Defining the Acute Kidney Injury and Repair Transcriptome

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Summary: The mammalian kidney has an intrinsic ability to repair after significant injury. However, this process is inefficient: patients are at high risk for the loss of kidney function in later life. No therapy exists to treat established acute kidney injury (AKI) per se: strategies to promote endogenous repair processes and retard associated fibrosis are a high priority. Whole-organ gene expression profiling has been used to identify repair responses initiated with AKI, and factors that may promote the transition from AKI to chronic kidney disease. Transcriptional profiling has shown molecular markers and potential regulatory pathways of renal repair. Activation of a few key developmental pathways has been reported during repair. Whether these are comparable networks with similar target genes with those in earlier nephrogenesis remains unclear. Altered microRNA profiles, persistent tubular injury responses, and distinct late inflammatory responses highlight continuing kidney pathology. Additional insights into injury and repair processes will be gained by study of the repair transcriptome and cell-specific translatome using high-resolution technologies such as RNA sequencing and translational profiling tailored to specific cellular compartments within the kidney. An enhanced understanding holds promise for both the identification of novel therapeutic targets and biomarker-based evaluation of the damage-repair process.

Keywords: Acute kidney injury, repair, transcriptome, TRAP, miRNA, development, cancer

The clinical syndrome of acute kidney injury (AKI) is characterized by an abrupt (within 48 h) decrease in kidney function, frequently caused by ischemia reperfusion injury (IRI), sepsis, or nephrotoxic insult.1-3 Despite advances in medical care, patients with AKI continue to have high morbidity and mortality; in-hospital mortality rates in critically ill patients with AKI approach 50% to 70%.3,4 Furthermore, survivors also have a strikingly higher risk of developing chronic kidney disease (pooled adjusted hazard ratio, 8.8; 95% confidence interval, 3.1-25.5), and end-stage renal disease (pooled adjusted hazard ratio, 3.1; 95% confidence interval, 1.9-5.0) compared with non-AKI patient groups.5

The histologic features of human ischemic AKI include loss of the brush border typical of the proximal tubular epithelium, sloughing of tubular epithelial cells into the lumen resulting in focal loss of tubular epithelial cells, infiltration of inflammatory cells, and the appearance of Tamm-Horsfall protein-rich casts in the urine.6 After AKI, a repair process restores renal tubular epithelium and kidney function. The cellular mechanisms of repair have been scrutinized intensively using mouse genetic approaches. Agreement is increasing that surviving cells within the renal tubular epithelium repair tubular damage in the mouse, and likely the human kidney (see article by Marcus Moeller in this issue). Whether repair is a general capacity shared by surviving cells, or a more specific function ascribed to a small subset of identifiable epithelial cells, has engendered considerable debate (see article by Marcus Moeller in this issue). It is clear that the reparative process is not as efficient or effective as desired: fibrosis is evident despite the reacquisition of biochemical parameters such as plasma creatinine removal, and progression to chronic kidney disease is a frequent long-term outcome.5

Fibrosis is associated with injury-invoked appearance of α-smooth muscle actin–positive myofibroblasts. In fibrosis, Yang et al7 suggested G2/M-arrested proximal tubular cells activate c-jun NH2-terminal kinase signaling, initiating production of profibrotic cytokines. In fibroblasts, hypermethylation of RAS protein activator like 1, an inhibitor of the Ras oncprotein, leads to prolonged fibroblast activation and fibrogenesis.8 Once triggered, myofibroblasts synthesize a distinct collagen I–rich extracellular matrix that may promote further fibrosis.

Initial suggestions that most fibrotic cells arise from an epithelial-to-mesenchymal conversion of renal tubule cells have been challenged; a revised view of an extratubular origin for myofibroblasts is supported by several fate-mapping studies. One view holds that...
perivascular fibroblasts (pericytes) are the chief culprit, whereas another associates fibrosis resident non-pericyte intertubular fibroblasts and bone marrow–derived fibroblasts. The origins of injury-associated myofibroblasts are discussed in article by Benjamin Humphreys.

Harnessing and enhancing the kidney’s intrinsic mechanisms of repair, and developing approaches to suppress and reverse renal fibrosis, are major goals of renal regenerative medicine. These strategies are founded, and dependent, on our detailed knowledge of the molecular and cellular events at play. New approaches to interrogate underlying mechanisms have enhanced resolution at the molecular level by enabling systematic, relatively unbiased, quantitative measurement of transcriptional and translational events. Further, the move from whole-organ analysis to a breakdown of responses in specific cellular compartments is increasing cellular resolution. These advances will facilitate the identification of new targets augmenting renal repair processes and suppress renal scarring.

Here, we provide a brief overview of the cellular responses initiated by AKI, with a particular focus on the repair processes after ischemic AKI, review studies that have performed whole-kidney or cell-specific gene/transcript expression analysis temporally in the setting of murine and human AKI, and discuss the role of next-generation RNA-sequencing (RNA-seq) and translating ribosome affinity purification (TRAP) profiling in transcriptional and translational analyses, respectively, of the renal repair process.

**BRIEF OVERVIEW OF CELLULAR RESPONSES AFTER ISCHEMIC AKI**

**Injury and Repair of Nephron**

**Renal Tubule Damage**

The proximal tubule is divided into three molecularly, histologically, and topographically distinct segments: S1, S2, and S3. The S3 segment, although highly developed in rodents, is not as pronounced in human beings. The epithelial cells in the straight S3 segment of the rodent proximal tubules located in the outer stripe of the outer medulla are exquisitely sensitive to ischemic insults. Histologically, the ischemic injury is readily discernible in this stripe in animal models of ischemic AKI induced by clamping of the renal pedicle. The S1 and S2 segments of the proximal tubule also respond to injury but the S3 segment shows the most marked cell loss after AKI in the mouse kidney. Although the medullary thick ascending limb (TAL) of the loop of Henle also resides in the outer medullary region, the TAL is relatively resistant to IRI. However, an AKI-like phenotype can be induced experimentally by targeting apoptosis specifically within the TAL. IRI regimens that effectively target the S3 segment of the proximal tubule (PT) have little effect on cells of the TAL. The differential sensitivities of adjacent tubular epithelial cell types may reflect a distinct ability of TAL cells to switch from oxidative to glycolytic metabolism, to mount anti-apoptotic response (activating extracellular signal-related kinase and BCI-2 proteins), and increased expression of insulin-like growth factor-1 (IGF-1) and hepatocyte growth factor (HGF).

Both proximal and distal tubules undergo cell death in human AKI although biopsy specimens of renal allografts show significantly greater apoptosis in distal tubules whereas proximal tubular epithelial cells show more marked proliferation. Focal areas of tubular epithelial cell loss in the TAL and proximal tubular S3 segment have been reported in patients with ischemic acute tubular necrosis.

Ischemia-induced renal tubular adenosine triphosphate depletion is likely an initiating insult in rodent IRI-associated AKI. Critical alterations in tubular dynamics, metabolism, and structure ultimately lead to necrotic and/or apoptotic cell death. These include depletion of cellular energy stores, loss of basolateral distribution of Na⁺K⁺-adenosine triphosphatase and β-integrins (loss of polarity), disruption of the actin cytoskeleton and adherent and tight-junctions (shedding of brush border and sloughing of cells), accumulation of intracellular calcium, accumulation of hypoxanthine, and generation of reactive oxygen species.

**Renal Tubule Repair**

Damaged renal tubular epithelium may be repaired by surviving epithelial cells, other cell types resident within the kidney, or cells that move into the injured organ. Only direct experimental analysis can distinguish among these possibilities; consequently, the most robust conclusions are founded on fate-mapping strategies using mouse genetics. By using approaches that label renal tubule cells exclusively, Humphreys et al argued that repair by surviving cells within the proximal tubule epithelium is a broad mechanism. Further analysis of clone size and differentiation markers suggests that repair in S1/S2 segments is not mediated by a rare stem cell but is general property of differentiated proximal tubule epithelial cells activated on injury. A contrasting view argues for repair from a small subset of CD24+, CD133+ cells that reside within human renal tubules.

**Non-Nephron Components of Injury and Repair**

**Macrophage, Leukocytes, and Neutrophils**

One of the earliest cellular responses to renal damage, seen within the first few hours after the triggering stimulus, is neutrophil and macrophage infiltration;
key aspects of the engagement of an innate immune response. Early monocyte/macrophage trafficking is facilitated by chemokine (C-C motif) ligand 2 (CCL2) (monocyte chemoattractant protein 1)/chemokine (C-C motif) receptor 2 (CCR2) and chemokine (C-X3-C motif) ligand 1 (CX3CL1)/chemokine (C-X3-C motif) receptor 1 (CX3CR1) chemokine signaling pathways. Macrophage infiltration peaks at 24 hours and persists for at least 7 days. This component of proinflammatory macrophages (M1) infiltrating the inflamed kidney is distinct from resident macrophages and dendritic cells.

As a consequence of IRI-triggered changes in the environment, macrophages may transition from an initial proinflammatory state to acquire anti-inflammatory, pro-reparative properties. By 24 hours after injury initiation in the mouse kidney, macrophages expressed high levels of inducible nitric oxide synthase (a marker of proinflammatory M1 macrophages), and low levels of arginase-1 (a marker of M2 macrophages or alternatively activated macrophages). Subsequently, over the ensuing 6 days, flow-sorted macrophages showed increasing levels of arginase-1 paralleled by decreasing levels of inducible nitric oxide synthase, indicative of an M1 to M2 transition within this population. Liposomal clodronate-mediated depletion of M2 macrophages negatively impacts repair assessed at day 5 and day 7 after injury initiation, suggesting that the transition to M2 macrophages is beneficial to the repair process.

How these macrophages augment repair is unclear although production and secretion of a pro-reparative Wnt ligand (Wnt7b) (Wingless-related integration site motif) ligand 1 (CX3CL1)/chemokine (C-X3-C motif) receptor 2 (CCR2) and chemokine (C-X3-C motif) ligand 1 (CX3CL1) chemokine signaling pathways. Macrophage infiltration peaks at 24 hours and persists for at least 7 days. This component of proinflammatory macrophages (M1) infiltrating the inflamed kidney is distinct from resident macrophages and dendritic cells.

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**Vascular Cells**
The circulatory system is not only the source of oxygen for the tubular nephron but the conduit for ingressing inflammatory cells; entry is enabled by the up-regulation of adhesion molecules and selectins, on the surface of endothelial cells. Ischemia-reperfusion injury induced alterations in renal microcirculation are thought to compromise endothelial function. The capillaries in the outer medulla are uniquely susceptible to ischemic insults because of various factors including disproportionately reduced blood flow in the outer medulla compared with total blood perfusion. The capillary plexus of the outer strip is relatively sparse, supplied from the small side branches that rise exclusively from the efferent arterioles of the juxtamedullary glomeruli.

Capillary rarefaction, a reduction in the number of arterioles and capillaries, is observed on IRI. A compromised microvascular density may exacerbate the initial hypoxia and potentially contribute to the progressive development of interstitial fibrosis as in the fibrotic scarring observed in Alzheimer's disease. According to the chronic hypoxia hypothesis, capillary rarefaction is an important factor driving a final common pathway to end-stage renal disease. The development of salt-sensitive hypertension and impaired urinary concentrating ability are other functional consequences of vessel drop-out.

**IMMEDIATE AND EARLY MOLECULAR RESPONSES TO AKI**

**Immediate-Early Damage Responses (Up to 4 Hours After Injury)**

Both proximal and distal tubular epithelial cells mount an acute transcriptional response to IRI. The earliest genes to be induced after in vivo injury (within 4 h after injury) the immediate-early genes include *Fos, Jun*, and *Egr1*. *Fos* is induced predominantly in the TAL. The latter observation suggests that the distal tubule, in addition to the proximal tubule, also senses the acute insult. Subsequent microarray-based gene expression profiling studies encountered a similar immediate-early response, including *Fos* and *Egr1*, after renal IRI. An oxidative stress–induced increase in the intracellular calcium ion concentration is one possible explanation for this hyperacute response. Interestingly, in vitro, rat proximal tubular epithelial cells up-regulate *Fos* and *Jun* messenger RNA (mRNA) within 15 minutes of oxidative stress, attaining peak responses within 30 minutes, and returning to basal levels within 3 hours. The precise biological role of this immediate response, and the interplay with ensuing transcriptional responses, is not well understood.

**Early Damage Responses (4 to 24 Hours After Injury)**

A number of molecular approaches, including a representational difference analysis of complementary DNA (cDNA), were used in the late 1990s and early 2000s to examine AKI responses at the molecular level, identifying two prominent injury indicators: *Haver1* (also known as the kidney injury molecule-1 [Kim-1]) and *Lcn2* (also known as the neutrophil gelatinase-associated lipocalin2 [NGAL]). A variety of rat and mouse models of AKI have sought common molecular themes and additional earlier biomarkers of injury responses. Collectively, these studies have contributed significantly to our understanding of the acute responses after kidney injury, and identified frontline biomarker candidates, several of which are undergoing clinical scrutiny for diagnostic efficacy.
These profiling studies show shared early robust molecular responses after AKI, irrespective of the underlying insult.\textsuperscript{36,37,41,42} These include hemeoxygenase-1 (\textit{Ho1}), Lcn2 (NGAL), Havcr1 (Kim1), annexin A2 (Anxa2), clustatin, and interleukin 6 (IL6). Induction of HO-1 and NGAL reflect the organ’s response to mitigate toxic effects of intracellular heme and free catalytic iron, respectively, resulting from renal insult including IRI. Heme oxygenase converts the pro-oxidant, proinflammatory, and pro-apoptotic heme to biliverdin, a reaction that produces cytoprotective molecules and endogenous toxic heme.\textsuperscript{44}

NGAL also is induced in renal tubules, providing a reservoir for excess iron.\textsuperscript{45} Iron in its free catalytic form is a mediator of renal IRI, triggering the induction of toxic reactive oxygen species generation.\textsuperscript{46} NGAL: siderophore:Fe protects against ischemic IRI via up-regulation of hemeoxygenase-1.\textsuperscript{47}

Increased KIM-1 after renal IRI facilitates the clearance of dead cells, conferring endocytic and phagocytic phenotypes on epithelial cells with resultant internalization of lipoproteins and epithelial cells.\textsuperscript{48} IRI leads to increased intracellular calcium and induces Annexin a2.\textsuperscript{49} Annexins are known to bind phospholipids in a Ca\textsuperscript{2+}-dependent manner, and participates in various membrane-related events such as exocytosis, endocytosis, apoptosis, and binding to cytoskeletal proteins.\textsuperscript{50} Although the precise biological role of annexin a2 in AKI has not been determined, its actions may contribute to inflammation by increasing IL-6 production, akin to its role in lupus nephritis.\textsuperscript{51} IL-6 is a key proinflammatory cytokine up-regulated in both ischemic and toxic models of AKI. IL-6 is likely a critical driver of renal and extrarenal inflammatory responses after injury.\textsuperscript{52} Proximal tubule injury activates macrophage-mediated production of IL-6, particularly within the outer medullary region.\textsuperscript{53}

Apoptosis is clearly one cellular mechanism, identified by a cDNA microarray-based gene expression profiling study, underlying early loss of renal tubule cells on ischemic IRI.\textsuperscript{56} Several pro-apoptotic genes are up-regulated within the first 24 hours of IRI: members of the extrinsic death receptor pathway (\textit{Fadd} and \textit{Daxx}) and the intrinsic mitochondrial apoptotic pathway (\textit{Bad} and \textit{Bak}), and the anti-apoptotic gene, \textit{Bcl2}. Renal IRI was attenuated significantly in Bcl-2 transgenic mice with pre-activation of Bcl2,\textsuperscript{54} and in BH3 interacting domain death agonist (Bid)-deficient mice.\textsuperscript{55} In contrast, loss of Bax inhibitor-1 enhanced injury.\textsuperscript{56}

The striking down-regulation of the majority of the genes involved in the mitochondrial metabolism machinery at 24 hours after IRI is a common theme among the significantly down-regulated gene sets in the aforementioned studies. The biological and functional consequences of such a response is poorly understood. Proximal tubular cells are highly enriched in mitochondria and one possible explanation is that it reflects the proximal tubular cells attempt to attain a more protective hypometabolic state.

Reactivation of developmental genes during tubule repair/regeneration after AKI is a more widely believed paradigm than the data to support this view. Comparative cDNA microarray profiling between early and late stages of nephrogenesis and adult mouse kidneys at 3, 12, and 24 hours after IRI has identified some correlated changes in genes encoding transcriptional components (\textit{Nmyc1} and \textit{Wt1}), and growth factors (\textit{Gdnf} and \textit{MdK}).\textsuperscript{57} Two key developmental pathways regulating nephrogenesis, the Wnt/\beta-catenin and Notch signaling pathways, are reported to be activated after AKI.\textsuperscript{28,58} Re-expression of \textit{Pax2}, a key transcriptional regulator in nephrogenesis, in injured tubular epithelial cells has been suggested to reflect a dedifferentiation of tubular epithelial cell.\textsuperscript{59}

However, it remains unclear whether re-expression of these IRI-activated genes reflects a similar regulatory action to their normal role in ontogeny of the kidney. Furthermore, they raise the interesting question as to what extent tubular epithelial cells truly dedifferentiate. Direct unbiased analysis of relevant cells using new approaches for genome-wide discovery likely will provide some clarity. Recent reports that human embryonic stem cells and induced pluripotent stem cells can be coaxed in vitro into nephrogenic programs opens the door to comparing human nephrogenesis with adult repair programs.\textsuperscript{60-62}

Although the analysis of organ-wide injury responses provides broad information, the kidney is a complex organ; in all likelihood, there is much to be learned from a closer examination of individual cell populations. Moreover, acute responses mediated by relatively rare, but biologically significant, cell populations will be lost among the responses of more abundant cellular compartments.

Fluorescent-activated cell sorting (FACS) cells is one approach to increase cellular resolution, although the necessary processes of cell isolation can trigger injury responses, exaggerating data variability and diminishing reproducibility.\textsuperscript{53} A new approach, TRAP, provides a useful alternative strategy.\textsuperscript{63,64} TRAP has the additional advantage of focusing on the actively translated mRNA population at any stage; importantly, general mRNA profiling does not always predict changes in protein abundance, indicating mechanisms favoring translation of specific mRNAs.\textsuperscript{57}

Recently, a generalized TRAP approach was developed and applied to the mouse kidney to obtain cell-specific molecular signatures in an IRI-invoked AKI model.\textsuperscript{66} Here, TRAP relies on affinity purification of translating ribosomes through an enhanced green fluorescent protein (eGFP)-tagged, L10a ribosomal protein subunit (L10a::eGFP), and subsequent profiling
of mRNAs stripped from the ribosome by microarray or RNA-seq. Cell type specificity is governed by the requirement for CRE-recombinase-mediated removal of a transcription-blocking cassette upstream of an L10a::eGFP cDNA cassette: cell type-specific CRE lines activate L10a::eGFP in distinct cell populations in the kidney (Fig. 1). In a recent study, distinct CRE lines enabled TRAP mRNA signatures to be generated for four critical cellular compartments in the kidney after IRI injury: the nephron, vascular, macrophage/monocyte, and interstitial mesenchyme (Fig. 2).

Intersection of TRAP data, with gene lists from various whole-organ gene expression profiling studies performed within 24 hours of AKI, show general responses, mounted by all four major cellular compartments, and cell-restricted responses were defined as responses noted in three, or fewer than three, cellular compartments (Tables 1 and 2, respectively).

**Figure 1.** TRAP RNA-seq work flow. pA = SV40 poly A; CAGGS = hybrid promoter composed of Cytomegalovirus early enhancer fused to hicken β-actin promoter; DAVID = Database for Annotation, Visualization and Integrated Discovery. (see Liu et al for full details regarding approach).
A number of common molecular responses provide diagnostic evidence of AKI themes independent of the AKI trigger. Approximately 20% of differentially expressed genes are shared in different models of AKI: Table 1 summarizes the shared responses at 24 hours. These include Lcn2, Ho1, Sphk1, p21, Cd44, Anxa2, Anxa3, Fosl1, and Clu.

Specific models also highlighted several molecular responses of interest that were not common to all AKI studies (Table 2). Such responses can be grouped further according to their site of activation, such as Adm, Myc, Tpm4, and Tnfrsf12a (nephron and interstitium/pericyte and endothelium); Anxa1, Cldn7, and Haver1 (nephron and interstitium/pericyte and myeloid-lineage cells); Fos (endothelium and myeloid lineage cells); Hspa1a and Tubb5 (nephron and interstitium/pericytes); Fgb and Gdf15 (only nephron); Cxcl1, Il6, and Tagln (only interstitial/pericyte); Dnajb9 and intercellular adhesion molecule-1 (only endothelium); and Vcam1 (only myeloid-lineage cells). TRAP unraveled large sets of unique responses in the vascular endothelium and interstitial/pericyte populations, in addition to the expected responses of genes associated with their respective compartments (Table 2).

A striking IRI feature of the TRAP Cdh5-L10a vascular endothelium compartment is evidence of pathway regulation for CXCR4, endothelin-1, Toll-like receptor, IL-8, thrombopoietin, and Janus kinase-STAT (JAK-STAT) signaling. These data highlight vasculature-associated molecular pathways not readily discernible in

<table>
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<tr>
<th>Studies</th>
<th>Gene Symbol</th>
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<th>AKI Models</th>
</tr>
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<tbody>
<tr>
<td>Yoshida et al42, Yuen et al37</td>
<td>Akap12</td>
<td>A kinase (regulatory subunit of protein kinase A) anchor protein (gravin) 12</td>
<td>IRI</td>
</tr>
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<td>Yoshida et al42, Yuen et al37</td>
<td>Anxa2</td>
<td>Annexin A2</td>
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<td>CD44 antigen</td>
<td>Cisplatin, IRI</td>
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<td>Cdkn1a</td>
<td>Cyclin-dependent kinase inhibitor 1A (P21)</td>
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<td>Early growth response 1</td>
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<td>Cludn7</td>
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a microarray-based analysis of total kidney RNA samples. Similarly, specific responses from infiltrating cells of the myeloid lineage (Lyz2-L10a) readily are shown by TRAP. These included IL-6, peroxisome proliferator-activated receptor, glucocorticoid, IL-17A, and extracellular signal-related kinase 5 signaling. Furthermore, the data identify chemokine receptors (Ccr1 and Cxcr2) and growth factors (Csf1) linked to IRI. The glutamate-leucine-arginine (ELR) motif containing CXC chemokines (ELR$^+$CXC) attract polymorphonuclear leukocytes to the sites of acute inflammation. Gene ontology also shows a significant enrichment of IRI-induced genes associated with hepatic fibrosis/hepatic stellate cell activation, suggesting parallels between kidney and liver in fibrotic programs, and furthermore, that fibrosis-inducing activities are present within Lyz2-derived myeloid lineages and Foxd1-derived, interstitial/pericytes lineages 24 hours after IRI, before histologically apparent fibrosis (late fibrosis).

For several of these early response genes additional data are available from genetic studies on their actions in AKI-induced renal dysfunction. In general, a significant acute injury response involves myeloid-lineage cells aggravating ischemic AKI. Genetic knockout of Cd44, Edn1, intercellular adhesion molecule-1, and Il6 protect against ischemic AKI. In contrast, AKI is worsened upon removal of Cdkn1a, Hmox1, and Atf3, arguing for a primary protective role of this group of factors.

### Table 2. Comparison of Responses in AKI Models Analyzed by Transcriptional and Translational Profiling: Cell Compartment–Specific Regulation of IRI and Cisplatin Invoked Responses Predicted Through Comparison With TRAP Responses Observed Within Distinct Cell Populations in the Mouse Kidney

<table>
<thead>
<tr>
<th>Studies</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
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<th>TRAP IRI</th>
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<td>Fgb</td>
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</tr>
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<td>Huang et al$^{41}$, Yuen et al$^{37}$</td>
<td>Myc</td>
<td>Myelocytomatosis oncogene</td>
<td>Cisplatin, IRI</td>
<td>✓ ✓ ✓</td>
</tr>
<tr>
<td>Yuen et al$^{37}$</td>
<td>Tnfrsf12a</td>
<td>Tumor necrosis factor–receptor superfamily, member 12a</td>
<td>IRI</td>
<td>✓ ✓ ✓</td>
</tr>
<tr>
<td>Yuen et al$^{37}$</td>
<td>Tpm4</td>
<td>Tropomyosin 4; predicted gene 7809</td>
<td>IRI</td>
<td>✓ ✓ ✓</td>
</tr>
<tr>
<td>Yuen et al$^{37}$</td>
<td>Adm</td>
<td>Adrenomedullin</td>
<td>IRI</td>
<td>✓ ✓ ✓</td>
</tr>
</tbody>
</table>

### INTERMEDIATE AND LATE MOLECULAR RESPONSES TO AKI: 48 HOURS OR LONGER AFTER INJURY

#### Chronic Inflammation and Extracellular Matrix Remodeling After Ischemic AKI

To date, relatively few published studies have examined whole-organ or cell-specific molecular signatures at later stages of the injury responses defined here as later than 48 hours after the insult, when evident repair is underway (Table 3). Despite differences in species, insults, molecular profiling platforms, and genes under investigation, a unifying theme is the inability of the postischemic kidney to return to a pre-injury histologic or basal molecular state. Persistence of the proinflammatory milieu remains a major feature in the later postischemic kidney.
exaggerated by distinct late proinflammatory molecular responses after injury. Of note, published studies have used unilateral clamping of the renal pedicle in studying later responses to ischemic AKI. This makes parallel assessment of kidney function problematic in unilateral versus bilateral IRI models. Consequently, drawing clear-cut conclusions on whether an observed invoked molecular response is equivalent to survival is only possible in bilateral injury models.

Figure 3 summarizes shared and unique molecular features between comparable gene expression profiling studies examining molecular responses in a repairing kidney after unilateral IRI injury. A gene list containing genes that remained continuously increased (>1.5-fold change) throughout the intermediate (48 h to day 7) and late phases (day 7 onward) was collated for both studies. Five genes were shared between the two studies and the remaining 26 and 25 genes, respectively, were specific to each study (Fig. 3). Interestingly, the shared genes suggest persistent inflammation (complement C3, and suppressor of cytokine signaling 3) and continuous extracellular matrix remodeling (matrix gla-protein and cathepsin S) after injury. Extracellular matrix turnover is mediated by a number of elastolytic proteinases, including Zn\(^{2+}/Ca^{2+}\)-dependent matrix metalloproteinases and cathepsin cysteine proteases, such as cathepsin S. C3 is a key component of the complement cascade, a fundamental innate defense system. C3 is situated at the crossroads of three major complement activation pathways, yielding several effector molecules with powerful inflammatory effects. Indeed, complement C3 influences long-term kidney transplant outcomes. Defense, immune, and inflammatory responses persist after renal IRI. Among gene ontology analysis of data, complement activation, chemotaxis, cell adhesion, and antigen presentation emerge at day 10 after injury.

The temporal profiling of chemokines using chemokine pathway–specific microarrays identified 14 new genes up-regulated on day 7 after unilateral IRI in the mouse, reflecting a changing component of infiltrating cell types or a changing activation profile of existing cells within the damaged kidney. Among this group are the CC chemokines (Ccl2, Ccl6, Ccl12, and Ccl17), whose actions are linked to the attraction of mononuclear cells to sites of chronic inflammation, and CX3CL1 (also known as fractalkine, a member of the CX3C family). Ccl2, also known as monocyte chemo-attractant protein 1, is a potent agonist for monocytes, dendritic cells, memory T cells, and basophils.

FACS sorted T cells infiltrating the posts ischemic kidney also display transcriptome changes as suggested by the T helper (Th)1-Th2-Th3 polymerase chain reaction array analysis. Four weeks after injury and/or insult, genes associated with co-stimulatory pathway for

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**Table 3. Kidney mRNA Profiling After AKI**

<table>
<thead>
<tr>
<th>Studies</th>
<th>Species</th>
<th>AKI Model</th>
<th>Time Points</th>
<th>Profiling Platforms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basile et al</td>
<td>Rat</td>
<td>IRI</td>
<td>D 35</td>
<td>Customized cDNA microarray</td>
</tr>
<tr>
<td>Krishnamoorthy</td>
<td>Rat</td>
<td>IRI</td>
<td>D 5</td>
<td>mRNA profiling</td>
</tr>
<tr>
<td>Ko et al</td>
<td>Mouse</td>
<td>uIRI</td>
<td>D 3, d 10, d 28</td>
<td>mRNA profiling</td>
</tr>
<tr>
<td>Tran et al</td>
<td>Mouse</td>
<td>LPS (septic AKI)</td>
<td>42 h</td>
<td>mRNA profiling</td>
</tr>
<tr>
<td>Stroo et al</td>
<td>Mouse</td>
<td>uIRI</td>
<td>24 h, 7 d</td>
<td>Chemokine pathway–specific microarray</td>
</tr>
<tr>
<td>Ko et al</td>
<td>Mouse</td>
<td>uIRI</td>
<td>6 h, d 3, d 10, d 28</td>
<td>Th1-Th2-Th3 RT2 profiler polymerase chain reaction array</td>
</tr>
<tr>
<td>Riss et al</td>
<td>Mouse</td>
<td>uIRI</td>
<td>D 1, d 2, d 5, d 7, d 14</td>
<td>Mouse cDNA microarrays</td>
</tr>
</tbody>
</table>

**Abbreviation:** uIRI, unilateral ischemia reperfusion injury.
MicroRNAs (miRNAs) play important roles in varied cell-biological processes including development, apoptosis, proliferation, and differentiation. When miRNAs are down-regulated globally in the cortices of the proximal tubule through the proximal tubule–specific knock-out of dicer, an enzyme critical for miRNA genesis, the kidney is reported to be less susceptible to renal dysfunction secondary to ischemic AKI.84 Emerging evidence suggests that a few miRNAs remain significantly increased late in the course of postischemic kidney (Table 4).81–83 Table 5 illustrates the shared and unique microRNA responses among the unilateral IRI and unilateral ureteral obstruction (UUO) models. The shared responses include miR-21, miR-20a, miR-119a-3p, and miR-146a. Whether their increase plays a part in the persistence of chronic inflammatory processes has not been examined directly. However, miR-146a is known to regulate innate immune and inflammatory responses via post-translational inhibition of key target genes.84 A 12-month-old, age-matched comparison microarray analysis for 511 miRNAs in kidneys of B6.MRLc1 mice (a model of inflammation-driven spontaneous CKD) versus C57BL6 mice showed the highest expression level (2.2-fold) of miR-146a in the kidneys of CKD mice.85 These observations raise the intriguing possibility that miR-146a may contribute to sustained inflammation after renal IRI. Interestingly, a similar differential expression profile in the immunodeficient RAG-2/common γ-chain double-knockout mice suggests a negligible contribution of the infiltrating lymphocytes to the overall miRNA signature of the postischemic kidney.81

Studies in the rat also show a persistently altered gene profile after AKI injury. Basile et al.86 sought to identify alterations in renal gene expression in the recovered rats 35 days after bilateral renal ischemia reperfusion injury: serum creatinine levels had returned to baseline a week after IRI. By using a customized cDNA microarray to examine 2,000 rat genes, 16 genes persistently were altered at 35 days. Among the 12 up-regulated genes, osteopontin (Opn), complement C4 (proinflammatory), and $S_{100}A_{4}$ remained up-regulated after serum creatinine levels normalized. The role of Opn was explored further in Opn-deficient mice: a reduction of natural killer cell infiltration was observed correlating with decreased tissue damage 5 days after IRI consistent with a negative impact of endogenous Opn in the natural repair process.87 However, a second study found no differences in functional or morphologic consequences of ischemic AKI up to 7 days after IRI in a unilateral injury model.88 Although both studies agree on reduced levels of infiltrating immune cells, the former highlights natural killer cells and the latter highlights macrophages. Differences in surgical models or mouse strains may underlie differences between these independent findings.

### Fibrosis After Ischemic AKI

The persistence of AKI biomarker up-regulation several weeks after normalization of serum creatinine levels after IRI injury could reflect early indications of a progression from AKI to CKD.73 NGAL/Lipocalin2-deficient mice are relatively protected against the development of renal lesions (tubular injury, interstitial fibrosis) after 75% nephron reduction surgery.89 Kim-1 expression correlates directly with interstitial fibrosis in human allografts,90 and increased urinary Kim-1 is an independent predictor of long-term renal graft loss.91 In mice, chronic expression of Kim-1 in renal epithelial cells in the absence of an insult led to progressive interstitial kidney inflammation with fibrosis.92 These mice developed a phenotype analogous to the clinical progression of human CKD including proteinuria, anemia, hyperphosphatemia, hypertension, and cardiac hypertrophy. These studies raise the

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**Table 4.** miRNA Expression Changes Linked to IRI-Induced AKI

<table>
<thead>
<tr>
<th>Study</th>
<th>Species</th>
<th>Injury</th>
<th>Time Point</th>
<th>Profiling</th>
<th>Key miRNAs</th>
<th>Biologics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Godwin et al81</td>
<td>Mouse</td>
<td>uIRI</td>
<td>D 4, h 3,</td>
<td>miRNA profiling (uParaflo</td>
<td>miR-21, miR-20a, miR-146a, miR-155, miR-18a; profibrotic; miR-21, miR-192, miR-199a-3p; potential urinary biomarker</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>d 7, d 14,</td>
<td>microfluidic array)</td>
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<td></td>
<td></td>
<td></td>
<td>d 28</td>
<td></td>
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</tr>
<tr>
<td>Chau et al83</td>
<td>Mouse</td>
<td>uIRI</td>
<td>D 10</td>
<td>miRNA profiling (Agilent</td>
<td>miR-21, miR-20a, miR-146a, miR-155, miR-18a; profibrotic; miR-21, miR-192, miR-199a-3p; potential urinary biomarker</td>
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<td>microarray)</td>
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<td></td>
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<tr>
<td>Saikumar et al82</td>
<td>Rat</td>
<td>IRI</td>
<td>D 5</td>
<td>miRNA profiling</td>
<td></td>
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</tbody>
</table>

Abbreviation: uIRI, unilateral ischemia reperfusion injury.
possibility that Kim-1 does not simply play a role in engulfment of apoptotic cells in early AKI but may trigger pathogenic proinflammatory and profibrotic effects. Removing Kim-1 activity at different periods after an AKI trigger will provide important insights into Kim-1 action, and the potential role of Kim-1 in progressive renal pathology.

The distinct wave of chemokines observed a week after injury (discussed earlier) also could play a role in fibrotic pathogenesis, a possibility supported by the close temporal association between chemokine expression and histologically apparent fibrosis at 2 weeks further strengthens this possibility. Mice deficient for the CX3CL1 receptor, Cx3CR1, showed significantly reduced infiltration of macrophages correlating with decreased fibrosis, particularly in the outer medullary region. Treatment with a CX3CR1-neutralizing antibody also reduced fibrosis. The contribution of chemokines to the reparative processes, including recruitment and cross-talk of immune cells, is likely to continue as a major focus in identifying the initiating and propagating factors in injury-related fibrotic disease.

miRNAs also may contribute to the progression of AKI to CKD. By using miRNA microarrays, Chau et al identified 14 miRNAs unique to UUO, 18 unique to IRI studies, and a set of 24 miRNAs upregulated in both UUO and unilateral IRI models of kidney damage 10 days after initiation of injury. This common set includes miRNA-214, miRNA-199a-3p, miRNA-21, miRNA-20a, and miRNA-146a, as discussed earlier for unilateral IRI-associated microarray changes. Interestingly, miRNA-21 knock-out mice had significantly less injury-induced fibrosis, and anti-miR-21 oligonucleotide treatment of the wild-type mice reduced fibrosis in the setting of unilateral IRI and UUO. Of the IRI-specific set, miRNA-192 is down-regulated rapidly and persistently after ischemic AKI. Loss of miR-192 correlates with tubulointerstitial fibrosis and a reduction in renal function in patients with established diabetic nephropathy, and transforming growth factor-β decreases miR-192 expression; suggesting that a similar transforming growth factor-β–driven repression of miRNA-192 after ischemic injury could promote kidney fibrosis. The miRNA action on their mRNA targets may modulate production of up to 30% of total cellular proteins, small changes in large networks from miRNA imbalance could have a major pathologic impact. However, identifying the most critical protein components mediating pathology in these networks will be a major challenge.

### Tubular Proliferation/Repair after Ischemic AKI

Recent gene expression profiling studies have provided insights into potential strategies and therapeutic targets to augment tubular proliferation responses. By using a lipopolysaccharide (LPS)-induced septic AKI model, Tran et al compared differential gene expression among kidneys with persistent injury versus those showing functional recovery 42 hours after LPS administration. Gene ontology analysis identified oxidative phosphorylation and mitochondrial dysfunction among the top three enriched pathways. More specifically, expression of peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (PGC-1α), a transcriptional regulator of mitochondria and oxidative metabolic programs, was down-regulated significantly in kidneys that failed to recover from LPS treatment. Proximal tubule–specific knockout of PGC-1α resulted in persistent renal dysfunction after LPS treatment, consistent with a role for PGC-1α in functional recovery from endotoxemia. Reduced expression of PGC-1α in tubular epithelium also is associated with mitochondrial dysfunction in cisplatin-induced proximal tubule injury. Collectively, these studies argue for a more comprehensive understanding of the mitochondrial enzyme machinery in tubular repair processes.

Cell proliferation is a marked, and likely essential, response to effective repair of AKI. Krishnamoorthy et al performed whole-genome expression profiling of rat cortex and medulla and identified fibrinogen (Fgα), Fgβ, and Fγy to be increased persistently after IRI throughout the study (end point, 5 days after IRI), with a tubular expression similar to Kim-1. Administration of Fgβ-derived Bβ15-42 peptide promoted tubular cell proliferation and protected against ischemia-induced AKI in the mouse.
Hypertension After AKI

As discussed earlier, inflammatory and immune-associated cellular responses are predominant within the transcriptome after AKI injury. Less clear, but likely of importance, is the pathophysiology that results from fluid and electrolyte disturbances, and hypertension associated with AKI.

Basile et al reported that the vasodilator kallikrein remains strongly down-regulated 35 days after renal IRI in the rat. The kallikrein system plays an important role in blood pressure regulation, salt sensitivity, and electrolyte excretion. A kallikrein-deficient rat strain manifests polydipsia and hypertension in response to increased sodium intake as well as progressive renal scarring. Mining the repair transcriptome with a particular focus on understanding hemodynamic and electrolyte derangements may provide valuable new insights into the pathophysiology of salt and volume overload, hyperkalemia, hypophosphatemia, urinary concentrating defects, metabolic acidosis, and hypertension that frequently is encountered after human AKI.

CELL-SPECIFIC GENE EXPRESSION PROFILING OF THE REPAIRING KIDNEY

The T-cell profiling study examining infiltrating cells through FACSs and polymerase chain reaction array analysis takes an expansive (multistage), cell type-specific focus on kidney damage and repair. The immune response is complex, varied immune cells have been shown to play a role in acute and chronic pathogenesis of AKI. Sifting anti-inflammatory, pro-reparative responses from deleterious proinflammatory and profibrotic triggers is a challenge, especially if the former reflects a rare cell population whose transcriptional signature is a minor component of a larger proinflammatory response in the whole organ.

For example, Foxp3+CD4+ regulatory T cells (Tregs) play a critical role in immune homeostasis including self-tolerance and their ability to suppress inflammation. Several lines of evidence suggest that Tregs may promote repair after ischemic AKI. Adoptive transfer of Tregs at 24 hours after injury, or IL-1/anti–IL-2 complex–mediated expansion of the intrinsic Treg pool, promote functional renal recovery exemplified by reduced serum blood urea nitrogen and creatinine levels in a treatment cohort 5 days after injury. Further, Treg depletion aggravates ischemic renal dysfunction.

HUMAN KIDNEY INJURY TRANSCRIPTOME AND REPAIR

Kidney transplantation can be considered a highly scrutinized in vivo model of human AKI in which the inciting insults can be identified precisely in a temporal manner, unlike other settings of human AKI. Immediately after kidney transplant, significant AKI causes delayed graft function, an independent predictor of allograft rejection and graft loss. To define the transcripts induced by human AKI and assess their impact on renal allograft outcomes, Famulski et al performed microarray-based gene expression profiling of kidney tissue from 26 kidney transplant patients identified as a “pure AKI” cohort. The controls consisted of a set of 11 age-matched pristine protocol biopsy specimens from a different transplant cohort. The transcript score (geometric mean of the fold-increase in the top 30 transcripts versus control nephrectomies) correlated with reduced graft function, renal recovery, and requirement for renal replacement therapy assessed at 6 months after AKI.

The intragraft molecular signature predominantly reflected the renal parenchymal response to AKI, and showed similarities to cancer, cell adhesion, cell movement, and re-expression of developmental programs. Responses significantly overlapped with those observed in IRI-induced AKI mouse models, indicating a broad conservation of molecular and cellular processes.

Interestingly, similar transcriptional signatures also were encountered in transplants with other causes of allograft dysfunction including chronic antibody-mediated graft injury and recurrent primary renal disease. Although the considerable similarity between the intergraft transcriptomes, irrespective of the underlying pathology, suggests that the human transplant kidney mounts a shared robust response to varied insults.

Induction of AKI-associated transcripts (predominantly parenchymal) was reported to be a better predictor of future graft loss than fibrosis, inflammation, or expression of collagen genes. Although these findings suggest that fibrosis may not be as significant a factor to the overall progression of AKI, it remains highly likely that fibrosis contributes to longer-term injury, notably postrecovery onset of CKD. Although there are procedural and analytic hurdles to overcome, transcriptomic profiling of renal allograft biopsy specimens could become a useful complement to current approaches for risk stratification of transplant patients.

GENE EXPRESSION PROFILING: REPAIR/REGENERATION VERSUS CANCER

Transcriptional profiling studies comparing unilateral IRI in the mouse at various time points with expression profiling of human renal cell carcinoma has drawn parallels between these distinct kidney insults. Of 361 differentially expressed genes identified in both conditions, the majority (77%) showed concordant expression changes, either up-regulation or down-regulation, suggestive of related biological processes: cell proliferation, cell...
migration, cell adhesion, and cell death gene ontology terms are shared between IRI and cancer samples.

**CONCLUSIONS AND FUTURE DIRECTIONS**

The move from biased (array-based) to unbiased (next-generation sequencing) assessment of the transcriptome will provide important new insights into both coding and noncoding components of injury/repair responses in AKI. Deep sequencing of RNA, RNA-seq, enables a complete survey of mRNAs and noncoding RNAs (e.g., miRNA and long noncoding RNAs) that serves not only for discovery-identifying novel genes and variant transcripts, but as a more rigorous measure of observed responses.

In the developing kidney, the Genito Urinary Development Molecular Anatomy Project (GUDMAP) initiative (www.GUDMAP.org) has produced a wealth of high-quality annotated data from direct visualization and indirect microarray-based analysis of gene expression. These data now are being complemented by high-quality RNA-seq data sets. These data already have produced numerous novel insights into already well-studied developmental processes. This bodes well for insights that will be obtained in less well-scrutinized events induced on AKI in the adult kidney.

Understanding how the various cell types in the kidney communicate to regulate the intrinsic repair mechanisms, and their contribution to postinjury fibrosis, remains a major challenge. Here, a continued focus on cell-type-specific signatures, and a broadening of analysis beyond early injury, is likely to provide important new insights. Clearly, the major clinical goal is to develop new analytic tools to diagnose both short- and long-term outcomes, and, at the same time, to develop new therapeutic strategies to improve existing repair processes and reduce the long-term risk of CKD after AKI.

**REFERENCES**


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