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Focal Adhesion Kinase Signaling Mediates Acute Renal Injury Induced by Ischemia/Reperfusion

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Renal ischemia/reperfusion (I/R) injury is associated with cell matrix and focal adhesion remodeling. Focal adhesion kinase (FAK) is a nonreceptor protein tyrosine kinase that localizes at focal adhesions and regulates their turnover. Here, we investigated the role of FAK in renal I/R injury, using a novel conditional proximal tubule-specific *fak*-deletion mouse model. Tamoxifen treatment of FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} mice caused renal-specific *fak* recombination (FAK^{ΔloxP/ΔloxP}) and reduction of FAK expression in proximal tubules. In FAK^{ΔloxP/ΔloxP} mice compared with FAK^{loxP/loxP} controls, unilateral renal ischemia followed by reperfusion resulted in less tubular damage with reduced tubular cell proliferation and lower expression of kidney injury molecule-1, which was independent from the postischemic inflammatory response. Oxidative stress is involved in the pathophysiology of I/R injury. Primary cultured mouse renal cells were used to study the role of FAK deficiency for oxidative stress *in vitro*. The conditional *fak* deletion did not affect cell survival after hydrogen peroxide-induced cellular stress, whereas it impaired the recovery of focal adhesions that were disrupted by hydrogen peroxide. This was associated with reduced c-Jun N-terminal kinase-dependent phosphorylation of paxillin at serine 178 in FAK-deficient cells, which is required for focal adhesion turnover. Our findings support a role for FAK as a novel factor in the initiation of c-Jun N-terminal kinase-mediated cellular stress response during renal

I/R injury and suggest FAK as a target in renal injury protection. (Am J Pathol 2011, 179:2766–2778; DOI: 10.1016/j.ajpath.2011.08.025)

Renal ischemia/reperfusion (I/R) injury is a potential life-threatening clinical problem and may occur during hypoperfusion of the kidney or after renal transplantation.¹ During renal I/R injury, proximal tubular epithelial cells may detach from the extracellular matrix, leading to intraluminal obstruction or cell excretion in the urine.² Considerable attention has been directed to the role of cell-extracellular matrix adhesion-mediated signaling during I/R injury.^{3–5} Understanding the basic molecular mechanisms that underlie disruption of cell adhesion during the course of I/R injury is essential for the development of therapeutic strategies that antagonize cell detachment and subsequent cell stress signaling to prevent acute renal failure or to promote tissue regeneration.

Adhesion of cells to the extracellular matrix is essential for the control of cell survival, proliferation, differentiation, and migration.⁶ Cell-extracellular matrix adhesions are mediated via engagement of integrins with their extracellular ligands and subsequent assembly of an intracellular multiprotein complex termed focal adhesion (FA) complex.^{6,7} FAs are prominently present at the basolateral membrane of proximal tubular epithelial cells *in vivo*⁵ and allow activation of divergent downstream signaling cascades that modulate various adhesion-dependent biological processes. I/R injury induces alterations in the F-actin cytoskeleton⁸ and redistribution of integrins³ and

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other FA-associated signaling proteins.⁵ Disruption of adhesion was associated with dephosphorylation of FA proteins during hypoxia in isolated tubules⁹ and I/R injury *in vivo*.⁵ The effect of disruption of FA-mediated signaling events on the pathogenesis of acute renal failure is however still unclear.

Focal adhesion kinase (FAK) is a nonreceptor protein tyrosine kinase that localizes at FAs and regulates FA dynamics.¹⁰ Integrin-mediated adhesion induces autophosphorylation of tyrosine residue 397 on FAK, providing a docking site for various adapter signaling proteins such as c-Src, p130Cas, and phospho-inositide-3 kinase.¹¹ Through these interactions, FAK is implicated in the regulation of signaling pathways that control cell adhesion dynamics,¹⁰ cell survival,¹² proliferation,¹³ and inflammatory responses.¹⁴ We have reported that in renal I/R injury FAs undergo drastic restructuring, associated with rapid dephosphorylation of FAK directly after the ischemic period followed by enhanced rephosphorylation of FAK during the reperfusion.⁵ We propose that FAK plays an important role in the pathophysiology of I/R-mediated renal failure.

Here, we investigated the role of FAK in I/R-induced renal injury *in vivo*. Because *fak* knockout mice are embryonically lethal,¹⁵ we created a Cre/LoxP system-based conditional proximal tubule-specific *fak* knockout mouse model by crossing floxed *fak* (FAK^{loxP/loxP}) mice¹⁶ with γ GT-Cre-ER^{T2} mice.¹⁷ Our data show that *in vivo* proximal tubular FAK deficiency attenuates I/R-induced renal injury. To study the role of FAK deficiency *in vitro*, we used FAK-deficient primary cultured renal cells and used an exposure model to hydrogen peroxide (H₂O₂) that mimics reperfusion-associated oxidative stress. H₂O₂-induced turnover of FA and activation of cell stress pathways were compared between FAK-expressing and FAK-deficient cells. The *in vitro* data suggest that FAK mediates FA turnover and thus weakens the stability of FAs during reperfusion after renal ischemia. FAK is also involved in the initiation of the c-Jun N-terminal kinase (JNK)-mediated cellular stress response to I/R injury, accompanied by early tubular epithelial injury responses that occur during the pathophysiological process associated with acute renal failure.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium/Ham's F12 (1:1), phosphate-buffered saline (PBS), and penicillin/streptomycin/amphotericin B were from Invitrogen (Carlsbad, CA). Fetal bovine serum was from Life Technologies (Grand Island, NY). Collagenase (Crude, type XI), epidermal growth factor, 1× insulin-transferrin sodium selenite supplement, cholera toxin, H₂O₂, and all of the other reagents not specially referred to were obtained from Sigma-Aldrich (St. Louis, MO).

Mice, Genotyping, and Recombination PCR

FAK^{loxP/loxP},¹⁶ γ GT-Cre-ER^{T2},¹⁷ and Rosa-Cre-ER^{T2}¹⁸ mice were maintained and bred at the animal facility of the Leiden University Gorlaeus Laboratories in accordance with institutional guidance and national health standards. The mice were regularly monitored and had free access to water and standard mice chow.

Mice were genotyped with PCR. Genomic DNA was isolated from ear cuts, and FAK^{loxP/loxP} and FAK^{wt/wt} bands (400 bp and 290 bp, respectively) were detected with 5'-GAGAATCCAGCTTTGGCTGTTG-3' and 5'-GAATGCTACAGGAACCAATAAC-3' primers and the following conditions: 5 minutes at 95°C (one cycle); 30 seconds at 94°C, 30 seconds at 62°C, and 1 minute at 72°C (35 cycles); and 5 minutes at 72°C. Cre-ER^{T2} bands were detected with 5'-GTTCAGGGATCGCCAGGCG-3' and 5'-GCTGGCTGGTGGCAGATGG-3' primers and the following conditions: 5 minutes at 95°C (one cycle); 1 minute at 94°C, 1 minute at 65°C, and 2 minutes at 72°C (30 cycles); and 10 minutes at 72°C. PCR products were separated on 2% agarose gels.

For each mouse used in the experiment, three cryosections (10 μ m) of the right, unaffected kidney were lysed for DNA extraction. DNA extracted from mouse kidney or cultured primary mouse renal cells were detected for the presence of *fak* recombination with the use of 5'-GACCTTCCAATTCTCATTCTCC-3' and 5'-GAATGCTACAGGAACCAATAAC-3' primers and the following conditions: 5 minutes at 95°C (one cycle); 30 seconds at 94°C, 30 seconds at 62°C, and 1 minute at 72°C (35 cycles); and 5 minutes at 72°C. PCR products were separated on 2% agarose gels.

Tamoxifen Treatment of Mice

Tamoxifen powder (300 mg; Sigma-Aldrich) was dissolved in 900 μ L of absolute ethanol and suspended in 5.1 mL of sunflower oil. This suspension (50 mg/mL) was sonicated twice for 30 seconds to generate a clear solution and stored in aliquots at -20°C. The solution was thawed at 37°C and administered orally to mice (100 μ L) by a feeding needle for 4 consecutive days followed by a period of 4 days without treatment, before analysis and the onset of the I/R injury.

Renal I/R Injury

Male mice (12 to 14 weeks of age) were anesthetized with 3% isoflurane in a mix of 60% N₂O and 40% O₂. A small incision was made over the left flank; the left renal pedicle was prepared and clamped with a B1 hemostatic clamp (Fine Science Tools, Heidelberg, Germany) for 35 minutes, whereas the right kidney was unaffected and served as internal control. After removal of the clamp, the incision was closed with nondegradable sutures. Animals were sacrificed at 24 hours after ischemia. Both kidneys were harvested and prepared as described below. Additional control kidneys were obtained from animals that underwent sham surgery without I/R injury. Each group consisted of a minimum of eight animals. All of the kid-

neys were briefly washed in ice-cold PBS to remove excess blood and sliced into two equal halves. One half of each kidney was frozen in liquid nitrogen and stored at -80°C . The other half was fixed in ice-cold Carnoy's solution [60% (v/v) absolute ethanol, 30% (v/v) chloroform, and 10% (v/v) glacial acetic acid] for 3 hours and thereafter transferred to 70% (v/v) ethanol and stored at 4°C . All experiments that used mice were approved by the local animal experimental committee of the Leiden University.

Isolation and Culture of Primary Mouse Renal Cells

For *in vitro* experiments, mouse renal cells were isolated from the kidneys of male FAK^{loxP/loxP}/Rosa-Cre-ER^{T2} mice (~25 g) or the FAK^{loxP/loxP} littermates. The kidneys were minced and digested in a collagenase solution in Hanks' HEPES balanced salt solution (137 mmol/L NaCl, 5 mmol/L KCl, 0.8 mmol/L MgSO₄, 0.4 mmol/L Na₂HPO₄, 0.4 mmol/L KH₂PO₄, 1.3 mmol/L CaCl₂, 4 mmol/L NaHCO₃, 25 mmol/L HEPES, 5 mmol/L D-glucose, pH 7.4) for 30 minutes at 37°C . The cell suspension was washed three times in Hanks' HEPES balanced salt solution. After washing, cells were resuspended in Dulbecco's modified Eagle's medium/Ham's F12 (1:1) containing 1% (v/v) fetal bovine serum, 0.5 mg/mL bovine serum albumin, 10 ng/mL epidermal growth factor, 10 ng/mL cholera toxin, 50 nmol/L hydrocortisone, 15 mmol/L HEPES, 2 mmol/L glutamine, $1\times$ insulin-transferrin sodium selenite supplement, and 1% (v/v) penicillin/streptomycin/amphotericin B and were maintained at 37°C in a humidified atmosphere of 95% air/5% CO₂. To induce *fak* recombination, cells were exposed to 4-hydroxy-tamoxifen (4-OHT; Sigma-Aldrich), and medium was refreshed every other day. After 7 to 9 days of culturing, confluent monolayer was subjected to 1 mmol/L H₂O₂ in serum-free medium for indicated time periods.

Histopathology and Immunohistochemistry

Fixed kidneys were embedded in paraffin in a standard fashion and sectioned (3 μm). Sections were deparaffinized, stained with H&E, and examined for tubular injury. Tubulo-interstitial injury was assessed in the cortex, outer stripe of the outer medulla (OSOM), and inner stripe of the outer medulla (ISOM) with the use of a semiquantitative scoring index as previously described.⁵ In short, tubular cast formation, tubular dilatation, and tubular degeneration (vacuolar change, loss of brush border, detachment of tubular epithelial cells, and condensation of tubular nuclei) were scored blind by two independent observers according to the following criteria: 0, normal; 1, <30%; 2, 30% to 70%; and 3, >70% of the pertinent area. After scoring all individual scores per kidney were added together to define the overall tubular damage in the kidney.

For immunohistochemistry of kidney injury molecule-1 (KIM-1), paraffin sections of the kidneys were stained as described previously.¹⁹ Briefly, after antigen retrieval, sections were blocked with normal goat serum (NGS;

Vector Laboratories, Burlingame, CA) and incubated with primary antibody for 1 hour. Sections were labeled with biotinylated goat anti-mouse/rabbit IgG, and staining was visualized with an avidin-biotinylated horseradish peroxidase complex. Nuclei were counterstained with toluidine blue. For KIM-1 staining the sections were scored blind by two independent observers according to the following criteria: 0, no staining; 0.5, one tubule; 1, very few tubules; 2, several tubules but not widespread; 3, widespread staining or entire OSOM; and 4, many tubules stained beyond OSOM.

For immunohistochemistry of CD45 and F4/80, cryosections (10 μm) were air dried and fixed in acetone for 10 minutes. After blocking with NGS, sections were incubated with rat anti-mouse CD45 (Abcam, Oxford, UK) for 1 hour and rat anti-mouse F4/80 (Abcam) overnight. Sections were then labeled with horseradish peroxidase-conjugated rabbit anti-rat IgG and counterstained with hematoxylin.

All of the stainings were examined with light microscopy (Leica DM6000B, Wetzlar, Germany).

Immunofluorescence

Cryosections were immunostained for FAK as described previously.⁵ Briefly, sections were fixed in ice-cold 4% buffered formaldehyde for 10 minutes and blocked in 5% (v/v) NGS for 1 hour. The sections were then incubated overnight at 4°C in a humidified chamber with rabbit polyclonal anti-FAK antibody (Millipore, Billerica, MA) and subsequently incubated with Alexa 488-labeled goat anti-rabbit IgG (Invitrogen). Rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) was used for F-actin staining. Sections were counterstained with Hoechst 33258 dye and mounted with Aqua-Poly/Mount (Polysciences, Warrington, PA).

For cleaved caspase-3 and Ki-67 stainings, 10- μm cryosections were air dried and fixed in 4% buffered formaldehyde. Sections were permeabilized in 0.2% Triton X-100 in PBS and blocked with 5% NGS in PBS with 0.05% Triton X-100. Then the sections were incubated overnight with rabbit polyclonal anti-cleaved caspase-3 (Asp175) antibody (Cell Signaling Technologies, Danvers, MA) or rabbit polyclonal anti-Ki-67 antibody (Novocastra, Newcastle, UK). Sections were subsequently incubated with cyanine-3-labeled goat anti-rabbit IgG (Invitrogen) and counterstained with Hoechst 33258 dye. All antibodies were diluted in PBS with 0.05% Triton X-100. Cleaved caspase-3-positive or Ki-67-positive cells were counted blind in the cortex, OSOM, and ISOM with the use of $40\times$ objective by two independent observers and expressed as the number of positive cells per high-power field.

Fixed cells on coverslips were permeabilized with 0.4% (w/v) Triton X-100 in PBS for 10 minutes, followed by three washes with PBS. After blocking with 5% (v/v) NGS and 1% (w/v) bovine serum albumin in PBS, cells were stained for anti-vinculin (Transduction Laboratories, Lexington, KY) and/or anti-P_{Ser178}-paxillin (Abcam) overnight at 4°C . Thereafter, cells were washed three times with PBS and subsequently incubated with cyanine-3-labeled goat anti-mouse IgG and/or Alexa 488-labeled goat anti-

rabbit IgG (Invitrogen), in combination with Hoechst 33258. Coverslips were mounted on glass slides with the use of Aqua-Poly/Mount.

Images were made with a Nikon E600 epifluorescence microscope (Nikon, Tokyo, Japan) or Bio-Rad Radiance 2100 confocal system with a $\times 60$ Plan Apo objective (NA 1.4; Nikon, Melville, NY). All images were processed with Image-Pro Plus version 5.1 (Media Cybernetics, Bethesda, MD).

Western Blot Analysis

Western blot samples were processed as described previously.⁵ Briefly, cryosections were lysed in Triton lysis buffer (20 mmol/L Tris, pH 7.4, 137 mmol/L NaCl, 2 mmol/L EDTA, 1% Triton X-100, 25 mmol/L β -glycerophosphate, and 10% glycerol) with protease inhibitors. Lysates were syringed four times through a 26-gauge needle, centrifuged, and immediately boiled in sample preparation buffer (125 mmol/L Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, and bromophenol blue) plus β -mercaptoethanol. Equal amounts of protein samples were separated by SDS-PAGE and transferred to nitrocellulose membrane (Whatman, Dassel, Germany). Blots were blocked either in 5% (w/v) bovine serum albumin in Tris-buffered saline/Tween 20 [0.5 mol/L NaCl, 20 mmol/L Tris-HCl, and 0.05% (v/v) Tween 20, pH 7.4], or I-block [0.2% (w/v) casein in Tris-buffered saline/Tween 20] for 1 hour at room temperature. Primary antibody incubation was performed overnight at 4°C with anti-P_{Tyr397}-FAK (BioSource International, Camarillo, CA); anti-FAK (clone77; Millipore); anti-P_{Ser178}-paxillin (Abcam); anti-P_{Tyr118}-paxillin (BioSource International); anti-paxillin (Transduction Laboratories); anti-P_{Thr183}/P_{Tyr185}-JNK; anti-JNK; anti-P_{Ser63}-c-Jun (Cell Signaling Technologies); anti-c-Jun (Oncogene Science, Uniondale, NY) and anti-tubulin (Sigma-Aldrich). Thereafter, blots were incubated with either horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in Tris-buffered saline/Tween 20, or alkaline phosphatase-conjugated secondary antibody (Applied Biosystems, Foster City, CA) in I-block for 1 hour at room temperature. Protein signals were detected with the ECL Plus reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK) by imaging with the Typhoon 9400 (GE Healthcare), or with Tropix Western-Star Kit by films. Densitometric analysis of the protein band intensity was performed with ImageJ v1.41o analysis software (<http://imagej.nih.gov/ij/>).

ELISA for MCP-1 and IL-6

Medium samples from cell culture were collected for the measurement of secreted IL-6 and monocyte chemoattractant protein 1 (MCP-1) with the use of mouse IL-6 and MCP-1 enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (eBioscience, San Diego, CA).

LDH Release Assay

Total cell death was measured by the release of lactate dehydrogenase (LDH) from cells in the culture medium as a measure of plasma membrane permeability, as previously described.²⁰ The percentage of free LDH leakage was calculated from the amount of LDH release caused by treatment relative to the amount of that released by 0.1% (w/v) Triton X-100 (ie, 100% release).

Statistics

Data are expressed as mean $\pm$ SEM. Statistical significance was determined with the Student's *t*-test, except for the semiquantitative scoring of H&E and KIM-1 stainings in which the Mann-Whitney *U*-test was applied for nonparametric data. Significant difference was set when the *P* value was <0.05 . All statistical analyses were performed with Graphpad Prism 4 (GraphPad Software, San Diego, CA).

Results

Conditional Renal Proximal Tubule-Specific Fak Knockout

The role of FAK during I/R-induced injury was studied in a conditional proximal tubule-specific *fak* knockout mouse model that used the Cre/LoxP technology. Mice with LoxP sites flanking the second exon of the *fak* gene (FAK^{loxP/loxP})¹⁶ were crossed with transgenic mice that express Cre recombinase under control of the γ GT promoter (γ GT-Cre-ER^{T2})¹⁷ to generate FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} offspring (Figure 1A).

We confirmed recombination of the *fak* alleles in primary cultured renal cells isolated from FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} and control FAK^{loxP/loxP}//Rosa-Cre-ER^{T2} mice. Cre recombinase in FAK^{loxP/loxP}//Rosa-Cre-ER^{T2} mice is ubiquitously expressed under the Rosa promoter, and the addition of the active metabolite 4-OHT induced complete recombination of the *fak* alleles coinciding with complete loss of FAK protein (Figure 1B). However, treatment of FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} renal cells with 4-OHT showed only partial recombination of the *fak* alleles. Cre expression driven by the γ GT promoter predominantly occurs in the proximal tubular cells of the S3 segment,¹⁷ which represent a small fraction of the primary renal cell culture. Consequently, no significant decrease in FAK protein level was observed from 4-OHT-treated FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} renal cells (Figure 1B).

To show *fak* recombination in animals, male FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} mice and their FAK^{loxP/loxP} littermates were treated with 5 mg of tamoxifen for 4 consecutive days to induce Cre recombinase activity. FAK^{loxP/loxP}//Rosa-Cre-ER^{T2} mice served as controls. Four days after the last treatment, kidney, liver, and spleen were harvested, and *fak* recombination was shown by PCR analysis. All examined tissues from FAK^{loxP/loxP}//Rosa-Cre-ER^{T2} mice showed almost complete recombination

Figure 1. Tamoxifen successfully recombines “loxed” *fak* alleles in the mouse kidney. **A:** Representative PCR genotype analysis showing the successful breeding for the offspring derived from FAK^{loxP/loxP} and γ GT-Cre-ER^{T2} strain crossbreeding (FAK^{loxP/loxP}// γ GT-Cre-ER^{T2}). **B:** Primary cultured renal cells from FAK^{loxP/loxP}, FAK^{loxP/loxP}//Rosa-Cre-ER^{T2}, and FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} mice were treated with 4-OHT. Genomic DNA was extracted and analyzed for recombination of the loxed *fak* alleles by PCR (upper panels). The recombination band appears at 327 bp. Protein level of FAK was detected by Western blot analysis (lower panels). Data show complete *fak* gene recombination and significant reduction of FAK protein in cells from FAK^{loxP/loxP}//Rosa-Cre-ER^{T2} animals, in contrast to the cells from FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} animals that only show partial recombination of *fak* gene, and no reduction of FAK protein was observed. **C:** Tissue specificity of *fak* recombination in FAK^{loxP/loxP}//Rosa-Cre-ER^{T2} and FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} mice was examined in kidney, liver, and spleen tissue. Animals were treated with either 5 mg of tamoxifen or vehicle daily for 4 days, and tissue samples were obtained 4 days after the last treatment. PCR results show that, in FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} animals, tamoxifen-induced *fak* recombination only occurs in the kidney. Similar to the case in cultured cells, the recombination is not complete. **D:** Cryosections of kidneys from tamoxifen-treated FAK^{loxP/loxP}, FAK^{loxP/loxP}//Rosa-Cre-ER^{T2}, and FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} mice were prepared for Western blot analysis to determine the protein level of FAK in kidney tissue. The results confirmed the observation from PCR that there is no obvious reduction of FAK in FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} animals. **E:** To determine the location of the FAK-deficient tubules in the kidney, cryosections of kidneys from tamoxifen-treated FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} mice and FAK^{loxP/loxP} littermates (FAK^{loxP/loxP} and FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} animals, respectively) were stained for FAK (green) and F-actin (red). Tubular FAK expression at the basolateral cell membrane was unchanged in FAK^{loxP/loxP} animals, whereas the loss of FAK expression was observed in FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} animals (proximal tubule outlined and indicated by asterisk). All sections were imaged with confocal laser scanning microscopy. Sections are representative of proximal tubules from three different mice. Original magnification: $\times 60$.

of the *fak* alleles as detected by the presence of a 327-bp recombination band after tamoxifen treatment (Figure 1C). In tamoxifen-treated FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} mice, the recombination band was weak, indicating partial recombination and consistent with our *in vitro* findings. More importantly, recombination was only observed in the kidney but not in liver or spleen tissue, showing the tissue specificity of *fak* recombination. Control FAK^{loxP/loxP} mice showed

no recombination in any of the tissues (data not shown). Reduced FAK expression was observed in the kidney from tamoxifen-treated FAK^{loxP/loxP}//Rosa-Cre-ER^{T2} mice but was less evident in FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} mice (Figure 1D). FAK-specific immunofluorescence staining of kidney tissue showed a lower FAK expression at basolateral membrane of proximal tubules after *fak* recombination in FAK^{loxP/loxP}// γ GT-Cre-ER^{T2} mice (FAK^{loxP/loxP} mice)

compared with FAK^{loxP/loxP} littermates (Figure 1E). Although the ubiquitous loss of renal FAK expression was observed in FAK^{loxP/loxP}//Rosa-Cre-ER^{T2} mice, the loss of FAK protein in FAK^{loxP/loxP}//γGT-Cre-ER^{T2} mice was mostly observed in proximal tubules that locate in the

OSOM but not observed in distal tubules in either OSOM or cortex (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). These data show that inducible renal proximal tubule-specific deletion of *fak* was achieved in FAK^{loxP/loxP}//γGT-Cre-ER^{T2} mice.

Figure 2. Histology and semiquantification of I/R-induced renal injury in FAK^{ΔloxP/ΔloxP} and FAK^{loxP/loxP} animals. FAK^{ΔloxP/ΔloxP} and FAK^{loxP/loxP} animals were subjected to 35 minutes of ischemia and 24 hours of reperfusion or sham surgery. **A:** H&E stainings were examined for tubular damage. **Large panels:** Low-magnification overview shows extensive tubular dilatation of tubule segments located in the cortical and corticomedullary areas of the kidney during I/R injury in FAK^{loxP/loxP} animals but less pronounced tissue injury in FAK^{ΔloxP/ΔloxP} animals. Sham-operated animals of both groups did not show signs of histologic injury. **Small panels:** High-magnification images show that tubular injury in FAK^{loxP/loxP} animals is present as cast deposition (indicated by asterisk), tubular dilatation (indicated by arrow), or degeneration of the tubular epithelium (indicated by open arrow). In contrast, FAK^{ΔloxP/ΔloxP} animals only display mostly dilatation of the tubules (arrow). All images are representative for the average injury of the experimental group. Original magnification: ×25 and ×5; zoom ×400. **B–C:** Tissue sections from FAK^{ΔloxP/ΔloxP} and FAK^{loxP/loxP} groups were scored double-blinded and semiquantitatively for incidence of cast, dilatation, and degeneration per region (ie, cortex, OSOM, and ISOM) to assess tubulointerstitial injury. Data are shown as total tubular injury score (**B**) and average score per histopathological marker per region (**C**), both indicating reduced tubular injury by I/R in FAK^{ΔloxP/ΔloxP} mice compared with FAK^{loxP/loxP} littermates. All data are expressed as mean ± SEM (*n* = 8 to 13 mice per group). **P* < 0.05.

Figure 3. Less KIM-1 is expressed after I/R in FAK^{ΔloxP/ΔloxP} mice compared with FAK^{loxP/loxP} littermates. **A:** Paraffin sections of the kidneys from FAK^{ΔloxP/ΔloxP} mice and FAK^{loxP/loxP} littermates either subjected to 35 minutes of ischemia and 24 hours of reperfusion or sham surgery were stained for KIM-1. Images are representative for average expression of each group. G, glomerulus. Original magnification: ×25 and ×100. **B:** KIM-1 expression was scored on a 0 to 4 scale that was based on the area per tissue showing specific staining as shown here. **C:** Sections were scored double-blinded for KIM-1 expression, showing that lower expression of KIM-1 was induced by I/R in FAK^{ΔloxP/ΔloxP} mice compared with FAK^{loxP/loxP} littermates. All data are expressed as mean ± SEM (*n* = 8 to 13 mice per group). **P* < 0.05.

Proximal Tubular FAK Deficiency Reduces Tubular Injury after Renal Ischemia

To study the role of FAK in I/R injury, FAK^{loxP/loxP}/γGT-Cre-ERT² mice and control FAK^{loxP/loxP} littermates were treated with tamoxifen with the use of the above-described procedure and subjected to unilateral ischemia for 35 minutes followed by 24 hours of reperfusion. Kidneys from both FAK^{ΔloxP/ΔloxP} and FAK^{loxP/loxP} animals that received sham surgery were used as controls and showed no histologic indications of tubular injury in the OSOM in which the S3 segments of proximal tubules are located (Figure 2A). These findings show that *fak* recombination itself does not lead to tubular injury or alter tubular structure. In contrast, kidneys subjected to ischemia from FAK^{ΔloxP/ΔloxP} and FAK^{loxP/loxP} animals displayed clear histologic signs of tubular damage associated with I/R injury (Figure 2A). Tubular injury was scored semiquantitatively in the cortex, OSOM, and ISOM of the kidney on the basis of the incidence of cast deposition, tubular dilatation, and degeneration of the tubular epithelium. A significantly higher degree of tubular injury after ischemia was detected in the FAK^{loxP/loxP} group than in the FAK^{ΔloxP/ΔloxP} group (Figure 2B). The occurrence of tubular dilatation during I/R injury was significantly reduced in the FAK^{ΔloxP/ΔloxP} animals (Figure 2C).

KIM-1 is a validated biomarker of renal parenchymal tissue injury. Expression of KIM-1 is absent in healthy kidney tissue but is strongly up-regulated at the brush border of proximal tubules after an ischemic insult.^{19,21}

KIM-1 was expressed in tubules of the OSOM and cortex of FAK^{loxP/loxP} kidneys after ischemia, whereas in the FAK^{ΔloxP/ΔloxP} kidneys KIM-1 was predominantly found in the proximal tubules of the OSOM only, indicating attenuated renal injury in FAK^{ΔloxP/ΔloxP} animals (Figure 3A). With the use of a semiquantitative scoring system for KIM-1 staining (Figure 3B), we found that FAK^{ΔloxP/ΔloxP} kidneys had significantly less KIM-1 staining during I/R injury than FAK^{loxP/loxP} kidneys (Figure 3C). These data show that loss of FAK reduces tubular injury as well as expression of KIM-1 associated with I/R injury.

Given the significantly reduced tissue injury in FAK^{ΔloxP/ΔloxP} animals, we anticipated a decrease in the onset of tubular cell apoptosis in these animals, because apoptosis is a characteristic consequence of I/R injury in renal tubular cells. The positive staining for cleaved caspase-3 was found significantly increased in FAK^{loxP/loxP} kidneys subjected to I/R injury, whereas FAK^{ΔloxP/ΔloxP} kidneys showed a trend toward a lower degree of tubular epithelial cell apoptosis (Figure 4, A and B). We also investigated the effect of proximal tubular *fak* deletion on post-I/R tubular cell recovery. Tubular cell proliferation is elevated after I/R injury as an initiating step of cell recovery to repair the tubular damage. With the use of Ki-67 as a cell proliferation marker, I/R injury induced high proliferation in FAK^{loxP/loxP} kidneys, whereas the number of Ki-67-positive cells was significantly reduced in FAK^{ΔloxP/ΔloxP} kidneys, indicating less proliferation in FAK^{ΔloxP/ΔloxP} animals (Figure 4, C and D).

Figure 4. Immunostaining and quantification of cleaved caspase-3 and Ki-67 in FAK^{ΔloxP/ΔloxP} and FAK^{loxP/loxP} animals. Cyrosections of the kidneys from FAK^{ΔloxP/ΔloxP} mice and FAK^{loxP/loxP} littermates either subjected to 35 minutes of ischemia and 24 hours of reperfusion or sham surgery were stained for cleaved caspase-3 (A) or Ki-67 (C). Images are representative for average expression of each group (indicated by arrows). Original magnification: $\times 40$. For each section, 5 to 10 different fields in the cortex, OSOM, and ISOM were analyzed to count the number of cleaved caspase-3-positive (B) or Ki-67-positive (D) cells in each region, and the average number of each group was expressed as the number of positive cells per high-power field (HPF). Results indicate a trend toward less cleaved caspase-3 staining and significantly reduced Ki-67 staining in FAK^{ΔloxP/ΔloxP} mice compared with FAK^{loxP/loxP} littermates. Data are expressed as mean $\pm$ SEM ($n = 8$ to 13 mice per group). * $P < 0.05$.

Expression of FAK Does Not Regulate Renal Inflammation during I/R Injury

Expression of pro-inflammatory chemokines by proximal tubular epithelium is a critical factor in the regulation of immune cell infiltration in the kidney during I/R injury²² and was found to be dependent on NF- κ B activation.²³ Recent studies have shown involvement of FAK in the regulation of NF- κ B.^{14,24} Because the extent of the inflammatory response is correlated to the extent of tubular injury during I/R injury, we performed immunostaining for the pan-leukocyte marker CD45 and the macrophage marker F4/80 to determine whether FAK deficiency also reduced the accumulation of inflammatory cells after renal ischemia. However, no significant difference in the number of CD45- and F4/80-expressing cells was found between kidneys from FAK^{loxP/loxP} and FAK^{ΔloxP/ΔloxP} animals after ischemia (see Supplemental Figure S2 at <http://ajp.amjpathol.org>).

To confirm our *in vivo* finding that FAK is not involved in the regulation of the inflammatory response after ischemia, we examined chemokine/cytokine production in

primary cultured FAK-expressing and FAK-deficient tubular epithelial cells. Cells were subjected to H₂O₂ to mimic the *in vivo* biochemical perturbation caused by the oxidative stress associated with reperfusion after renal ischemia.⁴ Culture medium samples were collected to determine the release of IL-6 and MCP-1. Although H₂O₂ caused a time-dependent increase in both IL-6 and MCP-1 levels, no difference between FAK-expressing and FAK-deficient cells was observed (see Supplemental Figure S3 at <http://ajp.amjpathol.org>). These results suggest that modulation of FAK expression does not influence pro-inflammatory cytokine production *in vitro* and does not result in alterations in immune cell infiltration in our *in vivo* model.

Loss of FAK Reduces the Rate of Focal Adhesion Recovery after Oxidative Stress

We have demonstrated that I/R injury modulates FA restructuring dynamics and phosphorylation of FAK.⁵ These findings indicated that FAK plays a critical role in

establishing recovery of cell adhesion during I/R injury. To provoke FA disruption we exposed primary cultured renal cells to H_2O_2 to mimic oxidative stress, an important *in vivo* biochemical cellular perturbation component during reperfusion. FAs were identified at different time points by immunostaining for the FA protein vinculin (Figure 5A). Analysis of culture supernatant fluid that was collected at each time point and analyzed for LDH release showed that no significant cell killing occurs after 8 hours of H_2O_2 exposure and that there is no difference in cell death between $FAK^{loxP/loxP}$ and $FAK^{\Delta loxP/\Delta loxP}$ cells during exposure (see Supplemental Figure S4 at <http://ajp.amjpathol.org>). After 2 hours of exposure to H_2O_2 , a 40% decrease in the number of FAs occurred in both FAK-expressing and FAK-deficient cells (Figure 5B). Recovery of the FA organization started after 2 hours but was slightly delayed in FAK-deficient cells and accompanied by a shape change in FAs compared with that in FAK-expressing cells (Figure 5, A and B). This suggests that the loss of FAK increases the stability of FAs during recovery and is in accordance with previous studies reporting a similar effect of FAK deficiency on increased FA size.²⁵

FAK Facilitates Activation of JNK-Associated FA Recovery via Phosphorylation of Ser178 Paxillin

Oxidative stress-induced cell injury during I/R injury involves activation of the stress kinase JNK.^{26,27} JNK phosphorylates the FA adapter protein paxillin at serine residue 178 (Ser178).²⁸ Because FAK is necessary for normal FA turnover, this paxillin phosphorylation site can recruit FAK and therefore is critical for regulation of FA dynamics.²⁹ We hypothesized that loss of FAK might affect the activation of JNK pathway as well as its interaction with paxillin signaling and, ultimately, affect the FA dynamics after oxidative stress in tubular epithelial cells. We found that H_2O_2 induced phosphorylation of JNK and its kinase substrate c-Jun after 30 minutes of exposure (Figure 6, A and B), which was associated with an increase of P_{Ser178} -paxillin expression (Figure 6C). No effect of H_2O_2 was observed on either P_{Tyr397} -FAK (Figure 6D) or P_{Tyr118} -paxillin (Figure 6C). In the absence of FAK, phosphorylation of JNK by H_2O_2 was significantly decreased (Figure 6A), which coincided with reduced phosphorylation of c-Jun (Figure 6B) as well as reduced P_{Ser178} -paxillin (Figure 6C). Although ERK and p38 MAPK phosphorylation occurred during *in vitro* oxidative stress, no difference was observed in the level of phosphorylation of both kinases between FAK-expressing and FAK-deficient cells (data not shown).

Under control conditions little P_{Ser178} -paxillin was observed at FAs, whereas H_2O_2 exposure resulted in a rapid increase in P_{Ser178} -paxillin at these sites with colocalization of vinculin (Figure 7A). In FAK-deficient cells however, P_{Ser178} -paxillin phosphorylation during oxidative stress was found to be much weaker (Figure 7B). In FAK-expressing cells the number of cells with FAs that are P_{Ser178} -paxillin positive increased rapidly to ~60% after 2 hours of H_2O_2 exposure, whereas this number was

Figure 5. FAK mediates FA recovery after H_2O_2 exposure. FAK-expressing and FAK-deficient ($FAK^{loxP/loxP}$ and $FAK^{\Delta loxP/\Delta loxP}$, respectively) primary renal cells were cultured on coverslips and treated with 1 mmol/L H_2O_2 for indicated time periods. **A:** The location of FAs was analyzed by immunofluorescent staining for vinculin and followed by confocal laser scanning microscopic analysis. Magnified images show shape changes in FAs during exposure. Original magnification: $\times 60$. **B:** For each condition and time point, five different fields on each coverslip were analyzed to quantify the percentage of vinculin-negative cells. Results indicate a slower recovery of FA after 2 hours of H_2O_2 exposure in FAK-deficient cells compared with FAK-expressing cells. Data are expressed as mean $\pm$ SEM ($n = 3$). * $P < 0.05$, significant difference between $FAK^{loxP/loxP}$ and $FAK^{\Delta loxP/\Delta loxP}$ cells.

significantly lower in FAK-deficient cells (Figure 7, B and C). These results indicate that resident JNK/ P_{Ser178} -paxillin signaling, which is involved in FA assembly in response to oxidative stress, is impaired in FAK-deficient cells.

Figure 6. FAK mediates JNK-dependent serine phosphorylation of paxillin after H₂O₂ exposure. FAK-expressing and FAK-deficient (FAK^{loxP/loxP} and FAK^{ΔloxP/ΔloxP}, respectively) primary renal cells were exposed to 1 mmol/L H₂O₂ for indicated time periods. Phosphorylation of P_{Thr183}/P_{Tyr185}-JNK (**A**), P_{Ser63}-c-Jun (**B**), P_{Ser178}-paxillin and P_{Tyr118}-paxillin (**C**), and P_{Tyr397}-FAK (**D**) was determined with phospho-specific antibodies. Western blot analyses show less activation of JNK/P_{Ser178}-paxillin signaling in FAK-deficient cells during H₂O₂-induced oxidative stress and are representative of three independent experiments. Densitometric analysis of the blots determined the ratio between phosphorylated and total protein (arbitrary units). Data are expressed as mean ± SEM (*n* = 3). **P* < 0.05.

Discussion

In this study we investigated the role of FAK in renal I/R injury with the use of selective and conditional deletion of *fak* in proximal tubules. We find that proximal tubule-specific deletion of *fak* protects against I/R-induced renal injury by decreasing tubular damage, which is associated with a decrease in KIM-1 expression and tubular cell proliferation. Our *in vitro* studies

show that oxidative stress-associated signaling is involved in the dynamic turnover of FAs and that this process is impaired in FAK-deficient cells. This is reflected by a reduced activation of the stress kinase JNK and the subsequent phosphorylation of the FA-associated adapter protein paxillin at Ser178.

Genetic *fak* knockout mice display embryonic lethality (at embryonic day 8.5).¹⁵ Because of its ubiquitous ex-

Figure 7. FAK mediates serine phosphorylation of paxillin at FAs after H₂O₂ exposure. FAK-expressing and FAK-deficient (FAK^{loxP/loxP} and FAK^{ΔloxP/ΔloxP}, respectively) primary renal cells were cultured on coverslips and treated with 1 mmol/L H₂O₂ for indicated time periods. **A:** The location of serine-phosphorylated paxillin and FAs after 2-hour exposure was analyzed by immunofluorescent staining of P_{Ser178}-paxillin and vinculin, respectively, and followed by confocal laser scanning microscopic analysis. Representative images show the colocalization of vinculin (red) and H₂O₂-induced P_{Ser178}-paxillin (green). Original magnification: ×60. **B:** Magnified images show expression of P_{Ser178}-paxillin during exposure. Original magnification: ×60. **C:** For each condition and time point, five different fields on each coverslip were analyzed to quantify the percentage of P_{Ser178}-paxillin-positive cells. Significant lower percentages of P_{Ser178}-paxillin-positive cells were detected in the FAK-deficient group from 30 minutes to 2 hours of H₂O₂ exposure, which is consistent with the observation from Western blot analysis. Data are expressed as mean ± SEM (*n* = 3). **P* < 0.05, significant difference between FAK^{loxP/loxP} and FAK^{ΔloxP/ΔloxP} cells.

pression pattern, deletion of *fak* in adulthood will result in a complex knockout phenotype in the context of I/R injury affecting tubular epithelial, endothelial, and inflammatory cell function. For example, FAK deficiency impaired pathogen-killing and decreased lifespan of neutrophils.³⁰ Nonspecific knockdown after systemic administration of small interfering RNA targeting FAK in mice resulted in tissue insulin resistance.³¹ Therefore, tissue-orientated Cre/LoxP system was used to induce a spatially conditional knockout of *fak* in the mouse.^{16,17} It was observed in our experiments that, although tamoxifen administration induced complete multiorgan *fak* recombination and significant reduction of renal FAK expression in FAK^{loxP/loxP}//*Rosa-Cre-ERT2* mice, the same treatment only resulted in partial renal *fak* recombination and exclusively proximal tubular FAK deficiency in FAK^{loxP/loxP}//*γGT-Cre-ERT2* mice. Nevertheless, to eliminate the potential effects of *fak* knockout on renal physiology as well as individual knockout mouse variability, the unilateral ischemia model was used in our experiments whereby the contralateral kidney from each animal was used as an internal control for injury. Because I/R injury predomi-

nantly affects cells of the proximal tubules,³² we aimed to study the role of FAK specifically in this segment of the nephron.

FAK deficiency significantly protected against renal injury on the basis of various injury markers. This suggests that FAK signaling is involved in the pathophysiology of I/R-induced acute renal failure. It fits our previous observation that FAK undergoes dephosphorylation directly after ischemia and thereafter is differentially hyperphosphorylated at different phosphorylation sites during the reperfusion phase.⁵ KIM-1 expression is an early marker of cellular injury that is specific for the proximal tubular cells and is induced by different types of injuries, including I/R injury.¹⁹ Because FAK deficiency reduced I/R-caused KIM-1 up-regulation, cell proliferation elevation, as well as renal tissue damage, we anticipated that the lack of FAK also alleviates the cellular stress response that is otherwise driven by the hyperphosphorylation of FAK during reperfusion.

In vitro studies have shown a central role for FAK in different cellular signaling pathways such as cell stress signaling,²⁸ survival,¹² and proliferation¹³ next to its role

in controlling cell adhesion dynamics¹⁰ and regulating NF- κ B-mediated expression of pro-inflammatory factors.¹⁴ However, establishing the role of FAK in tissue injury *in vivo* has been hampered by the difficulty to induce spatiotemporal knockdown of FAK expression. A recent study that used inducible, myocyte-specific knockdown of FAK showed a role for FAK in NF- κ B-dependent cell survival after myocardial infarction and the reduction of infarction size in control animals compared with mice with FAK-deficient myocytes.³³

Here, we propose a role for FAK in the initiation of tubular epithelial cell stress next to the control of dynamic FA reorganization. The *in vivo* results that FAK deficiency alleviated tubular damage, KIM-1 expression, and tubular cell proliferation during I/R injury, but did not affect inflammatory cell influx, suggest that tubular epithelial cell stress rather than renal inflammation is mediated by FAK. We did not examine whether FAK deficiency of the proximal tubular epithelium alters macrophage subset polarization by induction of differential expression of pro-inflammatory cytokines.³⁴ However, because macrophage influx significantly increases at day 3 after ischemia and peaks at day 7,³⁵ the involvement of macrophage in the injury process studied here may be expected to be restricted to a minimum. We were able to mimic the *in vivo* biochemical perturbation caused by the oxidative stress associated with reperfusion after renal ischemia with the use of H₂O₂ exposure on primary renal cells, although *in vivo* oxidants are usually composed of a variety of species. Previous studies have shown that reactive oxygen species mediates FA complex disassembly accompanied by FAK dephosphorylation and weakens FA-mediated cell matrix adhesion of tubular epithelial cells.⁴ We confirmed that H₂O₂ profoundly impaired FA stability by initiating FA turnover in both FAK-expressing and FAK-deficient cells. Although NF- κ B-mediated expression of MCP-1 and IL-6³⁶ increased in response to H₂O₂ exposure, this was independent of FAK expression. Whether oxidative stress is the critical component in regulating FA disturbance *in vivo* during renal I/R injury needs further investigation.

The stress kinase JNK is an important player in the cellular stress response to injury and is activated during I/R *in vivo*.^{26,37} Interestingly, our data showed that FAK deficiency suppressed oxidative stress-induced activation of JNK in renal cells. This is consistent with a model in which the protection against renal injury is because of reduced JNK activation.³⁸ We have previously demonstrated that active phosphorylated JNK is localized at FAs in renal cells on chemical-induced injury.³⁸ Previous studies also suggest a physical association between FAK and JNK via the adaptor protein JIP3.³⁹ FAK may therefore target JNK to FAs during oxidative stress, contributing to its localized activation and stress signaling. There is increasing evidence that JNK plays an important role in controlling FA dynamics.²⁸ These findings point to an interplay between FAK, JNK, and control of FA dynamics and a role for these proteins in the stress response during tubular epithelial injury.

Pharmacologic modulation of FAK activity in the kidney may represent a valid strategy to protect against I/R-

induced renal failure. FAK inhibitors have been studied for their potential as anticancer therapy and might therefore be "repurposed."⁴⁰

In conclusion, our data indicate a role for FAK signaling in proximal tubules during progression of I/R-induced acute renal injury. We propose a model whereby FAK is required for recruitment of JNK to FAs leading to JNK-mediated phosphorylation of paxillin at Ser178, thereby facilitating FA turnover during oxidative stress. This FAK/JNK/paxillin linkage could potentially be the basis for future targets for therapeutic intervention in acute renal failure.

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