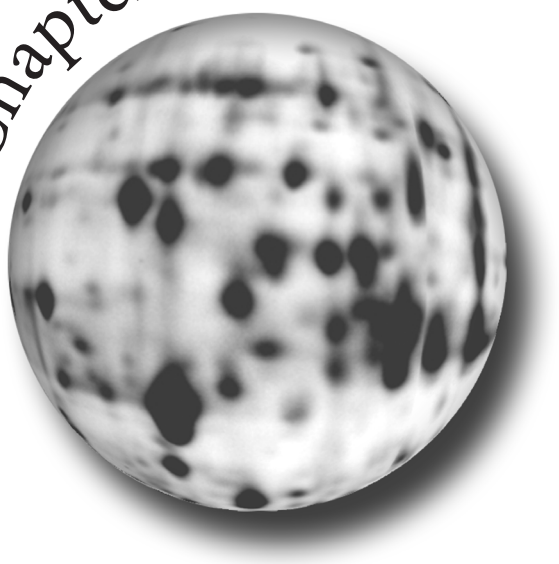
Molecular and cellular responses to renal injury : a (phospho)-proteomic approach

Graauw, M. de

An introduction to renal cell injury and regeneration

Chapter 1



AN INTRODUCTION TO RENAL CELL INJURY AND REGENERATION

Acute renal failure (ARF) is a rapid loss of renal function due to damage to the kidneys resulting from drug or toxicant exposure or renal ischemia/reperfusion. The regenerative capacity of the kidney is well documented and acute renal failure may be reversible with complete recovery of renal function. However, half of the patients do not respond to current therapies [1,2] and develop chronic renal failure and/or end-stage renal disease, a prognosis that has not changed over the past years [3]. These patients will require a lifelong dialysis or a kidney transplant in order to survive. To prevent the onset of ARF or improve the regenerative capacitiy of the kidney we need to understand on the one hand which events or factors cause renal cell injury and death, and on the other hand which mechanisms lead to renal cell repair and regeneration.

The observation that patients suffering from ARF have increased numbers of viable renal tubular cells in their urine indicates the importance of renal cell adhesion in the maintenance of tubular integrity and the involvement of cell adhesion in the onset of renal injury [4]. In addition, surviving renal cells that do not lose their cell adhesion are thought to contribute to the regeneration of renal function via proliferation and migration, suggesting an important role for cell adhesion in the regeneration of the kidney. This chapter will describe renal physiology and morphology in relation to renal injury and the changes that occur during renal injury and regeneration in cell adhesion structures of renal epithelial cells.

Renal morphology and physiology

Kidneys are responsible for preserving the body's internal environment. They maintain the total body salt, water, potassium and acid-base balance, while excreting toxins and other waste products. There are three mechanisms by which the kidneys accomplish the homeostasis in the internal environment, namely glomerular filtration, tubular reabsorption, and tubular excretion. The kidney can be divided into three segments: cortex, outer medulla and inner medulla (**Fig. 1**). Each human kidney consists of about a million nephrons, which stretch through these different parts of the kidney. In turn, one single nephron consists of several subunits, among which, the glomerulus, proximal tubule, distal tubule and the collecting duct, which all have their own specific transport property (**Fig. 1**). The renal tubule and collecting duct are composed of a single layer of cells surrounding a tubular lumen. The cell structure and function varies considerably from one segment to another, but each of them contributes to the transport function of the kidney. As a result of this transport function, cells within the different segments of the nephron may be extensively exposed to various toxins. The epithelial cells lining the proximal tubule are considered to be the major target for cellular injury, because of the reabsorption of large fluid volumes (including toxicants) and the high activity of several enzymes within these cells.

Renal cell injury is caused by ischemia and reperfusion (IR), exposure to nephrotoxic drugs, such as cisplatin, gentamicin and cyclosporine or exposure to toxicants, such as cysteine S-conjugates resulting in many cases in ARF. Renal proximal tubular

epithelial (RPTE) cell injury is characterized by adenosine triphosphate (ATP) depletion, mitochondrial dysfunction, impaired solute and ion transport, loss of brush border morphology, loss of cell polarity and cytoskeletal disruption [5,6]. Proximal tubular cells may loose their interaction to the basement membrane, leaving a denuded proximal tubule and causing cast formation in the tubular lumen (**Fig. 2**). Denudation of the basement membrane causes an increased back-leak of glomerular filtrate [7,8]. Together with tubular obstruction caused by detached cells, this will lead to impaired renal function. The finding that up to 100% of exfoliated tubular cells found in the urine of ARF patients were viable shows that exfoliation does not necessarily results in cell death [4]. However, when injury is too severe, irreversible cell injury occurs, which results in cell death. Two different types of cell death have been distinguished in ARF: necrosis and apoptosis [9,10]. Necrosis is characterized by swelling of the cell and organelles, with little changes in the nucleus and occurs in response to very harmful conditions. Apoptosis is a form of programmed cell death, which is characterized by cell shrinkage and condensation of nuclear chromatin resulting in formation of apoptotic bodies. These are rapidly removed via phagocytosis making it difficult to evaluate the importance of apoptosis in renal injury. Nevertheless, more and more evidence is obtained indicating that pathways generally associated with apoptosis, are important in renal tubular injury [11]. It is likely that the cascades that lead to apoptotic or necrotic cell death are activated almost simultaneously. However, during toxicant exposure the initiation of apoptosis may occur at exposure levels that are less severe than that needed to induce necrosis. RPTE cells that do not die or detach from the basement membrane are thought to contribute to renal regeneration [12]. These surviving cells migrate to the denuded areas, proliferate, re-polarize and/or dedifferentiate, and restore nephron structure and function [12,13]. Given the importance of cell adhesion in the process of renal injury (**Fig. 2**) this chapter will focus on the cell adhesion structures of renal proximal tubular epithelial cells in the context of molecular mechanisms of renal tissue injury.

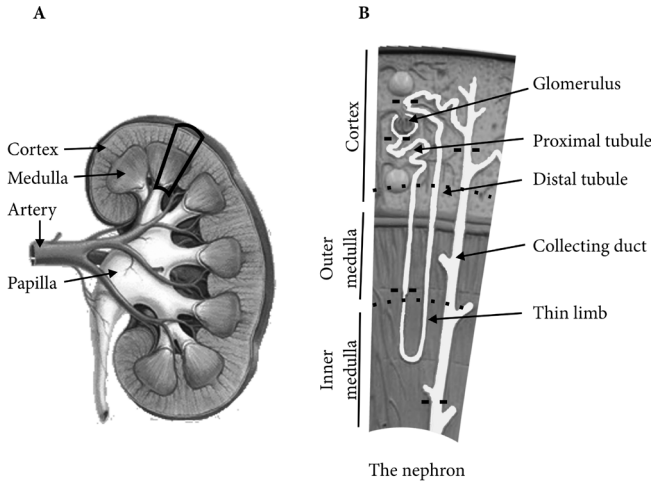


Figure 1. *Schematic representation of the kidney and nephron.* The kidney consists of a papilla, inner- and outer medulla and the cortex (A). Each kidney consists of about a million nephrons (B), which contain, the glomerulus, proximal tubule, descending and ascending thin limb, distal tubule and the collecting duct.

Adhesion molecules and the actin cytoskeleton in renal cell function

Tubular cell adhesion is established through cell attachment to the basement membrane via cell-extra-cellular matrix (ECM) interaction and to neighboring cells via cell-cell adhesion complexes (**Fig. 2**). These two adhesions are important in the maintenance of cell polarity [14]. Both the cell-ECM and cell-cell adhesion complexes are linked to the F-actin cytoskeleton through cytoskeletal/signalling adapter proteins (**Fig. 3**). In addition, the cell surface that contacts the fluid in the tubular lumen (apical cell membrane) has numerous F-actin rich microvilli, which collectively are called the brush border. These microvilli greatly increase the membrane surface area, thereby facilitating transfer of compounds between cells and the tubular fluid.

Cell-ECM adhesion complexes

Like other cell types RPTE cells require adhesion to the basement membrane for normal function. This adhesion is mediated by cellular integrins, which are heterodimeric transmembrane proteins consisting of an α and β subunit (**Fig. 4A**). Upon binding to the ECM, integrins cluster. Upon clustering, the cytoplasmic domain of integrins will become part of a large complex of signalling- and cytoskeletal-related proteins, together forming a specialized structure commonly referred to as focal adhesions (FAs) or focal contacts. These FAs mediate cell attachment to the ECM and serve as anchor points for actin filament tethering and remodeling. The FA complex consists of several cytosolic proteins, including talin, vinculin, paxillin and the non-receptor tyrosine kinase, focal adhesion kinase (FAK). The composition of the FAs and the cellular signalling in these complexes is mainly regulated via protein phosphorylation .

Upon binding of FAK to the integrins it is autophosphorylated on Tyr397, thereby creating a binding site for the SH2 domain of Src kinase [15] (**Fig. 4A**). This interaction results in activation of Src kinase by phosphorylation of Tyr416. Activated Src kinase will then phosphorylate FAK at multiple Tyr residues, among which are Tyr576, thereby enhancing FAK catalytic activity, and Tyr861, thereby creating additional interaction sites for SH2-containing proteins [16,17]. The FAK-Src complex is able to mediate phosphorylation of downstream targets such as the signalling adapter protein paxillin [18]. Moreover, stimulation of RPTE cells with growth factors, such as hepatocyte growth factor (HGF) or epidermal growth factor (EGF) enhance phosphorylation of FAK, thereby stimulating signalling through focal adhesions [19]. In contrast, loss of integrin-based cell-ECM interaction results in dephosphorylation of FAK [20], which plays a role in renal cell injury.

Cell-cell adhesion complexes

In addition to cell-ECM interactions, renal epithelial cells form multiple cell-cell adhesions. These cell-cell interactions consist of adherens junctions (AJ) and tight junctions (TJ) of which the AJ are better understood (**Fig. 4B**). The AJs are primarily composed of a family of transmembrane proteins called cadherins (*e.g.* epithelial E-cadherin, neural N-cadherin, placental P-cadherin and kidney K-cadherin) [21,22]. Cadherins have a single transmembrane-spanning region, an extracellular domain and an intracellular domain.

The extracellular part of a cadherin protein binds to the extracellular domain of a cadherin protein from an adjacent cell, thereby linking two cells in a zipper-like way [23]. At the intracellular surface, cadherins interact with the actin cytoskeleton via catenins (*e.g.* α-catenin, β-catenin, plakoglobin and p120 catenin). Within this complex, β-catenin binds directly to the F-actin cytoskeleton, which is required for formation of cell-cell junctions [24]. β-catenin forms a link between cadherin molecules and α-catenin. The stability of AJs is, amongst others, regulated via tyrosine phosphorylation of cadherin and catenin proteins. For example, expression of an inducible Src kinase in madin darby canine kidney (MDCK) cells results in phosphorylation of β-catenin and E-cadherin, which is associated with disassembly of the AJs [25]. Loss of cell-cell junctions via disruption of the cadherin/catenin complex results in loss of adhesiveness, which leads to RPTE cell detachment and in the end loss of renal tubular function [7,8].

F-actin cytoskeleton in cell adhesion

The F-actin cytoskeletal network plays an important role in many cellular processes and functions, such as cell motility, contraction, changes in cell shape and rigidity, cell division and signal transduction. Most processes that are regulated by the F-actin network depend on precise polarization and actin assembly and disassembly cycles [26]. The actin network is able to actively remodel to respond to external stimuli, such as growth factors, and to participate in regional events within a cell. For example, in cell movement the initial event is the formation of cell protrusions, such as filopodia or lamellipodia [27]. These structures are rich in F-actin and actin-binding proteins. The formation of lamellipodia is regulated by dissolution of the existing actin filament network and generation of a new actin cytoskeleton. In collaboration with actin-binding proteins, such as gelsolin, arp 2/3 complex and cofilin, actin filaments mediate the actin-dependent cellular processes.

Actin filaments are arranged into "higher order" forms, including the cortical actin network (actin gel), stress fibers (loose nonparallel bundles), and tight parallel bundles like those observed in the microvilli of renal epithelial cells [28]. Filamentous (F)-actin is build using G-actin monomers as building blocks (**Fig. 5**). During polymerization G-actin binds to ATP and is added to the barbed end of an existing filament where ATP is rapidly hydrolyzed. The polymerization of actin is catalyzed by the Arp 2/3 complex, which acts together with cortactin, downstream of a variety of receptors and signalling cascades. Disassembly of actin filaments (depolymerization) occurs primarily by dissociation at the pointed end, releasing G-actin bound to adenosine diphosphate (GDP) [28,29]. Together the actin filaments form a tightly regulated F-actin network, which controls proper RPTE cell function.

Several protein (complexes) are known to regulate the F-actin network, including the family of Rho GTPases [30]. Rac, Cdc42 and Rho are Rho-GTPases that regulate the formation of stress fibers, lamellipodia, and filopodia. Furthermore Rho GTPases have an important role in the regulation of cell adhesion, motility, polarity and intercellular adhesion [31]. Activated Rho-GTPases exist in a GTP-bound state, whereas inactive Rho-GTPases are bound to GDP [32] (**Fig. 3**). Rho-kinase, the downstream effector of Rho sig-

nalling is involved in the activation/phosphorylation of myosin light chain (MLC) and inactivation/phosphorylation of cofilin, which subsequently modulate stress fiber formation and assembly [33]. The formation of renal tubules is regulated by Rho-kinase, but occurs independent of MLC kinase activity [34,35]. In addition, ischemia/reperfusion-induced cytoskeletal reorganization is associated with up-regulation of both Rac and RhoA expression [36]. The precise role of Rho-GTPases in renal cell injury remains elusive.

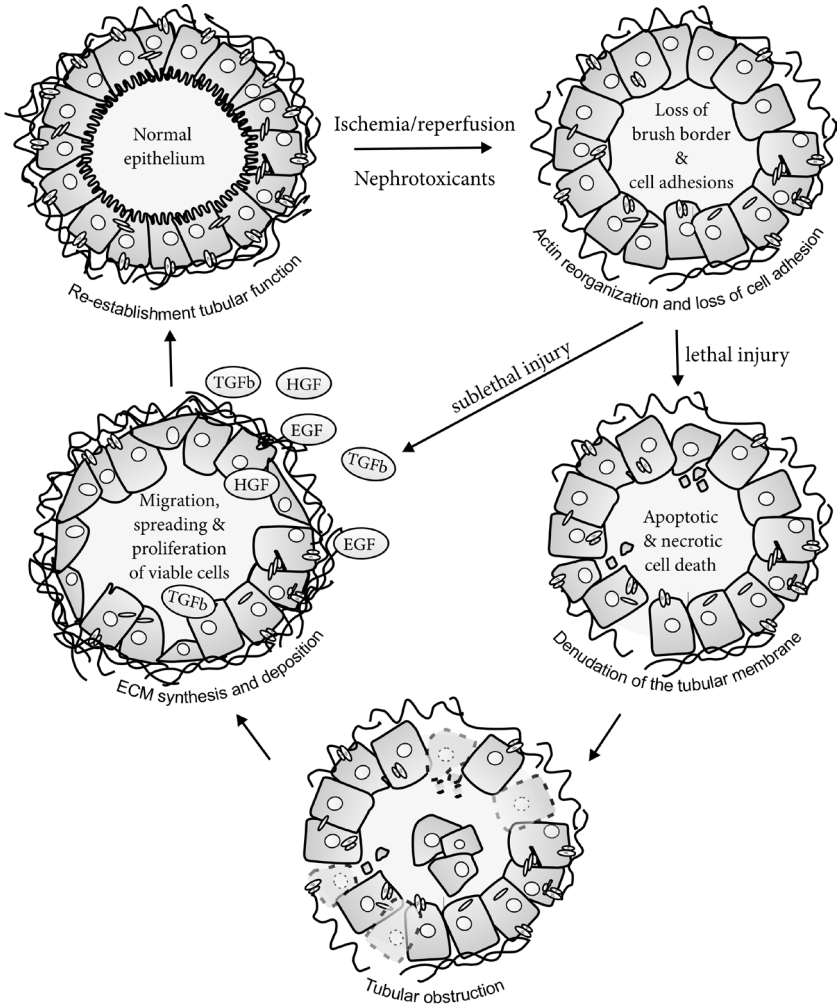


Figure 2. *Diagram of morphological changes occurring in the proximal tubulus during injury and regeneration*. Proximal tubular cells exposed to ischemia/reperfusion or nephro-toxicants undergo disruption of the F-actin cytoskeletal network, resulting in loss of brush border morphology, cell-ECM and cell-cell adhesion. Irreversible cell injury results in the onset of cell death via necrosis or apoptosis. During the regeneration phase, cells migrate, spread and proliferate to fill the denuded areas and regain normal kidney function. When severely damaged, the kidney will not be able to regenerate and chronic renal injury is obtained.

Induction of renal cell injury

Renal cell injury is mostly due to ischemia or exposure to toxicants that directly affect the kidney. These so-called nephrotoxicants include therapeutic agents such as antibiotics (*e.g.* gentamycin, cyclosporine), radiocontrast agents (*e.g.* iodothalmates and diatrizoates) and chemotherapeutics (*e.g.* cisplatin), or chemicals, such as halogenated cysteine S-conjugates. Damage to the proximal tubule resulting from ischemia reperfusion is selective to the S3-segment in the outer medulla region, whereas the site of proximal tubular injury caused by nephrotoxicants is dependent on the physico-chemical properties of the toxicant and the specific transport system needed for its uptake in the cells. In this thesis we have used two models to study the mechanism underlying the reorganization of renal cell adhesion and the F-actin cytoskeleton during renal cell injury. These include ATP-depletion due to ischemia-reperfusion and exposure to the model nephrotoxicant S-(1,2-dichlorovinyl)-L-cysteine (DCVC).

Ischemia-reperfusion-induced renal injury

IR-induced renal cell injury develops following a sudden drop in total or regional blood flow to the kidney [37] followed by a restoration of the blood flow during the reperfusion period. In organ transplantation IR results in damage that may affect cell viability and lead to organ failure. IR involves a complex cascade of events, including loss of ATP, derangement of the ionic homeostasis, production of reactive oxygen species (ROS), and cell death. Mitochondria play an important role in this process, since they generate the cellular energy in the form of ATP [38]. The absence of O_2 as a result of ischemia, leads to a break down of ATP. Reperfusion is characterized by an increase in the formation of ROS, a decrease in ATP production and cell death. Since the establishment and maintenance of the cell cytoskeletal and protein network is a dynamic and ATP dependent process, IR-induced ATP depletion rapidly results in changes in the actin cytoskeleton, loss of junctional complexes, and re-localization of cell adhesion proteins. IR injury and regeneration can be studied *in vivo* by clamping the renal artery of rats or mice for 30-45 minutes followed by different reperfusion periods or *in vitro* by depleting cellular ATP using chemicals (*e.g.* antimycin A or cyanide combined or not with deoxyglucose) [5,39].

Chemical-induced renal cell injury

The transport function of the kidney enables it to excrete waist products from our body. But when our body is exposed to drugs or toxic compounds, the high blood flow to the kidney and its ability to concentrate solutes cause exposure of the kidney to high concentrations of these toxic compounds resulting in injury. As mentioned above, several classes of nephro-toxicants have been described. In this thesis we have used the non-enzymatic product of trichloroethylene, S-(1,2-dichlorovinyl)-L-cysteine (DCVC), which requires bio-activation in order for toxicity to occur [40,41]. Thus, DCVC and other nephrotoxic cysteine S-conjugates kill cells after they are metabolized to reactive intermediates by a renal cysteine S-conjugate β-lyase, which is present at high concentration in the proximal tubular cells [42]. DCVC can therefore be used to selectively target RPTE cells and is often

used as a model compound to study the mechanisms underlying toxicant-induced renal cell injury both *in vivo* as well as *in vitro*. The reactive intermediates that are formed bind to cellular macromolecules (*e.g.* proteins and lipids), resulting in cellular toxicity. DCVC induces apoptosis in the renal proximal tubule cell line LLC-PK1 [43] and in primary cultured rat [44] or human proximal tubular cells [45] by a mitochondrial-dependent pathway. In addition, DCVC induces changes in the F-actin cytoskeletal network. *In vivo* DCVC induces loss of F-actin in the proximal tubulus, which is associated with loss of the brush border and loss of cells from the basement membrane [46]. *In vitro* DCVC causes detachment RPTE cells, which is associated with F-actin damage and changes in FAs and precedes the onset of apoptosis [20,47].

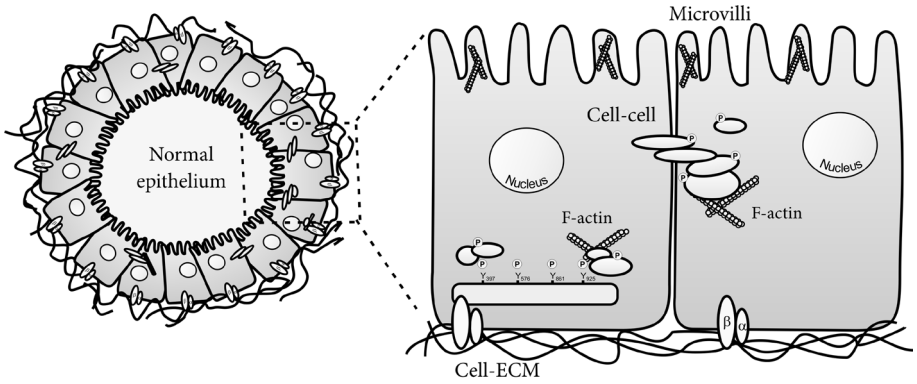


Figure 3. *Schematic representation of tubular cell adhesion.* Tubular cell adhesion is mediated via cell-extra-cellular matrix (ECM) interaction and via cell-cell adhesion complexes. Both complexes are linked to the F-actin cytoskeletal network. The apical cell surface contains a dense F-actin network and many signalling-related molecules.

Reorganization of adhesion molecules and the actin cytoskeleton in renal cell injury

Renal injury in associated with loss of cell-matrix and cell-cell interactions and reorganization of the F-actin cytoskeleton, resulting in RPTE cell detachment, formation of cellular aggregates in the tubular lumen, glomerular filtrate back-leak and loss of renal tubular function [7,8]. Over the past few decades several studies have provided evidence for the reorganization of integrin- and cadherin-based adhesions and the cytoskeletal network in renal cell injury and regeneration. Below the most essential findings are discussed.

Loss of cell-matrix adhesion

Loss of integrin-based cell-matrix interaction results in the onset and progression of renal cell injury. In 1993 Gailit *et. al.* demonstrated for the first time that renal oxidative stress disrupts the focal adhesions, which is associated with redistribution of the β3 integrin subunit from the basal to the apical side of cultured RPTE cells [7]. Since this initial finding, a number of studies have investigated the role of integrins in renal injury. Zuk *et. al.* demonstrated a role for β1 integrins in IR-induced injury [8]. These integrins newly

appeared on lateral borders in epithelial cells, thereby decreasing their concentration at the basolateral side of the cells, possibly contributing to RPTE cell exfoliation. Moreover, integrin antagonism with a GRGD peptide following oxidant-induced renal cell injury inhibited regeneration in rat RPTE cells [48]. Administration of HUTS-21 antibody, which recognizes an activation-dependent epitope of β1 integrins resulted in the preservation of renal functional in a rat IR model of renal injury [49]. In addition to the IR-induced cell injury, integrins play a role in toxicant-induced injury. In RPTE cells, DCVC exposure was associated with a decrease in basal localization of integrin subunits and an appearance of integrins on the apical membrane [50]. Taken together these studies have provided evidence, both *in vitro* as well as *in vivo*, for a role of integrins in renal injury. However, most studies, especially the *in vivo* studies, determined expression and localization of the integrin receptors and did not describe an effect of cell-matrix disruption on downstream signalling pathway.

As described above, integrin-mediated cell adhesion signalling through FA complexes is mainly regulated by protein phosphorylation. *In vitro* it has been demonstrated that phosphorylation of focal adhesion proteins is altered as a result of renal cell injury. Hypoxia in freshly isolated rabbit proximal tubules results in loss of tyrosine phosphorylation of several focal adhesion proteins including, FAK and paxillin [51]. During nephrotoxicant exposure in primary cultured rat RPTE cells both FAK and paxillin phosphorylation is lost [20]. So far, the effect of ATP depletion or nephrotoxic compounds on phosphorylation and activation of FAK and other FA proteins *in vivo* remains unclear.

Loss of cell-cell adhesion

Renal injury is associated with back-leak of glomerular filtrate. Postischemic injury can be classified as 'recovering from ARF' or 'sustained ARF' [52]. In sustained ARF more than 50% of the glomerular filtrate is lost due to back-leak, while no back-leak is observed in patients recovering from ARF. Kwon *et. al.* were the first to explore a structural basis for this sustaining back-leak [52]. Staining of renal allografts for different TJ or AJ markers (*e.g.* the zonula occludens complex ZO-1 and adherens complex, α, β, and γ catenins) revealed diminished intensity and redistribution of each protein from the cell-cell junctions. Thus, most likely impaired integrity of cell-cell adhesion in the proximal tubule provides a paracellular pathway through which filtrate leaks back in patients with sustained ARF.

Since this finding other studies have investigated the role of cell-cell interacting proteins and their phosphorylation status in renal injury. Exposure of the renal cell lines, LLC-PK1 and MDCK to the nephrotoxic compound cadmium results in reduced ATP levels followed by a decrease in trans-epithelial resistance (TER) and relocalization of cadherins and catenins [53]. Mouse RPTE cells subjected to ATP depletion have a diminished staining of E-cadherin and an increased tyrosine phosphorylation of β catenin and plakoglobin [39,54]. Cisplatin, which is an antitumour agent that may cause nephrotoxicity, causes the early loss of cell-cell adhesions. This is associated with altered localization of β-catenin in association with PKC-mediated phosphorylation of the actin-capping protein adducin [55].

In vivo studies show that E-cadherin is lost from the AJs, which is associated with cleaveage of the protein to an 80 kDa fragment [56]. In addition, cadmium-induced renal injury in rats is associated with a loss of N-cadherin, E-cadherin and β-catenin from the cell-cell junctions [57]. This loss was specific for the proximal tubule and could not be observed in other parts of the kidney.

Although a number of studies show that a toxic insult disrupts the cell-cell contacts in renal cells *in vitro*, the *in vivo* relevance of these findings is still unclear. Some evidence suggests that the disruption of cell-cell adhesion is associated with renal failure in patients, but the understanding of the cause and effect is still limited due to the lack of *in vivo* data. Regardless, patients suffering from ARF have increased numbers of viable renal tubular cells in their urine indicating cell detachment, including cell-cell dissociation [4].

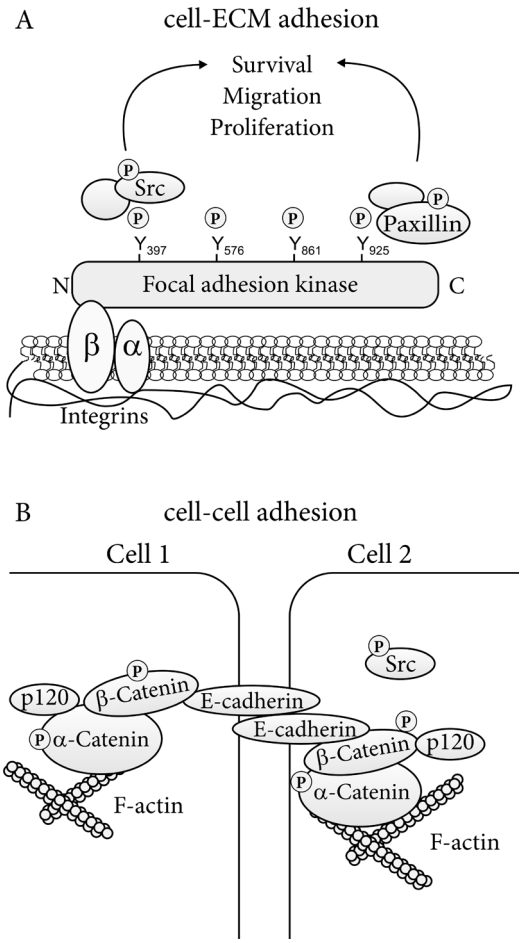


Figure 4. *Cell-ECM and cell-cell adhesion complexes.* At the cell-ECM interaction focal adhesions are formed consisting of integrins and adhesion molecules such as FAK, Src and paxillin (A). The cell-cell interaction contains cadherins, which link two cells in a zipper-like way, and catenins, which link the complex to the F-actin network (B).

Cytoskeletal reorganization

Alterations in the actin cytoskeleton have been shown to affect physiological processes in the kidney during and following ischemic and toxic injury (reviewed in [58]). Although IR-induced changes in the F-actin network are mostly caused by severe reductions in renal blood flow, even very short ischemic-periods can provoke changes in the surface polarity and adhesion complexes mediated by the actin cytoskeleton. The proximal tubule apical microfilaments (epithelial brushborder) are particularly sensitive to an ischemic insult [59]. Within the proximal tubule, ischemia results in very early loss of actin filaments in the epithelial brush border, with the majority of F-actin loss occurring within five minutes, while the glomerular or distal tubules remain unaffected. The recovery of the actin cytoskeleton after ATP repletion occurs early and may be necessary for reestablishment of polarity, which is essential for normal reabsorptive functions [60,61]. RPTE cells may also undergo toxicant-induced disruption of the F-actin network. Treatment of rats with the model nephrotoxicant DCVC results in loss of F-actin in the S3 segment of the proximal tubule in association with loss of the brush border and loss of cells from the basement membrane [46]. The high sensitivity of the microvillar F-actin is due to the rapid rate of ATP-dependent actin polymerization, thereby making it highly susceptible to ATP depletion [62]. The actin-binding protein cofilin seems to play an important role in ATP depletion-induced F-actin reorganization [63,64]. ATP depletion rapidly induces dephosphorylation/ activation and relocalization of cofilin. A constitutive inactive mutant protects LLC-PK1 cells against chemical anoxia-induced F-actin injury [63].

Alterations in the cytoskeletal organization are often accompanied by a modification in the polarized distribution of some membrane (transport) proteins. Ezrin, a phosphoprotein that mediates the binding of microvillar F-actin to the membrane, is dephosphorylated during ischemia and dissociates from the actin filaments [65 66]. Reoxygenation following an anoxia period causes recovery of the microvillar structure and reassociation of ezrin to the cytoskeleton and the brush border. Moreover, the actin-binding protein villin redistributes in parallel with F-actin from the apical to the basolateral plasma membrane during IR [60,67]. Repolarization of villin to the apical membrane begins within hours after reperfusion with enhanced apical localization over time during the period of regeneration, which is accompanied by the reestablishment of a normal actin distribution in the brush border.

Renal cell injury does not only involve changes in the apical F-actin, but also results in disruption of F-actin stress fibers and cortical F-actin. *In vitro* ATP depletion results in loss of stress fibers [68], while cisplatin-induced renal cell injury is associated with increased stress fiber formation [55]. The Rho GTPase family is mainly involved in the regulation of F-actin stress fibers. Expression of a constitutively active RhoV14 in LLC-PK1 cells prevented disruption of stress fibers and cortical F-actin during ATP depletion and enhanced the rate of stress fiber reassembly during recovery. Conversely, the Rho inhibitor C3 or dominant negative RhoN19 prevented recovery of F-actin assembly upon ATP repletion [60,69]. In addition, myosin II, an important effector in organizing basal actin structures, is rapidly inactivated during ATP depletion [60,68]. This inactivation precedes

dissociation of myosin II from actin stress fibers during ATP depletion. Myosin II activation is associated with reorganization of F-actin stress fibers during recovery from ATP depletion. Furthermore, myosin activation and actin stress fiber formation are Rho-kinase dependent [60,68].

Although many studies have shown that renal cell injury and apoptosis is preceded by changes in the F-actin cytoskeletal network and redistribution of some actin-binding proteins, the precise mechanisms underlying this F-actin disruption are still not clear. Moreover, the F-actin network is linked to cell-matrix and cell-cell interactions. The link between F-actin reorganization and the disruption of cell-ECM and cell-cell interaction as a result of IR or toxicant-induced cell injury is yet unclear. Few studies have studied the effect of ATP depletion or toxicant exposure in live cells, so that the precise time course of cellular injury can be established. ATP-depletion of cultured renal epithelial cells expressing an enhanced green fluorescent protein (EGFP)-actin show that assembly of lamellar actin is inhibited rapidly as cellular ATP levels are reduced, whereas disruption of actin in stress fibers is more gradual and persistent [70]. Actin that is associated with focal adhesions is largely resistant to ATP depletion, while cell-cell interactions are sites of actin filament assembly even when ATP levels are maximally decreased. The role of signalling complexes and actin binding proteins in these processes remains thus far unclear.

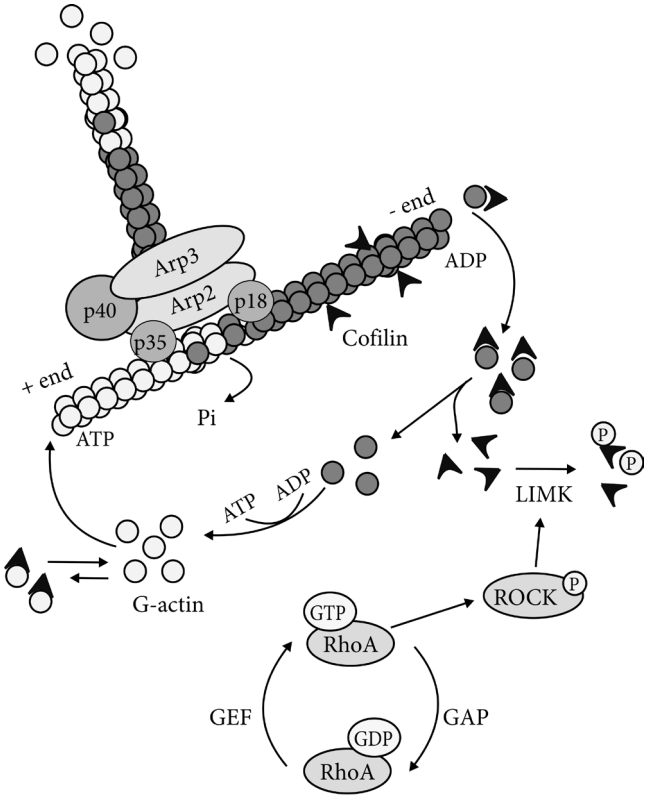


Figure 5. *Actin polymerization.* Actin filaments are build from ATP-bound G-actin monomers at the + end. Actin hydrolyzes its bound ATP to ADP + Pi. Cofilin promotes dissociation of G-actin-ADP from the – end of the F-actin filament. Cofilin binding to G-actin-ADP inhibits ADP/ATP exchange, thereby inhibiting re-polymerization. Phosphorylation of cofilin by LIM kinase (LIMK) causes it to dissociate from G-actin, thereby stimulating ADP/ATP exchange and actin polymerization. LIMK is activated through GTP-RhoA and Rho kinase (ROCK). The Arp2/3 complex binds to the side of an existing actin filament and nucleates assembly of a new F-actin fiber, thereby facilitating F-actin branching.

Activation of stress signalling pathways

Perturbation of normal epithelial cell function by IR or toxicant exposure results in the induction of diverse stress response pathways, including activation of the MAP kinase (MAPK) pathways and phosphorylation/expression of multiple small heat shock proteins [71,72]. The MAPKs are localized at focal adhesions, thus the possibility exists that disruption of cell adhesions after renal cell injury is in part responsible for the activation the MAPK stress signalling pathways.

The family of MAPKs consists of extracellular signal-regulated kinases (ERKs), which are activated downstream of tyrosine kinase receptors [73], and c-Jun amino-terminal kinase (JNK), and p38, which are responsive to cellular stresses [72]. Renal IR causes activation of JNK and p38 [74-76], but also a variety of nephrotoxicants cause JNK and p38 activation [77-79]. Activation of JNK in renal cell injury is associated with the onset of apoptosis. Downregulation of JNK in human kidney cells using antisense oligonucleotides prevents JNK activation and inhibits IR-induced apoptosis [80]. In addition, prevention of JNK phosphorylation results in reduced levels of apoptotic cells [76]. Sub-lethal renal cell injury, which does not lead to apoptosis, but results in a preconditioning of cells and protection against a subsequent, more severe stress prevents activation of JNK and p38 [81,82]. Activation of JNK and p38 may contribute to cellular apoptosis by phosphorylation of various transcription factors, thereby affecting gene expression and ultimately protein expression [83-86] or by phosphorylation of various cellular proteins, such as the anti-apoptotic protein Bcl-2 [87,88] or heat shock protein 27 (Hsp27) [89].

The small heat shock protein Hsp27 has an important role in the regulation of RPTE cell survival after injury. Several studies have shown that overexpression of Hsp27 in cells renders them more resistant towards stress-induced injury [90,91]. In the kidney, increased expression of Hsp27 is found during the recovery phase after injury [81]. In addition, overexpression of Hsp27 inhibits apoptotic cell death of LLC-PK1 cells caused by either oxidative stress or ATP depletion [82]. The protective function of Hsp27 seems to be dependent on the phosphorylation status of the protein. Overexpression of a non-phosphorylatable form of Hsp27 was much less effective in mediating protection and may even result in enhanced apoptosis [92-94]. The protective effect of Hsp27 is, amongst others, dependent on regulation of the actin microfilament stability [93], which is influenced by p38-mediated phosphorylation of Hsp27 [89].

The epithelial mesenchymal transition in chronic renal injury

Toxicant- or IR-induced renal failure may ultimately result in chronic progressive fibrosis of the kidney, which can lead to scarring of the kidney and disruption of renal function. The major players in renal fibrosis have been considered the resident fibroblasts and the infiltrated mononuclear cells. However, there is increasing evidence that new fibroblasts may be derived from tubular epithelial cells. This occurs through a process termed the epithelial-mesenchymal transition (EMT) [95,96]. EMT is characterized by disruption of epithelial junction complexes and loss of cell polarity, thereby transforming stationary epithelial cells into migratory mesenchymal cells. Normal epithelial cells interact with the

ECM through integrin-based complexes. The mesenchymal cells lose this domain-specific anchorage and acquire the ability to invade the ECM [97]. In embryonic development, EMT permits anchored epithelial cells to reorient in a developing organism and allows migration of epithelial cells to distant sites where they can form tissues such as the neural crest [98]. In mature tissue, EMT of resident epithelial cells is thought to occur during injury as an additional source of myofibroblasts/fibroblasts.

In recent years more and more data point to the involvement of EMT in injured kidneys. Initial reports demonstrated that fibroblast specific protein (FSP1 or S1004A) can be detected in tubular cells of injured kidneys in both acute and chronic disease [99]. S100A4 is a member of the S100 family of calcium binding proteins and is, amongst other cell types expressed in fibroblasts. Evidence for epithelial-derived fibroblasts in renal fibrosis comes from studies using transgenic mice genetically engineered to express the LacZ marker specifically in renal tubular epithelia [100]. This study demonstrated that more than one third of the renal fibroblasts are derived from renal tubular epithelium via a local EMT, suggesting that EMT of epithelial cells plays a crucial role in renal fibrosis. The importance of EMT in renal fibrosis is confirmed by studies using tissue-type plasminogen (tPA) null mice [27]. Mice lacking tPA were largely protected from development of interstitial fibrosis after obstructive injury. Ablation of tPA selectively blocks tubular EMT, but did not affect myofibroblastic activation from interstitial fibroblasts.

How EMT is activated in kidneys subjected to injury remains to be elucidated. However, *in vitro* studies have provided insight into the mechanisms involved in renal cellular EMT. Since *in vivo* the tubular cells will be exposed to a cocktail of many different factors, the *in vitro* studies may not always resemble the *in vivo* situation, but they will provide links to mechanisms involved in EMT. The main inducers of EMT can be categorized into growth factors (*e.g.* TGF-β, EGF and FGF), and enzymes (*e.g.* matrix metalloproteinases), which facilitate disruption of the basement membrane integrity [14]. In general, the main inducer of EMT is TGF-β. Mimicking the renal *in vivo* condition by using a Boyden chamber system with renal basement membranes shows that stimulation of RPTE cells with TGF-β increases migration of the cells across the membrane [20]. This is associated with increased production of matrix metalloproteinase (MMP) 2 and 9, which degrade the basal membrane.

In addition EMT processes are believed to be involved in renal regeneration processes. The EMT process is, at least in an early stage, reversible. Therefore it is hypothesized that EMT-derived fibroblasts could facilitate immediate repair of the injured kidney and potentially serve as a pool of vital cells with the capacity to repopulate the injured epithelium. Transformed cells may undergo re-differentiation to generate renewed epithelial cells if they are exposed to an appropriate trigger. For example HGF, a potent regenerative growth factor, may induce a mesenchymal to epithelial transition (MET) [13], which is consistent with the role of HGF in kidney development [6]. In addition, the growth factor bone morphogenic protein-7 (BMP-7), which plays an important role regulation of nephrogenesis associated with MET, can reverse renal injury [101]. Administration of BMP-7 in mice reverses transforming growth factor (TGF)-β-induced EMT and thereby reverses

chronic renal injury [101].

Both TGF-β and BMP-7 belong to the TGF-β superfamily. They signal through heteromeric complexes of transmembrane type I and type II serine/threonine receptors (reviewed in [12]). Within this complex type II tyrosine kinase activates the type I tyrosine kinase receptor, which subsequently phosphorylates different Smad proteins. TGF-β phosphorylates Smad 2 and 3, while the BMPs phosphorylate Smad 1, 5 and 8. The phosphorylated Smads form a complex with Smad 4, which than shuttles to the nucleus and regulates transcription. In addition to the Smad proteins, TGF-β is capable of activation a number of other proteins/signalling cascades among which, p38 MAPK, Akt/PKB, ILK and RhoA, thereby facilitating renal fibrosis. The role of TGF-β and BMP activated proteins in IR- or nephrotoxicant-induced renal injury remains to be elucidated.

Mechanisms of renal proximal tubular cell regeneration

Whether the kidney is capable of complete regeneration after renal injury depends mainly on the degree of injury (*e.g.* mild renal injury in the medulla or more severe injury that extends all the way to the renal cortex region). The four crucial processes for kidney regeneration after injury are migration, proliferation, differentation and repair of physiological functions (reviewed in [102]). These processes are influenced by various factors, including growth factors and cytokines. Depending on the combination of the expression and activity of these factors within the kidney, the regeneration phase will lead to complete recovery or progression towards an end-stage renal disease.

It is generally believed that renal regeneration starts with a migratory response of cells into the denuded areas. This is followed by a proliferative signal to replace lost cells, which will then differentiate and restore normal kidney function. Although migration has been described as a first step in repopulating the denuded tubuli, direct *in vivo* evidence is missing. The model used to study migration is a mechanical scrape technique, thereby simulating RPTE cell loss in 2 dimensional cultures. These studies demonstrated that migration of RPTE cells is influenced by various growth factors like EGF and TGF-β. EGF stimulates migration, whereas TGF-β inhibits cell migration [103]. Moreover, nephrotoxicants such as mercuric chloride and DCVC inhibited migration in such an assay. The proliferation and differentiation of RPTE cells is also under control of exogenous growth factors. These factors are released from injured cells, from cells present in the interstitium or from inflammatory cells. Although the growth factors EGF, IGF, FGF, HGF and TGF-β all contribute to the renal regeneration process, we will focus on the latter two since they are believed to be the most important growth factors in the control of renal regeneration.

Hepatocyte growth factor

HGF is present in the kidney in an inactive state and is proteolytically activated and released in response to certain stimuli, among which renal tissue injury [104]. HGF binds and activates the tyrosine kinase receptor c-Met, which results in phosphorylation and activation of several downstream pathways (reviewed in [105]). Administration of HGF to

mice protects against IR- [106] or cisplatin-induced renal injury [107] and stimulates renal regeneration. In addition, HGF prevents renal fibrosis and end-stage renal disease by inhibiting the injury-induced increase in synthesis and deposition of ECM components [108,109]. Moreover, treatment with anti-HGF antibodies in a mouse model for renal fibrosis results in markedly increased matrix deposits in the kidney [110].The level of HGF has been suggested to be a reliable indicator of kidney rejection. In a rat renal transplantation model, HGF levels rapidly increased during acute rejection, while recombinant HGF effectively protected the kidney from acute rejection [111]. These results suggest that HGF may be induced as a counter-response to renal injury and that HGF levels may be used as a reliable indicator for the diagnosis of acute rejection.

Transforming growth factor beta

Members of the TGF-β family of growth factors have been shown to be produced in kidney during its development. In renal injury and regeneration, levels of TGF-β mRNA are elevated in the outer medulla in tubules that appear incompletely regenerated [112] . In addition, a number of TGF-β responsive genes are transiently enhanced following induction of ischemic ARF in the rat [113]. These studies suggest that endogenous TGF-β serves to promote tissue regeneration following renal injury. However, sustained elevation of TGF-β is related to a perturbed regeneration process, eventually resulting in renal fibrosis [114] and progression of renal disease. Both in cell culture as well as in perfused rat kidney TGF-β stimulates excretion of various ECM proteins and tissue inhibitors of metalloproteinases (TIMP), while it decreases expression of matrix metalloproteinases (MMP). In this way, TGF-β provides an optimal environment for progressive ECM accumulation, which contributes to renal fibrosis [115]. The onset of renal fibrosis could be blocked by using anti-TGF-β antibodies. Neutralization of TGF-β attenuates renal failure progression in uremic animals [116]. In rats with chronic progressive anti-thymocyte serum nephritis the TGF-β/Smad signalling is up-regulated. TGF-β blockade by αT immunoglobulin suppresses the progression of renal scarring, at least in part, via inhibition of activated TGF-β/Smad signalling [117].

Reciprocal functions of TGF-beta and HGF

In renal disease progression and regeneration, TGF-β and HGF exert opposite effects. HGF promotes renal regeneration, while TGF-β antagonizes proper regeneration [110]. A balance in the activity of both signalling pathways is important during renal regeneration. When this balance shifts towards activation of TGF-β signalling, the increased production of matrix proteins may lead to renal fibrosis. HGF decreases the expression of tissue inhibitors of metalloproteinases (TIMP) and increases the expression of MMP-9 [118], while TGF-β regulates expression of these proteins the other way around [112]. TGF-β and HGF do not only control renal regeneration at the level of ECM composition, but also counteract each others effects at the level of cell scattering and tubulogenesis. For example, HGF stimulates branching morphogenesis in a tubulogenesis assay, while TGF-β counteracts the effects of HGF [119].

In conclusion, proper renal regeneration after an IR- or toxic insult is under control of a balanced HGF- or TGF-β-induced activation of signal transduction pathways. Increasing our understanding of signal transduction pathways that are activated by these growth factors is essential to understand how we can stimulate the renal repair process after renal injury.

Concluding remarks

It is clear that the disruption of cell adhesion complexes in association with reorganization of the actin cytoskeleton plays an important role in renal cell injury. Progress has been made in the identification and understanding of specific proteins and complexes that mediate cell adhesion and actin organization in the kidney. In addition to their potential role in cell detachment and the onset of apoptosis after injury, it is likely that these proteins are important in the restitution of the tubular epithelium resulting in renal regeneration. Many studies on the role of cell adhesions structures and the actin cytoskeleton in renal cell injury are performed *in vitro*. It is presently unclear whether these findings will correlate with *in vivo* renal dysfunction following injury. The *in vivo* investigation is necessary to fully understand the role of cell adhesion and the actin cytoskeleton in renal injury and regeneration. In addition, although the role of some adhesion proteins in renal cell injury has been established, many of these proteins remain unknown. Identification of these proteins and addressing their role in the renal injury and regeneration process will contribute to a further understanding of renal pathology.

AIM AND OUTLINE OF THIS THESIS

The general aim of the research presented in this thesis is to evaluate the changes in protein expression and phosphorylation that occur in association with changes in cell adhesion and cytoskeletal organization prior to or during renal cell injury and regeneration. To analyze the changes in protein expression and phosphorylation we have used 2D-Difference In Gel Electrophoresis (DIGE) (**chapter 4**) and 2D-phosphotyrosine blotting (**chapter 5 and 6**) respectively. Proteins were identified using mass spectrometric analysis. As a model for renal cell injury we have used an *in vivo* unilateral renal ischemia/reperfusion rat model (**chapter 3**) and renal proximal tubular cells treated with the model nephrotoxicant S-(1,2-dichlorovinyl)-L-cysteine (DCVC) (**chapter 4 and 5**).

The activity and function of many cellular proteins depends on their phosphorylation status. Identification of differentially phosphorylated proteins by means of phospho-proteomic analysis provides insight into the activation of signal transduction pathways, such as those involved in renal cell adhesion and actin organization. **Chapter 2** provides an overview of different phospho-proteomic techniques that are used to identify phosphorylated proteins. **Chapter 3** describes the differential protein tyrosine phosphorylation and F-actin reorganization that occurs during ischemia/reperfusion-induced renal injury and regeneration under *in vivo* conditions. This study focuses on the phosphorylation of the cell-matrix protein focal adhesion kinase (FAK) and its downstream partners. With the 2D-DIGE technique described in **chapter 4** proteins were identified that are differentially expressed prior to DCVC-induced renal cell death. Furthermore, **chapter 4** delineates the role of heat shock protein 27 (Hsp27) phosphorylation in cell adhesion and F-actin reorganization and renal apoptotic cell death. **Chapter 5** describes the identification of proteins that are differentially tyrosine phosphorylated, such as the major differentially phosphorylated protein actin regulatory protein Arp2. These proteins were identified using 2D-phosphotyrosine proteomics. Both progression and regeneration from renal injury may involve cell migration and the so-called epithelial to mesenchymal transition (EMT). In **chapter 6** several phosphotyrosine-proteins involved in the onset of EMT were identified using phospho-proteomics. Moreover, the role of the F-actin binding protein annexin A2 in actin organization and 3D branching morphogenesis is described. **Chapter 7** discusses the results of these studies and their implications for future research.

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