Angiotensin II down-regulates nephrin–Akt signaling and induces podocyte injury: role of c-Abl

Qian Yang[a], Yiqiong Ma[a], Yipeng Liu[a], Wei Liang[a], Xinghua Chen[a], Zhilong Ren[a], Huiming Wang[a], Pravin C. Singhal[b], and Guohua Ding[a]
[a]Division of Nephrology, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, China; [b]Renal Molecular Research Laboratory, Feinstein Institute for Medical Research, Hofstra North Shore-Long Island Medical School, Great Neck, NY 11021

ABSTRACT Recent studies have shown that nephrin plays a vital role in angiotensin II (Ang II)–induced podocyte injury and thus contributes to the onset of proteinuria and the progression of renal diseases, but its specific mechanism remains unclear. c-Abl is an SH2/SH3 domain–containing nonreceptor tyrosine kinase that is involved in cell survival and regulation of the cytoskeleton. Phosphorylated nephrin is able to interact with molecules containing SH2/SH3 domains, suggesting that c-Abl may be a downstream molecule of nephrin signaling. Here we report that Ang II–infused rats developed proteinuria and podocyte damage accompanied by nephrin dephosphorylation and minimal interaction between nephrin and c-Abl. In vitro, Ang II induced podocyte injury and nephrin and Akt dephosphorylation, which occurred in tandem with minimal interaction between nephrin and c-Abl. Moreover, Ang II promoted c-Abl phosphorylation and interaction between c-Abl and SH2 domain–containing 5′-inositol phosphatase 2 (SHIP2). c-Abl small interfering RNA (siRNA) and STI571 (c-Abl inhibitor) provided protection against Ang II–induced podocyte injury, suppressed the Ang II-induced c-Abl–SHIP2 interaction and SHIP2 phosphorylation, and maintained a stable level of nephrin phosphorylation. These results indicate that c-Abl is a molecular chaperone of nephrin signaling and the SHIP2-Akt pathway and that the released c-Abl contributes to Ang II–induced podocyte injury.

INTRODUCTION

Podocytes and their foot processes interposed slit diaphragm (SD) play a crucial role in establishing the selective permeability of the glomerular filtration barrier (Greka and Mundel, 2012). Podocyte injury is associated with proteinuria and the progression of glomerular diseases. Angiotensin II (Ang II) is a well-known risk factor for the initiation and progression of kidney disease (Ruster and Wolf, 2006, 2013). In addition to its hemodynamic effects on renal tissue, the direct effect of Ang II on podocyte injury has been documented

extensively (Yu et al., 2013; Shengyou and Li, 2014). Recent studies show that Ang II–induced cytoskeletal rearrangement and podocyte apoptosis contribute to the onset of proteinuria and the progression of renal diseases, but the exact molecular mechanism of Ang II–induced podocyte injury remains unknown.

Nephrin is a widely studied transmembrane protein in the SD region, and several SH2 or SH3 domain–containing proteins, such as podocin (Li et al., 2004), CD2AP (Huber et al., 2003), and Nck (Jones et al., 2006), bind the cytoplasmic domain of nephrin, which suggests that nephrin is a signaling molecule that transmits signals from the SD to the interior of podocytes. Previous studies showed that phosphorylated nephrin is crucial for promoting the survival and maintenance of the stress fibers in podocytes (Huber et al., 2003; Zhu et al., 2008). In concert with those reports, our previous studies demonstrated that Ang II is able to promote nephrin dephosphorylation and podocyte injury, but the nephrin signal transduction pathway deserves further study (Ren et al., 2012). Akt is the major component of the phosphoinositide 3-kinase (PI3K)/Akt pathway and plays an important role in regulating the cytoskeleton and the survival status of various cells

FIGURE 1: Ang II–induced podocyte injury in vivo and in vitro. (A) Representative transmission electron microscopic images of the ultrastructure of capillary loops in each group (original magnification, ×10,000). The black arrows indicate foot process fusion. The white asterisks show chromatin agglutination in the podocyte nuclei. cap, capillary lumen; podo, podocyte. Scale bar, 1900 nm. (B, C) Representative images of double immunofluorescence staining of WT1 and TUNEL in the kidney sections from each group (original magnification, ×400) and quantification of apoptotic podocytes. AngII, Ang II-infused group; Normal, normal saline–infused group. Scale bar, 20 μm. *$p < 0.05$ compared with the

(Fan et al., 2014; Shen et al., 2014). In addition, our previous study showed that podocyte apoptosis is increased after Akt inactivation induced by a PI3K inhibitor (Chen et al., 2009). Several studies have explored the relationship between nephrin phosphorylation and Akt activation (Huber et al., 2003; Zhu et al., 2008; Venkatareddy et al., 2011) and concluded that nephrin dephosphorylation is associated with Akt inactivation and podocyte injury. However, the molecular mechanism underlying nephrin–Akt signaling still needs to be identified.

c-Abl is a nonreceptor protein tyrosine kinase that is expressed in podocytes and contains several functional domains that interact with multiple signaling molecules (Panjarian et al., 2013). Among these functional domains, the SH2 and SH3 modules of c-Abl are regarded as interaction modules and allosteric inhibitors of the catalytic domain (Corbi-Verge et al., 2013). After activation, this protein participates in the regulation of diverse cellular events, including cytoskeletal rearrangement and apoptosis (Ba et al., 2005; Dudek et al., 2010; Mitra and Radha, 2010; Wang et al., 2013). In addition, our previous study showed that c-Abl mediates Ang II–induced podocyte injury (Chen et al., 2013). We hypothesize that c-Abl participates in nephrin–Akt signaling in podocytes. Therefore we examined the role of c-Abl in Ang II–induced podocyte injury and nephrin signal transduction.

RESULTS

Effects of Ang II on podocyte injury in vivo and in vitro

Ang II–infused rats developed severe proteinuria compared with saline-infused rats (Figure 1D). An electron microscopic analysis demonstrated the existence of diffuse foot process fusion (cytoskeletal rearrangement) and chromatin agglutination (podocyte apoptosis) in Ang II–treated rats (Figure 1A). A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to further confirm that Ang II can induce podocyte apoptosis in vivo. As shown in Figure 1, B and C, the Ang II–infused rats exhibited higher apoptotic rates than those observed in normal rats (10.52 ± 3.35 vs. 3.52 ± 1.06 on day 14; 15.26 ± 5.13 vs. 3.51 ± 1.14 on day 28). These findings are consistent with our previous observations.

To assess the effects of Ang II on cultured podocytes, we treated the cells with Ang II (10^{-7} M) at various time points (0, 0.25, 0.5, 3, 6, and 12 h). As shown in Figure 1, E and F, Ang II promoted cytoskeletal rearrangement in the podocytes in a time-dependent manner. The stress fibers in normal cells were disorganized after Ang II treatment, and peripheral actin rims and scattered actin fragments were observed in the Ang II–treated podocytes. Previous reports showed that reorganization of the podocyte cytoskeleton would affect cellular motility (Hsu et al., 2008), which would promote cell detachment and apoptosis. Therefore we further evaluated the effects of Ang II on podocyte migration and apoptosis. As shown in Figure 1, G–I, Ang II markedly provoked podocyte migration and apoptosis in a time-dependent manner.

Effects of Ang II on nephrin expression and Akt dephosphorylation in vivo and in vitro

Previous studies indicated that nephrin plays an important role in podocyte injury and that Akt signaling could participate in this process, but the signal transduction pattern has not been well characterized (Zhu et al., 2008; Hyvonen et al., 2010; Li et al., 2015). Phosphorylated Akt is regarded as the activated form. As shown in Figure 2A, the levels of nephrin and phosphorylated nephrin were significantly decreased in the glomeruli of Ang II–infused rats, and these decreases were accompanied by a reduction in the levels of phosphorylated Akt. Incubation of the cultured podocytes with Ang II significantly reduced the expression levels of nephrin and phosphorylated nephrin in a time-dependent manner (Figure 2B). Furthermore, the phosphorylated Akt level was also decreased after Ang II stimulation in vitro (Figure 2B). According to the quantitative results, the reduction in phosphorylated nephrin results from the combination of decreases in total nephrin expression and its dephosphorylation.

Effects of nephrin on Ang II–induced podocyte injury and Akt phosphorylation

To further verify that Akt signaling participates in nephrin signal transduction, we transfected pc-DNA3.1-NPHS1 into cultured podocytes. As shown in Figure 3A, pc-DNA3.1-NPHS1 transfection significantly reversed the nephrin down-regulation and dephosphorylation of nephrin and Akt observed in Ang II–treated podocytes compared with the effects observed in untransfected or pcDNA3.1-transfected podocytes. Nephrin overexpression partially weakened the F-actin disruption induced by Ang II (Figure 3B). Moreover, cell migration and apoptosis were partially rescued by the transfection of Ang II–stimulated podocytes with nephrin plasmid (Figure 3, C and D). These findings indicate that Akt serves as a downstream intermediate of nephrin signaling and contributes to Ang II–induced podocyte injury.

Effect of Ang II on the interaction of nephrin and c-Abl

Phosphorylated nephrin tends to interact with molecules containing SH2/SH3 domains (Barletta et al., 2003; Jones et al., 2006). Previous studies showed that c-Abl is an SH2/SH3 domain–containing nonreceptor tyrosine kinase involved in cell survival and cytoskeletal regulation. Immunofluorescence assays and coimmunoprecipitation were performed to test whether c-Abl plays a role in nephrin signal transduction in Ang II–treated podocytes. Double immunofluorescence staining revealed that c-Abl and nephrin were colocalized in a linear pattern along the glomerular capillary loops in vivo (Figure 4A) and were coexpressed in the cell membranes and cytoplasm of cultured podocytes (Figure 4C) in the absence of Ang II. According to the quantitative results presented in Figure 2, Ang II stimulation induced not only a decrease in the total expression of nephrin but also its dephosphorylation. Thus we hypothesize that Ang II treatment results in less phosphorylated nephrin available to colocalize with

normal group at the same time point. (D) Quantitative analysis of urinary protein excretion in the different groups ($n = 6$ for each group). *$p < 0.05$ compared with the normal group at the same time point. (E, F) FITC-phalloidin staining and quantification of cortical F-actin score of each group of differentiated mouse podocytes stimulated with 10^{-7} M Ang II for various time points (a–f, podocytes treated with Ang II at 10^{-7} M for 0, 0.25, 0.5, 3, 6, and 12 h, respectively). Scale bar, 10 µm. *$p < 0.05$ compared with the podocytes treated with Ang II for 0 h. (G, H) Representative migration results and quantification of podocytes treated with 10^{-7} M Ang II for various time points. Scale bar, 100 µm. *$p < 0.05$ compared with podocytes treated with Ang II for 0 h. (I) Flow cytometry analysis of the apoptotic rate of differentiated mouse podocytes treated with 10^{-7} M Ang II for various time points. *$p < 0.05$ compared with podocytes treated with Ang II for 0 h.
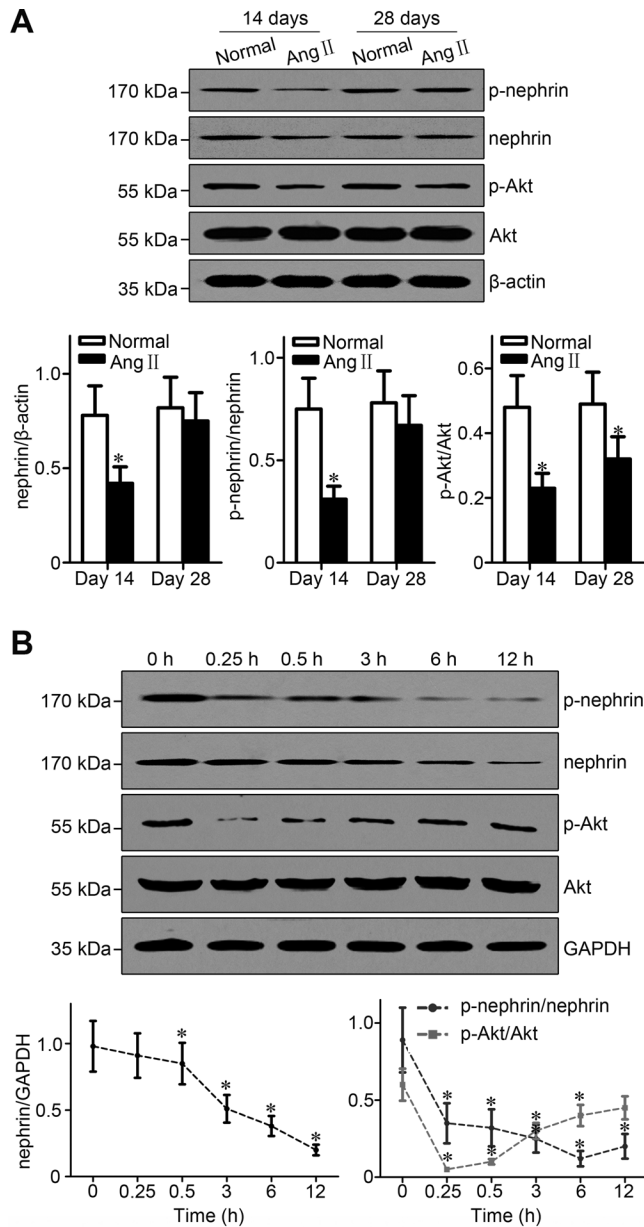
FIGURE 2: Ang II stimulation reduced the expression of nephrin, phospho-nephrin, and phospho-Akt in vivo and in vitro. (A) Representative Western blots of glomerular nephrin, phospho-nephrin, and phospho-Akt expression in the different groups. AngII, Ang II–infused group; Normal, normal saline–infused group. *$p < 0.05$ compared with the normal group at the same time point. (B) Representative Western blots of nephrin, phospho-nephrin, and phospho-Akt expression in 10^{-7} M Ang II–treated podocytes at various times. *$p < 0.05$ compared with podocytes treated with Ang II for 0 h.

c-Abl. As shown in Figure 4, A and C, nephrin–c-Abl colocalization was suppressed after Ang II stimulation both in vivo and in vitro. The same results were obtained in the immunoprecipitation assay. As shown in Figure 4, B and D, the presence of Ang II decreased the level of nephrin–c-Abl complex in the glomeruli and cultured cells. Moreover, Ang II stimulation resulted in a greater reduction in nephrin–c-Abl complex expression compared with the reduction in nephrin expression; thus the reduction in the interaction between nephrin and c-Abl is partially due to a reduction in nephrin expression.

Effects of Ang II on the phosphorylation of c-Abl and SH2 domain–containing 5'-inositol phosphatase 2 and their interaction

Previous observations suggested that c-Abl phosphorylation at tyrosine 412 is necessary for its activation and signal transduction (Brasher and Van Etten, 2000; Tammer et al., 2007). Thus, to determine whether c-Abl is activated in response to Ang II, we stimulated podocytes with Ang II (10^{-7} M) at various time points. The results from a Western blot analysis showed that incubation of the podocytes with Ang II significantly enhanced c-Abl phosphorylation in a time-dependent manner, with maximal phosphorylation at ~3 h (Figure 5A). Previous studies showed that lipid phosphatase SH2 domain–containing 5'-inositol phosphatase 2 (SHIP2) down-regulates the PI3K/Akt pathway by hydrolyzing phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) to phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2) and that this enzymatic activity is controlled by phosphorylation at tyrosine 986/987 (Batty et al., 2007; Prasad et al., 2009). Phosphorylated c-Abl is able to interact with SHIP2 and regulate its activity (Mokhtari et al., 2013). Thus we examined the interaction between c-Abl and SHIP2 by coimmunoprecipitation and SHIP2 phosphorylation using a Western blot assay and found that c-Abl was coimmunoprecipitated with SHIP2 in podocytes and that its binding to SHIP2 was significantly promoted by Ang II treatment for 30 min (Figure 5B). As shown in Figure 5C, similar to the results obtained for c-Abl, the SHIP2 tyrosine phosphorylation was notably increased in the Ang II–exposed podocytes in a time-dependent manner.

Effects of nephrin on the nephrin–c-Abl and c-Abl–SHIP2 interactions and c-Abl phosphorylation

To further characterize the transduction of the nephrin signal, we further examined the nephrin–c-Abl and c-Abl–SHIP2 interactions and c-Abl phosphorylation in pc-DNA3.1-NPHS1–transfected podocytes. As shown in Figure 6A, the nephrin–c-Abl interaction was enhanced in the nephrin-overexpressing podocytes. In addition, the Ang II–induced phosphorylation of c-Abl and its interaction with SHIP2 were markedly decreased by nephrin overexpression (Figure 6, A and B).

Effect of c-Abl on nephrin–Akt signal transduction

A recombinant plasmid (pcDNA3-Abl-His6-FLAG) was transfected into cultured podocytes to overexpress c-Abl. As shown in Figure 7A, c-Abl transfection elevated c-Abl expression by ~60% relative to the levels observed in the control cells. The expression of phosphorylated nephrin was not affected in the c-Abl plasmid–transfected cells with or without Ang II treatment (Figure 7A). c-Abl overexpression could mimic the effects of Ang II on SHIP2 phosphorylation and Akt dephosphorylation. Moreover, transfection with the c-Abl plasmid overtly enhanced the Ang II–induced c-Abl–SHIP2 interaction and SHIP2 phosphorylation accompanied by a further induction of Akt dephosphorylation (Figure 7, A and B).

In addition, c-Abl small interfering RNA (siRNA) and STI571 (a c-Abl inhibitor) were used to reduce c-Abl expression and phosphorylation, respectively. Ang II–promoted c-Abl phosphorylation was normalized by knockdown of c-Abl expression or STI571 treatment (Figure 8A). Furthermore, c-Abl siRNA or pretreatment with STI571 significantly decreased the Ang II–induced interaction of c-Abl with SHIP2 and SHIP2 phosphorylation while maintaining a stable level of nephrin phosphorylation (Figure 8, A and B).

Effect of c-Abl on Ang II–induced podocyte injury

Related in vitro experiments showed that c-Abl overexpression had a similar effect on podocyte injury as Ang II (Figure 7, C–E). In
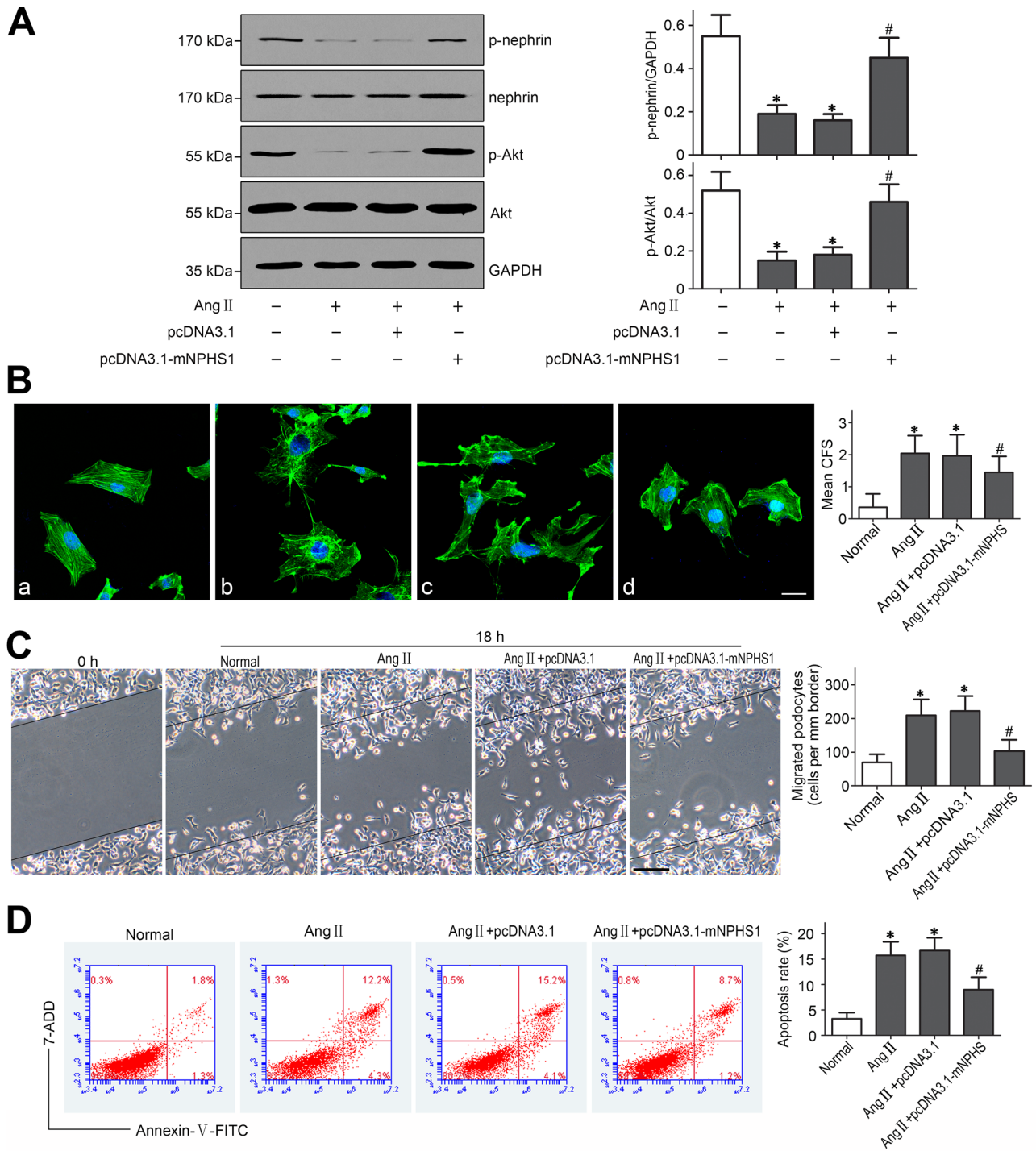
FIGURE 3: Nephrin overexpression attenuated Ang II–induced Akt dephosphorylation and podocyte injury. The podocytes were transfected with no plasmid, pcDNA3.1, or pcDNA3.1-NPHS1 and then stimulated with Ang II (10^{-7} M) for 1 h. Untreated and untransfected podocytes were defined as normal cells. (A) The phospho-nephrin and phospho-Akt expression levels were analyzed by Western blotting. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with cells treated with Ang II only. (B) FITC-phalloidin staining and quantification of the cortical F-actin score of each group. (a) Normal group, (b) Ang II–stimulated group, (c) pcDNA3.1+AngII–stimulated group, and (d) pcDNA3.1-NPHS1+Ang II–stimulated group. Scale bar, 10 μm. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only. (C) Representative migration results and quantification for the different groups. Scale bar, 100 μm. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only. (D) Flow cytometry analysis of apoptosis in the different groups. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only.
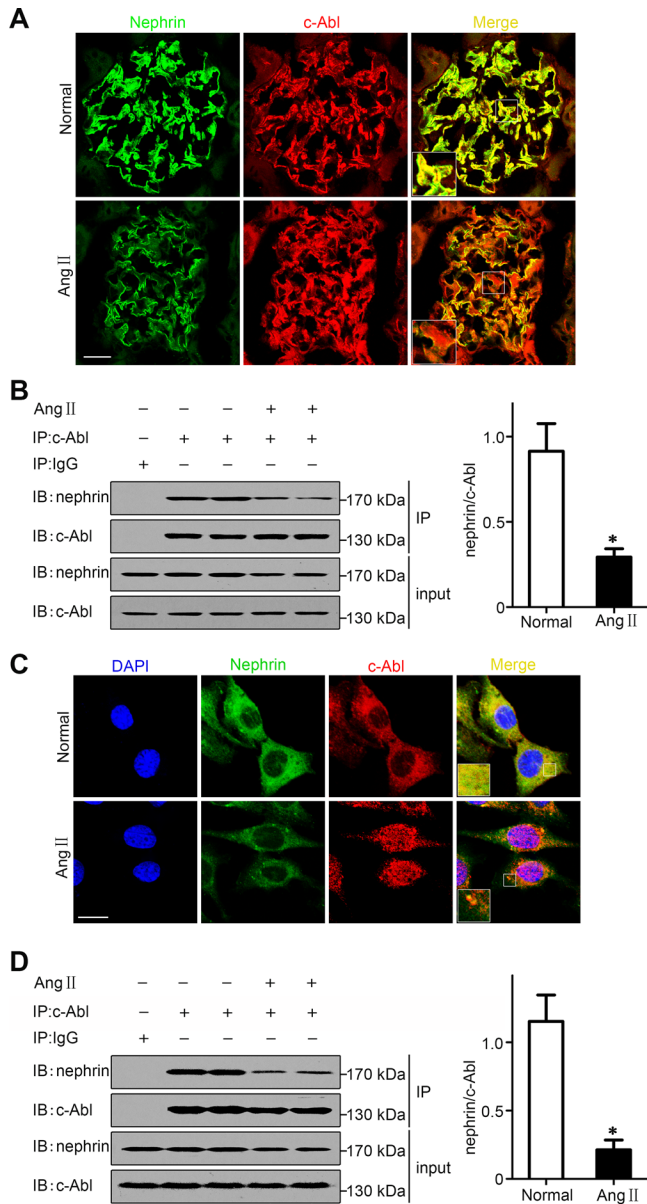
FIGURE 4: Effect of Ang II on the interaction of nephrin and c-Abl in vivo and in vitro. (A) Double immunofluorescence staining of glomerular nephrin and c-Abl in the different groups (original magnification, ×400). AngII, Ang II–infused group on day 14; Normal, normal saline–infused group on day 14. Scale bar, 20 μm. (B) Representative coimmunoprecipitation results of the interaction between nephrin and c-Abl in the glomeruli from the different groups. *$p < 0.05$ compared with the normal group. (C) Double immunofluorescence staining of nephrin and c-Abl in cultured podocytes in the presence or absence of Ang II (10^{-7} M for 1 h; original magnification, ×400). Scale bar, 10 μm. (D) Representative coimmunoprecipitation results of the interaction between nephrin and c-Abl in podocytes in the presence or absence of Ang II (10^{-7} M for 1 h). *$p < 0.05$ compared with normal cells.

addition, c-Abl plasmid transfection significantly augmented Ang II–induced F-actin disruption (Figure 7C). Moreover, c-Abl overexpression markedly enhanced Ang II–induced cell migration and apoptosis (Figure 7, D and E). In contrast, c-Abl siRNA or pretreatment with STI571 significantly ameliorated Ang II–induced podocyte injury (Figure 8, C–E).
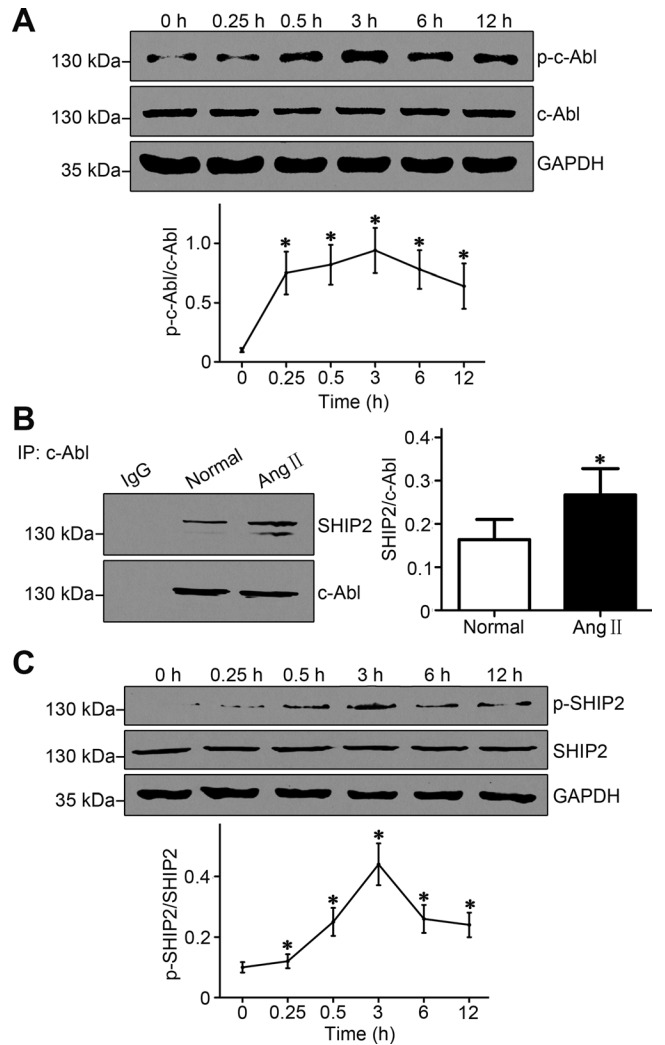
FIGURE 5: Effects of Ang II on the phosphorylation of c-Abl and SHIP2 and the c-Abl–SHIP2 interaction. (A) Representative Western blots of phospho–c-Abl expression in 10^{-7} M Ang II–treated podocytes at various times. *$p < 0.05$ compared with the podocytes treated with Ang II for 0 h. (B) Representative coimmunoprecipitation results of the interaction between c-Abl and SHIP2 in podocytes in the presence or absence of Ang II (10^{-7} M for 1 h). *$p < 0.05$ compared with normal cells. (C) Representative Western blots of the phospho-SHIP2 expression in 10^{-7} M Ang II–treated podocytes at various times. *$p < 0.05$ compared with the podocytes treated with Ang II for 0 h.

DISCUSSION

Ang II plays a vital role in the initiation and progression of proteinuric kidney diseases in general and podocyte injury in particular. Our previous studies demonstrated that Ang II exerts a direct effect on podocyte injury (Ding et al., 2002; Ren et al., 2012), but the mechanism remains elusive. In the present study, we found that c-Abl–mediated nephrin–Akt signaling contributes to Ang II–induced podocyte injury.

Nephrin signaling is important for maintaining podocyte SD structure and function. Verma et al. (2003) demonstrated that the Src-family kinase Fyn directly binds to and phosphorylates nephrin both in vivo and in vitro. Nephrin phosphorylation has been considered important for maintenance of the morphology and function of podocytes under physiological conditions. Fyn-knockout mice display abnormal foot processes and proteinuria (Jones et al., 2006). In
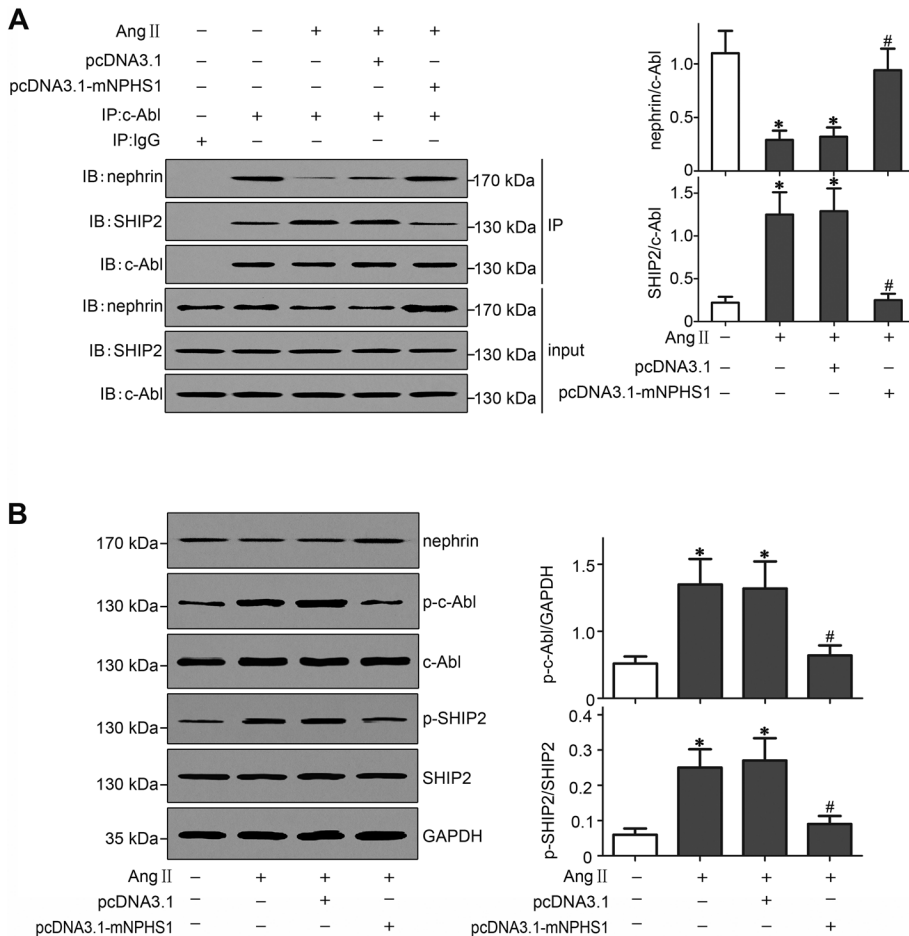
FIGURE 6: Effects of nephrin plasmid transfection on the interaction between nephrin and c-Abl, c-Abl and SHIP2 phosphorylation, and the c-Abl–SHIP2 interaction. The podocytes were transfected with no plasmid, pcDNA3.1, or pcDNA3.1-NPHS1 and then stimulated with 10^{-7} M Ang II for 1 h. Untreated and untransfected podocytes were defined as the normal group. (A) Representative coimmunoprecipitation results of the interaction between nephrin and c-Abl and the interaction between c-Abl and SHIP2 in the different groups. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only. (B) Representative Western blots of phospho–c-Abl and phospho-SHIP2 expression in the different groups. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only.

the puromycin aminonucleoside rat model, Li *et al.* (2006) detected decreased glomerular nephrin tyrosine phosphorylation, altered podocyte morphology, and proteinuria. The results of these studies support the hypothesis that the Ang II–induced down-regulation of phosphorylated nephrin is associated with podocyte injury and proteinuria. However, the downstream events in nephrin signal transduction remain unknown.

Akt activation is involved in the maintenance of cell stress fibers and survival status through the phosphorylation of a variety of substrates (Li *et al.*, 2006; Elloul *et al.*, 2014). In the present study, we found that the Ang II–induced down-regulation of phosphorylated nephrin is associated with Akt dephosphorylation and podocyte injury. However, some inconsistencies were found in the timing of this dephosphorylation; in particular, we observed a transient alteration of phospho-Akt and a persistent dephosphorylation of nephrin. To determine whether Akt is a downstream intermediate of nephrin signaling, we evaluated the levels of Akt phosphorylation in podocytes with altered nephrin expression. Nephrin overexpression attenuated the aforementioned Ang II–induced events, indicating

that Akt signaling is regulated by nephrin and that nephrin–Akt signaling contributes to sustaining the integrity of the actin cytoskeleton and podocyte survival. The difference between these alterations suggests that other mechanisms may be involved in the regulation of Akt signaling. Of interest, even though Akt phosphorylation was reversed, the podocyte injury appeared to be irreversible, which suggests that the Akt dephosphorylation-triggered downstream signal is persistent. Huber *et al.* (2003) demonstrated that nephrin expression facilitates the maintenance of Akt phosphorylation at least partially through the nephrin–CD2AP-PI3K pathway in detachment-induced podocyte apoptosis, which suggests that basal Akt activation exerts a protective effect on podocyte injury. Additional studies need to be performed to address the question of whether other mechanisms are involved in the regulation of nephrin–Akt signaling.

Previous studies showed that nephrin phosphorylation initiates molecular interactions with SH2 or SH3 domain–containing proteins, such as Neph1, podocin, and Nck, to thereby maintain the integrity of the actin cytoskeleton and ensure cell survival (Barletta *et al.*, 2003; Huber *et al.*, 2003; Jones *et al.*, 2006). c-Abl is an SH2/SH3 domain–containing nonreceptor tyrosine kinase involved in cell survival and cytoskeletal regulation (Hopkins *et al.*, 2012; Kalwa *et al.*, 2012; Wang *et al.*, 2013). However, it is not known whether c-Abl binds to nephrin. In the present study, the interaction between nephrin and c-Abl was detected in glomeruli and podocytes under physiological conditions. Ang II treatment attenuated this association, and this effect was accompanied by c-Abl phosphorylation. Previous reports showed that SH2/SH3 domains are important for c-Abl autoinhibition (Corbi-Verge *et al.*, 2013). c-Abl is present in two entirely distinct configurations under different conditions: an inactivated orbicular configuration with the tandem SH3/SH2 domains mechanically clamped to the N- and C-lobes of the catalytic domain, and an activated linear configuration with the catalytic domain exposed to tyrosine kinases (Nagar *et al.*, 2003; de Oliveira *et al.*, 2013; Panjarian *et al.*, 2013). We propose that when phosphorylated nephrin recruits c-Abl through its SH2/SH3 domains, the inactivated configuration of c-Abl is in a stable state, and, when dissociated from nephrin, c-Abl shifts to its linear configuration and is phosphorylated by tyrosine kinases.

SHIP2 is a well-known phosphatase that hydrolyzes PI(3,4,5)P3 to PI(3,4)P2, resulting in the down-regulation of Akt signaling (Pesesse *et al.*, 2001). Wisniewski *et al.* (1999) demonstrated that c-Abl binds directly to SHIP2 via its SH3 domain. In our studies, we observed that Ang II enhances the interaction between c-Abl and SHIP2. The inhibition of c-Abl activity by STI571 not only dissociated SHIP2 from c-Abl, but it also suppressed SHIP2 phosphorylation. Mokhtari *et al.* (2013) demonstrated that imatinib inhibits c-Abl–induced
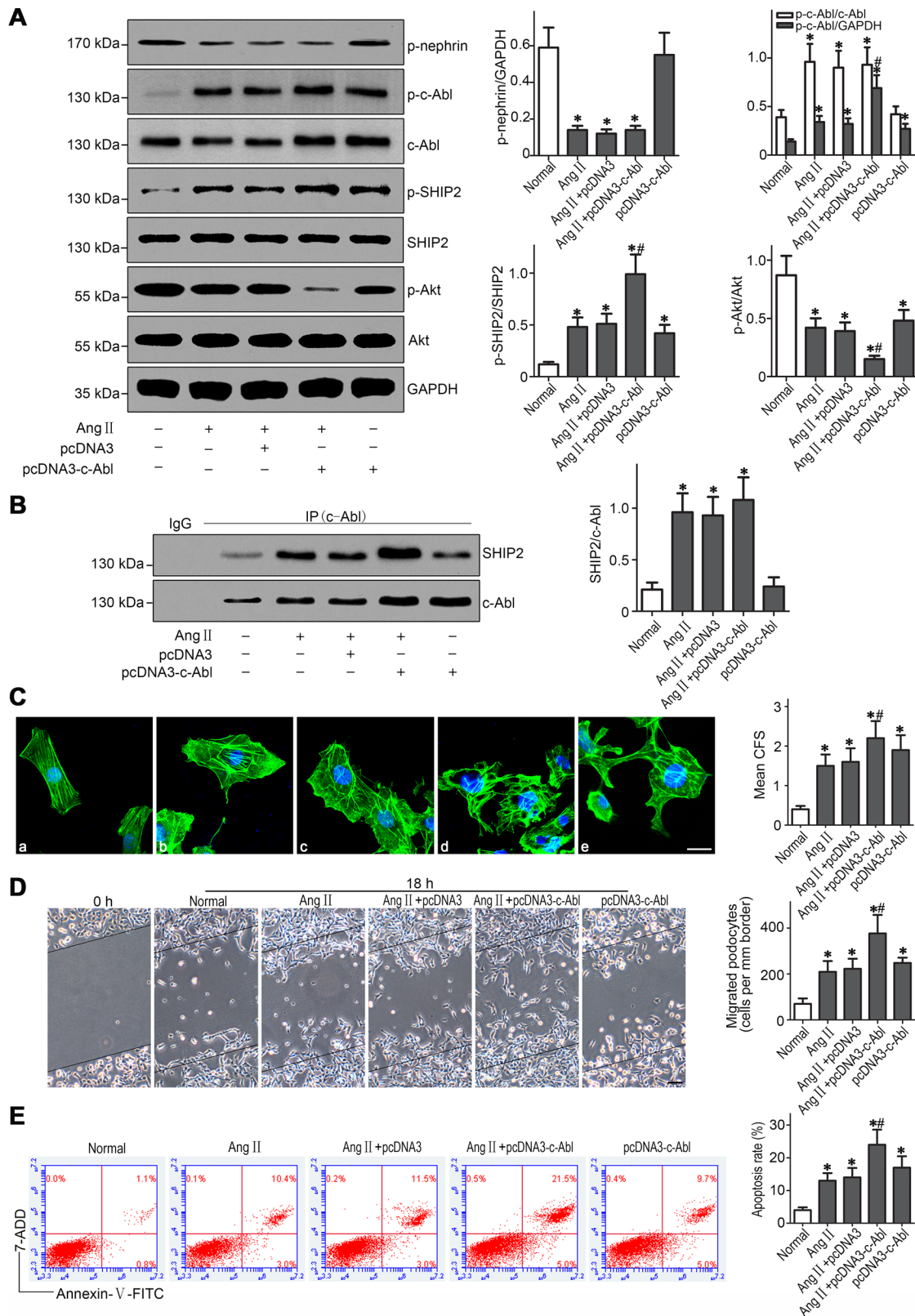
FIGURE 7: Effects of c-Abl plasmid transfection on nephrin–Akt signal transduction and Ang II–induced podocyte injury. The podocytes were transfected with no plasmid, pcDNA3, or pcDNA3-Abl-His6-FLAG and then stimulated with 10^{-7} M Ang II for 1 h. Untreated and untransfected podocytes were defined as the normal group. (A) Representative Western blots of phospho-nephrin, phospho–c-Abl, phospho-SHIP2, and phospho-Akt expression in the different groups. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only. (B) Representative coimmunoprecipitation results of the interaction between c-Abl and SHIP2 in the different groups. *$p < 0.05$ compared with the normal cells. #$p < 0.05$ compared with the cells treated with Ang II only. (C) FITC-phalloidin staining and

SHIP2 activation, which supports the hypothesis that SHIP2 activation is a downstream event of c-Abl signaling. Several studies found that SHIP2 overexpression can reduce Akt phosphorylation and promote cell injury in various cell lines, including podocytes (Hyvonen et al., 2010). An increasing number of reports support the notion that c-Abl promotes SHIP2 phosphorylation and contributes to Akt dephosphorylation and podocyte injury.

In conclusion, the present study shows that c-Abl is a molecular chaperone for nephrin signaling and the SHIP2–Akt pathway and that c-Abl released from nephrin is involved in Ang II–induced podocyte injury (Figure 9). These findings provide a basis for testing new therapeutic strategies for renin/angiotensin/aldosterone system–associated podocyte injury.

MATERIALS AND METHODS

Animals
Twenty-four male specific-pathogen-free Wistar rats weighing between 140 and 160 g were supplied by the Hubei Research Center of Experimental Animals (Hubei, China) and raised in a temperature- and humidity-controlled laminar flow room under an artificial light cycle with free access to tap water and standard rat chow. Rats embedded with an osmotic minipump (Model 2002 or 2004; Alzet, Cupertino, CA) were randomly assigned to the normal saline infusion group or the Ang II (Sigma-Aldrich, St. Louis, MO) infusion group, with Ang II administered at 400 ng/kg per minute for 14 or 28 d. We collected 24-h urine samples in metabolic cages and measured urinary proteins on days 7, 14, 21, and 28. The animals were killed on days 14 and 28. The kidneys were perfused with vanadate (a phosphatase inhibitor) before isolation and stored at –80°C for biochemical and renal pathological analyses.

Cell culture
Conditionally immortalized mouse podocytes were kindly provided by Peter Mundel (Massachusetts General Hospital, Boston, MA) and cultured under standard conditions. The medium consisted of RPMI 1640 (HyClone, Logan, UT) with 10% heat-inactivated fetal calf serum (Life Technologies, Carlsbad, CA), 100 U/ml penicillin G, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), and 10 U/ml recombinant mouse interferon-γ (Pepro Tech, Rocky Hill, NJ), and the cells were maintained at 33°C. To induce differentiation, the podocytes were cultured at 37°C for 10–14 d in the absence of interferon. The resulting differentiated podocytes were used in all subsequent experiments. The differentiated cells were stimulated with Ang II (10^{-7} M) for various times (0–12 h) or for 1 h. c-Abl was inhibited by the addition of STI571 (10 μM, 30 min; Enzo Life Sciences, Farmingdale, NY) before exposure to Ang II. All of the experimental results were verified in three separate cultures of podocytes.

Western immunoblotting
The total proteins from the glomeruli and podocytes were extracted with radioimmunoprecipitation assay (RIPA) buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) containing a protease inhibitor cocktail (Sigma-Aldrich) and centrifuged at 12,000 rpm and 4°C for 20 min. The supernatants were then mixed with loading buffer and boiled at 95–100°C for 5 min. Equal amounts of protein were separated through 10% SDS–PAGE and then transferred to nitrocellulose membranes (GE Healthcare, Fairfield, CT). The membranes were incubated overnight at 4°C with a primary antibody (nephrin guinea pig polyclonal antibody, 1:500 [PROGEN Biotechnik, Heidelberg, Germany]; p-nephrin(Y1217) rabbit monoclonal antibody, 1:1000 [Epitomics, Burlingame, CA]; c-Abl rabbit polyclonal antibody, 1:500 [Cell Signaling Technology, Boston, MA]; p-c-Abl(Y412) rabbit polyclonal antibody, 1:500 [Cell Signaling Technology]; SHIP2 mouse monoclonal antibody, 1:100 [Santa Cruz Biotechnology, Dallas, TX]; p-SHIP2(Tyr986/987) rabbit polyclonal antibody, 1:500 [Cell Signaling Technology]; (p-)Akt rabbit polyclonal antibody, 1:500 [Cell Signaling Technology]; β-actin mouse monoclonal antibody, 1:2000 [Antgene, Hubei, China]; and glyceraldehyde-3-phosphate dehydrogenase mouse monoclonal antibody, 1:1000 [Antgene]). An Alexa Fluor 680/790–labeled goat anti-rabbit/goat anti-mouse immunoglobulin G (IgG) antibody (1:10,000; LI-COR Biosciences, Lincoln, NE) was used as the secondary antibody, and the blots were visualized using a LI-COR Odyssey Infrared Imaging System.

Immunofluorescence assay
The frozen kidney sections were blocked with 5% bovine serum albumin for 30 min at room temperature. The cell-climbing film (cell growing on a glass slide) was fixed in 4% paraformaldehyde with 0.1% Triton X-100 for 30 min at 4°C. The sections were incubated with a mixture of guinea pig anti-nephrin polyclonal antibody (1:50; PROGEN Biotechnik) and rabbit anti–c-Abl polyclonal antibody (1:50; Cell Signaling Technology) or with fluorescein isothiocyanate (FITC)–phalloidin (2.5 μg/ml; Sigma-Aldrich) overnight at 4°C and then with FITC/tetramethylrhodamine–conjugated IgG as the secondary antibody at 37°C for 90 min in the dark. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Antgene) for 5 min. All microscopic images were recorded using a confocal microscope (Olympus, Japan). The F-actin cytoskeletal reorganization of each cell was scored using a scale ranging from 0 to 3 based on the degree of cortical F-actin ring formation (0, no cortical F-actin, normal stress fibers; 1, cortical F-actin deposits under half of the cell border; 2, cortical F-actin deposits exceeding half of the cell border; 3, complete cortical ring formation and/or total absence of central stress fiber; Hsu et al., 2008).

Coimmunoprecipitation
Coimmunoprecipitation experiments were performed according to the manufacturer's instructions (P2012; Beyotime, China). The total proteins from the cultured podocytes were extracted using lysis buffer (20 mM Tris, 150 mM NaCl, 1.0% Triton X-100, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5). A c-Abl rabbit polyclonal antibody (1:200; Cell Signaling Technology) was added to the protein samples, and these were then rotated overnight at 4°C. The mixture was then loaded with 40 μl of protein

quantification of the cortical F-actin score of each group. (a) Normal group, (b) Ang II–stimulated group, (c) pcDNA3+Ang II–stimulated group, (d) pcDNA3-c-Abl+Ang II–stimulated group, and (e) pcDNA3-c-Abl group. Scale bar, 10 μm. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only. (D) Representative migration results and quantification for the different groups. Scale bar, 100 μm. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only. (E) Flow cytometry analysis of apoptosis in the different groups. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only.
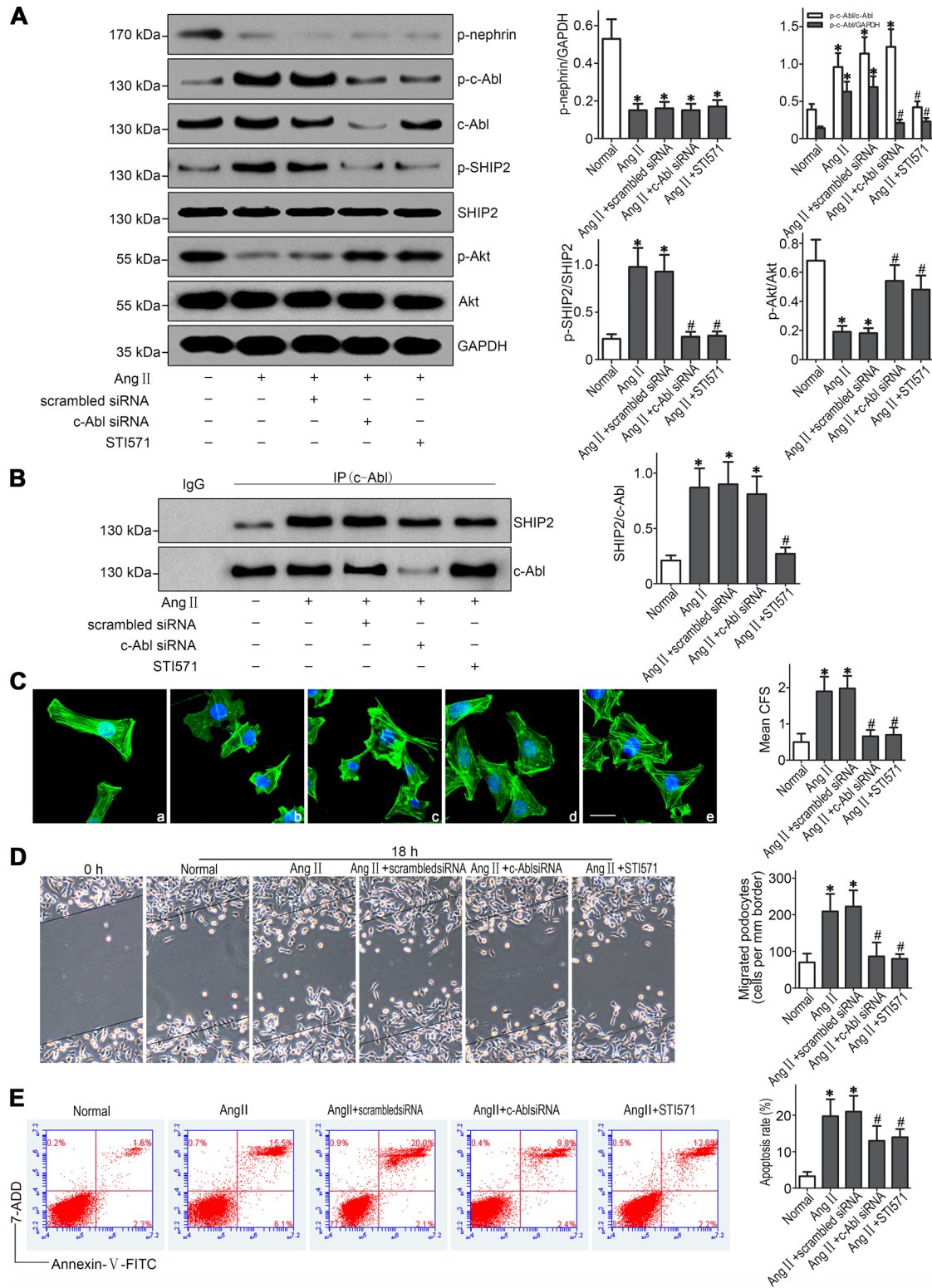
FIGURE 8: Effects of the knockdown or inhibition of c-Abl on nephrin–Akt signal transduction and Ang II–induced podocyte injury. Podocytes were transfected with no siRNA, scrambled siRNA, or c-Abl siRNA or pretreated with STI571 and then stimulated with 10^{-7} M Ang II for 1 h. Untreated and untransfected podocytes were defined as the normal group. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only. (A) Representative Western blots of phospho-nephrin, phospho–c-Abl, phospho-SHIP2, and phospho-Akt expression in the different groups. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only. (B) Representative coimmunoprecipitation results of the interaction between c-Abl and SHIP2 in the different groups. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only. (C) FITC-phalloidin staining and quantification of the cortical F-actin score of each group. (a) Normal group,
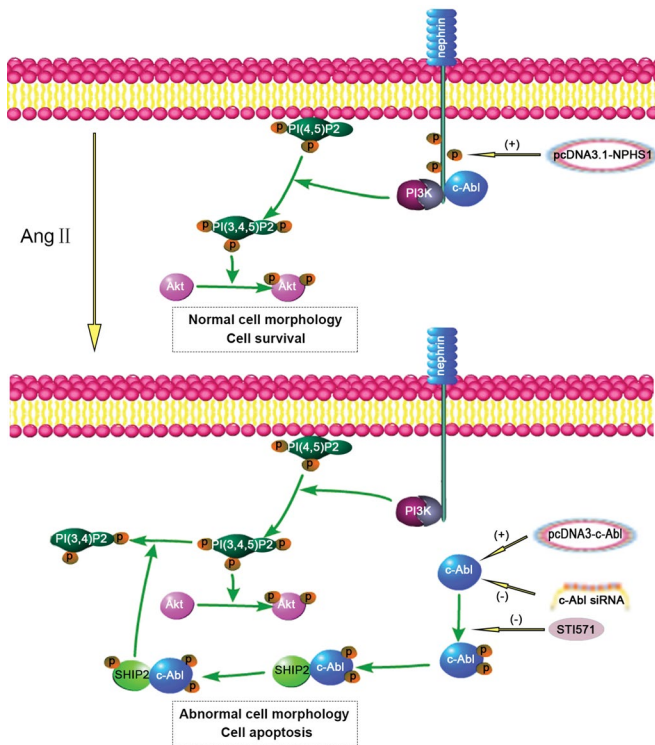
FIGURE 9: Schematic of the proposed signaling pathway. Under physiological conditions, nephrin is phosphorylated by the Src kinase Fyn to a certain degree in podocytes. Phospho-nephrin recruits c-Abl, resulting in autoinhibition of c-Abl activation. Phospho-nephrin is decreased in the presence of Ang II. After nephrin dephosphorylation, c-Abl is released from nephrin and activated. c-Abl activation results in the recruitment and phosphorylation of SHIP2. Phospho-SHIP2 dephosphorylates PI(3,4,5)P3 to PI(3,4)P2, which results in Akt dephosphorylation and ultimately podocyte injury.

was a gift from Benjamin Turk (Addgene plasmid #52684). The transfection of the c-Abl/nephrin plasmid was performed using the X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Briefly, 2×10^5 cells were seeded in a six-well plate and transfected with the complexes containing 2 μg of either c-Abl or nephrin plasmid (or a negative control with pcDNA3.1/pcDNA3) and 6 μl of the X-tremeGENE transfection reagent under normal growth conditions for 72 h. G418 (Sigma-Aldrich) was used to select the stably transfected cell lines.

Cell migration assay
Podocytes were seeded into six-well culture plates precoated with type 1 collagen (C3867; Sigma-Aldrich) and cultured under standard conditions. After the cells were prepared under control or experimental conditions, two wounds were made to each well using a sterile pipet tip. The detached cells were subsequently removed by gently washing with PBS. The cells were then cultured at 37°C for another 18 h. Images of the gaps were photographed 0 and 18 h after scraping with an inverted phase-contrast microscope. The number of cells crossing the 1-mm wound border was calculated. Three independent experiments were performed.

Apoptosis assay
Podocyte apoptosis in kidney tissue was assessed by double IF staining with WT-1 and TUNEL according to the manufacturer's instructions (Roche Applied Science, Basel, Germany). Briefly, antigen retrieval from the dewaxed paraffin sections (3 μm thick) was performed in high-pressure citrate buffer (0.01 mol/l, pH 6.0) for 10 min, and the retrieved antigen was blocked with 10% goat serum for 30 min at room temperature. The sections were then incubated with polyclonal anti–WT-1 antibody (1:100; Santa Cruz Biotechnology) overnight. After washing, Cy3-conjugated anti-rabbit IgG antibody (Boster, Hubei, China) was added for 60 min. The sections were then incubated with a mixture of terminal deoxynucleotidyl transferase (TdT) and FITC-dUTP for 60 min at room temperature. The omission of TdT was used as a negative control. The number of apoptotic podocytes in each glomerular cross-section was counted using the Weibel–Gomez method (Nicholas *et al.*, 2011).

The degree of apoptosis in the cultured podocytes was also evaluated by flow cytometry with annexin V-FITC and 7-aminoactinomycin D (7-ADD) double staining according to the manufacturer's instructions (FITC Annexin V Apoptosis Detection Kit with 7-AAD; BioLegend, San Diego, CA).

Statistical analyses
The quantitative data are presented as the means ± SEM, and the statistical analyses were performed using SPSS, version 17.0. Statistical comparisons of the groups were conducted with one-way analysis of variance, and the least significant difference test was used for multiple comparisons. Differences with $p < 0.05$ were considered statistically significant.

A+G–agarose, incubated for 3 h at 4°C, centrifuged at $2500 \times g$ and 4°C for 5 min and washed five times with phosphate-buffered saline (PBS). The beads were mixed with 1× Lane Marker Sample Buffer. After being boiled at 95–100°C for 5 min, the samples were analyzed by Western blotting for c-Abl, nephrin, and SHIP2 expression.

Transfection
The transfection of the podocytes with c-Abl siRNA (QIAGEN, Hilden, Germany) was performed according to the HiPerFect Transfection Reagent Handbook (QIAGEN). Briefly, 2×10^5 cells were seeded in a six-well plate and transfected with the complexes containing 10 nM c-Abl siRNA (or a negative control with scrambled siRNA) and 15 μl of HiPerFect transfection reagent under normal growth conditions for 24 h.

pcDNA3.1-mNPHS1 was kindly provided by L. B. Holzman (University of Michigan, Ann Arbor, MI). pcDNA3-Abl-His6-FLAG

(b) Ang II–stimulated group, (c) Scramble siRNA+Ang II–stimulated group, (d) c-Abl siRNA+Ang II–stimulated group, and (e) STI571+Ang II–stimulated group. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only. (D) Representative migration results and quantification for the different groups. Scale bar, 100 μm. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only. (E) Flow cytometry analysis of apoptosis in the different groups. *$p < 0.05$ compared with the normal cells; #$p < 0.05$ compared with the cells treated with Ang II only.

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