

Dedifferentiation of Immortalized Human Podocytes in Response to Transforming Growth Factor- β

A Model for Diabetic Podocytopathy

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OBJECTIVE—Diabetic nephropathy is associated with dedifferentiation of podocytes, losing the specialized features required for efficient glomerular function and acquiring a number of profibrotic, proinflammatory, and proliferative features. These result from tight junction and cytoskeletal rearrangement, augmented proliferation, and apoptosis.

RESEARCH DESIGN AND METHODS—Experiments were performed in conditionally immortalized human podocytes developed by transfection with the temperature-sensitive *SV40-T* gene. Cells were then cultured in the presence of transforming growth factor (TGF)- β 1 or angiotensin II in the presence or absence of a selective inhibitor of the TGF- β type I receptor kinase, SB-431542. Gene and protein expression were then examined by real-time RT-PCR and immunofluorescence, and correlated with changes observed in vivo in experimental diabetes.

RESULTS—Treatment of cells with TGF- β 1 resulted in dynamic changes in their morphology, starting with retraction and shortening of foot processes and finishing with the formation of broad and complex tight junctions between adjacent podocytes. This dedifferentiation was also associated with dose- and time-dependent reduction in the expression of glomerular epithelial markers (nephrin, *p*-cadherin, zonula occludens-1) and increased expression of mesenchymal markers (α -smooth muscle actin, vimentin, nestin), matrix components (fibronectin, collagen I, and collagen IV α 3), cellular proliferation, and apoptosis. The induction of diabetes in mice was also associated with similar changes in morphology, protein expression, and proliferation in glomerular podocytes.

CONCLUSIONS—In response to TGF- β and other TGF-dependent stimuli, mature podocytes undergo dedifferentiation that leads to effacement of foot processes, morphologic flattening, and increased formation of intercellular tight junctions. This simplification of their phenotype to a more embryonic form is also associated with reentry of mature podocytes into the cell cycle, which results in enhanced proliferation and apoptosis. These “pathoadaptive” changes are seen early in the diabetic glomerulus and ultimately contribute to albuminuria, glomerulosclerosis, and podocytopenia. *Diabetes* 60:1779–1788, 2011

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Diabetic kidney disease is associated with significant podocyte injury and dysfunction (1–3). Foot process retraction and flattening (known as effacement) enhances the loss of protein into the primary urine by altering the architecture of the slit pore and subpodocyte space (4) and reducing the ultrafiltration coefficient leading to glomerular hypertension (1). Podocytes are also responsible for the maintenance of the glomerular basement membrane, its charge barrier, and the shape and integrity of the glomerular capillary loop, all functions that are compromised in the diabetic glomerulus. In addition, mature podocytes can dedifferentiate, losing the specialized features required for efficient glomerular function, and in the process acquire a number of profibrotic (5–7), proinflammatory (3), and proliferative features (8).

A number of factors have been suggested as potential initiators of podocyte effacement in response to chronic hyperglycemia, including angiotensin II, advanced glycation end products, interleukin-1, and mechanical and oxidative stress (9–17). Each of these stimuli seems to require the induction of transforming growth factor (TGF)- β (18,19). It has been shown that exposure of differentiated podocytes to hyperglycemia in vitro results in upregulation of TGF- β expression (20), paralleling its upregulation in diabetic glomeruli (21). High glucose also augments the response of the podocyte to ambient levels of TGF- β (20). TGF- β is known to have concentration-dependent effects on podocyte differentiation (22) and apoptosis (22–24). In this article, we explore the mechanics of dedifferentiation in glomerular epithelial cells in high glucose using a conditionally immortalized differentiated human podocyte cell line and show that cultured podocytes undergo a dynamic range of functional and structural morphologic changes equivalent to those observed in vivo in diabetic glomeruli, which result from tight junction and cytoskeletal rearrangement, apoptosis, and augmented proliferation.

RESEARCH DESIGN AND METHODS

Cell culture models. Experiments were performed in conditionally immortalized human podocytes developed by transfection with the temperature-sensitive *SV40-T* gene (25). Podocytes were propagated and seeded at 33°C in RPMI with 25 mmol/L glucose with 10% FCS and 1×ITS media supplement (Sigma-Aldrich, St. Louis, MO), which contains 1.0 mg/mL insulin from bovine pancreas, 0.55 mg/mL human transferrin, and 0.5 μ g/mL sodium selenite. When cells had grown to ~60% confluence, they were transferred to 2% FCS media and incubated at 37.5°C for 10–14 days. Under these conditions, the podocytes undergo growth arrest, display the typical arborized pattern of foot process extensions, and express markers of mature podocytic differentiation in vivo, including Wilm’s tumor (WT)-1 and nephrin. Cells were then cultured

in RPMI with 25 mmol/L glucose in the presence or absence of TGF- β 1 (2, 5, or 10 ng/mL, R&D Systems, Minneapolis, MN) or angiotensin II (1 nM, Auspep, Parkville, Victoria, Australia) with or without the selective inhibitor of the TGF- β type I receptor kinase, SB-431542 (10 μ mol/L, TOCRIS, Ellisville, MO). **Live cell imaging.** Contraction of individual podocytes was observed using time-lapse video microscopy on the stage of an inverted phase-contrast microscope (Zeiss, Oberkochen, Germany). Images were recorded by time-lapse video intervals and stored as stacks, processed, and displayed as eight frames per second (ImageJ).

Immunofluorescence. Cells were grown on coverslips, washed twice with PBS, fixed in 4% paraformaldehyde for 20 min, permeabilized using 1% SDS, and incubated in a blocking buffer (1% BSA, 0.25% Triton \times 100 in PBS, pH 7.4). Primary and secondary antibodies were diluted in blocking buffer, and the cells with antibodies were incubated overnight at 4°C. Coverslips were then mounted onto glass microscope slides using Prolog Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA) or TO-PRO-3 (Invitrogen). F-actin was visualized by fluorescent phalloidin (Alexa Fluor 594 phalloidin, Invitrogen). Cells were viewed using an Olympus (Tokyo, Japan) BX61 fluorescence microscope, and images were captured on a Zeiss 510 Meta laser scanning confocal microscope (Zeiss) using LSM 510 software (version 3.2 SP2; Zeiss) or an Olympus BX61 fluorescence microscope. Primary antibodies used included the following: α smooth muscle actin (α SMA Clone 1A4; Dako, Cupertino, CA), or P-cadherin (R&D Systems), nestin (R&D Systems), zonula occludens-1 (ZO-1; Invitrogen), vimentin (Sigma, St. Louis, MO), α -tubulin (Sigma), collagen I (Southern Biotech, Birmingham, AL), collagen I (Southern Biotech), fibronectin (Sigma), nephrin (Santa Cruz Biotechnology, Santa Cruz, CA), synaptopodin (Santa Cruz Biotechnology), proliferating cell nuclear antibody (PCNA; Santa Cruz Biotechnology), and WT-1 (Santa Cruz Biotechnology). Secondary antibodies used for immunofluorescence detection included Alexa Fluor 488 (mouse anti-rabbit, goat anti-rabbit, and rabbit anti-mouse), Alexa Fluor 594 (rabbit anti-goat, rabbit anti-mouse), and Alexa Fluor 689 (donkey anti-goat). F-actin was stained with phalloidin red (Invitrogen). For nephrin staining, a specific antibody to monoclonal antibody 5-1-6 antigen was used, which is identical to rat nephrin (26).

Western blot analysis. Cells were homogenized in lysis buffer (20 mmol/L Tris at pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.5% sodium deoxycholate, 50 mmol/L NaF, and 2 mmol/L Na₃VO₄) containing 5% Protease Inhibitor Cocktail (Sigma). Protein quantity was determined by BCA protein assay kit (Pierce, Rockford, IL). Samples were run on 6–12% SDS-PAGE and transferred onto PVDF membranes by semidry transfer (Semi Dry Transfer Cell; Bio-Rad Laboratories, Inc., Hercules, CA). After transfer, all incubations were conducted on a rocking platform at room temperature. The membrane was blocked in 5% skim milk/Tris-buffered saline with Tween overnight and then incubated for 1 h with α SMA (1:2,000; Dako), connective tissue growth factor (1:1,000 Abcam, Cambridge, U.K.), P-cadherin (1:1,000 R&D Systems), ZO-1 (1:1,000 Invitrogen), vimentin (1:2,000 Sigma), collagen I (1:2,000 Southern Biotech), collagen IV (1:1,000 Southern Biotech), fibronectin (1:1,000 Sigma), synaptopodin (Santa Cruz Biotechnology), PCNA, p21Cip1 (1:1,000 Santa Cruz Biotechnology), and p27Kip1 (1:20,000 Sigma). The membrane was washed with Tris-buffered saline with Tween and then incubated with a peroxidase-conjugated goat anti-mouse, goat anti-rabbit, or mouse anti-goat secondary antibody (EnVision; Dako) for 1 h. Immunoreactivity was detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). Quantitation of blots was carried out using Quantity One software on the Chemidoc XRS imaging system (Bio-Rad Laboratories, Inc.).

Gene expression analysis. Gene expression was analyzed by real-time RT-PCR, performed as described previously (22) using the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7500; Perkin-Elmer, Foster City, CA). Fluorescence for each cycle was quantitatively analyzed by an ABI Prism 7500 Sequence Detection System (Perkin-Elmer). To control for variation in the amount of DNA that was available for PCR in the different samples, gene expression of the target sequence was normalized in relation to the expression of an endogenous control 18S ribosomal RNA (18S rRNA TaqMan Control Reagent kit, ABI Prism 7500; Perkin-Elmer). Triplicate experiments were performed, with six replicates. Results were expressed relative to control (untreated) cells, which was arbitrarily assigned a value of 1. Values are shown as mean \pm SEM, unless otherwise specified. $P < 0.05$ was considered significant (*t* test).

Assessment of proliferation, apoptosis, and detachment. To measure cellular proliferation, mature podocytes were seeded (1,000 cells/well) in 96-well plates and treated with TGF- β . Cell proliferation was monitored with a VICTOR3 V Multilabel Counter (Perkin-Elmer) at a wavelength of 490 nm using the Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). Apoptosis was estimated using the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega). Both were performed according to the manufacturers' protocols. To measure cell detachment, cells were grown in a 24-well culture

dish and treated with 10 ng/mL TGF- β for 24 h. Medium was removed, and cells were trypsinized until all cells were suspended, which was optically controlled. Hereafter, cells were left to settle again in culture medium containing TGF- β for 1 h. Cells in suspension and adherent cells were collected separately, and cell number was measured. Expression of cell-cycle mediators was determined by real-time RT-PCR and Western blotting, as detailed above.

Assessment of albumin permeability. The amount of fluorescein isothiocyanate (FITC)-labeled BSA that passed across a podocyte monolayer was measured, as described by Maruo et al. (27) with minor modifications. In brief, monolayers were grown on the surface of membrane filters (0.4- μ m pore; Corning Costar Corp., Cambridge, MA) on which FITC-BSA (10 mg/mL 100 μ g/mL; Sigma-Aldrich) was put into the top chamber. After incubation for different time periods, fluorescence in the basolateral compartment was measured using fluorescence spectroscopy (excitation = 490; emission = 525 nm). To quantify the transmembrane flux of FITC-BSA in micrograms per hour, we calculated the BSA concentrations in the lower chambers using fluorescent arbitrary units of the albumin solution added to the apical compartment, taking into account the volume of the basolateral compartment.

In vivo studies. To correlate changes observed in vitro with those seen in vivo, the expression of key proteins was examined after the induction of diabetes in apoE-KO mice with five daily injections of streptozotocin (55 mg/kg). Control mice received an equivalent volume of citrate buffer. This model of diabetes results in sustained chronic hyperglycemia (blood glucose >15 mmol/L) and accelerated diabetic nephropathy, with glomerular lesions similar to those observed in human diabetes (28). Control and diabetic mice were then followed for 10 weeks, during which time they had access to standard mouse chow and water ad libitum. After 10 weeks, animals were given a fatal overdose of anesthetic and exsanguinated by cardiac puncture. Both kidneys were removed and fixed in 4% paraformaldehyde in PBS for subsequent immunofluorescent histologic analysis using the techniques detailed above.

Statistical analyses. Values are shown as means \pm SEM unless otherwise specified. Statview (Brainpower, Calabasas, CA) was used to analyze data by unpaired Student *t* test or by ANOVA and compare using the Fisher protected least significant difference post hoc test. Nonparametric data were analyzed by Mann-Whitney *U* test. *P* values < 0.05 were considered significant.

RESULTS

Cell model and phenotype. This study used a conditionally immortalized differentiated human podocyte cell line, incubated in 2% FCS at 37.5°C for 14 days. Under these “nonpermissive” conditions, these cells show many of the specialized characteristics of mature podocytes, including dynamic motility, an aberrant appearance, interdigitating actin-rich foot processes, cortical f-actin, microvilli, and coated pits (Fig. 1, *inset*), as well as the expression of specialized proteins associated with slit-pores (e.g., nephrin, p-cadherin), filaments (e.g., nestin), and podocyte-specific transcriptional factors (e.g., WT-1) (Fig. 2).

Induction of dedifferentiation by TGF- β 1. Treatment of immortalized human podocytes with TGF- β 1 resulted in rapid changes in morphology and motility that were observed using time-lapse video microscopy (Fig. 3, Supplementary Video). The first visible change was retraction and shortening of foot processes and contraction of the podocyte cell body, which occurred rapidly after exposure to TGF- β 1 and was maximal at 60 min. During this time period, the specialized arrangement of F-actin containing filaments was significantly reorganized, with the peripheral “ring”-like expression seen in mature podocytes giving way to coarse filaments aligned along the cell axis that act to retract foot processes and compact the cell body (Fig. 3). This change was followed by flattening, broadening, and elongation of the cell. During this transition, the microvilli and coated pits that covered the mature podocyte surface were also lost, being replaced by the smooth and featureless landscape of the dedifferentiated phenotype (Fig. 1). The phenotypic transition was completed with the formation of broad and complex tight junctions between adjacent podocytes (a process inappropriately called “fusion”). This was associated with

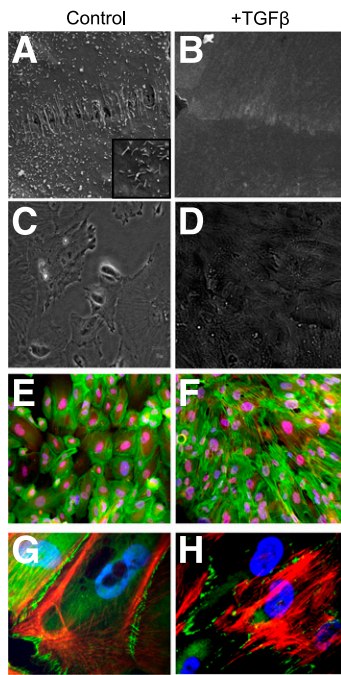


FIG. 1. Morphologic changes induced in immortalized human podocytes after treatment with TGF- β 1 (10 ng/mL, *right*) for 3 days when compared with control cells (*left*), as shown by scanning electron microscopy (A and B, magnification 100 \times , insert showing microvilli), light microscopy (C and D, magnification 100 \times), immunofluorescence staining for β -actin (green) and nestin (red) (E and F), and immunofluorescence staining for F-actin (red) and ZO-1 (green) with a blue nuclear counterstain (DAPI) (G and H). (A high-quality color representation of this figure is available in the online issue.)

reduced dynamic motility and increased expression of tight junction proteins, ZO-1, as well as a significant shift in their distribution, with the formation of continuous linear “zipper-like” structures (Figs. 2 and 3). Dedifferentiation

was also associated with dose- and time-dependent reduction in the gene expression of glomerular epithelial markers (nephrin, *p*-cadherin, and ZO-1) and increased expression of mesenchymal markers (α SMA, vimentin, and nestin) and matrix components (fibronectin, collagen I, and collagen IV α 3) (Fig. 4). Quantitatively similar changes were also observed at a protein level (Fig. 4) and on immunofluorescence staining (Fig. 2).

Finally, although mature podocytes are postmitotic, dedifferentiation induced after treatment with TGF- β 1 was associated with a time-dependent increase in cellular proliferation, as assessed by a proliferation assay, cell counting, and the induction of *PCNA* and cell-cycle regulators at a gene and protein level (Fig. 5). At the same time, treatment with TGF- β 1 also resulted in increased apoptosis, as assessed by the caspase 3/7 assay (Fig. 5).

Induction of dedifferentiation by angiotensin II. Angiotensin II also plays an important role in diabetic podocytopathy, because both ACE inhibitors and AT₁ receptor antagonists are able to attenuate podocyte foot process effacement and loss of nephrin expression in experimental models of diabetic nephropathy. In the study cells, angiotensin II was able to induce changes of dedifferentiation (Fig. 6), similar to those observed with TGF- β 1. Moreover, angiotensin II-dependent dedifferentiation was blocked by the selective inhibitor TGF- β type I receptor kinase, SB-431542 (Fig. 6).

Functional effects on albumin permeability. Despite significant morphologic changes, there was no evidence of increased podocyte detachment. On the contrary, increased tight junction formation between adjacent podocytes after chronic treatment with TGF- β 1 led to a time-dependent reduction in the detachment from the monolayer (data not shown). Consistent with this finding, the permeability of the podocyte monolayer to FITC-labeled albumin was also reduced by 38% after long-term treatment with TGF- β 1 for 3 days (control $26 \pm 2 \mu\text{g}/\text{min}$; TGF- β 1 $16 \pm 3 \mu\text{g}/\text{min}$, $P < 0.01$). However, an initial transient increase in albumin

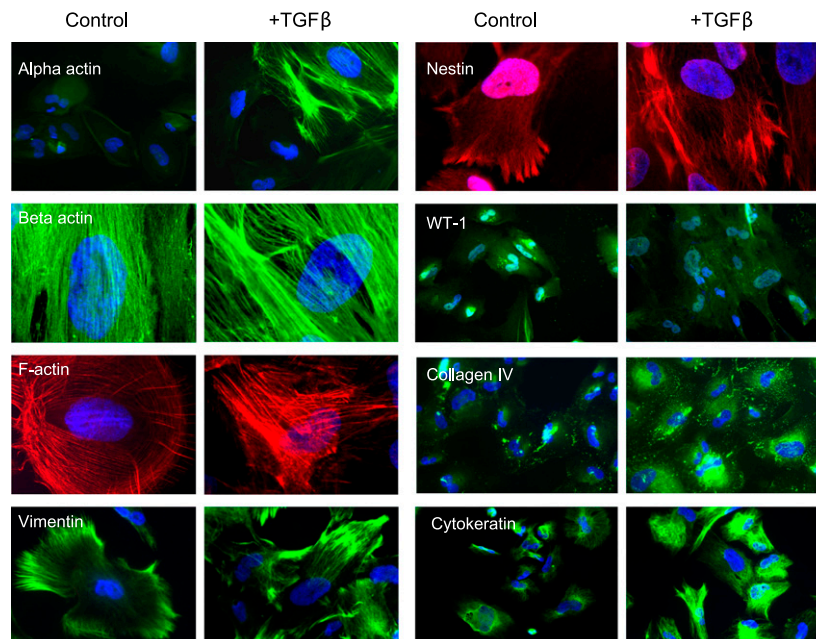


FIG. 2. Changes in the expression of key markers of differentiation in immortalized human podocytes in response to treatment with TGF- β 1 (10 ng/mL) for 3 days when compared with control cells. Immunofluorescence staining for α -actin, β -actin, F-actin, vimentin, nestin, WT1, collagen IV α 3, and cytokeratin, with a blue nuclear counterstain (DAPI). (A high-quality color representation of this figure is available in the online issue.)

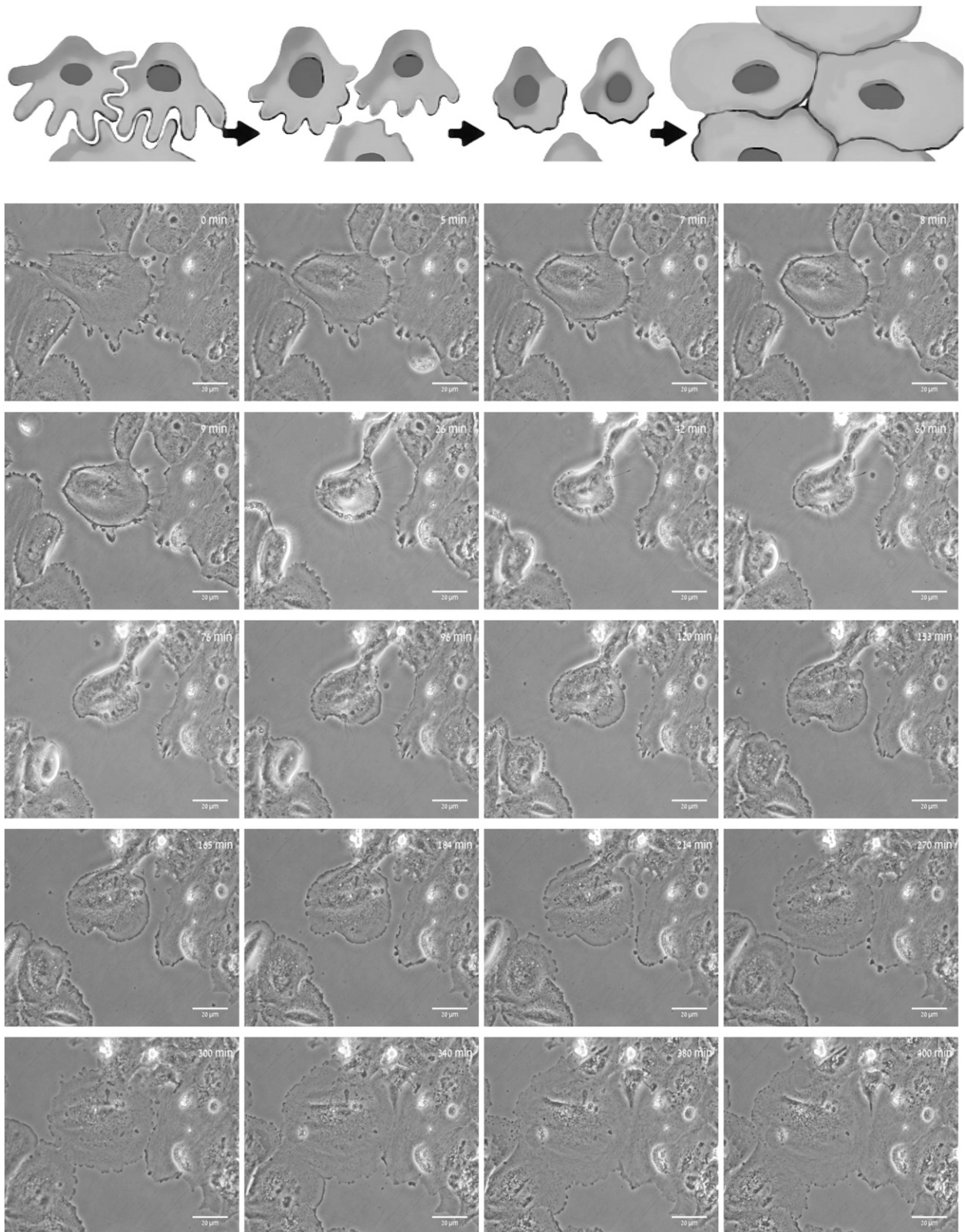


FIG. 3. Dynamic changes in the morphology of immortalized human podocytes after treatment with TGF- β 1 (10 mg/mL) over 6 h. A cartoon (*top*) illustrates changes observed in real-time video microscopy (*bottom*, Supplementary Video). Initial retraction and shortening of foot processes and contraction of the podocyte cell body are followed by flattening, broadening, and elongation of the cell and the formation of broad and complex tight junctions between adjacent podocytes. (A high-quality digital representation of this figure is available in the online issue.)

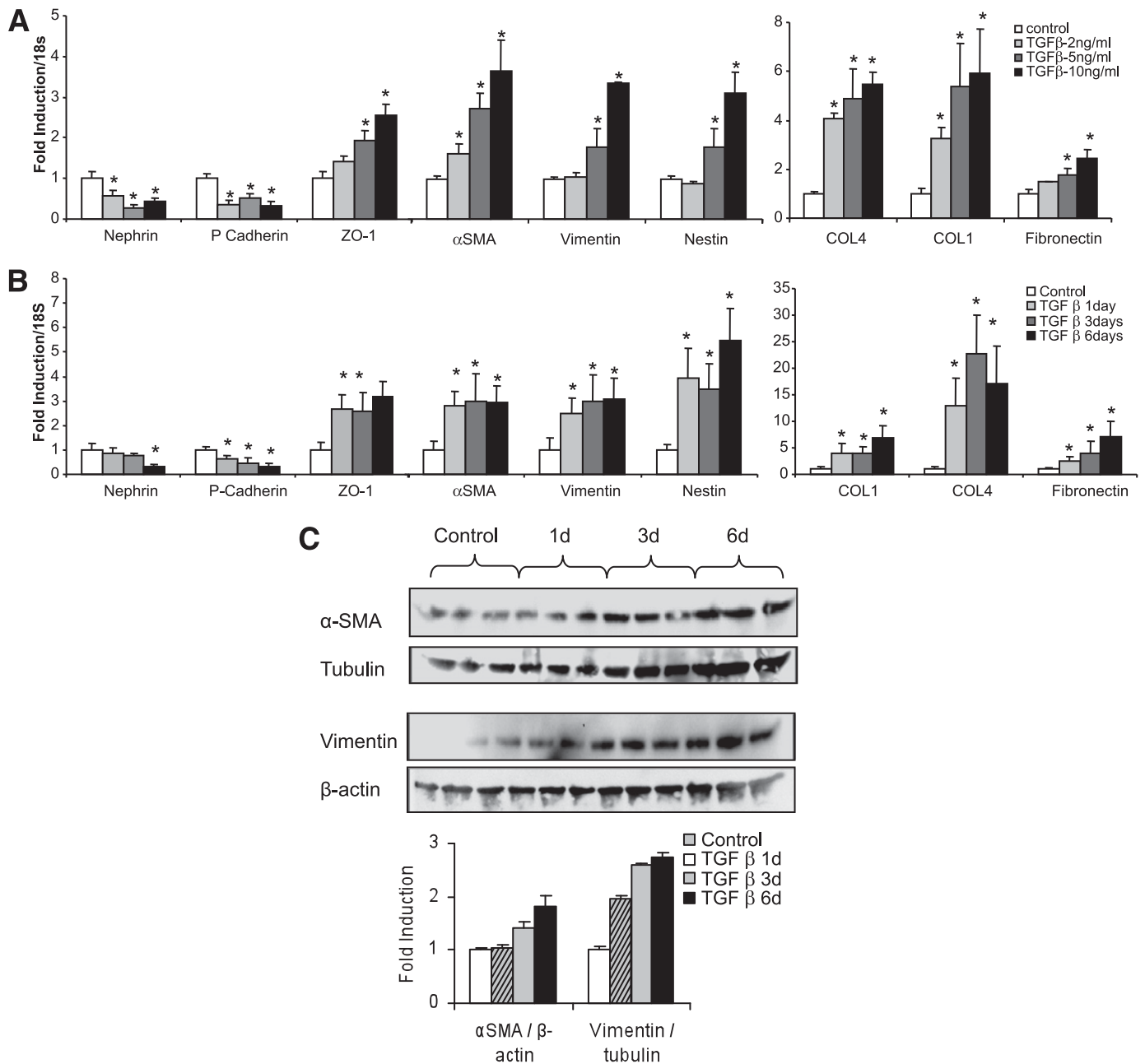


FIG. 4. The induction of dose-dependent (A) and time-dependent changes (B) in the expression of key target genes in immortalized human podocytes after treatment with TGF- β 1 (2–10 mg/mL) for 1–6 days, as measured by real-time RT-PCR ($n = 6/\text{group}$). C: Time-dependent changes in the expression of α -SMA and vimentin protein induced by TGF- β 1 (10 mg/mL), as measured by Western blotting and adjusted for actin and tubulin expression, respectively. * $P < 0.05$ vs. control.

permeability was noted after exposure to TGF- β 1 (30 min, control $17 \pm 2 \mu\text{g}/\text{min}$; TGF- β 1 $23 \pm 2 \mu\text{g}/\text{min}$, $P = 0.02$), as previously described by others (29), potentially reflecting the retraction of foot processes and contraction of the cell body that was observed on light microscopy, which preceded the subsequent spreading, flattening, and interconnection of adjacent podocytes observed at later time points (Fig. 3).

Podocyte dedifferentiation in the diabetic kidney. The induction of diabetes in mice was associated with changes in both morphology and distribution of protein expression in glomerular podocytes (Fig. 7). Most notably, the extensively arborized pattern of interlocking foot processes

was reduced in diabetic mice, with fewer, shorter, and broader foot processes observed on the immunofluorescent stain for the intermediate filament, nestin, as a marker for podocyte and foot processes. This change was associated with changes in the expression and orientation of f-actin, which change their circular configuration in control cells to linearize in diabetic podocytes and form stress fibers. Increased expression of mesenchymal markers, α -SMA, and vimentin was also observed in diabetic podocytes. In addition, changes in tight junction were also observed in diabetic podocytes with reduced expression of the slit-pore protein, nephrin. Finally, specific evidence of podocyte proliferation was observed in diabetic podocytes

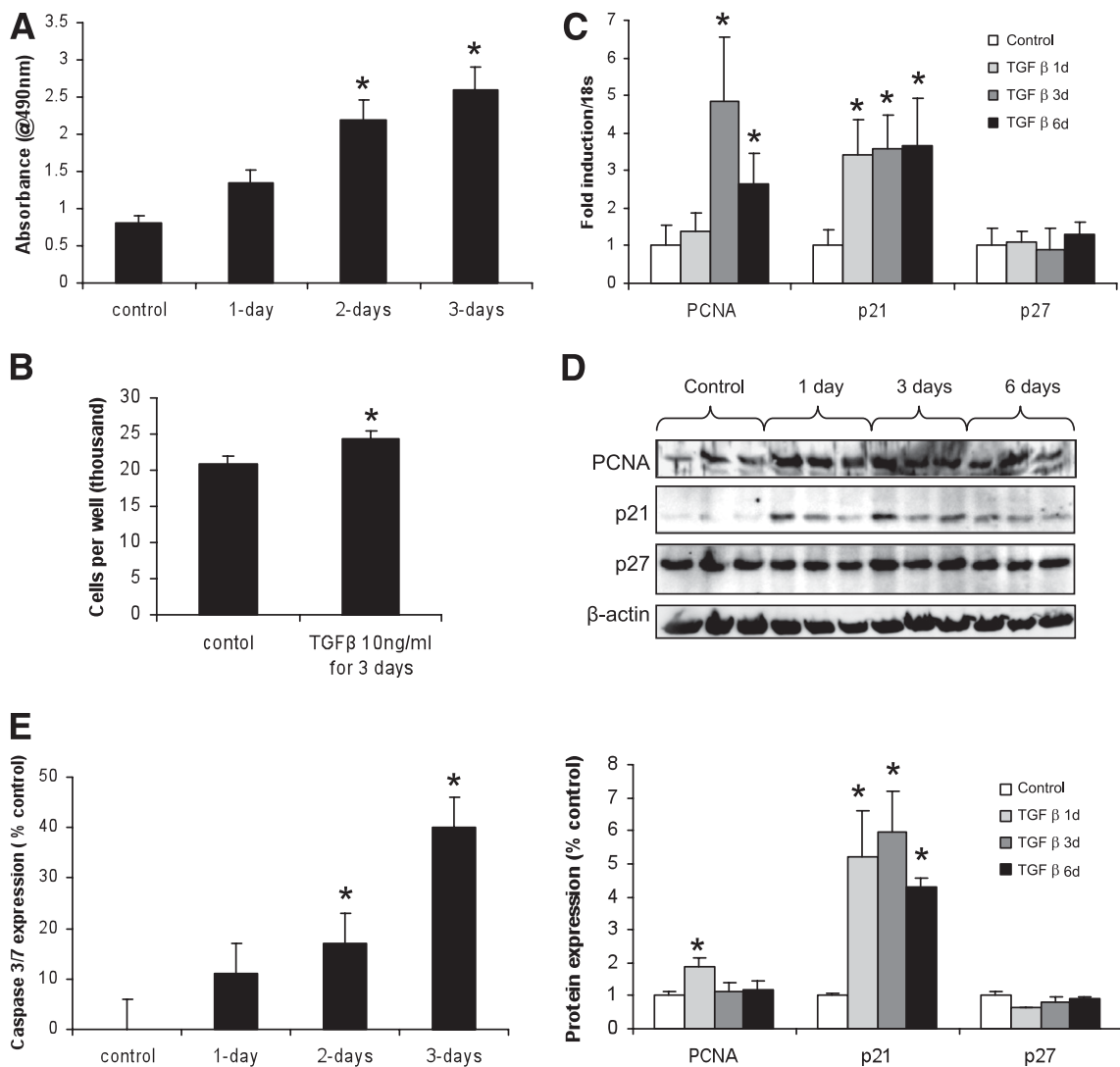


FIG. 5. The induction of a time-dependent increase in cellular proliferation in immortalized human podocytes after treatment with TGF-β1 (10 ng/mL) for 1–6 days as measured by proliferation assay (A), cell counting (B), and the induction of PCNA and cell-cycle regulators p21 and p27 at a gene level (C) and protein level (D), as measured by real-time RT-PCR and quantified by Western blotting, respectively. At the same time, treatment with TGF-β1 (10 ng/mL) for 1–3 days also resulted in increased apoptosis, as denoted by the caspase 3/7 expression (E). **P* < 0.05 vs. control.

in vivo, as evidenced by increased glomerular staining of proliferation markers, PCNA and Ki67, specifically within podocytes (Fig. 8).

DISCUSSION

The glomerular podocyte is believed to play a role in the development and progression of albuminuria and glomerulosclerosis associated with diabetes (1–3,30). Indeed, recent studies show that mice with specific deletion of the insulin receptor only from their podocytes develop significant albuminuria together with histologic features that recapitulate diabetic nephropathy, but in a normoglycemic environment (31). Such data place podocytes, and more particularly the dysregulation of their growth and differentiation, at the very center of the pathogenesis of nephropathy. In this study, we describe the morphologic and phenotypic transition of immortalized human podocytes in high glucose in response to TGF-β1 and angiotensin II, two important and codependent mediators of diabetic

nephropathy. We also documented a range of novel effects on podocyte differentiation, apoptosis, and proliferation changes that were analogous to those observed in vivo in diabetic glomeruli. Better understanding of these phenotypic changes provides important insights to the prevention and management of diabetic renal disease.

The changes in podocyte structure and function induced by TGF-β1 have been described as epithelial to mesenchymal transition (EMT) (8,10,13), because some profibrotic elements that characterize a mesenchymal phenotype are acquired, whereas some markers of glomerular epithelial cell differentiation are lost. However, what is occurring in podocytes in vitro or in vivo is not classic EMT, as recently defined by Zeisberg and Neilson (32) and Wang et al. (33). First, podocytes are embryonically derived from the metanephric mesenchyme. Although podocytes also share positional characteristics of epithelial cells (e.g., they sit on a basement membrane and line a cavity [Bowman’s space]), mature podocytes do not express E-cadherin and may be better considered pericytes, whose circumferential arms

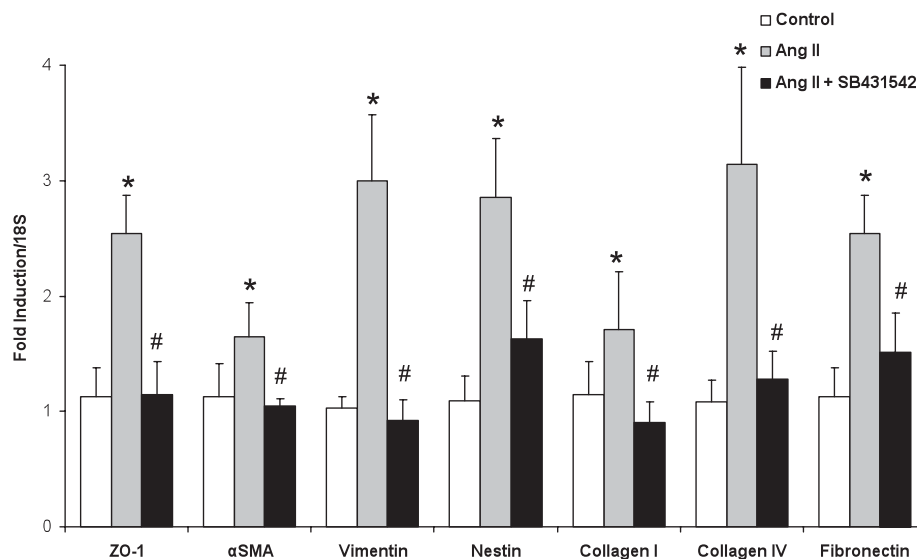


FIG. 6. The induction in the expression of key target genes in cultured human podocytes after treatment with angiotensin II (1 nM) for 3 days in the presence and absence of a selective inhibitor of the TGF- β 1 type I receptor kinase, SB-431542, as measured by real-time RT-PCR ($n = 6/\text{group}$). * $P < 0.05$ vs. control. # $P < 0.05$ vs. angiotensin II.

engirdle the vascular endothelium in both the brain and the glomerulus. Second, after exposure to TGF- β 1, epithelial markers are increased in podocytes (e.g., ZO-1, cytokeratin) associated with increased tight junction formation (34), rather than reduced, as observed in classic EMT, which facilitates cellular separation and invasion. In a reverse process of podocyte maturation, ZO-1 migrates and translocates from the basal to the lateral side of the podocyte (35). Third, TGF- β 1 and other stimuli of EMT act to suppress epithelial proliferation, whereas TGF- β 1 enhances podocyte proliferation in our human podocytes. Taken together, the phenotypic changes observed in our *in vitro* and *in vivo* models are more appropriately described as dedifferentiation: the regression of a specialized cell to a simpler, more embryonic, unspecialized form.

In common with EMT, both processes seem to be a means by which intrinsic cellular plasticity facilitates rapid structural and functional adaptations. Although effacement of podocytes is generally regarded as an abnormal response to injury, the formation of *de novo* tight junctions between podocytes may act to counteract the expansion of glomerular capillaries in response to injury. Indeed, we show in this study that the dedifferentiation of cultured podocytes results in increased podocyte connections with reorganized tight junctions, a flat cobblestone-like appearance that reduces albumin flux across the monolayer, as previously described in epithelial cells (36). However, although adaptive in the short-term, these phenotypic transitions may ultimately become maladaptive, where their chronic activation may aggravate glomerular fluid and shear stress, thereby leading to progressive organ dysfunction.

Our findings are consistent with the known actions of TGF- β and angiotensin II on podocyte differentiation and apoptosis under normal glucose conditions (19,22–24). However, with respect to ZO-1, diabetic mice have been reported to show decreased glomerular expression of this protein and a small but nonsignificant effect of hyperglycemia on primary rat glomerular epithelial cells (37). By contrast, we found that the expression of ZO-1 was increased by TGF- β in association with the increased

formation of tight junctions between adjacent dedifferentiated cells. It is possible that the loss of podocytes with diabetes confounds interpretation of some of these findings. Moreover, these results may also reflect the disparate actions of hyperglycemia and TGF- β on podocytes (20), as well as the various cell lines used in the different studies. Previous studies of podocytes in culture have been criticized because of lack of markers of mature podocyte differentiation (e.g., *p*-cadherin and nephrin). The conditionally immortalized human podocyte cell line established by Saleem et al. (25) used in our experiments does not share this problem (38,39); it shows both growth arrest and clear differentiation when exposed to “non-permissive” temperatures (37.5°C). However, the changes induced by TGF- β in this model suggest that some of the criticisms of earlier models may have been unfounded. For example, the regular, cobblestone-like polygonal phenotype with non-specific tight junctions and proliferating cells observed in constitutively immortalized (SV40) human podocyte lines was thought to demonstrate its unsuitability as an experimental model. More likely, this dedifferentiated phenotype reflects podocytopathy and dysfunction as occurs *in vivo*, because similar changes can be induced by pathogenic stimuli in the podocyte line used in the current study.

Mature podocytes are traditionally thought of as archetypal postmitotic cells, terminally differentiated with little or no capacity for regenerative replication. This has led to the misconception that podocyte proliferation cannot be seen in renal disease. However, proliferating podocytes are readily observed in experimental models of selective glomerular injury (40), because some podocytes reengage the cell cycle as an adaptive response to injury in the attempt to mitigate podocyte loss. Dedifferentiated podocytes can and do proliferate *in vitro* and *in vivo* in a range of human diseases, including HIV nephropathy, crescentic glomerulonephritis, and collapsing glomerulopathy. Our studies demonstrate for the first time that podocytes expressing proliferation markers are also observed in the diabetic glomerulus. Moreover, we show that TGF- β 1, a well-known mitogen that is increased in the diabetic

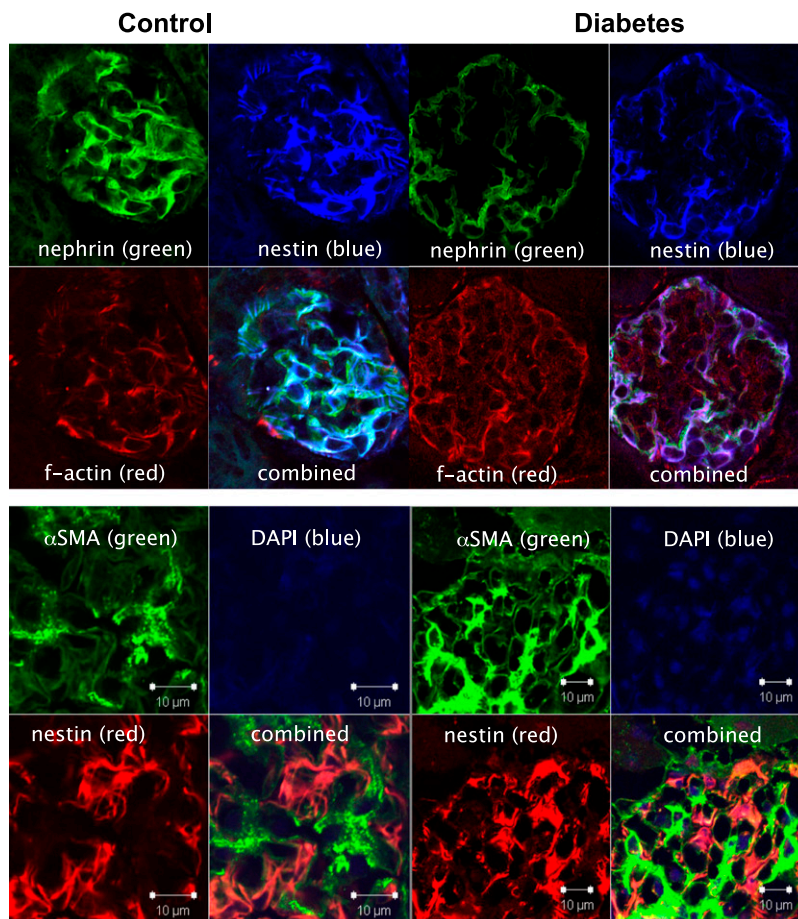


FIG. 7. Immunofluorescent staining of cortical glomeruli showing expression of the podocyte marker, nephrin (green), f-actin (red), and the intermediate filament, nestin (blue); merged staining from control and diabetic apoE-KO mice (*top*); and immunostaining of cortical glomeruli showing expression of α -SMA (green), nestin (red), DAPI (blue), and merged (*bottom*). (A high-quality color representation of this figure is available in the online issue.)

kidney, is also able to stimulate podocyte proliferation, in addition to its known effects on differentiation and apoptosis (22–24). Indirect evidence for podocyte proliferation in human diabetes comes from the observation that increased numbers of podocytes are seen in the urine (7,41), long before any reduction in any glomerular podocyte numbers. It is possible that podocyte proliferation has not been suspected in diabetes, because it is offset by detachment and apoptosis, meaning the net effect is one of a progressive but modest podocyte loss. Moreover, in advanced disease, there may be a critical threshold of podocyte depletion that defines the point of no return, beyond which proliferation and other measures to conserve this cell population also fail, and thus glomerulosclerosis becomes irreversible (42). The coordinate regulation of cell proliferation and death seems to provide an organism with a mechanism to control embryogenesis, as well as repair and regeneration. It is possible to speculate that dysregulated hyperplasia results in cellular and collapsing hyperplasia, whereas dysregulated apoptosis results in podocytopenia and segmental glomerulosclerosis by exposing the basement membrane to form synechiae. Indeed, in terminally differentiated neuronal cells, reentry into the cell cycle more often leads to apoptosis than proliferation, although both cellular processes are always stimulated.

The precise mechanism by which cells regain their ability to proliferate remains to be established. It probably reflects an epiphenomenon of the wholesale change in phenotype, rather than any specific change in proliferative capacity. However, it is possible that alterations in cell-cycle control proteins are important, including repression of p27 expression, a cyclin-dependent kinase inhibitor that promotes growth phase arrest in postmitotic cells like podocytes (43). Studies reducing the expression of p27 in other postmitotic cells have shown that cell-cycle reentry and repression of podocytes may be involved in podocyte proliferation in focal and segmental glomerulosclerosis. Other pro-proliferative mediators induced by TGF- β 1, including nuclear factor- κ B, may also play a role (44).

Although the dynamic changes in podocyte structure and function demonstrated in this article seem consistent with *in vivo* phenomena, several limitations should be considered. The use of recombinant TGF- β in our *in vitro* models may not reflect the complex array of growth factors and cytokines ambient in the diabetic glomerulus. The concentration of TGF- β used in our experiments is nonetheless consistent with that observed in the diabetic glomerulus (45), as well as in the plasma of patients with diabetes (46,47). Similarly, the angiotensin II dose used in our experiments (1 nM) is consistent with the angiotensin II concentration observed *in vivo* (48). Second, the behavior

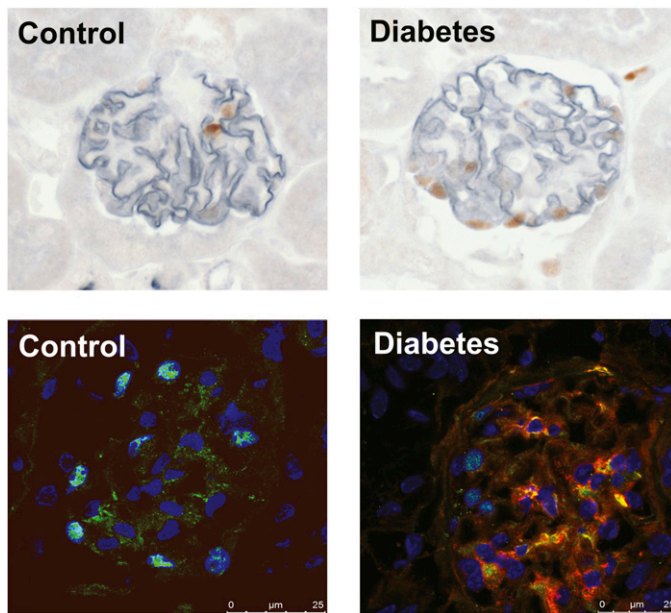


FIG. 8. Immunostaining of cortical glomeruli from control and diabetic apoE-KO mice. *Top:* Costaining for the podocyte marker, nephlin, and PCNA. *Bottom:* Immunofluorescent staining for the proliferation marker, Ki67 (green), WT-1 (red) to denote podocytes, and a merged signal (yellow) to denote proliferating podocytes. DAPI (blue) is used as a nuclear counterstain. (A high-quality color representation of this figure is available in the online issue.)

of podocytes in cell monoculture may not reflect their regulation within the glomerulus, which is substantially influenced by other cells and local hemodynamic factors on other pressures (oncotic pressure and shear stress). Third, although the accelerated renal lesion associated with diabetes in the apoE-KO mouse is more consistent with human nephropathy (28), it may not be fully representative of the human diabetic kidney.

In summary, the foot processes of podocytes are normally flexible, dynamic, and contractile structures, whose configuration depends on rearrangements of an actin cytoskeleton (49–53). In response to TGF- β and other TGF-dependent stimuli, mature podocytes undergo dedifferentiation that leads to effacement of foot processes, morphologic flattening, reduced motility, and increased formation of intercellular tight junctions. This simplification of their phenotype to a more embryonic form is also associated with reentry of mature podocytes into the cell cycle, which results in enhanced proliferation and apoptosis. These “pathoadaptive” changes are seen early in the diabetic glomerulus and potentially contribute to albuminuria, glomerulosclerosis, and podocytopenia.

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