THE ROLE OF THE AGE-DEPENDENT LOSS OF α(E)-CATENIN IN INCREASED ACUTE KIDNEY INJURY

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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and hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

First and foremost, I dedicate my dissertation work to my parents. Thank you for loving me unconditionally. Thank you for supporting my decision to pursuing my PhD in the US. Thank you for giving me chances to prove and improve myself. I am honored to have you as my parents.

I would also dedicate my dissertation work to my family, especially to my grandma who led me to the kingdom of medical science; to grandpa who opened my eye to the world; to aunt who instilled the importance of confidence and kindness.

Also, I dedicate my dissertation work to my brilliant and outrageously loving and supportive boyfriend, Andrew Peth, who cheered me on when I was discouraged, who wiped my tears away when I was stressed out and who has most importantly been 100% confident in my ability to get this done.

Finally, I dedicate my dissertation work to my friends. Thank you for supporting me throughout the process. Thank you for sharing happiness and sadness with me. Thank you for listening to all my complaints patiently. Thank you for cheering me up whenever I feel low.

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8-OHdG, 8-hydroxy-2'-deoxyguanosine

ACE, angiotensin-converting enzyme

ADH, antidiuretic hormone

AIF, apoptosis-inducing factor

AJ, adherens junctions

AKI, acute kidney injury

APC, adenomatous polyposis coli

ARF, acute renal failure

AT II, angiotensin II

BCL-2, B-cell lymphoma protein 2

BH3, BCL-2 homology 3

BSA, bovine serum albumin

BUN, blood urea nitrogen

Casc3, cancer susceptibility candidate 3

CCC, cadherin-catenin complex

CDK, cyclin-independent kinase

CIN, contrast-induced nephropathy

CKD, chronic kidney disease

CLP, cecal ligation puncture

CPN, chronic progressive nephropathy

EPO, erythropoietin

ER, endoplasmic reticulum

ESRD, end stage renal disease

FADD, Fas-associated death domain protein

FBS, fetal bovine serum

GFR, glomerular filtration rate

GSH, glutathione

GST, glutathione S-transferase

Hh, Hedgehog

HO-1, hemeoxygenase-1

HNE, 4-hydroxynonenal

IGF-1, insulin-like growth factor 1

IP, intraperitoneal

I/R, ischemia-reperfusion

LDH, lactate dehydrogenase

LPS, lipopolysaccharide

MOMP, mitochondria outer membrane permeabilization

MTs, microtubules

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NK, natural killer

NSAID, non-steroidal anti-inflammatory drugs

p-ERK, phosphorylated extracellular signal regulated kinase

PTH, parathyroid hormone

ROS, reactive oxygen species

RPF, renal plasma flow

Rplp1, Ribosomal Protein, Large, P1

RR, relative risk

RT, room temperature

Scr, serum creatinine

SF, serum free

SOD, superoxide dismutase

tBID, truncated BID

UO, urine output

Zag, zinc-α (2)-glycoprotein

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ABSTRACT

The aging kidney undergoes structural and functional alterations which make it more susceptible to acute kidney injury (AKI). Previous studies in our laboratory have shown that the aging kidney has a marked loss of α(E)-catenin in proximal tubular epithelium. α-Catenin, a key regulator of actin cytoskeleton, interacts with a variety of actin-binding proteins. Fascin 2 is an actin bundling protein that interacts with adhesion molecules and F-actin. In this work, we hypothesized that loss of α(E)-catenin leads to disruption of actin cytoskeleton which increases cisplatin-induced injury in aged kidney. A stable shRNA knock-down of α(E)-catenin was generated in NRK-52E cells (C2 cells); NT3 cells are the non-targeted control. We demonstrated that age dependent loss of α(E)-catenin in renal tubule epithelial cells facilitates the Fas mediated apoptotic signaling pathway in response to cisplatin-induced AKI injury. In addition, a cisplatin-induced loss of fascin 2 was observed in aged kidney. Overexpression of Fscn2 abolished increased cisplatin-induced apoptosis, mitochondrial dysfunction and oxidative stress in C2 cells compared with NT3 cells. In conclusion, this dissertation projects novel insight into understanding the increased incidence of AKI in aged kidney and identified a novel role of fascin 2 in renal epithelial cells, which depends on the functional interaction with α(E)-

catenin and F-actin. These findings may lay the groundwork for new therapeutic approaches to AKI in aged patients in the future.

CHAPTER 1

INTRODUCTION

Overview of Kidney Structure and Function

The kidneys are a pair of bean-shaped structures which are located just below and posterior to the liver in the peritoneal cavity [1]. Externally, there are three layers surrounding kidneys: the renal fascia, the perirenal fat capsule and the renal capsule. A cross section through kidney shows three interior structures: the outer cortex, the middle medulla and the renal pelvis (Figure 1.1A). The renal cortex surrounding the medulla is called a renal pyramid [2]. A nephron is the functional unit of kidney which regulates electrolyte concentrations and fluid volume by filtering the blood, reabsorbing nutrients and excreting waste as urine (Figure 1.1B). There are over one million nephrons in each kidney which can be categorized into two types: cortical nephrons (85%) and juxtamedullary nephrons (15%). The loops of Henle of the cortical nephrons are located at the junction between renal medulla and renal cortex, while juxtamedullary nephrons have their loop of Henle deep in the renal medulla. A nephron is composed of three parts: the glomerulus, the tubule and the associated capillary network.

The glomerulus, which is located at the beginning of a nephron, is a network of capillaries. It serves as the first step in urine formation by filtering solutes and water from blood and delivering them to renal tubule. The glomerular capillaries are connected to high-resistance arterioles at both ends which creates high hydrostatic pressure providing the driving force for blood plasma to be filtered into a cup-like sac named Bowman's capsule (Figure 1.1B). There are three barriers substances need to pass before entering

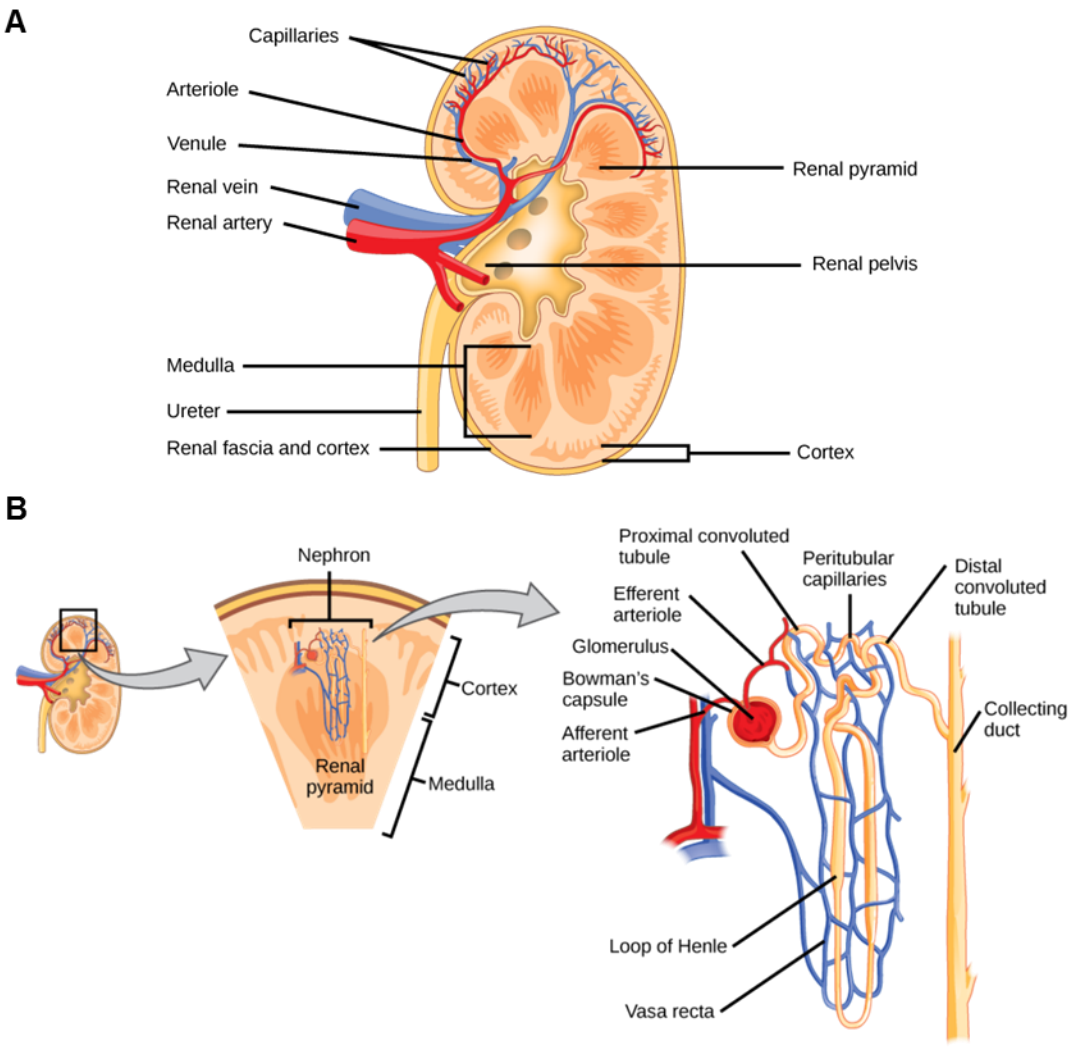


Figure 1.1 The structure of the kidney and nephron. (A) Externally, the kidney is surrounded by renal fascia, perirenal fat capsule and renal capsule. Internally, the kidney is filled with nephrons that filter blood and generate urine. (B) The nephron is the functional unit of the kidney which consists of glomerulus (filtration) and tubule (absorption and excretion).

From: http://goo.gl/q1gdx6

the tubule as glomerular filtrate: the glomerular capillary endothelial cells, glomerular basement membrane and podocytes. Since the basement membrane has negative charge and the diameter of a pore on glomerular membrane is around 8nm, small/positively charged molecules will pass more efficiently than large/negatively charged molecules [3].

The glomerular filtration rate (GFR), the rate at which all of the glomeruli filters the blood, provides a diagnostic measure of overall renal function, with decreased GFR serving as a hallmark of loss of renal function.

The renal tubule can be divided into three portions: the proximal tubule which is proximal to glomerulus, the loop of Henle which forms a loop going through the renal medulla and the distal tubule which empties the filtrate into collecting ducts (Figure 1.1B) [4]. Under normal conditions, the majority of the filtrate entering the proximal tubules is reabsorbed into the peritubular capillaries, including 65% of sodium and water and all filtered glucose and amino acids (Table 1.1). The reabsorption of sodium is driven by Na^+K^+-ATPase. Amino acids, glucose and PO_4^{3-} are reabsorbed via secondary active transport using the sodium gradient as the energy source. Water is reabsorbed passively via osmotic force. The loop of Henle receives filtrate from proximal tubule. The descending limb of the loop of Henle extends into the medulla and the ascending limb returns to the cortex. The primary function of the loop of Henle is to reduce solutes and the volume of water within the urine without changing its concentration by a countercurrent multiplier system which uses electrolyte pumps as source of energy. The reabsorption taking place in distal tubule is regulated by the endocrine system, which also plays important role in regulating blood pressure. The distal tubule secretes more PO_4^{3-} and reabsorbs more Ca^{2+} in presence of parathyroid hormone (PTH). Aldosterone stimulates the reabsorption of Na^+ and secretion of K^+. The last segment of the distal tubule and collecting duct are normally impermeable to water, they become permeable only in the presence of antidiuretic hormone (ADH). The epithelial cells lining the tubule

Table 1.1 Function of nephron components

Part	Function	Notes
Glomerulus	Filtration: water, dissolved substance Retention: plasma proteins and blood cells	• ~180 L/day of filtrate • Similar in composition to blood plasma without plasma proteins
Proximal Tubule	Active Reabsorption: glucose (100%), amino acids (100%), vitamins, HCO_3^- (90%), Na^+ (65%), K^+, PO_4^{3-}, Mg^{2+} and Ca^{2+} Passive Reabsorption: Urea, Cl^-, lipid-soluble material, H_2O (65%) Secretion: H^+, NH_4^+, creatinine, drugs and toxins	• PTH inhibits phosphate excretion • AT II stimulates Na^+, H_2O and HCO_3^- reabsorption
Descending Loop of Henle	Passive Reabsorption: H_2O (25%)	• Reabsorbs via medullary hypertonicity • Makes urine hypertonic
Ascending loop of Henle	Active Reabsorption: Na^+ (10-20%), K^+ and Cl^- Passive Reabsorption: Mg^{2+}, Ca^{2+}	• This region is impermeable to H2O • Urine becomes less concentrated as it ascends
Distal Tubule	Reabsorption: Na^+, Cl^-, Ca^{2+} and H_2O Secretion: H^+ , NH_4^+, creatinine, drugs and toxins	• PTH causes Ca^{2+} reabsorption • ADH stimulate the reabsorption of H_2O • Aldosterone stimulates the reabsorption of Na^+
Collecting Tubules	Reabsorption: Na^+(3% -5%), HCO_3^- and H_2O Secretion: K^+, H^+	• ADH stimulates the reabsorption of H_2O • Aldosterone stimulates the reabsorption of Na^+

PTH: Parathyroid hormone

AT II: Angiotensin II

ADH: Antidiuretic hormone

have a high density of mitochondria which produce ATP to facilitate the active transport of solutes [5].

The renal blood supply starts with branching of the abdominal aorta into the renal arteries and ends with the exiting of the renal veins to join the inferior vena cava. The renal arteries radiate out into numerous afferent arterioles entering the capillaries which supply the nephrons. Despite their small size, the kidneys receive about 20% of the

cardiac output and consume 25% of oxygen absorbed through the lungs [2]. The kidneys have an auto-regulatory system to keep blood flow and perfusion constant over a wide range of systemic blood pressures. The kidney changes its blood flow in response to changes of sodium concentration in the urinary via a mechanism called tubuloglomerular feedback. The urinary sodium level is sensed by macula densa cells which are located at the distal convoluted tubule. When the sodium level is moderately increased, the macula densa cells release ATP and reduces prostaglandin E2 release to juxtaglomerular cells [6,7]. Juxtaglomerular cells are specialized smooth muscle cells mainly in the wall of the afferent arterioles, and some in the efferent arterioles [8]. Juxtaglomerular cells decrease renin secretion which relaxes constricted vessels and thus lowers GFR. Further increase in urinary sodium level causes the release of nitric oxide to prevent excessive vasoconstriction. When the sodium level is decreased, more renin is released by juxtaglomerular cells which stimulates the renin-angiotension system and constricts the efferent arteriole to increase GFR [7]. Furthermore, juxtaglomerular cells also secrete renin in response to a decrease in systemic blood pressure and increased sympathetic nervous activity [8].

Importantly, renin released by juxtaglomerular cells also regulates systematic blood pressure and fluid balance via the renin-angiotensin system. Juxtaglomerular cells release renin into circulation in response to a drop of renal blood flow. Renin converse the angiotensinogen released by liver to angiotensin Ⅰ which is subsequently converted to angiotensin Ⅱ by the angiotensin-converting enzyme released by the lungs. Angiotensin Ⅱ constricts blood vessels leading to an increase of blood pressure. In addition, angiotensin Ⅱ also stimulates adrenal cortex to secrete aldosterone which increases the reabsorption of

sodium and water and excretion of potassium in renal distal tubule. Moreover, angiotensin Ⅱ stimulates posterior pituitary gland to release ADH which causes water reabsorption in collecting duct [9].

Besides renin, kidney secretes a variety of other hormones. The interstitial fibroblasts in the kidney releases erythropoietin in response to hypoxia. Erythropoietin stimulates the production of red blood cells in the bone marrow, induces angiogenesis and increases iron absorption [10]. Renal proximal tubule produces calcitriol, the activated form of vitamin D, which promotes the absorption of calcium and phosphate in intestine [11].

In conclusion, essential regulatory functions kidney performs are: 1) excretion of waste products of metabolism into urine (urea, uric acid, etc.); 2) reabsorption of vital nutrients (glucose, amino acids, water, electrolytes, etc.) 3) maintenance of acid-base homeostasis by regulating CO_3^{2-} and H^+ levels; 4) regulation of plasma osmolality via antidiuretic hormone (ADH); 5) regulation of blood pressure via the renin-angiotensin system; 6) production of hormones (calcitriol, erythropoietin, and renin) [5].

Aging: Structural and Functional Changes

During the last century, human lifespan has increased substantially, resulting in a large increase of elderly people over the next two decades [12]. Individuals aged 65 years or more represented 12.8% of US population in 2008 [13]. By 2030, the number of elderly people is expected to be 71 million, accounting for 21% of US population [14]. In fact, the elderly population is the most rapidly growing population segment in the western world

[15]. It is estimated that by 2025, there will be over 800 million individuals over the age of 65 worldwide [16]. Thus, the study of age-dependent pathophysiology, and translation of these findings to the clinic, is a significant challenge for biomedical sciences in the 21st century.

It is well established that aging is associated with structural and functional renal

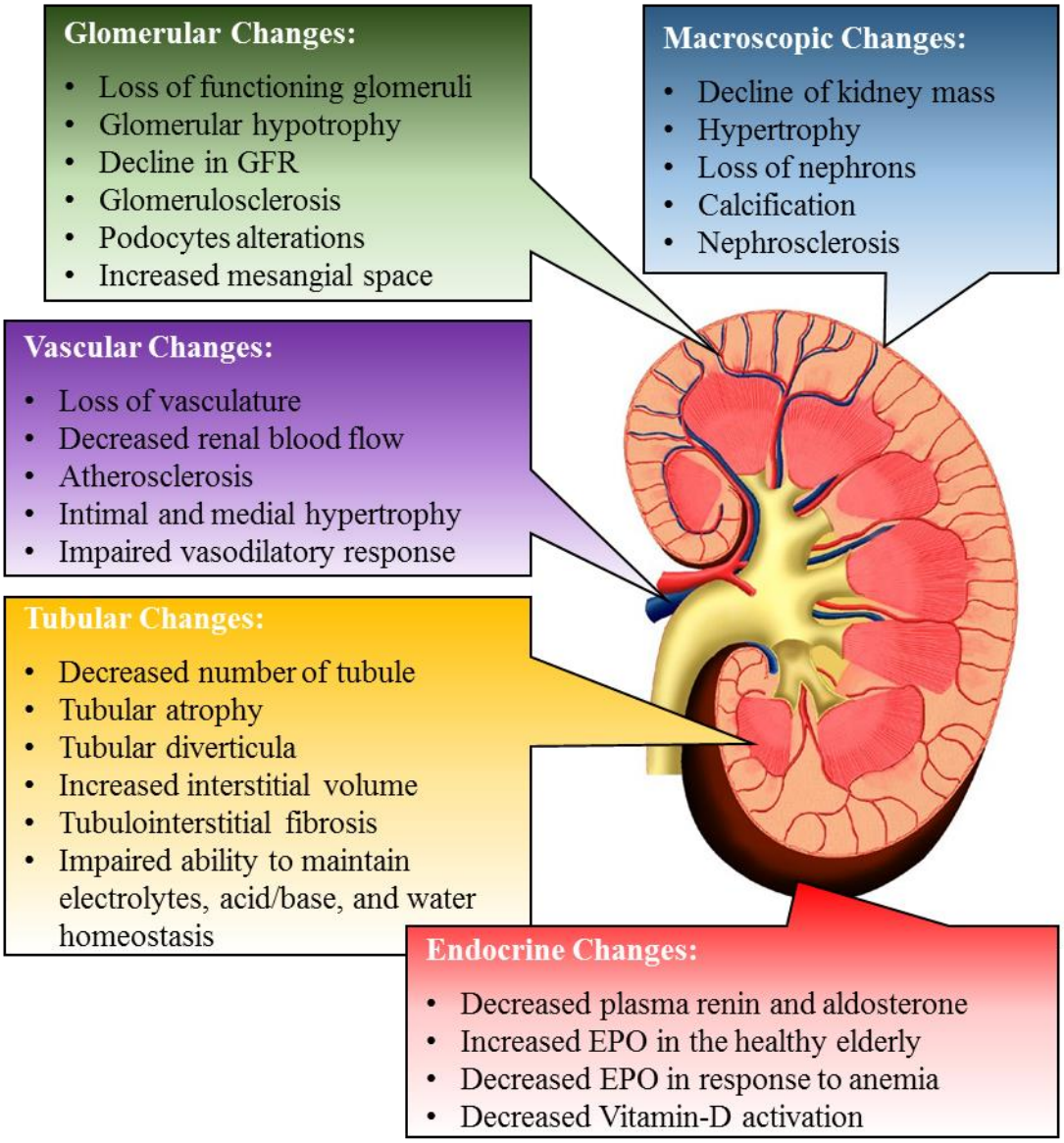


Figure 1.2 Age-related changes in the kidney. Several featured changes of glomerular, tubular, vascular and endocrine in aged kidney.

changes (Figure 1.2) [17]. It has been stated that "with the possible exception of the lung, the changes in kidney function with normal aging are the most dramatic of any human organ or organ system" [18,19]. The normal kidney loses about 20%–25% of its mass during aging, with the loss involving cortical glomeruli, tubules and vasculatures [20]. An age-dependent decline in kidney volume is not detectable by imaging, possibly due to the compensatory hypertrophy of functional nephrons [21,22]. In addition to nephron loss, aged kidney is also characterized by glomerulosclerosis, the scaring and hardening of the glomerulus, and tubulointerstitial fibrosis, a progressive deposition of extracellular matrix proteins on kidney parenchyma [23,24]. Moreover, there is an increasing incidence of nephrosclerosis with aging from 2.7% for people aged 18–29 years to 73% for people aged 70–77 years [25].

The aged kidney has parallel glomerular and vascular changes [26]. While the proportion of functioning glomeruli decreases with aging, the percentage of hyaline and sclerotic glomeruli increases [27]. Meanwhile, the number of podocytes decreases with age since these cells are unable to undergo cell division [28]. The thickening of glomerular basement membrane is another feature of aging kidney as well as mesangial expansion [29,30]. Glomerular obsolescence goes along with an altered control of glomerular hemodynamics which increases glomerular plasma flow and intra-capillary pressure, leading to glomerulosclerosis [31]. Most important of all, the aging kidney is characterized by decreased glomerular filtration rate (GFR). The seminal Baltimore longitudinal study demonstrated an average of 0.75 mL/min yearly decline in GFR in 254 men without hypertension or kidney disease [32]. A similar rate of decline (0.63 mL/min/year) was reported in a recent study based on 1203 living kidney donors [25]. The rate of decline in

GFR was tripled in subjects over 40 as compared with those under 40 in the Baltimore study [32]. A more recent study in healthy Chinese people described similar results [33]. However, approximately 30% of the individuals in this study had no GFR decline which indicates there is wide variability among individuals in the age related fall in GFR [32]. Similar changes are observed in renal plasma flow (RPF). The RPF declines by about 10% per decade after the fourth decade in the renal cortex, while medullary flow is relatively well preserved [34]. In addition, the aged kidney vasculature is also characterized by intimal and medial hypertrophy with overt atherosclerotic lesions [34]. Furthermore, aging renal vasculature also demonstrates impaired responses to vasodilatory stimuli and enhanced responses to vasoconstrictors [35,36].

The age-dependent loss of renal mass and renal function are more due to tubular changes than glomerular and vascular changes [37]. The number, length and volume of tubules decreases with age [23]. Tubular diverticula and atrophy, expanded interstitial volume and tubulointerstitial fibrosis are all hallmarks of aged kidney [38,39]. The reabsorption of sodium is enhanced in proximal tubule and impaired in distal tubule which allows maintenance of sodium balance in the elderly under steady-state conditions [40]. However, this functional reset limits the ability to excrete sodium in response to high salt intake and to conserve sodium in response to low salt intake which predispose elderly people to volume depletion, salt retention, hypertension, cardiovascular congestion and acute kidney injury (AKI) [41]. The activity of Na-K ATPase activity is also impaired in the elderly, putting them at higher risk of hyperkalemia [42]. The capacity for concentrating and diluting urine decreases with aging due to reduced expression of urea transporters, vasopressin-2 receptors and aquaporin 2/3 water channels [43–45]. Furthermore, the capacity

for generating ammonia and the activity of proton pump are greatly impaired in elderly people which make them more prone to develop acidosis in response to acid load [46,47].

The aged kidney also has impaired endocrine function. Plasma aldosterone and renin activity are reduced by approximately 50% in elderly subjects, especially under stress conditions [48]. Erythropoietin (EPO) levels are higher in the healthy elderly individuals, but significantly reduced in aged patients with anemia [49,50]. Elderly people are also more prone to develop vitamin D deficiency due to the impaired ability to synthesis 1, 25-dihydroxy vitamin D from 25-hydroxy vitamin D [51].

In conclusion, renal aging is a complicated, multifactorial process. All these changes discussed above may predispose the kidneys of the elderly to AKI as well as progressive chronic kidney disease (CKD).

Table 1.2 RIFLE criteria for AKI

Stage	GFR Criteria	SCr Criteria	UO Criteria
Risk	↓ GFR > 25%	↑ SCr × 1.5	UO < 0.5 ml/kg/h × 6h
Injury	↓ GFR > 50%	↑ SCr × 2	UO < 0.5 ml/kg/h × 12h
Failure	↓ GFR > 75%	↑ SCr × 3	UO < 0.3 ml/kg/h × 24h
Loss of Function	Complete loss of kidney function >4 weeks		
ESKD	Complete loss of kidney function >3 months		

GFR, Glomerular Filtration Rate
SCr, Serum Creatinine
UO, Urine Output
ESKD, End Stage Kidney Disease

Aging and AKI: Clinical Evidence

Acute kidney injury, previously called acute renal failure (ARF) [52], is defined as an abrupt onset of renal dysfunction ranging from minor loss of function to failure [53–55]. AKI is a common clinical complication that develops in approximately 4%–7% of hospitalized patients each year and the prognosis can be poor [56,57]. The severity of AKI is defined by RIFLE criteria which is an acronym for Risk, Injury, Failure, Loss of function and End stage kidney disease (Table 1.2) [53]. While the mortality rates for AKI are decreasing, the mortality range remains from 20%–35% [58,59] , almost 2 out of 3 afflicted patients died within 90 days after onset [60]. Thus, AKI remains a significant public health problem.

The causes of AKI are numerous including: 1) severe hypotension or volume depletion; 2) structural and functional damage to kidney caused by ischemic or nephrotoxic insult; 3) obstruction of urinary tract. Therefore, the causes of AKI are commonly classified into three categories: prerenal, intrinsic, and postrenal (Figure 1.3) [61].

Tubular damage due to ischemia is the most common causes of AKI[62]. Because of high metabolic activity and the structural characteristics of the renal vasculature, the kidney medulla is in a chronic hypoxic state. A small reduction in medullary blood flow may cause ischemic damage to renal tubule epithelium which induces AKI [63]. Ischemic injury induces alterations in renal tubule epithelial cell structure and metabolism including loss of polarity and brush border, redistribution of integrins and Na^+/K^+-ATPase, disruption of cytoskeleton, obstruction of tubules and initiation of cell death

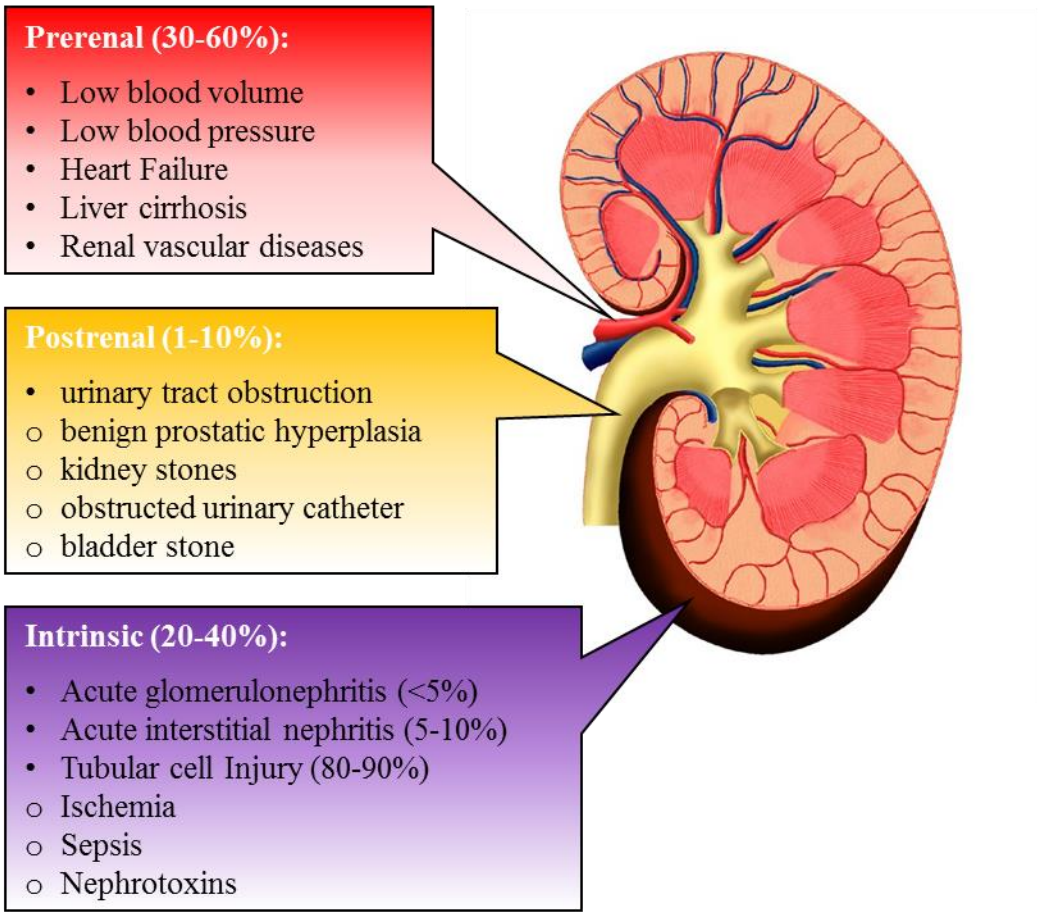


Figure 1.3 Causes of AKI. The causes of AKI are numerous and are commonly categorized into prerenal, intrinsic, and postrenal.

(Figure 1.4A) [61]. All these changes further decrease the function of tubular cell which ultimately results in decreased GFR through multiple mechanisms (Figure 1.4B) [61].

A relationship between AKI and aging has long been recognized. In 1972, studies suggested that 23.8% of patients with AKI were over the age of 60 and the following year it was shown that 13.7% were older than 70 [64,65]. In 1987, these percentages had risen to 76% and 46%, respectively [66]. The increasing prevalence of elderly AKI patients is supported by additional studies; Turney et al. showed that the median age of AKI patients was 41.25 years in the 1950s and increased to 60.5 in the 1980s [67]. In a study performed

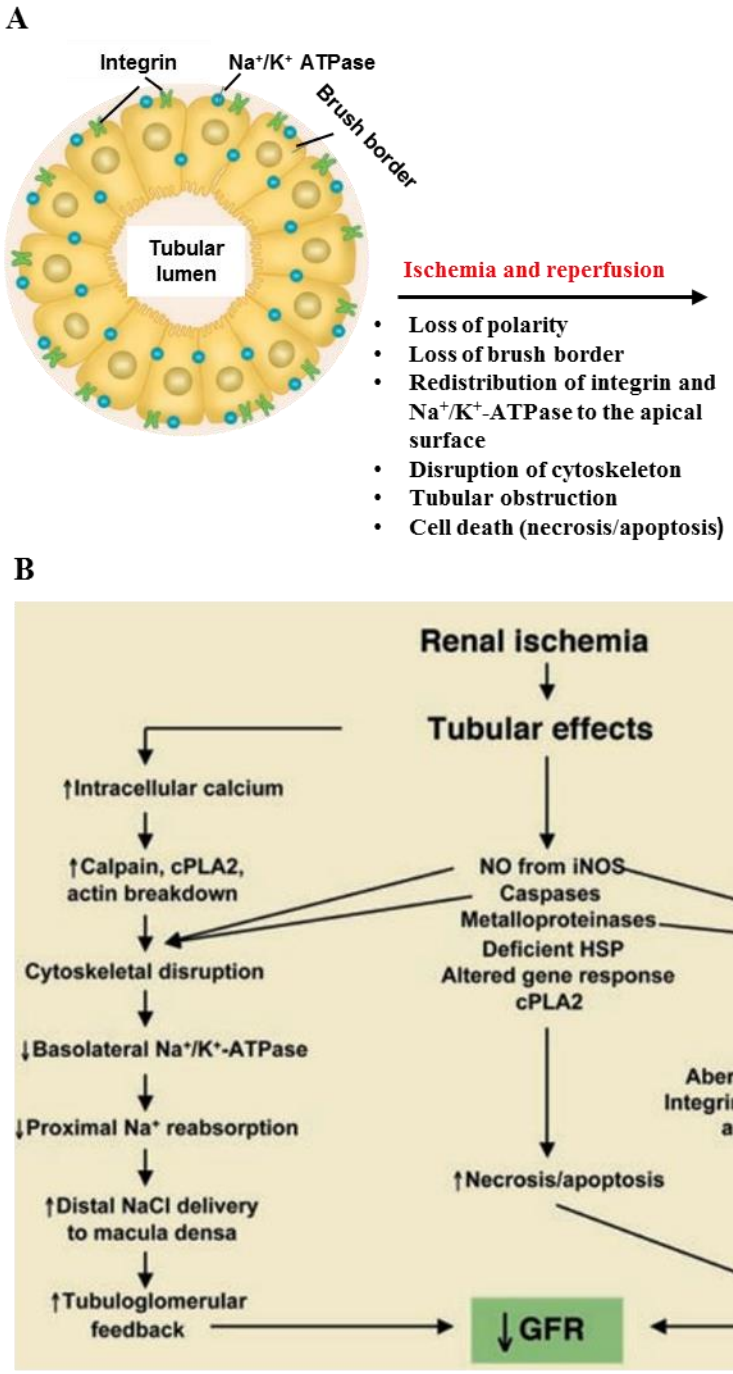


Figure 1.4 Effect of ischemia on renal tubule epithelial cells. (A) Morphological changes occur in the proximal tubules following ischemia and reperfusion. (B) Effects of ischemia on renal tubules in the pathogenesis of ischemic AKI. From: J Clin Invest. 2004 Jul 1; 114(1): 5–14.

by Pascual *et al.* in Spain, the incidence of AKI is 3.5 times higher in patients over 70

than those under 70; patients older than 80 years old were 5.0 times more likely to develop AKI [68]. Rosenfeld *et al*. performed a study showing the average age of those who succumbed to AKI was 71.9 ± 8.8 years old, demonstrating the importance of the relationship between aging and AKI [66]. Age above 65 years has also been shown to be an independent risk factor for AKI in a multinational, multicenter study [69]. Balardi and colleagues have shown that elderly patients (≥65 years) had ten times the incidence rate of AKI compared with those less than 65 years of age in Italy [70]. Xue et al. also established age as a risk factor for AKI; the incidence of AKI was 1.9% in patients younger than 65 and rose to 2.9% in those older than 85 [59]. Most recently, an increase in AKI in the elderly was seen following crush injury due to the earthquake in Wenchuan, China in 2008; incidence of the elderly patient with crush-related AKI was 2.6-fold higher than the younger patients [71].

Moreover, AKI that develops in the elderly is more severe and the patient is less likely to recover. Venkatachalam et al. showed that the percentage of elderly patients who did not recover renal function was 31.3% compared with 26% of younger cohorts [72]. Hospitalized AKI patients requiring dialysis are older than their counterparts who do not require dialysis (63.4 vs. 47.6 years) [73,74]. Data from a community-based cohort in California showed that the incidence of AKI requiring dialysis was 79 per 100,000 person-years in patients younger than 50 and 3545 in patients over 80 [75]. No increase in mortality has been consistently reported [76–79]; however, there are studies suggesting that mortality following AKI is increased in the elderly [64,80].

There is a growing recognition that AKI most often occurs on a background of CKD, a gradual loss of renal function over months or years. Approximately 35% of the

elderly US population has stage 3 CKD [58,81,82]. Elderly patients who developed AKI on a background of CKD are less likely to recover from AKI and more likely to progress to more advanced stage or even ESKD which contributes to the higher mortality rate [83,84]. The hazard ratio of developing ESKD for patients with both AKI and CKD is 13.0 relative to those only with AKI [85]. The two-year mortality rate is higher for those with AKI and CKD (64.3%) than those with AKI alone (54.3%) [85]. On the other hand, AKI also predisposes patients to CKD after tubular regeneration due to inflammatory responses, paracrine stimulation of myofibroblasts, epithelial cell senescence, and loss of cellular plasticity, all of which promote a pro-fibrogenic phenotype [86,87].

Older individuals more commonly develop atherosclerosis, hypertension, diabetes mellitus and heart failure, each of which can directly increase the risk of AKI [73]. These comorbidities can also increase the risk of AKI indirectly by leading to increased medication use in elderly patients as compared with younger patients [84]. Approximately 20% of the episodes of AKI are induced by nephrotoxic drugs and the incidence of drug-induced nephrotoxicity leading to AKI among elderly in the hospital can be as high as 66% [88,89]. AKI secondary to non-steroidal anti-inflammatory drugs (NSAIDs) is more common in the elderly [90]. More than 80% of patients with NSAID induced AKI are over the age of 60 [91]. In a study with patients aged 50–84 years, the relative risk (RR) for AKI was 3.2 in NSAID users and was increased dramatically when NSAIDs were used in combination with diuretics (RR 11.6) and calcium channel blockers (RR 7.8) [92]. The combination of NSAIDs and angiotensin-converting enzyme (ACE) inhibitors was also demonstrated to be associated with nephrotoxicity in elderly patients (>75 years) [93]. The incidence of aminoglycoside-induced nephrotoxicity is also elevated in the elderly and increased

injury in response to aminoglycosides in combination an ACE inhibitor in elderly patients has been reported [94–96]. While contrast-induced nephropathy (CIN) is a significant cause of AKI in hospitalized elderly patients [89,97], age per se may not be an independent predictor of contrast nephropathy [98]. It is expected, however, that CIN will remain an important cause of AKI in the elderly due to the increased use of contrast media in this population [99].

Aging and AKI: Experimental Models

Table 1.3 List of animal models for aki and corresponding clinical condition.

Animal Models	Clinical Conditions
Glycerol	Rhabdomyolysis
Ischemia-reperfusion	Hemodynamic changes induced AKI
NSAID, gentamicin, tobramycin, cisplatin, ifosfamide, etc.	Drug induced AKI
Uranium, potassium dichromate	Occupational hazard
S-(1,2-dichlorovinyl)-L-cysteine	Contaminated water induced AKI
Lipopolysaccharide	Sepsis induced AKI
Radiocontrast	Contrast induced AKI

Since the relationship between aging and AKI has been established for four decades, a number of experimental animal models have been developed to investigate AKI (Table 1.3). A number of animal studies in the 1980s indicated that the aging kidney has a greater susceptibility to both ischemic and toxic injuries [13].

Zager and his colleagues showed aging increases susceptibility to severe ischemic acute renal failure in the rat. In response to renal artery occlusion, the adolescent (3-4 months), mature (12-13 months), and aged (24-25 months) rats lost about 59%, 82%, and 94% of their renal function, respectively [100]. Beierschmidt et al. demonstrated an age-related increase in acetaminophen nephrotoxicity in male Fischer 344 rats, comparing rats at 2–4, 12–14 and 22–25 months of age [101]. Interestingly, baseline BUN, urine osmolality and urine volume were similar in all groups, suggesting that a major component of aging was increased sensitivity to insult as opposed to an overt loss of renal function. The nephrotoxicity of gentamicin is increased in the aging male rat with no alteration in the pharmacokinetics of the antibiotic [102]. These findings were verified with female rats, but suggested the lack of a relationship between the loss of renal function (decreased GFR) and tubular injury (necrosis or casts). In contrast, however, Miura et al. demonstrated that slices of kidney from old rats were more susceptible to in vitro anoxia (100% nitrogen) than slices from young counterparts as assessed by organic anion transport in the proximal tubules, leading the authors to conclude that the increased sensitivity to injury involves age-dependent alterations in the proximal tubules [103]. Previous studies in our laboratory also showed similar results that renal slices from aged Fischer 344 rats fed ad libitum, but not aged caloric-restricted male animals, were more susceptible to ischemic injury (100% nitrogen) when compared with slices from young animals as assessed by histological and biochemical evaluations [104]. These ex vivo studies again demonstrated that the proximal tubular epithelial cell damage had an inherent increase in susceptibility to injury. Aged Wag/Rij rats (23–26 months) are more sensitive to tobramycin, an aminoglycoside antibiotic, as evidenced by tubular necrosis and urinary NAG levels [105].

More recent studies have extended age-dependent AKI models to the mouse. The aging (46–49 weeks) male C57Bl/6 mice exhibited prolonged elevation of plasma creatinine and greater mortality after bilateral renal ischemia-reperfusion (I/R) induced AKI compared to the young (8–10 weeks) [106]. Star and colleagues developed a sepsis-induced AKI model by cecal ligation puncture (CLP) using aged (10.5–11 months) mice. Lipopolysaccharide (LPS) induced an increase in BUN and creatinine in the aged, but not young mice, setting the stage for the development of the more complex, clinically relevant CLP model [107]. These results indicate that laboratory models recapitulate the clinical scenario of age-related AKI in humans and allow for the elucidation of specific mechanisms.

In our study, cisplatin, a widely used nephrotoxicant-induced AKI model, was used to investigate the pathophysiological mechanism of AKI in aged kidney [108]. Cis-Diamminedichloroplatinum Ⅱ or cisplatin is a widely used and highly effective anticancer drug for the treatment of various solid tumors in the head, neck, breast, lung, ovary, testis and uterus [109]. However, the use of cisplatin is dramatically limited by its side effects including nephrotoxicity, gastrotoxicity, ototoxicity and myelosuppression [110]. Approximately 28-36% of patients experience AKI following the treatment with cisplatin which makes nephrotoxicity a dose-limiting side effect of cisplatin [111,112]. Elderly patients treated with cisplatin based chemotherapy have a significantly higher incidence of nephrotoxicity [113]. This was further confirmed by a study conducted in Indonesia showed that cisplatin-induced renal dysfunction was worse in aged patients [114]. Like most alkylating antineoplastic agents, cisplatin causes crosslinking of DNA, triggering apoptosis in tumor cells and other proliferating cells [115]. However, cisplatin can also

cause considerable injury to proximal tubular epithelial cells [116]. High concentrations of cisplatin in the kidney and adverse impacts on the renal transport system are the two major factors that cause cisplatin-induced nephrotoxicity [117]. The mechanism of cisplatin-induced nephrotoxicity has been studied for more than 30 years and recent studies suggest that local accumulation of cisplatin, intracellular conversion to nephrotoxins, inflammatory responses, oxidative stress, calcium overload, phospholipase activation, inhibition of mitochondrial respiratory chain, DNA damage and activation of apoptotic pathways, can partially explain this injury [117–119].

Apoptosis and AKI

Apoptosis, or programmed cell death, is a fundamental process needed to maintain homeostasis [120]. Too little apoptosis can promote autoimmune diseases and cancer; while excessive apoptosis can drive neurodegeneration and ischemic injury [121]. Two major pathways have been demonstrated to induce apoptosis: the mitochondria-mediated intrinsic pathway and the receptor-dependent extrinsic pathway which are distinguished by their initiating signals (Figure 1.5) [122]. The intrinsic pathway is triggered by cell stress-induced mitochondria outer membrane permeabilization (MOMP), resulting in the release of cytochrome c that activates caspase-9. The extrinsic pathway is initiated by the binding of apoptotic ligand to death receptors leading to the activation of caspase-8. The crosstalk between the extrinsic and intrinsic pathways occurs through caspase-8 [123]. High levels of caspase-8 directly cleaves caspase-3 leading to apoptosis, while low levels of caspase-8 cleaves BID to truncated BID (tBID), which translocates to the mitochondrial membrane to activate the intrinsic pathway by inducing MOMP [124,125]. Both intrinsic and extrinsic

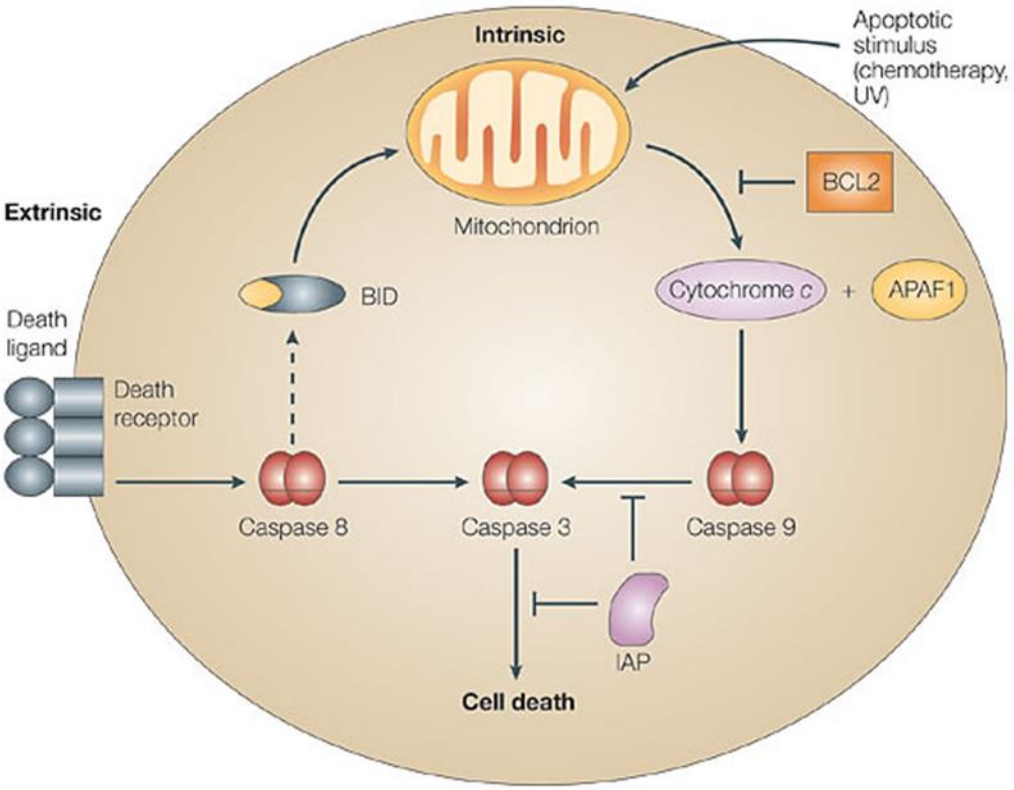


Figure 1.5 Apoptotic pathways. Apoptosis can be initiated by the death-receptor (extrinsic) pathway that acts through caspase 8 or mitochondrial (intrinsic) pathway that acts through caspase 9, but both pathways converge to activate the caspases 3. The crosstalk between the extrinsic and intrinsic pathways occurs through caspase-8. Low levels of caspase-8 cleaves BID to truncated BID (tBID), which translocates to the mitochondria membrane to activate the intrinsic pathway by inducing mitochondria outer membrane permeabilization (MOMP). Nature Reviews Drug Discovery. 2005; 4: 399-409.

pathways will ultimately cleave caspase-3 which initiates the morphological changes of apoptosis including cell shrinkage, membrane blebbing and DNA fragmentation [126]. In addition, apoptosis mediated by endoplasmic reticulum (ER) stress involving caspase 12, phosphorylated extracellular signal regulated kinase (p-ERK) and Ca^{2+}-independent phospholipase A2 may also play an important role in cisplatin-induced nephrotoxicity [127].

The B-cell lymphoma protein 2 (BCL-2) protein family plays important role in regulating apoptosis [128]. BCL-2 proteins are able to regulate the MOMP, promoting (BAX, BAK and BID) or inhibiting (BCL-2, BCL-X_L and MCL1) cytochrome c release [121]. Another BCL-2 protein family member, the BCL-2 homology 3 (BH3) is able to convey signals to initiate apoptosis [129]. Besides Its role in apoptosis, recently work showed decreased BCL-2 expression was accompanied by reduced proliferation and migration of the nasopharyngeal carcinoma cells [130].

The death receptor Fas, also called CD95, is a member of the TNF receptor superfamily [131]. When Fas encounters its ligand FasL, the receptor trimerizes in the cytoplasmic domain and recruits the Fas-associated death domain protein (FADD). The death effector domain on FADD is capable of recruiting, cleaving and activating caspase-8 [132]. FasL is most prevalent in natural killer (NK) cells and T lymphocytes [125]. In the kidney, FasL has been reported to be expressed in tubular epithelial cells, renal endothelial cells, renal fibroblasts and mesangial cells [133]. Evidence has shown that FasL plays a key role in mediating cisplatin-induced AKI [134]. The expression of Fas is significantly reduced in cisplatin-resistant ovarian cancer epithelial cells, and up-regulation of Fas reverses the development of cisplatin resistance [135]. Epigallocatechin-3-gallate, a green tea polyphenol, and amifostine ameliorate cisplatin nephrotoxicity by inhibiting Fas-dependent apoptosis [136,137]. FasL expression was increased dramatically in cisplatin exposed HeLa cells after centrocyte/centroblast marker 1 stimulation [138].

Increased cell death due to apoptosis is an intriguing hypothesis for enhanced cell injury with aging, as it represents a convergence between chronic renal dysfunction due to tubular loss and increased AKI in the aging kidney. As such, an increase in salicylate-

induced nephrotoxicity was seen in 12 month old rats, as compared to young controls (3 months) [139]. Interestingly, there was evidence of an increased sensitivity of the mitochondria in the proximal tubular epithelial cells to injury. Further evidence for a role of the mitochondria comes from a study of I/R injury in aged (27 months) Wistar rats [140]. Tubular cell apoptosis was increased in aged rats as compared to young controls; basal levels of cytosolic cytochrome C, active caspase-3/9 were elevated in the aging kidney and the up-regulation following I/R injury of caspase-3/9 was increased in aged rats [141]. Increased expression of Bax, a pro-apoptotic protein, caspase-3/9 and cytochrome C in the aging kidney have been observed in other studies [142,143]. On the other hand, the expression of Bcl-2, which is an apoptosis inhibitor, however, is decreased in aged rat kidney [142]. Moreover, the expression of p21, a cyclin-independent kinase (CDK) inhibitor which induces apoptosis, is increased in aged rats [144]. Higher levels of p53 and p21 were expressed in the aging male C57Bl/6 mice after bilateral renal I/R-induced AKI [145]. Therefore, increased apoptotic cell death could account, in part, for the increased AKI in the aging kidney.

α-Catenin: Role in Cell Adhesion

α-Catenin which bridges the cadherin-catenin complex (CCC) and actin cytoskeleton, is essential for maintaining the integrity of the intercellular adherens junctions (AJ) (Figure 1.6A) [146]. Cadherin is a calcium-dependent transmembrane glycoprotein which mediates cell–cell adhesion via homophilic binding of their extracellular domains of cadherins on adjacent cells [147]. Intracellularly, cadherin interacts directly with p120-catenin and β-catenin [148]. α-Catenin joins the complex by binding to β-

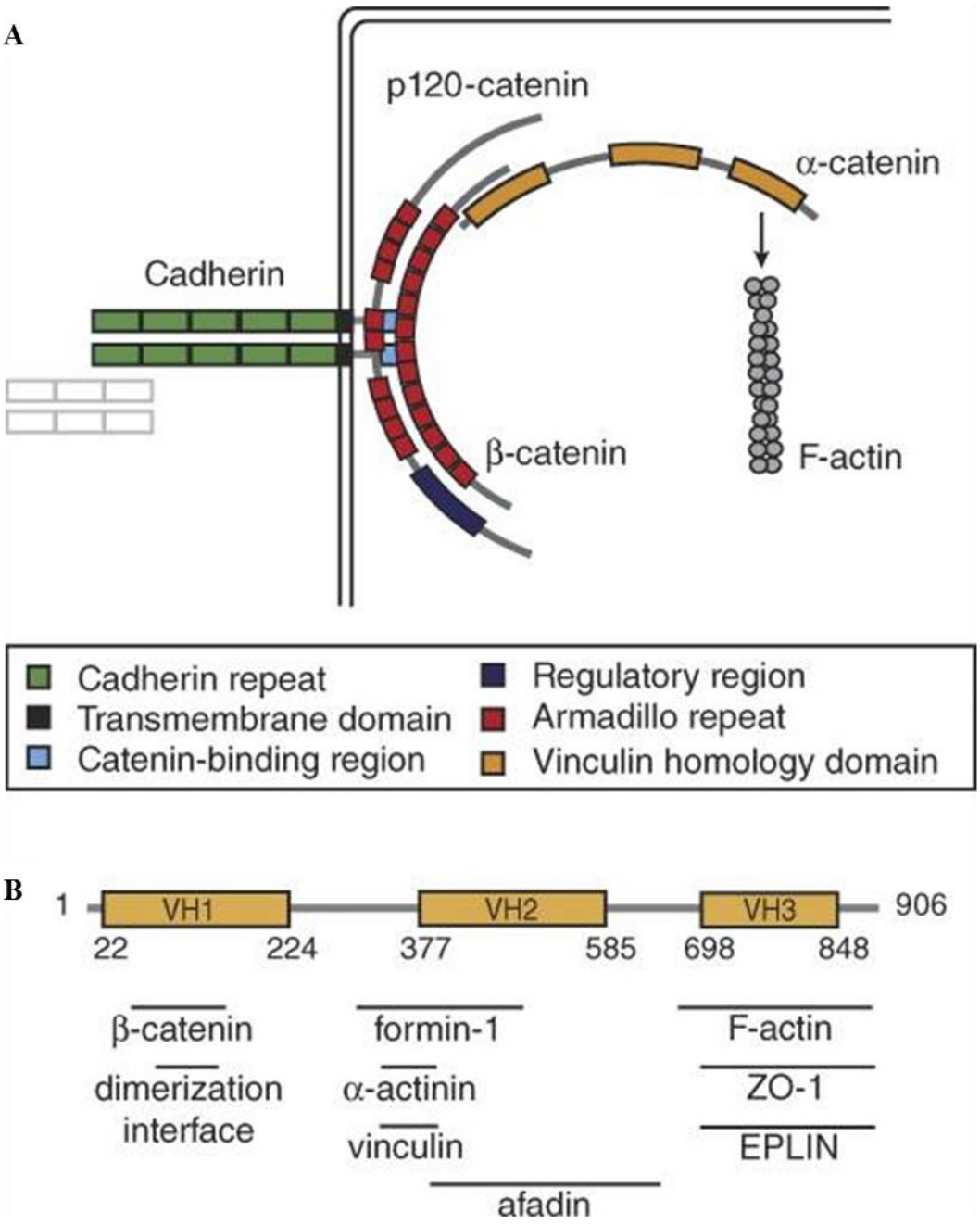


Figure 1.6 The role of α-catenin in cell–cell adhesion. (A) The cadherin–catenin complex (CCC). α-Catenin joins the complex by binding to β-catenin through its N terminus, while the C-terminal actin-binding domain recruits the actin cytoskeleton. (B) Key features of α-catenin, including the three vinculin homology (VH) domains and the binding sites of several actin-related proteins. From: J Cell Biol. 2011 Nov 14; 195(4): 543–552.

catenin through its N terminus, while the C-terminal actin-binding domain recruits the actin cytoskeleton [149]. Other binding partners of α-catenin includes formin-1, α-actinin, vinculin, afadin, ZO-1 and EPLIN (Figure 1.6B) [150].

In mammals, there are three forms of α-catenin and one close relative sharing identical amino acid sequence: α(N)-catenin is expressed primarily in neural tissue; α(E)-catenin is most prevalent in epithelial tissues; α(T)-catenin is restricted to heart tissue; and α-catulin is a ubiquitously expressed α-catenin-like protein. Vinculin is a more distant relative to α-catenin which is also ubiquitously expressed[151]. α(E)-Catenin (102 kDa) is the founding member of the α-catenin family. The human α(E)-catenin is encoded by gene CTNNA1 which localizes to chromosome 5q31 and consists of 16 coding exons and at least one 5' non-coding exon [152]. Mouse and human α(E)-catenin proteins are exceptionally highly conserved, sharing 99.2% identity [149].

For many years we explained the function of α-catenin by using a simple model in which cadherin, β-catenin, α-catenin bind one another as a ternary complex which binds F-actin with the C-terminal of α-catenin [153]. However, studies from Nelson and Weis laboratories challenges this conventional simple model. Their study showed that although isolated α(E)-catenin could bind F-actin or E-cadherin-β-catenin complex, these two binding events seemed to be mutually exclusive because they could not reconstitute the quaternary cadherin-catenin-F-actin complex on membrane patches[154]. Furthermore, α-catenin can homodimerize and the affinity for F-actin was higher for homodimers than monomers, which preferentially bind to the E-cadherin-β-catenin complex. These homodimers could inhibit Arp2/3-dependent actin polymerization at high concentrations in *vitro* [155]. Since β-catenin binding and homodimerization require the same binding site,

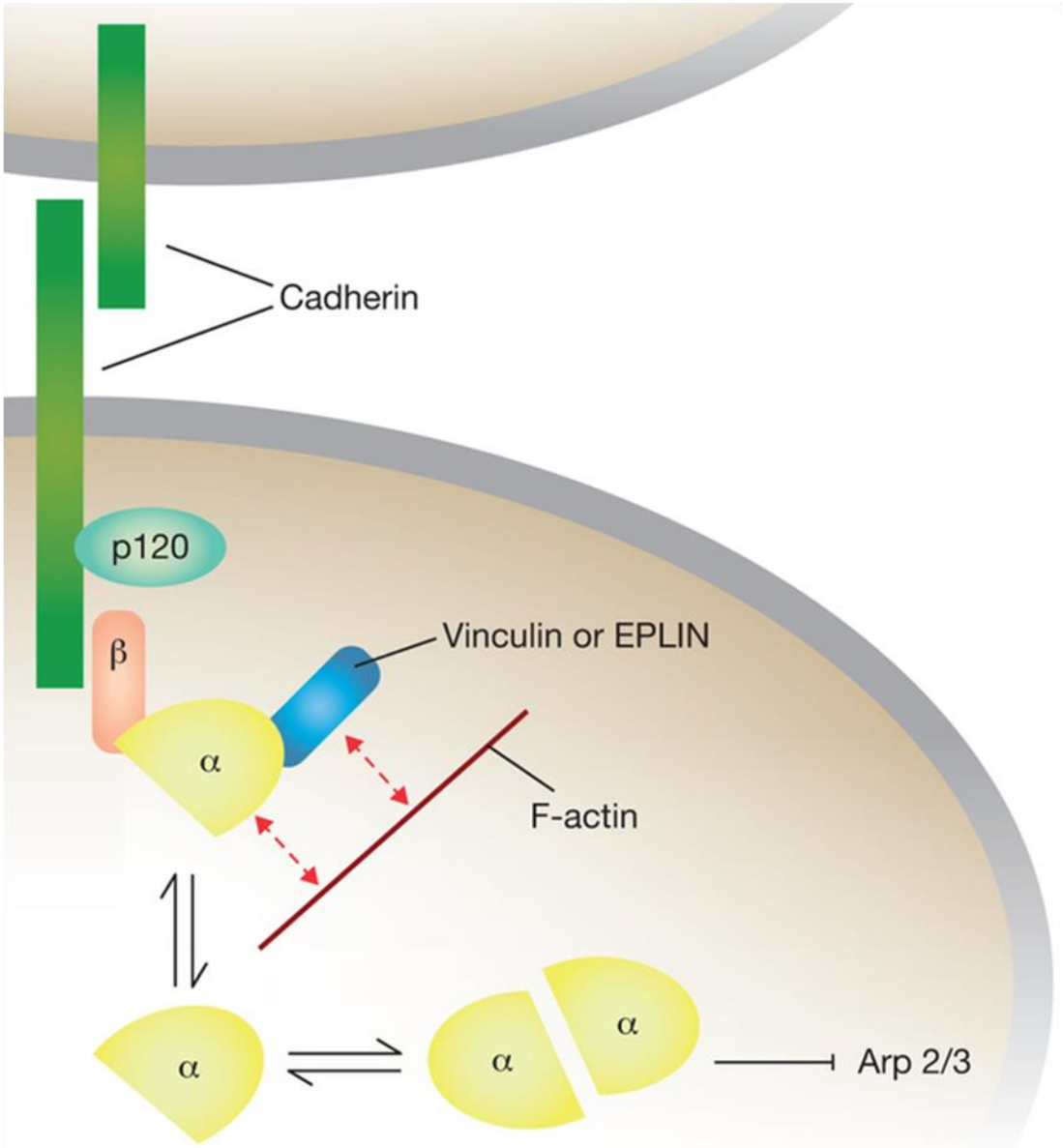


Figure 1.7 The α-catenin network. α-catenin exists in membrane-bound and cytosolic pools that undergo dynamic exchange. At the plasma membrane, it incorporates with the cadherin–catenin molecular complex. Cadherin-based α-catenin may interact with the actin cytoskeleton by binding F-actin. In the cytosol, α-catenin can homodimerize and regulate the cytoskeleton by inhibiting the Arp2/3 dependent actin polymerization. From: Nature Cell Biology. 2013; 15: 238–239.

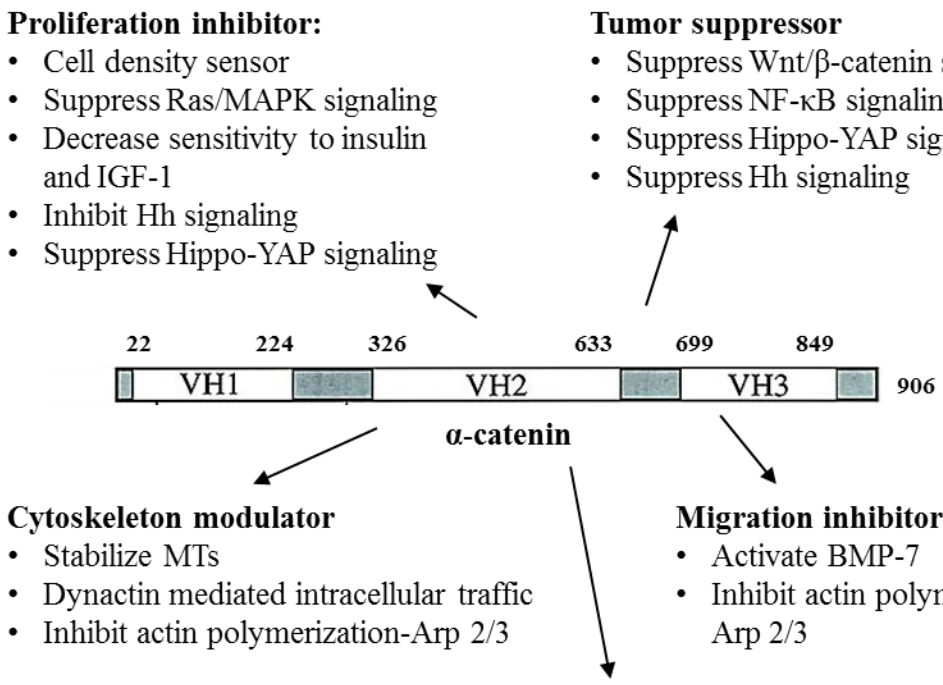
α-catenin was proposed to influence its binding partners allosterically and the ability of

cadherin to regulate cytoskeleton requires the release of α-catenin from CCC (Figure 1.7) [156].

Since E-cadherin-β-catenin bound α-catenin does not bind F-actin, it might be concluded that α-catenin's capacity to regulate cytoskeleton is unrelated to cadherin. However, a recent study showed force is required to form a stable bond between F-actin and CCC which explained why CCC-F-actin linkage could not be reconstituted in solution using purified proteins in previous studies [157]. Furthermore, evidence has shown that monomeric α-catenin works as a linking molecule between CCC and F-actin, whereas cytoplasmic dimeric α-catenin equilibrates with junctional monomeric α-catenin [158]. Importantly, although direct binding between α-catenin and F-actin can be formed *in vitro*, whether this occurs *in vivo* is still undetermined. Interestingly, studies also found that the E-cadherin-β-catenin bound α-catenin works as an intermediary helping other actin binding proteins like EPLIN, vanculin, α-actinin, afadin and formin-1 in linking the CCC to F-actin [159,160]. Besides recruiting F-actin to AJ dynamically, α-catenin also plays an important role in mechanotransduction [150]. Under low stress conditions, α-catenin's conformation allows an inhibitory region to block its vinculin binding site. Upon the application of force, α-catenin undergoes a conformational change which displace the inhibitory region from the vinculin binding site, allowing vinculin to bind and recruiting more F-actin to the CCC via vinculin [161].

α-Catenin: Roles Beyond Adherens Junctions

There is an increasing recognition that in addition to the well-established role in cell adhesion, α-catenin regulates multiple pathways controlling proliferation, migration and apoptosis in a cadherin-independent manner (Figure 1.8) [162–164]. Furthermore, several early studies suggested that a large cellular pool of α-catenin exists in cytosol [165,166]. Wnt signaling in *Xenopus* was attenuated by overexpression of α-catenin which indicates the cytosolic α-catenin may interact with β-catenin, playing roles distinct from α-catenin at



Apoptosis (Depends on the cellular context)	
Anti-apoptosis	Mammary glands
	Gastric and lung cancer (Suppress p53)
Pro-apoptosis	Central nervous system (Suppress NF-κB)
	Epithelial cancer (Activte DR4/DR5)
	Myeloid leukemia cell

Figure 1.8 Roles of α-catenin beyond adherens junctions. α-Catenin regulates multiple pathways including proliferation, migration and apoptosis in a cadherin-independent manner.

junctions [167].

The results of several studies support a role for α-catenin in cell proliferation. Hyperproliferation and defects in epithelial polarity were observed upon ablation of α-catenin in mouse epidermis, which may be mediated via increased sensitivity to insulin and insulin-like growth factor 1 (IGF-1) of these cells and the enhanced signaling of Ras and MAPK [163]. Lien et al. also showed the Hedgehog (Hh) signaling was up-regulated in the cerebral cortex of α(E)-catenin null brains which subsequently lead to hyperproliferation [168]. Deletion of α(E)-catenin in the hair follicle stem cell prohibited the translocation of Yap1 from nuclear to cytoplasm after cells achieve confluency [169,170]. Similar results were seen in E-cadherin deficient cells which indicates the adherens junctions may regulates cell proliferation through α-catenin via working as cell density sensor [171].

Besides suppression of cell proliferation, α-catenin also has profound effects on the actin and microtubule based cytoskeleton. α-Catenin is able to interact with F-actin directly or through actin binding proteins. Nelson and Weis showed that homodimers of α-catenin can inhibit actin polymerization via interact with Arp 2/3 [149]. Depletion of cytosolic pool of α(E)-catenin caused cells increased migration and increased F-actin-Arp 2/3 complex [166]. Chimeric fusion of α-catenin to cell membrane was able to stabilize microtubules (MTs) in noncentrosome cytoplasts of fibroblast-like cells in a cadherin independent manner [172]. The downstream effector is unclear, but it has been suggested that formin-1 may be a reasonable candidate since this protein has a similar effect on microtubule status and it can bind α(E)-catenin. Another study from Lien's group showed that loss of α-catenin rather than AJ allows lysosomes to travel significantly faster than

wild type cells which supports an adhesion-independent role of α-catenin in intracellular traffic mediated by dynactin [173].

Mis-localization or loss of α-catenin has been reported to be a more severe prognosis of cancer progression than loss of E-cadherin in several tumors [174]. α-catenin had been show to interact with the adenomatous polyposis coli (APC) which leads to nuclear translocation of APC-β-catenin complex inhibiting the transcription of Wnt, a well-known signaling pathway promoting tumor formation and progression [175]. Interestingly, α-catenin has be shown to suppress NF-κB signaling only in E-cadherin-negative basal like breast cancer cells, but not the E-cadherin positive cells [176]. Genetic deletion of α(E)-catenin in the progenitor cells and hair follicle stem cell lead to skin tumorigenesis by promoting nuclear location and transcriptional activity of YAP [169]. Conditional deletion of α-catenin in the mouse brain leads to the activation of Hh signaling which enlarged the mutant's head with massive hyperplasia [168].

The role of α-catenin in apoptosis is controversial. Increased apoptosis has been reported in α-catenin deleted mammary glands of mice [177]. In addition, increased expression of p53 was coupled to decreased α-catenin expression in both gastric and lung cancer [178]. However, in epidermis or central nervous systems, deletion of α-catenin has been reported to have a protective effect from apoptosis by up regulating NF-κB [179]. α-catenin knockdown in epithelial cancer cells also attenuates DR4/DR5 mediated apoptosis [180]. In a myeloid leukemia cell line, reintroducing α-catenin leads to a decrease in cell proliferation and increase in apoptosis [181]. Hence, the role of α-catenin in cell death may depend on the cellular context.

Our laboratory has demonstrated that the expression of α(E)-catenin is decreased in the aging kidney [182]. Given the importance of this complex in establishing cell polarity and regulating the actin cytoskeleton, this deficiency may inhibit the complete recovery of the tubular epithelium from injury in the aging kidney. This was supported by our recent studies which demonstrated that loss of α(E)-catenin expression leads to down-regulation of BMP-7 and N-cadherin, decreasing repair in renal tubule epithelial cells due to alterations in cell migration [183,184].

Fascin：An Actin Binding Protein

Fascin, a actin-binding and bundling protein, plays important role in maintenance and stability of parallel filamentous actin bundles, regulating cell proliferation, adhesion, migration and apoptosis in different cellular contexts [185,186]. There are 3 isoforms of fascin: Fascin-1, encoded by FSCN1, is mainly expressed in mesenchymal and nervous tissues; Fascin-2, encoded by FSCN2, is most prevalent in retinal cells; FSCN3, encoding for fascin-3, is restricted to testis [187]. Fascin is principally expressed in fibroblasts, neuronal cells, vascular endothelia cells as well as dendritic cells [188]. Recent studies in our lab showed fascin 2 is also expressed in renal tubule epithelial cells (Chapter 4).

While fascin has been shown to regulate actin bundles assembly in different cellular contexts, more specific roles for fascin has been demonstrated recently in the formation and turnover of cell adhesive structures [189]. Fascin binds to β-catenin on its E-cadherin binding site which colocalized with cadherin at cell-cell borders and dynamic cell leading edges [190]. However, α-catenin is absent in the β-catenin-fascin complex,

possibly because the steric conformational or phosphorylation/acetylation constraints may preclude simultaneous association of β-catenin with fascin and α-catenin, or a weak association of α-catenin with the complex may lessen its resolution under co-immunoprecipitation conditions. Furthermore, fascin has been show to play important role in cell proliferation, migration and apoptosis. Decreased adhesion dynamics and cell migration were observed in fascin knockdown human colon carcinoma cells [191]. Up-regulated fascin expression enhances cholangiocarcinoma RBE cell proliferation, migration and invasion [192]. Increased FSCN1 expression is observed in gastric cancer tissues and cell lines. Up-regulation of FSCN1 promoted proliferation invasion, but suppressed apoptosis of gastric cancer cells [193].

In summary, relationship between AKI and aging has long been recognized. AKI in the elderly is more severe and patients are less likely to recover, presumably due to increased apoptosis of renal tubular epithelial cells [194–197]. Previous studies from our laboratory showed marked loss of α(E)-catenin in proximal tubular epithelium in aged kidney [108,198]. α-Catenin, a tension-sensing, key regulator of the actin cytoskeleton, interacts with a variety of actin-binding proteins [199–201]. Fascin 2 is an actin bundling protein that interacts with adhesion molecules and F-actin [185,202]. Many previous studies have focused on fascin 2 expressed in retina [203], whereas its role in renal tubular epithelium is undetermined. Given the relationships between α(E)-catenin, fascin and actin cytoskeleton, along with their demonstrated role in cell apoptosis, studies in this dissertation were designed to investigate the role of age-dependent loss of α(E)-catenin in predisposing the aged kidney to cisplatin-induced AKI. Specifically, we hypothesized that age dependent loss of α-catenin leads to disruption of the actin cytoskeleton via

fascin 2 which contributes to the increased apoptosis of renal tubular epithelial cells to cisplatin injury.

In order to study the effects of reduced α(E)-catenin, cell lines with stable knockdown of α(E)-catenin (C2 cells) were generated by Sigma-Aldrich in NRK-52E cells as previously described by our laboratory [183,184]; NT3 cells are the non-targeted control. Open reading frame clones of mouse Fscn1 and rat Fscn2 in the pCMV6 Entry vector were used to generate stable overexpressed cell lines in C2 (C2\Fscn1, C2\Fscn2). Non-targeted vector controls were generated in both NT3 and C2 cells (NT3\V, C2\V).

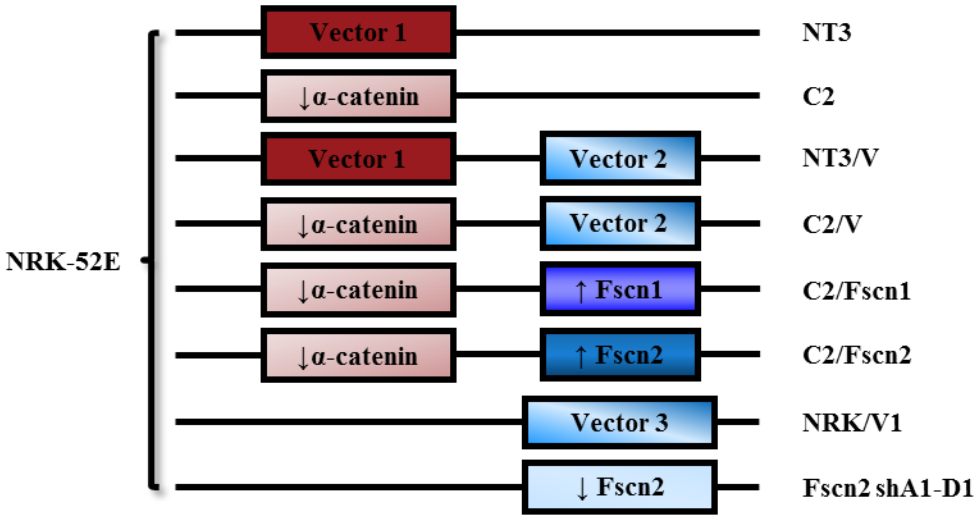


Figure 1.9 Schematic of the cell lines used in this dissertation. Sigma-Aldrich created stable cell lines in NRK-52E cells using lentiviral shRNA to knock down α(E)-catenin (*Ctnna1*). Single-cell clones were isolated for the non-targeted control (NT3) and α(E)-catenin targeted cells (C2). Open reading frame clones of mouse *Fscn1* and rat *Fscn2* were used to generate stable overexpressed cell lines in C2 (C2\Fscn1, C2\Fscn2). Non-targeted vector controls were generated in both NT3 and C2 cells (NT3\V, C2\V). Stable Fscn2 knockdowns (Fscn2 shA1-D1) and vector control (NRK\V1) were generated using shRNA with NRK-52E cells. Vector 1: Plko.1-Puro, Sigma; Vector 2: pCMV-6-Entry, OriGene; Vector 3: pGFP-V-RS, OriGene.

Stable Fscn2 knockdowns (Fscn2 shA1-D1) and vector control (NRK\V1) were also generated using shRNA in the pGFP-V-RS vector with NRK-52E cells to investigate the role of Fscn2 in increased cisplatin-induced AKI in aged kidney (Figure 1.9).

SUMMARY OF RATIONALE AND HYPOTHESIS FOR ALL EXPERIMENTS

During the last century, human lifespan has increased substantially which will result in a large increase of elderly people over the next two decades. Acute kidney injury (AKI) is a common clinical complication with significant mortality ranging from 20-35%. Evidence has shown that AKI in the elderly is more severe and patients are less likely to recover, presumably due to increased apoptosis of renal tubular epithelial cells [194–197]. Previous studies from our laboratory showed marked loss of α(E)-catenin in proximal tubular epithelium in aged kidney [108,198]. α-Catenin, a tension-sensing, key regulator of the actin cytoskeleton, interacts with a variety of actin-binding proteins [199–201]. Fascin 2 is an actin bundling protein that interacts with adhesion molecules and F-actin [185,202]. Many previous studies have focused on fascin 2 expressed in retina [203], whereas its role in renal tubular epithelium is undetermined. Given the relationships between α(E)-catenin, fascin and actin cytoskeleton, along with their demonstrated role in cell apoptosis, studies in this dissertation were designed to investigate the role of age-dependent loss of α(E)-catenin in predisposing the aged kidney to cisplatin-induced AKI. Specifically, we hypothesized that **AGE DEPENDENT LOSS OF α-CATENIN LEADS TO DISRUPTION OF THE ACTIN CYTOSKELETON VIA FASCIN 2 WHICH CONTRIBUTES TO THE INCREASED APOPTOSIS OF RENAL TUBULAR EPITHELIAL CELLS TO CISPLATIN INJURY.**

Chapter 2

Rationale: Cisplatin is one of the most potent and widely used antitumor drugs. However, the use of cisplatin is limited by its major side effect, nephrotoxicity. Evidence has shown an increased incidence and severity of AKI in the elderly. Previous studies from our laboratory demonstrate a decrease in α(E)-catenin expression in aged kidney.

Hypothesis: It is hypothesized that age-dependent loss of α(E)-catenin increases cisplatin-induced nephrotoxicity.

Chapter 3

Rationale: The aging kidney undergoes structural and functional alterations that make it more susceptible to drug-induced AKI. Previous studies in our lab have shown the expression of α(E)-catenin is decreased in aged kidney and loss of α(E)-catenin potentiates cisplatin-induced apoptosis, but not necrosis, in a caspase-dependent manner, in renal tubular epithelial cells (NRK-52E cells). However, the specific apoptotic pathway underlying the increased AKI in aged kidney is undetermined.

Hypothesis: It is hypothesized that age-dependent loss of α(E)-catenin potentiates Fas-mediated apoptotic pathway in renal tubule epithelial cells.

Chapter 4

Rationale: It is well established that aging is associated with structural and functional renal changes that make the kidney more susceptible to AKI. Previous studies have

shown that the aging kidney has a marked loss of α(E)-catenin in proximal tubular epithelium. α-Catenin, a key regulator of actin cytoskeleton, interacts with a variety of actin-binding proteins. Fascin 2 is an actin bundling protein that interacts with adhesion molecules and F-actin.

Hypothesis: It is hypothesized that age-dependent loss of α(E)-catenin leads to disruption of actin cytoskeleton via fascin 2 which makes renal tubule epithelial cells more susceptible to cisplatin injury.

CHAPTER 2

LOSS OF α(E)-CATENIN POTENTIATES CISPLATIN-INDUCED NEPHROTOXICITY VIA INCREASING APOPTOSIS IN RENAL TUBULAR EPITHELIAL CELLS

This research was published in Toxicological Sciences.

2014; 141(1):254-62

Abstract

Cisplatin is one of the most potent and widely used antitumor drugs. However, the use of cisplatin is limited by its side effect, nephrotoxicity. Evidence has shown an increased incidence and severity of AKI in the elderly. Previous studies from our laboratory demonstrate a decrease in α(E)-catenin expression in aged kidney. In this study, we investigated whether the loss of α(E)-catenin may increase cisplatin nephrotoxicity. To study the effects of reduced α(E)-catenin, a cell line with stable knockdown of α(E)-catenin (C2 cells) was generated with renal tubule epithelial cells (NRK-52E); NT3 is non-targeted control. C2 cells exhibited a significant loss of viability as determined by MTT assay compared with NT3 cells after cisplatin challenge, but showed no difference in lactate dehydrogenase (LDH) leakage. Increased caspase3/7 activation and PARP cleavage was observed in C2 cells after cisplatin treatment. Z-VAD, a pan-caspase inhibitor, abolished the difference in susceptibility between NT3 and C2 cells. Interestingly, the expression of α(E)-catenin was further decreased after cisplatin treatment. Furthermore, in vivo data demonstrated a significant increase in serum creatinine at 72 hr after a single dose of cisplatin in 24-month-old rats, but not in 4-month-old rats. Increased expression of KIM-1 and in situ apoptosis were also detected in aged kidney after cisplatin challenge. Taken together, these data suggest that loss of α(E)-catenin increases apoptosis of tubular epithelial cells which may contribute to the increased nephrotoxicity induced by cisplatin in aged kidney.

Key words: Aging, Cisplatin, α-catenin, Apoptosis, AKI

Introduction

Acute kidney injury (AKI), a common clinical complication, is characterized by a rapid and progressive loss of renal function [56,204]. Evidence has shown that there is an increased incidence and severity of AKI in the elderly [205]. Approximately 20% of AKI cases are induced by nephrotoxic drugs and the incidence of drug induced nephrotoxicity among elderly who developed AKI in the hospital can be as high as 66% [117].

Cis-Diamminedichloroplatinum Ⅱ or cisplatin is a widely used and highly effective anticancer drug for the treatment of various solid tumors in the head, neck, breast, lung, ovary, testis and uterus [109]. However, the use of cisplatin is dramatically limited by its side effects including nephrotoxicity, gastrotoxicity, ototoxicity and myelosuppression [110]. Approximately 28-36% of patients experience AKI following the treatment of cisplatin, which makes nephrotoxicity a dose-limiting side effect of cisplatin [111,112]. Like most alkylating antineoplastic agents, cisplatin causes crosslinking of DNA, triggering apoptosis in tumor cells and other proliferating cells [115]. However, cisplatin can also cause considerable injury to proximal tubular epithelial cells [116]. The mechanism of cisplatin-induced nephrotoxicity has been studied for more than 30 years and recent studies suggest that local accumulation of cisplatin, intracellular conversion to nephrotoxins, inflammation response, oxidative stress, DNA damage and activation of apoptotic pathways, can partially explain this injury [117–119].

α-Catenin, which works at the interface between the cadherin-β-catenin complex and F-actin, is very important in the relationship between the adherens junction (AJ) and cytoskeleton which is essential for cell adhesion [158]. There are three forms of α-catenin:

α(E)-catenin is mainly expressed in epithelial tissues; α(N)-catenin is most prevalent in neural tissues and α(T)-catenin is restricted to the heart and testes [206]. Recent studies indicate that besides a simple structural function, α-catenin is involved in multiple pathways controlling membrane and actin dynamics, cell proliferation, migration and apoptosis [204]. Our laboratory has reported a dramatic decrease of α-catenin expression in proximal tubular epithelium in aged male Fisher 344 rats [182]. Decreased expression of α-catenin was coupled with an increase in p53 expression in both lung and gastric cancer [178]. In addition, an increase in apoptosis was observed in α-catenin deleted mouse mammary glands [177]. In this study, the role of α(E)-catenin in cisplatin-induced apoptosis was determined in a cell line (C2 cells) that has stable knockdown of α(E)-catenin. C2 cells are characterized by decrease cell-cell aggregation, increased monolayer permeability and decreased repair in a wound healing assay due to migration deficits [183,184]. The hypothesis that loss of α(E)-catenin increases susceptibility to cisplatin was examined in series of experiments.

Methods

Materials:

cis-Diamineplatinum(II) dichloride (Sigma-Aldrich Cat #P4394); N-cadherin antagonist peptide (GenScript, SWELYYPLRANL-NH_2); N-cadherin antagonist control peptide (GenScript, SRELYYPLRANL-NH2); Caspase Inhibitor I (Z-VAD (OMe)-FMK) (CalBiochem Cat #627610); Mercury(II) chloride (Sigma-Aldrich Cat #203777); Staurosporine (CalBiochem Cat #569396); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Cat #M2128); neutral red (Sigma Cat #N4638).

Animals:

Male Fisher 344 rats (4, 20 and 24 month old) were obtained from the NIA colony. Animals were randomly assigned to the cisplatin treated group and saline control group with n=5 each. Animals received a single intraperitoneal (IP) injection of 2.75 mg/kg cisplatin, 2 mg/kg mercuric chloride or equal volume of saline as control. Animals were placed in metabolic cages overnight before harvesting. On the day of the experiment (72 hr after cisplatin injection; 48 hr after mercury injection), rats were anesthetized with ketamine (80-120 mg/kg)/xylazine (5-10 mg/kg) via IP injection. Urine was collected and a cardiac puncture was performed to obtain blood. Kidney tissue was fixed in 4% paraformaldehyde overnight and stored in 70% ethanol. All animal experiments and care were approved by the Animal Care and Use Committee in accordance with the NIH (Protocol number #AUP 6752).

Cell culture:

Cells were plated at a density of 5×10^4 cells/cm^2 and cultured in DMEM/F12 (1:1) with L-glutamine and HEPES (Gibco Cat #11039-021) supplemented with 10% fetal bovine serum (FBS) (Altanta Biologicals Cat #S11150), 5 μg/ml puromycin dihydrochloride (Sigma Cat #P9620) and incubated at 37°C in 5% CO_2. Cells were harvested with TrypLE^{TM} Express (Gibco Cat #12604-021) and pelleted at 1250 rpm for 5 min. at room temperature (RT). Single cell colonies were grown to confluence and passaged to larger plates. The cell lines (NT3 and C2) were used within 20 passages of establishing a clonal cell line, as described by our laboratory [183,184].

Cell viability:

Cell viability was assessed by the MTT assay based on the ability of mitochondria to convert soluble MTT to its insoluble purple formazan. Cells were seeded in 96 well flat bottom tissue culture plates (Sigma Cat #Z707910) at a density of 5×10^4 cells/cm^2. After 24hr, culture media was replaced by serum free (SF) media supplemented with desired treatments. Three hours before harvest, 10 μl of 5 mg/ml MTT (Sigma Cat #M2128), dissolved in DPBS (Gibco Cat #14190-144), was added to each well. Upon harvesting, cells were washed with cold DPBS and dissolved by adding 50 μl solubilization solution (10% Triton X-100, 0.1N HCl in isopropanol). The plates were read at 570/690 nm on the Synergy HT Multi-Detection Microplate Reader (BioTek, Winooski, VT). The results are expressed by percent viability as [$Abs_{570\text{-}690}$ treated/$Abs_{570\text{-}690}$ control×100].

Cell viability was also estimated by neutral red assay which stains lysosomes red, in live cells. Briefly, confluent cultures of NT3 and C2 cells, in 96 well plates, were challenged with cisplatin in SF media for 24 hr. Three hours before harvest, 10 µl of 500 µg/ml neutral red (Sigma Cat #N4638), dissolved in DPBS, was added to each well. After incubation, the media was aspirated and cells were fixed with 50 µl fixative solution (1% formaldehyde, 1%$CaCl_2$) for 5 min. Then the fixative solution was aspirated, the plates were dried in RT and cells were dissolved by adding 100 µl solubilization solution (1% acetic acid, 50% ethanol) for 15 min. The plates were read at 540 nm on the Synergy HT Multi-Detection Microplate Reader. The results are expressed by percent viability as [Abs_{540} treated/Abs_{540} control×100].

LDH assay:

Confluent cultures of NT3 and C2 cells in 96 well plates, were challenged with cisplatin in SF media for 24 hr. Cytotoxicity was determined by LDH assay. The LDH assay was performed using Pierce™ LDH Cytotoxicity Assay Kit (Pierce Cat #88953) according to the manufacturer's instructions.

Caspase 3/7 activity assay:

Confluent cultures of NT3 and C2 cells in 96 well plates were challenged with cisplatin in SF media for 24 hr. Caspase 3/7 activity was determined by Caspase-Glo® 3/7 Assay kit (Promega Cat #G8091) according to the manufacturer's instructions.

Western blot:

Subconfluent cells were washed twice with ice-cold DPBS and lysed with lysis buffer (10mM Tris-HCl, 1% SDS) containing Halt™ Protease/Phosphatase inhibitors (Thermo Scientific Cat #78444). Cells were scraped and incubated on a rocker for 15 min at 4°C. Cells were further disrupted by pipette 15 times and spun at 12,000 g for 15 min at 4°C. Protein concentration was determined by NanoDrop 2000c Spectrophotometer (Thermo Scientific, Waltham, MA) at 280 nm.

The following antibodies were used: anti-caspase-3 (Cell Signaling Cat #9662), anti-caspase-7 (Cell Signaling Cat #9492), anti-PARP (Cleaved-Asp214) (Sigma Cat #SAB4500487), anti-α-catenin (BD Transduction Laboratories™ Cat #610194), anti-N-cadherin (BD Transduction Laboratories™ Cat #610920) and anti-β-actin (Sigma Cat #A2228). Goat-anti-mouse HRP conjugate and Goat-anti-rabbit HRP conjugate (Jackson ImmunoResearch Laboratories, Cat #115035003 and 305035003) were used at 1:20,000 dilutions. Blots were developed using SuperSignal West Femto Chemiluminescent Substrate (Pierce Cat #34095), imaged using the ChemiDoc™ imaging system (Bio-Rad, Hercules, CA), and quantitation performed using the ImageLab 3.0 software (Bio-Rad, Hercules, CA).

Real-Time PCR:

From $5x10^6$-$1x10^7$ cells were harvested and suspended in 1 ml PBS. RNA was isolated using the RNeasy mini kit (Qiagen Cat #74104) with on-column DNase digestion. RNA concentration and quality was determined by NanoDrop 2000c Spectrophotometer. cDNA was generated from 2 µg RNA using the High Capacity cDNA Synthesis Kit (Life Technologies Cat #4368814) following the kit protocol. Quantitative PCR was performed

in duplicate using 50 ng cDNA/reaction via Taqman assays with SsoFast™ Probes Supermix with ROX (Bio-Rad Cat #172-5251) and the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The following cycling conditions were used: 95°C for 20 sec., then 95°C for 1 sec and 60°C for 20 sec repeated 40 times.

Commercially available TaqMan primer sets were used to assess α(E)-catenin (Rn01406769_mH) (Life technologies Cat #4331182). Relative quantitation was performed using the Pfaffl method normalized to Gapdh.

In situ apoptosis

Paraffin-embedded kidney sections were used to detect the *in situ* apoptosis. The *in situ* apoptosis assay was performed using *In situ* Apoptosis Detection Kit (Genway Cat #40-831-160019) according to the manufacturer's instructions. To quantify the *in situ* apoptosis, the positively stained area was measured using the point tool of CellSense.

Statistics:

Results are expressed as mean ± S.E. A two-way analysis of variance (ANOVA) was performed with the exception of Figure 1A&E and Figure 4A in which a one-way ANOVA was performed, followed by post-hoc t-tests with the Bonferoni correction using the statistical software GraphPad Prism 6 (GraphPad Software, La Jolla, CA). The differences were considered statistically significant when $p<0.05$.

Results

Initial experiments demonstrated that decreased cell density increased the sensitivity to cisplatin challenge, indicating that cell-cell adhesion increases cell resistance to cisplatin injury (Figure 2.1A). Because α-catenin is well known to play important role in cell adhesion [206], a clonal α(E)-catenin knockdown cell line (C2) was generated in NRK-52E cells using lentiviral shRNA; the NT3 cell line is the non-targeted control. Confluent cultures of NT3 and C2 cells were challenged with cisplatin for 24 hr. C2 cells exhibited a significant loss of viability at all concentrations of cisplatin as compared with NT3 cells (Figure 2.1B&C). A time course assay was also performed using 150 μM cisplatin. C2 cells have significantly lower cell viability than NT3 cells at early (6-12 hr) and late (21-48 hr) time points following cisplatin treatment (Figure 2.1D). The increased cell viability in the early time points (6-12 hr) can be caused by the protective cellular stress response [207]. As previously demonstrated by our laboratory, the expression of N-cadherin is also decreased in C2 cells [184]. In order to exclude the potential effect of decreased N-cadherin expression in the increased sensitivity of C2 cells, NRK-52E cells were treated with an N-cadherin antagonist peptide (H-SWELYYPLRANL-NH2) alone or in the presence of 150 μM cisplatin. Another peptide (H-SRELYYPLRANL-NH2) was used as a control [208]. The N-cadherin antagonist peptide did not increase the sensitivity of NRK-52E cells to cisplatin (Figure 2.1E). These results indicate that loss of α(E)-catenin, rather than loss of N-cadherin, increases cell susceptibility to cisplatin injury.

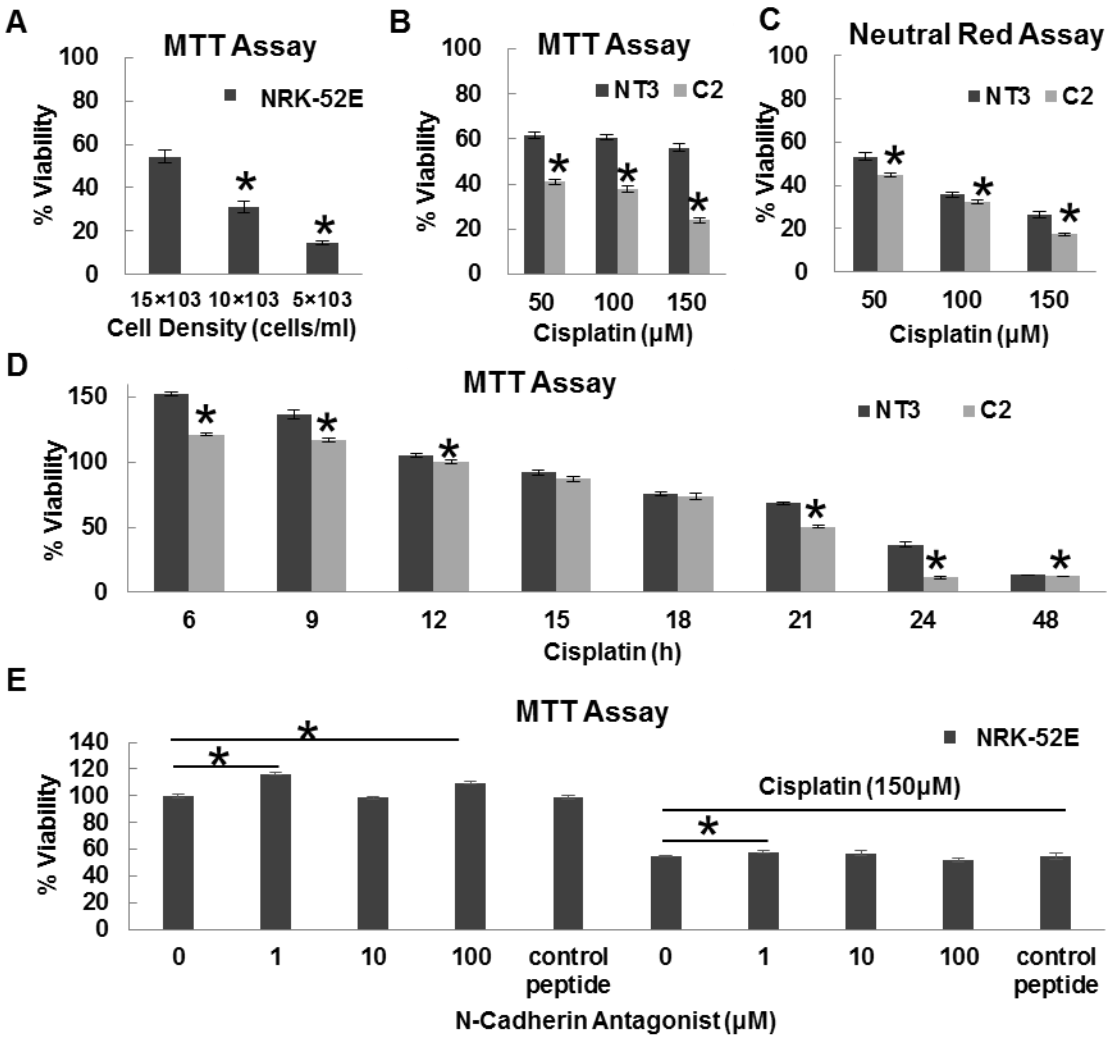


Figure 2.1. Effect of decreased α(E)-catenin on susceptibility to cisplatin injury. (A) Cell viability was determined by MTT assay in cultures of NRK-52E cells seeded with the indicated densities and challenged with 150 μM cisplatin in SF media for 24 hr. The densities correspond with 100%, 75% and 50% confluence. The results are presented as percent viability of untreated control with corresponding cell density in SF media. The asterisks indicate significant differences from the 15×10^3 group (n=16). Cell viability determined by MTT (B) and neutral red (C) assays in confluent cultures of NT3 and C2 cells treated with the indicated concentrations of cisplatin in SF media for 24 hr. (D) Cell viability determined by MTT assay upon treatment for the indicated time periods with 150 μM cisplatin. The asterisks in B, C, and D indicate significant differences between NT3 and C2 cells for each treatment (n=24). (E) Cell viability determined by MTT assay in NRK-52E cells treated with the indicated concentrations of cadherin antagonist (IC_{50}=0.09 μM) alone or in the presence of 150 μM cisplatin. Control peptide was used at a concentration of 1 mM. (n=16). The results in B, C, D, and E are presented as percent viability of untreated control in SF media.

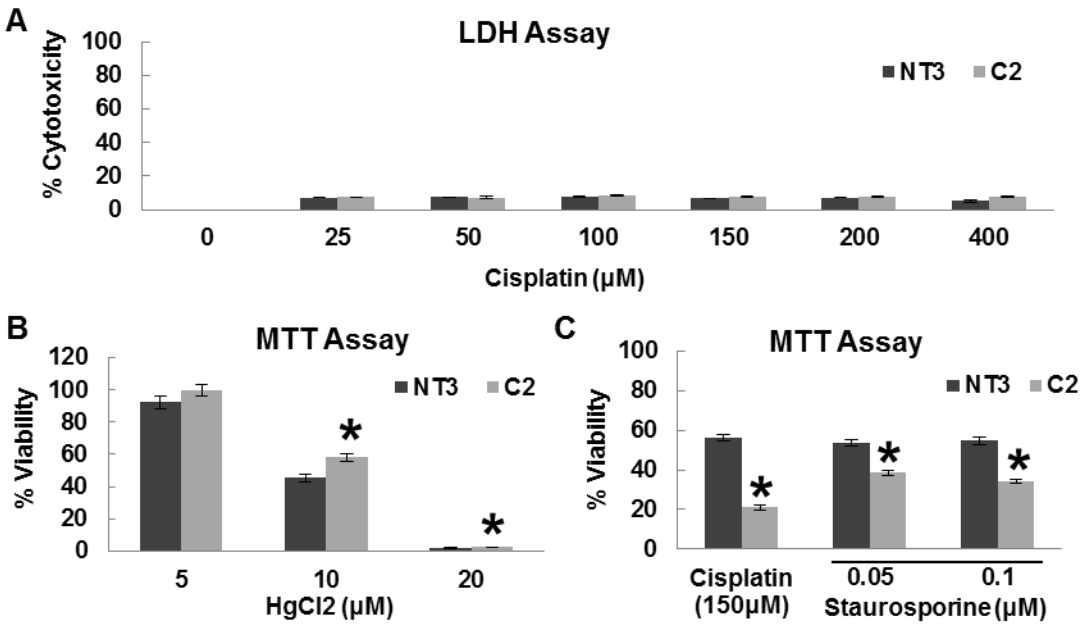


Figure 2.2. Susceptibility of NT3 and C2 cells to injuries induced by different chemicals. (A) Cytotoxicity was determined by LDH assay in confluent cultures of NT3 and C2 cells treated with the indicated concentrations of cisplatin in SF media for 24 hr. The results are presented as the percent cytotoxicity of the maximum LDH release control (n=6). Cell viability was determined by MTT assay in cells treated with the indicated concentrations of mercury (B), cisplatin, and staurosporine (C). The results are presented as percent viability of untreated control in SF media (B and C). The asterisks indicate significant differences between NT3 and C2 cells of each treatment (n=24).

Apoptosis and necrosis are two major mechanisms of cell death [209]. LDH leakage, a marker of necrosis, was not different in C2 cells as compared to NT3 after 24 hr of cisplatin challenge (Figure 2.2A). This suggests that the increased susceptibility of C2 cells to cisplatin is not due to necrosis, an interpretation that is supported by the finding that C2 cells are not more sensitive to inorganic mercury, which induces necrosis in proximal tubular epithelial cells [210] (Figure 2.2B). However, staurosposine, a commonly used drug to induce apoptosis in cell culture [211], decreased viability in C2 cells to a greater extent than NT3 cells (Figure 2.2C). Taken together, these observations suggest that the increased susceptibility of C2 cells to cisplatin is more likely to be caused by apoptosis rather than necrosis.

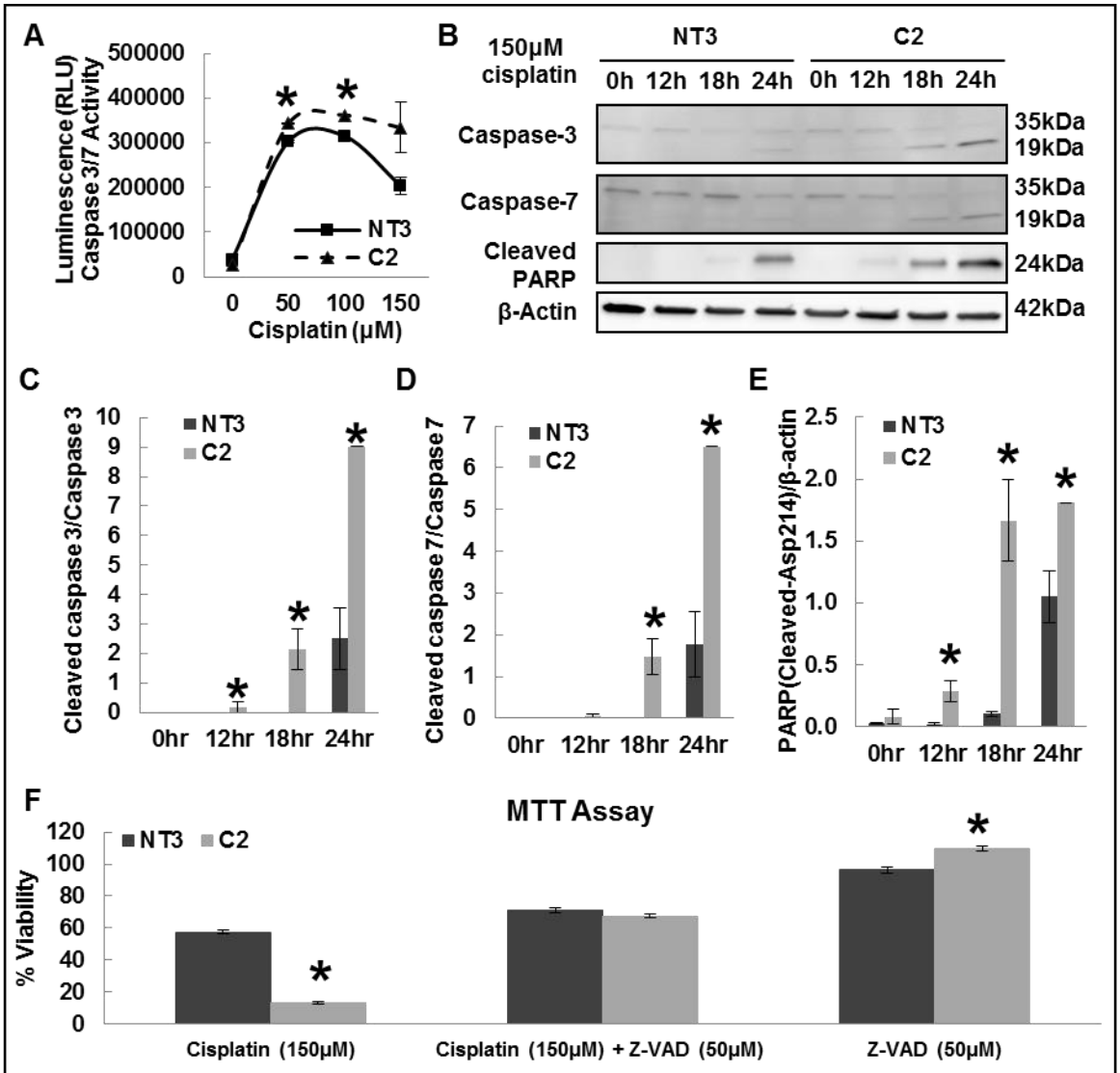


Figure 2.3. Apoptosis induced by cisplatin in NT3 and C2 cells. (A) Caspase 3/7 activity in confluent cultures of cells treated with the indicated concentrations of cisplatin in SF media for 24 hr, was determined by luminescent assay. The asterisks indicate the significant differences between NT3 and C2 cells of each treatment (n=8). Immunoblot analysis (B) and quantification of cleaved caspase 3/caspase 3 (C), cleaved caspase 7/caspase7 (D) and PARP (Cleaved-Asp214)/ β-actin (E) in whole cell lysates (70 μg protein) obtained from cells treated with 150 μM cisplatin for the indicated time periods. The β-actin blot serves as the loading control. The significant difference between C2 and NT3 at each time point is indicated by asterisk (n=2). (F) Cell viability determined by MTT assay in cells treated with 150 μM cisplatin alone, in the presence of 50 μM Z-VAD or 50 μM Z-VAD alone in SF media for 24 hr. The results are presented as percent viability of untreated control in SF media. The asterisks indicate significant differences between NT3 and C2 cells of each treatment (n=24).

In order to study the role of apoptosis in the increased susceptibility of C2 cells to

cisplatin, a Caspase-Glo® 3/7 Assay was performed to detect the activity of caspase 3/7, which plays key effector roles in apoptosis. The basal caspase activity in C2 cells is not significantly different from that in NT3 cells. However, after cisplatin treatment, increased caspase 3/7 activity was observed in C2 cells as compared to NT3 cells (Figure 2.3A). This finding was confirmed by western blot (Figure 2.3B) which revealed increased caspase3/7 activation (Figure 2.3C&D) and PARP cleavage (Figure 2.3E) in C2 cells at 18 hr of cisplatin treatment. Importantly, the susceptibility difference between NT3 and C2 cells to cisplatin was abolished by Z-VAD, a pan-caspase inhibitor (Figure

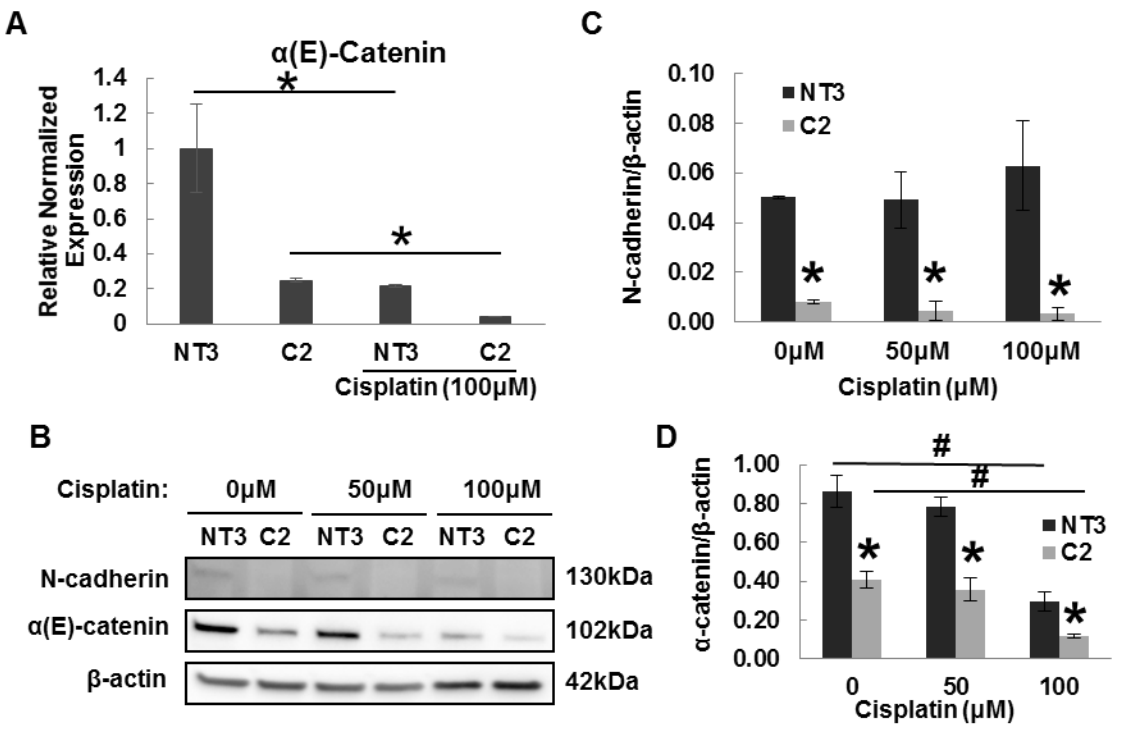


Figure 2.4. The effect of cisplatin on α-catenin and N-cadherin expression. (A) qPCR for α(E)-catenin in NT3 and C2 cells with or without 100 μM cisplatin treatment for 24hr. The result is normalized to untreated NT3 cells. The significant differences are indicated by asterisks (n=3). Immunoblot analysis (B) and quantification of N-cadherin/ β-actin (C) and α-catenin/β-actin (D) in whole cell lysates (40 μg protein) obtained from cells treated with the indicated concentrations of cisplatin in SF media for 24 hr. The β-actin blot serves as loading control. The significant differences between C2 and NT3 are indicated by asterisks (n=2). The pound indicates significant difference from the untreated control group (n=2).

2.3F). Together, these data identify apoptosis as the underlying mechanism of the increased susceptibility of C2 cells to cisplatin injury.

Another interesting finding is that cisplatin significantly suppressed mRNA (Figure 2.4A) and protein (Figure 2.4B&D) expression of α-catenin. After challenge with 100 μM cisplatin for 24 hr, α(E)-catenin levels were at 20% in the NT3 cells. The expression of α(E)-catenin in C2 cells also decreased even further after cisplatin treatment. Similar reduction of α-catenin protein expression was observed by western blot. However, the expression of N-cadherin did not change after the cisplatin challenge (Figure 2.4B&C). These results demonstrate that α-catenin is also a target of cisplatin-induced cell injury.

Consistent with previous studies [182], no significant difference was observed between 4 and 24-month-old male Fisher 344 rats in either serum creatinine (Figure 2.5A) or blood urea nitrogen (BUN) levels (data not shown). However, a significant increase in serum creatinine was seen at 72 hr after a single dose of cisplatin in aged rats, but not in young rats. No significant difference was seen in BUN levels (data not shown), this is probably because BUN is less sensitive than serum creatinine as a biomarker of AKI [212] . Interestingly, when aged (20 month) and young rats were challenged with mercuric chloride, no significant difference was seen (Figure 2.5B). The aged rat expressed higher level of KIM-1 compared with young animal in control groups which is consistent with previous reports from our laboratory [213] . A significant increase in urine KIM-1 was detected after the cisplatin challenge in both young and aged rats (Figure 2.5C). Interestingly, the expression of α(E)-catenin in the tubules of young rats was not influenced by cisplatin. However, α(E)-catenin expression was further decreased in aged kidney after cisplatin injury (Figure 2.5D&F). Conversely, the expression of N-cadherin

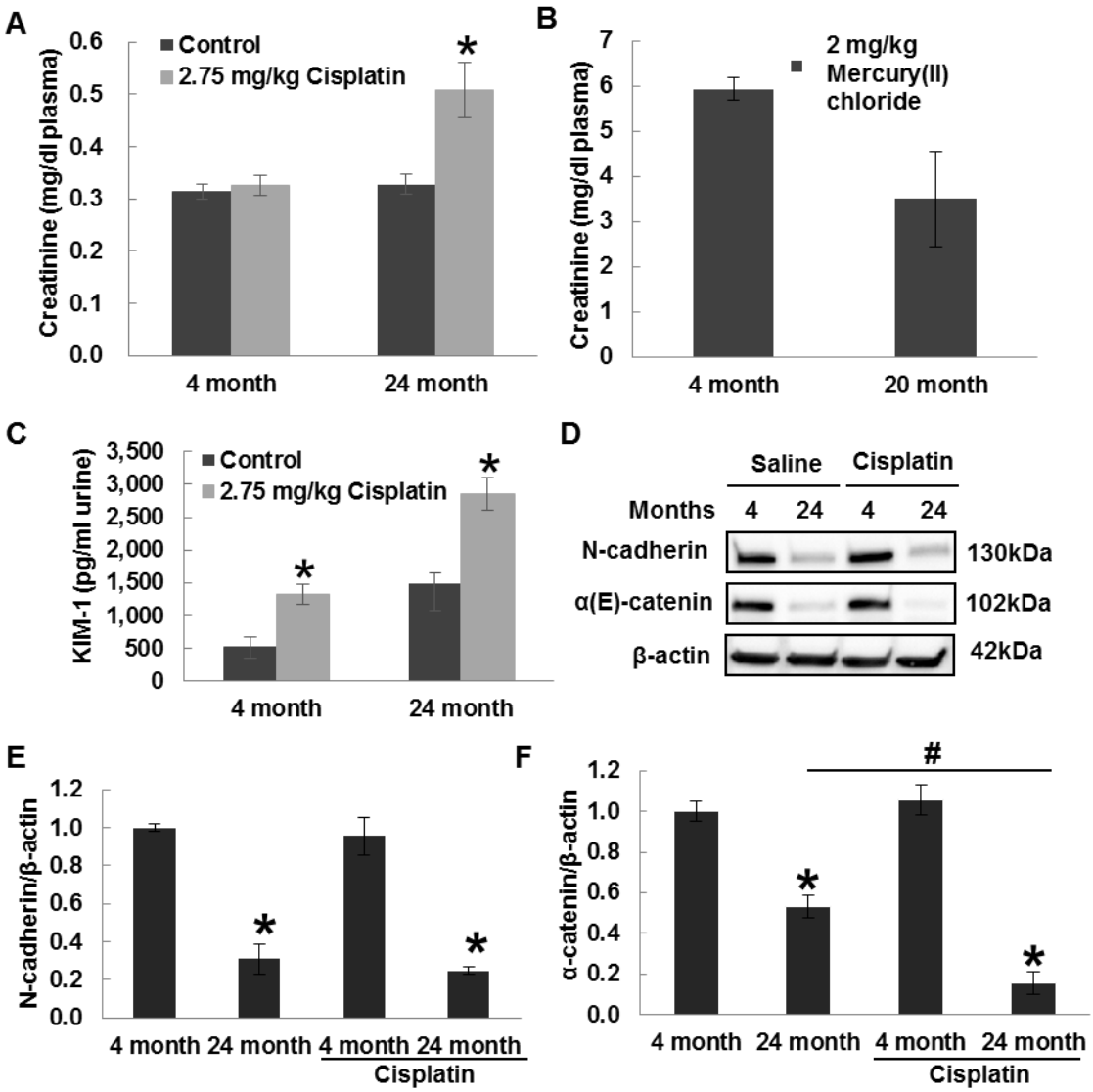


Figure 2.5. Cisplatin or mercury(II) chloride induced changes in young and aged kidney. Serum creatinine levels were measured after 72 hr of cisplatin treatment (A) or 48 hr of mercury(II) chloride treatment (B). Urine KIM-1 levels were measured after 72 hr of cisplatin treatment (C). Equal volume of saline is used as control. The asterisk indicates significant differences from the control group (n=5). Immunoblot (D) and quantification of N-cadherin/ β-actin (E) and α-catenin/β-actin (F) in kidney tissue lysates (100 µg protein) obtained from young and aged rats after 72 hr of cisplatin treatment. The asterisk indicates significant differences between young and aged group of each treatment (n=5). The pound indicates significant difference from the untreated group (n=5).

was not affected by cisplatin in neither young nor aged kidney (Figure 2.5D&E). *In situ* apoptosis was detected by TUNEL assay (Figure 2.6A). The aged kidney exhibited higher level of *in situ* apoptosis compared to the young kidney. The *in situ* apoptosis was

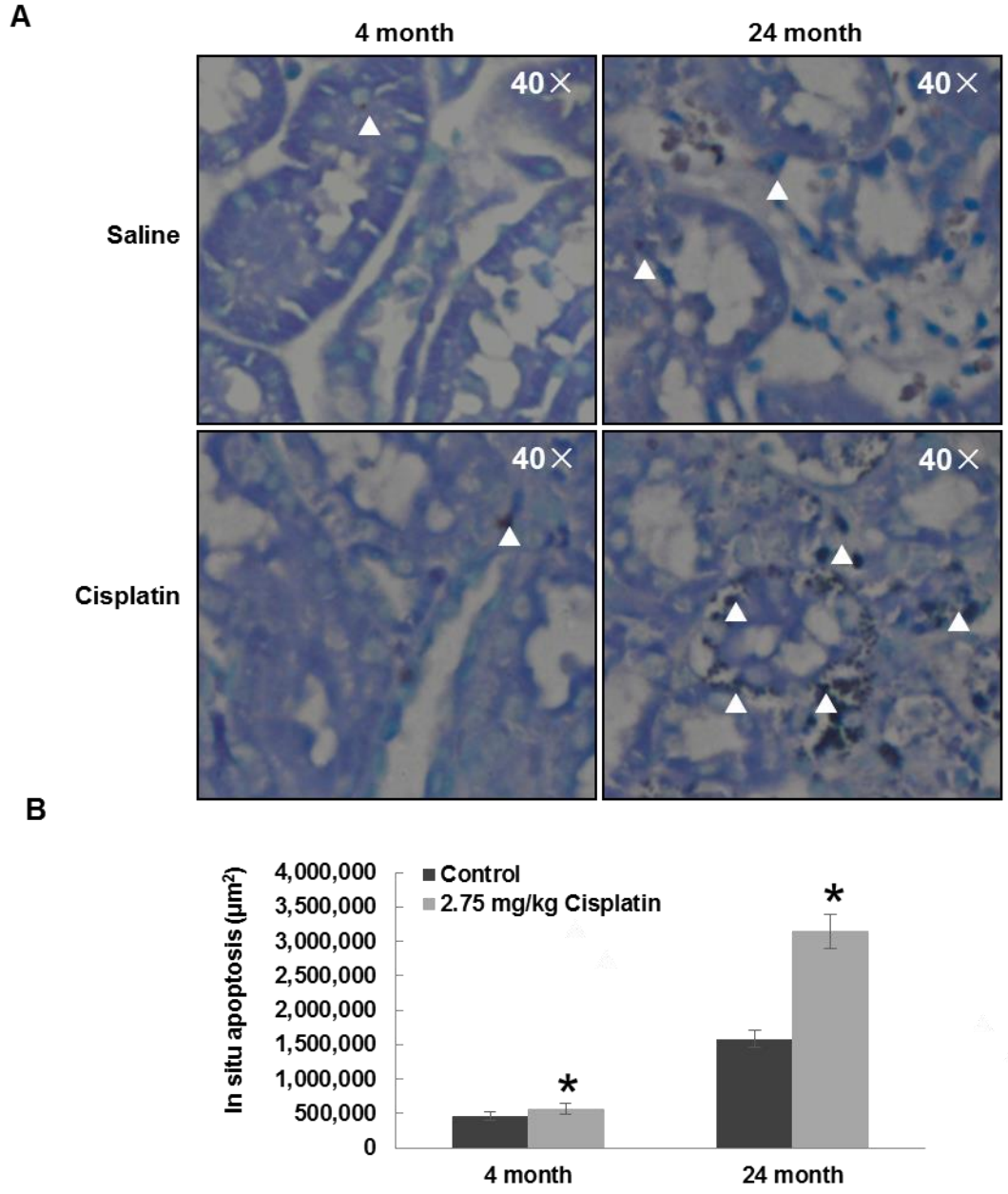


Figure 2.6. *In situ* apoptosis induced by cisplatin in aged kidney. *In situ* apoptosis was detected (A) and quantified (B) via TUNEL assay. The white arrow heads point the apoptotic cells. The asterisks indicate significant difference from the untreated group (n=5).

increased to a larger extent by cisplatin in aged group than young group (Figure 2.6B). These data demonstrate that aged kidney, which is marked by loss of α-catenin, is more susceptible to cisplatin injury, but not necrosis.

Discussion

For decades, α-catenin has been viewed as a simple linkage molecule between cadherin-β-catenin complex and actin cytoskeleton mediating cell-adhesion in a cadherin dependent manner [204]. Recent studies, however, have revealed cadherin-independent functions of α-catenin [214]. Mis-localization or loss of α-catenin has been reported to be a more severe prognosis of cancer progression than loss of E-cadherin in several tumors [174]. Moreover, α-catenin is also involved in cell proliferation, apoptosis, and actin cytoskeleton dynamics in a cadherin-independent manner [204,214]. The role of α-catenin in apoptosis is contentious. On one hand, deletion of α-catenin in the central nervous system or epidermis was reported to cause a decrease in apoptosis due to up-regulation of NFκB [179]. Reintroducing α-catenin into a myeloid leukemia cell line resulted in a decrease in proliferation and increase in apoptosis [204]. On the other hand, evidence has shown a decreased expression of α-catenin coupled with an increase in p53 expression in both lung and gastric cancer [178]. In addition, an increase in apoptosis was observed in α-catenin deleted mouse mammary gland [177]. Hence, whether loss of α-catenin increases or decreases apoptosis may depend on the cellular context. In our study, C2 cells exhibited a significant loss of viability as compared with NT3 cells after the cisplatin treatment (Figure 2.1B, C&D), but showed no cytotoxicity (LDH leakage) difference (Figure 2.2A). These results indicate that the increased susceptibility of C2 cells to cisplatin is not due to necrosis. Furthermore, increased caspase3/7 activation and PARP cleavage was observed in C2 cells after cisplatin treatment. Z-VAD, a pan-caspase inhibitor, abolished the susceptibility difference between NT3 and C2 cells to cisplatin (Figure 2.3). Taken

together, our study is the first to provide evidence that loss of α(E)-catenin increases cisplatin-induced apoptosis of renal tubular epithelial cells.

Interestingly, the mRNA and protein expression of α-catenin was further decreased in both NT3 and C2 cells after the cisplatin challenge (Figure 2.4). Correspondingly, our in vivo study showed a further decrease of α(E)-catenin expression at 72 hr after a single dose of cisplatin in aged kidney, but α(E)-catenin expression was not altered in young rats (Figure 2.5D&F). This results indicate that loss of α(E)-catenin during aging is exacerbated by further loss following cisplatin injury, which initiates further apoptosis of renal tubular epithelial cells, leading to increased injury.

The health of aging population is a critical issue for the 21st century. There will be more than 70 million Americans over the age of 65 by 2030 according to the U.S. Census Bureau [215]. Aging can change the structure and function of several organs, which increases the incidence of many diseases. With the exception of lung, the change in kidney with normal aging, is most dramatic among all human organ systems [18]. Aging related changes in the kidney may not be obvious under normal conditions. However, these changes may contribute to renal dysfunction under stress or chemical insults, including drug-induced AKI [205]. This is consistent with our in vivo study which shows no significant difference between 4 and 24-month-old male Fisher 344 rats in basal serum creatinine level. However, after 72 hr of an IP dose of cisplatin treatment, increased serum creatinine level were observed in aged rats but not young ones (Figure 2.5A).

Cisplatin is a widely used chemotherapeutic agent with broad-spectrum against a variety of tumors. However, its clinical usage is dramatically limited by a dose-limiting side effect, nephrotoxicity [216]. The mechanism underlying cisplatin-induced

nephrotoxicity involves several factors, including DNA damage, oxidative stress, inflammatory response and apoptosis [111]. Two major pathways of apoptosis have been studied in cisplatin-induced nephrotoxicity: the intrinsic and extrinsic pathways [117]. Both pathways will ultimately activate caspase 3/7 leading to the morphological changes of apoptosis including cell shrinkage, membrane blebbing and DNA fragmentation [119]. The intrinsic pathway, involving the mitochondria, has been reported to play a key role in cisplatin-induced renal tubular cell death. After exposure to cisplatin, pro-apoptotic proteins BAX and BAK are activated, altering the integrity of mitochondrial membrane. As a result, cytochrome C and apoptosis-inducing factor (AIF) will be released from the mitochondria, which leads to the caspase dependent or independent apoptosis pathway correspondently [217]. Because our study showed that pan-caspase inhibitor completely abolished the susceptibility difference between NT3 and C2 cells to cisplatin (Figure 2.3C), loss of α-catenin may only influence the caspase dependent apoptosis pathway. In addition, apoptosis mediated by endoplasmic reticulum (ER) stress involving caspase 12, phosphorylated extracellular signal regulated kinase (p-ERK) and Ca2+-independent phospholipase A2 may also play an important role in cisplatin-induced nephrotoxicity [127]. Furthermore, evidence has shown that the inhibition of p38-MAPK can attenuate cisplatin-induced nephrotoxicity in mice [218]. The extrinsic pathway is mainly initiated by activating cell death receptors by binding TNF-α or Fas, which cause the recruitment and activation of caspase 8/10 leading to the activation of caspase 3 or the mitochondrial apoptosis pathway [117,119]. Our future studies will be focused on which specific pathway is influenced by the loss of α(E)-catenin causing the increased susceptibility of the aged kidney to apoptosis.

In conclusion, this study showed that loss of α(E)-catenin increases cisplatin-induced apoptosis of renal tubular epithelial cells. Considering the loss of α(E)-catenin in aged kidney, this result could partially explain the increased nephrotoxicity induced by cisplatin in the aged kidney.

CHAPTER 3

LOSS OF α(E)-CATENIN PROMOTES FAS MEDIATED APOPTOSIS IN TUBULAR EPITHELIAL CELLS

This research was published in Apoptosis.

2015; 20(7):921-9

Abstract

The aging kidney undergoes structural and functional alterations which make it more susceptible to drug-induced acute kidney injury (AKI). Previous studies in our lab have shown that the expression of α(E)-catenin is decreased in aged kidney and loss of α(E)-catenin potentiates AKI-induced apoptosis, but not necrosis, in renal tubular epithelial cells (NRK-52E cells). However, the specific apoptotic pathway underlying the increased AKI-induced cell death is not yet understood. In this study, cells were challenged with nephrotoxicant cisplatin to induce AKI. A ~5.5-fold increase in Fas expression in C2 (stable α(E)-catenin knockdown) relative to NT3 (non-targeted control) cells was seen. Increased caspase-8 and -9 activation was induced by cisplatin in C2 as compared to NT3 cells. In addition, decreased Bcl-2 expression and increased BID cleavage and cytochrome C release were detected in C2 cells after cisplatin challenge. Treating the cells with cisplatin, in combination with a Bcl-2 inhibitor, decreased the viability of NT3 cells to the same level as C2 cells after cisplatin. Furthermore, caspase-3/-7 activation is blocked by Fas, caspase-8, caspase-9 and pan-caspase inhibitors. These inhibitors also completely abolished the difference in viability between NT3 and C2 cells in response to cisplatin. These results demonstrate a Fas-mediated apoptotic signaling pathway that is enhanced by the age-dependent loss of α(E)-catenin in renal tubule epithelial cells.

Key words: Aging, AKI, α(E)-catenin, Apoptosis, Fas

Introduction

Aging is a major challenge facing all scientists and doctors today because of the substantial increase in the human lifespan during the last century [219]. By 2050, it is expected that the number of individuals aged 60 or more will double, accounting for 11%, currently, to 22% of world's population [220]. Several structural and functional alterations occur in the aging kidney which makes aging a major risk factor for acute kidney injury (AKI) [13]. Clinical studies performed in Spain showed the incidence of AKI is 3.5 times higher in aged patients (≥70 years) compared with those less than 70 years old [68]. In addition, the increased medication use in elderly patients can also increase the incidence of AKI since nephrotoxic drugs are the cause for approximately 20% of AKI cases [117]. In our study, cisplatin, a widely used nephrotoxicant-induced AKI model, was used to investigate the pathophysiological mechanism of AKI in aged kidney [123].

α-catenin, which bridges the E-cadherin-β catenin complex and actin cytoskeleton, is essential for maintaining the integrity of the intercellular adherens junction [221]. There are three forms of α-catenin: neural (N), epithelial (E) and testis/heart (T) [151]. There is an increasing recognition that in addition to the well-established role in cell adhesion, α-catenin regulates multiple pathways controlling cell density, polarity, proliferation and apoptosis [162–164]. Previous studies in our lab have shown the expression of α(E)-catenin is dramatically decreased in proximal tubular epithelium cells in aged male Fisher 344 rats [182]. The decreased expression of α(E)-catenin is coupled with increased cisplatin-induced apoptosis, rather than necrosis, in a caspase dependent manner [198].

The intrinsic and extrinsic pathways are two major caspase dependent ways to induce apoptosis, which are distinguished by the initiating signal origination [117]. The

intrinsic pathway is triggered by cell stress induced mitochondria outer membrane permeabilization (MOMP), resulting in the release of cytochrome c which activates caspase-9. The extrinsic pathway is initiated by the binding of apoptotic ligand to death receptors leading to the activation of caspase-8. Both intrinsic and extrinsic pathways will ultimately cleave caspase-3/7 which initiates the morphological changes of apoptosis [126]. In this study, the specific apoptotic pathway promoted by decreased α(E)-catenin will be identified by using a stable α(E)-catenin knockdown cell line (C2 cells) generated in NRK-52E cells. NT3 cells will be used as the non-targeted control [183,184]. For the first time, this study provides evidence that age-dependent loss of α(E)-catenin increases the susceptibility to acute kidney injury by facilitating the Fas mediated apoptosis pathway in renal tubule epithelial cells.

Methods

Materials:

The following chemicals were used in the experiments. cis-Diamineplatinum(II) dichloride (Sigma-Aldrich Cat #P4394); Kp7-6 (CalBiochem Cat #341291); Pentoxifylline (Sigma-Aldrich Cat #P1784); Z-IETD-FMK (CalBiochem Cat #218759); Z-LEHD-FMK (CalBiochem Cat #218761); Z-VAD (OMe)-FMK (CalBiochem Cat #627610); ABT-199 (Selleckchem Cat # S8048).

Animals:

Male Fisher 344 rats (4-, and 24-month-old) were obtained from the NIA colony. Animals were randomly assigned to the cisplatin treated group and saline control group with n=5 each. Animals received a single intraperitoneal (IP) injection of 2.75 mg/kg cisplatin, or an equal volume of saline as control. Animals were placed in metabolic cages overnight before harvesting. 72 h after cisplatin injection, rats were anesthetized with ketamine (80–120 mg/kg)/xylazine (5–10 mg/kg) via IP injection. Kidney tissue lysates were obtained to perform western blot. All animal experiments and care were approved by the University of Missouri Animal Care and Use Committee in accordance with NIH (Protocol number #AUP 6752).

Cell Culture:

C2 (single cell clone of stable a(E)-catenin knockdown) and NT3 (vector control) cells were generated and cultured as previously described [15, 16]; and used within 20

passages of establishing the clonal cell line for all studies. For these experiments, cells were plated at a density of 5×104 cells/cm2 and cultured in DMEM/F12 (1:1) with L-glutamine and HEPES (Gibco Cat #11039-021) supplemented with 10% fetal bovine serum (FBS) (Altanta Biologicals Cat #S11150), 5 µg/ml puromycin dihydrochloride (Sigma Cat #P9620) and incubated at 37°C in 5% CO2. Cells were harvested with TrypLETM Express (Gibco Cat #12604-021) and pelleted at 1500 rpm for 5 min. at room temperature (RT).

RT² Profiler PCR Array

Total RNA was extracted using the RNeasy mini kit (Qiagen Cat #74104) and subjected to cDNA synthesis using the RT² Easy First Strand Kits (Qiagen Cat # 330421). Genes associated with apoptosis were assessed by real time RT² Profiler Rat Apoptosis PCR Array (Qiagen Cat# PARN-012Z) following the kit protocol.

MTT Assay:

Cells were seeded in a 96 well flat bottom tissue culture plate (Sigma Cat #Z707910) at a density of 5×104 cells/cm2. After 24h, culture media was replaced by serum free (SF) media supplemented with desired treatments. Three hours before harvest, 10 µl of 5 mg/ml MTT (Sigma Cat #M2128), dissolved in DPBS (Gibco Cat #14190-144), was added to each well. Upon harvesting, cells were washed with cold DPBS and dissolved by adding 50 µl solubilization solution (10% Triton X-100, 0.1N HCl in isopropanol). The plates were read at 570/690 nm on the Synergy HT Multi-Detection

Microplate Reader (BioTek, Winooski, VT). The results are expressed by percent viability as [Abs570-690 treated/Abs570-690 control×100].

Real-Time PCR:

1x107 cells were harvested and suspended in 1 ml PBS. RNA was isolated using the NucleoSpin (Clotech Cat #740955) followed by determining the RNA concentration using NanoDrop 2000c Spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was generated from 2 µg RNA using the High Capacity cDNA Synthesis Kit (Life Technologies Cat #4368814). Quantitative PCR was performed in duplicate using 50 ng cDNA/reaction via Taqman assays with SsoFast™ Probes Supermix with ROX (Bio-Rad Cat #172-5251) and the CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The following cycling conditions were used: 95°C for 20 sec., then 95°C for 1 sec and 60°C for 20 sec repeated 40 times. Commercially available TaqMan primer sets were used to assess Fas (Rn00685720_m1) (Life technologies Cat #4331182) and TNF-α (Rn01525859_g1) (Life technologies Cat #4453320). Relative quantitation was performed using the Pfaffl method [1] normalized to Casc3.

Caspase Activity Assay:

Confluent cultures of NT3 and C2 cells in 96 well plates were challenged with desired treatments. Caspase activity was determined by Caspase-Glo® 3/7 Assay (Promega Cat #G8091), Caspase-Glo® 8 Assay (Promega Cat #G8201) and Caspase-Glo® 9 Assay (Promega Cat #G8211), according to the manufacturer's instructions.

Western Blot:

Subconfluent cells were washed twice with ice-cold DPBS and lysed with lysis buffer (10mM Tris-HCl, 1% SDS) containing HaltTM Protease/Phosphatase inhibitors (Thermo Scientific Cat #78444). Cells were scraped and incubated on a rocker for 15 min at 4°C. Cells were further disrupted by pipetting 15 times and spun at 12,000 g for 15 min at 4°C. Protein concentration was determined by NanoDrop 2000c Spectrophotometer at 280 nm.

The following antibodies were used: Cytochrome c Release Assay Kit (GeneTex Cat #GTX85531), TNF-α (NOVUS Cat #NB600-587), BID (NOVUS Cat #NB100-56106), Bcl-2 (Cell Signaling Cat #2876), Fas (Abcam Cat #15285), FasL (Abcam Cat #82419) and anti-β-actin (Sigma Cat #A2228). Goat-anti-mouse HRP conjugate and Goat-anti-rabbit HRP conjugate (Jackson ImmunoResearch Laboratories, Cat #115035003 and 305035003) were used at 1:20,000 dilutions. Blots were developed using SuperSignal West Femto Chemiluminescent Substrate (Pierce Cat #34095), imaged using the ChemiDocTM imaging system (Bio-Rad, Hercules, CA), and quantitation performed using the ImageLab 3.0 software (Bio-Rad, Hercules, CA).

Statistics:

Results are expressed as mean ± S.E. A two-way analysis of variance (ANOVA) was performed with the exception of Fig.2A, 2C, 2D and 3A in which a one-way ANOVA was performed, followed by post-hoc t-tests with the Bonferoni correction using

the statistical software GraphPad Prism 6 (GraphPad Software, La Jolla, CA). The differences were considered statistically significant when $p<0.05$.

Results

Target genes involved in apoptosis were assessed by RT² Profiler PCR Array in NT3 and C2 cells. The gene expression (fold-change) in C2 cells relative to NT3 cells is depicted by the heat map with up-regulation in red and down-regulation in green (Fig 3.1). The up regulated genes include Fas, TNF-α related genes, caspases and pro-apoptotic Bcl-2 family members. The down regulated genes include Card 10, II10 and Birc3 which are mainly anti-apoptotic [222].

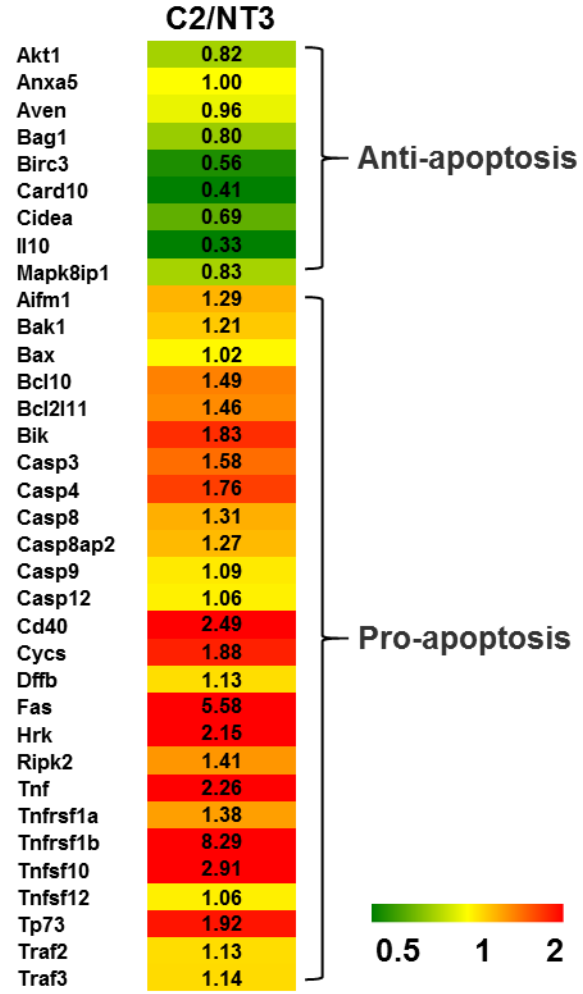


Fig 3.1 Apoptosis gene expression profiling of NT3 and C2 cells Apoptosis PCR Array analysis of mRNA from NT3 and C2 cells. A heat map shows the gene expression fold changes of C2 cells relative to NT3 cells with up-regulation in red and down-regulation in green.

Fas and TNF-α are two major death receptors which mediate the extrinsic apoptosis pathway [126]. Real-time PCR revealed the Fas mRNA was elevated 5.5-fold in C2 Cells relative to NT3 cells (Fig 3.2A), which is consistent with the PCR Array result (Fig 3.1). In addition, increased protein expression of Fas ligand (FasL) was detected in C2 cells rather than NT3 cells after cisplatin treatment (Fig 3.2B&C). The protein expression of Fas was consistently higher in C2 cells than in NT3 cells before and after cisplatin treatment (Fig

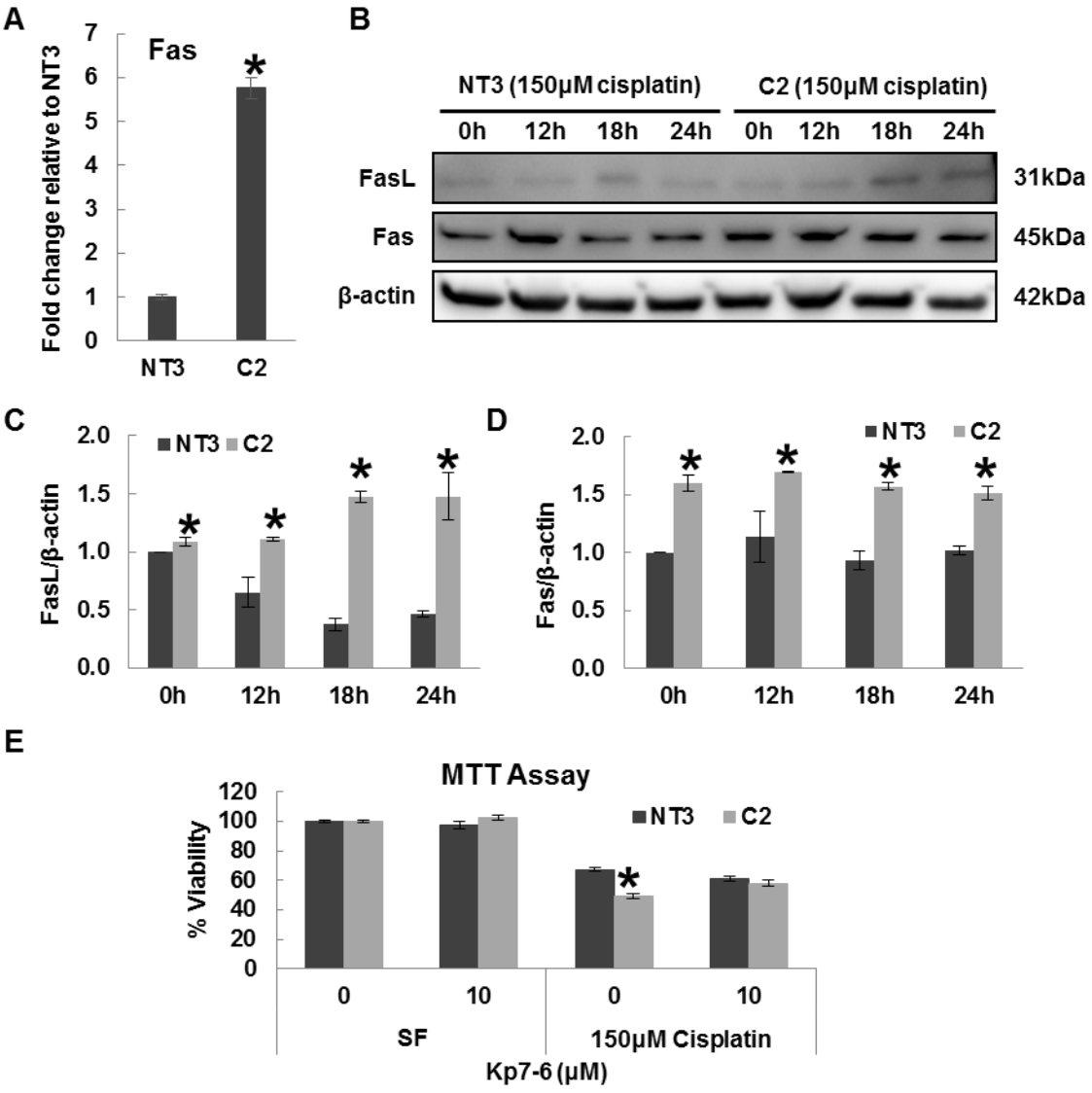


Fig 3.2 The role of Fas in cisplatin induced injury in NT3 and C2 cells (A) Real time PCR of Fas in NT3 and C2 cells. The result is normalized to NT3 cells (N=3). Immunoblot analysis (B) and quantification of Fas (C) and FasL (D) in whole cell lysates of NT3 and C2 cells (N=3). The β-actin blot serves as the loading control. (E) Cell viability determined by MTT assay in cells treated with indicated concentrations of Kp7-6 alone or in the presence of 150 μM cisplatin for 24 h (N=3). The result is presented as the viability percentage of untreated control in SF media. The asterisks indicate significant differences between NT3 and C2 cells.

3.2B&D). Confluent cultures of NT3 and C2 cells were challenged with cisplatin in combination with Kp 7-6 (FasL/Fas antagonist) for 24h. C2 cells exhibited a significant loss of viability after cisplatin injury as compared with NT3 cells and the susceptibility

difference between NT3 and C2 cells to cisplatin was completely abolished by Kp 7-6

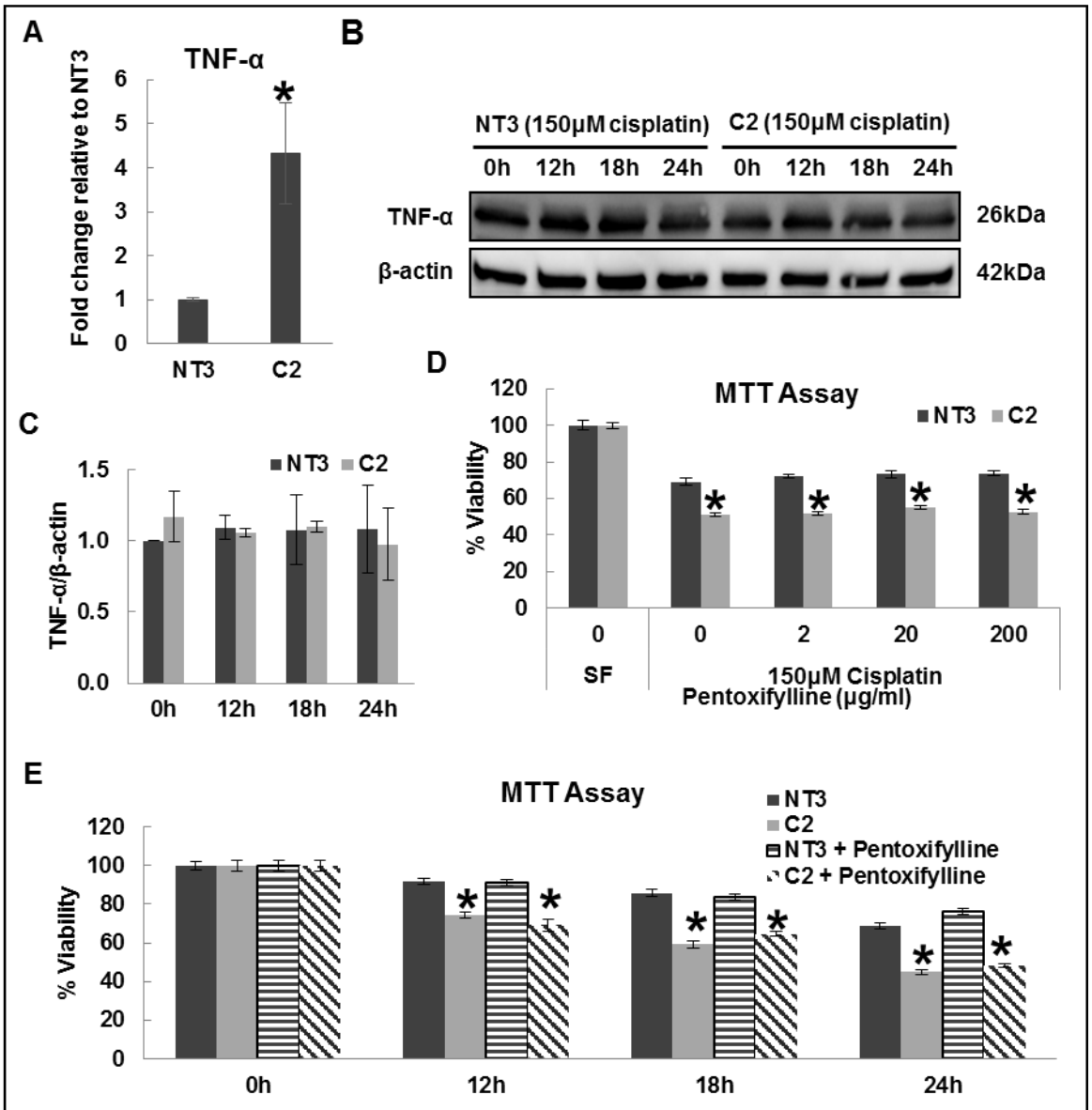


Fig 3.3 The role of TNF-α in cisplatin induced injury in NT3 and C2 cells (A) Real time PCR of TNF-α in NT3 and C2 cells. The result is normalized to NT3 cells (N=3). Immunoblot analysis (B) and quantification of TNF-α (C) in whole cell lysates obtained from cells treated with 150 μM cisplatin for indicated time periods (N=2). The β-actin blot serves as the loading control. (D) Cell viability determined by MTT assay in cells treated with indicated concentrations of pentoxifylline and 150 μM cisplatin for 24 h (N=3). (E) Cell viability determined by MTT assay in cells treated with 150 μM cisplatin alone or in combine with 200 μg/ml pentoxifylline for the indicated time. The results are presented as the viability percentage of untreated control in SF media. The asterisks indicate significant differences between NT3 and C2 cells.

(Fig 3.2F). Although C2 cells showed higher mRNA expression of TNF-α (Fig 3.3A), no difference of TNF-α protein expression was observed between NT3 and C2 cells (Fig 3.3B&C). Furthermore, pentoxifylline, a TNF-α inhibitor, failed to rescue C2 cells from the decreased viability as compared with NT3 cells in response to cisplatin (Fig 3.3D&E). Taken together, these results indicate Fas, rather than TNF-α, mediates the increased cisplatin-induced apoptosis in C2 cells.

Caspase-8, a member of the cysteine proteases, is known to be directly recruited by the Fas-associated death domain (FADD) [223]. A Caspase-Glo 8 Assay was performed to

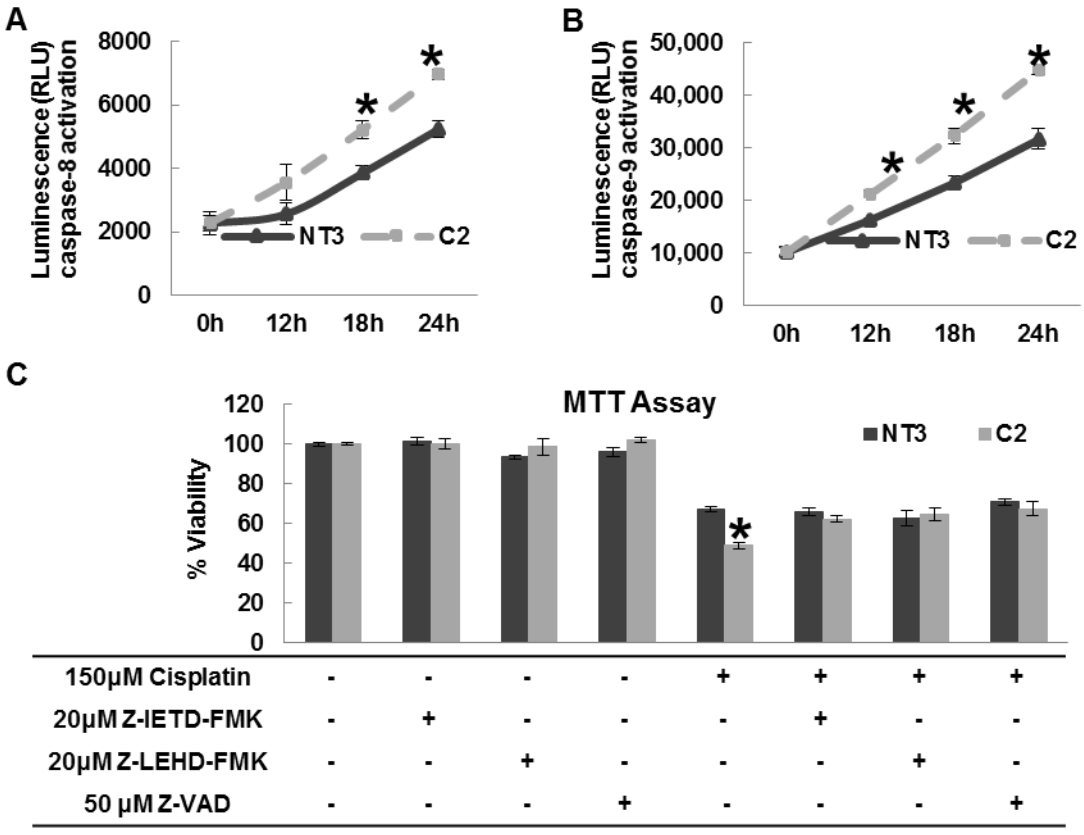


Fig 3.4 The role of caspases in cisplatin induced injury in NT3 and C2 cells The activities of caspase-8 (A) and caspase-9 (B) in confluent cultures of cells treated with 150 μM cisplatin for indicated time periods were determined by luminescent assay (N=2). (C) Cell viability determined by MTT assay in cells treated with indicated combinations of cisplatin, Z-IETD-FMK, Z-LEHD-FMK and Z-VAD (N=3). The asterisks indicate significant differences between NT3 and C2 cells.

assess the activity of caspase-8. No significant difference was detected in the basal caspase-8 activity between NT3 and C2 cells. However, C2 cells showed higher caspase-8 activation than NT3 cells after cisplatin treatment in a time-dependent manner (Fig 3.4A). Interestingly, similar results were observed in a Caspase-Glo 9 Assay (Fig 3.4B). Treating the cells with Z-IETD-FMK (caspase-8 inhibitor), Z-LEHD-FMK (caspase-9 inhibitor) and Z-VAD (pan-caspase inhibitor) abolished the susceptibility difference between NT3 and C2 cells to cisplatin (Fig 3.4C). Since caspase-9 is an initiator caspase, which plays a critical role in intrinsic apoptosis pathway [224], these data suggest the involvement of mitochondria in the pathway.

Members of the Bcl-2 family govern MOMP and have either anti-apoptotic (Bcl-2) or pro-apoptotic (BID) function [225]. Western blot was performed to measure the protein expression of Bcl-2 members (Fig 3.5A). Increased BID cleavage was induced by cisplatin in C2 relative to NT3 cells (Fig 3.5C). Correspondingly, lower Bcl-2 expression was observed in basal C2 cells. After cisplatin treatment, the expression of Bcl-2 was further decreased in C2 cells, but not in NT3 cells (Fig 3.5D). This result was consistent with our in vivo study. Aged kidney showed increased BID cleavage (Fig 3.5E&F) and decreased Bcl-2 expression (Fig 3.5E, F&G) compared to young kidney. Cisplatin-induced further increase of BID cleavage and decrease of Bcl-2 expression in aged kidney. However, the BID cleavage and Bcl-2 expression in young kidney were not influenced by cisplatin. Cytosolic cytochrome c was also measured (Fig 3.5A) which revealed that cisplatin induced more cytochrome c release in C2 than NT3 cells (Fig 3.5B). Importantly, ABT-199, a Bcl-2 inhibitor, decreased the viability of NT3 cells in a dose-dependent manner and completely abolished the viability difference between NT3

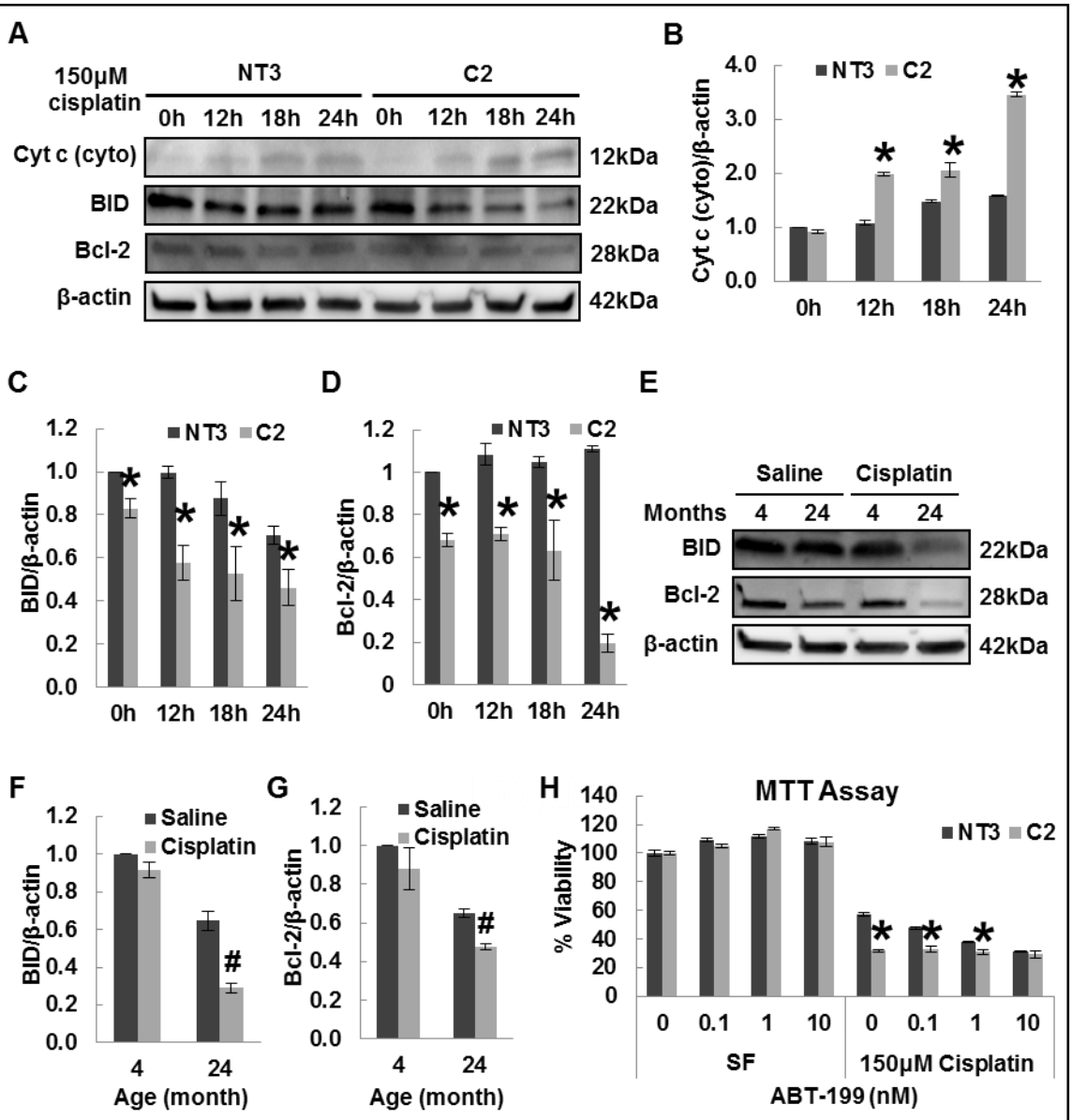


Fig 3.5 The role of mitochondria in cisplatin induced injury in NT3 and C2 cells Immunoblot analysis (A) and quantification of cytosolic cytochrome c (B), BID (C) and Bcl-2 (D) in whole cell lysates obtained from cells treated with 150 μM cisplatin for indicated time periods (N=2). The β-actin blot serves as the loading control. Immunoblot (E) and quantification of BID (F) and Bcl-2 (G) in kidney tissue lysates obtained from 4- and 24-months-old rats at 72 h after saline or 2.75 mg/kg cisplatin IP injection. The pound indicates the significant difference between saline and cisplatin treated group (N=5). (H) Cell viability determined by MTT assay in cells treated with indicated concentrations of ABT-199 alone or in the presence of 150 μM cisplatin for 24 h (N=3). The asterisks indicate the significant differences between NT3 and C2 cells.

and C2 cells at the dose of 10nM (Fig 3.5H). These results demonstrate the key role that

mitochondria played in the apoptosis pathway and the decreased viability of C2 cells to cisplatin may be due to decreased anti-apoptotic Bcl-2 expression.

Caspase-3 and caspase-7 are effector caspases that can be activated by either the extrinsic (caspase-8) or intrinsic (caspase-9) pathway leading to apoptosis [119]. Previous studies in our lab showed increased caspase-3/7 activation in C2 cells after cisplatin treatment [198]. A Caspase-Glo 3/7 Assay was used to measure the activity of caspase-3/7 (Fig 3.6). Z-IETD-FMK, Z-LEHD-FMK and Z-VAD completely blocked caspase-3/7 activation induced by cisplatin in NT3 and C2 cells, which indicates that caspase-3/7 is downstream of both caspase-8 and 9 in the pathway. Interestingly, treating the cells with cisplatin in combination with Kp 7-6, a FasL/Fas antagonist, selectively decreased the cisplatin-induced caspase-3/7 activation in C2 cells to the level observed in NT3 cells. These data demonstrate that loss of α(E)-catenin promotes apoptosis by increasing drug-induced Fas signaling pathway in renal tubule epithelial cells.

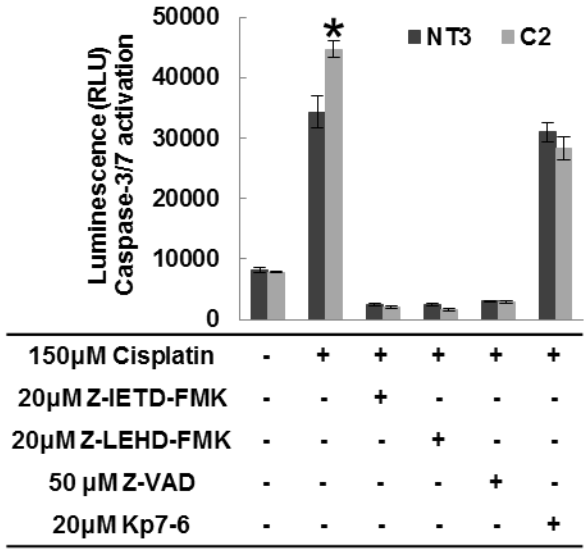


Fig 3.6 Caspase-3/7 are the downstream of the Fas mediated apoptosis pathway Caspase-3/7 activities in confluent cultures of cells treated with indicated combinations of cisplatin, Z-IETD-FMK, Z-LEHD-FMK, Z-VAD and Kp7-6 for 24 h, were determined by luminescent assay. The asterisks indicate the significant differences between NT3 and C2 cells.

Discussion

These results provide the first evidence that, in renal tubule epithelial cells, loss of α(E)-catenin increases the susceptibility to acute injury by enhancing a Fas mediated apoptosis pathway.

α-Catenin, previously known as a linking protein between the cadherin-β-catenin complex and actin cytoskeleton, has recently been shown to exert other functions [150]. Our study focused on the possible role of α-catenin in regulating cell death since renal tubular epithelium cell death is a hallmark of AKI [226]. Increased apoptosis has been reported in α-catenin deleted mammary glands of mice [177]. In addition, increased expression of p53 was coupled with decreased α-catenin expression in both gastric and lung cancer [178]. However, in epidermis or central nervous systems, deletion of α-catenin has been reported to have a protective effect from apoptosis by up regulating NF-κB [179]. α-catenin knockdown in epithelial cancer cells attenuates DR4/DR5 mediated apoptosis [180]. In a myeloid leukemia cell line, reintroducing α-catenin leads to a decrease in cell proliferation and increase in apoptosis [204]. Hence, the role of α-catenin in cell death may depend on the cellular context. Our laboratory generated a stable α-catenin knockdown cell line (C2 cell) in NRK-52E cells. A previous study showed C2 cells are more susceptible to staurosporine, a commonly used drug to induce apoptosis, but not inorganic mercury which induces necrosis in renal epithelial cells [198]. Increased susceptibility to cisplatin was also detected in C2 cells, and data suggests that the increased susceptibility is due to increased apoptosis rather than necrosis [198].

Apoptosis, or programed cell death, is a fundamental process needed to maintain homeostasis [120]. Two major pathways have been demonstrated to induce apoptosis: the

mitochondrial mediated intrinsic pathway and the receptor involving extrinsic pathway [122]. Treating the cells with cisplatin induced more caspase-8 and caspase-9 activation in C2 cells, which indicates both the intrinsic and extrinsic pathways are involved (Fig 3.4A&B). The crosstalk between the extrinsic and intrinsic pathways occurs through caspase-8 [123]. High levels of caspase-8 directly cleaves caspase-3/7 leading to apoptosis, while low levels of caspase-8 cleaves BID to truncated BID (tBID), which translocates to the mitochondria membrane to activate the intrinsic pathway by inducing MOMP [125,227]. Our studies showed that Z-LEHD-FMK, the caspase-9 inhibitor, is able to abolish the viability difference between NT3 and C2 similar to Z-IETD-FMK, the caspase-8 inhibitor (Fig 3.4C). This demonstrates that the cisplatin induced elevation of caspase 8 activity in C2 cells initiated the mitochondria mediated apoptosis pathway rather than leading to apoptosis directly. Concurrently, increased BID cleavage and cytochrome C release were detected in C2 cells after the cisplatin injury as compared to NT3 cells (Fig 3.5A, B&C). Importantly, the expression of Bcl-2, which is an apoptosis inhibitor, was decreased in C2 cells (Fig 3.5D). Meanwhile, increased BID cleavage and decreased Bcl-2 expression were also detected in aged rat kidney. The cisplatin challenge induced further increase of BID cleavage and decrease of Bcl-2 in C2 cells and aged kidney, but not in NT3 cells or young kidney. ABT-199, a Bcl-2 inhibitor, decreased viability of NT3 cells to the extent of C2 cells in response to cisplatin (Fig 3.5H). These results demonstrate that the increased susceptibility of C2 cells to cisplatin is due to the increased activity of the caspase-8/mitochondria/caspase-9 pathway, and the decreased expression of Bcl-2, which works as a suppressor in this apoptosis pathway. Apoptosis can also be induced by

endoplasmic reticulum (ER) stress [127]. However, this possibility was excluded because caspase-12 expression is not different between young and aged kidney (data not shown).

The receptor Fas, also called CD95, is a member of TNF receptor superfamily [131]. When Fas encounters its ligand FasL, the receptor trimerizes in the cytoplasmic domain and recruits the Fas-associated death domain protein (FADD). The death effector domain on FADD is capable of recruiting, cleaving and activating caspase-8 [132]. FasL is most prevalent in natural killer (NK) cells and T lymphocytes [125]. In the kidney, FasL has been reported to be expressed in tubular epithelial cells, renal endothelial cells, renal fibroblasts and mesangial cells [133]. Evidence has shown that FasL plays a key role in mediating cisplatin-induced AKI [134]. The expression of Fas is significantly reduced in cisplatin-resistant ovarian cancer epithelial cells, and up-regulation of Fas reverses the development of cisplatin resistance [135]. Epigallocatechin-3-gallate, a green tea polyphenol, and amifostine ameliorate cisplatin nephrotoxicity by inhibiting Fas conducted apoptosis [136,137]. FasL expression was increased dramatically in cisplatin exposed HeLa cells after centrocyte/centroblast marker 1 stimulation [138]. In our study, increased expression of Fas was detected in C2 cells (Fig 3.1). Kp 7-6, a Fas/FasL antagonist, abolished the cisplatin-induced viability difference between NT3 and C2 cells (Fig 3.2E). Most importantly, caspase-3/7 activity was decreased by Kp7-6 in C2 cells, but not in NT3 cells (Fig 3.6). These data confirmed that increased Fas mediated apoptosis is the cause of increased susceptibility of C2 cells to acute injury. A possible scheme of the Fas apoptotic signaling pathway is shown in Fig 3.7.

In conclusion, this study showed that age-dependent loss of α(E)-catenin in renal tubule epithelial cells facilitate the Fas mediated apoptotic signaling pathway in response

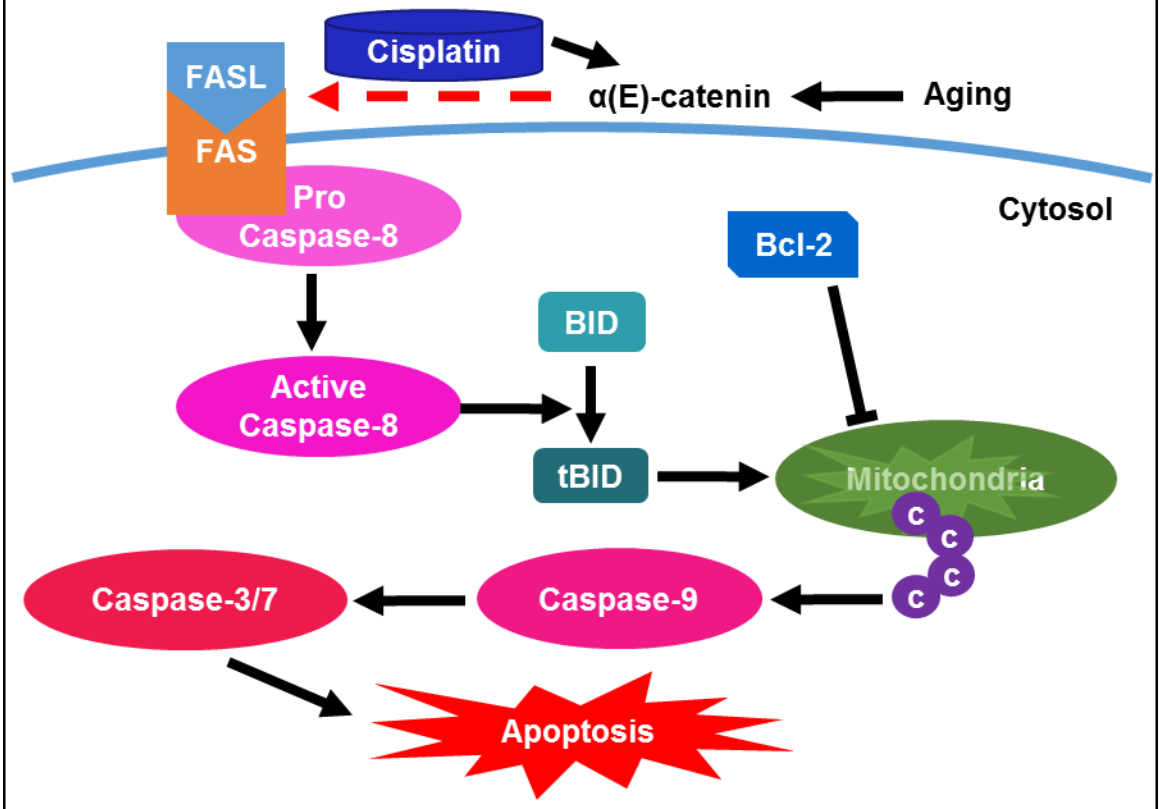


Fig 3.7 Loss of α(E)-catenin promotes Fas mediated apoptosis pathway The expression of α(E)-catenin is decreased in aged kidney. Cisplatin causes further decrease of α(E)-catenin and promotes the Fas mediated apoptosis pathway. The pathway starts with the activation of caspase-8 and the cleavage of BID. The truncated BID (tBID) translocates to the surface of the mitochondria inducing cytochrome c release into the cytosol. The cytosolic cytochrome c activates caspase-9, followed by the activation of downstream caspase-3/-7. Bcl-2 is a negative regulator which inhibits the release of cytochrome c from mitochondria.

to cisplatin-induced AKI injury. This result may explain, in part, the increased incidence of AKI in aged kidney and shed light on the development of prospective treatments to AKI in aged patients.

CHAPTER 4

FSCN2 AMELIORATES CISPLATIN-INDUCED APOPTOSIS IN AGED KIDNEY

Abstract

Previous studies have shown that the aging kidney has a marked loss of α(E)-catenin in proximal tubular epithelium. α-Catenin, a key regulator of actin cytoskeleton, interacts with a variety of actin-binding proteins. Fascin 2 is an actin bundling protein that interacts with adhesion molecules and F-actin. In this study, we hypothesize that loss of α(E)-catenin leads to disruption of actin cytoskeleton which increases cisplatin-induced injury in aged kidney. A stable shRNA knock-down of α(E)-catenin was generated in NRK-52E cells (C2 cells); NT3 cells are the non-targeted control. A cisplatin-induced loss of fascin 2 was observed in aged kidney and C2 cells. Overexpression of Fscn2 abolished increased cisplatin-induced apoptosis, mitochondrial dysfunction and oxidative stress in C2 cells compared with NT3 cells. Furthermore, there is an inverse correlation between the Fscn2 level and the susceptibility of tubular epithelial cells to cisplatin injury. Hence, our data demonstrates that fascin 2 plays an important role in preventing cisplatin-induced acute kidney injury in aged kidney.

Key words: actin, aging, α-catenin, Fscn2, apoptosis, cisplatin

Introduction

During the last century, human lifespan has increased dramatically, contributing to a substantial increase in the number of elderly people over the next two decades [13]. It is well established that aging is associated with structural and functional renal changes which make it more susceptible to acute kidney injury (AKI) [17]. Balardi and colleagues have shown that elderly patients (≥65 years) had ten times the incidence rate of AKI compared with those less than 65 years of age in Italy [70]. Xue et al. also established age as a risk factor for AKI; the incidence of AKI was 1.9% in patients younger than 65 which rose to 2.9% in those older than 85 [59]. Moreover, AKI that develops in the elderly is more severe and less likely to recover with delayed, or decreased, repair. Arora et al. demonstrated that recovery, determined by normalized serum creatinine, from AKI was 3-times as long in elderly (mean 67.1 years) compared to young (32.3 years); 32 days versus 11.4 days, respectively [79]. In meta-analysis of 17 studies, it was found that a higher percentage of surviving elderly patients did not recover renal function as compared to younger patients [228]. While proliferation is important in the repair of the kidney following injury, migration of tubular epithelial cells also plays a critical role in the recovery process following injury [194–197].

α-Catenin, which connects the cadherin-β-catenin complex to F-actin, is important in the relationship between the adherens junction (AJ) and cytoskeleton which is essential for cell adhesion [158]. Besides linking cadherin/catenin complex to the cytoskeleton, α-catenin also interacts with a variety of actin-binding proteins, including α-actinin and vinculin, as well as actin itself [199–201]. There are three forms of α-catenin: neural (N), epithelial (E) and testis/heart (T) [206]. Recent studies indicate that in addition to the well-

established role in cell adhesion, α-catenin is also involved in multiple pathways controlling membrane and actin dynamics, cell proliferation, migration and apoptosis [204]. Our laboratory has reported a dramatic decrease of α(E)-catenin expression in proximal tubular epithelium in aged male Fisher 344 rats [182]. To further study the role of α(E)-catenin in aged kidney, stable shRNA knock-down of α(E)-catenin was generated in a renal tubule epithelial cell line (NRK-52E cells). Single cell clones were selected for a non-targeted (NT3) control and a targeted knock-down (C2). C2 cells are characterized by increased monolayer permeability, increased cisplatin-induced apoptosis, decreased cell-cell aggregation, and decreased repair in a wound healing assay due to migration deficits [108,183,184].

Fascin, an actin-binding and bundling protein, plays an important role in maintenance and stability of parallel filamentous actin bundles, regulating cell proliferation, adhesion, migration and apoptosis [185,186]. Fascin is highly conserved from Drosophila to humans [229]. There are 3 isoforms of fascin: fascin 1, encoded by Fscn1, is mainly expressed in mesenchymal and nervous tissues; fascin 2, encoded by Fscn2, is most prevalent in retinal cells; Fscn3, encoding for fascin 3, is restricted to the testes [189]. While fascin has been shown to regulate actin bundle assembly, more specific roles for fascin have been demonstrated recently in the formation and turnover of cell adhesive structures which suggests fascin can be a potential mediator between α(E)-catenin and F-actin [189]. In terms of cell death, it has been shown that the upregulation of fascin1 prevents apoptosis and may be important for tumor cell survival [193]. Furthermore, up-regulated fascin expression enhances cholangiocarcinoma rat brain endothelial cell proliferation, migration and invasion [192]. Studies in our lab demonstrated the expression

of *Fscn2* was decreased in the aging kidney. Given the relationship between α(E)-catenin, fascin and actin cytoskeleton, along with their demonstrated roles in cell proliferation and apoptosis, the current studies were designed to test the hypothesis that age-dependent loss of α-catenin leads to disruption of the cytoskeleton via fascin, leading to renal dysfunction.

Methods

Animals

Male Fisher 344 (F344) rats were obtained from the NIH colony. Twenty-four months caloric restricted (RS) and 4, 20 and 24 month ad libitum (AL) rats were purchased. A 10% caloric restriction begins when rats are 14 weeks old. The restriction is then increased to 25% at 15 weeks and 40% at 16 weeks of age. Rats are maintained on a 40% caloric restriction throughout their life. On the day of the experiment, rats were anesthetized by intraperitoneal injection of ketamine (80-120 mg/kg)/xylazine (5-10 mg/kg). Kidneys were collected and 1 mm cross sections were snap frozen in liquid nitrogen and or embedded in Tissue-Tek OCT compound (Andwin Scientific) and frozen. The remaining kidney tissue was fixed in 4% paraformaldehyde overnight and stored in 70% EtOH prior to embedding in Paraplast-Plus (Oxford Labware). The Animal Care and Use Committee of the University of Missouri in accordance with the National Institutes of Health approved all experimental procedures (Protocol number #AUP 6752).

Cell Culture

Cells were plated at a density of 5×104 cells/cm2 and cultured in DMEM/F12 (1:1), containing L-Glutamine and HEPES (Gibco), supplemented with 5-10% fetal bovine serum (FBS) (Altanta Biologicals) plus antibiotic and incubated at 37°C with 5% CO2. Cells were harvested with TrypLE Express (Gibco) and pelleted at 1500 rpm for 5 min at room temperature. Cell lines with stable knockdown of α(E)-catenin (C2 cells) were generated by Sigma-Aldrich in NRK-52E cells as previously described by our laboratory [183,184]; NT3 cells are the non-targeted control. Cells were grown in the presence of 5

µg/ml puromycin (Sigma-Aldrich) for lentivirus maintenance. Open reading frame clones of mouse Fscn1 and rat Fscn2 in the pCMV6 Entry vector (Origene) were used to generate stable overexpressed cell lines in C2 (C2\Fscn1, C2\Fscn2). Non-targeted vector controls were generated in both NT3 and C2 cells (NT3\V, C2\V). Cells were grown in 5 µg/ml puromycin and 200 µg/ml geneticin (Gibco) for vector maintenance. Stable Fscn2 knockdowns (Fscn2 shA1-D1) and vector control (NRK\V1) were generated using shRNA in the pGFP-V-RS vector (Origene) with NRK-52E cells and selected with 5 µg/ml puromycin for vector maintenance.

Real-Time PCR

Tissue (20-40 mg) was minced in a -80°C cold block, homogenized in 100 µl lysis buffer, followed by the addition of 500 µl lysis buffer. Cells were harvested, re-pelletted in PBS, then resuspended in 350 µl lysis buffer. RNA was isolated using NucleoSpin RNA Miniprep Kit (Machery-Nagel) following the kit protocol. RNA concentration was determined by spectrophotometry with Nanodrop 2000c (Thermo Scientific). cDNA was generated from 2 µg RNA using the High Capacity cDNA Synthesis Kit (Life Technologies) following the kit protocol. The levels of Was, Mylk1, Mylk2, Iqgap, Nck2, Pak4, Fscn2, Fscn1, Ctnna1 and cancer susceptibility candidate 3 (Casc3) (Life Technologies) were determined by Taqman assays (Applied Biosystems) with Bull'sEye TaqProbe 2x qPCR MasterMix (MidSci) and the CFX96 Touch system (Bio-Rad) with the following cycling conditions: 95°C for 10 min, then 40 times at 95°C for 15 sec and 60°C for 60 sec. Relative quantitation was performed using the Pfaffl method normalized to Casc3 [230].

Western Blot

Subconfluent cells were washed twice with ice-cold DPBS (Gibco) and lysed with lysis buffer (10 mM Tris-HCl, 1% SDS) containing HaltTM Protease/Phosphatase inhibitors (Thermo Scientific). Cells were scraped and incubated on a rocker for 15 min at 4°C. Cells were further disrupted by pipetting 15 times and spun at 12,000 g for 15 min at 4°C. Protein concentration was determined by NanoDrop 2000c Spectrophotometer at 280 nm. The following antibodies were used: Fascin1 (NOVUS), Fascin2 (NOVUS), BID (NOVUS), BCL-2 (Cell Signaling), Cleaved PARP (Sigma), α(E)-catenin (GeneTex) and anti-β-actin (Sigma). Goat-anti-mouse HRP conjugate and Goat-anti-rabbit HRP conjugate (Jackson ImmunoResearch Laboratories) were used at 1:20,000 dilutions. Blots were developed using SuperSignal West Femto Chemiluminescent Substrate (Pierce), imaged using the ChemiDocTM imaging system (Bio-Rad), and quantitation performed using the ImageLab 3.0 software (Bio-Rad).

Immunofluorescence and Phalloidin Staining

Cells were grown on 2-well glass chamber slides (Ibidi) overnight. Cells were washed with PBS, fixed in 2% paraformaldehyde for 10 min, permeabilized with 1% TritonX-100 for 10 min and blocked with 1% bovine serum albumin (BSA) for 1 h. Cells were incubated in primary antibody or stained with 200 ng/ml FITC phalloidin (Sigma) in blocking buffer overnight on rocker at 4°C. Cells were subsequently washed 2x in PBS, incubated in conjugated secondary antibody for 2 h on the rocking plate at RT (this step was omitted during phalloidin staining), shaken dry, and counterstained with

Fluoroshield with DAPI (Sigma). Cells were imaged on an Olympus IX51 microscope (Olympus) with a 40x or 60x oil immersion lens with a UC50 digital camera using cellSense software (Olympus) at identical exposure times. The following antibodies were used: Fascin1 (NOVUS), Fascin 2 (NOVUS), α(E)-catenin (GeneTex), Goat-anti-mouse FITC conjugate (Sigma), Goat-anti-rabbit TRITC conjugate (Sigma), Goat-anti-mouse TRITC conjugate (Sigma).

MTT Assay

Cells were seeded in a 96 well flat bottom tissue culture plate (MidSci) at a density of 5×104 cells/cm^2. After 24 h, culture media was replaced by SF media supplemented with desired treatments. Three hours before harvest, 10 µl of 5 mg/ml MTT (Sigma), dissolved in DPBS, was added to each well. Upon harvesting, cells were washed with cold DPBS and dissolved by adding 50 µl solubilization solution (10% Triton X-100, 0.1N HCl in isopropanol). The plates were read at 570/690 nm on the Synergy HT Multi-Detection Microplate Reader (BioTek). The results are expressed as percent viability [Abs570-690 treated/Abs570-690 control×100].

Caspase Activity Assay

Confluent cultures of NT3 and C2 cells in 96 well plates were challenged with desired treatments. Caspase activity was determined by Caspase-Glo® 3/7 Assay (Promega) according to the manufacturer's instructions.

ATP Detection Assay:

Confluent cultures of cells in 96 well plates were challenged with desired treatments. Cellular ATP was determined by Mitochondrial ToxGloTM Assay (Promega Cat #G8001), according to the manufacturer's instructions.

Oxygen Consumption Assay:

Oxygen consumption was measured polarographically at 25℃ using a Clark-type electrode in the medium used for swelling measurements supplemented with 1mM MgCl2 and either 5mM glutamate/ 5mM malate or 10mM sussinate. State 3 was initiated by adding 2μM ADP to the reaction mixture. State 4 is started by adding 2μM oligomycin to the reaction mixture. Respiratory ratio= state 3/state 4.

Oxidative Stress Assay:

Confluent cultures cells in 96 well plates were challenged with desired treatments. Oxidative stress was determined by HNE Adduct Competitive ELISA kit (Cell Biolabs, INC Cat # STA-838), according to the manufacturer's instructions.

Statistical Analysis

All experiments were independently performed in triplicate at a minimum. All data were expressed as mean ± S.E. An analysis of variance (ANOVA) were performed followed by post-hoc t-tests with the Bonferoni correction using the statistical software GraphPad Prism 6 (GraphPad Software, La Jolla, CA). The differences were considered statistically significant when $p<0.05$.

Results

Decreased Expression of *Fscn2* in Aged Kidney

The expression of *Fscn2* mRNA was decreased at 20 and 24 months in aged animals, whereas life-long caloric restriction prevented the loss of *Fscn2* in aged kidney (Figure 4.1A). Interestingly, at the protein level, fascin 2 expression decreased only slightly with aging. However, the expression of fascin 2 was significantly decreased in aged kidney after cisplatin challenge, without a corresponding loss of expression in the young kidney (Figure 4.1, B and C). These results were further confirmed on a cellular level. Basal fascin 2 expression is similar between NT3 and C2 cells. Challenging the cells with cisplatin induced a decrease of fascin 2 expression in C2, but not NT3 cells (Figure 4.1, D and E).

Colocalization of Fascin 2 and α(E)-catenin in Renal Tubule Epithelial Cell

Next, we investigated the localization of fascin 2 in NRK-52E cells. Fascin2 is expressed and localized within the cytosol and along the cell membrane. Co-staining with FITC-phalloidin to visualize the F-actin cytoskeleton indicated that fascin 2 mostly accumulated around actin stress fibers, actin bundles that line the cell periphery and filopodia (Figure 4.2A). Interestingly, a co-localization of fascin 2 and α(E)-catenin was also observed (Figure 4.2B). Although there is no direct evidence showing fascin 2 localizes at adherens junctions (AJs), it may play role in cell adhesion [231–233]. However, fascin 2 was not detectable in anti-α(E)-catenin immunoprecipitates (data not shown) which indicates fascin 2 and α(E)-catenin do not interact directly. Alternatively, fascin 2

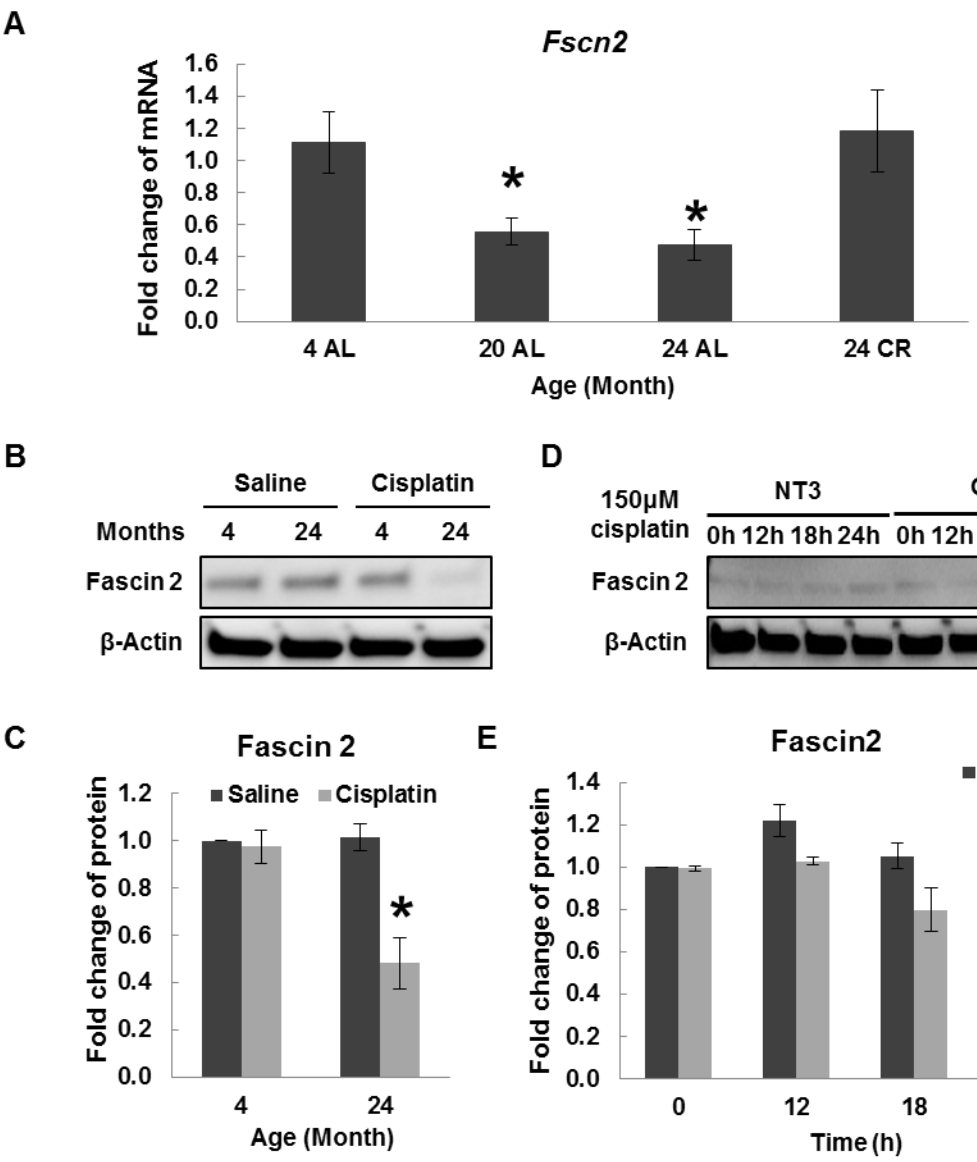


Figure 4.1 The expression of *Fscn2* is decreased in aged kidney. (A) Relative mRNA levels of *Fscn2* were measured by real-time PCR. The result is normalized to 4 AL sample. *P<0.05 vs 4 AL group. Immunoblot analysis (B) and quantification (C) of fascin 2 protein expression in whole tissue lysates. The β-actin blot served as the loading control. *P<0.05 vs 4 month saline group. Immunoblot analysis (D) and quantification (E) of fascin 2 protein expression in whole cell lysates. The β-actin blot served as the loading control. *P<0.05 vs NT3 0 h group.

may have a weak association with α(E)-catenin which would lessen its detection under co-immunoprecipitation conditions. Collectively, these results suggested that fascin 2 and α(E)-catenin may regulate the actin skeleton in renal tubular epithelial cells.

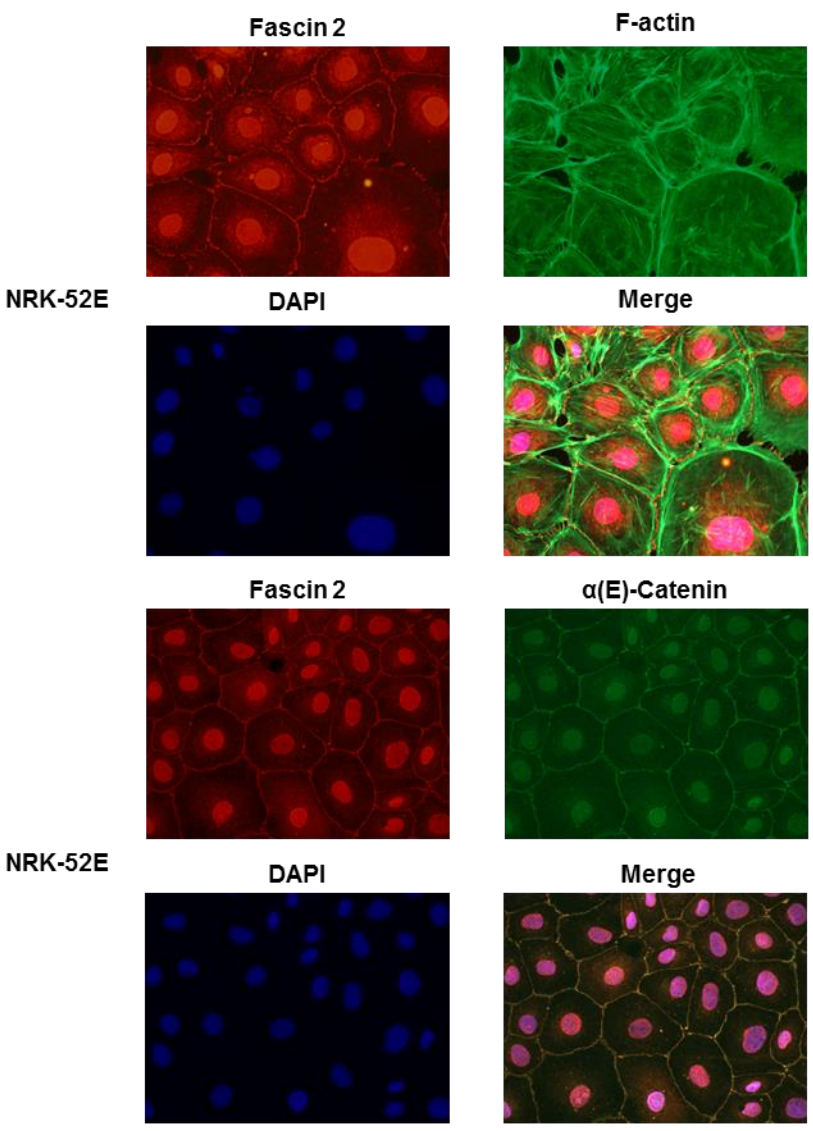


Figure 4.2 Fascin 2 is colocalized with α(E)-catenin in NRK-52E cells. (A) Representative immunofluorescence image (60x) of NRK-52E cells treated with rabbit anti-Fascin2 (red), FITC-phalloidin (green) and DAPI (blue). (B) Representative immunofluorescence image (60x) of NRK-52E cells treated with rabbit anti-Fascin2 (red), mouse anti-α(E)-catenin (green) and DAPI (blue).

Fscn2, as well as *Fscn1*, Decreased the Susceptibility of C2 Cells to Cisplatin-induced Apoptosis

Previous studies in our laboratory have demonstrated that cisplatin-induced apoptosis was enhanced by age-dependent loss of α(E)-catenin in renal tubule epithelial

cells [108,198]. In this study, we assessed whether *Fscn1* or *2* could rescue cells from the

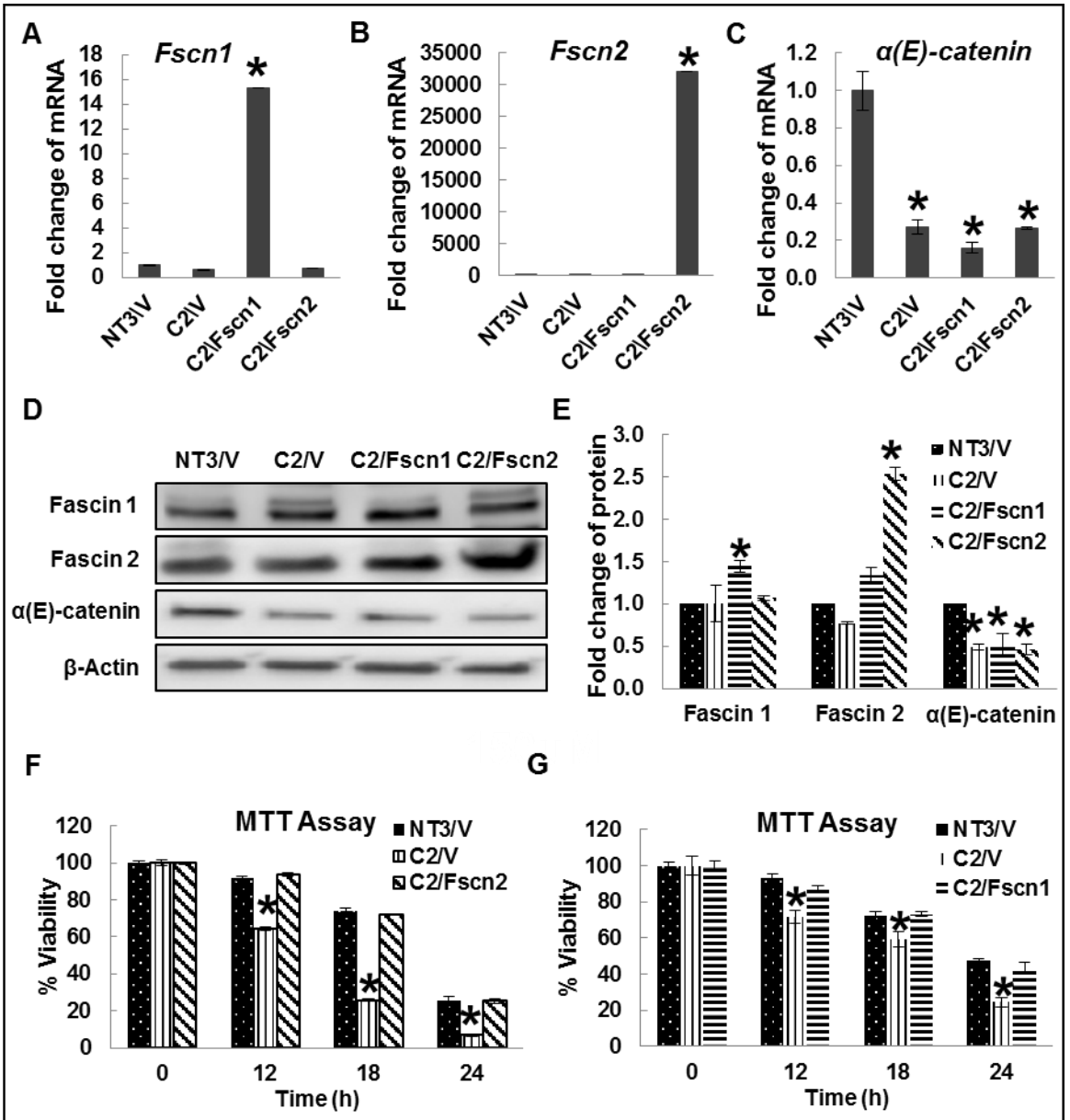


Figure 4.3 Overexpression of *Fscn1* or *Fscn2* abolishes the increased susceptibility of C2 cells to cisplatin via facilitating actin polymerization. Relative mRNA levels of *Fscn1* (A), *Fscn2* (B) and α(E)-catenin (C) were measured by real-time PCR. The result is normalized to the corresponding expression of each gene in NT3/V cells. Immunoblot analysis (D) and quantification (E) of fascin 1, fascin 2 and α(E)-catenin protein expression in whole cell lysates. The β-actin blot served as the loading control. (F and G) Cell viability was determined by the MTT assay in cells treated with 150 μM cisplatin for the indicated time periods. The results are presented as the percent viability of untreated NT3/V cells in SF media. *P<0.05 vs NT3/V group.

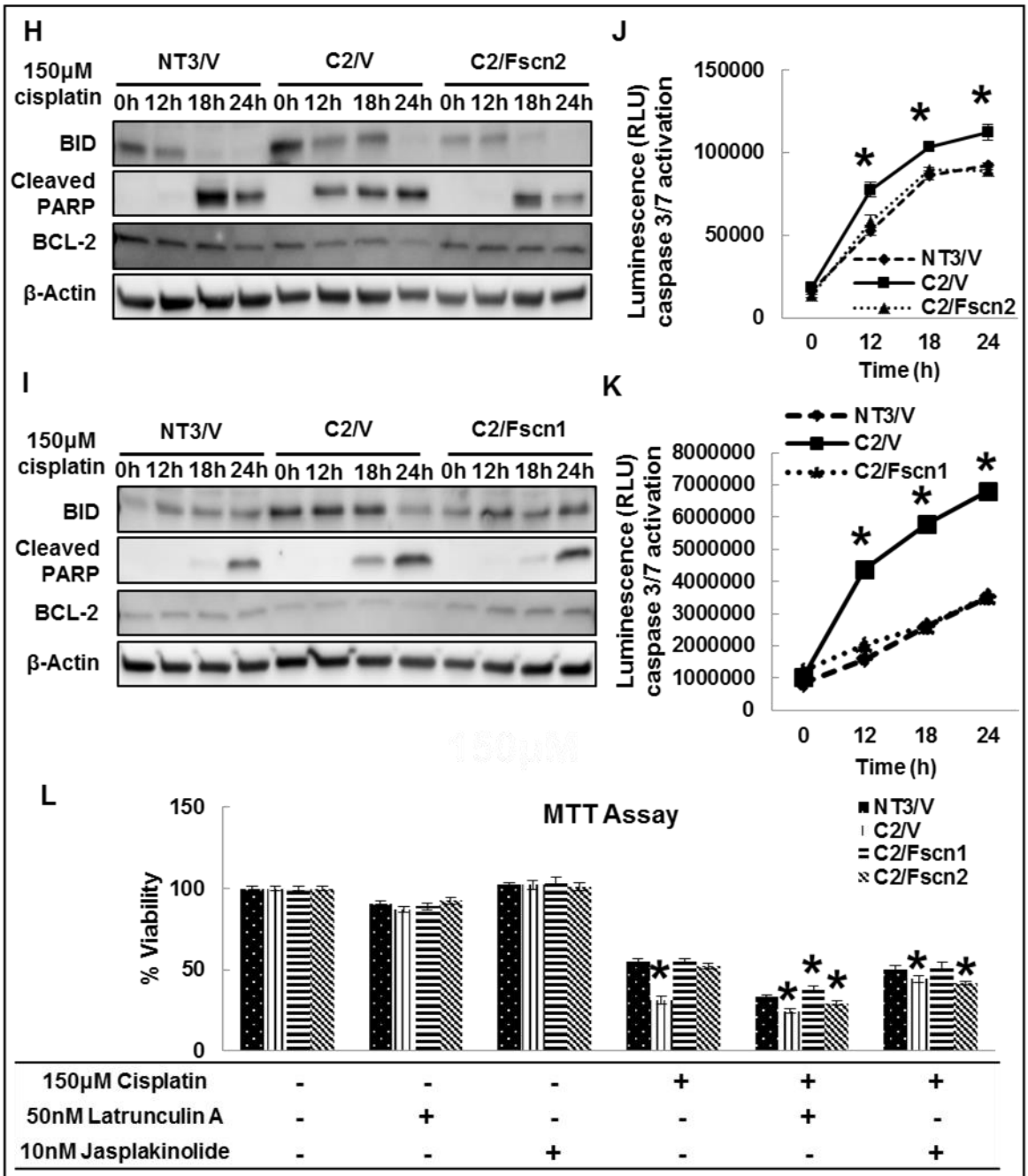


Figure 4.3 Overexpression of *Fscn1* or *Fscn2* abolishes the increased susceptibility of C2 cells to cisplatin via facilitating actin polymerization(Continued). (H and I) Immunoblot analysis of BID, cleaved PARP and BCL-2 protein expression in whole cell lysates. The β-actin blot served as the loading control. (J and K) The activities of caspase 3/7 in cells treated with 150 μM cisplatin for indicated time periods were determined by luminescent assay. (L) Cell viability was determined by the MTT assay in cells treated with indicated treatment for 24 h. The results are presented as percent viability of untreated NT3/V cells in SF media. *P<0.05 vs NT3/V group.

increased apoptosis in C2 cells in response to cisplatin. Fascin 1 is a highly homologous

family member with fascin 2 which also regulates actin bundling in a different cell lines [234]. We over expressed full-length human *FSCN1* and rat *Fscn2* in C2 cells (C2/Fscn1 and C2/Fscn2) without increasing the expression of α(E)-catenin (Figure 4.3, A-E). The MTT assay was performed to measure cell viability after cisplatin challenge. C2/V cells exhibited a significant loss of viability after cisplatin injury as compared with NT3/V cells (Figure 4.3, F and G). In addition, increased BID and PARP cleavage and decreased BCL-2 expression was induced by cisplatin in C2/V relative to NT3/V cells (Figure 4.3, H and I). Furthermore, C2/V exhibited higher caspase 3/7 activity in response to cisplatin as compared with NT3/V cells (Figure 4.3, J and K). Surprisingly, overexpression of either *Fscn1* or *Fscn2* was able to completely abolish those differences (Figure 4.3, F-K). Importantly, latrunculin A, a compound that inhibits actin polymerization [235], decreased the viability of C2/Fscn1, C2/Fscn2 and NT3/V to the same extent as C2/V in response to cisplatin. Correspondingly, stabilization of actin polymerization with Jasplakinolide [236] rescued C2/V to control (NT3) values (Figure 4.3L). Collectively, these findings suggested that increased actin stress fiber formation, via *Fscn1* or *Fscn2*, increases resistance to cisplatin-induced apoptosis.

Fscn2 Rescues Mitochondrial Dysfunction in C2 Cells

Abnormal mitochondria DNA and increased levels of reactive oxygen species (ROS) have been observed in aged kidney **[237]**. Several studies suggest that cisplatin accumulates in renal mitochondria, hampering respiratory chain and increasing ROS production which lead to apoptosis of renal tubular epithelial cells **[238]**. Consistent with these observations, our study showed that cisplatin impaired ATP production in a dose-

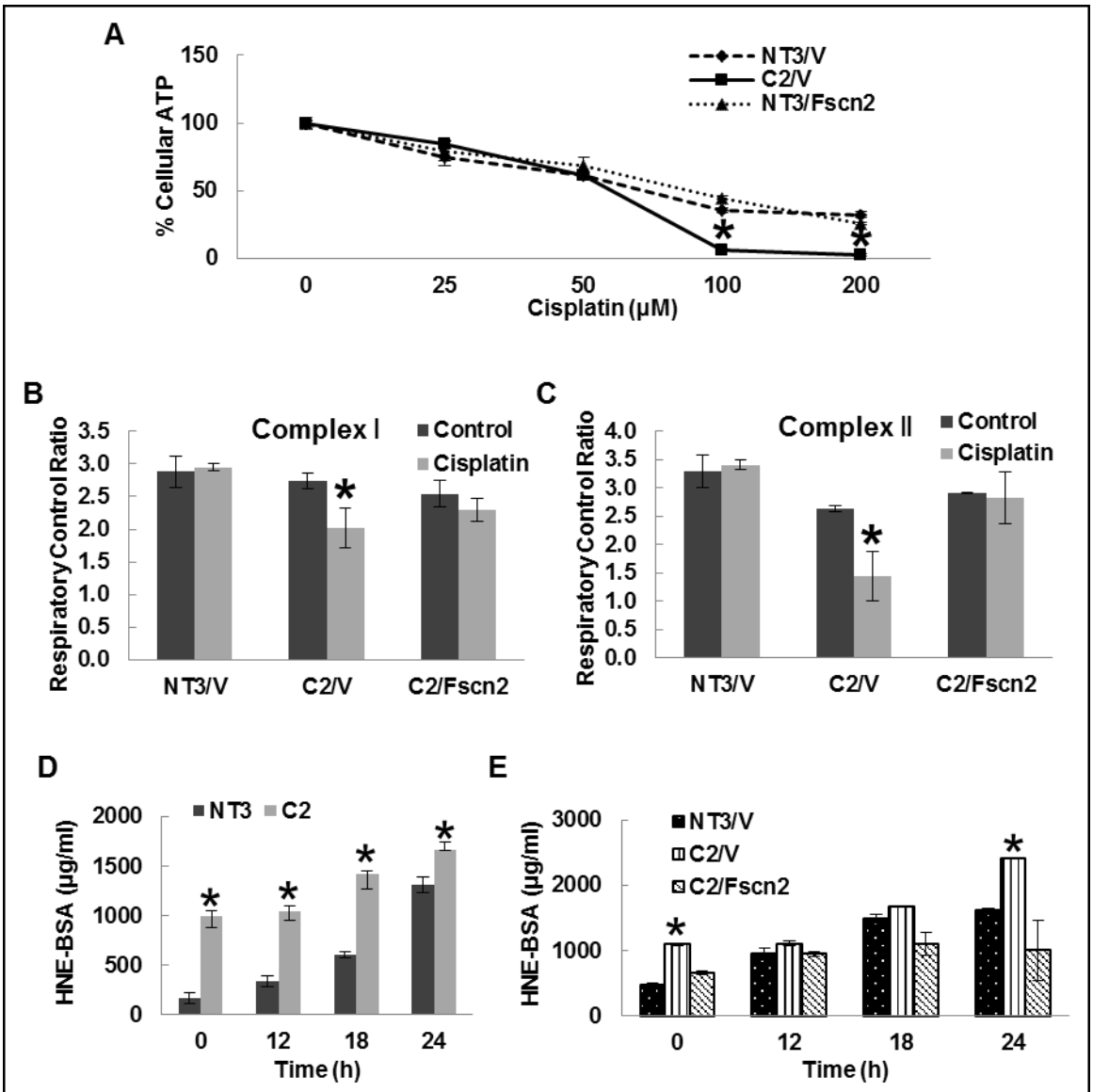


Fig 4.4 Overexpression of Fscn2 rescues mitochondrial dysfunction in C2 cells. (A) Cellular ATP in confluent cultures of cells treated with indicated concentrations of cisplatin for 24 h. (B and C) Oxygen consumption ratio of mitochondrial complex Ⅰ and Ⅱ in cells in SF medium or treated with 150 μM cisplatin for 24 h. (D and E) Oxidative stress in confluent cultures of cells treated with 150 μM cisplatin for indicated time. *P<0.05 vs NT3/V group.

dependent manner (Figure 4.4A). C2/V exhibited lower ATP levels in response to cisplatin as compared with NT3/V cells and overexpression of Fscn2 was able to completely abolish these differences (Figure 4.4A). Cisplatin induces the loss of mitochondrial complex Ⅰ and Ⅱ functions in C2/V cells, but not in NT3/V cells. Fscn2

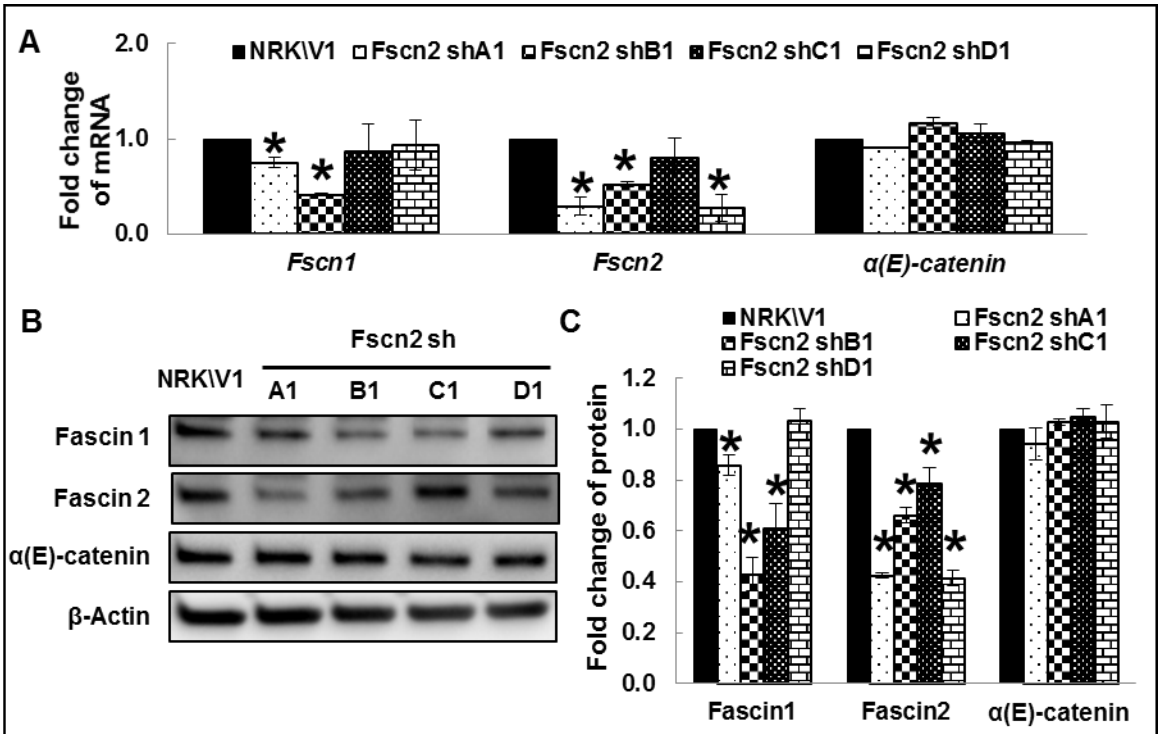


Figure 4.5. ***Fscn2*** **knockdown affects apoptosis in the NRK cells following cisplatin treatment.** (A) Relative mRNA levels of *Fscn1*, *Fscn2* and α(E)-catenin were measured by real-time PCR. The result is normalized to corresponding *Fscn1* or *Fscn2* expression in NRK/V1 cells. Immunoblot analysis (B) and quantification (C) of fascin 1, fascin 2 and α(E)-catenin protein expression in whole cell lysates. The β-actin blot served as the loading control. *P<0.05 vs NRK/V1 group.

overexpression rescued these deficits in C2/V cells in response to cisplatin (Figure 4.4B&C). Growing evidence demonstrates that mitochondria complex Ⅰ is the major generator of superoxide radicals in mammalian mitochondria [239]. This is consistent with our finding that more oxidative stress as measured by the 4-hydroxynonenal (HNE) assay, was induced by cisplatin in C2 cells than NT3 cells (Figure 4.4D). Overexpression of Fscn2 in C2 cells significantly reduced the HNE level in response to cisplatin treatment (Figure 4.4E). Therefore, these data demonstrated that *Fscn2* reduces cisplatin-induced mitochondrial dysfunction in the aging kidney.

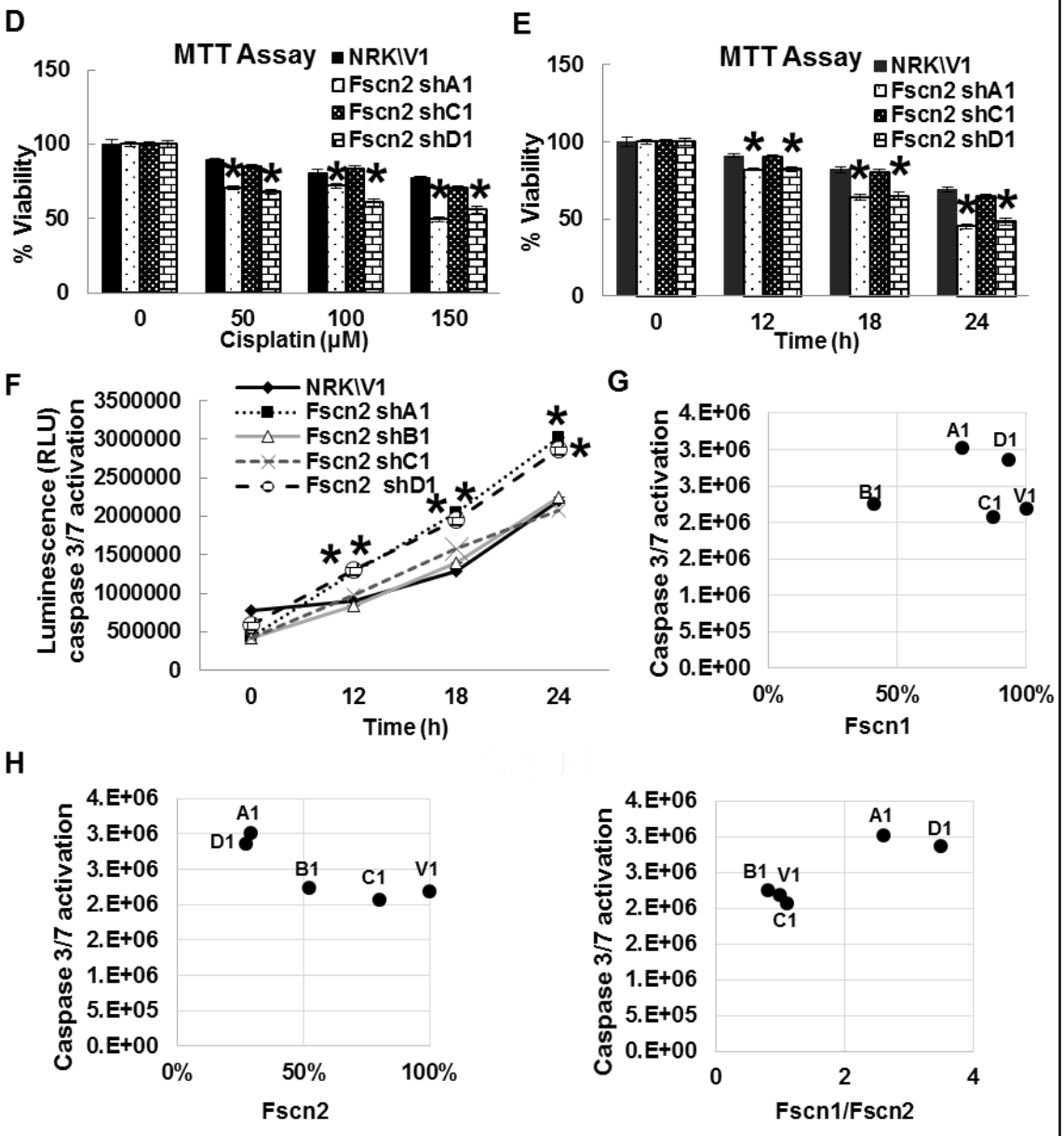


Figure 4.5. *Fscn2* knockdown affects apoptosis in the NRK cells following cisplatin treatment (Continued). (D) Cell viability was determined by the MTT assay in cells treated with indicated concentrations of cisplatin for 24 h or (E) 150 μM cisplatin for the indicated time periods. The results are presented as the percent viability of untreated NRK/V1 cells in SF media. (F) The activities of caspase 3/7 in cells treated with 150 μM cisplatin for the indicated time periods were determined by luminescent assay. Scatter graphs showing the correlation of cisplatin induced caspase 3/7 activation with either *Fscn1* expression (G), *Fscn2* expression (H) or *Fscn1*/*Fscn2* ratio (I). *P<0.05 vs NRK/V1 group.

Fscn2 Deficiency Increases the Susceptibility of Tubular Cells to Cisplatin-induced Apoptosis

To further confirm the role of Fscn2 in tubular epithelial dysfunction, several shRNA vectors were designed to knock down Fscn2 expression in the parental NRK-52E cell line. All these cell lines have varied expression of Fscn1 and Fscn2 while the α(E)-catenin level remained constant (Figure 4.5, A-C). shC1 did not elicit substantial knockdown of Fscn2 (80%) or Fscn1 (87%) and, as expected, viability following cisplatin challenge was not affected with this construct (Figure 4.5, D and E). The shA1 and shD1 constructs knocked down Fscn2 dramatically (29% in shA1, 27% in shD1), with slight change in Fscn1 (75% in shA1, 93% in shD1), and showed increased susceptibility to cisplatin as compared with NRK/V1 control cells (Figure 4.5, D and E). Of note, all of the shB1 cells, which significantly reduced both *Fscn2* (52%) and *Fscn1* (41%), died following cisplatin challenge (data not shown). Consistent with these observations, higher caspase 3/7 activities were observed in shA1 and shD1 cells than other cell lines following the cisplatin treatment (Figure 4.5F). Furthermore, the scatter graph confirmed that when *Fscn2* expression was lowered to 30%, significantly increased caspase 3/7 activities in response to cisplatin were seen (Figure 4.5, G-I). Taken together, the data suggest that the loss of *Fscn2* increases the susceptibility of tubular epithelial cells to cisplatin injury.

Discussion

A relationship between AKI and aging has long been recognized. AKI in the elderly is more severe and patients are less likely to recover, presumably due to impaired proliferation and migration of renal tubular epithelial cells [194–197]. Previous studies from our laboratory showed marked loss of α(E)-catenin in proximal tubular epithelium in aged kidney [108,198]. α-Catenin, a tension-sensing, key regulator of the actin cytoskeleton, interacts with a variety of actin-binding proteins [199–201]. Fascin 2 is an actin bundling protein that interacts with adhesion molecules and F-actin [185,202]. While many previous studies have focused on the function of fascin 2 in retina [203], its role in renal tubular epithelium is undetermined. In this study, a stress-induced loss of fascin 2 was observed in aged kidney and C2 cells. Overexpression of Fscn2 abolished the increased cisplatin-induced apoptosis, mitochondrial dysfunction and oxidative stress in C2 cells compared with NT3 cells. Moreover, there was an inverse correlation between Fscn2 levels and the susceptibility of tubular epithelial cells to cisplatin injury. Thus, Fscn2 ameliorated cisplatin-induced apoptosis in renal tubule epithelial cells of aged kidney.

It was shown that disruption of the actin cytoskeleton is also associated with apoptosis in kidney injury. There is alterations in the actin cytoskeleton precede apoptosis in cisplatin-induced nephrotoxicity [240]. Furthermore, a large amount of evidence indicates changes to the dynamics of actin cytoskeleton cause release of reactive oxygen species from mitochondria and subsequent cell apoptosis [241]. Overexpression of gelsolin, an actin binding protein that regulates actin filament assembly and disassembly, maintains the mitochondrial membrane potential and reduces cytochrome c release by closing the voltage-dependent anion channel in the mitochondrial outer membrane [242]. Staurosporin

has been shown to phosphorylate cofilin, an actin binding protein that disassembles actin filaments. The activated cofilin then relocates into mitochondria, triggers the release of cytochrome c and initiates apoptosis. Importantly, the actin-binding domain of cofilin is essential for this pro-apoptosis function [243]. Although the specific mechanisms still remain to be studied, these studies suggested a link between actin cytoskeleton, mitochondria and apoptosis.

Fascin is a small globular protein which selectively cross links actin filaments that have been arranged in a parallel orientation into tightly packed actin bundles [244]. These bundles play a critical role in the formation and organization of a variety of highly contractile and dynamic subcellular structures, including lamellipodia, filopodia and stress fibers [245]. The formation of structures such as filopodia and stress fibers play key roles in cell survival [246,247]. Lai and colleagues demonstrated that microRNA-133a levels are inversely related to Fscn1, i.e., when miR-133a is up-regulated, proliferation and migration are inhibited, whereas apoptosis of gastric cancer cells is promoted and this process can be reversed by up-regulation of Fscn1 [193]. Moreover, fascin is a suppressor of caspase-associated anoikis in colon adenocarcinoma cells [248]. Importantly, there is evidence that filopodia actin bundles can be recycled into stress fibers for use in cytoskeletal tension and retraction. Fascin containing filopodia are tethered by adhesion complexes which initiate stress fiber assembly in motile fish keratocytes. In addition, untethered filopodia actin bundles incorporate stress fiber with myosin Ⅱ and integrate in lamellipodia [249]. Furthermore, the association of fascin with tropomyosin and the competition between fascin, caldesmon and tropomyosin for F-actin may also play an important role in the interconversion of filopodial bundles and stress fibers [250,251].

In vitro and in vivo investigations have shown that the actin-bundling activity of fascin within filipodia exhibits a rapid turnover rate [252,253]. A previous model established by Rock and coworkers suggests that F-actin cross-linked by fascin is restricted to filaments in a parallel orientation and is very stable in absence of free fascin [244]. When fascin is released from one of its binding sites, it will rebind that site before the second site releases. The free actin-binding domain has a faster on rate for fascin than the off rate of the fascin-bound actin-binding domain which makes actin bundles very stable (Figure 4.6A). When competing free fascin 1 or fascin 2 molecules are present, they can compete for the transiently available binding site while bound fascin enters a singly bound state. The singly bound fascin, with no second binding site available, dissociates from actin binding sites quickly allowing fascin 1 or fascin 2 to form a new link (Figure 4.6B). This model has been validated by previously reported data (in vitro and in vivo) [252,253]. Based on our results, we hypothesize that fascin 2 has higher affinity for F-actin than fascin1; and fascin 1 is only able to compete with fascin 2 when the Fscn1:Fscn2 ratio is greater than 30:1. In NT3/V and C2/Fscn2 cells, the Fscn1:Fscn2 ratio is less than 30:1 and actin is bundled with fascin 2 (Figure 4.6, A and C). However, in C2/V cells, the decreased expression of fascin 2 enables fascin 1 to compete for the binding site. The F-actin is bundled with both fascin 1 and fascin 2 (Figure 4.6, B and C). Since fascin 1 has lower affinity for F-actin than fascin 2, F-actins bundled by fascin 1 may generate less force than F-actins bundled by fascin 2. Actin bundles in this case are very unstable because of the unbalanced forces which makes the cells more susceptible to cisplatin injury. This contrasts with C2/Fscn 1 cells in which fascin 2 is completely replaced by fascin 1 and the actin bundle structure is stable. This accounts for similarities between fascin 1 and 2

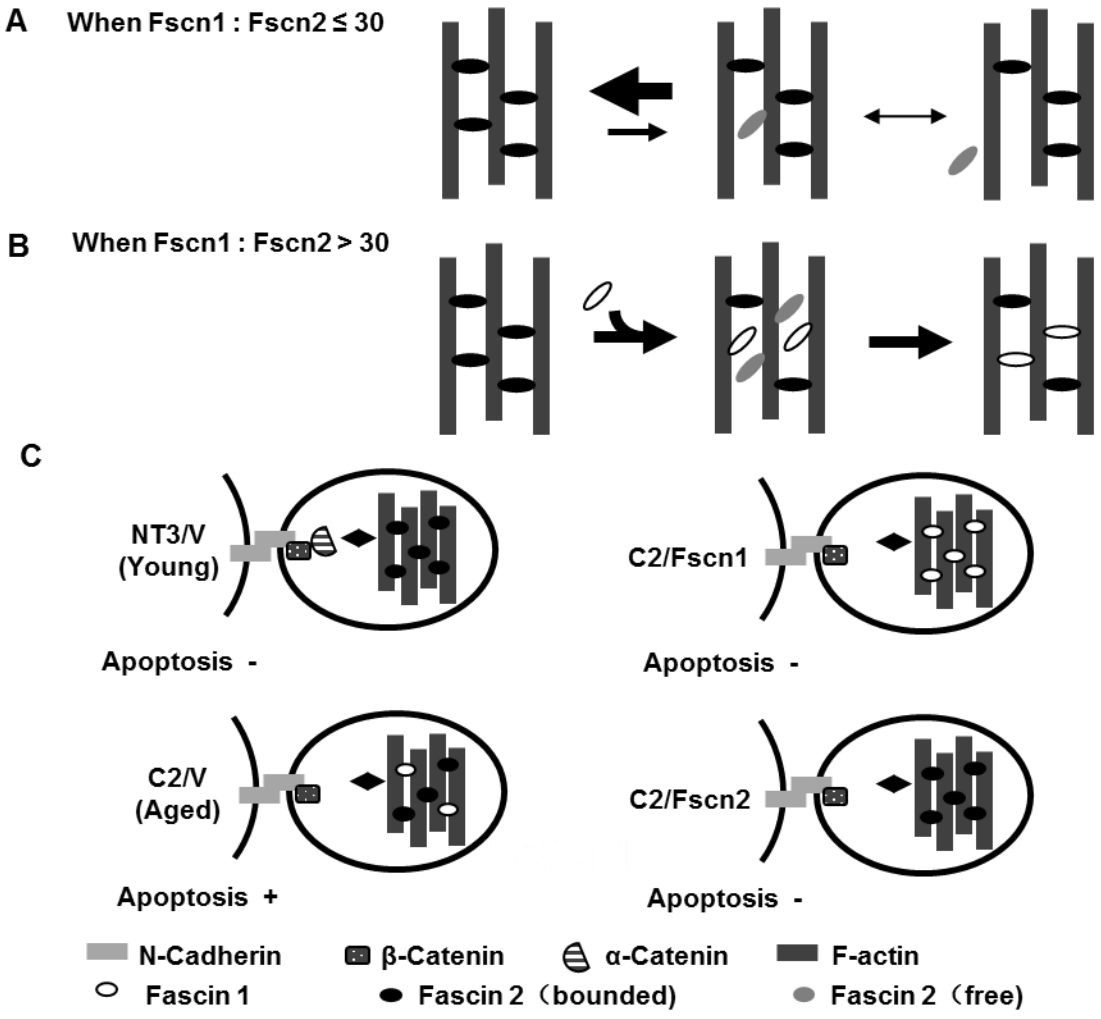


Figure 4.6 Models of fascin mediated actin bundle formation and function. Fascin 2 has higher affinity for actin than fascin1. (A) When *Fscn1* : *Fscn2* ≤ 30, Fascin 2 are bound to two actin filaments (black ovals) stabilizing actin bundles. These proteins can toggle to a state (gray ovals) where they are only bound to a single filament. Because the actin site is restrained, the single bound fascin 2 can readily rebind the second filament. This rebinding rate is faster than the dissociation rate in the single bound state, yielding bundles that are very stable. (B) When *Fscn1* : *Fscn2* > 30, fascin 1 (white oval) can occupy actin sites near single bound fascin 2, preventing its rebinding. This leads to dissociation of the fascin 2 and replacement with fascin 1. (C) In NT3/V and C2/Fscn2 cells, *Fscn1* : *Fscn2* < 30:1, all the actin are bundled with fascin 2; in C2/V cells, the decreased expression of fascin 2 makes fascin 1 able to complete for the binding site. F-actins bundled with both fascin 1 and fascin 2 are very unstable. These cel have more apoptosis in response to cisplatin. In C2/Fscn 1 cells, fascin 2 is completely replaced by fascin 1. This actin bundle structure is stable which makes the cells have similar apoptosis level in response to cisplatin compared to NT3/V cells.

with respect to both protection from apoptotic signals. This model was further confirmed by our fascin 2 knockdown experiments. The shA1 and shD1 construct have an Fscn1:Fscn2 ratio greater than 30:1 (58:1 in shA1, 98:1 in shD1), and was associated with increased apoptosis in response to cisplatin. The Fscn1:Fscn2 ratio in shB1 and shC1 is roughly 30:1, thus the wild type function is maintained.

In summary, we have shown that Fscn2 facilitates cell ameliorates cisplatin-induced AKI in aged kidney. This newly identified role of Fscn2 in renal epithelial cells, which depends on the functional interaction with α(E)-catenin and F-actin, may lay the groundwork for new therapeutic approaches to AKI in aged patients in the future. Furthermore, there are many different actin cross-linking proteins which also interact with α-catenin. Understanding the properties and functions of these cross-linking proteins will lead to a more complete understanding of the role of actin cytoskeleton in the loss the renal function in aged kidney.

CHAPTER 5

CONCLUSION

Three decades have passed since the first study appeared indicating that the aging kidney was characterized by increased susceptibility to nephrotoxic injury. Data from these experimental models is strengthened by clinical data demonstrating that the aging population has an increased incidence and severity of AKI, a considerable public health problem associated with significant morbidity and mortality rates. Unfortunately, there is no effective therapy currently available for AKI. It is clear, however, that the increased susceptibility of the aging kidney to injury is complex and, most likely, cannot be accounted for by a single mechanism. Cisplatin is one of the most potent and widely used antitumor drugs. However, AKI limits the use of cisplatin and other platinum-based therapeutics [119]. Previous data from our laboratory demonstrated that aging is associated with loss of the N-cadherin/α-catenin complex [182]. In this dissertation work, the role of the age-dependent loss of α-catenin in predisposing the aged kidney to cisplatin-induced nephrotoxicity was investigated (Chapter 2). In addition, we delineated the cell death signaling pathway enhanced by the age-dependent loss of α(E)-catenin in renal tubule epithelial cells (Chapter 2 and 3). Furthermore, a potential role for actin cytoskeleton in regulating the susceptibility of renal tubular epithelial cells to cisplatin injury was proposed (Chapter 4). Accordingly, three hypotheses were tested 1) Age-dependent loss of α(E)-catenin increases susceptibility to cisplatin injury, 2) Fas-mediated apoptotic signaling pathway is enhanced by the age-dependent loss of α(E)-catenin in renal tubule epithelial cells, and 3) age-dependent loss of α(E)-catenin leads to disruption of actin

cytoskeleton via fascin 2 which makes renal tubule epithelial cells more susceptible to cisplatin injury.

α-Catenin is a actin binding and bundling protein which associates the cadherin-catenin complex with actin cytoskeleton [221]. In addition to the classical role α-catenin has in cell adhesion, recent data suggest that α-catenin may also function as a force transducer and a regulator of cell signaling and motility [150]. This study furthers our understanding of the role α-catenin plays beyond AJs via investigating the influence age-dependent loss of α-catenin has on cisplatin-induced AKI. To study the effects of reduced α(E)-catenin, a renal tubule epithelial cell line with stable knockdown of α(E)-catenin (C2 cells) was used; NT3 is non-targeted control. Our data showed that C2 cells exhibited a significant loss of viability (MTT and neutral red assays) as compared with NT3 cells after the cisplatin treatment, but showed no difference in LDH leakage, a marker of necrosis (Figure 2.1 and 2.2). These results indicate that the increased susceptibility of C2 cells to cisplatin is due to apoptosis, not necrosis. This was confirmed by the observation that increased caspase3/7 activation and PARP cleavage was detected in C2 cells after cisplatin treatment. Z-VAD, a pan-caspase inhibitor, abolished the susceptibility difference between NT3 and C2 cells to cisplatin (Figure 2.3). Interestingly, the expression of α(E)-catenin was further decreased after cisplatin treatment and a similar result was obtained in vivo (Figure 2.4 and 2.5). These results indicate that decreases in α(E)-catenin expression during aging is exacerbated by further loss following cisplatin injury, which initiates further apoptosis of renal tubular epithelial cells, leading to increased injury. Furthermore, in vivo data demonstrated a significant increase in serum creatinine at 72 hr after a single dose of cisplatin in 24-month-old rats, but not in 4-

month-old rats. Increased expression of KIM-1 and in situ apoptosis were also detected in aged kidney after cisplatin challenge (Figure 2.5 and 2.6). Taken together, the first set of studies suggest that loss of α(E)-catenin increases apoptosis of tubular epithelial cells which may contribute to the increased nephrotoxicity induced by cisplatin in aged kidney.

Apoptosis, or programmed cell death, plays important roles in embryonic development and homeostasis maintenance [254]. Intracellular damage and some physiological cues activate caspases which induces cells to enter apoptosis [255]. Caspases can be further divided into two groups based on the timing of activation during apoptosis: initiator caspases (-8 and -9) and effector caspases (-3 and -7) [256]. Initiator caspases activated by death signals form dimers and launch the apoptotic signaling cascade. Effector caspases are activated by cleavage of their Asp residue and induce morphological changes [256]. Importantly, cleavage of cytoskeletal proteins will be induced during apoptosis which leads to the loss of cell shape. In addition, focal adhesion complexes are also cleaved during apoptosis, which results in the detachment of apoptotic cells from their neighbors [257]. Death receptor-mediated pathway and mitochondrial-mediated pathways are two major signaling cascades that lead to apoptosis, as have already been discussed (Introduction). The second part of this dissertation was to identify the specific apoptosis signaling pathway that was induced by age-dependent loss of α(E)-catenin in renal tubule epithelial cells. The expression of Fas was increased by 5.5 fold in C2 cells relative to NT3 cells (Fig 3.1). Kp7-6, a Fas/FasL antagonist, abolished the cisplatin-induced viability and caspase 3/7 activity difference between NT3 and C2 cells (Fig 3.2 and 3.6). Increased caspase 8/9 activation, decreased Bcl-2 expression, increased BID cleavage and increased cytochrome c release was induced by

cisplatin in C2 as compared to NT3 cells (Figure 3.4 and 3.5). Treating the cells with cisplatin, in combination with ABT-199, a Bcl-2 inhibitor, decreased the viability of NT3 cells to the same level as C2 cells after cisplatin (Figure 3.5H). These data support the hypothesis that increased Fas-mediated apoptosis is the cause of increased susceptibility of C2 cells to cisplatin. A proposed scheme of the Fas apoptotic signaling pathway is shown in Fig 3.7.

While the aforementioned results suggest enhanced apoptosis in stressed aged kidney, the increased susceptibility of aged kidney to AKI may not be accounted for by a single mechanism. α(E)-catenin is known to compete with Arp2/3 in regulating actin polymerization. Benjamin and his colleagues proposed that the interaction between α-catenin and β-catenin enriches α-catenin at AJs, facilitating its dimerization. Since β-catenin binding and homodimerization require the same binding site, α-catenin is thought to dissociate from β-catenin first. α-Catenin dimer then bind to F-actin, preventing its interaction with Arp 2/3 and promoting the formation of F-actin bundles by itself or by interacting with other actin bundling proteins such as fascin [258]. This model predicts that the defects arising from loss of α-catenin function can be suppressed by: 1) the concurrent inhibition of Arp 2/3 activity; 2) enhancing the expression of actin bundling proteins. A previous study showed that defects observed in α-catenin knockout embryos mutant can be ameliorated by reducing Arp 2/3 activity [155]. In this dissertation, we tested the second hypothesis by overexpressing fascin 2 in α(E)-catenin knockdown cells. Fascin, the most abundant actin bundling protein, mainly localizes to filopodia and invadopodia [252]. It is a key element in stabilizing and maintaining parallel actin bundles, which plays an important role in regulating of cell adhesion, proliferation, migration and

invasion [185]. Fascin regulates migration of a number of cells including mouse melanoblasts, urothelial carcinoma, hepatic stellate cells and glioma cells [202,259–261]. Migration of non-small-cell lung cancer can be inhibited by microRNA-145 via suppressing fascin 1 [262]. In terms of cell death, it has been shown that the upregulation of fascin1 prevents apoptosis, which may be important for tumor cell survival [263]. In this study, a stress-induced loss of fascin 2 was observed in aged kidney and C2 cells (Figure 4.1). Previous work has shown fascin binds to and colocalizes with β-catenin at the leading edge of epithelial cells [190]. Co-localization of fascin 2 and α(E)-catenin was also observed (Figure 4.2). However, fascin 2 was not detectable in anti-α(E)-catenin immunoprecipitates (data not shown). Furthermore, overexpression of *Fscn2* abolishes the increased susceptibility of C2 cells to cisplatin-induced apoptosis and there is an inverse correlation between the Fscn2 level and the susceptibility of tubular epithelial cells to cisplatin injury (Figure 4.3 and 4.5). We also found that aging is associated with disorganization of the actin cytoskeleton in renal tubular epithelium. Similar results were seen in C2 cells which exhibited a significant loss of actin stress fibers and filopodia (data not shown). It is well known that fascin localizes to filopodia tips bundling F-actin in motile cells. More recent study has shown that fascin also regulates formation of actin stress fibers [264–266]. This study further confirmed the role of fascin in regulating actin stress fiber in that overexpression of *Fscn2* rescued actin stress fiber expression in C2 cells (data not shown). Importantly, latrunculin A, an actin polymerization inhibitor, decreased the viability of C2/Fscn2 and NT3/V to the same extent as C2/V in response to cisplatin. Correspondingly, stabilization of actin polymerization with Jasplakinolide, rescued C2/V to control (NT3) values (Figure 4.3). Collectively, these findings suggested

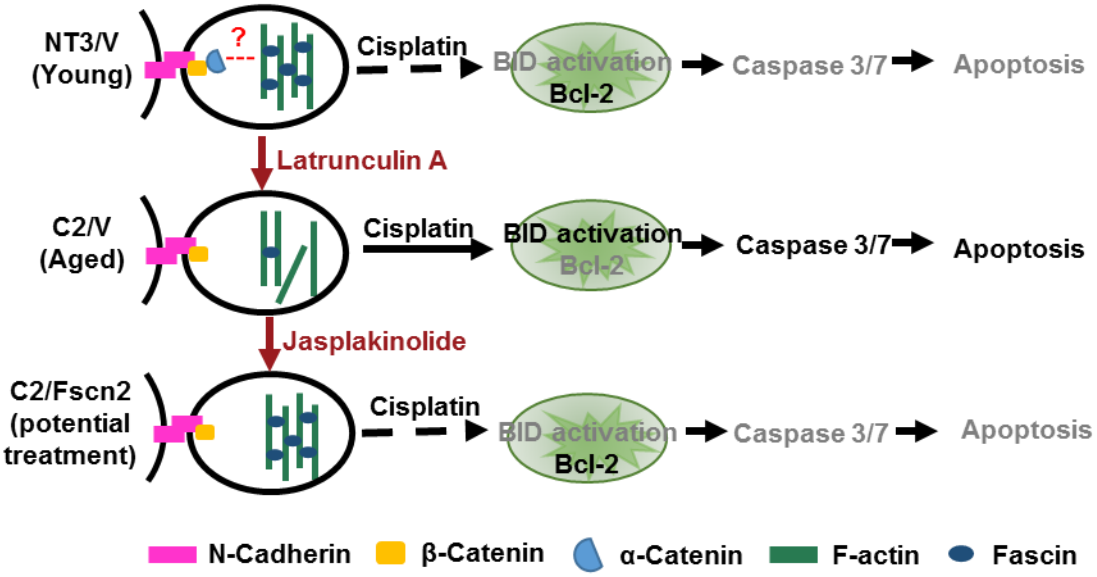


Figure 5.1 Fascin facilitates actin bundling and increases resistance to cisplatin-induced apoptosis. C2/V cells (or aged kidney) has decreased α-catenin and fascin expression compared to NT3/V cells (or young kidney) which compromises the stability of actin structure making to cell more susceptible to cisplatin-induced apoptosis. Overexpression of Fscn2 in C2 cells stabilized F-actin without α-catenin which increases the resistance to cisplatin injury. latrunculin A, an actin polymerization inhibitor, increased the susceptibility of NT3/V cells to cisplatin. Jasplakinolide increases the resistance of C2/V cells to cisplatin injury via facilitating actin polymerization.
(Grey, down-regulated/inhibited; Black, up-regulated/activated).

that increased actin stress fiber formation, via fascin, increases resistance to cisplatin-induced apoptosis (Figure 5.1).

Mitochondria, the powerhouse of a cell, generates energy via the production of ATP and oxidative phosphorylation [267]. The kidney is particularly vulnerable to mitochondrial damage because 1) it consumes large amounts of energy; 2) mitochondrial toxins accumulates in kidney because it is a major excretory route [268]. Impaired mitochondria function has been associated with aging because mitochondria DNA is more vulnerable to accumulation of mutations over time [268]. Abnormal mitochondria DNA and increased levels of reactive oxygen species (ROS) have been observed in aged kidney which may partially explain the high prevalence of CKD in the elderly [237]. Further

evidence for a role of the mitochondria comes from a I/R injury study in aged (27 months) Wistar rats [140]. Tubular cell apoptosis was increased in aged rats as compared to young controls; levels of cytosolic cytochrome C and active caspase-3/9 were elevated in the aging kidney and the up-regulation following I/R injury of caspase-3/9 was increased in aged rats [141]. Increased expression of Bax, a pro-apoptotic protein, as well as caspase-3/9 and cytochrome C in the aging kidney have been observed in other studies [142,143]. The expression of Bcl-2, which is an apoptosis inhibitor, however, is decreased in aged rat kidney [142]. Moreover, several studies suggest that cisplatin accumulates in renal mitochondria, hampering respiratory chain, increasing ROS production and causing the release of pro-apoptotic molecules, which lead to apoptosis of renal tubular cells [238]. Our current study showed that C2 cells had increased BID cleavage, decreased Bcl-2 expression, increased cytochrome c release and increased caspase-9 activation than NT3 cells in response to cisplatin (Figure 3.4 and 3.5). This result was consistent with *in vivo* data that aged kidneys have increased BID cleavage and decreased Bcl-2 expression compared to young kidney. Cisplatin induced a further increase of BID cleavage and decrease of Bcl-2 expression in aged kidney, but not in young kidney (Figure 3.5). Importantly, ABT-199, a Bcl-2 inhibitor, decreased the viability of NT3 cells in a dose-dependent manner and completely abolished the viability difference between NT3 and C2 cells at the dose of 10nM (Fig.3.5). In addition, ATP production was impaired with cisplatin treatment in a dose dependent manner (Figure 4.4). C2/V exhibited lower ATP production in response to cisplatin as compared with NT3/V cells and overexpression of *Fscn2* was able to completely abolish these differences (Figure 4.4A) Furthermore, cisplatin induces the loss function of mitochondrial complex Ⅰ and Ⅱ in C2/V cells, but not

in NT3/V cells. *Fscn2* overexpression rescued C2/V cells from the dificits in mitochondrial function induced by cisplatin treatment (Figure 4.4B&C). Therefore, increased mitochondria damage could account, in part, for the increased AKI in the aging kidney.

Oxidative stress, a condition in which the balance between prooxidant and antioxidant is disturbed (Figure 5.2), induces oxidative damage and compromises cell viability [269]. The main intracellular source of ROS under normal conditions is the leakage of electrons from the mitochondria electron transport chain during oxidative phosphorylation [270]. Numerous factors and agents including cisplatin can also induce significant generation of ROS [271]. Hence, oxidative stress can be another mechanism

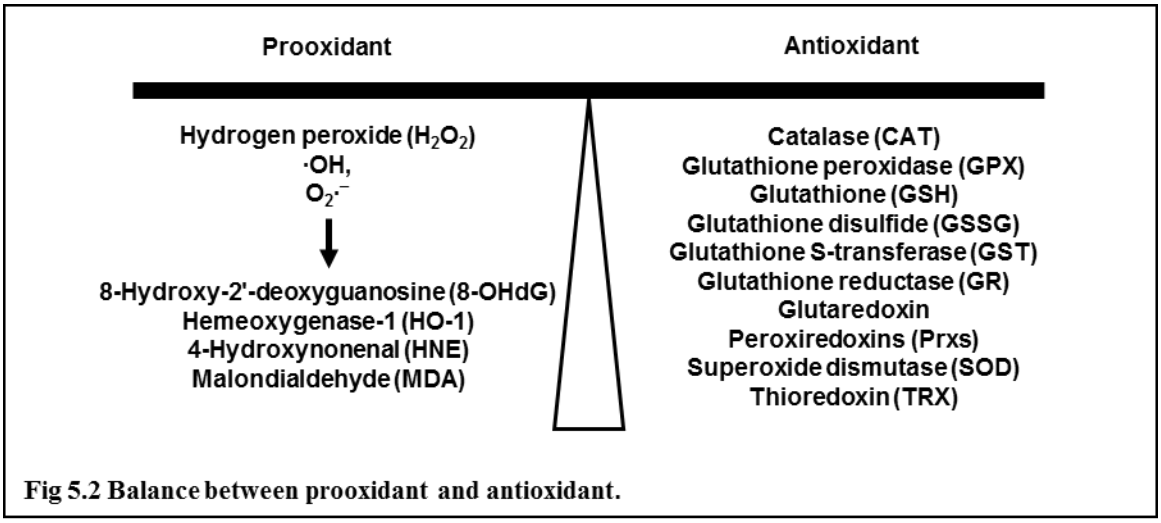


Fig 5.2 Balance between prooxidant and antioxidant.

accounting for the increased susceptibility of aged kidney to cisplatin-induced AKI. However, the data regarding the role of antioxidants in age-dependent AKI is ambiguous. Although glutathione S-transferase (GST) activity is lower (45% of control) in aged rat kidneys compared to middle-aged and young rats, renal glutathione (GSH) levels are not decreased in the aging rat kidney [101]. Previous data from our laboratory have confirmed that basal GSH levels are not lower in the aging kidney, but levels are depleted more

rapidly when challenged with acute stress [104]. Several studies suggest that deficiencies within other antioxidant defense systems may play a role in the aging kidney. A decrease in catalase levels (41% of young) was seen in male Wistar rats at 15 months as compared to 10 and 2.5 month rats; no difference was seen at 10 months as compared to 2.5 [272]. Total plasma antioxidant potential is reduced in aging rats; this may explain the marked increase in 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in the kidney following I/R injury in aged animals [273]. In a study comparing young (3 months), middle-aged (12 months) and aged male (24 months) Wistar rats, lipid peroxidation was elevated at baseline in middle-aged and aged rats which showed more severe I/R injury and 30 days supplementation with vitamin E attenuated I/R injury in these rats [274]. Vitamin E supplementation, however, could represent a sub-chronic adaptation, rather than a direct protective effect against acute injury. Furthermore, superoxide dismutase (SOD) attenuated I/R injury in aged rats to a greater extent than the young ones [275]. The induction of hemeoxygenase-1 (HO-1) was blunted in aging mice (12 months old) following I/R injury and the injury was worse in the aging mice [276]. Interestingly, HO-1 was localized to interstitial macrophages, suggesting that they may have a renoprotective role. Overexpression of Sirt1, a NAD-dependent protein deacetylase, in the proximal tubule cell maintains peroxisome number and reduces renal reactive oxygen species levels which rescued cell apoptosis induced by cisplatin [277]. In accordance with previous studies that repeatedly indicated oxidative stress is increased in aged kidney, our work shows that cisplatin induced more 4-hydroxynonenal (HNE) in C2 cells in comparison with NT3 cells (Figure 4.4D), overexpression of Fscn2 in C2 cells significantly reduced the HNE level in response to cisplatin treatment (Figure 4.4E). Growing evidence

demonstrates that mitochondria complex Ⅰ is the major generator of superoxide radicals in mammalian mitochondria [239]. This is consistent with our finding that cisplatin induces the loss of mitochondrial complex Ⅰ function in C2/V cells, but not in NT3/V cells (Figure 4.4B). Furthermore, mitochondria is not only the main source of ROS, it's also the major target of elevated ROS exposure which causes oxidative damage to mitochondrial DNA leading to cell apoptosis [278]. However, the mechanism by which mitochondrial DNA damage leads to cell apoptosis still remains to be defined. Moreover, evidence has shown that oxidative stress can induce apoptosis in both mitochondrial-dependent and mitochondrial-independent manner [269]. ROS has shown to promote the binding of tBID to voltage-dependent anion channel via oxidation of cardiolipin [279]. ROS overexposure causes oxidation of proteins, nucleic acids and lipids which leads to loss of mitochondrial transmembrane potential and initiates mitochondrial permeability transition [280]. The oxidation of cardiolipin also decreases its affinity for cytochrome c, which facilitates its release from the inner mitochondrial membrane [239]. In the mitochondrial-independent pathway, cellular oxidative stress has been shown to cause dissociation of the GST-JNK complex and JNK pathway activation leading to apoptosis [281]. In addition, Fas is able to sitimulate NADPH oxidase to generate H_2O_2 and $O_2\cdot^-$ [282]. Taken together, these data suggest that decreased antioxidants and increased oxidative stress may play a role in age-dependent AKI. However, significant work will be required to elucidate the specific pathways, as well as potential cell-specific effects.

Last but not least, clinical evidence suggests that AKI is also associated with delayed, or decreased, repair in the elderly and there has been a recent surge of work examining aging and kidney restitution [72,142]. Baraldi et al. suggested that complete

recovery was reduced in the elderly patients [70]. Arora et al. demonstrated that recovery from AKI, as determined by time to normalization of serum creatinine, was three-times as long in elderly compared to young patients (32 vs. 11.4 days, respectively) [79]. Schmitt et al. examined data from 17 studies of AKI and found that a higher percentage of surviving elderly (>65 years) patients did not recover renal function as compared to younger patients; the risk rate was 1.28 (95% confidence interval of 1.06–1.55) [142]. Fortunately, data from animal studies are in agreement with the clinical findings. In a seminal study, Cantley and coworkers demonstrated that zinc-α(2)-glycoprotein (Zag), an inhibitor of epithelial cell proliferation, is elevated (6.4-fold) in proximal tubular epithelial cells from aged mice (19–24 months) [283]. Overexpression of Zag decreased proliferation of proximal tubular epithelial cells *in vitro* and, importantly, knockdown of Zag in the kidneys of aged mice using siRNA increased proliferation following I/R injury in vivo. Additionally, Zag knockdown increased peritubular deposition of collagen IV which was hypothesized to attenuate recovery.

Several mechanisms may underlie the decreased repair potential of the aging kidney, such as decreased DNA synthesis and an imbalance of growth factor expression. Another factor in the decreased ability of the aging kidney to repair following injury may be the decreased expression of components of the cadherin/catenin complex that mediates cell-cell adhesion in the proximal tubule. Our laboratory has demonstrated that the expression of α(E)-catenin is decreased in the aging kidney [184,284]. Given the importance of this complex in establishing cell polarity and regulating the actin cytoskeleton, this deficiency may inhibit the complete recovery of the tubular epithelium in the aging kidney. This was supported by our recent study which demonstrated that loss of α(E)-

catenin expression leads to down-regulation of BMP-7 and N-cadherin, decreasing repair in renal tubule epithelial cells due to alterations in cell migration [284,285]. We further investigated the role of α(E)-catenin, F-actin and fascin 2 in cell migration (data not shown). C2/V cells exhibited decreased healing in a scratch assay as compared to NT3/V cells, in the presence of mitomycin C which blocks cell proliferation. Overexpression of *Fscn2* was able to rescue this deficit. Immunofluorescence staining of fascin 2 in migrating cells subjected to a wound healing assay showed an organized network of actin stress fibers at the leading edge of migrating NT3 cells. However, only about 62% of C2 cells formed a migrating line at 4 h after the scratch. Less fascin 2 was expressed in cytosol around the actin stress fiber in C2 cells with a further decrease of fascin 2 expression in those C2 cells failing to form the migration line. These data suggest that deficient repair, and its role in AKI, represent a novel and fruitful area to be explored in the aging kidney.

In summary, AKI in the elderly is multifactorial which involves many converging mechanisms. The data presented in this dissertation demonstrates that (Figure 5.3):

1) Aged kidney, marked by loss of α(E)-catenin, is more susceptible to cisplatin injury via apoptosis.

2) Loss of α(E)-catenin increases cisplatin-induced apoptosis in renal tubular epithelial cells.

3) Fas-mediated apoptotic signaling pathway is enhanced by the age-dependent loss of α(E)-catenin in renal tubule epithelial cells.

4) The expression of fascin 2 was decreased in aged kidney under stress.

5) Loss of Fscn2 increases the susceptibility of renal tubular epithelial cells to cisplatin injury.

6) Overexpression of *Fscn2* in α(E)-catenin knockdown cells, increases their resistance to cisplatin-induced apoptosis via facilitating actin polymerization.

7) Loss of α(E)-catenin is associated with mitochondrial dysfunction in renal tubular epithelial cells, which can be rescued by overexpression of *Fscn2*.

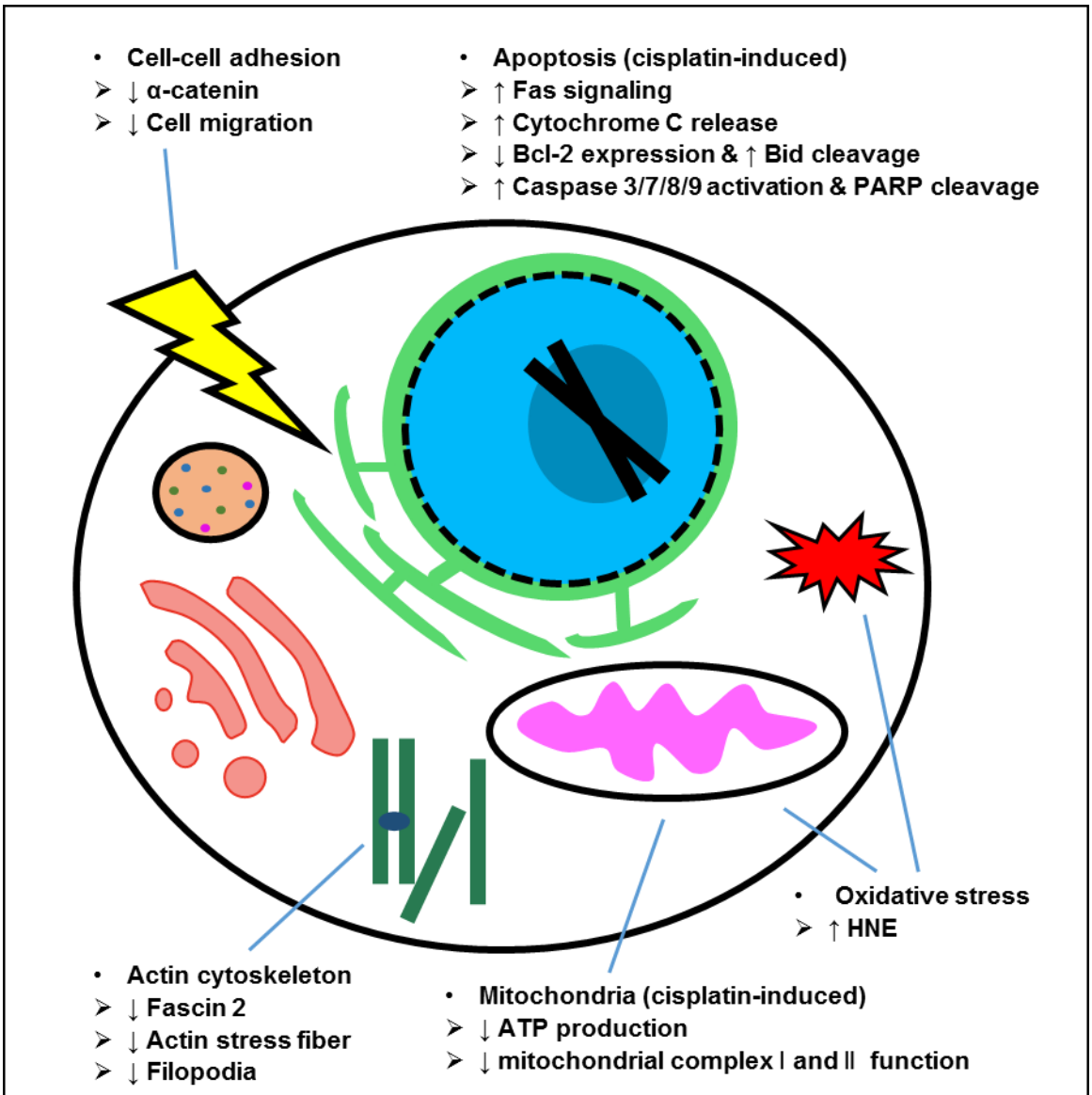


Figure 5.3 Summary of the mechanisms demonstrated in this dissertation underlying the increased incidence of AKI with aging.

8) Loss of α(E)-catenin increases oxidative stress in renal tubular epithelial cells, *Fscn2* overexpression abolishes the increased oxidative stress.

9) Loss of α(E)-catenin exhibited impaired cell migration; Fscn2 was able to rescue this deficit (data not shown).

There are several implications of these studies. First, anti-oxidants and agents that facilitate actin polymerization have the potential to be used as supplementary treatment to reduce the cisplatin-induced nephrotoxicity. However, traditional drugs like Jasplakinolide have unacceptable off target effects because they cannot distinguish between cytoskeleton, smooth muscle, cardiac forms of actin. Since fascin 2 is more selectively expressed than F-actin, developing a pharmacological or genetic therapy targeting at renal tubular fascin 2 to stabilize actin bundles will be better way to minimize drug induced nephrotoxicity. Second, because of the critical role renal tubule epithelium plays in AKI, manipulating renal tubule epithelial cell fate is of great importance for enhancing therapeutic efficiency to treat AKI. Our study indicates that focal adhesions are impaired and actin cytoskeleton is disrupted in aged kidney. The impacts of nanotopography on cell behavior across different lineages have been recognized since 1964. Cellular structure in the nanoscale range (9nm) has been shown to retain some effect. Thus, nanotopographical manipulation of focal adhesion formation and F-actin organization can be applied to tissue engineered scaffolds to improve resistance of aged kidney to exogenous insults.

It is important to note that, the studies in this dissertation have some caveats in interpretation. First of all, we used male Fisher 344 rats as our animal model showing that the aging kidney has a greater susceptibility to cisplatin. Evidence has shown there is a

high incidence rate of chronic progressive nephropathy (CPN) in senescent rats (81%, 12-24 month old) and male rats are more susceptible to CPN than females [286]. Histological features of CPN include kidney enlargement caused by dilated tubules filled with proteinaceous casts, while aged human kidney is characterized by shrunken size with tubular atrophy. In addition, unlike CKD in aged human kidney, CPN does not involve vascular changes and inflammation is not a key player in CPN [287]. Because the pathological characteristics of CPN does not fulfil the key hallmarks of human kidney senescence, male rats may not represent the optimal model to investigate xenobiotic-induced injury. Second, our data regarding to the role of α-catenin and fascin2 in the increased cisplatin-induced AKI are mostly obtained in cell culture *in vitro* without complete confirmatory data *in vivo*. Third, our *Fscn2* knockdowns using shRNA in the pGFP-V-RS vector with NRK-52E cells are not very selective and stable. The expression of *Fscn1* was also suppressed in these cell lines and a restoration of *Fscn2* expression was observed after 3~4 passages. Other knockdown methods like the CRISPR-Cas system can be used to address this issue. Last, although this is the study demonstrating the role of *fscn2* in renal tubule epithelial cells, our data supports the existing evidence suggesting that fascins play a role in linking alpha-catenin to the actin cytoskeleton, and further agrees with past literature showing an effect of fascins on migration and apoptosis. This existing past work detracts somewhat from the novelty of the current findings, although this is the first data showing the role of *fscn2* in acute kidney injury.

Thus, future work should focus on developing transgenic mouse models to confirm the conclusions we drew from current study. However, knockout mice in which many genes involved in development were targeted, including α-catenin, are embryonic lethal,

precluding the study of these genes in pathophysiology. Hence, development of an inducible, organ-specific transgenic model will avoid this limitation [288]. Since floxed α-catenin and proximal tubule inducible Cre mice are available from Jackson Laboratories, these mice can be used to generate an inducible, proximal tubular epithelial specific α-catenin$^{-/-}$ mouse model. Likewise, an inducible, proximal tubular epithelial specific Fscn2$^{+/+}$ mouse model should be generated to see whether overexpression of *Fscn2* can protect aged kidney from cisplatin-induced AKI.

In addition, clearer mechanistic insight into the relationships among between alpha-catenin, fascin 2, actin cytoskeleton and apoptosis, particularly within the context of stressed and aged kidney cells, still remain to be studied. Potential signaling pathways for future study are proposed (Figure 5.4). Our study has shown aging is associated with a

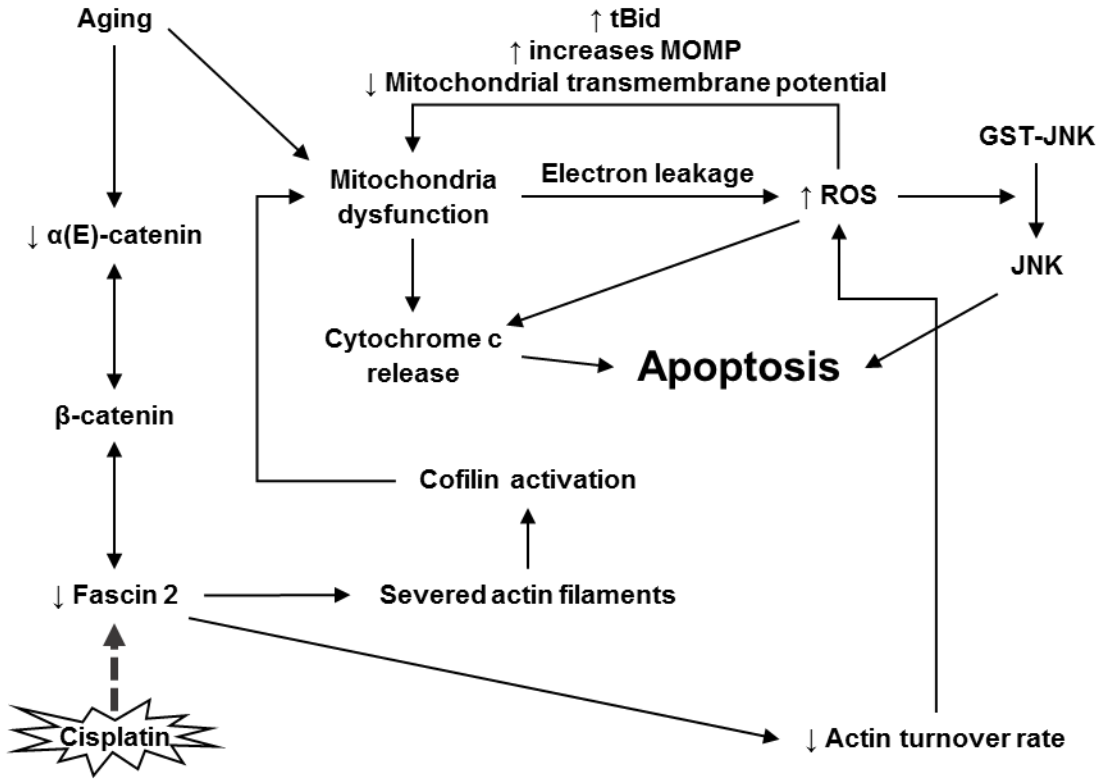


Figure 5.4 Potential signaling pathways for future study.

loss of α-(E)catenin in kidney and thus a decreased expression of fascin 2. Since both α-(E)catenin and fascin 2 interacts with β-catenin, β-catenin may play as a mediator between α-(E)catenin and fascin 2. Evidence has shown that fascin crosslinks a limited number of actin filaments into tight bundles, limiting the thickness of the stress fibers and preventing bundled actin severing by cofilin [264]. Here we propose that decreased expression of fascin allows cofilin to disassemble actin bundles and thus be activated. Activated cofilin will translocate to mitochondria and induce cytochrome c release leading to apoptosis [243]. In addition, fascin depletion also decreases actin turnover rate which triggers an increase of ROS level in cytosol [241]. Moreover, impaired mitochondria function has been associated with aging because mitochondria DNA is more vulnerable to the accumulation of mutations over time. Since the leakage of election from mitochondria electron transport is the main intracellular source of ROS under normal conditions, oxidative stress levels are increased with aging. Oxidative stress enhances the binding of tBID, decreases the potential of mitochondrial transmembrane, increases MOMP and facilitates the release of cytochrome c. Oxidative stress also causes dissociation of the GST-JNK complex and JNK pathway activation which leads to apoptosis independent of mitochondria. Furthermore, there are many different actin cross-linking proteins which also interact with α-catenin. Understanding the properties and functions of these cross-linking proteins will lead to a more complete understanding of the role of actin cytoskeleton in the loss the renal function in aged kidney.

The kidneys, especially the proximal tubule, play critical role in xenobiotic elimination [289]. Given prospective drug compounds in early clinical trials have extremely high failure rate due to unexpected nephrotoxicity, it is imperative to develop renal tubule

models that are more relevant to humans to better predict the safety, toxicity and efficacy of drugs. Current preclinical methods of evaluating renal toxicity relies on 2D cell culture or animal models with laboratory animal species. However, conventional 2D cell culture cannot capture the complexity of 3D physiological system adequately. Numerous studies have shown that cells cultured in 3D models have different response to drugs from those cultured in 2D models [290]. Evidence has shown epithelial cell lines from animal or human kidney have transformed characteristics, loss of renal tubule transporters and changes in metabolism [291]. Another drawback with conventional cell culture models is that they lack the interaction between kidney cell types, while renal vasculature plays critical role in drug disposition. Animal models with laboratory animal species demonstrate interspecies differences in drug absorption and disposition [289]. Recently Nortis developed a 3-D cell culture chip technology which allows for creation of a confluent tube of cells by injection primary human renal epithelial cells within a flowing chamber[289]. This microphysiological system reflects human physiology more accurately than traditional models. Thus, using novel 3-D cell culture models to access mechanisms of kidney injury and predict renal handling of endogenous and exogenous intoxicants should be considered in the future.

In conclusion, renal aging is a complex multifactorial process which predisposes to AKI in the elderly population. The studies in this dissertation are innovative that they 1) provide the first data to link loss of α-catenin to increased susceptibility of aged kidney to AKI; 2) identified a novel role of fascin 2 in renal epithelial cells, which depends on the functional interaction with α(E)-catenin and F-actin; 3) suggested that facilitating F-actin bundling may represent a potential intervention for treatment of AKI. These studies

represent a significant advance in attempts to manage the increasing burden of renal diseases in the expanding geriatric population. Hopefully, a deeper understanding of the mechanisms underlying AKI in elderly patients will lead to progression in the development of preventive and protective interventions that decrease the dialysis-requiring AKI and potentiate the resolution of AKI.

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