Sustained Activation of EGFR Triggers Renal Fibrogenesis after Acute Kidney Injury

Jinhua Tang,* Na Liu,* Evelyn Tolbert,† Murugavel Ponnusamy,† Li Ma,† Rujun Gong,† George Bayliss,† Haidong Yan,* and Shougang Zhuang*

From the Department of Nephrology,* Shanghai East Hospital, Tongji University School of Medicine, Shanghai, China; and the Department of Medicine,† Rhode Island Hospital, Alpert Medical School of Brown University, Providence, Rhode Island

Severe acute kidney injury (AKI) is frequently accompanied by maladaptive repair and renal fibrogenesis; however, the molecular mechanisms that mediate these acute and chronic consequences of AKI remain poorly understood. In this study, we examined the role of epidermal growth factor receptor (EGFR) in these processes using waved-2 (Wa-2) mice, which have reduced EGFR activity, and their wild-type (WT) littermates after renal ischemia. Renal EGFR phosphorylation was induced within 2 days after ischemia, increased over time, and remained elevated at 28 days in WT mice, but this was diminished in Wa-2 mice. At the early stage of postischemia (2 days), Wa-2 mice developed more severe acute renal tubular damage with less reparative responses as indicated by enhanced tubular cell apoptosis, and reduced dedifferentiation and proliferation as compared to WT animals. At the late stage of postischemia (28 days), Wa-2 mice exhibited a less severe renal interstitial fibrosis as shown by reduced activation/proliferation of renal myofibroblasts and decreased deposition of extracellular matrix proteins. EGFR activation also contributed to cell cycle arrest at the G2/M phase, a cellular event associated with production of profibrogenetic factors, in the injured kidney. Collectively, these results indicate that severe AKI results in sustained activation of EGFR, which is required for reparative response of renal tubular cells initially, but eventually leads to fibrogenesis. (Am J Pathol 2013, 183: 160–172; http://dx.doi.org/10.1016/j.ajpath.2013.04.005)

Acute kidney injury (AKI) is a serious clinical problem with mortality approaching 50%. After injury, the kidney can either be completely repaired, leaving no lasting evidence of damage, or incompletely repaired, resulting in renal fibrosis. Currently, approximately one-third of patients with AKI progress to chronic kidney disease (CKD), which is characterized by tubular atrophy, proliferation of renal interstitial myofibroblasts, persistent tubulointerstitial inflammation, and excessive deposition of extracellular matrix proteins.1,2 As AKI is a major cause of CKD and AKI can also exacerbate CKD and hasten development of end-stage renal disease,3–5 understanding the molecular events that determine physiological repair and pathological fibrosis of the kidney may lead to therapeutic interventions that promote renal repair and prevent development of renal fibrosis.

Emerging evidence suggests that tyrosine kinase receptors play important roles in renal epithelial repair and regeneration. It has been reported that specific epidermal growth factor receptor (EGFR) deletion in the renal proximal tubule or treatment with erlotinib, a specific EGFR inhibitor, delayed renal function recovery following ischemia/reperfusion (I/R) injury.6 Similarly, Waved-2 (Wa-2) mice expressing a point mutation in EGFR that reduces receptor tyrosine kinase activity by 90% resulted in a slow recovery of renal function after AKI.7 On the other hand, activation of EGFR with exogenous EGF or heparin-binding EGF-like growth factor (HB-EGF) can accelerate tubular cell proliferation and promote renal functional recovery after acute ischemic injury.8,9 These studies suggest that EGFR activation is involved in the regulation of renal repair and functional recovery after AKI.

Supported by NIH grant DK-085065 (S.Z.), National Nature Science Foundation of China grants 81270778 (S.Z.), 81170638 (H.Y.), and 81200492 (N.L.), and the Pudong New District Foundation of China (PWZxk2010-02).
However, several studies have also indicated that EGFR activation contributes to the development and progression of renal fibrosis after chronic kidney injury. For example, transgenic mice overexpressing a kidney tubule-specific, dominant-negative EGFR construct attenuated tubulointerstitial fibrotic lesions in the kidney after subtotal renal ablation or following chronic infusion of angiotension II (Ang II). Pharmacological inhibition of EGFR with gefitinib prevented the decline of renal function and reduced the development of renal vascular and glomerular fibrosis in a rat model of N^G^'-nitro-L-arginine methyl ester (L-NAME)-induced hypertension. Moreover, genetic and pharmacological blockage of EGFR attenuated renal fibrosis induced by unilateral ureteral obstruction (UUO) injury.

EGFR is a transmembrane receptor with intrinsic tyrosine kinase activity and is expressed in both renal epithelial cells and renal interstitial fibroblasts. Ligand binding to EGFR induces it to phosphorylate specific tyrosine residues within its cytoplasmic domains, which serve as docking sites for signaling molecules, and subsequently initiating activation of multiple intracellular signaling pathways, such as the extracellular signal-regulated kinase1/2 (ERK1/2) pathway, the phosphoinositide-3-kinase (PI3K)/Akt pathways and the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways. Activation of these pathways triggers expression of numerous genes needed for several biological cellular responses, including cell survival, proliferation, dedifferentiation, and fibrosis.

Although EGFR has been shown to mediate both tubular reparative and fibrotic responses after injury, all those observations were made separately in different acute or chronic kidney injury models. To date, a fundamental unanswered question is whether EGFR is activated in both the early and late phases of injury and mediates both repair and fibrotic responses in the same animal models. In addition, it is also unclear whether EGFR exerts these functional effects by acting in different cell types. In this study, we attempted to address these issues by using a mouse model of severe AKI induced by unilateral I/R injury. Our data indicated that severe I/R injury induces a sustained EGFR activation, which is essential for the tubular cell regenerative response at the early stage of reperfusion, but eventually leads to the activation/proliferation of renal interstitial myofibroblasts and development of renal fibrosis in this model.

Materials and Methods

Antibodies and Reagents

Antibodies against p-EGFR, p-STAT3, STAT3, p-Akt, Akt, and vimentin were purchased from Cell Signaling Technology (Dancers, MA). Antibody to Pax-2 was purchased from Invitrogen (Carlsbad, CA). Antibodies to EGFR, proliferating cell nuclear antigen (PCNA), GAPDH, collagen I(A2), and fibronectin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against p-histone H3 was purchased from Abcam (Cambridge, MA). Antibody to neutrophil gelatinase-associated lipocalin (NGAL) was purchased from R&D Systems (Minneapolis, MN). Antibody to α-smooth muscle actin (α-SMA) and α-tubulin, all secondary antibodies, and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Wa-2 Mice and Unilateral I/R Model

Wa-2 mice were maintained on a C57BL/6Jei × C3H/HeSnJ background (Jackson Laboratory, Bar Harbor, ME). The unilateral I/R model was established in 6- to 8-week-old male Wa-2 mice and their wild-type (WT) littermates, with six mice in each group. Mice were anesthetized with 75 mg/kg ketamine (i.p.) and 50 mg/kg dexmedetomidine (Dexdomitor) (i.m.). A flank incision was made on the left side; the renal arteries and veins were isolated from the surrounding tissue by blunt dissection and then occluded with a non-traumatic vascular clamp (85-g pressure; Roboz Surgical Instruments, Gaithersburg, MD) for 45 minutes at 37°C. The sham-operated kidney was used as the control, in which the renal pedicles were isolated, but no clamps were applied. At days 2, 7, 14, or 28 after surgery, animals were sacrificed and the kidneys were collected and then horizontally cut into two pieces. One piece of the kidney was fixed in formaldehyde and used for immunofluorescence, immunochemistry, and Masson’s trichrome staining; another piece was snap-frozen in liquid nitrogen and used for immunoblot analysis. Because the healthy contralateral kidney can complement the function of the injured kidney, there is usually no azotemia in this model. Thus, renal function was not measured.

Tubular Injury

Tubular injury was scored on a scale from 0 to 3, where 0 = normal, 1 = injury <30%, 2 = injury 30% to 60%, 3 = injury >60%. To detect cell death, kidneys were removed at day 2 after I/R injury. The TUNEL staining was performed according to the protocol provided by Roche Molecular System (Branchburg, NJ).

Immunoblot Analysis

Immunoblot analysis for tissue samples was performed according to our previous protocols. The densitometry analysis of immunoblot results was conducted with ImageJ software version 1.45 (NIH, Bethesda, MD).

Immunohistochemical and Immunofluorescent Staining

Immunofluorescence staining was performed according to the procedure described in our previous studies. Renal tissues were fixed in 4.5% buffered formalin, dehydrated, and then embedded in paraffin. For immunofluorescent staining, the tissue sections were rehydrated and labeled.
with antibodies, including primary antibodies Pax-2 (1:50), PCNA (1:50), p-EGFR (1:50), α-SMA (1:200), p-histone H3 (1:250), p-STAT3 (1:100), and p-Akt (1:100), and then exposed to fluorescein isothiocyanate or Texas Red–labeled secondary antibodies (Invitrogen). PAS staining was performed in the Department of Pathology at Rhode Island Hospital. Masson’s trichrome staining was performed according to the procedure described by the manufacturer (Sigma-Aldrich). For quantitative assessment of renal fibrosis, the collagen tissue area (blue color) was measured using Image Pro-Plus software version 7 (Media-Cybernetics, Silver Spring, MD) by drawing a line around the perimeter of positive staining area, and the average ratio to each microscopic field (×100) was calculated and graphed.

**Statistical Analysis**

All data were presented as means ± SEM for each group. Comparisons between intergroups were made using one-way analysis of variance followed by Tukey’s test. Statistically significant differences were considered at $P < 0.05$.

**Results**

**Phosphorylation and Expression of EGFR in the Kidney after I/R Injury in WT and Wa-2 Mice**

As an initial step toward understanding the role of EGFR in renal repair and renal fibrosis, we examined the phosphorylation and expression of EGFR in the kidney after I/R injury in Wa-2 mice and their WT littermates. To induce the severe kidney injury that is able to trigger fibrogenesis and to ensure enough survival animals (at least six) for analysis, a model of unilateral renal ischemia for 45 minutes was used. As shown in Figure 1, A–C, I/R injury induced phosphorylation of EGFR in the kidneys of WT mice, which was slightly increased at day 2, remained at similar levels at day 7, peaked at day 14, and remained elevated at 28 days. Total EGFR was also increased at day 2 and then gradually increased until 28 days after ischemia. However, the up-regulation of phosphorylated EGFR (p-EGFR) was not only due to the increased level of total EGFR because the ratio of p-EGFR to total EGFR was still increased in a time-dependent manner during the course of I/R injury (Figure 1B). In Wa-2 mice, renal p-EGFR levels were significantly inhibited at both the early (day 2) (Figure 1, D and E) and the late phase (day 28) of the injury (Supplemental Figure S1). The renal EGFR level in Wa-2 mice was not altered at day 2 (Figure 1, D and F), but was slightly reduced at day 28 after ischemic injury (Supplemental Figure S1). Immunofluorescent staining showed that p-EGFR was primarily located in renal tubular cells in the early phase (day 2) of I/R injury (Figure 1B). In Wa-2 mice, renal p-EGFR levels were significantly inhibited at both the early (day 2) (Figure 1, D and E) and the late phase (day 28) of the injury (Supplemental Figure S1). The renal EGFR level in Wa-2 mice was not altered at day 2 (Figure 1, D and F), but was slightly reduced at day 28 after ischemic injury (Supplemental Figure S1). Immunofluorescent staining showed that p-EGFR was primarily located in renal tubular cells in the early phase (day 2) of I/R injury (Figure 1G), but it was also observed in interstitial myofibroblasts as indicated by co-localization of α-SMA and p-EGFR (Supplemental Figure S2C). Further, myofibroblasts are mostly localized in the area with severely damaged renal tubules, but rare to find in areas with less tubular damage (Supplemental Figure S2, A–C) at 2 days after I/R injury. The number of cells expressing both α-SMA and p-EGFR or α-SMA or p-EGFR alone were markedly reduced in the kidneys of
Wa-2 mice (Supplemental Figure S2D). These data illustrated that the kidney exposed to severe ischemic injury led to EGFR activation, which occurs predominately in tubular cells and also in myofibroblasts in the early phase of AKI. Further, EGFR activation is required for activation of renal interstitial fibroblasts.

Reduction of EGFR Kinase Activity Potentiates the Kidney Damage in the Early Phase of I/R-Induced AKI

To determine the role of EGFR activation in I/R-induced AKI, changes in kidney morphology, apoptosis, and expression of the injury marker neutrophil gelatinase associated lipocalin (NGAL), were assessed at 2 days after ischemia. Tubular dilation, swelling, necrosis, luminal congestion, and hemorrhage were present in the kidneys of WT mice subjected to I/R injury, but these changes were more severe in Wa-2 mice (Figure 2A). Scoring of kidney sections for histopathological damage to the tubules showed an increase in renal damage in Wa-2 mice as compared with WT mice (Figure 2B). Sham-operated WT and Wa-2 mice incurred no tubular injury (Figure 2, A and B). The TUNEL staining showed more positivity in renal tubular cells of Wa-2 mice compared with WT mice after I/R injury (Figure 2, C and D). NGAL was not detected in control kidneys by immunoblot analysis, but its expression level was increased in the kidney of WT mice after I/R injury and further increased in the injured kidneys of Wa-2 mice (Figure 2, E and F). Immunofluorescent staining showed that NGAL expression is limited to renal proximal tubular cells (data not shown). Taken together, these data indicate that EGFR activation is necessary for renoprotection in the early phase of AKI.

EGFR Activation Is Required for Renal Tubular Cell Dedifferentiation and Proliferation in the Early Phase of I/R-Induced AKI

Fate mapping studies indicated that renal repair after AKI is carried out mainly by proliferation of dedifferentiated intrinsic renal tubular cells. Although EGFR has been shown to be involved in renal tubular cell proliferation after AKI, little is currently known about the signaling mechanism that regulates renal tubular cell dedifferentiation in response to injury in vivo. Our previous studies showed that EGFR activation plays a regulatory role in primary cultures of renal tubular cell dedifferentiation after...
oxidant injury. Here, we further examined whether EGFR also mediates this process in injured kidney at 2 days after ischemia. As shown in Figure 3, A and B, Pax2, a hallmark of dedifferentiation of renal epithelial cells located in nuclei, is absent in healthy adult tubules, but was re-expressed in the kidneys of WT mice after I/R injury. Its expression was markedly decreased in the kidneys of Wa-2 mice. Similarly, vimentin, another marker of renal tubular cell dedifferentiation, was also re-expressed in the kidneys of WT mice subjected to I/R injury and significantly decreased in Wa-2 mice (Figure 3, C and D).

Dedifferentiated renal tubular cells acquire the ability to proliferate and migrate, and proliferation of intrinsic renal epithelial cells has been recognized as the predominant mechanism of renal regeneration. We further examined the role of EGFR in renal tubular proliferation in the injured kidney by assessing expression of PCNA, a marker of cell proliferation, at 2 days after ischemia. Immunofluorescence staining showed that PCNA was mainly expressed in the nucleus of proximal tubular cells, and the number of PCNA-positive cells was increased in the injured kidneys of WT mice but significantly decreased in Wa-2 mice (Figure 3, E and F). A very small number of PCNA-positive cells was also observed in the kidneys of WT control mice, reflecting the minimal turnover rate of renal tubular cells under physiological conditions. Western blot analysis confirmed such changes in the kidneys of WT and Wa-2 mice after I/R injury (Figure 3, G and H). Therefore, these results indicated that EGFR activation is required for renal tubular cell dedifferentiation and proliferation following I/R injury.
EGFR Activation Is Required for STAT3 and Akt Phosphorylation in the Early Phase of I/R-Induced AKI

Because activation of STAT3 and Akt signaling pathways has been reported to be associated with cell survival and proliferation, we examined the phosphorylation status of renal STAT3 and Akt in WT and Wa-2 mice in the early time course of I/R-induced AKI. Phospho-Akt (p-Akt) and phospho-STAT3 (p-STAT3) were not detectable in sham-operated kidneys, and their levels were increased in the kidney of WT mice subjected to I/R injury. However, I/R-induced expression of p-Akt and p-STAT3 was significantly suppressed in the kidneys of Wa-2 mice (Figure 4, A, B, and D). Total STAT3 and Akt were also increased in the injured kidneys of WT mice, but remained at the same levels in the kidneys of Wa-2 mice (Figure 4, A, C, and E). These data suggest that EGFR may regulate renal regenerative responses through activation of, at least, the STAT3 and Akt signaling pathways.

EGFR Activation Contributes to Renal Fibrogenesis at the Late Phase of I/R Injury

AKI can progress to CKD, which is characterized by activation of renal interstitial fibroblasts (as indicated by expression of α-SMA) and expression of extracellular matrix proteins (ie, fibronectin and collagen I). We investigated whether EGFR activation is associated with renal fibrogenesis after AKI. As shown in Figure 5, A–D, basal levels of collagen I and α-SMA were detected in the kidneys of WT mice. After I/R injury, their expression levels were slightly elevated at 2 days, further increased at 7 days, and reached the maximum at 14 and 28 days (Figure 5, A–C). Fibronectin was not detectable in control kidneys. After I/R injury, its expression was induced at 14 days and continuously elevated up to 28 days after I/R injury in the kidney of WT mice (Figure 5, A and D). By contrast, the expression level of fibronectin was significantly reduced, and collagen I and α-SMA were suppressed to basal levels at 28 days in Wa-2 mice subjected to chronic I/R injury (Figure 5, E–H). Consistent with these observations, I/R injury also induced expression of interstitial collagen fibrils in the kidney of WT mice at 14 and 28 days, which was reduced in Wa-2 mice as demonstrated by Masson’s trichrome staining (Figure 6, A and B, and Supplemental Figure S3).

Transformation of interstitial fibroblasts into α-SMA-positive myofibroblasts is the main mechanism for production of extracellular matrix proteins in the evolution of CKD. We therefore examined localization and expression of α-SMA and p-EGFR in the kidneys of both WT and Wa-2 mice at 28 days after I/R injury. p-EGFR was colocalized with most of the α-SMA-positive cells (Figure 6, C and D) and also expressed in the renal tubular cells of the injured kidney (Supplemental Figure S1), indicating that p-EGFR is expressed in both renal interstitial myofibroblasts and tubular epithelial cells in the late phase of ischemic injury. Taken together with diminished expression of α-SMA, collagen I, and fibronectin in the injured kidneys of Wa-2 mice, this suggests that EGFR plays a critical role in mediating activation of renal interstitial myofibroblasts and renal fibrogenesis after prolonged I/R injury.

EGFR Activation Is Required for Expression of PCNA in Renal Myofibroblasts after I/R Injury

Another feature of renal fibrogenesis is proliferation of renal interstitial myofibroblasts. Because EGFR activation has been demonstrated to be associated with proliferation of cultured renal fibroblasts in vitro, we here examined whether EGFR activation also contributes to their proliferation in vivo after I/R injury by measuring expression of PCNA. Western blot analysis indicated that PCNA was barely detectable in the kidney of the sham-operated WT mice: I/R injury induced its expression, and none was detected in Wa-2 mice (Figure 7, A and B). Consistent with this observation, immunostaining showed a large number of PCNA-positive cells in the kidneys of WT mice after I/R injury, as indicated by expression of p-EGFR.
injury, and a smaller number of PCNA-positive cells was in the kidney of Wa-2 mice. In the early phase of AKI, we observed that PCNA is mostly expressed in the nucleus of tubular cells (Figure 3E). In contrast to this, PCNA was mainly expressed in the cytoplasm of interstitial renal myofibroblasts 28 days after I/R injury, which is indicated by its colocalization with α-SMA (Figure 7, C and D). To confirm the localization of PCNA in renal interstitial myofibroblasts, cultured NRK-49F cells were also stained with antibodies against PCNA, α-SMA, and DAPI. As shown in Supplemental Figure S4, PCNA is localized in the cytoplasm of NRK-49F cells.

EGFR Activity Contributes to Renal Epithelial Cell Arrest in the G2/M Phase of Cell Cycle after I/R Injury

It has been shown that renal tubular cells arrested in the G2/M phase demonstrate a prominent profibrotic phenotype and result in increased production of profibrotic growth factors/cytokines.37 Phosphorylation of histone H3 (p-H3) at serine 10 is a hallmark of cells in G2/M phase.38 To understand the role of EGFR in this process, we examined the expression of p-H3 by immunoblot analysis and immunostaining in WT and Wa-2 mice subjected to I/R injury. As shown in Figure 8, A and B, a large number of renal tubular cells labeled with p-H3 were detected in WT mice at 28 days after I/R injury; the number of p-H3-positive cells was significantly decreased in the injured kidney of Wa-2 mice. Consistent with this observation, immunoblot analysis showed that p-H3 was barely observed in sham-operated mice, but its expression was increased in the kidney of WT mice after I/R injury and decreased in Wa-2 mice (Figure 8, C and D). Thus, activation of EGFR is essential for driving renal epithelial cell cycle to arrest at G2/M phase after I/R injury.

EGFR Mediates the Phosphorylation of STAT3 and Akt after Prolonged I/R Injury

STAT3 has been shown to be activated in the obstructed kidney and is associated with the development and progression of renal fibrosis in a murine model of UUO.21,34 The Akt pathway was also shown to mediate HB-EGF–induced heart fibrosis and proliferation of cardiac fibroblasts.39 Thus, we examined whether EGFR also mediates the activation of these signaling pathways at the
later stage of I/R injury. As shown in Figures 9 and 10, phosphorylated levels of STAT3 and Akt were significantly increased at 28 days after I/R injury in WT mice. By contrast, phosphorylated STAT3 and Akt levels were diminished in the injured kidney of Wa-2 mice. Immunofluorescent co-staining of p-STAT3 and α-SMA indicated that p-STAT3 was mainly expressed in renal interstitial myofibroblasts (Figure 9D), which was consistent with its localization in UUO-injured kidneys.34 Further, co-staining of p-Akt with α-SMA was also observed in the injured kidneys of WT mice (Figure 10D). Expression levels of both were markedly reduced in the kidneys of Wa-2 mice (Figures 9 and 10). These data, combined with localization of p-EGFR in renal myofibroblasts at the later stage of AKI, suggest that EGFR may mediate activation and proliferation of renal myofibroblasts through activation of STAT3 and Akt signaling pathways.

**Discussion**

AKI can lead to complete or incomplete tubular repair, depending on the severity of the insult. After mild injury, renal repair usually results in a return to a normal structural and functional state, but severe injury is frequently accompanied by the development of renal fibrosis.40,41 The molecular mechanism that determines renal tubular repair and the fibrotic responses to injury remains poorly understood. Here, we took an advantage of a murine model of unilateral severe I/R-induced AKI to determine whether EGFR plays a pivotal role in these processes. Our results indicate that severe I/R injury induced a sustained activation of EGFR, which is required for renal regenerative responses (tubular cell dedifferentiation and proliferation) in the early phase, but is in turn, a critical mediator of renal fibrogenesis in the later phase of AKI. Thus, we suggest that EGFR exerts dual roles in the course of severe AKI and that sustained EGFR activation is a critical molecular event that leads to development of renal tubulointerstitial fibrosis.

Ischemic injury is a common cause of AKI. Following injury, some tubular epithelial cells die by apoptosis and necrosis. The surviving tubular cells undergo dedifferentiation, proliferation, and migration, finally re-differentiating into mature tubular epithelial cells.42 Dedifferentiation of renal epithelial cells is the initial step of the renal regenerative response after injury. Although our in vitro studies have shown that EGFR activation regulates dedifferentiation of renal tubular cells in primary cultures of renal tubular cells after oxidant injury,24 the regulatory mechanism in vivo remains unclear. In this study, we
demonstrated the role of EGFR signaling in mediating this process as evidenced by re-expression of vimentin and Pax2 (two dedifferentiation markers expressed in mesenchymal cells, but not in the mature nephron) in injured renal tubular cells of WT mice and reduced expression in Wa-2 mice after AKI. Similarly, we found that PCNA, a marker of cell proliferation, was markedly elevated in the kidneys of WT mice after I/R injury and decreased in Wa-2 mice. Because proliferation of renal epithelial cells is considered a cellular response secondary to dedifferentiation and the predominant mechanism of renal repair, our data suggest that EGFR activation contributes to renal regeneration after severe injury.

It has been documented that the reparative response is usually incomplete in the kidney subjected to severe injury and is mostly accompanied by fibrogenesis. In this study, we observed that renal expression of collagen I and fibronectin, as well as α-SMA, was up-regulated over time, with the maximum at 28 days after I/R injury in WT mice; however, their expression levels were largely suppressed in the kidney of Wa-2 mice. In addition, we also demonstrated that EGFR is responsible for proliferation of renal interstitial cells.

Figure 7 Expression and location of PCNA after chronic I/R injury in WT and Wa-2 mice. A: The kidney tissue lysates were subjected to immunoblot analysis with specific antibodies against PCNA and α-tubulin. B: The expression levels of PCNA and α-tubulin were quantified by densitometry and normalized with α-tubulin. Data are means ± SEM (n = 6). Representative immunoblots are from two samples from six animals in each group. C: The kidney tissues collected at day 28 were used for co-staining with antibodies to PCNA and α-SMA, and overlay with DAPI. D: Positive staining (×400) of both PCNA and α-SMA, or α-SMA, or PCNA alone were counted in 10 high-power fields and expressed as means ± SEM. Means with different symbols are significantly different from one another, P < 0.05.

Figure 8 Expression of p-histone H3 in WT and Wa-2 mice after chronic I/R injury. A: Photomicrographs (×200) illustrate p-histone H3 (Serine 10) staining of the kidney tissues collected at day 28 after sham and I/R injury in WT and Wa-2 mice. B: Epithelial cells with positive staining for p-histone H3 were counted in 10 high-power fields and expressed as means ± SEM. C: Lysates of kidney tissues collected at day 28 after sham and I/R injury were subjected to immunoblot analysis with specific antibodies against p-histone H3 and α-tubulin. D: The expression levels of p-histone H3 were quantified by densitometry and normalized with α-tubulin. Data are means ± SEM (n = 6). Representative immunoblots are from two samples from six animals in each group. Means with different symbols are significantly different from one another, P < 0.05.
myofibroblasts in kidneys after long-term I/R injury. This suggests that EGFR mediates renal fibrogenesis under this pathological condition. Consistent with our observation, other studies also showed that EGFR activation is involved in the development/progression of renal fibrosis in different animal models after chronic injury. It has been reported that increased EGFR activation was associated with interstitial fibrosis and tubular atrophy after prolonged ischemia or chronic infusion of Ang II\textsuperscript{10,11}; activation of EGFR promotes transforming growth factor β (TGF-β)-dependent renal fibrosis\textsuperscript{43}; and pharmacological or genetic inhibition of EGFR inhibits renal deterioration and fibrogenesis induced by Ang II, UUO, or 5/6 renal nephrectomy.\textsuperscript{10,11,13} Thus, it seems that EGFR functions as a double-edged sword, regulating repair and fibrosis.

The dual role of EGFR in renal regeneration and renal fibrosis may be associated with the degree and length of its activation. In contrast to slight and transient EGFR phosphorylation induced by acute and mild injury,\textsuperscript{7} chronic and severe kidney injury usually induces prolonged activation.\textsuperscript{13,43} In agreement with this observation, we demonstrated that severe ischemic injury (renal artery clamping for 45 minutes) induced a sustained increase in p-EGFR, which occurred 2 days reperfusion of post-ischemic injury, gradually increased further and remained elevated 28 days after ischemic injury. At this point, increased deposition of extracellular matrix and overexpression of collagen I, fibronectin, and α-SMA were clearly detected in the injured kidney. Similar to this observation, we recently observed that UUO injury induces a persistent activation of EGFR, which is required for renal fibrosis.\textsuperscript{13} Although p-EGFR is mainly expressed in renal tubular cells soon (2 days) after I/R injury, it is expressed in both renal tubular and renal interstitial myofibroblasts later after I/R injury (28 days). Furthermore, we observed an increased number of renal interstitial cells coexpressing PCNA and α-SMA in the kidney, which further emphasizes the importance of EGFR in regulating activation/proliferation of renal myofibroblasts. The mechanism that regulates sustained EGFR activation in the fibrotic kidney is not completely understood, but persistent production of EGFR ligands or down-regulation of the machinery responsible for EGFR activation/degradation may be involved in this process. In this regard, it was recently reported that reactive oxygen species–dependent phosphorylation of Src is associated with prolonged activation of EGFR in cultured renal epithelial cells exposed to TGF-β1.\textsuperscript{43} Src may exert this effect through phosphorylation of c-Cbl and/or Cool, two signaling molecules that participate in the regulation of EGFR endocytosis and degradation.\textsuperscript{44,45} Further studies are needed to address which EGFR ligands are persistently produced during the course of chronic kidney damage after ischemic injury.

It has been reported that renal tubular cells arrested in the G2/M stage of the cell cycle after severe or sustained kidney injury acquire the ability to produce profibrogenic cytokines such as TGF-β1 and connective tissue growth factor (CTGF).\textsuperscript{37} This suggests that inhibition of epithelial cells arrested at G2/M phase may help prevent fibrogenesis and attenuate progression of an acute injury to a chronic one. In this study, we observed that p-histone-H3, a hallmark of

**Figure 9** Expression and location of STAT3 after chronic I/R injury in WT and Wa-2 mice. A: The kidney tissue lysates were subjected to immunoblot analysis with specific antibodies against p-STAT3, STAT3, and α-tubulin. B: The expression levels of p-STAT3 and STAT3 were quantified by densitometry, and the ratio between p-STAT3/STAT3 was calculated. C: Total STAT3 level was normalized with α-tubulin. Data are means ± SEM (n = 6). Representative immunoblots are from two samples from six animals in each group. D: The kidney tissues collected at day 28 were used for co-staining with antibodies to p-STAT3, α-SMA, and DAPI. E: Positive staining (×400) cells of both p-STAT3 and α-SMA, or α-SMA, or p-STAT3 alone were counted in 10 high-power fields and expressed as means ± SEM. Means with different symbols are significantly different from one another. \( P < 0.05 \).
cells arrested at the G2/M stage, was highly expressed in renal tubular cells of WT mice after I/R injury and largely inhibited in Wa-2 mice, suggesting that sustained EGFR activation is capable of promoting renal fibrogenesis through conversion of renal epithelial cells to a profibrotic phenotype.

It is interesting to note that PCNA is expressed in the nuclei of renal tubular cells, but in the cytoplasm of renal myofibroblasts. It has been documented that PCNA is a crucial factor in DNA synthesis and repair. Its biological function in the cytoplasm of renal myofibroblasts is not clear. Two recent studies showed that PCNA localized in the cytoplasm of neutrophils is able to interact with procaspases, preventing their activation and thereby promoting cell survival. In this context, the localization of PCNA in renal myofibroblasts may also enhance their ability to survive and render them more resistant to antifibrotic treatments. The cellular mechanism that controls localization of PCNA to the nucleus or cytoplasm of different cell types is unclear; our data indicated that genetic inactivation of EGFR markedly reduced expression levels of PCNA in both renal tubular cells and myofibroblasts. This suggests that EGFR activation is required for biological function of PCNA. Further studies are needed to address the functional role of cytoplasmic PCNA in renal interstitial myofibroblasts.

Phosphorylation of EGFR leads to activation of several important signaling pathways, including STAT3 and Akt. It has been shown in many cell types that STAT3 and Akt activation is associated with cell survival and proliferation. We demonstrated that phosphorylation (activation) of STAT3 and Akt is increased in both the early and late phases following I/R injury in the kidneys of WT mice, and their phosphorylation was markedly attenuated in the kidneys of Wa-2 mice, suggesting an important role for EGFR in modulating their activation in both phases. Previous studies have shown that active Akt and STAT3 are primarily located in renal tubular cells, highlighting the important role of these signaling pathways in transducing EGFR activation in renal tubular repair and regeneration. In the later phase of post I/R injury, p-STAT3 and p-Akt were predominately located in renal myofibroblasts. These data, together with our recent observation that p-EGFR is expressed in myofibroblasts of fibrotic kidney after UUO injury, suggest that activation of these two pathways is key to EGFR-mediated regulation of renal myofibroblast activation and proliferation. Because EGFR activation contributes to PCNA expression, and PCNA is mostly located in the cytoplasm of renal interstitial myofibroblasts, it may also modulate renal myofibroblast survival. As described above, cytoplasmic PCNA has been identified as a key regulator of cell lifespan.

The development of fibrosis after acute tubular injury has important clinical consequence. In recent years, progression of acute injury to chronic disease due to abnormal repair mechanisms has gained a great deal of attention. The dual roles of EGFR in renal repair and fibrosis in severe AKI suggest that EGFR may be an important therapeutic target. Although the potential beneficial effect of EGFR activation in renal structural and functional recovery after acute injury has been reported in animal studies, our previous and current studies clearly indicate that prolonged activation of...
EGFR is implicated in renal fibrogenesis. This suggests a limit to using growth factors to enhance renal regeneration after severe AKI and may also explain why a clinical trial using insulin-like growth factor-1 (IGF-1) in patients with AKI failed to promote renal function.\textsuperscript{50} As with EGFR activation, induction of IGF-1 receptor activation with IGF-1 also exerts a profibrotic effect in addition to promoting growth in renal tubular cells.\textsuperscript{51}

\section*{Conclusion}

In summary, our studies demonstrated that severe I/R injury induces EGFR activation, which is essential for initiating the kidney’s regenerative responses through tubular cell dedifferentiation and proliferation. Sustained EGFR activation leads to renal fibrogenesis by inducing renal myofibroblast activation and proliferation in late phase following ischemic injury. The different effects of EGFR may be associated with the degree and length of EGFR activation and expression in different cell types. Understanding the molecular mechanism triggering prolonged EGFR activation following severe kidney injury may help to develop strategies for accelerating renal regeneration and preventing renal fibrogenesis.

\section*{Supplemental Data}

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2013.04.005.

\section*{References}