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

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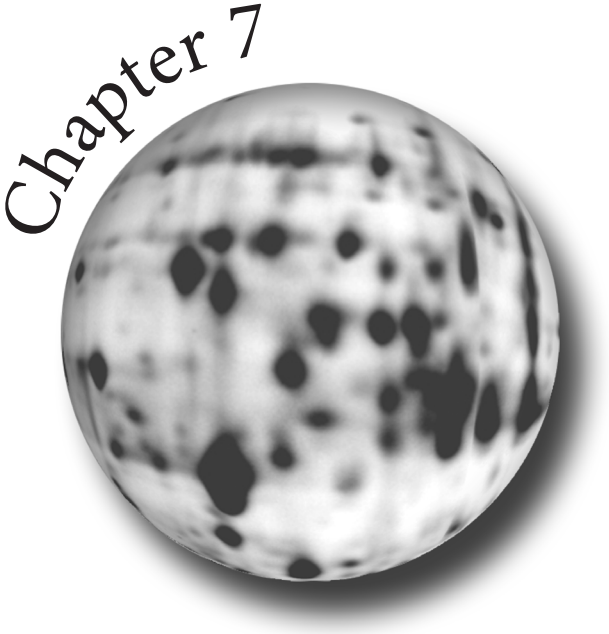
General discussion



GENERAL DISCUSSION

Renal injury can arise in a variety of clinical situations, such as a sudden drop in blood flow as a result of transplantation (ischemia) or exposure to nephrotoxic medicine, such as anti-cancer drugs. Many patients suffering from renal injury develop renal end-stage disease. Despite the increasing knowledge regarding the causes and effects of renal injury, therapy remains generally non-specific and supportive (*e.g.* dialysis) and mortality remains high. The research described in this thesis was designed to identify new signalling pathways involved in renal cell injury and regeneration and understand their role in this process. In this chapter the usefulness of proteomic analysis for the identification of such pathways and the major conclusions concerning the function of the identified proteins are discussed.

Differential protein phosphorylation in renal injury

The proximal epithelial cells are the major target in renal injury. At the cellular level, one of the earliest and most critical events in the pathogenesis of renal injury is disruption of the actin cytoskeletal network in the RPTE cells. This disruption is associated with loss of cell polarity, weakening of cell-cell junctions and cell-ECM adhesion. When severely injured these disturbances result in loss of epithelial barrier function as well as apoptotic cell death, cast formation and tubular obstruction. The disruption of the actin cytoskeleton early after ischemia, or exposure to a nephrotoxicant seems to be a key event in the onset of renal injury as well as injury of other organs that are subjected to ischemia (*e.g* heart, lung, brain). Inhibitors of the Rho kinase pathway attenuate the development of renal damage [1] and inhibitors of the myosin light chain kinase, which stimulates actin-myosin interaction, block ischemic injury in lung [2].

Although a link exists between F-actin reorganization and cell-adhesion loss, research has mainly focused on the effect of IR injury on integrin assembly [3,4]. The changes in downstream signalling pathways remain unclear. To understand the changes in F-actin and cell adhesion proteins in the renal injury and regeneration process we set-up an ischemia-reperfusion model, which allows us to study these changes in detail (**chapter 3**). Focal adhesions (FAs), rich in tyrosine phosphorylation, were present at the basolateral membrane of RPTE cells. These FAs contained FAK, paxillin and talin which co-localized with F-actin stress fibers. Protein tyrosine phosphorylation was lost directly after ischemia, which was associated with reorganization of the FAs and actin cytoskeleton. During reperfusion, phosphorylation increased in conjunction with an increase in FA size and formation of stress fibers. Several known focal adhesion proteins, including FAK were differentially phosphorylated, thereby activating different signalling pathways. The increased phosphorylation that was observed after 24 h of reperfusion correlated with increased FA size and stress fiber formation. The differential phosphorylation of focal adhesion proteins occurring after 30 minutes of ischemia are early events in the ischemic period. In rats subjected to 30 minutes of renal ischemia no cell death or hardly any cell detachment was observed. This indicates that the differential phosphorylation events and reorganization of the actin cytoskeleton and focal adhesions that take place after injury

do not necessarily result in cell death (**chapter 3**). Such early changes correlate with the finding that exfoliated RPTE cells found in urine in both laboratory animals and patients after ARF are still viable. Thus, disturbances in the cell-matrix adhesion complexes after ischemia are involved in cell detachment and renal injury, but uncoupled from cell death [5]. In I/R-induced injury *in vivo* we did not find a clear correlation between differential protein phosphorylation and cell death. However, *in vitro* differential tyrosine phosphorylation was associated with cell death induced by DCVC (**chapter 5**). Using phospho-proteomics we have identified some of these differentially phosphorylated proteins. In addition, we identified differentially expressed proteins in response to DCVC-induced cell death (**chapter 4**). Similar strategies could be used to identify the proteins with a change in tyrosine phosphorylation or expression in response to I/R injury. Thus far proteomic studies using *in vivo* material have focused on constructing maps of kidney cortex and medulla to detect differential protein expression in the different renal segments under normal conditions [6,7]. In addition, biomarker studies have been performed by comparing proteomic profiles of urine obtained from, for example, renal transplant patients with no rejection and those with acute rejection [8-10]. Identification of proteins that are differentially expressed or phosphorylated in kidneys subjected to I/R has not been done before. In a preliminary study using 2D electrophoresis of renal tissue sections from I/R treated animals we could show differential expression of several proteins. The actin-binding protein ezrin was identified as a protein with increased expression after I/R (unpublished observation). Optimization of this technique or related proteomic techniques (**chapter 2**) is required to identify a larger group of proteins involved in I/R injury.

Proteomic analysis of renal cell injury preceding apoptosis

Apoptotic cell death contributes to functional deterioration of organs, while interference with the apoptotic program results in protective effect on renal function [11,12]. The onset of apoptosis is preceded by activation of stress response signalling pathways and impairment of both extra-cellular matrix (cell-ECM, focal adhesions) and cell-cell interactions (adherens junctions) in association with alterations in protein kinase activity. This is generally associated with changes in protein expression and phosphorylation. To increase our understanding of these events in relation to renal cell injury, different proteomic techniques were used in this thesis to detect changes in protein expression and tyrosine phosphorylation profiles between control RPTE cells and cells destined for apoptosis (**chapter 4 and 5**). In the past several years, proteome analysis has been used to study changes in protein expression during apoptosis. This has mainly provided information on late apoptotic events, such as cleavage of proteins by caspases. However, less is know about initial changes in protein expression and modification of proteins prior to caspase activation. In a first study we have used two-dimensional difference gel electrophoresis (2D-DIGE) to determine early changes in the stress response pathways that precede focal adhesion disorganization linked to the onset of apoptosis of renal epithelial cells (**chapter 4**). We identified proteins that were either alternatively expressed or post-translationally modified in a MAPK-dependent manner after treatment of the renal epithelial cell line

LLC-PK1 with the model nephrotoxicant DCVC. Proteins included those involved in metabolism, i.e. aconitase and pyruvate dehydrogenase; and those related to stress responses and cytoskeletal reorganization, ***i.e.*** cofilin, Hsp27 and alphaB-crystallin. In addition to the DCVC-induced activation of MAPK-dependent stress responses we assessed a role for differential tyrosine phosphorylation in DCVC-induced RPTE cell injury (**chapter 5**). DCVC-induced cell detachment and apoptosis was preceded by lamellipodia formation. The DCVC-induced effects were enhanced by the protein tyrosine phosphatase inhibitor vanadate, and almost completely prevented by the protein tyrosine kinase inhibitor genistein. Using 2D phospho-tyrosine proteomics we identified proteins with a change in tyrosine phosphorylation, which included, amongst others, the actin regulatory protein Arp2 and heat-shock protein 60. These studies support previous findings where DCVC causes drastic changes in the organization of the F-actin network [13,14]. In addition, these studies support the importance of the F-actin cytoskeleton in the contribution to renal cell injury.

Stress response pathways, cell adhesion and the cytoskeleton

During renal cell injury stress signalling pathways are activated, which involve the family of MAPKs, including JNK, p38 and ERK. Ischemia/reperfusion activates all three MAPKs [15-17]. *In vitro* as well as *in vivo* studies using either antisense or pharmacological inhibitors, suggest that sustained activation of JNK after RPTE cell injury promotes cell death [18,19]. Moreover, sub-lethal injury that results in pre-conditioning prevents the activation of JNK and p38 after an additional I/R insult [20,21]. Inhibition of p38 with SB203580 in DCVC-exposed RPTE cells resulted in accelerated loss of FAs in association with cell detachment and an increase apoptosis (**chapter 4**). Inhibition of JNK with SP600125, which prevented DCVC-induced cell detachment, also prevented the onset of apoptosis. These data show a relationship between cytoskeletal reorganization, loss of FA and apoptosis, which is mediated by stress kinase activation. In addition, this relationship is mediated through DCVC-induced protein tyrosine phosphorylation since the phosphatase inhibitor vanadate promoted cytoskeletal reorganization, loss of FA and apoptosis, while the kinase inhibitor genistein prevented this (**chapter 5**).The accelerated loss of FA and cell death caused by p38 inhibition is correlated with a decrease in phosphorylation of Hsp27. Overexpression of a phospho-defective mutant resulted in accelerated loss of FA and cell death in response to DCVC. The focal adhesions are involved in cell migration. Phosphorylation of Hsp27 has been shown to enhance cell migration and inhibition of Hsp27 phosphorylation using a small synthetic molecule (KRIBB3) or RNAi-mediated knock-down inhibits cell migration *in vitro* [22] possibly via the role of Hsp27 in focal adhesion assembly/disassembly. This suggests that phosphorylation of Hsp27 may not only protect cell against apoptosis, but may also be involved in the regeneration process after renal injury so that viable cell can migrate into the denuded areas of proximal tubules subjected to injury.

Heat shock protein in renal cell injury

Heat shock proteins (Hsps), or stress proteins, are molecular chaperones responsible for protein processing and protection against cellular injury through the prevention of inappropriate peptide interactions [23]. In addition, these proteins may function by interacting with the cytoskeleton to protect against and assist recovery from cellular injury. Heat shock proteins are often found to be upregulated in the kidney after renal injury [24,25]. In LLC-PK1 cells overexpression of Hsp72 confers resistance against oxidative injury and cisplatin toxicity [26]. Pretreatment of rats with a low-dosis of cyclosporine, which induced expression of Hsp70 prevents subsequent I/R injury [27]. Moreover, hyperthermic preconditioning, which induces strong and long lasting expression of Hsp32, Hsp72, and Hsp90 expression in rat kidneys, resulted in improved renal functions after cold ischemia and renal transplantation [28].

In our 2D-DIGE study we have identified Hsp27, which is a small heat shock protein, as a protein involved in renal cell injury and apoptosis (**chapter 4**). Although Hsp27 has been found to be upregulated in renal injury [29] we did not detect any difference in total cellular level of Hsp27 after DCVC treatment. Instead, changes in the two-dimensional protein profile of Hsp27 were primarily due to increased phosphorylation of Hsp27, which explained a pI shift to the acidic region. Phosphorylation of Hsp27 was mediated by p38 activation since SB203580 inhibited it (**chapter 4**). Inhibition of p38 accelerated the reorganization of focal adhesions after DCVC treatment; this was associated with a more rapid cell detachment and onset of apoptosis. In a similar fashion, stable overexpression of an Hsp27 phosphorylation defective mutant in LLC-PK1 cells also increased the susceptibility toward reorganization of focal adhesions caused by DCVC as well as the induction of apoptosis (**chapter 4**). These data show the importance of Hsp27 phosphorylation in the maintenance of cell adhesion and possibly F-actin organization. In ATP-depleted cells, Hsp27 was associated closely with F-actin at lateral cell boundaries and with aggregated actin within the cell body. Less Hsp27 interaction with actin was found during recovery [30]. Phosphorylation of Hsp27 can modulate actin filament dynamics. Early during stress, phosphorylation-induced conformational changes in the Hsp27 oligomers regulate the activity of the protein at the level of microfilament dynamics, resulting in both enhanced stability and accelerated recovery of the filaments [31]. In addition, protein phosphatase inhibitors and heat preconditioning prevent Hsp27 dephosphorylation, F-actin disruption and deterioration of morphology in ATP-depleted cells [32]. Our data show that DCVC-induced early p38 activation results in a rapid phosphorylation of Hsp27, which is a requirement for proper maintenance of cell adhesion, thus suppressing renal epithelial cell apoptosis. Given the reorganisation of the cytoskeleton and focal adhesions during I/R injury *in vivo* (**chapter 3**) and the effect of phospho-Hsp27 on DCVC-induced cytoskeletal and focal adhesion reorganization (**chapter 4**), there may well be an important role for the phosphorylation of Hsp27 *in vivo*. Thus far such an involvement has not been established.

Cofilin/ADF and the Arp2/3 complex in renal injury

In addition to Hsp27, we found differential expression of the actin regulatory proteins cofilin and b-crystallin (**chapter 4**) and differential phosphorylation of the actin regulatory protein Arp2 (**chapter 5**) in response to DCVC-induced cell injury. Although we did not yet determine the role of these proteins in DCVC-induced injury, others have shown an involvement of these proteins in renal cell injury and actin reorganisation, suggestion a possible role for cofilin and the Arp2/3 complex in DCVC-induced renal injury. The breakdown of apical F-actin containing microvilli of the proximal tubule cell is an early-occurring hallmark of ischemic acute renal failure [33] since the high turn-over rate of actin polymerization in these microvilli renders the actin cytoskeleton highly susceptible to ischemic injury. The actin-binding protein ADF/cofilin plays a critical role in the apical microvilli breakdown in response to ischemia. Ischemia-induced cell injury resulted in dephosphorylation and activation of this actin-binding protein [34]. Prior to ischemia-induced microvillar destruction ADF/cofilin was activated and relocalized to the apical domain [35]. In addition, ADF/cofilin mediates actin cytoskeletal alterations in LLC-PK cells during ATP depletion [36]. Cofilin was dephosphorylated and thereby activated in response to ATP-depletion resulting in actin depolymerization and cell injury. These studies suggest that DCVC-induced cytoskeletal reorganization could be associated with activation/dephosphorylation of cofilin and expression of an inactive, phospho-mimicking (S3E) mutant of cofilin may render DCVC-exposed RPTE cells less susceptible to F-actin reorganization and cell death.

In general, actin dynamics is controlled by the Arp2/3 complex, the Wiskott-Aldrich syndrome protein (WASp), the capping protein, profilin, and the actin-depolymerizing factor ADF/cofilin [37]. These proteins co-operate in the assembly of branched actin filament networks. For example, protrusion of the lamellipod relies upon the combined actions of the Arp2/3 complex and cofilin for generation of filamentous actin [38]. Using 2D phospho-tyrosine blotting we identified Arp2 as the major differentially phosphorylated protein in response to DCVC-induced cell injury (**chapter 5**). Arp2 was located in the lamellipodia that were formed prior to the onset of apoptosis. The formation of dynamic lamellipodia and Arp2 phosphorylation were under direct control of tyrosine kinases, since both were blocked by inhibition of tyrosine kinases. Although nothing is known on the tyrosine phosphorylation of Arp2, the protein itself has been shown to be involved in apoptosis. Together with dephosphorylated/activated cofilin, the Arp2/3 complex co-localises in apoptotic blebs during etoposide-induced apoptotic cell death [39]. In addition, the activation of the Arp2/3 complex is under tight control of the actin-binding protein cortactin [40]. In turn the activity of cortactin is regulated by several protein tyrosine kinases, among which the tyrosine kinase Src [41]. We propose a possible role for tyrosine phosphorylation of the Arp2/3 complex in the regulation of F-actin reorganization either alone or together with the actin-binding protein cofilin that precedes the onset of renal cell apoptosis caused by nephrotoxicants.

As alluded to above, the structure and function of the actin cytoskeleton is rapidly altered by exposure to various toxicants or in response to ischemia. We have shown that

2D-DIGE and phospho-tyrosine blotting can be used to identify specific patterns of protein expression and/or post-translational modifications in relation to renal cell injury (**Fig. 1**). The protein identifications in these studies point to the more common observation of alterations in the cytoskeletal structure that take place during the early stages of exposure to toxic chemicals or in pathological stages, such as ischemia [42].

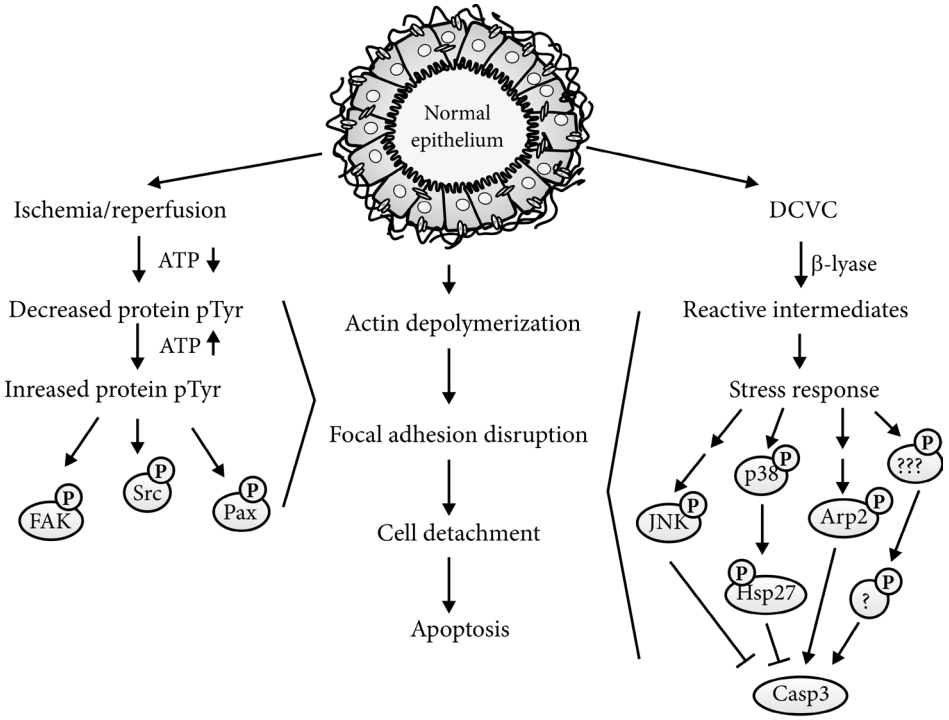


Figure 1. *Overview of differentially phosphorylated or expressed proteins in renal cell injury.* The identified proteins and possible signalling pathways are translated to the kidney *in vivo*. Different pathways are activated after exposure of renal cell to DCVC or ischemia/reperfusion, which is associated with F-actin reorganization, focal adhesion dissociation and the onset of apoptosis cell death.

Proteomic analysis of the EMT process

The epithelial-to-mesenchymal transition plays an important role in the progression of renal injury and is thought to stimulate renal regeneration [43,44]. Thus far, direct evidence for the involvement of EMT in renal regeneration is lacking. Tubular epithelial cells can acquire a mensenchymal phenotype and enhanced migratory capacity, which enables them to transit from the renal tubular microenvironment into the interstitial space. Here the cells can escape cell death and either contribute to the progression of renal fibrosis or serve as a pool of viable cells to regenerate the kidney. The EMT process is characterized by loss of cell polarity, F-actin reorganization and disappearance of cell-cell interactions

and is controlled by receptor tyrosine kinases, *i.e.* c-Met, that in turn activate downstream tyrosine kinases such as Src family members. As a result, changes occur in the tyrosine phosphorylation status of cytoskeletal and cell adhesion proteins. Since therapeutic options to inhibit progression of chronic renal disease are still not available in the clinic, modulation of the EMT process offers a novel therapeutic target to potentially inhibit renal fibrosis or even stimulate regeneration. To increase our understanding of the regulatory pathways that are involved in the EMT process we have identified proteins that are differentially tyrosine phosphorylated prior to the loss of cell-cell interaction and the onset of cell scattering and EMT (**chapter 6**).

Phosphorylation of annexin A2 in renal fibrosis

The major differentially phosphorylated protein was annexin A2, which is known to be phosphorylated on Tyr23 by Src kinase [45]. Expression of a phospho-mimicking mutant of AnxA2 resulted in cell scattering and 3D branching morphogenesis independent of the scatter factor HGF (**chapter 6**). The induction of cell scattering by phosphorylation of AnxA2 is regulated via activation of the actin binding protein cofilin. In cell expressing Y23E-AnxA2, cofilin was dephosphorylated when cell obtained a scattered morphology. This scattering was inhibited by co-expression of a phospho-mimicking mutant of cofilin (S3E). In the kidney, AnxA2 is expressed in the renal cortex [46]. In response to I/R injury the expression of AnxA2 is increased during the reperfusion phase [47]. Moreover, activation of Src kinase increased during the reperfusion phase (**chapter 3**). In addition, I/R-induced cell injury is associated with dephosphorylation and activation of cofilin [34] resulting in microvilli breakdown. Together this suggests that phospho-AnxA2 may contribute to renal cell injury early after an ischemic period via dysregulation of the actin cytoskeletal network in the microvilli or may contribute to renal fibrosis since it enhances EMT and migration (**chapter 6**). The Y23E-AnxA2 mutant itself promoted tubulogenesis formation, suggesting that phosphorylation of AnxA2 may promote renal regeneration. However, during I/R injury and regeneration many growth factors are released, among which HGF. Combining Y23E-AnxA2 and HGF resulted in completely disorganized 3D structures, suggesting that phospho-AnxA2 *in vivo* will not stimulate regeneration, but rather enhance renal fibrosis (**Fig. 2**) (**chapter 3**).

Extrinsic regulators of EMT, a possible role for AnxA2

EMT in the kidney is regulated by numerous growth factors, such as the EMT promoters FGF, EGF and TGFβ and the EMT suppressors like BMP7 and HGF [48]. TGFβ has been identified as the main inducer of EMT in the kidney and in other organs. Cultured cells exposed to EGF or FGF exhibit only a mild EMT transition, whereas EGF and FGF have a dramatic synergistic affect when combined with TGFβ [49,50]. TGFβ-induced EMT appears to be primarily dependent on intact Smad signalling, since overexpression of inhibitory Smad-7 abolishes Smad-2 phosphorylation and tubular cell phenotypic conversion [51]. Besides Smad signalling, TGFβ is capable of activating several other signal transduction pathways in tubular epithelial cells, including the p38/MAPK pathway, PI3 kinase/PKB

pathway, the Rho-GTPase RhoA and β-catenin [52-55]. The TGFβ-induced EMT is negatively modulated by the endogenous regulators HGF and BMP7. Both growth factors block the phenotypic conversion of tubular epithelial cells induced by TGFβ [56,57].

Preliminary data of RNAi-mediated AnxA2 knockdown cells exposed to TGFβ indicate that AnxA2 is involved in the TGFβ-induced cell scattering pathway, since these RNAi cells did not undergo an EMT in response to TGFβ. Although EMT is an important process in embryogenesis and AnxA2 is involved in EMT, AnxA2 null mice develop normal [58]. Given the possible involvement of AnxA2 in TGFβ-mediated EMT and the role of TGFβ in renal fibrosis, AnxA2 null mice, which have no expression of AnxA2 in the kidney may be less susceptible to I/R-induced injury. The mechanism underlying this inhibition remains to be investigated. However, AnxA2 is involved in receptor recycling via regulation of endocytosis and exocytosis [59,60], suggesting a possible involvement of AnxA2 in TGFβ (receptor) internalization as well.

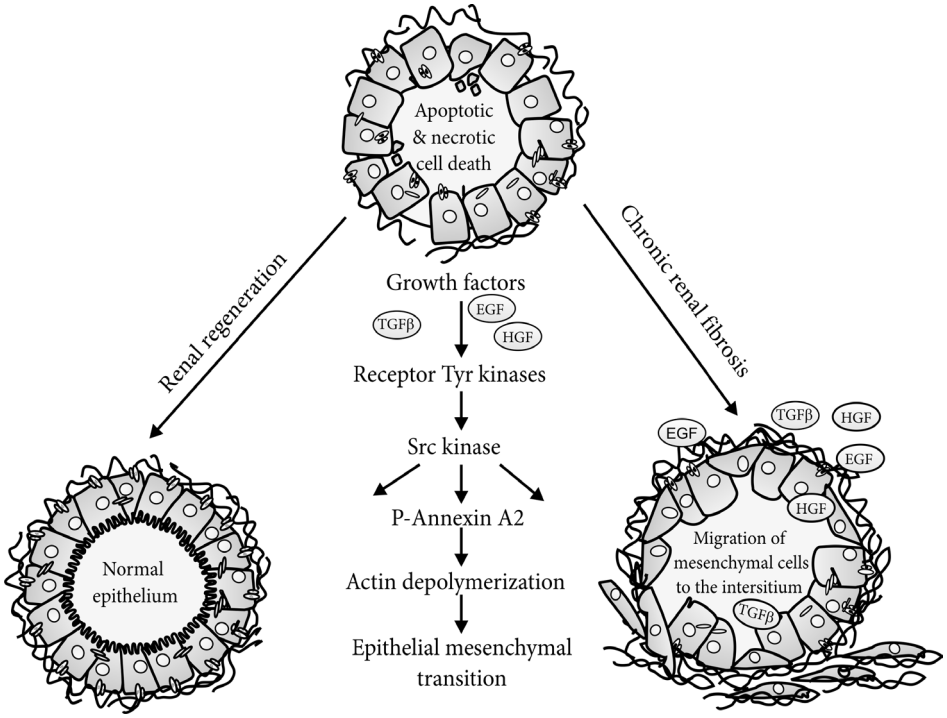


Figure 2. *Possible contribution of AnxA2 phosphorylation in renal regeneration or progression.* Annexin A2 tyrosine phosphorylation is involved in EMT-related processes, which can contribute to renal regeneration or the progression or renal injury.

Conclusions

In this thesis we have identified proteins that are differentially expressed and/or phosphorylated during the different stages of renal cell injury and regeneration. The identified proteins and possible signalling pathways are translated to the kidney *in vivo* and

summarized in **figure 1** and **2**. Renal injury can be caused by either ischemia/reperfusion or exposure to nephro-toxicants such as DCVC. DCVC is metabolized to reactive metabolites by the β-lyase enzyme. This initiates the onset of stress response pathways such as the p38/Hsp27 and JNK pathway and allows tyrosine phosphorylation of proteins such as the actin-binding protein Arp2. Activation of these pathways is associated with F-actin reorganization, such as formation of stress fibers and lamellipodia and may result in the onset of apoptosis. Ischemia results in ATP depletion, which is associated with a drop in tyrosine phosphorylation and reorganization of the F-actin cytoskeletal network. ATP repletion because of blood flow restoration results in a bi-phasic increase in tyrosine phosphorylation of focal adhesion proteins like FAK, Src and Paxillin and is associated with F-actin stress fiber formation and increased focal adhesion size (**Fig. 1**). Depending on the degree of renal injury, the kidney regenerates or is destined for chronic renal injury like fibrosis. Both processes can be regulated by the epithelial-to-mesenchymal transition, which involves activation of receptor tyrosine kinases with downstream activation of Src kinase. The Src kinase phosphorylates multiple proteins on Tyr residues, among which AnxA2 resulting in EMT (**Fig. 2**).

Altogether, the protein identifications in these studies point to the more common observation of alterations in the cytoskeletal structure that take place during renal cell injury and regeneration. Assessing a role for these proteins in renal injury will increase our insight into the molecular and cellular understanding of renal cell injury and EMT processes in relation to the progression of chronic kidney disease.

Future perspectives

With regard to our results on mechanisms of DCVC-induced apoptosis (**chapter 4 and 5**) future research should resolve whether the phosphorylation of Arp2 is indeed involved in DCVC-induced apoptosis or other processes involving F-actin reoganization. This can for example be investigated in a similar way as described for the AnxA2 phospho-mimicking studies (**chapter 6**). As described above, the identification of the proteins described in this thesis is associated with the changes in F-actin organization that take place in response to renal cell injury and regeneration. To further increase our understanding of cytoskeletal reorganization and differential phosphorylation in relation to cytotoxicity and cell death, screens should be developed to identify large groups of differentially phosphorylated proteins in cells treated with different toxicants or under pathological circumstances, such as ischemia. The 2D gel electrophoresis techniques described in this thesis are much too time-consuming for this purpose, but many high throughput techniques have been developed (as described in **chapter 2**), such as LC/MS in conjunctions with phospho-enrichment. These techniques would also make it possible to identify the proteins that are differentially phosphorylated in response to I/R injury (**chapter 3**). Alternatively, RNAi-mediated knockdown approaches could be used to specifically knockdown kinases to study their involvement in the reorganization of F-actin and cell adhesion that takes place prior to renal cell death. This can also be done in a high-throughput assay using RNAi kinase libraries in conjunction with multi-well microscopy tecchnologies.

With regard to the findings for phosphorylation of annexin A2 (**chapter 6**), its role in F-actin reorganization should be further investigated. How does AnxA2 change the actin polymerization process? In addition, the role of AnxA2, but possibly also its close family member AnxA1, in receptor internalization should be further studied. Thus far, the possible role AnxA2 in renal injury has mainly been studied using different cell culture systems. The AnxA2 null mice would be a nice *in vivo* model to study the role of AnxA2 in renal ischemia/reperfusion or in toxicant-induced renal injury.

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