Research Article

Tacrolimus ameliorates podocyte injury by restoring FK506 binding protein 12 (FKBP12) at actin cytoskeleton

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Abstract
FKBP12 was identified as a binding protein of tacrolimus (Tac). Tac binds to FKBP12 and exhibits immunosuppressive effects in T cells. Although it is reported that Tac treatment directly ameliorates the dysfunction of the podocyte in nephrotic syndrome, the precise pharmacological mechanism of Tac is not well understood yet. It is also known that FKBP12 functions independently of Tac. However, the localization and the physiological function of FKBP12 are not well elucidated. In this study, we observed that FKBP12 is highly expressed in glomeruli, and the FKBP12 in glomeruli is restricted in podocytes. FKBP12 in cultured podocytes was expressed along the actin cytoskeleton and associated with filamentous actin (F-actin). FKBP12 interacted with the actin-associated proteins 14-3-3 and synaptopodin. RNA silencing for FKBP12 reduced 14-3-3 expression, F-actin staining, and process formation in cultured podocytes. FKBP12 expression was decreased in the nephrotic model caused by adriamycin (ADR) and the cultured podocyte treated with ADR. The process formation was deteriorated in the podocytes treated with ADR. Tac treatment ameliorated these decreases. Tac treatment to the normal cells increased the expression of FKBP12 at F-actin in processes and enhanced process formation. Tac enhanced the interaction of FKBP12 with synaptopodin. These observations suggested that FKBP12 at actin cytoskeleton participates in the maintenance of processes, and Tac treatment ameliorates podocyte injury by restoring FKBP12 at actin cytoskeleton.

Keywords
actin cytoskeleton, calcineurin inhibitor, glomerular epithelial cell, immunofilin, nephropathy, proteinuria

Abbreviations: ADR, Adriamycin; CatL, cathepsin L; CN, calcineurin; CNI, calcineurin inhibitor; CsA, cyclosporin A; CyPA, cyclophilin A; F-actin, filamentous actin; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FKBP12, FK506 binding protein 12; FSGS, focal segmental glomerulosclerosis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBM, glomerular basement membrane; HEK, human embryonic kidney; IF, immunofluorescence; IP, immunoprecipitation; KD, knock down; LPS, lipopolysaccharide; MCNS, minimal change nephrotic syndrome; NFAT, nuclear factor of activated T cell; NMS, normal mouse serum; NRS, normal rabbit serum; NS, not significant; PAGE, polyacrylamide gel electrophoresis; PAN, puromycin aminonucleoside; RECA1, rat endothelial cell antigen-1; RFP, red fluorescent protein; RIPA, radioimmunoprecipitation assay; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; SDS, sodium dodecyl sulfate; Synp, synaptopodin; Tac, tacrolimus; TRITC, tetramethylrhodamine isothiocyanate.
1 | INTRODUCTION

Glomerulus, a ball-shaped structure composed of capillaries, is a filtration unit of the kidney. The glomerular capillary wall functions as a barrier, preventing the leakage of plasma protein into the urine. The glomerular capillary wall consists of three layers: an endothelial cell, a glomerular basement membrane (GBM), and a glomerular epithelial cell (podocyte). A podocyte is characterized as a specialized structure of the interdigitating foot processes, covering the outer side of the GBM. The foot processes composed of actin filaments play an essential role in maintaining the structure of the glomerular capillary wall. Podocyte dysfunction is a common feature of renal injury in various types of renal disease, including focal segmental glomerulosclerosis (FSGS). Sustained podocyte damage leads to rearrangement of the actin cytoskeleton, foot process effacement, and subsequently the emergence of proteinuria. Therefore, podocyte protection is of considerable significance to prevent or delay the progression of nephrotic syndrome.

Calcineurin inhibitors (CNIs) such as tacrolimus (Tac), which is also known as FK506, and cyclosporin A (CsA) are widely used immunosuppressive agents. It is understood that CNIs decreased the production of cytokines, such as IL-2 and IL-4, by inhibiting the activation of a nuclear factor of an activated T cell (NFAT), a substrate of calcineurin in T cells. It is also reported that CNIs ameliorated proteinuria in patients with steroid-resistant nephrotic syndrome, including FSGS. Sharma et al. demonstrated that CNI has a direct protective effect on glomeruli by measuring glomerular albumin permeability with their in vitro assay. It is also reported that the activation of NFAT in podocytes may be a critical pathogenic event in FSGS and that CNI ameliorated the dysfunction of podocytes by inhibiting the activation of NFAT. On the other hand, Faul et al. showed that the beneficial effect of CNI on proteinuria results from the stabilization of the actin cytoskeleton in podocytes without affecting NFAT activity. They showed that CNI ameliorated podocyte injury in the lipopolysaccharide-induced nephrotic state by preventing synaptopodin dephosphorylation. However, the precise pharmacological mechanism of how CNI ameliorates proteinuria is unclear.

Although Tac and CsA do not have high similarity in their molecular structure, both have anti-phosphatase activity and function as immunosuppressants. The reason why the reagents have common pharmacological activity is not well explained. Tac and CsA bind to FK506 binding protein 1A (FKBP12) and cyclophilin A (CyPA), respectively, and then they gain their pharmacological activity. Both FKBP12 and CyPA are immunophilin family proteins, which suggests that these reagents may exhibit their common activity by modifying the function of their binding partner, immunophilins. It is known that these immunophilins function independently of the agents. Immunophilins are endogenous cytosolic peptidyl-prolyl isomerases and are reported to be expressed in various organs and tissues. For example, FKBP12 binds to TGF-β type 1 receptor and inhibits its signaling in fibroblasts. Colucci et al. reported that FKBP12 inhibits hepcidin expression by binding to the BMP type 1 receptor in hepatocytes. Although CyPA is primarily located intracellularly, it can be secreted into the extracellular environment in various cell types. The secreted form of CyPA mediates intracellular signal communication and is also identified to be a potent chemoattractant for leukocytes in vitro. Leong et al. reported that CyPA promotes inflammation in acute kidney injury. However, the localization and the physiological function of FKBP12 and CyPA in kidneys are not well investigated, and how these immunophilins are associated with the podocyte protective effect of CNIs is unclear.

In the present study, first, we analyzed the distribution of these immunophilins. Although CyPA in glomeruli was expressed in podocytes and a part of mesangial cells, FKBP12 expression in glomeruli was restricted in podocytes. Studies with human cultured podocytes showed that FKBP12 was localized along the actin cytoskeleton and associates with filamentous actin (F-actin). Immunoprecipitation (IP) assay showed that FKBP12 interacted with 14-3-3 and synaptopodin. The downregulation of 14-3-3, the alteration in the structure of F-actin, and the deteriorated process formation were observed in FKBP12 knockdown (KD) podocytes. FKBP12 expression was clearly decreased in podocyte injury models. Tac treatment restored the expression of FKBP12 at F-actin and ameliorated the deterioration of process formation in the injured podocyte. The treatment of Tac to normal podocytes increased the expression of FKBP12 at F-actin in processes. Tac enhanced the interactions of FKBP12 with synaptopodin and 14-3-3. These results suggest that FKBP12 at actin cytoskeleton participates in the maintenance of processes. Tac treatment ameliorates podocyte injury by restoring FKBP12 at actin cytoskeleton.

2 | METHODS

2.1 | Animals

Specific pathogen-free 7-week-old female Wistar rats (Charles River Japan, Atsugi, Japan) were used. All animal experiments were conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Procedures for the present study were approved.
by Animal Committee at Niigata University School of Medicine (Niigata, Japan; permit numbers SA00710), and all animals were treated according to the guidelines for animal experimentation of Niigata University.

2.2 | Podocyte cell culture

Human cultured podocyte was donated by Dr Moin A. Saleem (University of Bristol, Bristol, UK). Cultivation of conditionally immortalized human podocytes was conducted as reported previously. A conditionally immortalized human podocyte cell line was demonstrating nephrin and podocin expression. In brief, podocytes were maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, Japan). For propagation of podocytes, cells first were cultivated at 33°C and maintained for 2 weeks at 37°C to induce differentiation. Human cultured podocytes were cultured with Adriamycin (ADR) 5 μg/ml for 24 h and then harvested for immunofluorescence (IF), reverse transcription-polymerase chain reaction (RT-PCR), and western blot. Human cultured podocytes were cultured with Tac 10 μg/ml or placebo for 24 h and then harvested for IF, RT-PCR, and western blot. Tac and placebo were kindly provided by Astellas Pharmaceutical Co., Ltd. (Osaka, Japan).

2.3 | RNA silencing analysis

The siRNA sequences targeting human FKBP12 (GenBank accession number: NM_000801.4) and human 14-3-3β (GenBank accession number: NM_003404.4) were synthesized by ThermoFisher Scientific Ink (Massachusetts, USA). The sense and antisense strands of siRNA were used. The sequence of the sense strand was 5′-CCCCGGGAGAGAAGGAAUA-3′, corresponding to regions in exon 3 of FKB12. The sequence of the sense strand was 5′-GGGCAAGAGUACCUGAGAGAU-3′, corresponding to regions in exon 3 of YWHAB (14-3-3β). BLAST searches of selected sequences revealed no significant homology to other human genes. Negative control siRNA of nonspecific nucleotide sequence was purchased from ThermoFisher Scientific Ink. Before treatment, human cultured podocytes and human embryonic kidney (HEK) 293 cells were cultured to a density of 70%–80% at 37°C, and then they were treated with the siRNA using HiPerFect Transfection Reagent (QIAGEN Inc.), according to the manufacturer’s instructions. Cells were harvested for 48 or 72 h after siRNA treatment for RT-PCR, IF, and western blot analyses.

2.4 | Induction of ADR-induced nephropathy in rats

ADR-induced nephropathy was prepared by intravenous injection with 6 mg/kg BW, basically according to the method described previously. Rats were sacrificed, and kidneys were removed. The materials were used for IF, RT-PCR, and western blot. Small tissue samples were used for IF studies. To prepare a set of glomerular RNA for each time point, glomeruli were isolated from the remaining kidney tissue pooled from three rats each by a sieving method, and glomerular RNA was prepared with Trizol (Life Technologies, Inc., Gaithersburg, MD, USA).

2.5 | Effect of Tac in ADR-induced nephropathy

ADR-induced nephropathy was prepared by intravenous injection with 6 mg/kg BW, basically according to the method described previously. Rats were injected with the ADR and treated with 1 mg/kg BW of Tac or placebo daily and then were sacrificed on day 28 after ADR injection. The kidney from each animal was cut into portions and used for the IF study. Human cultured podocytes were cultured with the ADR and treated with Tac or placebo before induction of the podocyte injury and then harvested for IF, RT-PCR, and western blot.

2.6 | RT-PCR and real-time RT-PCR

A semiquantitative RT-PCR analysis and real-time PCR analysis with the RNA of rat glomeruli and human cultured podocyte was performed basically according to the method described previously. The sequences of the primers used for this study are provided in Table S1. For semiquantitative RT-PCR, the band intensity was determined by image analysis using Bio Doc-It System and densitometry software, Lab Works 4.0 (UVP Inc., Upland, CA, USA). The results were corrected for the amount of mRNA in the sample by dividing by the intensity of the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For real-time PCR, cDNA and specific primers were mixed with an SYBR Premix Ex Taq (Takara, Otsu, Japan). PCR reactions were run on a Smart Cycler System (Takara). C values of the gene targets were normalized to GAPDH. Fold change in expression of target genes compared with the control was calculated using the 2−ΔΔCT method with GAPDH. The sequences of the primers were shown in Table S1.
2.7 IF analysis

IF was performed basically according to the method previously reported. The antibody against FKBP12 was prepared in a rabbit immunized with a specific peptide. The following antibodies were used as the glomerular cell markers, mouse anti-rat endothelial cell antigen-1 (RECA-1) antibody, mouse monoclonal antibody to Thy1.1 (OX7), mesangial cell marker, mouse monoclonal antibody to nephrin, slit diaphragm marker (mAb 5-1-6), mouse monoclonal antibody to podocalyxin, a podocyte apical marker, mouse monoclonal antibody to α3-integrin, and a podocyte basal marker, mouse monoclonal antibody to synaptopodin, a podocyte foot process marker. A rabbit anti-CyPA antibody was purchased from Proteintech, Tokyo, Japan. The suppliers of other antibodies used in this study are provided in Table S2. Fluorescein isothiocyanate-conjugated swine anti-rabbit IgGs and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG1 were used as secondary antibodies. To detect actin fiber in cultured podocytes, Rhodamine-Phalloidin was used according to the method of the previous report. To evaluate the FKBP12 and CyPA staining in the rat glomeruli, the staining was graded as follows: continuous staining of >75% was score 4; 75%–50% was score 3; 50%–25% was score 2; and 25%–0% was score 1. A score was assigned to each glomerulus, and 30 glomeruli of each rat were analyzed. The data are shown as mean ± standard deviation (SD).

2.8 Western blot analysis

Western blot analysis was performed basically according to the method described previously. In brief, rat glomeruli and human cultured podocytes were solubilized with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (2% SDS, 10% glycerol, 6% mercaptoethanol in 62.5 mmol/L Tris-HCl [pH 6.8] with protease inhibitors). The cells were also solubilized sequentially with 1% Triton X-100 and radioimmunoprecipitation assay (RIPA) buffer (0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 150 mmol/L NaCl, and 10 mmol/L EDTA in 25 mmol/L Tris-HCl [pH 7.2]) and separated into Triton X-100-soluble fractions (fraction1), Triton X-100-insoluble/RIPA-soluble fractions (fraction2), and RIPA-insoluble fractions (fraction3). The RIPA-insoluble fraction was solubilized with an SDS-PAGE sample buffer. The solubilized material was subjected to a polyvinylidene fluoride transfer membrane (Pall Corporation, Pensacola, FL, USA). After exposure to the primary antibodies, alkaline phosphatase-conjugated secondary antibodies were used. The reaction was developed with an alkaline phosphatase chromogen kit (Biomedica, Foster City, CA, USA). The band intensity was quantified by image analysis using Image J software.

2.9 Assay with HEK293 cell transfection and IP assay

Assays with HEK293 cell transfection were performed as previously described. HEK293 cells were transfected with monomeric red fluorescent protein (RFP)-FKBP12 (Addgene, Cambridge, MA, USA, Plasmid #67514) and synaptopodin-HA by the calcium phosphate method. The coding sequence of full-length synaptopodin was generated by PCR and subcloned into the pKH3 vector (Addgene). IP assays were performed basically according to the method described previously. The cells were solubilized with the SDS-PAGE sample buffer. The solubilized materials were analyzed by the western blot analysis. HEK293 cells were cultured with Tac 30 μg/ml or placebo for 30 min and then harvested for the interaction assay.

2.10 Actin binding assay

Actin binding assay was performed using the Actin Binding Protein Biochem Kit (Cytoskeleton Inc., Denver, CO, USA). For low-speed pellet assay, 40 μg non-muscle actin was polymerized for 1 h with the indicated concentration of polymerization buffer. The polymerized F-actin and non-muscle actin were incubated for 30 min with the lysate of human cultured podocytes. F-actin was separated via centrifugation for 90 min at 16 000 g. The pellet fractions were solubilized with the SDS-PAGE sample buffer. The solubilized materials were analyzed by the western blot analysis.

2.11 Statistical analyses

Statistical significance was evaluated using the unpaired t-test or Mann–Whitney U-test. Values were expressed as the mean ± SD. Differences at p < .05 were considered significant. Data were analyzed using Graphpad Prism 5.0 software (Graphpad Software, San Diego, CA, USA).

An antibody list and primer information are described in Tables S1 and S2, respectively.
3 | RESULTS

3.1 | Expression of FKBP12 and CyPA in several organs and tissues

First, mRNA expression of FKBP12 and CyPA in several major organs was analyzed. mRNA expression of FKBP12 and CyPA was detected in the cerebrum. mRNA expression of FKBP12 in lymphoid tissues, thymus and spleen, was lower than that of CyPA. FKBP12 and CyPA mRNA were abundantly expressed in epithelial tissues, lung and liver, and in the heart. In the kidney, mRNA expression of FKBP12 was lower than that of CyPA. Although CyPA mRNA was equally expressed in all fractions of kidney samples, the expression of FKBP12 mRNA in glomeruli was higher than that in the cortex and medulla (Figure 1).

3.2 | Localization of FKBP12 and CyPA in normal glomeruli

Next, to analyze the localization of FKBP12 in glomeruli, the antibody against FKBP12 was prepared in a rabbit immunized with a specific peptide. Clear positive staining was detected in glomeruli with the anti-FKBP12 antibody (Figure S1A). A positive band of approximately 12 kDa was detected in normal rat glomerular and cortical lysates with the anti-FKBP12 antibody (Figure S1B). We confirmed the specificity of the antibody by the HEK293 cells transfected with the human FKBP12-RFP construct. In western blotting with the anti-FKBP12 antibody and anti-RFP antibody, a positive band of FKBP12-RFP (approximately 40 kDa) was detected in the transfected cells. The positive band of endogenous FKBP12 (12 kDa) was detected by the anti-FKBP12 antibody in both the transfected and un-transfected cells (Figure S1C).

Then, we analyzed the precise localization of FKBP12 and CyPA in glomeruli. A dual-labeling IF study was carried out with glomerular-cell markers: (1) the endothelial cell marker RECA-1; (2) the mesangial cell marker Thy1.1; and (3) the podocyte marker synaptopodin. FKBP12 staining was clearly apart from that of RECA-1 and Thy1.1, and no staining co-localized with these markers was detected, indicating that FKBP12 was restrictedly expressed in the podocytes. Then, to analyze the subcellular localization of FKBP12 in podocytes, the dual-labeling IF study with podocyte markers was performed. In podocytes, major portions of the FKBP12 staining were co-localized with synaptopodin, a marker for foot processes, and a part of the FKBP12 was co-stained with nephrin, a slit diaphragm

![Figure 1](image-url)
FKBP12 is co-localized with 14-3-3β and associates with F-actin in podocytes

Next, expressions of FKBP12 and CyPA were analyzed in human cultured podocytes. Although no difference in mRNA expression of CyPA was detected in undifferentiated and differentiated podocytes, FKBP12 mRNA expression in the differentiated podocytes was higher than that in the undifferentiated cells (Figure 3A). It is suggested that FKBP12 is related to the differentiation of podocytes. To analyze the subcellular localization of FKBP12 and CyPA, western blot analysis with sequentially solubilized lysates of the cultured podocytes was performed. FKBP12 was detected in F1 (Triton X-100-soluble fraction) and F2 (Triton X-100-insoluble/RIPA-soluble fraction), not in F3 (RIPA-insoluble fraction). CyPA was detected in all fractions. Although CyPA was equally expressed in F1 and F2, the expression of FKBP12 in F2 was higher than that in F1. A small amount of CyPA was also detected in F3. 14-3-3 was detected in F1 and F2, and the expression of 14-3-3 in F2 was higher than that of F1 as well as FKBP12 (Figure 3B). These results suggested that the subcellular distribution of FKBP12 in the podocytes is similar to that of actin-associated protein 14-3-3. In immunostaining of the cultured podocytes, clear fibrous staining of FKBP12 toward the tip end of processes was detected in the cytoplasm. CyPA staining was broadly detected in the cytoplasm of podocytes (Figure 3C).

We further analyzed the precise localization of FKBP12 in podocytes. In dual-labeling IF with F-actin, the fibrous staining of FKBP12 was co-localized with F-actin in the processes of cultured podocytes (Figure 3D). In actin-binding assay, both FKBP12 and 14-3-3 were detected in the sample incubated with F-actin, indicating that FKBP12 associates with F-actin as well as 14-3-3 (Figure 3E). In dual-labeling IF of FKBP12 with 14-3-3β, major portions of FKBP12 staining were co-localized with 14-3-3β in glomeruli, suggesting that FKBP12 interacts with 14-3-3β (Figure 3F).

3.4 | FKBP12 interacts with the actin-associated proteins 14-3-3β and synaptopodin

Next, the interaction of FKBP12 with the actin-associated proteins was analyzed using the HEK293 cell transfection system. IP assay with lysate of the cells transfected with FKBP12 showed FKBP12 interacted with endogenous 14-3-3 (Figure 4A). The interaction of FKBP12 with synaptopodin (Synp) was detected in the HEK cells co-transfected with FKBP12 and Synp (Figure 4B). The interaction of 14-3-3 with Synp was detected in the HEK cells transfected with Synp (Figure 4C). Because it is reported that 14-3-3β interacts with Synp to regulate the actin cytoskeleton in podocytes, the specific antibody for 14-3-3β was used for the interaction of FKBP12. The interaction of FKBP12 with 14-3-3β was also detected in the lysates of cultured podocytes (Figure 4D). Because 14-3-3β is expressed in HEK293 cells, it was verified whether the interaction of FKBP12 with Synp was mediated with endogenous 14-3-3β of HEK cells. The interaction of FKBP12 with Synp was analyzed in the HEK cells in which endogenous 14-3-3β was knocked down with siRNA. The interaction of FKBP12 with Synp was not altered by the treatment of 14-3-3β siRNA, indicating that FKBP12 interacts with Synp independently of the expression of 14-3-3β in podocytes (Figure 4E). Furthermore, we analyzed the triadic relationship between FKBP12, 14-3-3β and Synp. No difference in the amount of endogenous 14-3-3β in the precipitate of FKBP12 was detected between the cells transfected with synaptopodin and the cells not transfected (Figure 4F upper panel). No difference in the amount of endogenous 14-3-3β in the precipitate of synaptopodin was detected between the cells transfected with FKBP12 and the cells not transfected (Figure 4F lower panel). These results indicated that the interaction of FKBP12 with 14-3-3β is not competitive to the interaction of Synp with 14-3-3β.
FIGURE 2 Localization of FKBP12 and CyPA in glomeruli. (A) Dual-labeling immunofluorescence (IF) findings of FKBP12 (green) with glomerular cell markers (red) in adult rat glomeruli. The staining of FKBP12 was clearly apart from that of RECA-1 and Thy1.1, indicating that FKBP12 is not expressed in endothelial cells or mesangial cells, and FKBP12 is restricted in podocytes. In podocytes, major portions of the FKBP12 staining were co-stained with synaptopodin, a foot process marker. A part of FKBP12 staining was co-localized with nephrin, podocalyxin, and integrin-α3 (arrows). (B) Dual-labeling IF findings of CyPA (green) with glomerular cell markers (red). The staining of CyPA was apart from that of RECA-1. A part of the CyPA staining was co-localized with Thy1.1. Some portions of the CyPA were co-localized with synaptopodin, nephrin, podocalyxin, and integrin-α3 (arrows). (C) Dual-labeling IF findings of FKBP12 (green) with nephrin (red) in neonatal rat glomeruli. FKBP12 staining was clearly detected in a maturing podocyte, and the staining was restrictedly detected at the basal side of the podocyte as a continuous fine granular pattern slightly before nephrin appeared (arrowhead). The staining of FKBP12 was co-localized with that of nephrin in the capillary loop stage. Scale bar, 20 µm.
FIGURE 3 FKBP12 is co-localized with 14-3-3β and associates with F-actin in podocytes. (A) mRNA expression of FKBP12 and CyPA in undifferentiated and differentiated human cultured podocytes. Although no difference in mRNA expression of CyPA was detected between the undifferentiated podocytes and the differentiated podocytes, FKBP12 mRNA expression in the differentiated cultured podocytes was higher than that in the undifferentiated cells. The semiquantitative data of the expressions of FKBP12 and CyPA corrected by that of GAPDH are expressed as means ± SD (n = 4; ***p < .005, t-test). (B) Western blot analysis of sequentially solubilized cell lysates of the cultured podocytes. Clear bands of FKBP12 were observed in F1 (Triton X-100-soluble fraction) and F2 (Triton X-100-insoluble/RIPA-soluble fraction). The band of FKBP12 in F2 was higher than that in F1. Bands of CyPA were equally detected in F1 and F2, and a weak one was observed in F3 (RIPA-insoluble fraction). Clear bands of 14-3-3 were detected in F1 and F2. The band of 14-3-3 in F2 was higher than that in F1. The distribution of each fraction in total fractions was evaluated by the densitometric analyses. The data are expressed as a percentage of each fraction in total fractions (F1, F2, and F3) (means ± SD, n = 5; ***p < .005, t-test). (C) Immunostaining of FKBP12 and CyPA in human cultured podocytes. Clear fibrous staining of FKBP12 toward the tip end of processes was detected in the cytoplasm in podocytes. Although weak fibrous staining of CyPA was detected, CyPA staining was broadly detected in the cytoplasm of podocytes. Scale bar, 20 μm. (D) Dual immunostaining of FKBP12 and F-actin in cultured podocytes. The fibrous staining of FKBP12 was co-localized with F-actin in the processes of podocytes. Scale bar, 20 and 10 μm. (E) Actin binding assay with the cell lysate of cultured podocytes. The specific bands of FKBP12 and 14-3-3 were detected in the sample incubated with F-actin. No band was detected in the sample incubated with monomeric actin (mono-Actin). (F) Dual immunostaining of FKBP12 and 14-3-3β in glomeruli. FKBP12 was co-localized with 14-3-3β in glomeruli. Scale bar, 20 μm.
3.5 Deranged F-actin structure, impaired process formation, and decreased expression of dephosphorylated 14-3-3β are detected in FKBP12 KD podocytes

We analyzed the function of FKBP12 in podocytes using RNA silencing. mRNA expression of FKBP12 was clearly decreased in the cultured podocytes treated with FKBP12 siRNA for 48 h (25.3%). Protein expression of FKBP12 was decreased in the cells treated with the siRNA (50.5%). mRNA and protein expression of CyPA was not altered in the cells treated with FKBP12 siRNA (Figure S2). F-actin staining was clearly decreased in the cells treated with FKBP12 siRNA. The proportion of the cells forming
processes was decreased by the siRNA (Figure 5A). The findings indicated that FKBP12 plays a role in the maintenance of F-actin and the formation of processes. Although the expression of phosphorylated 14-3-3 was not altered in the cells treated with FKBP12 siRNA, the expression of total 14-3-3β was decreased (63.0% ± 7.6%) (Figure 5B). The results showed that FKBP12 plays a role in the maintenance of the expression of dephosphorylated 14-3-3β.

### 3.6 | Expression of FKBP12 is decreased in ADR-induced podocyte injury

Next, we investigated the expression of FKBP12 in a nephrotic model, ADR nephropathy (a mimic of FSGS). The staining intensities of nephrin and podocin were clearly decreased on day 28 of ADR nephropathy when severe proteinuria was detected (Figure 6A). Although the alteration of mRNA in FKBP12 and nephrin was not detected on day 7, the mRNA expressions of FKBP12 and nephrin were decreased on day 28 of ADR nephropathy. The alternation of CyPA mRNA was not remarkable on days 7 and 28 (Figure 6B). The protein expression and staining intensity of FKBP12 were decreased on day 28. Those of CyPA were not altered on day 28 (Figure 6C,D). In puromycin-aminonucleoside (PAN) nephropathy (a mimic of minimal change nephrotic syndrome [MCNS]), mRNA expression, protein expression, and staining intensity of FKBP12 were also decreased on day 10 when proteinuria peaked (Figure S3).

Next, we investigated the expression of FKBP12 in human cultured podocytes treated with ADR. F-actin staining was clearly decreased in the cultured podocytes treated with ADR. The proportion of the cells forming processes was decreased by the ADR treatment. The decrease of nephrin mRNA was detected in the injured podocytes (Figure 6E). The protein expression and staining intensity of FKBP12 were clearly decreased in the cells treated with ADR. The alteration in protein expression of CyPA was not detected in the injured podocytes (Figure 6F,G). These results suggested that the rearrangement of F-actin resulted from the down-regulation of FKBP12 is involved in the pathogenesis of nephropathy.

### 3.7 | Tac treatment suppresses the decrease of FKBP12 in ADR-induced podocyte injury

The effect of Tac on FKBP12 expression in the podocyte injury models was analyzed. On day 28 of ADR nephropathy, the decrease of nephrin and podocin staining in glomeruli was suppressed by Tac treatment (Figure 7A). Tac treatment suppressed the decrease of FKBP12 staining in the ADR nephropathy (Figure 7B). The protein expression and staining intensity of CyPA in the ADR nephropathy were not altered by Tac treatment (Figure S4). Tac treatment suppressed the decrease of F-actin staining in the cultured podocytes treated with ADR. The proportion of cells forming processes was decreased in the podocytes treated with ADR. Tac treatment suppressed the decrease in the cells treated with ADR. The decrease of nephrin mRNA in the injured podocytes was suppressed by Tac treatment (Figure 7C). The decrease of FKBP12 expression in the injured podocytes was suppressed by the Tac treatment (Figure 7D). Tac treatment restored the FKBP12 at F-actin in the processes of podocytes (Figure 7E).

### 3.8 | Tac treatment to normal cultured podocytes increases FKBP12 expression at the tip end of processes and enhances process formation

Next, the effect of Tac on normal cultured podocytes was analyzed. The proportion of cells forming processes clearly increased in the podocytes treated with Tac, although the staining intensity of F-actin was not altered (Figure 8A). A dual-labeling IF study showed that the expression of FKBP12 at the tip end of processes was increased by Tac treatment to the cells (Figure 8B). Western blot analysis with sequentially solubilized lysates of the cultured podocytes showed that the proportion of FKBP12 in F1 to total FKBP12 was increased by Tac treatment. The treatment decreased the proportion of FKBP12 in F2 to total FKBP12. The treatment also increased the proportion of 14-3-3β in F1 to 14-3-3β. The treatment decreased the proportion of 14-3-3β in F2 to 14-3-3β. The alteration of total expression of FKBP12 and 14-3-3β was not detected in the podocytes treated with Tac. The treatment did not alter the proportion of CyPA in F1 and F2 and the expression of total CyPA (Figure 8C). These findings suggested that Tac shifts FKBP12 and 14-3-3β to the tip end of processes in podocytes.

### 3.9 | Tac enhances the interactions of FKBP12-synaptopodin and 14-3-3β-synaptopodin without affecting phosphorylation of 14-3-3 and synaptopodin

Next, the effect of Tac on the interactions of FKBP12-Synp and 14-3-3β-Synp was analyzed with HEK cells co-transfected with FKBP12 and Synp. The interactions were enhanced by Tac treatment (Figure 8D). Finally, the
effect of Tac on phosphorylation of 14-3-3 and Synp was analyzed with the HEK cells co-transfected with Synp and FKBP12. Transfected Synp was detected as an approximately 130 kDa band. The same size band detected by the anti-phosphoserine antibody is considered to show phosphorylated Synp. Phosphorylation of 14-3-3 and Synp was not altered by Tac treatment (Figure 8E). The findings indicated that Tac enhances the interactions of FKBP12-14-3-3β and 14-3-3β-Synp without affecting phosphorylation levels of 14-3-3 and Synp.
4 | DISCUSSION

It has been reported that Tac and CsA ameliorate proteinuria by the direct protective effect on podocytes in non-inflammatory nephrotic syndrome. Tac and CsA bind to immunophilins in the cytoplasm, and the complexes can inhibit calcineurin. The expression and the role of immunophilins in kidneys are not fully understood yet. The effect of CNIs on the function of immunophilins is also unclear. In the present study, we investigated the expression and the role of FKBP12 in podocytes and analyzed the effect of Tac on FKBP12 in podocytes.

First, we analyzed the expression of FKBP12 and CyPA in some major organs. In the kidney, the CyPA was equally expressed in glomeruli, cortex, and medulla, indicating CyPA is broadly expressed in glomeruli and tubules. By contrast, although the expression of FKBP12 in kidneys was lower than that in other organs, the FKBP12 was highly expressed in glomeruli (Figure 1). In dual-labeling IF studies with glomerular cell markers showed that CyPA was expressed in podocytes and a part of mesangial cells. By contrast, the FKBP12 is exclusively expressed in podocytes (Figure 2), which suggests that Tac has more selective effects on podocytes than CsA. Although Tac and CsA are commonly used immunosuppressive agents as CNIs, some differences in the effect and the toxicity between these drugs are reported. The differences in the distributions of the binding partner of these drugs, FKBP12 and CyPA, might be associated with the different effects of Tac and CsA.

Next, we further analyzed the subcellular localization of FKBP12 in podocytes. The dual-labeling IF studies with podocyte markers showed that FKBP12 is dominantly co-localized with Synp, the actin-associated protein of foot processes (Figure 2). FKBP12 was highly expressed in differentiated cultured podocytes with processes. The subcellular distribution assay showed that the distribution of FKBP12 was similar to that of actin-associated protein 14-3-3 in the cultured podocytes. In IF studies, FKBP12 was localized along F-actin in the cultured cells. Actin binding assay revealed the association of FKBP12 with F-actin. Furthermore, dual-labeling IF study revealed that FKBP12 was co-localized with 14-3-3β in glomeruli (Figure 3). These results suggest that FKBP12 interacts with 14-3-3 and is localized along F-actin to maintain the processes in podocytes. To investigate this hypothesis, FKBP12 KD podocytes were analyzed. Alteration in the structure of F-actin and deteriorated process formation were observed in the FKBP12 KD cells (Figure 5). These findings indicated that FKBP12 is essential for maintaining F-actin and processes in podocytes. We are the first to find a new function of FKBP12 for maintaining actin fibers.

Next, we analyzed the interaction of FKBP12 with 14-3-3 and synaptopodin. The IP assay with HEK transfection system revealed that FKBP12 interacts with 14-3-3β, and the interaction was confirmed by the analysis with cultured podocytes (Figure 4). 14-3-3 proteins bind to phosphorylated serine/threonine-containing motifs on various binding partners. Faul et al. reported that the 14-3-3 interacts with phosphorylated Synp, and the interaction is critical in podocyte. The 14-3-3 proteins also bind to non-phosphorylated proteins. It is reported that if 14-3-3 is phosphorylated, 14-3-3 is dissociated from its binding partners. Maeda et al. reported that upregulation of CaMKIV phosphorylates 14-3-3 in FSGS, which results in the release and degradation of Synp in podocytes. Zhang et al. also reported that 14-3-3β is dissociated from Synp in ADR nephropathy. In the present study, we showed that dephosphorylated 14-3-3, which can bind to FKBP12 and synaptopodin, clearly decreased in FKBP12-deleted cultured podocytes (Figure 5). These findings suggested that FKBP12 functions for maintaining F-actin structure by stabilizing the expression of 14-3-3 and synaptopodin.
dephosphorylated 14-3-3 and the interaction of 14-3-3 with Synp.

Synp is a proline-rich actin-binding protein that is highly expressed in the foot processes of podocytes. In podocytes, gene silencing of Synp causes the loss of F-actin and RhoA activity, which clearly indicates that Synp is one of the critical molecules in podocyte. In the present study, the analysis of HEK transfection revealed that FKBP12 also interacts with Synp independently of the expression of 14-3-3β (Figure 4). Herein, we identified

(A) Podocin Merge Nephrin

(B) Normal ADR day7 ADR day28

FKBP12 CyPA Nephrin GAPDH

(C) Normal ADR

FKBP12 CyPA Actin

10kDa 15kDa 37kDa

(D) Normal ADR

FKBP12 CyPA

(E) Normal ADR

F-actin

(F) Vehicle ADR

FKBP12 CyPA Actin GAPDH

10kDa 15kDa 37kDa

(G) Vehicle ADR

FKBP12 Merge F-actin

Nephrin GAPDH
novel interactions of FKBP12 with 14-3-3 and Synp. It is plausible that the direct interaction of FKBP12 with Synp also participates in the maintenance of F-actin and the processes of podocytes.

To elucidate the pathogenic significance of FKBP12 in nephrotic models, we also investigated the expression of FKBP12 in ADR-induced podocyte injury. Although CyPA expression was not altered, the expression of FKBP12 was clearly decreased in ADR nephropathy, a mimic of FSGS, and in human cultured podocytes treated with ADR (Figure 6). We also detected the decrease of FKBP12 in PAN nephropathy, a mimic of
MCNS (Figure S3). These observations suggested that the downregulation of FKBP12 at actin cytoskeleton alters the structure of actin fibers in the injured podocytes. The downregulation of FKBP12 participates in the initiation phase of pathogenesis in podocyte injury. We also investigated the effect of Tac on FKBP12 expression in the podocyte injury models. Tac treatment restored the expression of FKBP12 at F-actin in the processes of the injured podocytes. The treatment suppressed the decreases of F-actin staining and process formation (Figure 7). These observations indicated that Tac treatment ameliorates podocyte injury by restoring FKBP12 at F-actin in the processes of podocytes.

It is reported that CsA ameliorated podocyte injury by blocking the disruption of the 14-3-3-Synp complex through inhibiting dephosphorylation of Synp. The interaction of 14-3-3-Synp is regulated by phosphorylation in an opposite manner. It is suggested that dephosphorylated 14-3-3 is captured by phosphorylated Synp. Phosphorylation of 14-3-3 and/or dephosphorylation of Synp lead to the disruption of the interaction. The phosphorylation of Synp is antagonistically regulated by PKA/CaMKII and CN. The phosphorylation level of 14-3-3 is regulated by PKC, Akt, JNK, and CaMKIV. It is suggested that phosphorylation levels of 14-3-3 and Synp are strictly regulated by several kinases and phosphatases in podocytes. Interestingly, the present study revealed that Tac enhanced the interactions of FKBP12-Synp and 14-3-3-Synp independently of phosphorylation states of 14-3-3 and Synp (Figure 8). Although the mechanism of how Tac enhanced the interactions is not well explained, one possible explanation is that Tac enhanced the interactions by affecting the isomerase activity of FKBP12. FKBP12, a member of the immunophilin family protein, has peptidyl-prolyl isomerase activity. It is reported that peptidyl-prolyl isomerases modulate the accessibility of neighboring molecules by transitioning between the cis- and trans-isomer of proline. Synp contains many prolines that could be isomerized by FKBP12. Tac might enhance the interactions of Synp by affecting the isomerase activity of FKBP12.

We have previously reported that calcineurin (CN) is dominantly expressed at the slit diaphragm area of normal podocyte as an inactive form. The present study showed that FKBP12 was localized at F-actin in foot processes of podocytes, and the expression at slit diaphragm was not evident, suggesting that FKBP12 functions independently of CN in the physiological state. It is reported that FKBP12 does not interact with cain, an endogenous CN inhibitor. The report also showed that FKBP12 functions without affecting the activity of CN.

In the clinical field, Tac is widely used as an immunosuppressive agent, mainly in a transplantation area, and its toxicity to several organs limited the dose and the duration of the treatment. It is reported that Tac has toxicity to tubular epithelial cells and glomerular endothelial cells. In this study, 1 mg/kg BW of Tac was adopted as a dose for in vivo experiment. It is reported that no evident morphological alterations were detected in rats treated with 10 mg/kg BW. We have previously reported that mesangial alteration was ameliorated by the Tac treatment and that no alterations in glomerular endothelial cells or tubular epithelial cells were observed in rats treated with 1 mg/kg BW of Tac. Some other studies also reported that no evident morphological alterations in the kidney were detected in rats treated with 1 mg/kg BW of Tac. These reports suggested that the effect of Tac on podocyte detected in this study is the effect without causing damages in glomerular endothelial cells or tubular epithelial cells.

In conclusion, FKBP12 in glomeruli is restrictedly expressed in podocytes. In podocyte, FKBP12 is expressed along the actin cytoskeleton and associates with F-actin. FKBP12 interacts with 14-3-3 and Synp to maintain F-actin. FKBP12 plays a role in maintaining the expression of dephosphorylated 14-3-3. In the pathological state, FKBP12 is downregulated, and the decrease of FKBP12 at actin altered the structure of actin fibers. Tac treatment enhances the interactions of

FIGURE 7 Tacrolimus (Tac) treatment suppresses the decrease of FKBP12 in adriamycin-induced podocyte injury. (A) Dual-labeling immunofluorescence (IF) findings of podocin (green) and nephrin (red) in adriamycin (ADR) nephropathy treated with Tac. The decrease of nephrin and podocin staining in glomeruli was suppressed by Tac treatment on day 28 of ADR nephropathy. The score is shown as means ± SD (n = 4; *p < .05, U test). Scale bar, 20 μm. (B) IF staining of FKBP12 in ADR nephropathy treated with Tac. Tac treatment suppressed the decrease of FKBP12 staining in ADR nephropathy (n = 4; *p < .05, U test). Scale bar, 20 μm. (C) F-actin staining and expression of nephrin mRNA in the injured podocyte treated with Tac. Tac treatment suppressed the decrease of F-actin staining in the cultured podocyte treated with ADR (n = 4; *p < .05, U test). The proportion of the cells forming the processes to total cells was decreased in the podocytes treated with ADR. Tac treatment suppressed the decrease in the injured podocytes (arrow) (n = 3; *p < .05, t-test). The decrease of nephrin mRNA in the injured podocytes was suppressed by Tac treatment. Scale bar, 20 μm. (D) Western blot findings of FKBP12 in the injured podocyte treated with Tac. The protein expression of FKBP12 was decreased in the injured podocytes. The decrease was suppressed by Tac treatment (n = 3; *p < .05, t-test). The decrease of nephrin mRNA in the injured podocytes was suppressed by Tac treatment. Scale bar, 20 μm. (E) Dual-labeling IF findings of FKBP12 (green) with F-actin (red). Tac treatment restored the FKBP12 at F-actin in processes of the podocytes (arrowhead). Scale bar, 20 μm.
FKBP12-Synp and 14-3-3-Synp and restores FKBP12 at actin cytoskeleton. Tac treatment ameliorates the decrease of process formation in the injured podocyte. A schematic diagram of the putative model is shown in Figure 9. In the present study, we demonstrated that Tac treatment ameliorates podocyte injury by restoring FKBP12 at actin cytoskeleton. We propose another novel pharmacological mechanism that Tac protects podocyte in a calcineurin-independent manner.
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DISCLOSURES
All the authors declared no competing interest.

AUTHOR CONTRIBUTIONS
Hidenori Yasuda and Hiroshi Kawachi designed the experiments. Hidenori Yasuda performed major parts of the experiments and wrote the manuscript. Yoshiyasu Fukusumi supervised the experiments and contributed to in vivo experiments. Veniamin Ivanov and Ying Zhang contributed to the preparation of key reagents and materials. All authors were involved in data analysis. Hiroshi Kawachi conceived and directed the project and wrote the manuscript.

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