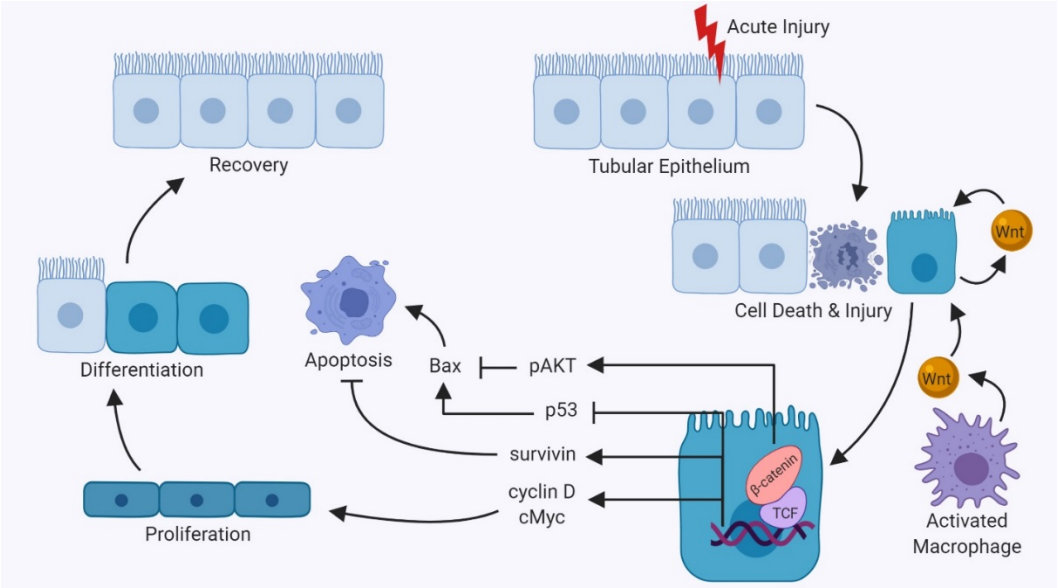

Figure 2.2 Effect of Wnt signaling in acute kidney injury.
Acute injury to the kidneys results in death and injury to tubular epithelial cells. These damaged cells secrete both Wnts and pro-inflammatory cytokines, resulting in recruitment of macrophages, which further contribute to Wnt pathway activation. Activation of Wnt/β-catenin signaling in tubular epithelium has many downstream effects which primarily result in proliferation or prevention of apoptosis. Apoptosis is prevented through the inhibition of pro-apoptotic Bax, mediated by β-catenin-dependent phosphorylation of Akt and/or inhibition of p53. Wnt/β-catenin signaling also promotes cell survival through increased expression of survivin. The target genes activated by Wnt/β-catenin signaling include cyclin D and cMyc, both of which are pro-proliferative proteins. Proliferation helps to replace cells killed during the injury and differentiation of these injured and replacement cells leads to recovery. This figure was created with Biorender.com.

cycle progression[90]. After the IRI model, proximal tubule cells with Wnt4 protein expression also co-stained for proliferating cell nuclear antigen (PCNA), suggesting that Wnt/β-catenin activity was present in proliferating proximal tubule cells[73]. In addition, overexpression of Wnt4 and β-catenin increased the protein expression of cyclin D1 and promoted cell cycle progression in LLC-PK1 cells[73]. A single dose of lithium given on day 3 after cisplatin-induced AKI resulted in greater expression of cyclin D1 and c-Myc, another pro-proliferative protein, in renal tubules and improved recovery from AKI[82]. However, it is possible that this increase in cyclin D1 is mediated by GSK-3β independent of β-catenin. Another group showed that selective deletion of Wnt7b in macrophages caused increased G2/M arrest in tubule cells after IRI, indicating that macrophage-derived Wnt7b promotes epithelial progression through the cell cycle[58].

Several lineage tracing studies have shown that the proliferating renal tubule cell population after AKI has increased Wnt/β-catenin activity. Cells with augmented β-catenin activity were followed using a tamoxifen-inducible Cre under the axin2 promoter that was crossed with the mT/mG ("tomato") reporter mouse[91] in which β-catenin activity (i.e. active Axin2 promoter) was marked with GFP[92]. After AKI induced by intramuscular injection of glycerol, large clones of cells with active β-catenin (i.e. GFP+) proliferated to repair the injured tubules[92]. Another group used the β-catenin/TCF/LacZ reporter mouse and confirmed that limited β-galactosidase (β-gal) staining, indicative of β-catenin signaling, was present in the uninjured mouse kidney but widespread staining was present in tubules 3 days after glycerol-induced AKI[93]. In addition, many of these β-gal positive cells also expressed CD24, a marker of renal progenitor cells[93]. Infusing CD24+ cells from embryonic mice into injured mouse showed integration of these exogenous CD24+ cells into the proximal tubule, a response that was inhibited by pre-treatment with a Wnt/β-catenin inhibitor[93]. Given the role of Wnt/β-catenin signaling in the developing kidney, it is tempting to speculate that this signaling pathway is important for maintenance of a unipotent renal progenitor cell that is responsible for repair after

AKI. It remains unclear whether there is a certain subset of renal tubule cells with the ability to activate Wnt/β-catenin signaling after injury or if all tubules can potentially turn on this signaling in response to injury. It is also uncertain which biological functions mediated by Wnt/β-catenin signaling (e.g. maintenance of “stemness”, proliferation, anti-apoptosis) regulate this repair process.

Most of the literature attribute Wnt/β-catenin’s protective effects on acutely injured tubules to either modulation of cell death or augmented proliferation. However, there are other β-catenin-dependent cellular effects that may also promote repair after AKI. Wnt/β-catenin promotes epithelial de-differentiation and migration, key responses of surviving tubule cells that then proliferate to cover the denuded basement membrane[80]. The β-catenin-dependent de-differentiation is likely mediated, in part, by the transcription factor Snail which is known to suppress E-cadherin expression[94]. Although this β-catenin/Snail signaling has been shown to induce de-differentiation in cancer and other organs, β-catenin’s effects on epithelial differentiation and migration have not been well-defined in the context of AKI[95]. In summary, there is consensus that Wnt/β-catenin signaling protects renal tubules in AKI though the exact mechanisms may still be debatable.

2.2.3 Acute Kidney Injury to Chronic Kidney Disease Progression and Wnt/β-catenin

Recent data suggests that there is a continuum between AKI and CKD. Although some patients have restoration of their serum creatinine following AKI, it is well-recognized that patients with severe AKI are at increased risk for the development of CKD. In addition, patients with CKD due to diabetes, hypertension or other insults are at increased risk for AKI. The mechanisms of AKI to CKD progression are not completely understood, but most of the attention has focused on persistent tubule injury and/or microvascular impairment.

There is strong preclinical data to suggest that the persistently injured proximal tubule may promote development of CKD through the production of profibrotic growth factors and inflammatory cytokines[22]. These tubule cells remain de-differentiated, and the growth factors they produce can have detrimental effects on the surrounding vasculature and interstitial cells (e.g. fibroblasts/pericytes, inflammatory cells). There is also evidence that these chronically injured tubules are cell cycle arrested, likely in G2/M which is associated with a phenotype that secretes large amounts of fibrotic growth factors like TGF-β and CTGF[96–98]. Several groups have targeted the proximal tubules specifically using the diphtheria toxin murine model. This model takes advantage of the fact that mice are not susceptible to diphtheria unless they express the human diphtheria receptor. When this human receptor is expressed in a cell-specific manner, the administration of diphtheria results in cell death limited to those cells expressing the receptor. Targeting the proximal tubules with the diphtheria toxin was sufficient to cause AKI to CKD progression if the severity was high or if repeated injury occurred[21,99].

Given the importance of the renal tubules in AKI to CKD progression and the vital role of Wnt/β-catenin signaling in this cellular compartment, a logical question is how Wnt/β-catenin signaling modulates AKI to CKD progression. One group addressed this and demonstrated that overexpression of Wnt1 by hydrodynamic-based gene transfer 5 days after IRI worsened fibrosis 6 days later[100]. Although this exogenous Wnt1 was expressed primarily in tubules, Wnt expression led to greater fibroblast activation and matrix production. The same group then showed that the inhibitor ICG-001 reduced fibrosis 11 days after IRI. This is the first paper to really look at the role of Wnt/β-catenin signaling in AKI to CKD which is sorely needed. There are a few caveats worth mentioning. The overexpression of Wnt1 appeared effective based upon the staining, but it is unclear how much β-catenin signaling occurred as a result and how these levels compare to Wnt/β-catenin signaling in pathophysiological states. As illustrated by renal development, too little and too much signaling is detrimental, suggesting that the effect

may vary with the level of β-catenin signaling. Additionally, the ICG-001 inhibitor, widely used as a β-catenin inhibitor, is a small molecule that binds to cyclic AMP response element-binding protein (CBP), a transcriptional co-activator of β-catenin[85]. This was shown to inhibit β-catenin/TCF-mediated responses rather than blocking all β-catenin-dependent responses and may augment β-catenin/FoxO-dependent responses[101]. Additionally, inhibition of CBP-dependent responses that are β-catenin-independent cannot be ruled out and have been reported by others[102]. This study took a valuable first step towards defining the role of Wnt/β-catenin signaling in AKI to CKD. However, given the caveats with ICG-001 and remaining questions about the amount and location of β-catenin signaling with the Wnt1 transgene, additional studies are warranted.

Addressing the question about which cells may be mediating Wnt/β-catenin's deleterious effects in AKI to CKD, β-catenin was selectively deleted in a subset of fibroblasts (Gli1-Cre). These conditional knockout mice had attenuated inflammation and tubular injury after IRI-induced AKI[103]. This effect was thought to be due to enhanced hepatocyte growth factor (HGF) in the mice lacking β-catenin in Gli1+ fibroblasts. Although this study looked at AKI at 1 day after IRI rather than AKI to CKD, it shows that, despite the beneficial effect of systemically augmenting Wnt/β-catenin in AKI, this signaling pathway may be deleterious in certain fibroblast populations.

The microvasculature is recognized as an important mediator of AKI to CKD progression. Early studies showed that IRI injury damaged peritubular capillaries with a 30% reduction in capillary density by microfil analysis from 4-40 weeks after injury[104]. There is not much data on Wnt/β-catenin signaling in the renal microvasculature in the context of AKI to CKD. However, several studies suggest that β-catenin signaling may play an important role in the injured renal peritubular capillaries. The Wnt/β-catenin agonist Dkk3 was present in the conditioned media of renal microvascular endothelial cells in vitro and induced a mesenchymal

transition in these endothelial cells[105]. Dkk3 was also detected in the tubular and endothelial compartments after adriamycin-induced nephropathy, a model of glomerular injury. In a rat model of kidney transplant chronic allograft nephropathy, increased nuclear β-catenin staining in endothelial cells suggest that this pathway is activated[106]. Rats with streptozotocin-induced diabetes had increased expression of β-catenin and decreased expression of CD31, an endothelial marker, in glomerular endothelial cells[107]. These data suggest that β-catenin signaling may impair endothelial function and promote de-differentiation, but none of them genetically manipulated the Wnt/β-catenin pathway specifically in the microvasculature. However, other studies using human umbilical vein endothelial cells (HUVECs) showed that β-catenin signaling stimulated endothelial cell proliferation and survival, possibly through the angiogenic factor interleukin-8[108]. The Wnt/β-catenin pathway has been described as pro-angiogenic, potentially through downstream vascular endothelial growth factor (VEGF) and/or matrix metalloproteinase (MMP)7, but most of these studies were either done with in vitro model systems or in the context of cancer metastasis rather than response to injury[109]. Thus, more research is needed to determine the role of endothelial Wnt/β-catenin signaling in AKI to CKD progression.

2.3 Wnt/β-catenin Signaling in Chronic Kidney Injury

There is much more data on the role of Wnt/β-catenin signaling in AKI than in AKI to CKD transition. As renal injury exists on a continuum between acute and chronic, an understanding of how this signaling pathway affects CKD has important treatment implications. Furthermore, growth factor pathways that are beneficial in AKI (e.g. epidermal growth factor) may be deleterious in CKD[110,111]. Many systemic inhibitors of Wnt/β-catenin ameliorate fibrosis in rodent models of CKD, implying that this pathway promotes fibrosis (Table 2)[112–115]. Some studies used the ICG-001 inhibitor and concerns about this approach were discussed above.

Table 2-2 Pharmacologic and genetic mediators of Wnt/β-catenin signaling in chronic kidney disease.

Pharmacologic	Mechanism	Effect	Reference
ICG-001	Competitively binds CBP to prevent β-catenin/CBP binding to TCF	Reduced fibrosis in models of IRI, UUO, and *in vitro* models of CKD	98,109
DKK-1	Binds to LRP5/6 to prevent dimerization with Frizzled and Wnt binding	Reduced fibrosis and pericyte activation in UUO models of CKD	71,116
Klotho	Binds to and sequesters Wnt ligands	Reduced fibrosis in UUO models of CKD	117
XAV939	Stabilizes Axin to activate destruction complex and stimulate β-catenin degradation	Reduced myofibroblast activation in *in vitro* models of CKD	113,118
sFRP4	Binds Wnt and Frizzled to disrupt signaling functions	Reduced fibrosis in UUO models of CKD	113
C59	Binds Porcupine to prevent acylation and secretion of all Wnts	Reduced fibrosis in UUO models of CKD	111
Pyrvinium	Inhibits axin degradation, which stimulates β-catenin degradation	Reduced fibrosis and myofibroblast markers in AngII injury *in vitro*	119
Genetic	**Mechanism**	**Effect**	**Reference**
SLC34a1-Cre Wnt1 Overexpression	Inducible and sustained Wnt1 production only in proximal tubular epithelium	Caused interstitial fibrosis by activation of myofibroblasts without induction by injury	113
Ksp-Cre β-catenin Stabilization	β-catenin stabilized (i.e. activated) in tubular epithelium by removing phosphorylation sites that target β-catenin for degradation	Caused increased inflammatory infiltration in protein overload injury model	120
Wn9a Overexpression	Overexpression of Wnt9a by delivery of DNA expression plasmids	Increased tubular cell senescence and worsened fibrosis in IRI model of CKD	115
Ksp-Cre Wntless Deletion	Wntless ablated in tubular epithelium, preventing secretion of Wnts	Reduced fibrosis and fibroblast activation in UUO and UIRI models of CKD	114
Gli1-Cre β-catenin Stabilization	β-catenin stabilized (i.e. activated) selectively in Gli1+ fibroblasts	Activation of myofibroblasts in uninjured kidney	73

However, others used either a secreted frizzled-related protein that competes with Wnt ligands for receptor binding or inhibited porcupine, which promotes Wnt secretion and receptor binding, and both approaches showed a protective effect[114,115]. These pre-clinical studies suggest that

Wnt/β-catenin signaling promotes fibrosis in CKD models, but do not inform about the cell-specific effects. Future studies that look at cell-autonomous β-catenin signaling may provide more information about the cell-specific roles of this signaling pathway in CKD.

2.3.1 Tubular Epithelial Wnt/β-catenin Signaling and CKD

Tubular epithelial β-catenin signaling is particularly important because of this cellular compartment's role in AKI to CKD progression. Several groups have generated transgenic mice in which the epithelial cells overexpress Wnt ligands. Overexpression of Wnt1 by proximal tubules was sufficient to cause tubulointerstitial fibrosis by paracrine signaling in fibroblasts/pericytes and without epithelial injury[121]. Similarly, Wnt9a overexpression by hydrodynamic tail injection after IRI increased tubular senescence and worsened fibrosis[120]. Both of these studies demonstrate the potential for Wnt signaling to cause tubulointerstitial fibrosis, the hallmark of CKD. However, given that Wnt is a soluble factor, it is difficult to attribute all the effects to epithelial signaling. The Wnt1 overexpressing mice had interstitial fibrosis and increased signaling in this compartment without epithelial injury. In addition, the levels of Wnt in the transgenic models may have exceeded those even in injury states which may be relevant if there is a dose-dependent effect. It is likely that injured tubules are important producers of Wnt ligands, but many of their deleterious effects may be mediated by paracrine signaling on fibrobasts/pericytes. Consistent with this, tubule-specific but not fibroblast-specific ablation of Wntless, a transmembrane protein that is required for the secretion of Wnt proteins, reduced fibroblast activation and fibrosis[122].

A more direct approach to target epithelial Wnt/β-catenin signaling is to alter the β-catenin activity specifically in epithelial cells. Tubule-specific deletion of β-catenin did not affect tubulointerstitial fibrosis development after the UUO model of injury[123]. These conditional knockout mice did have enhanced fibroblast survival due to reduced MMP7-mediated Fas ligand expression in fibroblasts. This data suggests that epithelial β-catenin does not play a major role in fibrosis induced by UUO. This is not too surprising as this model is an excellent fibrosis rodent model but is not ideal for epithelial injury/repair as it progressively destroys the renal parenchyma. Another group stabilized β-catenin in tubule epithelial cells and, in a model of protein overload induced by uninephrectomy and bovine serum albumin (BSA) injections, noted increased macrophage infiltration and blood urea nitrogen (BUN) levels but no fibrosis[124]. These data suggest that augmented epithelial β-catenin signaling may induce macrophage infiltration through increased tubular cytokine expression[124]. More studies are warranted to assess how epithelial β-catenin affects other models of injury including AKI to CKD.

2.3.2 Wnt/β-catenin Signaling in Renal Fibroblasts/Pericytes and CKD

Tubulointerstitial fibrosis consists of extracellular matrix (ECM) proteins like collagen and fibronectin, and myofibroblasts are the main producers of ECM. Although there is some debate about the origin of myofibroblasts, most agree that resident fibroblasts and pericytes are stimulated by growth factors to transform into myofibroblasts. Wnt1 and Wnt4 have both been shown to promote myofibroblast transformation from fibroblast and pericyte precursors in vitro[121,125]. As mentioned above, overexpression of tubule-derived Wnt1 had a profound effect on interstitial cells driving myofibroblast activation and proliferation[121]. Consistent with this, stabilization of β-catenin (i.e. activation) specifically in Gli1+ interstitial fibroblasts led to myofibroblast differentiation in the uninjured murine kidney[125]. Many other studies that systemically inhibit Wnt/β-catenin and reduce fibrosis are associated with reduced numbers of

myofibroblasts[94,115,126]. These studies taken together with those that directly modulate β-catenin signaling in myofibroblast precursors provide strong evidence for a fibroblast/pericyte-dependent profibrotic effect of Wnt/β-catenin in CKD.

2.3.3 Wnt/β-catening Signaling in Inflammatory Cells and CKD

Inflammatory cells are another important component and modulator of both AKI and CKD. Most of the attention has focused on macrophages, which are subtyped into many different subsets with different functions. Broadly speaking, they have been divided into the proinflammatory M1 (classically activated) and profibrotic M2 (alternatively activated)[127]. In AKI, the M1 macrophage phenotype initially predominates and clodronate depletion of macrophages at this stage is beneficial. By contrast, the M2 phenotype is considered reparative in the context of AKI and targeting this population at a later stage after AKI worsened renal recovery[58,128]. In CKD, the M2 phenotype has been implicated in promoting fibrosis in CKD through the production of TGF-β, PDGF, and galectin-3, and human biopsy samples with persistent macrophage infiltration portend a worse prognosis[129–131]. Wnt3a, given with TGF-β or IL-4, potentiated the M2 differentiation of macrophages in vitro[117]. Furthermore, mice with inducible deletion of β-catenin in macrophages had fewer M2 macrophages as well as reduced fibrosis after the UUO model of injury[117]. Thus, Wnt/β-catenin signaling in macrophages promotes M2 differentiation, an effect that may be beneficial in AKI but detrimental in CKD.

The role of T cells in CKD is not as well-studied, but regulatory T cells (Tregs), which suppress the activity of reactive effector T cells, may play a role in chronic kidney disease[118]. A group recently showed that using ICG-001, a blocker of β-catenin/TCF/LEF interactions, re-directs β-catenin signaling to FoxO-mediated transcription. In the context of injury (UUO and IRI), recombinant TGF-β1 plus ICG-001 increased β-catenin/FoxO signaling leading to Treg-mediated anti-inflammation and renoprotection[101]. Upon Treg depletion, the benefit of ICG-001

and TGF-β1 was abrogated. This immunosuppressive effect of β-catenin/FoxO required TGF-β1, but other effects of β-catenin/FoxO in the injured kidney have yet to be explored. In summary, β-catenin had divergent effects on macrophages and Tregs in the context of chronic injury. However, it may be that the transcriptional signaling partners (e.g. TCF/LEF versus FoxO) determine whether β-catenin signaling in inflammatory cells is beneficial or detrimental in CKD.

2.4 Modulating Wnt/β-catenin in AKI: Challenges and Opportunities

The preclinical data overwhelmingly suggest a strong protective role for Wnt/β-catenin signaling in AKI. Although one study did show a deleterious effect of β-catenin signaling in fibroblasts, this probably does not outweigh the beneficial effects in renal tubules in AKI based on the studies which used systemic inhibitors. Furthermore, there is data to suggest that augmenting Wnt/β-catenin signaling is beneficial when present both at the time of injury and when activated after the initial injury. The putative mechanisms whereby Wnt/β-catenin signaling protects injured renal tubules focus on either cell survival/anti-apoptosis or its proliferative effects on epithelial cells. It is possible that the protection against apoptosis accounts for the attenuated severity of injury when the Wnt/β-catenin pathway is activated at or before injury, and the pro-proliferative effects mediate the improved renal recovery when the pathway is modulated after injury.

There are a few challenges to Wnt/β-catenin activation in AKI related both to the pharmacologic strategy as well as issues regarding prevention versus treatment and the target population in AKI. The best approach to activate Wnt/β-catenin signaling in AKI is not clear. Both lithium and GSK-3 inhibitors, commonly used in preclinical trials, have β-catenin-independent effects. There may be long-term concerns about using GSK-3 inhibitors, though their use in treatment or prevention of AKI would presumably be of relatively short duration. The

bigger challenge is knowing which patient population is most likely to benefit: should it be for prevention of AKI or treatment of AKI? Although the patient population targeted for prevention of AKI is more homogeneous (e.g. cardiopulmonary bypass patients), most AKI is not preventable, so treatment has the potential to offer more benefit. Improved phenotyping of AKI, which has diverse etiologies and therefore is unlikely to respond to a one-drug-fits-all approach, should help guide more targeted subpopulations likely to benefit. More studies are warranted to answer these questions as well as the ideal duration and dose of treatment.

2.5 Modulating Wnt/β-catenin in AKI to CKD: Challenges and Opportunities

As most human AKI is difficult to prevent, treatment of severe AKI to prevent progression to CKD/ESRD is an attractive approach[119]. Although AKI causes a huge burden acutely in terms of mortality and hospitalization duration, there is also a huge effect on health and healthcare costs once AKI progresses to CKD. The clear concern with augmenting Wnt/β-catenin in AKI to CKD progression is the detrimental effects of this pathway that have been demonstrated in rodent CKD models. The general paradigm with Wnt/β-catenin signaling is that it is beneficial in AKI but detrimental in CKD. However, it may be that β-catenin signaling has more to do with location than duration. As recently shown, deleting β-catenin in fibroblasts was beneficial in AKI, suggesting that β-catenin mediates different effects in AKI based upon the target cell. In AKI, β-catenin's salutary effects on epithelial cells likely outweigh its adverse effects on fibroblasts. It is likely that β-catenin signaling always mediates detrimental effects on fibroblasts as this data is strong in CKD models. However, β-catenin signaling in fibroblasts likely contributes more to the effects of systemic Wnt/β-catenin modulation in CKD than it does in AKI. Thus, the beneficial effect of increasing β-catenin activity in AKI may be mediated by epithelial cells whereas in CKD, the detrimental effect may be driven by fibroblasts. However, there are legitimate concerns about chronic stimulation of β-catenin in epithelial cells as well. In summary,

preclinical data support a protective role for Wnt/β-catenin agonists in AKI. However, more studies are needed to determine if this pathway should be augmented in AKI to CKD. If so, the optimal treatment duration as well as dose that can be both safe and effective will also need defining. Although more work is needed, this unmet medical need of treating AKI to CKD progression merits the investment of resources.

Chapter 3

Cadherin-11 is expressed in the injured proximal tubule

Text for Chapter 3 was adapted from **Huffstater T**, Raddatz MA, Riley LA, Noll NA, Fogo AB, Gewin LS, Merryman WD. Cadherin-11 mediates kidney injury through alpha-1 antitrypsin. *In revision.*

3.1 Abstract

Introduction: Cadherin-11 has been shown to be a mediator of multiple fibrotic diseases, including pulmonary fibrosis, rheumatoid arthritis, and calcific aortic valve disease. Recent studies have identified CDH11 as a potential biomarker for chronic kidney disease (CKD), as its expression was increased in mouse models of renal injury as well as in human biopsies and urine from CKD patients. In other fibrotic diseases, CDH11 expression has been identified in fibroblasts, hematopoietic cells, and injured epithelial cells. However, the precise cell types that express CDH11 in the injured kidney remain unidentified.

Methods: To assess the expression of CDH11 in the injured kidney, we examined multiple models for inducing chronic kidney injury in mice. These were unilateral ureteral obstruction (UUO), the classic model of tubulointerstitial fibrosis; uninephrectomy with angiotensin II administration (Unx/AngII), a chronic hypertension model; and aristolochic acid nephropathy (AAN), a proximal tubule-specific AKI to CKD model. To identify CDH11 expression, qPCR and immunofluorescence staining were used for each model.

Results: CDH11 is minimally expressed in healthy adult kidneys, which was confirmed using qPCR and immunofluorescence staining. CDH11 expression was significantly increased in models of kidney injury compared to uninjured mice. *Cdh11*$^{-/-}$ mice had virtually no expression

of CDH11 compared to *Cdh11*$^{+/+}$ counterparts in each injury model. Injured proximal tubules were identified as the source of CDH11 expression in both mouse and human tissue samples.

Conclusions: The primary source of CDH11 expression in kidney injury is not fibroblasts or hematopoietic cells but injured proximal tubule epithelial cells.

3.2 Introduction

In rheumatoid arthritis, CDH11 is expressed primarily in fibroblast-like synoviocytes and mediates tissue remodeling, leading to the degradation of joint tissue. In a model of dermal fibrosis, both macrophages and fibroblasts were shown to express CDH11, which allowed for adhesion between these two cell types and mediated their interaction.[132,133] In calcific aortic valve disease, CDH11 is expressed in myofibroblasts and increases sensitivity of these cells to mechanical tension, exacerbating valve stenosis, hardening, and inflammation.[42,43] Switching from CDH2 to CDH11 is evident in pulmonary fibrosis, where CDH11 is expressed in myofibroblasts, macrophages, and alveolar epithelial cells, particularly those undergoing EMT.[38,134]

Although recent studies have shown increased CDH11 expression in models of renal fibrosis, the cell types responsible for this expression remain unknown.[51] Many different cell types play a role in the initiation and progression of CKD. Injured tubular epithelial cells produce profibrotic and proinflammatory cytokines, including TGF-β1 and IL-6, respectively. In the presence of profibrotic cytokines, fibroblasts or pericytes become activated into myofibroblasts, highly secretory cells that contribute to the pathological accumulation of ECM components. Immune cells recruited by proinflammatory cytokines also secrete TGF-β1 and continue to recruit other inflammatory cells, perpetuating myofibroblast activation and inflammation. As described above, CDH11 expression has been found in cells contributing to each step of this

process. Therefore, we evaluated these three cellular populations for expression of CDH11, using three models of kidney injury: UUO, AAN, and Unx/AngII. We injured both wild type (*Cdh11*$^{+/+}$) and genetic knockout (*Cdh11*$^{-/-}$) mice in these studies to confirm that CDH11 expression was increased after injury and to identify the cell types responsible for CDH11 expression in the kidney.

3.3 Methods

Mice

All mouse experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee before their commencement. Mice used in this study were transgenic mutants on a mixed background, either wild type (*Cdh11*$^{+/+}$) or global genetic knockout of CDH11 (*Cdh11*$^{-/-}$).

Animal Studies

CDH11 mutant mice were aged 10-12 weeks and induced with kidney injury either by the UUO, AAN, or Unx/AngII models. UUO was induced by obstruction of the ureter, as previously described.[135] Briefly, mice were anesthetized with 2% isoflurane inhalation. A small incision was made over the right flank and the right kidney was externalized using gentle pressure. A 5-0 silk suture was used to ligate the ureter distal to the renal pelvis and the kidney was returned to the cavity. The muscle layer was closed using absorbable sutures and the skin was closed using wound clips. Mice were sacrificed 7 days after the surgery.

AAN was induced by intraperitoneal (IP) injection of aristolochic acid (AA) at 2-3 mg/kg in six doses over 2 weeks. Mice were sacrificed 4 weeks after the final injection.

For the Unx/AngII model, mice were anesthetized with 2% isoflurane inhalation, a small incision was made over the right flank, and the right kidney was externalized using gentle pressure as in the UUO model. The adrenal gland was gently separated from the upper pole of the kidney and the renal fat pads were pulled away. A 5-0 silk suture was used to ligate the renal vein, artery, and ureter. The kidney was then excised, and the stump moistened with sterile saline before reinsertion to the cavity. The muscle was closed as in the UUO model, then an osmotic pump (Alzet Micro-Osmotic Pump; pumping rate 0.11 µL/hr, duration 28 days, reservoir volume 100 µL) containing angiotensin II (AngII) was inserted before closing the skin with wound clips. The pump delivered AngII at a rate of 800 ng/kg/min and was replaced every 4 weeks. Mice were sacrificed 4 or 8 weeks after the surgery.

For bone marrow transplantations, male *Cdh11*$^{+/+}$ or *Cdh11*$^{-/-}$ mice were euthanized for bone marrow isolation. Male *Cdh11*$^{+/+}$ mice aged 5-6 weeks were irradiated using a Cesium 137 source. Irradiation was performed in two dosages of ~6Gy, totaling 12 Gy (1200 Rad). Each dose was separated by 4 hours, and total time exposed to radiation per dose was less than 5 minutes. After receiving the second radiation dosage, mice were housed in sterile caging, provided sterile chow, and given acidified, sterile, antibiotic-treated water for two weeks following irradiation. This is a widely-used method to prevent infection, which was necessary to keep the animals alive and healthy as the bone marrow graft begins to repopulate their immune cell populations. 3 million cells of isolated donor marrow were given to recipient mice via a 100 µL retro-orbital injection while anesthetized with isoflurane. Slight pressure was applied to the skin dorsal and ventral to the eye, causing a slight bulge. Syringe of donor marrow was inserted at a shallow angle (about 30°) into the medial canthus and injected into the retro-orbital sinus. Following a 6 week reconstitution period, kidney injury was induced using the UUO model as described above. Mice were sacrificed 7 days following the surgery.

Euthanasia was performed through CO_2 inhalation and cervical dislocation in accordance with Vanderbilt University Medical Center's Division of Animal Care guidelines.

Quantitative Polymerase Chain Reaction

The upper pole of the kidney was used for quantitative polymerase chain reaction (qPCR) in each experimental animal. The kidneys were dissected under RNase-free conditions and immediately snap frozen. Samples were then thawed and homogenized in Trizol reagent using a bead beater and lysis matrix tubes (Lysis Matrix D, MP Biomedicals). RNA was isolated using the Direct-zol RNA Miniprep Kit (Zymo, R2050) and cDNA was synthesized using the Superscript IV system (Invitrogen, 18091050). Real time qPCR was performed using SYBR Green PCR Supermix (Bio-Rad, 1708882), the primers listed in **Table 3-1**, and a Bio-Rad CFX96 C100 thermocycler. *Gapdh* was used as a housekeeping gene. Data presented was calculated using the delta delta Ct method and statistical analysis was run on delta delta Ct values.

Table 3-1 qPCR primer sequences

Gene Target	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Gapdh	ATGACAATGAATACGGCTACAG	TCTCTTGCTCAGTGTCCTTG
Cdh11	TCACTATCAAAGTCTGTGGCTG	CAAACAGCACAACGATGACC

Immunofluorescence

Upon dissection, a central section of each mouse kidney was excised and submerged in 10% neutral buffered formalin (NBF) and stored at 4°C overnight before being processed and embedded in paraffin by the VUMC Translational Pathology Shared Resource. Slides containing sections from human biopsies were provided by the Fogo lab. Unstained slides were subjected to paraffin removal followed by antigen retrieval using citrate buffer with a pH of 6.0 (Abcam,

ab93678). Slides were heated at 95°C for 20 minutes, then allowed to cool before rinsing in PBS and continuing with staining protocol. Tissue was blocked in 5% bovine serum albumin in PBS-/- for one hour. Slides were then stained with primary antibodies against CDH11 (1:100; Invitrogen, 717600), αSMA conjugated to Cy3 (1:400; Sigma-Aldrich C6198), or LTL (1:200; Vector Laboratories, FL-1321) at 4°C overnight. Slides were rinsed, stained with AlexaFluor 647 secondary antibody (1:400; Invitrogen, A-21245) for one hour at room temperature, then mounted in ProLong Gold with DAPI (Thermo Fisher Scientific, P36941).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9. A 2-tailed Student *t* test was used for comparisons of two groups. Applicable tests and significance are labeled in figure captions with $p \leq 0.05$ as the cutoff for data to be considered significantly different.

3.4 Results

3.4.1 CDH11 expression in the kidney increases after injury

CDH11 expression was significantly increased in the UUO and AAN models of chronic kidney injury ($p \leq 0.0001$ and $p \leq 0.001$, respectively) compared to uninjured mice (Figure 3.1A,B), but not after injury with the Unx/AngII model (Figure 3.1C). *Cdh11*$^{-/-}$ mice had significantly diminished expression of CDH11 compared to *Cdh11*$^{+/+}$ counterparts in the UUO ($p \leq 0.0001$), AAN ($p \leq 0.001$), or Unx/AngII ($p \leq 0.05$) injury models (Figure 3.1A-C).

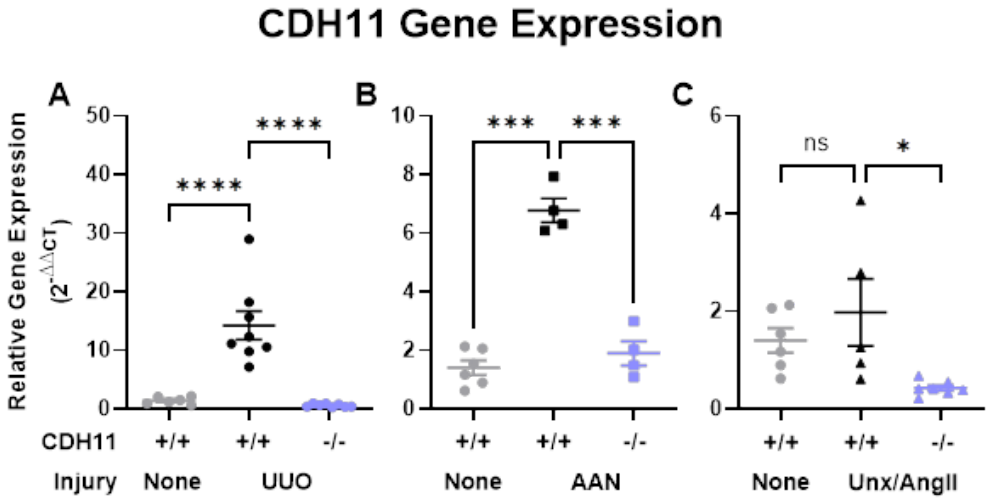

Figure 3.1 Renal CDH11 expression after injury
Expression of CDH11 in the kidney was significantly increased in *Cdh11*$^{+/+}$ mice after injury by UUO or AAN (left, center), but not after 8 weeks of injury with the Unx/AngII model (right). Expression of CDH11 in *Cdh11*$^{-/-}$ mice was significantly lower than that of *Cdh11*$^{+/+}$ mice after injury in all three injury models. Injured mice were normalized to uninjured *Cdh11*$^{+/+}$ mice in each injury model. The same uninjured mice, in gray, were used for comparison in each injury model. * indicates $p \leq 0.05$; *** indicates $p \leq 0.001$; **** indicates $p \leq 0.0001$ by 2-tailed Student t test.

3.4.2 CDH11 is not expressed in hematopoietic cells in the kidney

To further understand the role of CDH11 in kidney injury, we defined which cells were expressing CDH11 in the injured kidney. As described above, CDH11 is expressed in inflammatory cells in some cardiopulmonary diseases, so we investigated hematopoietic cells for CDH11 expression using bone marrow transplantation experiments. The bone marrow of Cdh11+/+ mice was replaced with that of either Cdh11+/+ or Cdh11-/- mice, and the mice were then injured by UUO and sacrificed after 7 days. There was no difference in CDH11 gene

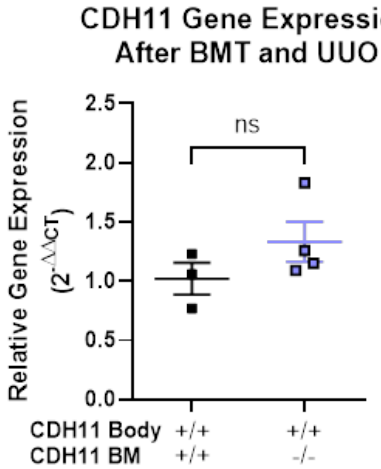

Figure 3.2 CDH11 gene expression after UUO in bone marrow transplantations
CDH11 deletion from bone marrow did not affect expression of CDH11 in the kidney, as mice with *Cdh11*$^{+/+}$ bone marrow did not have higher expression of CDH11 than mice with *Cdh11*$^{-/-}$ bone marrow 7 days after injury by UUO.

expression regardless of the genotype of bone marrow in the mice (Figure 3.2), indicating that the invading hematopoietic cells were not responsible for CDH11 expression in our model.

3.4.3 CDH11 is expressed in injured proximal tubules in mice and humans

CDH11 expression has also been found in fibroblast-like cells. We therefore used immunofluorescence to assess expression of CDH11 in fibroblasts in the kidney *ex vivo*. The staining for CDH11 did not colocalize with fibroblasts as expected, but instead localized to cortical tubules in the UUO model (Figure 3.3A). We verified that CDH11 expression localizes to injured proximal tubules by co-staining kidneys injured by Unx/AngII with CDH11 and LTL

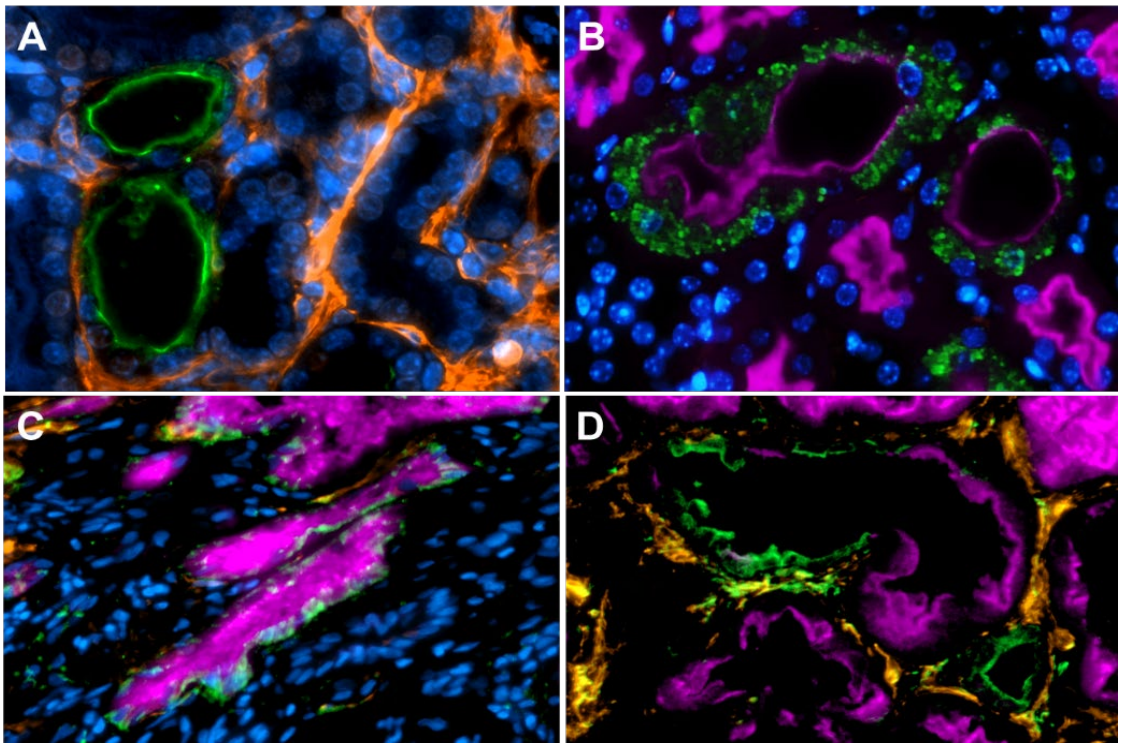

Figure 3.3 CDH11 is expressed in proximal tubules
Kidney sections from mice (A,B) or humans (C,D) were stained for DAPI (blue), CDH11 (green), αSMA (orange), and LTL (purple). CDH11 expression was not co-localized with myofibroblast cells, marked with αSMA staining, but was on tubular structures in the cortex of kidneys injured by UUO (A). In mice injured by 4 weeks of Unx/AngII, CDH11 localized to cells that also expressed LTL, a marker of proximal tubules (B). In human kidneys, CDH11 also colocalized with LTL (C,D).

(Figure 3.3B). CDH11 expression co-localized with LTL-positive tubules, consistent with proximal tubular expression, and this was confirmed in human samples, using biopsies from patients with severe fibrosis (Figure 3.3C,D). Thus, CDH11 is expressed in the proximal tubule cells of both murine and human chronically injured kidneys.

3.5 Discussion

We discovered that CDH11 is expressed in injured proximal tubule epithelial cells in both mouse and human kidneys. While expression of CDH11 was significantly increased in wild type mice in both the UUO and AAN models compared to uninjured controls, there was not a significant increase in the Unx/AngII model. The Unx/AngII model is a much slower, more mild injury than the other two models, so it's possible that CDH11 expression was not yet elevated

much above baseline in the *Cdh11*$^{+/+}$ mice. However, there was still significantly reduced expression of CDH11 in the *Cdh11*$^{-/-}$ mice, as expected. CDH11 expression in the injured *Cdh11*$^{-/-}$ mice was not only significantly lower than that of the injured *Cdh11*$^{+/+}$ mice but was also lower than that of the uninjured *Cdh11*$^{+/+}$ mice ($p \leq 0.001$). The effects of this reduced CDH11 expression do not seem to have any effects in the uninjured *Cdh11*$^{-/-}$ mice, but certainly have positive effects following an injury, as will be described in later chapters.

CDH11 expression has been identified in fibroblasts, hematopoietic cells, and epithelial cells (particularly those undergoing EMT) in other fibrotic diseases. Because the idea of whether PTECs undergo EMT is somewhat contentious and unclear in the kidney field, epithelial cells were the last place we looked for CDH11 expression. However, after observing no differences in CDH11 expression or injury in the bone marrow transplanted mice and the specific staining pattern in our immunofluorescence images, we realized CDH11 was likely expressed on tubular cells of some kind. In both the UUO and AAN models, the injury to the kidneys was severe enough that the tubules had taken on a more dilated structure, due to dedifferentiation following an injury. Due to this dedifferentiation, the proximal tubules were no longer expressing LTL, so we were not able to identify expression of both CDH11 and LTL in the same cells. The concentration of CDH11 staining seemed to be primarily focused in the cortex of the kidneys, so we next used immunofluorescence on kidneys from the Unx/AngII model. Due to the much milder nature of this injury, we were able to identify expression of CDH11 in tubules that were also expressing LTL, marking them as proximal tubules.

The expression pattern of CDH11 in the UUO and AAN models was also different than that in the Unx/AngII model. As is clear in Figure 3.3A, the CDH11 staining appears to be on the periphery of the cells, particularly concentrated around the inner edge of the tubules where the brush border would be on healthy proximal tubule cells. In the Unx/AngII model, on the other hand, Figure 3.3B shows a much more punctate, intracellular appearance to the staining. We

believe this is because the tubules in Figure 3.3B have only just begun to become stressed by the injury model and are in the process of ramping up their CDH11 expression. Then, once the cells become more injured and dedifferentiate, the tubules would lose their expression of LTL as CDH11 is migrated to the cell membranes. However, we acknowledge it is difficult to assess the exact nature of the staining in proximity to the cells in tissue sections such as these, as they are 3D structures (albeit very thin ones). To solidify this idea, we would need to stain cultured proximal tubule cells and look for similar patterns of expression and use confocal microscopy, ideally in both *ex vivo* and *in vitro* samples. This would be challenging, however, because it is notoriously difficult to achieve expression of markers associated with differentiation and maturity, such as LTL, in PTECs cultured *in vitro*.

The expression of CDH11 in PTECs is particularly exciting due to the outsized role PTECs play in not only initiating, but also progressing kidney injury. Due to their high metabolic activity, PTECs are highly susceptible to hypoxic conditions, and the vast array of transporters expressed in PTECs makes them more receptive to some cytotoxic compounds, such as AA. Other studies have established that injury to the proximal tubule specifically is sufficient to induce progression to CKD.[97,136,137] Additionally, after an injury to the kidney has occurred, the injured and dedifferentiated proximal tubules continue to play a role by excreting excess amounts of pro-fibrotic and -inflammatory cytokines such as TGF-β1 and IL-6. Since CDH11 expression only occurs when PTECs are injured, it follows that CDH11 plays a role either in exacerbating the injury and encouraging apoptosis or dedifferentiation, or it plays a role in PT recovery and regeneration. The following chapters will discuss the effects of CDH11 inhibition in the context of kidney injury, which we expected to be fairly substantial after identifying that CDH11 is expressed by such an influential population of cells.

Chapter 4

Genetic ablation of CDH11 reduces renal injury, fibrosis, and inflammatory signaling

Text for Chapter 4 was adapted from **Huffstater T**, Raddatz MA, Riley LA, Noll NA, Fogo AB, Gewin LS, Merryman WD. Cadherin-11 mediates kidney injury through alpha-1 antitrypsin. *In revision.*

4.1 Abstract

Introduction: Inhibition of CDH11 has been shown to improve outcomes in models of several fibrotic diseases. CDH11 inhibition reduced collagen deposition in dermal fibrosis, pulmonary fibrosis, and CAVD. In myocardial infarction, CDH11 ablation also improved mortality and improved ejection fraction, and in CAVD inhibition of CDH11 reduced contractility of interstitial cells and diminished IL-6 secretion. CDH11 expression is known to be increased in models of kidney injury, but the role of CDH11 or the effects of inhibiting CDH11 remain unknown.

Methods: To determine how CDH11 expression affects tubular injury, fibrosis, and inflammation, we injured *Cdh11*$^{+/+}$ and *Cdh11*$^{-/-}$ mice using the UUO, AAN, and Unx/AngII injury models described in Chapter 3. These mice were evaluated for renal function, kidney injury, and tubulointerstitial fibrosis using cellular and molecular analysis methods.

Results: Genetic knockout of CDH11 resulted in improved outcomes in all three models of kidney injury. CDH11 ablation improved mortality and renal function, reduced tubulointerstitial fibrosis, and diminished expression of pro-inflammatory cytokines.

Conclusions: Inhibition of CDH11 is beneficial in multiple models of kidney disease, cementing its role as a mediator of kidney injury, not just a biomarker.

4.2 Introduction

Inhibition of CDH11 improves outcomes in murine models of fibrotic diseases such as rheumatoid arthritis, pulmonary fibrosis, liver fibrosis, dermal fibrosis, myocardial infarction, and aortic valve disease.[47,50,132,138–140] CDH11 inhibition has been shown to improve mortality, reduce deposition of collagen, and diminish inflammatory signaling in several models of fibrotic disease. However, the role of CDH11 in CKD has not been investigated and it is unknown whether CDH11 is a mediator of kidney injury.

CKD is a fibrotic disease that arises after an injury to the kidney, which can be triggered in many ways, including prolonged hypertension, exposure to nephrotoxic drugs, or AKI. While CKD has many etiologies and many cell types play a role, proximal tubule epithelial cells (PTECs) play a crucial role in the initiation and progression of CKD. This is because PTECs are the most metabolically active cells in the kidney, making them especially vulnerable to injury. Injured PTECs have been shown to produce both profibrotic and proinflammatory cytokines, including TGF-β1 and IL-6, respectively. This results in activation of myofibroblasts and recruitment of immune cells to the site of the injury. Such processes are important for repair and regeneration, but when an injury is severe or sustained, they become dysregulated. This accumulation of invading cells and ECM results in tubulointerstitial fibrosis and chronic inflammation, which promote dedifferentiation of PTECs and hypoxia, causing further death of PTECs and other epithelial cells.[12]

Based on this data and the fact that the effects of CDH11 inhibition in other fibrotic diseases, such as reduced collagen deposition and inflammatory signaling, we evaluated the

role of CDH11 in models of kidney injury. We used the models that have been previously described, as they all cause different types of injury. The UUO model is the classic model of tubulointerstitial fibrosis, as it rapidly causes expansion of myofibroblasts and buildup of ECM in the injured kidney. Renal function is not impacted in this model because the mice retain one functional kidney. The AAN model is a PTEC-specific model of AKI to CKD transition. Injections with AA result in an acute injury to the PTECs, which can internalize the highly cytotoxic AA due to transporters that are not present in other cell types. The acute injury then transitions into a more fibrotic injury over time. Finally, the Unx/AngII model is a much slower injury more comparable to the development of human CKD. The loss of one kidney results in nephron reduction, which would not normally cause injury, but the infusion of AngII increases stress on the remaining kidney by raising the blood pressure. We hypothesized that CDH11 inhibition would improve outcomes in each of these models.

4.3 Methods

Mice

All mouse experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee before their commencement. Mice used in this study were transgenic mutants on a mixed background, either wild type (*Cdh11*$^{+/+}$) or global genetic knockout of CDH11 (*Cdh11*$^{-/-}$).

Animal Studies

CDH11 mutant mice were injured using the UUO, AAN, and Unx/AngII models as described in Chapter 3. Blood was collected 7 days after the final injection of AA, and 4 weeks after surgery for the AAN and Unx/AngII models, respectively. Mice were sacrificed 7 days after surgery for the UUO model, 4 weeks after the final injection of AA for the AAN model, and 8

weeks after surgery for the Unx/AngII model. Euthanasia was performed as described in Chapter 3.

Renal Function Measures

Renal function was determined by measuring blood urea nitrogen (BUN) in plasma samples collected from mice in the AAN or Unx/AngII models. Blood was collected from the submandibular vein into lithium heparin coated tubes (Fisher Scientific, 22043975), then centrifuged at 1.6 rcf for 15 minutes at 4°C. Plasma was then collected and stored at -80°C until analysis. BUN was measured using the BUN Colorimetric Detection Kit (ThermoFisher, EIABUN).

Quantitative Polymerase Chain Reaction

qPCR was performed as described in Chapter 3, using the primers listed in **Table 3-2**. *Gapdh* was used as a housekeeping gene. Data presented was calculated using the delta delta Ct method and statistical analysis was run on delta delta Ct values.

Table 4-1 qPCR primer sequences

Gene Target	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Gapdh	ATGACAATGAATACGGCTACAG	TCTCTTGCTCAGTGTCCTTG
Havrc1	AAACCAGAGATTCCCACACG	GTCGTGGGTCTTCCTGTAGC
Tgfβ1	CCTGGGTTGGAAGTGGATC	TTGGTTGTAGAGGGCAAGG
Il6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA

Histology

Upon dissection, a central section of each mouse kidney was excised and submerged in 10% neutral buffered formalin and stored at 4°C overnight before being processed and embedded in paraffin by the VUMC Translational Pathology Shared Resource. Unstained sections were submitted to paraffin removal followed by staining with PicroSirius Red (Fisher

Scientific, 5030077). Images were taken in brightfield at 4x, then compiled using Image Composite Editor. Compiled images of kidney sections were analyzed using previously described custom MatLab code created by Matthew Bersi, PhD, which analyzed the area fraction of collagen and healthy tissue.[141,142]

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9. A 2-tailed Student *t* test was used for comparisons of two groups. A log-rank Mankel-Cox test was used for comparisons between survival curves. Applicable tests and significance are labeled in figure captions with $p \leq 0.05$ as the cutoff for data to be considered significantly different.

4.4 Results

4.4.1 Inhibition of CDH11 improves mortality and renal function

Although the UUO and Unx/AngII models had little to no mortality, the AAN model had significant mortality associated with CDH11 expression. CDH11 deletion significantly improved survival ($p \leq 0.05$) 4 weeks after initiating aristolochic acid (AA) injections, as Cdh11+/+ mice had a mortality rate of about 25%, compared to 0% for Cdh11-/- mice (Figure 4.1A). Renal function was also improved in both the Unx/AngII and AAN models, reflected by a significant reduction in plasma BUN levels ($p \leq 0.001$ and $p \leq 0.05$, respectively) in *Cdh11*$^{-/-}$ mice compared to *Cdh11*$^{+/+}$ counterparts (Figure 4.1B,C). Although the change was not significant, there was also a trend in *Cdh11*$^{-/-}$ mice having slightly lower expression of KIM-1 (*Havrc1*), a marker of PTEC injury, in the UUO and Unx/AngII models (Figure 4.1D).

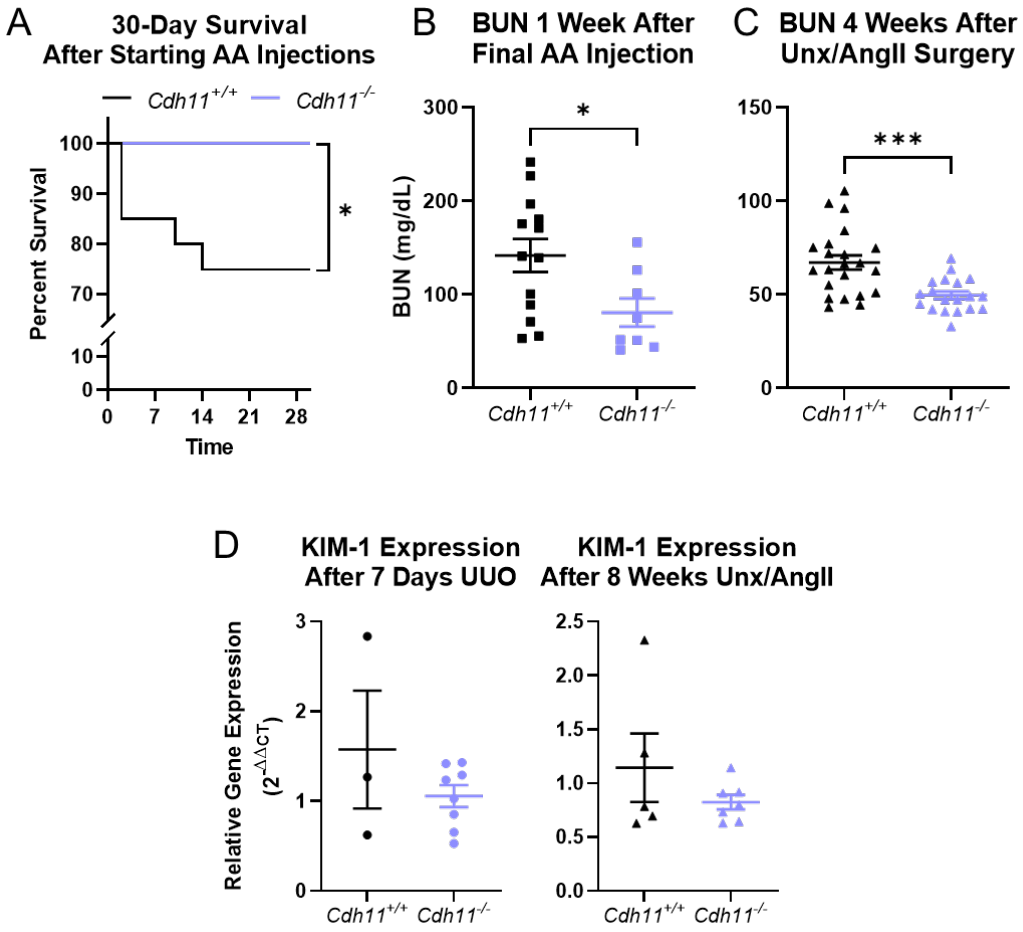

Figure 4.1 Genetic inhibition of CDH11 improves mortality and renal function
Cdh11$^{-/-}$ mice had significantly reduced mortality than *Cdh11*$^{+/+}$ mice over the course of 30 days after starting injections with AA (A). Plasma BUN levels were significantly reduced in *Cdh11*$^{-/-}$ mice compared to *Cdh11*$^{+/+}$ mice 1 week after final AA injection or 4 weeks after surgery in the AAN and Unx/AngII models, respectively (B,C). Although not significant, there may also be reduced expression of KIM-1 (*Havrc1*) in *Cdh11*$^{-/-}$ mice compared to *Cdh11*$^{+/+}$ mice after injury by UUO or Unx/AngII (D). * indicates $p \leq 0.05$; *** indicates $p \leq 0.001$ by Mankel-Cox log-rank test (A) or Student t test (B,C).

4.4.2 CDH11 inhibition reduces fibrosis and inflammatory signaling

Cdh11$^{-/-}$ mice had a reduction in tubulointerstitial fibrosis compared to *Cdh11*$^{+/+}$ counterparts in the UUO and Unx/AngII models. This is apparent in the significant reduction in collagen deposition 7 days after injury with UUO, measured by Picro Sirius Red staining (Figure 4.2A,B). This was complimented by a reduction in pro-fibrotic and pro-inflammatory signaling

markers TGF-β1 and IL-6, respectively, in both the UUO and Unx/AngII models. In the UUO model, TGF-β1 and IL-6 were significantly reduced ($p \leq 0.05$ and $p \leq 0.01$, respectively) 7 days after injury (Figure 4.2C). In the Unx/AngII model, only IL-6 was significantly reduced ($p \leq 0.05$),

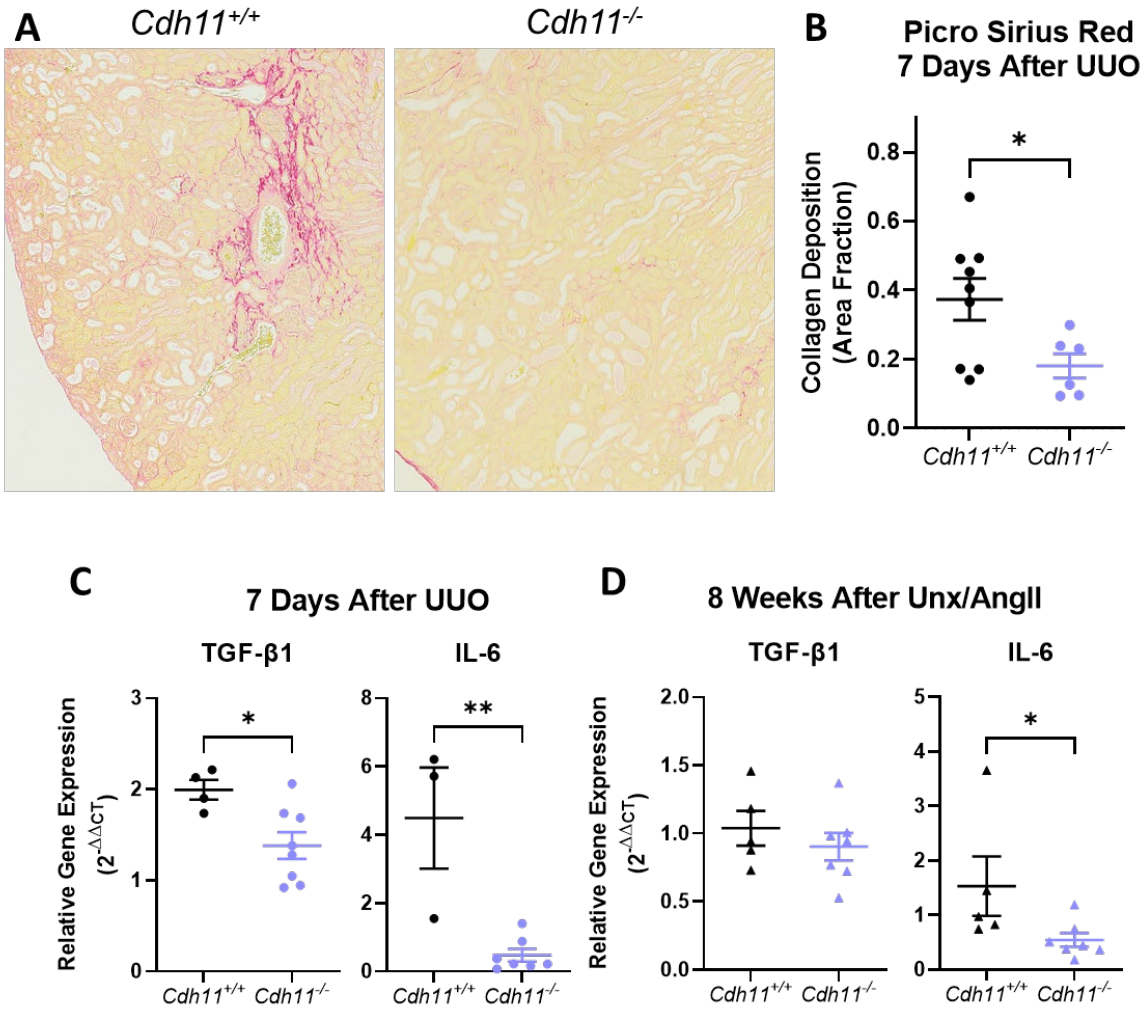

Figure 4.2 Knockout of CDH11 reduces fibrosis and inflammatory signaling
Inhibition of CDH11 resulted in significant reduction of ECM deposition, measured by a reduction in collagen staining using Picro Sirius Red, 7 days after injury with the UUO model (A,B). *Cdh11*$^{-/-}$ mice also had reduced expression of pro-fibrotic and pro-inflammatory markers TGF-β1 and IL-6 at 7 days after UUO (C). In the Unx/AngII model, *Cdh11*$^{-/-}$ mice had significantly reduced expression of IL-6 (D). * indicates $p \leq 0.05$; ** indicates $p \leq 0.01$ by Student t test. Images taken at 4X magnification.

but TGF-β1 may exhibit a downward trend (Figure 4.2D). These data clearly show a reduction in tubulointerstitial fibrosis and inflammatory signaling when CDH11 is knocked out.

4.5 Discussion

We have shown that genetic knockout of CDH11 is beneficial in three models of kidney injury, and results in improved renal function, reduced kidney injury, less collagen deposition, and diminished gene expression of profibrotic and proinflammatory cytokines. The results on renal function in the AAN model are particularly interesting, as the time point at which the blood was collected for measuring BUN was fairly early. The fact that CDH11 ablation improves renal function just one week after the final injection of AA implies that CDH11 may play a role in AKI. This was surprising to us, as most literature on CDH11 is in the context of fibrosis or cancer, rather than acute stages or forms of disease. However, expression of CDH11 by PTECs may partially explain the ability of CDH11 to play a role in AKI, as PTECs are usually the primary cell population that is affected by the injury. CDH11 ablation also improved renal function in the Unx/AngII model, indicating that it may also play a role in CKD, although the time point measured for BUNs was also on the early side for this model. These data combined with the fact that CDH11 is expressed in PTECs seem to suggest that genetic knockout of CDH11 reduces PTEC injury or death. We were therefore surprised not to see a greater difference in expression of KIM-1 between the *Cdh11*$^{+/+}$ and *Cdh11*$^{-/-}$ mice. However, this may simply be due to high variability in expression or measurement of this gene, so adding additional mice to these studies may clarify the issue further. Additionally, KIM-1 expression is fairly transient, so if the mice had been sacrificed at earlier time points, it's possible we would have seen a reduction of expression in the *Cdh11*$^{-/-}$ mice.

This leads us to discuss the Unx/AngII model, where CDH11 expression did not seem to be elevated in the injured *Cdh11*$^{+/+}$ mice compared to uninjured mice in Chapter 3 (Figure 3.1C). It is clear from the renal function and gene expression data in Figure 4.1 and Figure 4.2 that genetic knockout of CDH11 influences injury severity in this model. Because the Unx/AngII model is so slow, with a chronic buildup of injury over time, it's possible that CDH11 expression

is more transient in this model than in the UUO or AAN models. To identify whether this is the case, mice could be sacrificed at different time points over the course of the Unx/AngII injury and evaluated for CDH11 expression. Additionally, perhaps a higher dose of AngII would create a more severe injury state where changes in CDH11 expression would be more obvious. Regardless of the reason for the lack of difference in CDH11 expression between uninjured and injured *Cdh11*$^{+/+}$ mice in the Unx/AngII, the significantly reduced expression in the *Cdh11*$^{-/-}$ mice is beneficial.

We were glad to see that genetic knockout of CDH11 resulted in a reduction in collagen deposition in the UUO model, which is the classic model of tubulointerstitial fibrosis. Reduced collagen deposition is a common theme with CDH11 inhibition in other models of fibrotic disease, so we were pleased to see that recapitulated in our model of kidney fibrosis. This could imply that the role of CDH11 in kidney injury is similar to its role in other fibrotic diseases, despite the fact that it is expressed in renal epithelial cells rather than fibroblasts or hematopoietic cells. As described in Chapter 1, CDH11 is a transmembrane protein, so it is capable of interacting with both extracellular and intracellular binding partners and signaling pathways. The fact that CDH11 expression is on epithelial cells in the kidney model, but still plays a significant role in reduction of collagen deposition implies that either 1) CDH11 is preserving the PTECs thoroughly enough that they are substantially less injured and secreting fewer profibrotic factors to activate myofibroblasts, or 2) CDH11 is mediating interaction of injured PTECs with other cell types in the kidney such that injured PTECs have less influence on disease severity and progression. The mechanism of CDH11 in kidney injury could certainly also be a combination of these two things, but due to the general lack of information on CDH11 signaling, it is difficult to speculate. Alternatively, given that the mice used in these studies had a genetic ablation of CDH11 present throughout the injury, it is also possible that knockout of CDH11 makes PTECs much more resistant to injury stimuli in the first place. Future chapters

will explore inhibition of CDH11 after injury has been initiated and the possible mechanisms associated with CDH11 expression in kidney injury.

Chapter 5

Pharmacological targeting of CDH11 improves outcomes in murine kidney injury models

Text for Chapter 5 was adapted from **Huffstater T**, Raddatz MA, Riley LA, Noll NA, Fogo AB, Gewin LS, Merryman WD. Cadherin-11 mediates kidney injury through alpha-1 antitrypsin. *In revision.*

5.1 Abstract

Introduction: The functional domain of CDH11 is extracellular, which makes it attractive as a potential therapeutic drug target. A functional blocking antibody for CDH11 has been used successfully in other models of fibrotic disease, such as MI, where administration of the CDH11 blocking antibody significantly reduced mortality and improved cardiac function following MI. Therapeutic options for CKD and AKI remain woefully inadequate, and the potential of CDH11 as a drug target for kidney disease has not been evaluated.

Methods: C57BL/6 mice were injured using the three injury models described in Chapter 3. Mice were administered the CDH11 blocking antibody (SYN0012) or an isotype control (IgG2a) either prophylactically or after injury had been established. Measures of renal function, fibrosis, and inflammatory signaling were evaluated.

Results: Functional inhibition of CDH11 using SYN0012 produced many of the same results as the genetic knockout of CDH11 described in Chapter 4. Prophylactic administration of SYN0012 improved renal function in the AAN model, as well as reduced fibrotic and renal injury markers in the UUO model. Treatment with SYN0012 after injury had been established in the

Unx/AngII model also resulted in improvements in renal function and reduced expression of kidney injury, fibrotic, and inflammatory markers.

Conclusions: CDH11 may be a powerful drug target for both preventing and treating kidney disease. While AKI is difficult to predict, preventative treatment would certainly be possible in some situations for CKD, and the ability to treat kidney injury would be groundbreaking.

5.2 Introduction

Pharmaceutical interventions for kidney injury largely consist of medicines that help to manage symptoms of declining renal function, rather than halting or reversing kidney damage. However, some preventative measures are possible, particularly for CKD, if kidney decline is identified at an early stage or the patient has comorbidities that enhance the likelihood of CKD, such as hypertension. These preventative measures primarily include diet changes and strategies to manage blood pressure (such as medication, exercise regimens, sleep schedules, and stress management techniques). If the patient is diligent, these can be very successful in preventing the progression of CKD. However, most patients don't learn they have CKD until the disease has progressed to an irreversible state, and the damage to the kidneys is severe. If patients progress to ESRD, they may also have to undergo periodic dialysis to rid themselves of the waste the kidneys can no longer filter out or get a kidney transplant. These procedures are extremely expensive, time consuming, and uncomfortable, as dialysis and surgery have their own risks and side effects.

CDH11 is a transmembrane protein, meaning it has extracellular, intracellular, and transmembrane domains. The function of CDH11 relies on its ability to form bonds on the extracellular side of the membrane, which then can transmit signals to the intracellular side of

the cell.[43,49,50,143] This extracellular domain makes CDH11 highly attractive as a potential drug target, as pharmaceuticals do not need to enter the cell to be effective. The CDH11 blocking antibody SYN0012 has been used in models of other fibrotic diseases, such as myocardial infarction, CAVD, and atherosclerosis, where it was highly effective at inhibiting CDH11 function and improving outcomes.[42,140,144] SYN0012 was evaluated in a Phase IIb clinical trial by Roche, and had a very good safety profile, with minimal side effects.[133] This could indicate that targeting CDH11 in the context of kidney disease may be relatively low risk, but CDH11 inhibition has not been previously investigated in this context.

5.3 Methods

Mice

All mouse experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee before their commencement. Mice used in this study were wild type C57BL/6 (The Jackson Laboratory, 000664).

Animal Studies

C57BL/6 male mice were injured using the UUO, AAN, and Unx/AngII models as described in Chapter 3. Blood was collected 7 days after the final injection of AA, and 4 weeks after surgery for the AAN and Unx/AngII models, respectively. Mice were sacrificed 7 days after surgery for the UUO model, 4 weeks after the final injection of AA for the AAN model, and 12 weeks after surgery for the Unx/AngII model. Euthanasia was performed as described in Chapter 3.

SYN0012 or isotype control (IgG2a) were administered at 10 mg/kg once per week for all models. In the UUO and AAN models, SYN0012 and IgG2a were given prophylactically. For the UUO model SYN0012 or IgG2a were given 1 week prior to the surgery, and for the AAN

model SYN0012 or IgG2a were given on the day of the first injection of AA and weekly thereafter. In the Unx/AngII model, SYN0012 or IgG2a were given 4 weeks after the surgery once injury was established, and every week thereafter until the end of the study at 12 weeks (for a total of 8 weeks of SYN0012 or IgG2a treatment).

Renal Function Measures

Renal function was determined by measuring BUN in plasma samples collected from mice in the AAN or Unx/AngII models as described in Chapter 4.

Quantitative Polymerase Chain Reaction

qPCR was performed as described in Chapter 3, using the primers listed in **Table 5-1**. *Gapdh* was used as a housekeeping gene. Data presented was calculated using the delta delta Ct method and statistical analysis was run on delta delta Ct values.

Table 5-1 qPCR primer sequences

Gene Target	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Gapdh	ATGACAATGAATACGGCTACAG	TCTCTTGCTCAGTGTCCTTG
Havrc1	AAACCAGAGATTCCCACACG	GTCGTGGGTCTTCCTGTAGC
Tgfβ1	CCTGGGTTGGAAGTGGATC	TTGGTTGTAGAGGGCAAGG
Col1α1	CATAAAGGGTCATCGTGGCT	TTGAGTCCGTCTTTGCCAG
Il6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
Tnfα	AGACCCTCACACTCAGATCA	TGTCTTTGAGATCCATGCCG

Histology

Upon dissection, a central section of each mouse kidney was excised and submerged in 10% neutral buffered formalin (NBF) and stored at 4°C overnight before being processed and embedded in paraffin by the VUMC Translational Pathology Shared Resource. Unstained sections were submitted to paraffin removal followed by staining with either Masson's Trichrome (MilliPore Sigma, HT15) or PicroSirius Red (Fisher Scientific, 5030077). For Masson's

Trichrome, images were taken at 10x. For PicroSirius Red, images were taken in brightfield at 4x, then compiled using Image Composite Editor. Compiled images of kidney sections were analyzed using MatLab code created by Matthew Bersi, PhD, which analyzed the area fraction of collagen and healthy tissue.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9. A 2-tailed Student *t* test was used for comparisons of two groups. Applicable tests and significance are labeled in figure captions with $p \leq 0.05$ as the cutoff for data to be considered significantly different.

5.4 Results

5.4.1 Inhibition of CDH11 mitigates renal injury and fibrosis after UUO

To evaluate the efficacy of pharmacological CDH11 inhibition in the UUO model, SYN0012 or IgG2a were administered one week prior to UUO surgery. This dosing regimen was chosen to ensure the drug had ample time to inhibit CDH11 function given how rapidly the UUO model causes injury. Administration of SYN0012 significantly reduced gene expression of the PTEC injury marker KIM-1 (*Havrc1*) and the profibrotic markers TGF-β1 and Col1α1 (all $p \leq 0.05$) at 7 days following a UUO compared to mice treated with IgG2a (Figure 5.1A-C). SYN0012 administration also resulted in a significant reduction in collagen deposition ($p \leq 0.05$)

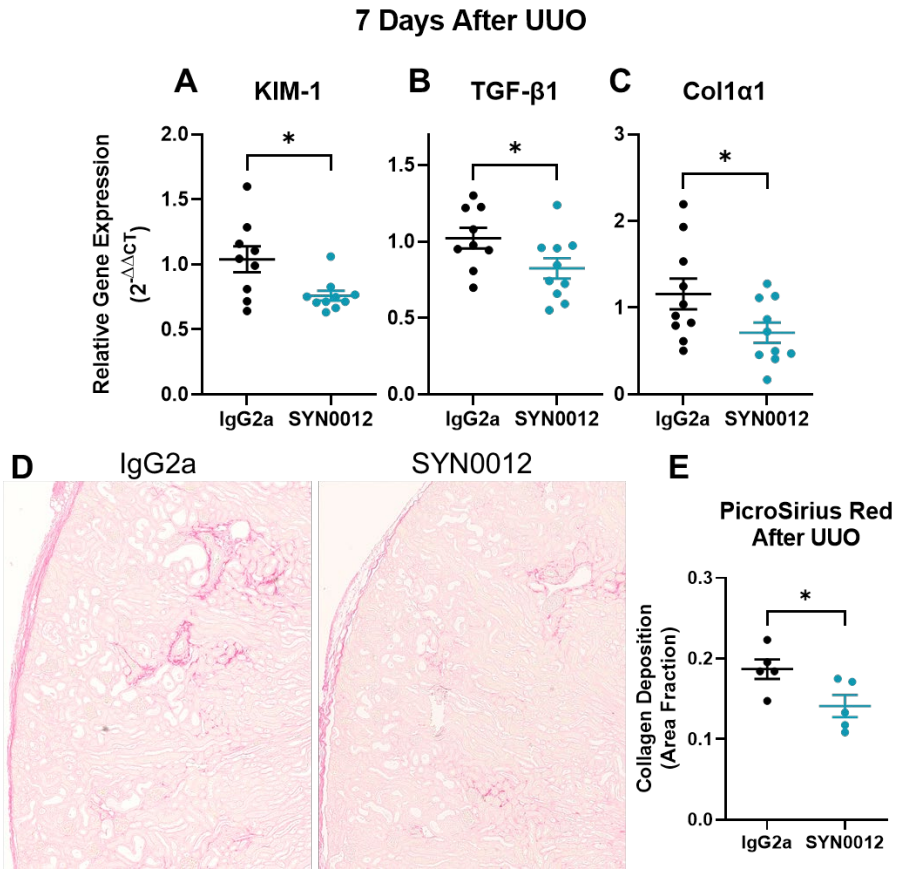

Figure 5.1 Inhibition of CDH11 with SYN0012 reduces renal injury and fibrosis after UUO
Administration of the CDH11 blocking antibody SYN0012 results in a significant reduction in KIM-1 (A), TGF-β1 (B), and Col1α1 (C) compared to mice given IgG2a at 7 days after injury with the UUO model. SYN0012 also resulted in a significant reduction in collagen deposition, determined by quantification of images of PicroSirius Red stained kidney sections (D,E). * indicates $p \leq 0.05$ by Student t test. Images taken at 4X magnification.

7 days after the UUO compared to mice given IgG2a, measured by image quantification of PicroSirius Red staining (Figure 5.1D,E). This data demonstrates that inhibition of CDH11 with a functional blocking antibody can reduce kidney injury and fibrosis.

5.4.2 Inhibition of CDH11 improves renal function in AAN model

To evaluate the efficacy of pharmacologic inhibition of CDH11 in the AAN model, SYN0012 or IgG2a were administered on the day of the first injections of AA and continued them each week throughout the study. Mice treated prophylactically with SYN0012 had significantly reduced plasma BUN levels ($p \leq 0.05$) compared to mice treated with IgG2a at 7 days following the final injection of AA, indicating CDH11 inhibition improved renal function

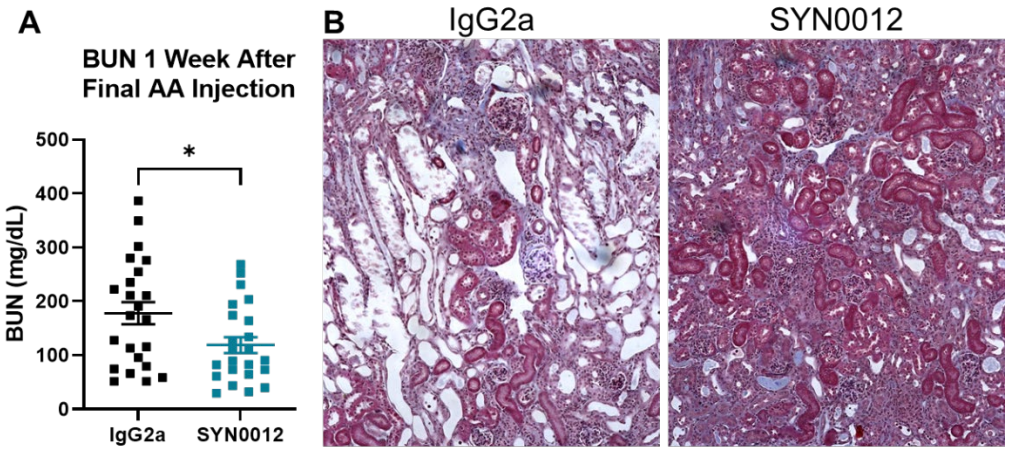

Figure 5.2 SYN0012 administration improves renal function and morphology after AA injections

Administration of SYN0012 significantly reduced plasma BUN levels compared to mice given IgG2a at 1 week following the final AA injection (A). SYN0012 administration also resulted in improvement in morphology and tubule structure 4 weeks after the final AA injection by evaluation of sections stained with Masson's Trichrome (B). * indicates $p \leq 0.05$ by Student t test. Images taken at 10X magnification.

(Figure 5.2A). Additionally, mice given SYN0012 had dramatically preserved morphology compared to mice given IgG2a, which had substantial loss of tubule structures at 4 weeks after the final injection of AA (Figure 5.2B).

5.4.3 Treatment with SYN0012 reduces renal injury and fibrosis after establishment of injury with Unx/AngII model

To identify whether inhibition of CDH11 would be useful as a treatment after injury has been established, rather than solely as a prophylactic, we administered SYN0012 or IgG2a 4 weeks after the Unx/AngII surgery. Injections of SYN0012 and IgG2a were continued every week for an additional 8 weeks before mice were sacrificed following 12 weeks of injury. Administration of SYN0012 resulted in a significant reduction in plasma BUN levels ($p \leq 0.05$) just 4 weeks later (8 weeks total injury) compared to mice treated with IgG2a (Figure 5.3A). SYN0012 administration also significantly reduced gene expression of PTEC injury marker KIM-1, profibrotic marker TGF-β1, and inflammatory marker TNF-α ($p \leq 0.05$) compared to mice given IgG2a (Figure 5.3B,C). Additionally, while not significant, the profibrotic marker Col1α1

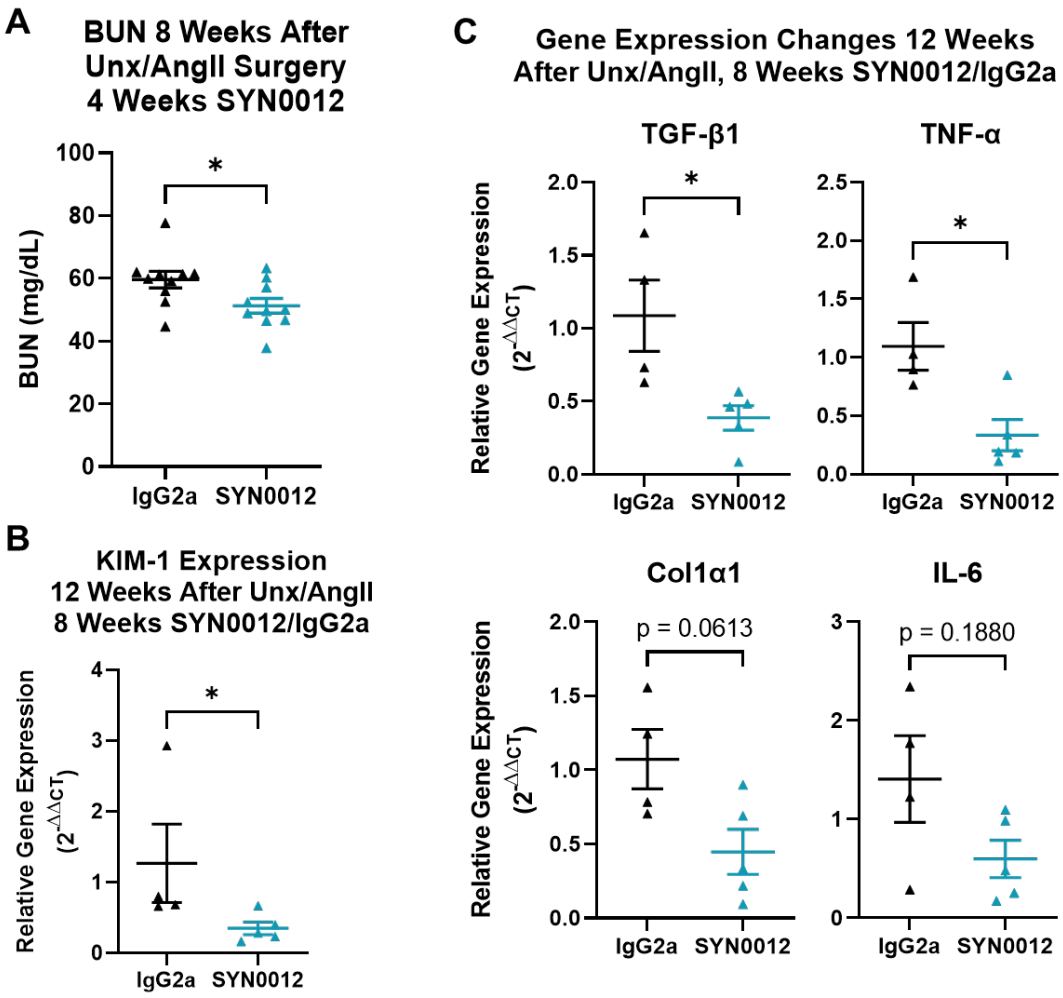

Figure 5.3 SYN0012 mitigates renal injury when administered 4 weeks after initiation of Unx/AngII injury model

Administration of SYN0012 for 4 weeks after 4 weeks of injury initiation significantly reduced plasma BUN levels compared to mice given IgG2a (A). Expression of the PTEC injury marker KIM-1 was also significantly reduced in the mice treated with SYN0012 compared to those given IgG2a (B). SYN0012 administration also significantly reduced expression of profibrotic and proinflammatory cytokines TGF-β1 and TNF-α (C). While not significant, there may also be a downward trend in expression of Col1α1 and IL-6 in mice treated with SYN0012 compared to those given IgG2a (C). * indicates $p \leq 0.05$ by Student t test.

and the inflammatory marker IL-6 also had gene expression that trended towards reduction with treatment of SYN0012 compared to mice given IgG2a (Figure 5.3C).

5.5 Discussion

We have demonstrated that CDH11 can be effectively inhibited pharmacologically using the functional blocking antibody SYN0012 and that this inhibition recapitulates many of the

results seen in the genetic knockout models. In some instances in the kidney injury models we used in this study, it appears as though the CDH11 blocking antibody had a stronger effect than genetic knockout of CDH11. We believe it is unlikely that the antibody is more efficient than an actual genetic deletion, and we attribute this difference instead to the different strains of mice used in these studies. The mixed background that the transgenic *Cdh11*$^{+/+}$ and *Cdh11*$^{-/-}$ come from tends to be relatively hardy compared to the C57BL/6 mice obtained from commercial sources. Differences in how injuries manifest in different strains of mice have been well documented in the field of nephrology and are certainly enough to explain these results.

Prophylactic administration of SYN0012 was delivered a week before the surgery in the UUO model and on the first day of injections in the AAN model. Due to the intensity and rapidity with which the UUO causes kidney damage, we wanted to ensure that SYN0012 would have ample time to permeate the tissue and inhibit CDH11 as soon as its expression was increased after the surgery. We were pleased with the substantial effect SYN0012 administration had on reducing kidney injury, fibrosis, and inflammatory signaling in the UUO model, but for the same reasons we administered SYN0012 a week ahead of the surgery, we're not sure SYN0012 administration would show efficacy as a treatment in this model. Regardless, this data clearly demonstrates that pharmacologic inhibition of CDH11 is sufficient to hamper fibrotic processes in kidney disease, similar to the effects seen in other fibrotic diseases such as CAVD, dermal fibrosis, pulmonary fibrosis, or MI.

The results in the UUO model identify CDH11 inhibition as a potential preventative strategy for patients at risk of developing CKD. The results of prophylactic SYN0012 treatment in the AAN model suggest that inhibiting CDH11 could also mitigate the severity of AKI. While the AAN model we use is generally an AKI to CKD model, the time point when blood was collected for measurement of plasma BUN levels lies in the acute phase of the injury. These results recapitulate those seen in the AAN model with CDH11 transgenic mice. The combination

of these two instances further strengthens the evidence of a role for CDH11 not only in CKD, but also in AKI. While patients usually aren't diagnosed with AKI until several days after the injury occurred, there are specific instances, such as cardiothoracic surgery, where there is higher risk of AKI and preventative action may be warranted. Even so, inhibition of CDH11 as a preventative therapy would likely be far more applicable to patients developing CKD. In this situation, patients with elevated blood pressure, diabetes, or weight management difficulties could be prescribed with CDH11-inhibiting pharmaceuticals to prevent further damage to the kidneys. Such a proactive treatment strategy could only be employed if the preventative drugs were abundantly safe, and SYN0012 had minimal side effects in a Phase IIb clinical trial.[133] The realities of administering a biologic such as SYN0012 for preventative therapy are challenging, but the safety of SYN0012 could speak to the general safety of CDH11 inhibition if small molecule options became available.

While the prophylactic administration studies were promising, the truly exciting data is the Unx/AngII treatment study. Here, SYN0012 was administered 4 weeks after Unx/AngII surgery, so the injury was initiated before CDH11 inhibition began. As described above, one of the primary clinical difficulties of AKI and CKD is that patients are not diagnosed until the damage to the kidneys is substantial. Therefore, the ability of CDH11 inhibition to improve

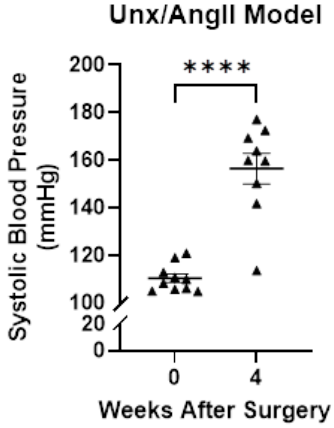

Figure 5.4 Systolic blood pressure 4 weeks after Unx/AngII surgery
4 weeks after Unx/AngII surgery, C57BL/6 mice had a substantial increase in systolic blood pressure. At baseline, the average blood pressure of these mice was approximately 110 mmHg, while 4 weeks after surgery it was approximately 160 mmHg. **** indicates $p \leq 0.0001$ by Student t test.

outcomes after initiation of injury is very clinically relevant to human disease. In addition, the Unx/AngII model in particular is more physiologically relevant for most patients who develop CKD than the UUO or AAN models. If symptoms could be identified at the early stages of CKD, perhaps treatment with a CDH11 inhibitor could prevent further progression or even reverse the damage enough for regeneration to occur. While the Unx/AngII model develops slowly, we ensured that mice were experiencing hypertensive injury before beginning administration of SYN0012. At 4 weeks following the Unx/AngII surgery, mice had significantly elevated ($p \leq 0.0001$) systolic blood pressure (Figure 5.4). If this experimental model were applied to human patients, SYN0012 (or a more convenient small molecule drug that also inhibits CDH11) could be administered to hypertensive patients or patients with some measure of decline in renal function. Reduction of renal injury, fibrosis, and inflammatory signaling as seen in this murine model would certainly also benefit hypertensive patients. However, such a strategy would depend upon CDH11 inhibition being safe, and more understanding regarding the role of CDH11 in disease is needed before such treatments could be safely applied in the clinic. The next chapter will discuss genetic alteration effects of CDH11 inhibition and potential mechanisms through which CDH11 mediates kidney injury.

Chapter 6

CDH11 mediates renal injury through alpha-1 antitrypsin suppression

Text for Chapter 6 was adapted from **Huffstater T**, Raddatz MA, Riley LA, Noll NA, Fogo AB, Gewin LS, Merryman WD. Cadherin-11 mediates kidney injury through alpha-1 antitrypsin. *In revision.*

6.1 Abstract

Introduction: Little is known about the functional role of CDH11 in fibrotic disease. CDH11 can form homotypic bonds with other CDH11s, but it has also been shown to interact with and alter several other proteins, both intracellularly and extracellularly. In some fibrotic diseases, such as rheumatoid arthritis or CAVD, CDH11 is known to have a role in mechanosensitive pathways, but in other contexts it also participates in molecular signaling pathways. Thus, the signaling of CDH11 in kidney injury remains unclear.

Methods: Kidneys from *Cdh11*$^{+/+}$ and *Cdh11*$^{-/-}$ mice injured with the AAN and Unx/AngII injury models were collected for analysis with RNA sequencing. Immortalized PTECs were treated with siRNA to knock down CDH11 in the absence of injury and evaluated for changes in protein expression. Deidentified patient records were analyzed for a relationship between genes altered by CDH11 expression and development of CKD.

Results: RNA sequencing revealed 51 genes that had significantly altered expression in both injury models when CDH11 was ablated. Through literature analysis and contextualization with other *in vivo* data, alpha-1 antitrypsin (AAT) was identified as a likely target through which CDH11 mediates kidney injury. Expression of AAT was significantly elevated in *Cdh11*$^{-/-}$ mice compared to *Cdh11*$^{+/+}$ counterparts, and AAT expression is inversely correlated with CDH11

expression *in vitro*. Patients with AAT deficiency (AATd) have significantly elevated risk of developing CKD.

Conclusions: AAT has never been described in association with CDH11 or CKD. Expression of AAT seems to be beneficial in multiple disease states, so this relationship with CDH11 is very interesting. Inhibition of CDH11 likely results in improved outcomes in models of kidney injury due to increased expression and production of AAT.

6.2 Introduction

There is relatively little information on downstream signaling targets of CDH11, particularly in the context of kidney disease. In other fibrotic diseases, such as CAVD and MI, many of the effects of CDH11 inhibition can be attributed to changes in tissue stiffness or migration of fibroblasts and immune cells, which largely have to do with the mechanosensitive aspects of CDH11 signaling.[42,49,140] CDH11 forms very strong homotypic bonds with other CDH11 proteins and connects intracellularly to the cytoskeleton. CDH11 is also the only member of the cadherin family known to participate in focal adhesions, allowing it to alter signaling based on sensing the extracellular environment.[143] CDH11 has also been shown to interact with profibrotic and proinflammatory signaling pathways, as CDH11 expression both induces and is induced by TGF-β1, and CDH11 expression stimulates IL-6 expression. CDH11 is also known to form a complex with β-catenin on the intracellular side of the membrane, effectively sequestering β-catenin in the cytosol.[145] This may inhibit canonical Wnt/β-catenin and other β-catenin-mediated signaling pathways, as it prevents translocation of β-catenin to the nucleus.

Many, if not all, of the signaling pathways CDH11 has been associated with are also known to play a role in kidney injury. For example, PTECs have been shown to alter their

differentiation *in vitro* in response to different substrate stiffnesses.[146] Additionally, TGF-β1, IL-6, and β-catenin (see Chapter 2) all have been shown to have profound effects on kidney injury. Trying to elucidate potential mechanisms of CDH11 in kidney injury become more complicated when the results from Chapters 4 and 5 are considered. For example, CDH11 expression would generally decrease Wnt/β-catenin signaling by sequestering β-catenin in the cytosol, so inhibition of CDH11 could reactivate Wnt/β-catenin signaling that would normally be silenced in PTECs after injury. Most studies show that Wnt/β-catenin signaling is beneficial in AKI but detrimental in CKD, although there is some speculation on the effects of Wnt/β-catenin signaling in CKD based on which cell types it's happening in (see Chapter 2).[147] However, the results of Chapters 4 and 5 suggest that inhibition of CDH11 is beneficial in both AKI and CKD conditions. For these reasons, a deeper understanding of the transcriptional and molecular signaling changes associated with CDH11 inhibition is needed, particularly in the context of kidney injury.

6.3 Methods

Mice

All mouse experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee before their commencement. Mice used in this study were transgenic mutants on a mixed background, either wild type (*Cdh11*$^{+/+}$) or global genetic knockout of CDH11 (*Cdh11*$^{-/-}$).

Animal Studies

Male CDH11 mutant mice were injured using the AAN and Unx/AngII models as described in Chapter 3. Mice were sacrificed 2 weeks after the final injection of AA for the AAN model, and 4 weeks after surgery for the Unx/AngII model. Euthanasia was performed as described in Chapter 3.

RNA Sequencing

Kidneys were dissected under RNase-free conditions and immediately snap frozen. Samples were then thawed and homogenized in Trizol reagent using a bead beater and lysis matrix tubes (Lysis Matrix D, MP Biomedicals). RNA was isolated using the Direct-zol RNA Miniprep Kit (Zymo, R2050) RNA integrity was measured with an Agilent Bioanalyzer prior to library preparations.

The Vanderbilt Technologies for Advanced Genomics (VANTAGE) center performed library preparation, sequencing, and read alignment. Briefly, cDNA libraries were generated using NEBNext Ultra II Directional RNA Library Prep kits (New England BioLabs, E7760) then sequenced on an Illumina NovaSeq 6000 using 150bp paired-end chemistry. Sequencing quality was assessed using FastQC v. 1.0.0. Reads were aligned to mouse genome mm10 using a STAR based aligner and gene counts were quantified, both using Illumina's DRAGEN RNA Pipeline v. 3.6.3. Gene count data was imported to R using tximport for further analysis.

Differential gene expression analysis was performed using the R package DEseq2 using Cook's outliers to filter low gene counts and $\alpha = 0.05$. GO and over-representation analysis was performed with the R package clusterProfiler using the respective *enrich* function with default parameters. Gene sets were considered over-represented if $p \leq 0.05$. Visualizations were generated using a combination of enrichplot and ggplot2 in R. RNA sequencing data generated in this manuscript have been deposited in GEO (Gene Expression Ominbus) of NCBI under accession number GSE189682.

Cell Culture

Immortalized PTECs were obtained from the Gewin lab, which were purified as previously described.[148,149] Cells were cultured at 33°C in DMEM/F12 supplemented with 5%

FBS, 1% penicillin/streptomycin, and 10 μg/mL recombinant murine interferon γ to induce activation of the simian virus 40 T antigen.

Transfection

24 hours prior to beginning the experiment, cells were moved to 37°C and media was changed to DMEM/F12 supplemented only with 5% FBS. 2 hours prior to beginning the experiment, media was exchanged for warmed OptiMEM (Gibco, 31985-070). siRNA non-targeting or CDH11 knockdown (UAUCACAAAGAAUUGGUUC, starts at Cdh11 677) constructs were administered at a concentration of 25 μM with Lipofectamine 3000 reagent. 10 μg of empty or previously described CDH11 overexpression plasmid was administered to PTECs in 10-cm dishes, with Lipofectamine 3000 and P3000 reagents (Invitrogen, L3000-015).[150] For both siRNA and plasmid transfections, transfection was performed overnight, with media changed back to DMEM/F12 with 5% FBS in the morning. Cells were then maintained for 72 hours before being collected with RIPA buffer with protease inhibitors.

Western Blot

Cells were lysed in RIPA buffer with protease inhibitors and frozen at -80°C. Protein lysates were denatured using β-mercaptoethanol and heat (10 minutes at 95°C). 10% polyacrylamide gels were used for gel electrophoresis to separate proteins. Proteins were transferred to activated PVDF membranes and blocked with 5% milk to prevent non-specific antibody binding. Membranes were incubated serially in primary antibody followed by fluorescently tagged secondary antibodies. Membranes were imaged using a Digital Chemidoc MP imaging system and ImageJ was used to analyze the images and perform densitometry. Antibodies used were α-tubulin for normalizing total protein, CDH11 (Cell Signaling, 4442), and AAT (Abcam, ab231093).

Human Chronic Kidney Disease Analysis

Using the Synthetic Derivative, a de-identified version of the electronic medical record at Vanderbilt University Medical Center (VUMC), we identified 10,242 patients who were first tested for alpha-1 antitrypsin deficiency (AATd) at 20 years of age or older at VUMC. AATd test results were reported as the identification of two alleles. These identities were extracted and mutant alleles quantified for regression analysis. Analysis of incident chronic kidney disease (CKD) was performed using a Cox proportional hazards model including age, sex, body mass index, self-reported race, ethnicity, hypertension, diabetes, and individual numbers of Z, F, and S alleles. Patients with AAT alleles other than the reference M, or mutant Z, F, or S were excluded from the study. Individual patients were censored at time of transplant of any organ, development of hepatorenal syndrome, or last recorded follow-up. CKD endpoint, censorship criteria, and disease covariates were defined by two relevant ICD codes, with the timing defined at the first occurrence. Disease covariates were included as present if they occurred at any time prior to or during the study. The incident analysis was censored at 8 years, after which less than 10% of study participants remained. Analyses were performed using the statistical programming language R, version 4.0.2.[151]

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9. A 2-tailed Student *t* test was used for comparisons of two groups. Simple linear regression was used for determining correlation between two factors. Applicable tests and significance are labeled in figure captions with $p \leq 0.05$ as the cutoff for data to be considered significantly different.

6.4 Results

6.4.1 Ablation of CDH11 results in many genetic differences in AAN and Unx/AngII models

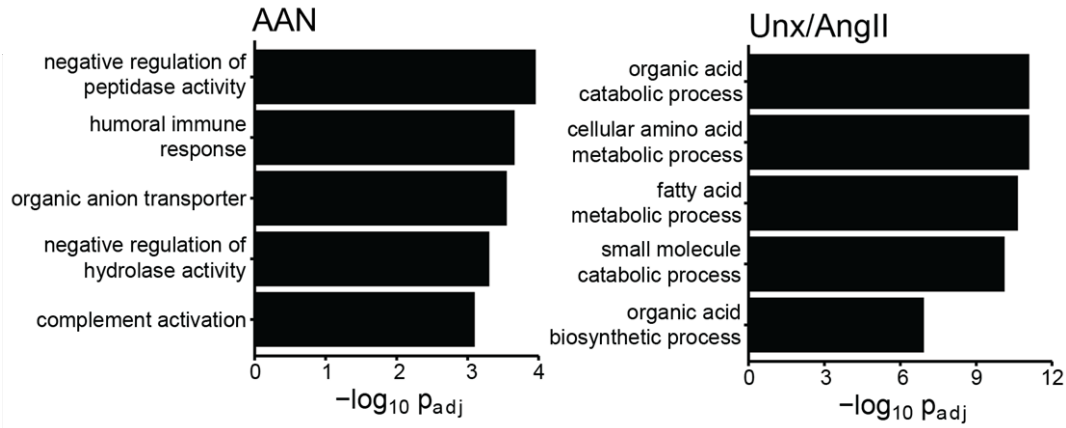

Figure 6.1 GO over-representation analysis identified enrichment of several gene categories when CDH11 was ablated in AAN and Unx/AngII models
In the AAN injury model, the gene clusters that were most significantly changed had to do with enzyme regulation and immune cell recruitment (left). In the Unx/AngII injury model, the most significantly altered gene clusters all had to do with metabolism, mostly of organic, amino, or fatty acids (right).

RNAseq analysis identified 384 and 5181 gene expression changes in *Cdh11*$^{-/-}$ mice compared to *Cdh11*$^{+/+}$ mice injured by AAN and Unx/AngII, respectively. Using GO over-representation analysis, we identified significant enrichment of several gene categories when CDH11 was ablated in each injury model (Figure 6.1). The most significantly changed clusters of genes in the AAN injury model largely had to do with enzyme regulation, including regulation of peptidase and hydrolase activity and immune cell recruitment, such as humoral immune response and complement activation. In the Unx/AngII injury model, gene clusters associatd with metabolism were the most altered, including catabolic and metabolic processes associated with organic, cellular amino, and fatty acids.

6.4.2 Genetic knockout of CDH11 resulted in 51 shared genetic changes in AAN and Unx/AngII models

As the changes reported by the GO over-expression analysis did not point to a common underlying mechanism with knockout of CDH11, we examined individual genes that were significantly different in each comparison and changed in the same direction to generate a CDH11-specific list of genetic alterations. We identified CDH11-dependent gene expression changes that were common to both models, and discovered 51 shared genetic changes associated with CDH11 knockout (Figure 6.2A). The top 5 most up- and down-regulated genes

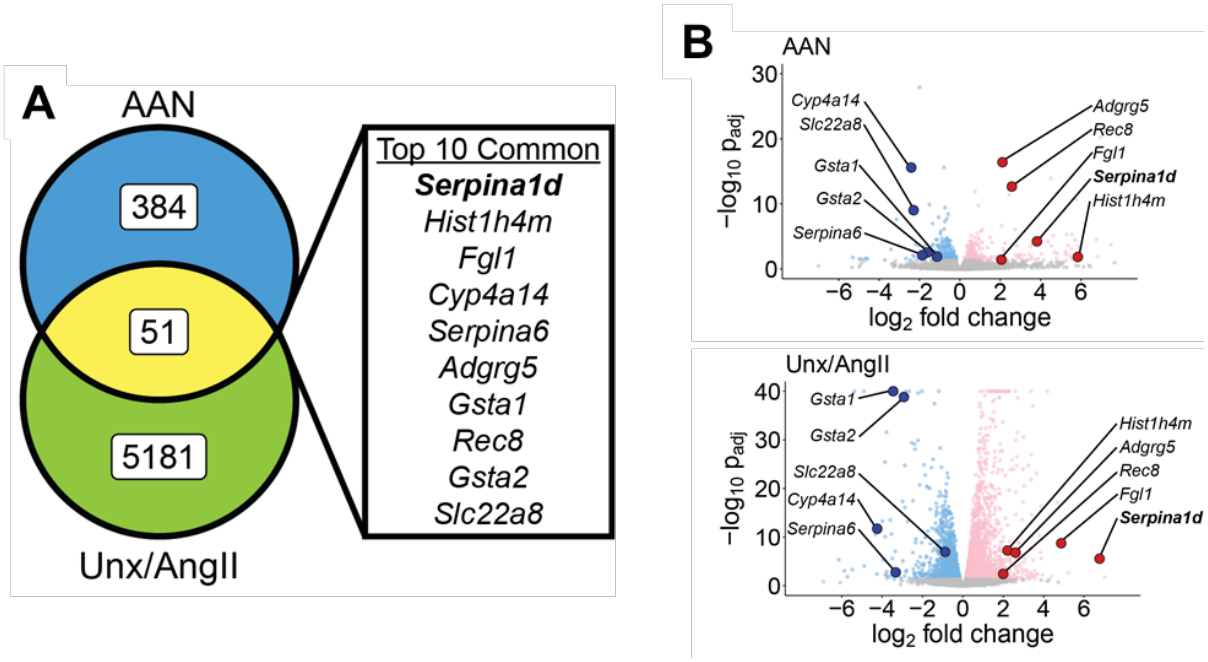

Figure 6.2 CDH11 ablation results in 51 shared genetic alterations in AAN and Unx/AngII models

In comparing individual genes in the AAN and Unx/AngII models, 51 genes were significantly altered in the same direction in both models. The top 5 most up- and down-regulated genes were evaluated for relevance to kidney injury and potential interaction with CDH11.

(*Serpina1d*, *Hist1h4m*, *Fgl1*, *Adgrg5*, and *Rec8* upregulated; *CYP4a14*, *Serpina6*, *Gsta1*, *Gsta2*, and *Slc22a8* downregulated) from this combined analysis were then evaluated in the literature for relevance to kidney disease and comparison with the effects we had seen in our *in vivo* injury models with CDH11 knockout, as described in Chapter 4 (Figure 6.2B). From this analysis, we identified alpha-1 antitrypsin (AAT) as a standout mechanistic candidate, as it has been implicated in epithelial injury, fibrosis, and inflammation in other diseases.

6.4.3 AAT expression is inversely related to CDH11 expression *in vivo* and *in vitro*

To confirm the results of the RNAseq analysis, we evaluated protein expression of AAT in the kidney both *in vivo* and *in vitro*. At 4 weeks after the final injection of AA, the *Cdh11*$^{-/-}$ mice had significantly increased protein expression of AAT ($p \leq 0.001$) compared to *Cdh11*$^{+/+}$ mice (Figure 6.3A). Additionally, expression of AAT in the injured *Cdh11*$^{-/-}$ mice was much more similar to the levels of AAT expression in the uninjured kidneys of both *Cdh11*$^{+/+}$ and *Cdh11*$^{-/-}$

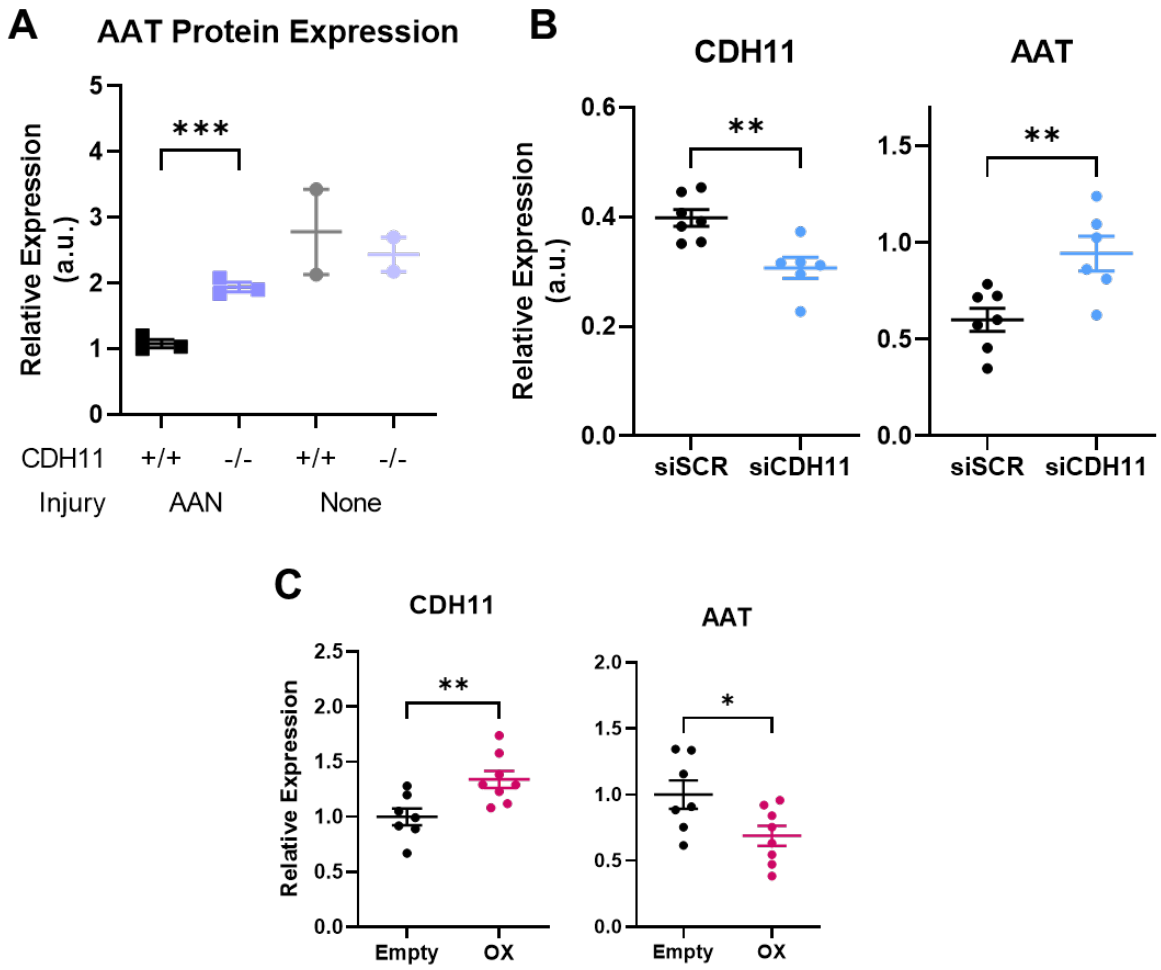

Figure 6.3 Protein expression of AAT is inversely related to CDH11 expression *in vivo* and *in vitro*

In kidneys injured with the AAN model, *Cdh11*$^{-/-}$ mice had significantly elevated protein expression of AAT compared to that of *Cdh11*$^{+/+}$ counterparts (A). When CDH11 is knocked down in PTECs *in vitro* using siRNA, the expression of AAT is increased while CDH11 expression is increased (B). When PTECs were administered a CDH11 overexpression plasmid, expression of AAT was decreased when CDH11 expression increased (C). * indicates $p \leq 0.05$; ** indicates $p \leq 0.01$; *** indicates $p \leq 0.001$ by Student t test.

mice (Figure 6.3A). To evaluate whether the difference in AAT expression was due to actual changes in CDH11 expression or reduced level of injury in the *Cdh11*$^{-/-}$ mice, we performed siRNA experiments on immortalized PTECs *in vitro*. In these experiments, we applied an siRNA CDH11 knockdown or scramble construct to cultured PTECs and evaluated the cells for expression of both CDH11 and AAT. We found that protein expression of CDH11 was decreased ($p \leq 0.01$) and protein expression of AAT was increased ($p \leq 0.01$) in the cells treated with CDH11 knockdown siRNA compared to those treated with non-targeting siRNA (Figure 6.3B). Additionally, to reflect the state of injury we see *in vivo* more accurately, we administered a plasmid designed to overexpress CDH11 to the cultured PTECs. Here we found that we were able to significantly increase protein expression of CDH11 ($p \leq 0.01$) and decrease ($p \leq 0.05$) AAT protein expression (Figure 6.3C).

6.4.4 Patients with AATd have substantially increased risk of developing CKD

Finally, to assess the clinical relevance of AAT expression in kidney injury, we evaluated deidentified records of patients tested for the human genetic disease alpha-1 antitrypsin deficiency (AATd). In this disease, Z alleles prevent secretion and function of AAT. Patients homozygous for the Z allele typically have 10-20% the normal concentration of circulating AAT. We analyzed incident CKD, defined by ICD codes, in patients genotyped for AAT deficiency. Both heterozygous (HR = 1.85 [95% CI: 1.44 - 2.38]) and homozygous Z allele genotypes (5.35 [3.07 - 9.31]) were associated with significantly higher risk of CKD in a dose-dependent fashion (Figure 6.4A). In sensitivity analysis, risk ratios were elevated for all stages of CKD (Figure 6.4B). As a secondary outcome, we assessed the association of AAT genotype with creatinine and BUN at the time of diagnosis among those with relevant clinical laboratory values. BUN values were significantly higher in both heterozygous and homozygous Z allele patients (median [IQR] vs median [IQR]) (Figure 6.4C). Creatinine values were higher only in the heterozygous

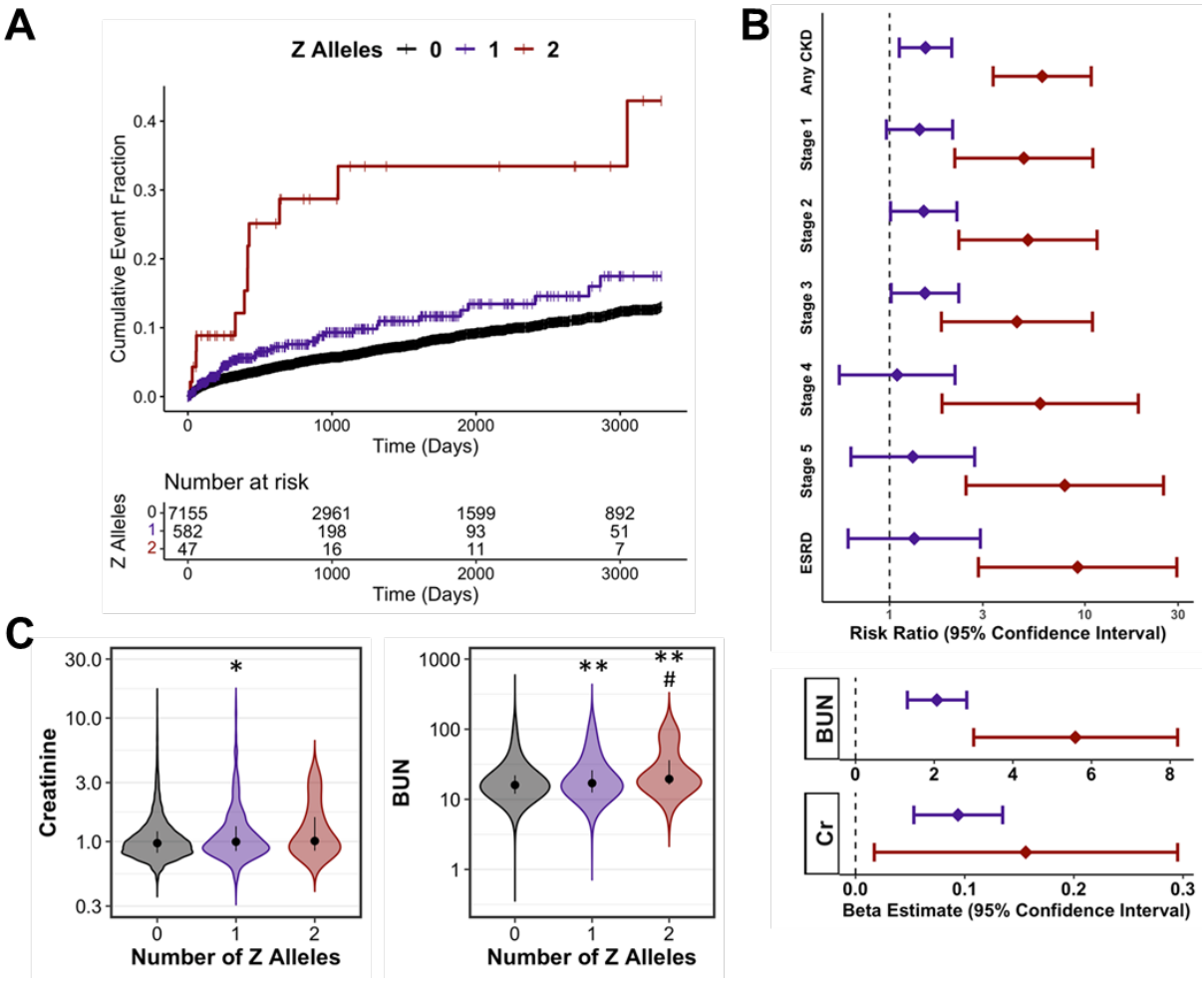

Figure 6.4 Patients with alpha-1 antitrypsin deficiency have increased risk of developing CKD

Patients with allele mutations in the AAT gene had substantially higher risk of developing CKD in their lifetime, defined by ICD codes, in a dose-dependent fashion (A). In sensitivity analysis, risk ratios were elevated for all stages of CKD including ESRD (B). Clinical laboratory values creatinine and BUN were also evaluated in patients with AATd (C).

group. In fully adjusted linear regression models, both heterozygous and homozygous Z alleles were associated with an increase in both BUN (Beta [95% CI]) and creatinine (Beta [95% CI]).

6.5 Discussion

We identified over 50 genes that had altered expression in kidneys of *Cdh11*$^{-/-}$ mice compared to those of *Cdh11*$^{+/+}$ mice in two models of kidney injury. Because the models of kidney injury used in this work cause kidney damage in such different ways, we evaluated

multiple models to identify changes that were not particular to a certain type of injury, but specific to CDH11 expression and signaling. The validity of this strategy is emphasized by the significant difference in the types of gene clusters identified as altered in the GO over-representation analysis (Figure 6.1). While it would have been interesting if knockout of CDH11 expression caused particular gene clusters to be significantly altered in both injury models, we were not concerned that these did not show much overlap because the two injury models are so different. This brought us to evaluate genetic alterations on the level of individual genes, were we saw significant overlap of gene expression changes in both models. It should be noted that more genetic changes were identified in the Unx/AngII model overall because the sequencing core mistakenly measured far more reads (or deeper read depth) in these samples than in the AAN samples (Figure 6.2). The difference in the number of altered genes therefore does not reflect how much of an effect CDH11 had on the injury, but rather this difference in read depth. Nonetheless, we were pleased to see that 51 genes were significantly altered in the same direction (up in both or down in both) in both injury models when CDH11 was ablated. This suggests that regardless of the specific cause of kidney injury, inhibition of CDH11 has substantial effects on gene expression and signaling.

Of the 51 genes that were significantly altered in *Cdh11*$^{-/-}$ kidneys, we identified AAT (*Serpina1d*) as the most likely to play a role in mitigating kidney injury with CDH11 inhibition. This is because AAT has been thoroughly studied in many other fibrotic and inflammatory diseases, and increased expression of AAT or even treatment with exogenous AAT seems to be universally beneficial. In fact, AAT can even cause some of the same effects as inhibition of CDH11 in certain pathologies. For example, treatment with exogenous AAT suppressed collagen-induced arthritis in a mouse model of rheumatoid arthritis, and dampened expression of IL-6 in fibroblasts, thus reducing infiltration of immune cells into the joint.[152] AAT has also been associated with several other fibrotic diseases, including pulmonary and liver fibrosis.[153,154]

However, AAT is also implicated in more acute tissue injury, such as acute myocardial infarction.[155] In one study, it was suggested that an insufficient rise in AAT levels after acute cardiac injury was associated with significantly worse clinical outcomes, and another showed that AAT had protective effects on cardiomyocytes in a preclinical model of acute myocardial ischemia-reperfusion injury. Additionally, in a preclinical model of acute liver failure, administration of AAT corresponded with a significant reduction in hepatocyte apoptosis, diminished infiltration of inflammatory cells, and improved overall survival.[156] This suggests that increased expression of or treatment with exogenous AAT may be beneficial in both chronic and acute injury pathologies, which mimics the results seen with CDH11 inhibition in Chapters 4 and 5. Throughout the literature, expression of AAT appears to be beneficial in many specific conditions where CDH11 has been identified as detrimental. Therefore, the inverse relationship of CDH11 and AAT identified in this study may be a piece of the puzzle for how CDH11 inhibition mitigates kidney injury.

In the context of kidney injury, there are a few studies that suggest AAT is renoprotective.[157–160] Interestingly, most of these studies use models that are usually considered models of AKI, rather than CKD. We believe the focus on AKI is because the classic function of AAT is inhibition of neutrophil elastase and impairment of neutrophil invasion. Neutrophils tend to be on the early side of immune responses, so evaluating AKI for changes in neutrophil behavior makes sense. However, AAT is a protease inhibitor with a diverse array of targets, including neutrophil elastase, cathepsin G, proteinase 3, caspases (including caspase-3), and metalloproteinases including TNF-α-converting enzyme (ADAM-17).[156,161–163] We also found that many of these enzymes that are inhibited by AAT are detrimental in the context of kidney disease. For example, ADAM-17 signaling in PTECs has been shown to drive kidney fibrosis in preclinical models, and inhibition of ADAM-17 was identified as a therapeutic target for CKD.[164] Inhibition of neutrophil elastase has also been shown to be beneficial in models of CKD. AAT

has also been suggested to have antioxidant effects, which are also beneficial in models of acute and chronic kidney injury.[163,165,166] One group, using the UUO model of kidney fibrosis, identified that treatment with AAT significantly attenuated fibrosis and inflammation in the affected kidney.[160] In summary, AAT has been shown to have anti-apoptotic, antifibrotic, and anti-inflammatory effects, all of which are known to be beneficial in kidney injury and correspond with the results from the *in vivo* models of kidney injury used in the experiments described in Chapters 4 and 5. This strengthens the beneficial impact of CDH11 inhibition, as this results in increased expression of AAT in the kidney. These findings are further emphasized by the clinical relationship we identified between AATd and CKD, where reduction in functional AAT significantly increased the risk of developing all stages of CKD.

Chapter 7

Summary, broader impacts, and future directions

7.1 Summary and broader impact

This work investigated the role of CDH11 in kidney injury. Both AKI and CKD are increasing in prevalence among adults in the United States. AKI is associated with extended hospital stays, increased mortality and morbidity, and is an independent risk factor for the development of CKD later in life.[15,16] AKI can be generally described as a sudden loss of renal function, which is usually attributed to damage to or death of tubular epithelial cells. Such an injury sets off several cellular and molecular events, including recruitment of inflammatory cells, activation of myofibroblasts, and regeneration of injured tubular epithelial cells. However, if the injury is severe or sustained, these events, which are meant to constitute the recovery process, become dysregulated and can lead to fibrosis and inflammation, the hallmarks of progression to CKD.[20,21,28,167] CKD is likewise a risk factor for AKI and currently affects one of every seven adults in the United States.[6,7] Despite these substantial public health burdens, treatments for both AKI and CKD are limited. Kidney disease is highly prevalent worldwide, and treatment options are relatively limited given its global impact.

Recent studies identified increased CDH11 expression in mouse models of kidney fibrosis as well as in biopsies and urine samples of humans with CKD.[51] Additionally, inhibition of CDH11 has been shown to significantly improve outcomes in rheumatoid arthritis, myocardial infarction, pulmonary fibrosis, dermal fibrosis, and calcific aortic valve disease in mice.[38,43,44,48,50,168] However, whether CDH11 acted as a mediator of kidney injury had not previously been investigated. We used three distinct models to induce kidney injury in mice and

evaluate expression of CDH11 and the effects of its inhibition. We found that expression of CDH11 increases following an injury, and that the cells responsible for this expression are PTECs. We also showed that the expression of CDH11 in PTECs occurs in both mouse and human kidneys.

Next, we evaluated the role of CDH11 in kidney disease using transgenic mice. We found that *Cdh11*$^{-/-}$ mice had improved renal function, reduced kidney injury, reduced tubulointerstitial fibrosis, and diminished inflammatory signaling. These results were recapitulated in a model of pharmacologic CDH11 inhibition. In C57BL/6 mice, we inhibited CDH11 using a functional blocking antibody and also saw improved renal function, reduced kidney injury, reduced tubulointerstitial fibrosis, and diminished inflammatory signaling compared to the mice given an isotype control. In both genetic and pharmacologic inhibition of CDH11, plasma BUN levels were reduced compared to mice with uninhibited CDH11 activity, and in some cases, expression of the proximal tubule injury marker KIM-1 was also diminished with CDH11 inhibition. These results imply that inhibition of CDH11 has a protective effect in PTECs. Additionally, inhibition of CDH11 also resulted in a reduction of collagen deposition and diminished gene expression of profibrotic signaling factor TGF-β1, as well as reduced gene expression of proinflammatory signaling cytokine IL-6. These data together show that CDH11 inhibition is renoprotective and can significantly reduce kidney injury and tubulointerstitial fibrosis.

We also investigated the genetic expression effects of CDH11 inhibition using RNA sequencing. In this way, we identified 51 genes that were significantly altered in two models of kidney injury in *Cdh11*$^{-/-}$ mice compared to *Cdh11*$^{+/+}$ mice. Among these, we identified AAT as likely to have a mechanistic relationship with CDH11. This is because AAT is beneficial in many cases where inhibition of CDH11 is also beneficial. Additionally, increased expression of AAT or treatment with exogenous AAT has anti-apoptotic, antifibrotic, and anti-inflammatory effects

similar to the renoprotective and fibrosis/inflammatory reduction effects we found with inhibition of CDH11. Finally, using analysis of deidentified medical records of patients tested for AATd, we discovered that lack of functional AAT significantly increases the risk of developing CKD.

Taken together, this work has identified CDH11 as an important mediator of kidney injury and a potential therapeutic target for prevention and treatment of AKI and CKD. This work also demonstrated novel relationships between CDH11 and AAT and between AAT and CKD, which will undoubtedly be significant in continuing the search for better therapies for people suffering from kidney injury.

7.2 Future directions

This work advanced our understanding of CDH11 in the context of kidney injury, but also raised several important questions that could be used to direct future research in this field. As described above, we identified expression of CDH11 in PTECs, showed that inhibition of CDH11 improves outcomes in multiple models of kidney injury, and discovered previously undescribed associations between CDH11 and AAT and between AAT and CKD. Future studies should further characterize the efficacy of pharmacologic inhibition of CDH11, determine exactly how CDH11 and AAT are related, and evaluate mechanisms by which AAT is beneficial in kidney injury.

The first of these topics is likely the easiest to design experimentally. We showed that CDH11 inhibition has significant effects in preventing and reducing kidney injury and fibrosis. However, this work did not investigate whether inhibition of CDH11 is capable of reversing kidney damage or initiating renal regenerative processes. Future studies could expand upon the treatment studies explored in Chapter 5 by administering CDH11 inhibition after an injury and evaluating whether the mice get better or if CDH11 inhibition simply prevents further damage to

the kidneys. The use of an inducible Cre mouse may also be useful in such experiments, although developing the necessary mouse lines for such an experiment may be challenging.

Another primary question that was raised as a result of the findings described above is how does CDH11 affect expression of AAT? We believe it's unlikely that CDH11 and AAT directly interact, because the findings in the RNAseq data reflect changes in gene expression, not protein activity, although direct protein interactions are not out of the question. To explore this question, we evaluated both AAT and CDH11 in the protein interaction database HitPredict.[169] HitPredict is a database of experimentally determined protein-protein interactions. From this analysis, we identified multiple potential pathways between CDH11 and AAT that are based solely on known protein interactions (Figure 7.1). The shortest potential pathway of

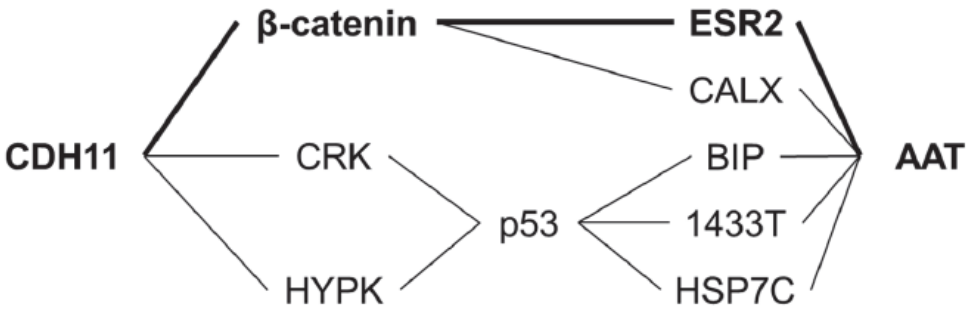

Figure 7.1 Protein-protein interactions, identified using HitPredict,[169] link CDH11 and AAT

known protein interactions between CDH11 and AAT involves β-catenin and estrogen receptor β (ESR2).[170–173] In addition, we also realized that 10 additional genes (of the 51 that were significantly altered in both models) are known to closely interact with AAT, CDH11, β-catenin, or ESR2. However, such a signaling pathway has not been explored, and the diagram depicted in Figure 7.1 likely does not contain all the possible protein interactions between CDH11 and AAT and may not include the mechanistic link between these two proteins either. Nevertheless, we believe this could guide initial experiments into determining how CDH11 signaling affects AAT expression. For example, β-catenin is known to be sequestered by CDH11 in the cytosol, so perhaps when CDH11 is inhibited, β-catenin translocation to the nucleus affects transcription factor binding and initiates expression of AAT. To explore this possibility, future studies could

begin by comparing the fraction of β-catenin in the cytosol versus the nucleus in *Cdh11*$^{-/-}$ and *Cdh11*$^{+/+}$ PTECs. If more β-catenin is present in the nuclei of *Cdh11*$^{-/-}$ PTECs, then β-catenin signaling may be responsible for the increase in AAT expression seen in the *Cdh11*$^{-/-}$ mice and cells. Similar experiments could be performed for other portions of the pathways shown in Figure 7.1, but if these do not elucidate the mechanism of CDH11 and AAT interaction, more broad investigation of signaling directly downstream of CDH11 or general stress pathways may be necessary.

Finally, while there appear to be many overlaps in the effects of CDH11 inhibition and augmentation of AAT, this work did not investigate specific mechanisms by which AAT improves kidney injury. While we suspect other groups are working on this topic based upon recent literature in the area, it is certainly a topic that warrants further investigation. Particularly given the significant relationship between AATd and development of CKD in clinical data, AAT signaling pathways may represent another important targeting strategy for development of kidney injury therapies. AAT has been shown to inhibit caspases associated with apoptosis, which could prevent damage to PTECs and thereby limit the initial injury and mitigate initiation of profibrotic and proinflammatory signaling cascades. AAT also has potent anti-fibrotic and anti-inflammatory effects, due to its inhibition of enzymes such as metalloproteinases and ADAM-17. However, it is unclear which of these processes is relevant in the context of kidney injury.

The work presented in this dissertation contributes to the understanding of CDH11 in kidney injury. These studies applied a variety of experimental designs, cellular and molecular techniques, and analysis strategies to further our understanding of CDH11 inhibition in three murine models of kidney disease. We described novel relationships between CDH11 and AAT and between AAT and CKD, providing the groundwork for future studies to further investigate these proteins in a therapeutic context for the treatment of kidney diseases.

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