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Src family kinases regulate renal epithelial dedifferentiation through activation of EGFR/PI3K signaling

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Abstract

Dedifferentiation, a process by which differentiated cells become mesenchymal-like proliferating cells, is the first step in renal epithelium repair and occurs *in vivo* after acute kidney injury and *in vitro* in primary culture. However, the underlying mechanism remains poorly understood. In this report, we studied the signaling events that mediate dedifferentiation of proximal renal tubular cells (RPTC) in primary culture. RPTC dedifferentiation characterized by increased expression of vimentin concurrent with decreased expression of cytokeratin-18 was observed at 24 h after the initial plating of freshly isolated proximal tubules and persisted for 72 h. At 96 h, RPTC started to redifferentiate as revealed by reciprocal expression of cytokeratin-18 and vimentin and completed at 120 h. Phosphorylation levels of Src, epidermal growth factor receptor (EGFR), AKT ([a target of phosphoinositide-3-kinase (PI3K)] and ERK1/2 were increased in the early time course of culture (<72 h). Inhibition of Src family kinases (SFKs) with PP1 blocked EGFR, AKT and ERK1/2 phosphorylation, as well as RPTC dedifferentiation. Inhibition of EGFR with AG1478 also blocked AKT and ERK1/2 phosphorylation and RPTC dedifferentiation. Although inactivation of the PI3K/AKT pathway with LY294002 inhibited RPTC dedifferentiation, blocking the ERK1/2 pathway with U0126 did not show such an effect. Moreover, inhibition of SFKs, EGFR, PI3K/AKT, but not ERK1/2 pathways abrogated RPTC outgrowth and SFK inhibition decreased RPTC proliferation and migration. These findings demonstrate a critical role of SFKs in mediating RPTC dedifferentiation through activation of the EGFR/PI3K signaling pathway.

Keywords

Src family kinases; dedifferentiation; epidermal growth factor receptor; proximal tubular cells

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Introduction

The ability of the kidney to recover from functional or structural injury has long been appreciated by clinicians. The kidney can completely recover from an ischemic or toxic insult if renal injury is not too severe. Recently, two research groups have demonstrated that intrarenal cells, but not bone marrow stem cells contribute significantly to restore tubular epithelium during repair of the postischemic kidney in adult mice (Duffield et al., 2005; Lin et al., 2005). Further, proximal renal tubular cells (RPTC) have been identified to be the major cell type responsible for repopulation of tubular cells after acute kidney injury (AKI) (Duffield et al., 2005; Lin et al., 2005). Since dedifferentiation is an initial and critical step during renal regeneration after injury, understanding the mechanism of epithelial dedifferentiation would offer new strategies for improving recovery following AKI.

To date, the mechanism by which RPTC undergo dedifferentiation after AKI *in vivo* is unknown. Like the *in vivo* tubular repair after AKI, renal epithelial cells in primary culture can also dedifferentiate into mesenchymal-like proliferating phenotype and then redifferentiate spontaneously to restore their epithelial state (Elberg et al., 2008; Smith et al., 2006). Therefore, the primary culture of RPTC provides an ideal system to study the mechanism leading to RPTC dedifferentiation/redifferentiation. Using this system, we have recently shown that activation of the epidermal growth factor receptor (EGFR) is required for RPTC dedifferentiation following oxidant injury (Zhuang et al., 2005). Conversely, inactivation of EGFR promotes RPTC redifferentiation (Hallman et al., 2008). EGFR is a tyrosine kinase receptor that can be activated by ligand dependent and independent mechanisms. Ligand-independent activation of EGFR is termed receptor transactivation that can be induced by some non-specific stimuli such as oxidative and osmotic stresses (Fischer et al., 2004; Kuper et al., 2009; Zwick et al., 1999). The mechanism of EGFR transactivation has been studied in a variety of cell types. Our and other groups have reported that Src family kinases (SFKs) can mediate EGFR activation in response to different stimuli including oxidant injury (Jamroz-Wisniewska et al., 2008; Wu et al., 2002; Zhuang et al., 2008; Zhuang and Schnellmann, 2004).

SFKs are a group of non-receptor tyrosine kinases, which are activated in response to a variety of growth factors/cytokines and some environmental stresses such as oxidants and osmotic shock and play a role in regulating proliferation, migration, adhesion and dedifferentiation in different cell types (Kefalas et al., 1995; Okutani et al., 2006; Thomas and Brugge, 1997). Among nine members in this family, three of them (Src, Fyn and Lyn) are expressed in renal epithelial cells (Thomas and Brugge, 1997) (Xing et al., 2008). SFKs regulate cellular functions through activation of multiple intracellular signaling pathways including phosphoinositide-3-kinase (PI3K/AKT) and extracellular signaling-regulated kinase1/2 (ERK1/2). Our recent studies revealed that Src mediates RPTC proliferation through a mechanism involved in the activation of the phosphoinositide-3-kinase/Akt pathway (Xing et al., 2008). He et al., demonstrated that Src-mediated activation of ERK1/2 is involved in the regulation of glomerular podocyte dedifferentiation and proliferation (He et al., 2004). However, it remains unclear whether SFK, phosphoinositide-3-kinase/AKT and ERK1/2 signaling pathways mediates the regulation of renal tubular cell dedifferentiation.

The purpose of this study was to determine the functional role of SFKs and associated signaling events in RPTC dedifferentiation in primary culture. Our results demonstrated that SFKs-mediated activation of the EGFR/PI3K/AKT signaling pathway plays a critical role in RPTC dedifferentiation whereas the ERK1/2 pathway is not involved in this process.

Materials and Methods

Reagents

PP1, PP3, AG1478 and LY294002 were purchased from Biomol (Plymouth Meeting, PA). Antibodies to phospho-Src (Tyr⁴¹⁶), phospho-Akt, phospho-ERK1/2, phospho-EGFR, AKT, Src, EGFR, ERK1/2 were purchased from Cell Signaling Technology (Danvers, MA). Anti-vimentin or cytokeratin-18 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma (St Louis, MO).

Isolation and culture of mouse renal proximal tubules

Mouse renal proximal tubules were isolated by the method as described by Nowak et al., (Nowak et al., 2003). Both kidneys were removed and dissected under sterile conditions. Cortical tissue was cut off and finely minced with a scalpel blade. Minced tissue was incubated for 30 min at 37°C (with shaking) in digestion medium consisting of Hanks' solution, collagenase type 4 (140 U/ml), and soybean trypsin inhibitor (0.75 mg/ml). Following sedimentation of undigested tissue, the supernatant containing cortical tubules were collected and centrifuged for 2 min $\times$ 50 g at 4°C. The pellet was washed with Hanks' solution, centrifuged for 2 min $\times$ 50 g at 4°C, washed again with DMEM/F-12, centrifuged, and resuspended in DMEM/F-12 medium. Cortical tubules were purified by gradient centrifugation in a 40% Percoll/60% DMEM/F-12 at 36,000 g $\times$ 20 min at 4°C. The band containing renal proximal tubules were collected and washed twice with DMEM/F-12, and the final pellet was resuspended in DMEM/F-12. The culture medium was a 50:50 mixture of DMEM/F-12 nutrient mix without phenol red and pyruvate, supplemented with 15 mM NaHCO₃, 15 mM HEPES, 1 mM glucose, and 5 mM lactate. Human transferrin (5 μ g/ml), selenium (5 ng/ml), hydrocortisone (50 nM), and bovine insulin (5 μ g/ml) were added to the medium immediately before media change.

Determination of cell proliferation

Cell proliferation was measured by BrdU incorporation assay and cell counting. RPTC (1×10^5) were cultured in a 12-well plate. After various treatments, BrdU incorporation assay was performed using a colorimetric BrdU cell proliferation enzyme-linked immunosorbent assay kit (Roche Applied Science, Penzberg, Germany). Briefly, BrdU labeling solution was added to cells and then incubated for 4 h. At the end of incubation, the labeling medium was removed, the cells were fixed, and the DNA was denatured. After addition of the anti-BrdU-peroxidase conjugate, the immune complexes were detected by subsequent reaction with tetramethylbenzidine as substrate for 20 min. The reaction product was quantified using a Spectramax M5 plate reader. In a separate 12-well plate, cells were trypsinized and cell counting was conducted using the hemocytometer.

Migration assay

To measure RPTC migration, RPTC were grown to confluence, and monolayers were wounded with a rubber policeman to produce a linear 4-mm swipe. After being washed once with PBS, cells were incubated in the medium for 24 h in the presence of 0.5 µg/ml mitomycin C to block any proliferation. Cell migration was determined using a microscope and camera, and the wound area was calculated using National Institutes of Health Image software.

Preparation of cell lysates and immunoblot analysis

After treatments, RPTCs were washed twice with phosphate-buffered saline without Ca^{2+} and Mg^{2+} and harvested in lysis buffer (0.25 M Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, and 0.5% 2-mercaptoethanol). Cells were disrupted by sonication for 15 s and the protein concentration was determined with the BCA protein assay kit (Pierce, Rockford, IL). 20 mg of protein from each sample were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. After treatment with 5% skim milk at 4 °C overnight, membranes were incubated with various antibodies for 1 h and then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) for 1 h. Bound antibodies were visualized after chemiluminescence detection on autoradiographic film. Representative immunoblots from three experiments are shown for each immunoblot result.

Statistical analysis

Each experiment was conducted three times. Data are presented as means $\pm$ SD and were subjected to one-way ANOVA. Multiple means were compared using Tukey's test. The differences between two groups were determined by Student's *t*-test. $P < 0.05$ was considered a statistically significant difference between mean values.

Results

Dedifferentiation of renal epithelial cells in primary cultures of renal tubular cells

Primary cultured renal tubular cells from isolated renal tubules can convert to the dedifferentiated phenotype and then proliferate and migrate, which is similar to the process of renal tubular cell regeneration *in vivo* after injury (Bonventre, 2003b). As such, this system can be used to study the mechanism of renal epithelial cell dedifferentiation. To establish the primary culture of RPTC, proximal tubules were isolated from the adult mouse kidney, plated in plain dishes and then cultured in a defined medium. One day after plating, RPTC started to grow out from open ends of tubular fragments as spindle-shaped cells that were well spread over the plates and then continued to grow to form cellular islands surrounding the remnant tubules over time. After 7 days, islands of cellular outgrowth became progressively larger to form a confluent monolayer of epithelial cells (Figure 1A).

Dedifferentiation is characterized by increased expression of vimentin and decreased expression of cyokeratins. Vimentin, a cytoskeleton filament that is expressed only in mesenchymal cells after birth (Bachmann et al., 1983; Holthofer et al., 1984), can be re-expressed in epithelial cells during the recovery phase that follows ischemia or nephrotoxic

tubular necrosis (Gossrau et al., 1989; Grone et al., 1987; Nouwen et al., 1994; Wallin et al., 1992; Witzgall et al., 1994) and *in vitro* in primary culture (Franke et al., 1979; Hatzinger et al., 1988). Therefore, its expression in adult renal tubular cells is considered as a marker of cellular dedifferentiation. In contrast, cytokeratin-18 is a primary keratin that is only expressed in differentiating epithelia and thus its expression is a feature of terminally differentiated epithelial cells (Baer et al., 2006). To monitor the dynamic changes in the expression of vimentin and cytokeratin-18 in primary cultures of RPTC, we harvested freshly isolated proximal tubules from mouse kidneys and RPTC grown out of proximal tubules on day 1, 2, 3, 4, 5, 7 after plating, and then conducted immunoblot analysis using antibodies against vimentin and cytokeratin-18. As shown in Figure 1B, abundant vimentin and cytokeratin-18 were detected in isolated tubules. Following plating, their expression altered reciprocally: vimentin expression was maintained at high levels for the first three days and then gradually decreased to a basal level on day 7, the time by which cells were confluent. In contrast, cytokeratin-18 levels declined to a low level at 24 h after plating, minimum at 48 h and then gradually restored. On day 5 and 7, its expression levels returned to that in isolated tubules before plating. These results clearly illustrated that increased expression of vimentin is induced in the process of tubule isolation whereas decreased expression of cytokeratin-18 occurs during the culture. The increased expression of vimentin concurrent with decreased expression of cytokeratin-18 between 24 and 72 h after plating suggests that the dedifferentiated phenotype of RPTC is predominant during this time frame, which allows us to explore the role of signaling events that regulate RPTC dedifferentiation.

SFK activity is required for RPTC dedifferentiation

It has been reported that the SFK activity is required for dedifferentiation of podocytes (Chuang and He, 2009), parotid acinar cells (Fujita-Yoshigaki et al., 2008) and hepatocytes (Godoy et al., 2009). Here we examined the role of SFKs in RPTC dedifferentiation using a Src selective inhibitor, PP1. As a negative control, PP3, a structurally related but biologically inactive isomer was also included. As shown in Figure 2A, RPTC were grown out of proximal tubules on day 3 in tubules exposed to the vehicle (DMSO). In the presence of PP1, RPTC outgrowth was inhibited in a dose dependent manner with a complete blockade at 20 μ M. In contrast, 20 μ M PP3 did not affect RPTC outgrowth. Immunoblot analysis showed that vimentin expression was completely blocked and cytokeratin-18 expression was upregulated in PP1-treated RPTC. In comparison, expression levels of these two proteins were not altered in cells exposed to PP3. These data suggest that the SFK activity is required for RPTC dedifferentiation and outgrowth.

Since SFK activity is positively regulated by phosphorylation of at tyrosine 416 in the catalytic domain (Leu and Maa, 2003) and our recent studies showed that c-Src mediates cell proliferation in a murine proximal tubular cell line (Xing et al., 2008), we examined the kinetic expression of phospho-Src (p-Src) at Tyr 416 in RPTC and the effect of PP1. Figure 3A shows that p-Src was expressed in isolated proximal tubules, maintained for the first three days after plating when the dedifferentiated phenotype predominates, and then decreased. On day 7, p-Src levels declined to basal levels. PP1 treatment inhibited Src phosphorylation. At 20 μ M, it reduced the p-Src to 20% of the control level (RPTC without PP1 exposure). In comparison, 20 μ M PP3 treatment did not affect Src phosphorylation

(Figure 3B, C). Total Src expression levels did not change under these experimental conditions (Figure 3B). These data suggest that RPTC dedifferentiation is associated with c-Src activation.

SFKs mediate activation of EGFR, Akt and ERK1/2

Our previous studies showed that EGFR activity is involved in RPTC dedifferentiation following oxidant injury (Zhuang et al., 2005) and other studies indicated that the PI3K/AKT and ERK1/2 pathways mediate dedifferentiation in hepatocytes and podocytes, respectively (Godoy et al., 2009; He et al., 2004). To determine the downstream signaling molecules that mediate the dedifferentiation function of SFKs in RPTC, we examined the effect of SFK inhibition on activation of EGFR, AKT and ERK1/2. As shown in Figure 4A, phosphorylated EGFR and AKT were observed in isolated renal tubules and RPTC in the early course of culture after plating (<4 days). At the later time course of the culture (day 4–7), phosphorylation levels of these proteins decreased. ERK1/2 phosphorylation was also detected in the isolated tubules but kept at constant levels through the time course of the culture until day 7. Treatment with PP1, but not PP3 inhibited phosphorylation of all these proteins (Figure 4B)

We next examined whether EGFR mediates AKT and ERK1/2 activation in RPTC. As shown in Figure 4C, treatment with AG1478, a specific EGFR inhibitor, blocked AKT and ERK1/2 phosphorylation whereas total protein levels of these two proteins did not change. Therefore, EGFR activation is also required for the activation of the PI3K/AKT and ERK1/2 pathways.

Collectively, these results indicated that Src mediates activation of EGFR, which in turn, activates the PI3K/AKT and ERK signaling pathways in cultured mouse RPTC.

Inhibition of EGFR and the PI3K/AKT pathway blocks RPTC dedifferentiation

To evaluate the role of EGFR and the PI3K/Akt and ERK1/2 pathways in renal epithelial cell dedifferentiation, pharmaceutical inhibitors, AG1478, LY294002 and U0126, were directly added to the culture at 24h after plating of proximal tubules. AG1478 is a specific inhibitor of EGFR. LY294002 and U0126 are selective inhibitors for the PI3K/AKT and ERK1/2 pathways, respectively. Figure 5A shows that treatment of cells with either AG1478 or LY294002, resulted in a complete inhibition of RPTC outgrowth. In contrast, U0126 did not show such an inhibitory effect. Consistent with this observation, AG1478 and LY294002, but not U0126, inhibited the expression of vimentin and increased cytokeratin-18 expression. AG1478, LY294002, U0126 blocked phosphorylation of EGFR, Akt and ERK1/2, respectively. Total levels of EGFR, Akt and ERK1/2 did not change in these experimental settings (Figure 5B, D, C). These data, together with the results obtained in Figure 4, suggest that the EGFR-PI3K pathway may play a critical role in transducing Src activation to renal epithelial dedifferentiation.

Src is required for RPTC proliferation and migration after plating

Given the importance of Src in mediating RPTC dedifferentiation, we further examined the role of Src in RPTC proliferation and migration in primary cultures of mouse RPTC. Figure

6 demonstrated that treatment of cells with PP1 dramatically inhibited RPTC proliferation as evaluated by measuring BrdU incorporation and counting cell numbers. To evaluate the role of Src in RPTC migration, a wounding healing assay was performed. A “wound” was made in confluent RPTC using a rubber policeman and then incubated for 24 h in the absence or presence of PP1. RPTC migrated into the denuded area and partially filled the wound area 24 h following injury. In the presence of PP1, RPTC migration was inhibited (Figure 7). These data suggest that Src also mediates RPTC proliferation and migration.

Discussion

Mature renal proximal tubular cells (RPTC) are quiescent and exhibit a differentiated phenotype. In response to injury, or upon *in vitro* culture, RPTC undergo dedifferentiation and then re-enter the cell cycle to proliferate (Bonventre, 2003a; Franke et al., 1979; Hatzinger et al., 1988; Witzgall et al., 1994). Although the mechanism of tubular cell proliferation has been extensively investigated *in vitro* and *in vivo*, little is known about the mechanism of renal tubular cell dedifferentiation. In this study, we examined the signaling mechanism of dedifferentiation in the primary culture of mouse proximal tubular epithelial cells. Our results revealed that Src, EGFR and AKT are activated during proximal tubule isolation and subsequent culture in the early time course and inhibition of each of them blocked RPTC dedifferentiation. Furthermore, SFK activity was required for activation of EGFR, which, in turn, mediated activation of AKT. Thus, we suggest that the SFKs/EGFR/PI3K/Akt signaling pathway plays a critical role in RPTC dedifferentiation.

Epithelial dedifferentiation is characterized by increased expression of vimentin and decreased cytokeratin-18 and E-cadherin. *In vivo* studies have indicated that renal ischemia/reoxygenation injury results in RPTC vimentin expression, which was detected within day 1, apparent by day 2, and absent by day 16 after injury (Witzgall et al., 1994). Concomitant with vimentin expression, proliferating cell nuclear antigen (a marker of proliferating cells) was expressed in those cells (Witzgall et al., 1994). Consistent with the *in vivo* animal studies, our results revealed that vimentin was abundantly expressed in the isolated proximal tubules and RPTC grew out of tubules within 72 h after plating. Given that mature renal tubules do not express vimentin in the normal kidney, these results suggest that the signal initiated during the isolation process triggers expression of vimentin and elicits the phenotypic switch of renal epithelial cells from the quiescent, “differentiated” state to a dedifferentiated and proliferating state. In addition, it is interesting to notice that in the freshly isolated tubules, increased expression of vimentin was not accompanied by decreased cytokeratin-18 expression, suggesting that vimentin expression is an early response following injury and occurs prior to cytokeratin down-regulation. Co-expression of vimentin and cytokeratin has also been reported in some cells of the regenerating pancreatic duct epithelium after partial pancreatectomy (Ko et al., 2004).

The signals that activate epithelial dedifferentiation after injury are less studied. Previously, we have demonstrated that EGFR is activated and involved in RPTC dedifferentiation following oxidant injury (Zhuang et al., 2005). In the current study, we have extended this observation and revealed that activation of SFKs is essential for RPTC dedifferentiation in primary culture and that EGFR-induced activation of the PI3K/AKT pathway mediates this

action of SFKs. These statements are supported by observations as follows: First, increased phosphorylation of Src, EGFR and AKT were detected in isolated tubules and RPTC grown out of tubules after plating; Second, inactivation of SFKs blocked EGFR phosphorylation and inactivation of EGFR blocked AKT phosphorylation. Third, inhibition of EGFR and the PI3K/AKT pathway resulted in suppression of vimentin expression and RPTC outgrowth. Since all those signaling molecules were activated in isolated proximal tubules, we suggest that the signaling for dedifferentiation starts during the isolation process. The mechanism underlying tubular isolation that resulted in activation of these enzymes is not clear. It is possible that mechanical injury that occurs during the isolation procedure (related to enzymatic digestion, centrifugation, and mechanical manipulation) results in the production of reactive oxygen species and then activates the Src and other signaling molecules. In this regard, it has been reported that hepatocytes suffered from cell injury as a result of the isolation procedure causes production of reactive oxygen species (Frances et al., 2007) and we have previously shown that exposure of RPTC to oxidative stress results in Src activation (Zhuang and Schnellmann, 2004).

Whereas our studies suggest that SFKs mediate RPCT dedifferentiation, the responsible SFK isoform (s) remains unclear. An early study showed that active Src is highly expressed in regenerating tubular cells in a rat model with ischemia/reperfusion injury (Takikita-Suzuki et al., 2003). Our recent studies demonstrated that among three SFKs (Src, Fyn and Lyn) expressed in RPTC, only Src is required for cell proliferation (Xing et al., 2008). These studies, together with the inhibitory effect of PP1 on dedifferentiation, suggest that Src may play an essential role in mediating RPTC dedifferentiation. However, since PP1 does not discriminate between different members of this family (Hanke et al., 1996), we can not rule out the possibility whether other SFKs members are also involved in this process.

It is well established that phenotypic change of RPTC from the differentiated to the dedifferentiated state accompanies their proliferation and migration. It is possible that SFKs-mediated signaling may also play a role in regulating these processes. We examined the role of SFKs in RPTC outgrowth, proliferation and migration in the presence or absence of PP1. Indeed, blockade of SFKs inhibited these events. In addition, we observed that blocking either EGFR or the PI3K/AKT pathway also inhibited RPTC outgrowth. Since cell proliferation and migration are the two major factors that contribute to the movement of cells away from the edge of the explants (Boland et al., 1996) and stimulation of EGFR by exogenous EGFR ligand, epiregulin also promotes both proliferation and migration in RPTC through a PI3K/Akt dependent mechanism (Zhuang et al., 2007), these results suggest that EGFR-dependent activation of the PI3K/AKT pathway may act downstream of Src to mediate these regenerative responses.

Dedifferentiation is a process similar to epithelial-mesenchymal transition. However, unlike trans-differentiation of epithelial into a fibroblast phenotype, dedifferentiated RPTC usually become a fully functional epithelial cells through a process termed re-differentiation (Hallman et al., 2008). How the dedifferentiated RPTC undergo redifferentiation is currently unknown. Since effective tubule repair requires cells that ultimately downregulate the dedifferentiation responses and redifferentiate to an epithelial phenotype, we speculated that suppression of the signaling molecule that mediates dedifferentiation may contribute to this

process. As such, we monitored phosphorylation of Src, EGFR and Akt in primary cultures of RPTC through the time course from dedifferentiation to redifferentiation and revealed that formation of redifferentiated phenotype (increased cytokeratin-18 expression) was accompanied by dephosphorylation of these signaling molecules. Therefore, RPTC may have an intrinsic ability to turn down or terminate activation of the Src-EGFR-PI3K/AKT or other signaling pathways that are associated with dedifferentiation once RPTC become the differentiated phenotype. However, if these molecules are persistently activated in RPTC under some pathological conditions, trans-differentiation of epithelial into a fibroblast phenotype might occur. In this context, it has been reported that transient activation of EGFR following short-term renal ischemia is associated with renal regeneration whereas its prolonged activation after chronic renal ischemia resulted in renal interstitial fibrosis (Terzi et al., 2000; Toubeau et al., 1994; Wang et al., 2003). Therefore, identification of the factors and mechanisms that promote redifferentiation rather than progression to a fibroblast would help to develop new therapeutic approaches for improving patient outcomes following AKI.

In summary, this study illustrates that the Src/EGFR/PI3K/AKT signaling pathway is critically involved in regulating RPTC dedifferentiation. Since the majority of studies suggest that replacement of renal epithelial cells after acute injury occurs via proliferation and migration of existing tubular cells and epithelial de-differentiation is the first step for initiating other regenerative events, this study provides novel insights into the mechanism of renal regeneration.

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Figure 1. RPTC outgrowth and dedifferentiation following proximal tubule plating
 (A) Renal proximal tubules plated (d0) and cultured for 1 to 7 days (d1–d7) and then photographed. (B) Tubules and/or cells were harvested at time points as indicated and cell lysates were prepared and subjected to immunoblot analysis using antibodies to vimentin, cytokeratin-18, or actin. (C) Expression levels of vimentin or cytokeratin-18 were quantified by densitometry and expressed as the percentage of their expression on day0. Values are means $\pm$ SD of 3 independent experiments. Values with asterisks are significantly different from controls ($P < 0.05$).

Figure 2. Effect of PP1 and PP3 on RPTC outgrowth and dedifferentiation

Isolated tubules were cultured for 24 h followed by exposure to DMSO, PP1 (10, 20 μM) or PP3 (20 μM) for an additional 48 h. (A) Microphotographs showing the effect of Src inhibition on RPTC outgrowth; (B) Tubules and/or cells were harvested and cell lysates were prepared and then subjected to immunoblot analysis using antibodies to vimentin, cytokeratin-18 or actin and (C) Expression levels of vimentin or cytokeratin-18 were quantified by densitometry and expressed as the percentage of controls. Values are means ±

SD of 3 independent experiments. Values with asterisks are significantly different from controls ($P < 0.05$).

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Figure 3. The kinetics of Src activation in RPTC following tubule isolation and plating and the effect of PP1 and PP3

Tubules/cells were harvested at time points as indicated (A) or isolated tubules were cultured for 24 h followed by exposure to DMSO, PP1 (10, 20 μ M) or PP3 (20 μ M) for an additional 48 h (B). Cell lysates were prepared and subjected to immunoblot analysis with antibodies to phospho-Src (Tyr-416), or total Src. Expression levels of p-Src in Figure 3B were quantified by densitometry and expressed as the percentage of controls (C). Values are means $\pm$ SD of 3 independent experiments. Values with asterisks are significantly different from controls ($P < 0.05$).

Figure 4. The kinetics of EGFR, AKT and ERK1/2 activation in RPTC following tubule isolation and plating and the effect of PP1 and PP3

Tubules/cells were harvested at time points as indicated (A) or isolated tubules were cultured for 24 h followed by exposure to DMSO, PP1 (20 μ M), PP3 (20 μ M) (B) or AG1478 (C) for an additional 24 h (B, C). Cell lysates were prepared and subjected to immunoblot analysis with antibodies to phospho-EGFR (Tyr-1068), EGFR, phospho-AKT (Ser-473), AKT, phospho-ERK1/2 (Thr202/Tyr204) or ERK1/2. Representative immunoblots from at least 3 experiments are shown.

Figure 5. Effect of EGFR, PI3K/AKT or ERK1/2 pathway inhibitors on RPTC outgrowth and dedifferentiation

Isolated tubules were cultured for 24 h followed by exposure to DMSO, AG1478 (10 μ M), LY294002 (20 μ M) or U0126 (10 μ M) for an additional 24 h. (A) Microphotographs showing the effect of the indicated inhibitors on RPTC outgrowth. (B, C and D) Tubules and/or cells were harvested and cell lysates were prepared and then subjected to immunoblot analysis using antibodies to vimentin, cytokeratin-18, p-EGFR, EGFR, p-AKT, AKT, p-ERK1/2, ERK1/2 or actin. Representative immunoblots from at least 3 experiments are shown.

Figure 6. Effect of Src inhibition on RPTC proliferation

Isolated tubules were cultured for 24 h followed by exposure to DMSO, PP1 (20 μ M) or PP3 (20 μ M). Cell proliferation was determined by BrdU incorporation (A) and by counting cell numbers (B). Data are expressed as means $\pm$ SE, $n = 5$. Values with asterisks are significantly different from controls ($P < 0.05$).

Figure 7. Effect of Src inhibition on RPTC migration

Confluent monolayers of RPTC were scraped to create mechanical injury (A) and then incubated for 24 h with DMSO (B), 20 μ M PP1 (C) or 20 μ M PP3 (D). (E) RPTC migration into the wounded area was quantified by measuring the migration of cells from the wound edge and expressed as percentage of DMSO-treated cultures. Each value represents means $\pm$ SE, $n > 8$. Values with asterisks are significantly different from controls ($P < 0.05$).