Methods

Mouse model of ADR-induced proteinuria

Female BALB/c mice weighing about 20 g and aged 8 weeks were obtained for the induction of ADR nephropathy from Oriental Yeast Co., Ltd. (Tokyo, Japan). ADR (doxorubicin hydrochloride; Wako, Osaka, Japan) diluted with 0.9% saline at a dose of 11 mg/kg body weight (BW) was injected once via the tail vein of each non-anesthetized mouse. Age-matched control female BALB/c mice were injected with an equal volume of PBS only. All mice were weighed and urine samples were collected once every few days. Urinary albumin/creatinine ratio (ACR) was measured using an immunoassay (DCA 2000 Systems; Siemens Medical Solutions Diagnostics, Tarrytown, NY) with a Bayer DCA 2000+ chemical analyzer (Bayer Diagnostics, Elkhart, IN) [22]. After anesthesia with sodium pentobarbital (100 mg/kg body weight; Dainippon Sumitomo Pharma, Osaka, Japan), five mice per day were euthanized on days 3, 7, 14, and 28 after the injection of ADR. All mice were housed under specific pathogen-free conditions using standard animal cages with free access to standard chow and drinking water.

Antibodies

Polyclonal rabbit anti-WT-1 antibody (Santa-Cruz Biotechnology Inc, CA), monoclonal mouse anti-WT-1 antibody (Dako Corporation, CA), monoclonal mouse

anti-GAPDH antibody (Abcam, Cambridge, UK), and polyclonal rabbit anti-cleaved caspase-3 antibody (Cell Signaling, Danvers, MA) were purchased for immunohistochemistry and WB analysis. Antibody against podocalyxin was a gift from Denka Seiken CO., LTD. To generate antibody against human dendrin, rabbits were immunized with a hemocyanin-conjugated peptide (single letter code, RHSQTLPRPWAPGGTGWC) corresponding to the amino acids of human dendrin. The anti-serum was affinity-purified with the corresponding peptide. Rabbit polyclonal antibodies against dendrin [20] and synaptopodin [23] have been described previously.

Plasmid constructs

Human dendrin cDNA (OriGene Technologies, Inc, MD) was cloned into pEGFP-C1 (BD Biosciences Clontech) and verified by DNA sequencing. FLAG- and GFP-tagged rat dendrin constructs have been described previously [20].

Renal histology and Immunohistochemistry

For the histological study, the mouse kidneys were fixed by perfusion fixation using 4% paraformaldehyde (PFA). The fixed kidneys were embedded in paraffin for light microscopy (LM) study. The 3 μm-thick sections were stained using the periodic acid-Schiff (PAS) technique. The sections were observed under LM (Olympus BX41;

Olympus). In PAS stained light microscopic sections, at least 100 midsections of glomerular areas and Bowman's capsule areas were measured using a digitizer KS-400 Imaging System as described previously [24].

For electron microscopy (EM), after perfusion fixation with 4% PFA, small blocks of kidney were fixed with 2% glutaraldehyde and postfixed in 1% osmium tetroxide (OsO_4). The samples were dehydrated in graded ethanol and embedded in epoxy resin. Ultrathin sections (80-100 nm thick) were stained with uranyl acetate and lead citrate and examined by electron microscopy (H-7100; Hitachi, Tokyo, Japan) at 75 kV.

For the immunofluorescence study, kidneys were fixed with the same agents as for LM, placed in OCT compound, frozen in liquid nitrogen and stored at -80 ℃. Immunostaining of podocyte-associated molecules (dendrin, WT-1 and synaptopodin) was performed. Double-stained immunofluorescence studies of the podocyte-associated proteins and post-staining with DAPI were performed as described previously [20]. The sections were observed under a confocal laser microscope (Olympus FV1000; Olympus, Tokyo, Japan). To examine the number of podocytes in glomeruli, double-positive cells in WT-1 and DAPI staining were counted in more than 100 glomeruli.

Human tissue samples

To show the location of dendrin in the nucleus in podocytes, triple staining for dendrin, podocalyxin or synaptopodin and DAPI was performed in rat kidney tissues and human kidney tissues. Tissue samples were obtained from diagnostic renal biopsies performed at Juntendo University Hospital. We investigated the samples from patients who had MCD (n=3), membranous nephropathy (MN) (n=3), FSGS (n=4), and lupus nephritis (LN) (n=4) and were manifesting nephrotic-range proteinuria. As control human samples, we used biopsy samples from the patients with minor glomerular abnormalities (n=5). The study was conducted under informed consent and was approved by the ethics committee on human research of the Juntendo University Faculty of Medicine.

Immunoblotting

To check the expression of dendrin, glomeruli were isolated from the kidneys using a graded sieving method [25]. WB analyses for dendrin, and for GAPDH as the internal control, were performed. Isolated glomeruli were lysed and immunoblotting was performed as described previously [25]. To evaluate the expression of cleaved caspase-3, cultured podocytes were treated with ADR (0.25 μg/ ml) for 0, 3 and 6 hours and WB was performed.

TUNEL assay

To detect apoptotic cells *in vivo*, an ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon international Inc., Temecula, CA) was purchased for the TUNEL assay. The TUNEL assay was performed according to the manufacturer's instructions. TUNEL-positive cells in the non-sclerotic parts of the glomeruli were counted.

Cell culture and treatment with ADR

Conditionally immortalized mouse podocytes were cultured as described previously [23]. To evaluate the reaction to ADR *in vitro*, the cells were treated with 0.01 μg/ml to 1.0 μg/ml of ADR in regular medium for 24, 48 and 72 hours. Immunofluorescence microscopy of cultured podocytes was performed as described previously [20]. Immunostaining of cultured podocytes was performed with anti-dendrin and anti-synaptopodin antibodies and DAPI. For the quantitative analysis of the ADR-mediated nuclear import of dendrin, 2000 cells were counted by confocal microscopy for each experiment.

Measurement of apoptosis by counting apoptotic nuclei with hypoploid DNA

The population of cells with hypoploid DNA was measured as described previously [26]. Briefly, the cells were centrifuged at 1,500 rpm for 5 min and the supernatant was decanted. The cells were chilled at 4 °C, then fixed at a concentration of 1-2 x 10^6

cells/ml in 70% ethanol at -20 ℃. Maintaining cells at -20 ℃ for 20 minutes yielded equivalent results. Prior to staining, cells were centrifuged at 1500 rpm for 5 minutes, washed twice with PBS and decanted. The fixed cells were re-suspended by light vortexing in 100 µl of 1 mg/ml RNase (Sigma Chemical Co., St. Louis, MO) solution at 37 ℃ for 20 minutes and were stained with 1 µl of propidium iodide (PI) (500 mg/ ml; Sigma Chemical Co., St. Louis, MO)[20].

Measurement of apoptosis by annexin V and PI staining

Apoptotic cells were identified by staining with annexin V and PI [20]. Briefly, cells (6×10^5) were treated with 0.25 µg/ml of ADR for 48 hours, then washed and suspended in a binding solution containing annexin V-biotin, at dilutions recommended by the manufacturer. After 30 minutes, the cells were washed and streptavisin-allophycocyanin (APC; 5 µg/ ml) was added. Following 20 minutes, PI (1 µg/ ml) was added. At least 10,000 cells were analyzed with FACS per sample. Early apoptosis, late apoptosis and whole apoptosis were determined as the percentages of annexin V+/ PI-, annexin V+/ PI+ and annexin V+ cells.

Statistical analysis

All values were expressed as the mean ± SE. Statistical significance (defined as $P<0.05$) was evaluated using a Tukey-Kramer test, Fisher's PLSD or t-test.

Results

ADR causes nephrosis and glomerulosclerosis in mice.

In ADR-injected mice, proteinuria significantly increased on day 7 (mg/ g· Cr, ACR control versus day 7: 16.87 ± 0.77 vs 184.38 ± 42.41, p< 0.05, n=8) and on day 28 (mg/ g· Cr: 162.63 ± 50.48, p< 0.05 vs control, n=8). There were two peaks of albuminuria in ADR-injected mice (**Figure 1A**). Hematuria was not detected in any nephrotic mice throughout the study period. Histopathologically, partial glomerulosclerosis (15 to 75% area of sclerosis in a glomerulus) and expansion of the mesangial area were observed from day 7. On day 28, global sclerosis was observed in some glomeruli (**Figure 1B**). Sclerotic glomeruli were significantly increased on day 28 (%: sclerotic glomerulus ratio: 3.26 ± 1.25, p< 0.05 versus control, each 100 glomeruli in n=3) (**Figure 1C**). To check glomerular hypertrophy on day 28, we compared the glomerular area with a control. The ratio for the glomerular and Bowman's capsular areas (GB) on day 28 (%: 78.25 ± 5.15, p< 0.05 versus control, n=3) was significantly increased compared with the GB ratio for the control (%: 76.45 ± 4.62, n=3) (**Figure 1D**). Under EM, FP effacement was observed from day 3 (**Figure 1E**) and developed gradually up to day 28. All forms of nephrotic syndrome are characterized by abnormalities of the molecular reorganization of SD in podocytes [1,27,28].

Injection of ADR in mice causes loss of podocytes from the GBM

A loss of podocytes from the GBM reportedly leads to glomerulosclerosis [4,9-11]. ADR injection was used to assess the podocyte loss from the GBM. To count podocyte numbers in the glomeruli, double-positive cells of WT-1 and DAPI were counted in each 100 non-sclerotic glomeruli (**Figure 2A and B**). Podocyte loss was detected from day 15. On day 28 after ADR injection, the number of podocytes (WT-1 positive cells) dropped significantly below the control level (WT-1 positive cell numbers of controls versus day 28: 13.47± 0.25 vs, 11.77 ± 0.27, $p< 0.001$ vs. control, each 50 glomeruli in n=3) (**Figure 2C**).

ADR induces podocyte apoptosis *in vivo* and *in vitro*

To detect apoptotic cells *in vivo*, TUNEL staining was performed. The most TUNEL-positive cells in glomeruli were detected on day 3 (%; counted TUNEL-positive cells / total glomeruli of control versus day 3: 0.14± 0.14 vs, 0.91± 0.16, $p< 0.05$ vs, control, each 100 glomeruli in n=3) (**Figure 3A, B**). The number of TUNEL-positive cells in the glomeruli gradually decreased after day 3. To evaluate whether the addition of ADR directly causes podocyte apoptosis *in vitro*, cultured podocytes were treated with 0.01 μg/ml to 1.0 μg/ml of ADR for 24, 48 and 72 hours. Measurement of apoptotic podocytes, by counting apoptotic nuclei with hypoploid DNA, showed that ADR induced podocyte apoptosis in a dose-dependent manner (%;

apoptotic nuclei of control versus 0.25 μg/ml ADR: 6.05 ± 2.37 vs, 65.41 ± 14.20, 0.5 μg/ml ADR: 88.24 ± 7.69, 1.0 μg/ml ADR: 95.34 ± 1.51, p< 0.0001 each vs, control, n=3) (**Figure 3C**) and in a time-dependent manner (%; apoptotic nuclei of control versus 48 hours after 0.25 μg/ml ADR treatment: 8.10 ±1.97 vs. 63.75 ±16.95, 72 hours: 97.55± 0.52, p< 0.0001 each vs. control, n=3) (**Figure 3D**). Furthermore, measurement of apoptosis by annexin V and PI staining also showed that ADR induced podocyte apoptosis (%; counted apoptotic nuclei for late apoptosis of the control versus 48 hours after 0.25 μg/ml ADR treatment: 3.07 ±1.02 vs 28.20 ± 3.64, for whole apoptosis of the control versus 48 hours after 0.25 μg/ml ADR treatment: 6.37 ±1.03 vs. 36.11 ± 4.48, p< 0.0001 each vs control, n=3) (**Figure 3E, F**). To detect whether podocyte apoptosis is associated with an apoptotic signal with caspase-3, WB analysis for cleaved caspase-3 was performed. The expression of cleaved caspase-3 increased in cultured differentiated podocytes with 0.25 μg/ml ADR treatment for 6 hours, compared with the control (**Figure 3G**). To examine whether apoptotic cells in the glomeruli of ADR induced nephrotic mice are podocytes, immunostaining with cleaved caspase-3 and podocyte marker synaptopodin was performed on day 3 after ADR injection. The staining of cleaved caspase-3 merged with that of synaptopodin.

Dendrin was localized in the podocyte nucleus and in the SD area of mice with ADR nephrosis.

To investigate the localization of dendrin in injured podocytes, immunostaining and WB analyses were performed using ADR-induced nephrotic kidneys. The expression pattern of dendrin showed a dotted pattern in glomeruli on days 7 and 15. An accumulation of dendrin was found in the podocyte nuclei, especially on day 3 after ADR injection. The expression of dendrin was almost restored on day 28 (**Figure 4A**). In the WB analysis for dendrin with GAPDH as the internal control, the expression of dendrin started to decrease on day 7. The expression was restored on day 28 (**Figure 4B**). In immunostaining with the nuclear podocyte marker WT-1 and dendrin, an accumulation of dendrin in the podocyte nuclei was found on day 3 after ADR injection (**Figure 4C**). Double-positive cells of dendrin and WT1 were counted in each period. A number of dendrin-positive nuclei were detected from day 3. On day 3 after ADR injection, the number of nuclear dendrin-positive podocytes (WT-1 positive cells) increased significantly compared to the number in the controls (% dendrin positive nuclei number / WT-1 positive cell number of controls versus day 3 in glomerulus :12.9 ± 1.9 vs, 23.4 ± 2.2, $p< 0.01$ versus control, each 30 glomeruli in n=3) (**Figure 4D**). To test whether ADR directly induced relocation of dendrin to the nuclei, the number of dendrin-positive nuclei in cultured podocytes was counted after 6 hours of incubation with ADR. The number of dendrin-positive nuclei in podocytes injured by ADR was significantly increased compared with control podocytes (%; nuclear control dendrin versus dendrin in podocytes injured by ADR: 8.81 ± 0.90 vs,

26.20 ± 3.23, p=0.001, assessed with 2000 cells, n=3) (**Figure 5A, B**).

Nuclear dendrin was detected in several human glomerular diseases, but not in MCD.

To examine the presence of translocated dendrin to podocyte nuclei in human glomerular diseases, we produced anti-human dendrin antibody and did immunostaining of dendrin on human kidney biopsy specimens. Anti-human dendrin antibody was designed to detect not only human dendrin, but also rat dendrin. Using WB analyses, the expression of dendrin was clearly detected in human and rat dendrin over-expressing HEK cells and in isolated rat glomeruli, but not in whole kidneys (**Supplementary Figure 1A, B**). After immunostaining, a linear pattern of dendrin was detected in normal rat kidneys (**Supplementary Figure 1C**). The staining pattern of dendrin almost merged with that of synaptopodin, but not with that of podocalyxin, because podocalyxin was located on the podocyte cell body and on primary FPs. Triple staining of human glomerulopathy with dendrin, podcalyxin and DAPI was then performed. Podocalyxin staining was used to detect the nuclear localization of dendrin, because the podocyte nucleus should be detected within the podocalyxin positive area. In MCD, dendrin staining showed both linear and dotted patterns in the capillary area (**Figure 6A**). Some dendrin-positive nuclei in glomeruli were detected in biopsy specimens from FSGS (**Figure 6B**). Dendrin-positive nuclei

in the glomeruli were also detected in specimens from LN (**Figure 6C**) and MN (**Figure 6D**). The mean number of dendrin-positive nuclei per glomerulus were (1.60 ± 0.51, 1.00 ± 0.58, 11.25 ±2.78, 6.33 ±0.67 and 13.00 ± 4.95 in samples from control kidney biopsy specimens (n=5), MCD (n=3), FSGS (n=3), MN (n=3) and LN (n=4), respectively (Figure7). Samples in FSGS, MN and LN, showed significantly higher numbers of dendrin-positive nuclei per glomerulus than control samples ($p<0.05$).

Discussion:

Guo et al. showed that ADR induces transcripts of TGF-β 1 and connective tissue growth factor and production of AGE and reactive oxygen species (ROS) 3 days after ADR injection in mice [29]. Koshikawa et al. also showed there was a marked increase in phosphorylated p38 MAPK in the podocytes of ADR nephropathy on day 3 [30]. In the present study, apoptotic cells increased the most and foot-process effacements in glomeruli were detected on day 3, when urinary albumin excretion was still within a normal range. Interestingly, a significantly higher number of dendrin-positive podocyte nuclei were detected on day 3 after ADR injection in mice. Furthermore, a 6-hr treatment with ADR, not inducing podocyte apoptosis, promoted relocation of dendrin to the nuclei of cultured podocytes. It is conceivable that some intracellular signaling cascades will give rise to serial reactions of the genes and proteins, including dendrin, that alter barrier function and cause podocyte apoptosis leading to overt proteinuria and podocyte loss.

To detect podocyte nuclei in mouse kidneys, anti-WT-1 antibody is usually used. However, the mouse monoclonal WT-1 antibody that gave a cytoplasmic stain of human glomeruli was not used, as Murea et al. reported [31]. Instead, anti-podocalyxin antibody and DAPI staining to detect podocyte nuclei in human kidney biopsy specimens was used in the present study.

Some reports suggest that podocyte apoptosis causes podocyte loss and leads to

glomerulosclerosis in human glomerulopathy [9,12]. In MCD, there was no podocyte loss and glomerulosclerosis [1]. Duner et al. reported that nuclear dendrin was not detected in MCD, although dendrin are re-distributed within podocytes [21]. In the present study, most dendrin was located at the SD area in MCD. However, it was possible to detect dendrin clearly located to the podocyte nucleus in FSGS, MN and LN. Podocyte apoptosis has reportedly caused podocyte loss and led to glomerulosclerosis [1]. These results suggest that relocation of dendrin to the podocyte nucleus as a response to injury might be related to podocyte apoptosis and the loss of podocytes from the GBM in human glomerulopathy, but not in MCD. The amount of nuclear dendrin could be an indicator of disease activity and progression to glomerulosclerosis and end stage renal disease (ESRD) in renal biopsy. In future studies, it will be necessary to investigate the relationship between nuclear dendrin and disease progression and activity in human glomerular disease.

The SD represents a signaling platform that contributes to the regulation of podocyte function in health and disease [32]. Both CD2AP and nephrin, SD-associated proteins, interact with the p85 regulatory subunit of phosphatidylinositol 3-kinase *in vivo*, stimulating antiapoptotic Akt signaling [2,33,34]. Podocytes lacking CD2AP are more susceptible to apoptosis, and mice lacking CD2AP show increased podocyte apoptosis [4,35]. Reportedly, the C-terminus of dendrin interacts with CD2AP [20]. Thus, it is possible that CD2AP regulates localization and nuclear translocation of

dendrin in injured podocytes. However, it is still unknown how nuclear dendrin provokes apoptosis in injured podocytes. Now, we are trying to elucidate how nuclear dendrin helps to promote podocyte apoptosis.

This study demonstrated that severe podocyte injury, which ADR causes in mice, induced translocation of dendrin to the podocyte nucleus before urinary albumin excretion, and podocyte loss and glomerulosclerosis developed. Moreover, nuclear dendrin was found in patients with glomerulopathies (FSGS, MN and LN), but not in MCD. These results suggest that the location of dendrin in the nucleus is a candidate novel marker to estimate the number of podocyte loss and to predict progression to glomerulosclerosis.

Acknowledgments

We thank Prof. Peter Mundel (University of Miami, Miller School of Medicine) for helpful discussions; Dr. Yutaka Kanamaru (Juntendo University) for his technical assistance and helpful discussions; and, Ms. Kaori Takahashi, Mr. Junichi Nakamoto, Mr. Mitsutaka Yoshida and Mr. Alehandro Oliva (Juntendo University) for their excellent technical assistance. This work was supported by research grants from the Takeda Science Foundation, Kanae Foundation for the Promotion of Medical Science and Kowa Life Science Foundation to K.A.; by a Grant–in-Aid for Scientific Research (Grant-in–Aid for Young Scientists (Start-up): (19890213) to K.A.; by Challenging Exploratory Research:(21659217) to K.A.; by a Grant-in–Aid for Young Scientists (B):(21790821) to E.A.; by a projected research grant from Juntendo University to MT; by a grant-in-aid (No. 12146205) to E.K for Scientific Research, by a grant-in-aid (No. 18076005) to T.U. for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan, and by a grant-in-aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare (No. 16271401) to T.U.

Figure legends

Figure 1: ADR induced nephrosis and caused glomerulosclerosis in mice.

A single dose (11 mg/ kg BW) of ADR was injected via the tail vein to achieve a nephrotic state. Urine samples were collected from all mice once every few days. (**A**) ADR induced massive albuminuria (ACR: urinary albumin creatinine ratio), with two significant peaks on days 7 and 28 (n=5-10, P< 0.01). The columns represent the mean ± SE of each value from different samples. Asterisks show that the difference in the urinary albumin creatinine ratio, from that on day 0, was significant. (**B**) Partial glomerulosclerosis was observed from day 7 and gradually increased. At day 28 after ADR injection, there were many casts and wide-ranging glomerulosclerosis compared with the control. Original magnifications were x 100 (for lower magnification) and x 400 (for higher magnification). (**C**) The incidence of sclerotic glomeruli among the total glomeruli per section on day 28 after ADR injection showed a significantly higher rate than that on day 0 (each 100 glomeruli in n=3, p< 0.05). Asterisks show that the difference in the sclerotic glomerulus/total glomerulus ratio, compared with day 0, was significant. (**D**) There was a significant increase in GB ratio between day 0 and day 28 after ADR injection (n=3, p< 0.05). Asterisks show that the difference in the GB ratio compared with day 0 was significant. The columns represent the mean ± SE of each value from different samples. (E) Kidneys with ADR-induced nephropathy showed FP effacement by EM. There was no FP effacement in the control glomerulus. FP

effacement (**arrows**) was observed from day 3 and was markedly increased from day 7 to day 28. Original magnifications were x 3,000 and x 10,000, Scale bar, 5μm.

Figure 2: Injection of ADR caused loss of podocytes in mice.

(**A, B**) Mean podocyte numbers per glomerulus in the controls and on day 28 are shown for both DAPI positive and WT-1 positive cells (**asterisks**). Original magnification was x 1,000. (**C**) A significant decrease in the number of podocytes was detected on day 28. After triple staining with WT-1, synaptopodin and DAPI, WT-1-positive cells on day 28 were significantly lower than the control level (each 50 glomeruli in n=3, P< 0.01). The columns represent the mean ± SE of each value from different samples.

Figure 3: ADR induces podocyte apoptosis *in vivo* and *in vitro*.

(**A**) TUNEL-positive cells in glomeruli (**B**) showed the highest numbers on day 3. Asterisks show that the difference in the TUNEL positive cells/total glomerulus compared with day 0 is significant. The columns represent the mean ± SE of each value from different samples (n=3, p< 0.05). (**C**) Analysis of hypoploid nuclei in cultured differentiated podocytes after treatment with 0.01 μg/ml to 1.0 μg/ml of ADR or control for 48 hours. Treatment with 0.25 μg/ml to 1.0 μg/ml of ADR induced significant apoptosis (n=3, P<0.0001). (**D**) Analysis of hypoploid nuclei after treatment

with 0.25 μg/ml of ADR for 24, 48 and 72 hours and control. Treatment with ADR for 48 and 72 hours induced significant apoptosis (n=3, p<0.0001). Asterisks show that the difference in the percentage (apoptotic cells/all cells) compared with the controls was significant. The columns represent the mean ± SE of each value from different samples. (**E, F**) Analysis of annexin V/PI double-positive populations in cultured differentiated podocytes after treatment with 0.25 μg/ml of ADR for 24 and 48 hours. This method also showed that treatment with ADR for 48 hours induced significant podocyte apoptosis (n=3, p<0.0001). Annexin V-positive cells (PI-negative), PI-positive cells (Annexin V-negative) and double-positive cells indicate early apoptotic cells, necrotic cells and late apoptotic cells, respectively. Asterisks show the differences from the controls in the percentage of late apoptosis/all cells, early apoptosis/all cells and whole apoptosis/all cells, which were significant. The columns represent the mean ± SE of each value from different samples. (**G**) To evaluate the expression of cleaved caspase-3, cells were treated with ADR (0.25 μg/ ml) for 0, 3 and 6 hours. The expression increased at 6 hours after ADR treatment per WB analysis. (H) The localization of cleaved caspase-3 in ADR-injected kidneys (day3) was investigated by immunostaining.

Figure 4: Expression and localization of dendrin in ADR-injected mice.

(**A**) The expression and localization of dendrin in control and ADR-injected kidneys

(days 3, 7, 15 and 28) were investigated by immunostaining. Significant nuclear localization of dendrin in podocytes (asterisks) was detected on day 3. Original magnification was x 1,000. **(B)** In the WB for dendrin and GAPDH, reduction of dendrin due to ADR nephropathy was observed on days 7 and 15, whereas GAPDH showed no reduction. **(C)** To count the number of dendrin positive-nuclei in podocytes, double staining of dendrin and WT1 was performed. The nuclear localization of dendrin (asterisks) in podocytes was significant on day 3. **(D)** The numbers of dendrin-positive nuclei and WT-1-positive nuclei per glomerulus were significant on day 3 (30 glomeruli each in n=3, $P< 0.05$). Asterisks show that the difference in the ratio, compared with the controls, was significant. The columns represent the mean ± SE of each value from different samples.

Figure 5: ADR induced nuclear import of dendrin in cultured podocytes.

(A) ADR induced nuclear import of dendrin after serum starvation for 24 hours. Nuclear translocation of dendrin can be completed in 6 hours. **(B)** Quantitative analysis of ADR-induced nuclear import of dendrin. For details, see ***Results***.

Figure 6: Kidney sections from several human glomerulopathies showed the nuclear relocation of dendrin.

(**A- D**) To show nuclear dendrin in human glomerular diseases, triple staining for