Forced expression of VEGF-A in podocytes influences mesangial cell number and endothelial cell differentiation in the mouse glomerulus

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Abstract

Background Glomerular podocyte-derived vascular endothelial growth factor (VEGF) is indispensable for the migration and proliferation of glomerular endothelial cells. In contrast, podocyte-specific Vegf overexpression leads to the collapse of glomerular tufts; however, the mechanisms underlying this outcome have not yet been reported.

Methods To further clarify the effects of elevated levels of Vegf expression on glomerular cells, we established a dual transgenic mouse line in which Vegf is exclusively and inducibly expressed in podocytes under the control of the "Tet-on system" (Podocin-rtTA/TetO-Vegf164 mice).

Results Macroscopic and microscopic examination of Podocin-rtTA/TetO-Vegf164 animals following Vegf induction identified the presence of prominent cortical hemorrhages. In addition, the endothelial cell number was increased along with enlargement of the sub-endothelial spaces. We also observed impaired endothelial fenestration and aberrant plasmalemmal vesicle associated protein-1(PV-1) expression. In contrast, the mesangial cell number markedly decreased, resulting in a glomerular tuft intussusceptive splitting defect. Furthermore, whereas platelet-derived growth factor B (PDGF-B) expression in the glomerular cells of Podocin-rtTA/TetO-Vegf164 mice was somewhat increased, phospho-PDGF receptor immunoreactivity in the mesangial cells was significantly decreased when compared to wild-type animals.

Comclusion Taken together, the results of this study indicate that the upregulation of podocyte VEGF decreases the number of mesangial cells, likely owing to an inhibition of PDGF-B-mediated signaling.

Keywords Glomerular development, Inducible transgenic mice, Plasmalemmal vesicle associated protein-1, Platelet-derived growth factor, POEMS syndrome

Introduction

Vascular endothelial growth factor (VEGF), known as an endothelium specific growth factor, has a prerequisite role for both vasculogenesis and angiogenesis [1, 2]. VEGF is secreted from surrounding pericytes and promotes the proliferation, differentiation, and survival of endothelial cells [3]. VEGF also increases vascular permeability and induces the remodeling of the interstitial matrix [3, 4].

In the kidneys, *Vegf* is constitutively expressed in podocytes [5] and plays an important role in the development of glomerular endothelial cells and in the maintenance of their integrity, as evinced by the loss of endothelial cells in mice carrying a podocyte-specific null mutation of *Vegf* as reported by Eremina et al. [6]. In addition, bevacizumab, an anti-VEGF antibody, has been reported to cause injury of the glomerular endothelial cells, leading to the overt proteinuria [7]. Therefore, the VEGF in podocytes is essential for the normal development and maintenance of glomerular capillary tufts.

Considering these known functions of VEGF, the mechanism for the observed loss of endothelial cells following VEGF downregulation is well understood. However, it remains unclear how VEGF overexpression affects the development of glomerular cells. Whereas it has been reported that podocyte-specific VEGF overexpression leads to a collapsing glomerulopathy [6], on the other hand, Veron et al. recently reported that excess VEGF in podocytes mainly affected podocytes *per se*; for example, foot process effacement in addition to the absence of the slit diaphragm, thickening of the glomerular basement membrane, and glomerulomegaly [8]. In particular, phenotypic change of mesangial cells associated with VEGF upregulation has not been reported to date. The question of the potential effects of VEGF overexpression on these cells is of clinical significance, as an upregulation of VEGF has been observed in many

glomerular diseases including diabetic nephropathy [9] and POEMS syndrome [10], and is considered to be likely involved in the development of the kidney injury associated with these disorders.

To address this issue, we therefore established transgenic mice with inducible podocyte-specific Vegf overexpression and analyzed the influence of elevated levels of podocyte-VEGF on each glomerular cell type (mesangial and endothelial cells).

Methods

Generation of Podocin-rtTA/TetO-Vegf164 transgenic mice

All mouse protocols were approved by the Committee for Animal Use and Experimentation of the Jikei University School of Medicine. (approval number: 24-004)

A 2.8 kb *Vegf164* cDNA encoding the entire coding region was obtained by reverse transcription-polymerase chain reaction (RT-PCR) using total mouse kidney RNA and the following primers:

5'-AAACCATGAACTTTCTGCTCTCTTGGGT-3'and 5'-TCACCGCCTTGGCTTGTCACA-3'.

After the sequence was verified, the cDNA fragment was inserted downstream of the bi-directional TetO gene promoter of the vector pBI-G containing the *LacZ* gene (631004, TaKaRa Bio, Shiga, Japan). 5 μg of the transgene, which was excised using the restriction enzymes *Not*I and *Sal*I, was injected into 283 fertilized eggs obtained from matings between C57BL/6 and DBF1 mice as described previously [11]. Of the resultant offspring, 13 mice were found to carry the transgene upon genotyping of tail DNA. These mice, designated TetO-Vegf164, were crossed with Podocin-rtTA mice (kindly gifted by Prof. Jeffrey B Kopp [12]) to obtain dual transgenic mice (Podocin-rtTA/TetO-Vegf164). Genotyping was performed by PCR using primers specific for each transgene as follows: podocin, 5'-CGCACTTCAGTTACTTCAGGT-3' and 5'-GCTTATGCCTGATGTTGATGA-3'; LacZ, 5'-TCTGCTTCAATCAATCAGCGTGCC-3' and 5'-GCCGTCTGAATTTGACCTGA-3'. Transgenic mice were maintained by sib mating and F2–F6 generation animals were used in this study.

To induce exogenous *Vegf164* in podocytes during embryonic development, pregnant mice were administered doxycycline at a concentration of 2 mg/L from embryonic day 10.5–14.5 until birth. VEGF-A concentration was

measured in urine samples using the Quantikine VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction.

Histology

Kidneys were dissected from mice at birth, fixed in 4% paraformaldehyde, and embedded in paraffin. Alternatively, samples for frozen sections were mounted in O.T.C compound (Sakura, Torrance, CA, USA). From these, 4 to 6 μm thick paraffin or frozen sections were layered onto poly-L-lysine coated slide glass and subjected to various staining procedures as described below.

Transmission electron microscopy (TEM) was performed as described previously [11].

LacZ staining and western blot analysis

An X-gal (4-Cl-5-Br-3-indolyl-β-galactosidase) assay was performed to assess the expression of the *LacZ* gene as described previously [11].

Western blot analysis for lacZ was performed according to the method described previously [13] using an anti β-galactosidase antibody (1:2000, Z3781, Promega, Madison, WI, USA).

Immunohistochemistry

Primary antibodies used for immunohistochemistry were as follows: rabbit anti-WT-1 (1:100, sc192, Santa Cruz Biotechnology, Dallas, TX, USA), rat anti-PV-1 (1:100, 550563, BD Biosciences, San Diego, CA, USA), rat anti-CD31

(1:100, 550274, BD Biosciences), mouse anti-desmin (1:200, M0760, Dako, Carpinteria, CA, USA), mouse anti-α-smooth muscle actin (SMA) (1:50, M0851, Dako), and rabbit anti-phospho PDGF receptor-β (1:500, ab16864, Abcam, Cambridge, UK). Rabbit anti-collagen IVα4 and rabbit anti-laminin α5 and β2 antibodies were kindly provided by Mr. Yamato Kikkawa (School of Pharmacy, Tokyo University of Pharmacy and Life Science, Tokyo, Japan) [14].

The percentage of αSMA-positive glomeruli among all glomeruli was measured on the sagittal section with maximum area from each kidney and the percentage of phospho-PDGF receptor-β positive glomeruli among to all αSMA-positive glomeruli was assessed in the adjacent sections.

Laser capture micro dissection and quantitative real-time PCR (qRT-PCR)

The expression level of *Vegf-a* and *Pdgf-b* mRNA was assessed by qRT-PCR after laser capture micro dissection. In brief, glomeruli were laser-cut from 6.0 μm frozen sections using a Leica LMD7000 system (Wetzlar, Germany). A total of 15 glomeruli were collected from 12-week-old induced-transgenic mice and wild-type littermates. qRT-PCR was performed as described previously [13], using the primers for *Vegf-a* (Mm00437306_m1), *Pdgf-b* (Mm00440677_m1), and 18s rRNA (Mm03928990_g1) purchased from Life Technologies.

Statistical analysis

All values are expressed as medians (range). Statistical analysis was performed using the Mann-Whitney U test for single comparisons. A p-value of <0.05 was considered to be a significant difference.

Results

Macroscopic and microscopic inspection of kidneys from Podocin-rtTA/TetO-Vegf164 mice

Podocin-rtTA/TetO-Vegf164, but not wild-type, mice expressed lacZ exclusively in podocytes when pregnant mice were administered doxycycline (Fig. 1A, B). Western blot analysis of whole kidney extracts showed that β-galactosidase was present in Podocin-rtTA/TetO-Vegf164 mice upon induction, but not in wild-type mice (Fig. 1C).

Podocin-rtTA/TetO-Vegf164 mice exhibited significantly higher concentrations of VEGF (median 48.4 pg/mL, 44.1–167.8 pg/mL, n = 6) in urine samples at P0 than wild-type mice (median 36.6 pg/mL, 28.5–53.0 pg/mL, n = 8, $p < 0.05$) (Fig. 1D).

Macroscopically, the dual transgenic mice exhibited numerous hemorrhages on the cortical area of the kidney (Fig. 1E, F), a phenotype similar to that previously reported [6]. In addition, light microscopy showed that many tubules were filled with red blood cells both in the cortex and medulla. Microaneurysms had formed (Fig. 1I) and hemorrhages were seen in the Bowman's space of some glomeruli (Fig. 1H). Obvious morphological abnormalities were not observed in the other organs of the transgenic animals.

Overexpression of Vegf164 causes phenotypic changes in endothelial cells

Ultra-structural analysis of Podocin-rtTA/TetO-Vegf164 mice by TEM found that the endothelial cells with cuboidal structure increased in number within the larger capillary lumens (Fig. 2A, B). In the dual transgenic mice, the fenestration of endothelial cells was lacking along the peripheral capillary tufts (Fig. 2C-F). In addition, the sub-endothelial space was markedly enlarged when compared to the wild-type (Fig. 2C-F). Immunofluorescence for

PV-1, a structural protein required for the formation of the stromal diaphragms of caveolae, revealed universally positive staining of the glomerulus in Podocin-rtTA/TetO-Vegf164 mice, whereas wild-type glomeruli located in the deep cortical area were almost negative (Fig. 2G, H).

Although the foot process effacement and absence of a slit-diaphragm was present to a degree in the transgenic mice (Fig. 2D), the cell body of the podocytes *per se* appeared to be indistinguishable between the dual transgenic and wild-type mice.

Decreased numbers of mesangial cells in the glomeruli of Podocin-rtTA/TetO-Vegf164 mice

Analysis of the podocyte, endothelial, and mesangial cell populations found that Podocin-rtTA/TetO-Vegf164 mice had similar or slightly fewer positive cells for Wilms' tumor 1 (WT1), a marker of podocytes, when compared to the normal glomeruli of wild-type mice (Fig. 3A, B). Similarly, the immunoreactivity of CD31, a marker of endothelial cells, was also comparable between dual transgenic and normal glomeruli (Fig. 3C, D). In contrast, the intensity of staining with desmin, a mesangial cell marker, was remarkably decreased in the glomeruli of Podocin-rtTA/TetO-Vegf164 mice when compared to wild-type (Fig. 3E, F). Notably, the mesangial cells were already low in number in the glomeruli at the capillary loop stage, as α-SMA positive cells were rarely identified in the dual transgenic mice (Fig. 3G, H).

The measurement of the percentage of αSMA-positive glomeruli among all glomeruli revealed a significant decrease in Podocin-rtTA/TetO-Vegf164 mice (n = 7, median 51.3%, 48.2–53.7%) compared to wild-type mice (n = 7, median 72.1%, 63.0–87.5%, $p < 0.01$). As it has been reported that a defect in a mature GBM, *e.g.*, laminin α5, caused a deficiency of mesangial cells [14], we then examined whether GBM development was impaired in

Podocin-rtTA/TetO-Vegf164 mice. Staining for collagenIVα4 and laminin α5 and β2 revealed that the signals of these mature-type components were compatible between dual transgenic and wild-type mice (Fig. 3I-N). However, the structure of the GBM from the Podocin-rtTA/TetO-Vegf164 mice was obviously aberrant, exhibiting a loss of intussusceptive splitting (Fig. 3J, L, N), the process governed by mesangial cells [14].

Expression level of Pdgf-b and immunoreactivity of phospho-PDGF receptor-β in glomeruli from Podocin-rtTA/TetO-Vegf164 mice

As PDGF-B has an essential role in the development of mesangial cells [15], we next evaluated the expression of *Pdgfb* in the glomerulus by qRT-PCR following laser capture micro dissection. The expression of *Vegfa* increased by 4.5-fold in Podocin-rtTA/TetO-Vegf164 mice when compared to their wild-type littermates. Additionally, the expression level of *Pdgfb* in the dual transgenic mice was also markedly increased (3.9-fold) (Fig. 4B). We then examined the immunoreactivity of phosphorylated PDGF receptor β-positive glomeruli (Fig. 4C, D) in adjacent sections to those subjected to immunostaining for αSMA (Fig. 4E, F). This demonstrated that the percentage of phospho-PDGF receptor-positive glomeruli among all αSMA-positive glomeruli was significantly smaller in Podocin-rtTA/TetO-Vegf164 mice (n = 7, median 50.0%, 16.3–72.5%) than in wild-type mice (n = 7, median 83.9%, 71.4–95.5%, $p < 0.01$) (Fig. 4G).

Discussion

In this study, we found for the first time, to our knowledge, that the Podocin-rtTA/TetO-Vegf164 mice exhibited a deficiency of mesangial cells, in addition to marked hemorrhage, formation of microaneurysms, and a defect in endothelial differentiation in the glomeruli. These phenotypes resemble those reported by Eremina et al. [6] but are somewhat different from those described by Veron et al. [16]. Whereas podocytes and their foot processes were not predominantly affected, only minor structural alternations, *e.g.*, partial foot process effacement, were observed in the current study. These discrepancies might result for the following reasons. In our study, *Vegfa* was up-regulated by approximately 4-fold (Fig. 4B), whereas the previous studies demonstrated 2-fold elevated *Vegf* expression [16]. In addition, the genetic background of our mice was not the same as the other models [16]. Furthermore, the exact time of kidney collection after birth might have differed between the present and previous studies [8, 16].

It has been previously reported that the pericytes are clearly important for vessel maturation and stabilization [17]. In the absence of pericytes, the microvessels leak, hemorrhage, and form multiple microaneurysms. Furthermore, the lack of pericytes causes over-proliferation and a defect of differentiation in endothelial cells [19]. Thus, although excess podocyte VEGF *per se* acts on glomerular endothelial cells, it is possible to postulate that the glomerular hemorrhage, formation of microaneurysms, and defective endothelial differentiation observed in this study might be attributed, as least in part, to a deficiency of mesangial cells.

A number of growth factors and extracellular matrixes are known to be important for the development of mesangial cells. For example, a null mutation of *Pdgfb* or its receptor, *Pdgfrb*, caused a loss of mesangial cells [15, 19]. In addition, lack of the G domain of laminin α5 of the GBM resulted in the total absence of mesangial cells and a defect in the

convolution of capillary tufts [14]. In our study, however, the glomerular expression of *Pdgfb* did not decrease but rather increased in Podocin-rtTA/Tet-O-Vegf164 mice when compared to their wild-type littermates (Fig. 4). Similarly, laminin α5 was distinctly present along with other mature-type components of the GBM in Podocin-rtTA/TetO-Vegf164 mice (Fig. 3).

It has been shown that mesangial cells express VEGF receptor-2 (Flk1), as do glomerular endothelial cells [20]. In addition, a recent report demonstrated that the VEGF-mediated activation of VEGF receptor-2 suppresses PDGF receptor-β signaling through the assembly of a receptor complex consisting of PDGF receptor-β and VEGF receptor-2 in vascular smooth muscle cells/pericytes, thereby inhibiting vessel maturation and stabilization [21]. We observed a similar phenomenon in this study, wherein although the degree of *Pdgfb* expression was higher, the immunoreactivity of phosphorylated PDGF receptor-β was somewhat less in α-SMA positive glomeruli from Podocin-rtTA/Tet-O-Vegf164 mice when compared to wild-type mice. Several possible explanations for the inhibition of PDGF-mediated signaling in mesangial cells from Podocin-rtTA/Tet-O-Vegf164 mice can be considered. First, similar to the occurrence in vascular smooth muscle cells/pericytes [21], excess VEGF might suppress PDGF receptor-β-mediated signaling in glomerular mesangial cells as well. Second, excess VEGF might modulate other signaling cascade factors such as Notch2 and BMPs, which have been reported to regulate the development of mesangial cells [11, 22]. However, the effect of these cascades on PDGF receptor-β-mediated signaling has not yet been clarified.

The results of our study suggest that the Podocin-rtTA/Tet-O-Vegf164 mouse model is relevant to some human kidney diseases, *e.g.*, diabetic nephropathy [23] and POEMS syndrome, a rare plasma dyscrasia characterized by monoclonal gammopathy and various combinations of polyneuropathy, organomegaly, endocrinopathy, and

dermatological changes [10]. In particular, the latter manifests excess VEGF, which is thought to be a possible causative factor for the development of several lesions [24]. Kidney injury is sometimes involved and typically features mesangioproliferative glomerulonephritis with widening of the subendothelial and mesangial space [24]. The lesions are similar to those seen in the mouse model used in this study. Therefore, the findings of this study might be informative with respect to the pathogenesis of this syndrome.

In conclusion, the phenotype observed following the up-regulation of VEGF in podocytes caused marked hemorrhage, the formation of microaneurysms, and a defect in endothelial differentiation in Podocin-rtTA/TetO-Vegf164 mouse model, all of which might be, at least in part, attributed to a deficiency of mesangial cells. Further studies are needed to clarify the mechanism by which excess VEGF leads to the loss of mesangial cells during glomerulogenesis, considered to be associated with the pathogenesis of some glomerular diseases in humans.

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Conflict of Interest The authors have declared that no conflict of interest exists.

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Figure captions

Fig. 1 Podocyte-specific overexpression of Vegf164. (A, B) Promoter activity of *Tet-O* was measured by lacZ staining of the kidneys from wild-type (**WT**) (A) and Podocin-rtTA/Tet-O-Vegf164 mice (**Tg**) (B) at P0 following doxycycline administration. (C) Western blot of kidney lysates showed a clear band for β-galactosidase only in the dual transgenic mice administered doxycycline. (D) Urinary VEGF-A concentrations in wild-type and the dual transgenic mice treated with doxycycline. The horizontal bars and rectangles represent the ranges and the interquartile ranges, respectively. The horizontal bars in the rectangles represent the medians. (E-I) Representative images of macroscopic (E, F) and microscopic (G-I) examination of the kidneys from wild-type (E, G) and Podocin-rtTA/Tet-O-Vegf164 mice (F, H, and I) at P0. Microaneurysms were seen in some glomeruli (arrows in H). Magnifications: ×10 (E, F); ×400 (A, B, G, H, and F)

Fig. 2 Electron microscopy and PV-1 expression in Podocin-rtTA/ TetO-Vegf164 mice. (A-H) Representative transmission electron microscopic images of glomeruli from wild-type (**WT**) (A, C, and E) and Podocin-rtTA/TetO-Vegf164 mice (**Tg**) (B, D, and F). In the dual transgenic mice, the glomerular tufts showed microaneurysms (indicated by * in B) and the endothelial cells increased in number (arrows in B). Loss of endothelial fenestration (arrowheads in C) and dilatation of sub-endothelial spaces (depicted by ** in D) were observed compared to the wild-type. (E, F) Immunofluorescence for plasmalemmal vesicle associated protein-1 (PV-1) (green) and CD31 (red) in glomeruli. The endothelial cells of Podocin-rtTA/Tet-O-Vegf164 mice were positive for PV-1. DAPI (blue) was used for nuclear staining. Scale bars = 10 μm in (A, B) and 500 nm in (C, D). Magnification: ×400 (E, F). EC; endothelial cell, fp; foot process. White lines depict the Bowman's capsule of the glomerulus in (E, F)

Fig. 3 Component cells and structures of the glomerular basement membrane from wild-type (WT) and Podocin-rtTA/TetO-Vegf164 (Tg) mice. Representative immunostaining for WT-1 (A, B), CD31 (C, D), desmin (E, F), αSMA (G, H), collagenIV α4 (I, J), laminin α5 (K, L), and laminin β2 (M, N) at P0. White broken lines depict the Bowman's capsule of the glomerulus in (C, D, E, and F). Magnification: ×400 (A-N)

Fig. 4 Expression of *Vegfa* and *Pdgfb* in glomeruli and immunostaining for phosphorylated PDGF receptor-β. (A, B) Quantitative RT-PCR analysis of *Vegfa* and *Pdgfb* mRNA expression in glomeruli from wild-type (WT) and induced Podocin-rtTA/TetO-Vegf164 mice (Tg) isolated by laser capture microdissection. (C-F) Representative glomeruli stained for phosphorylated PDGF receptor-β (C, D), or αSMA (E, F) are shown. Images (C, E) or (D, F) are from adjacent sections. Arrowheads in (E) and (F) indicate the relative immunoreactivity of phosphorylated PDGF receptor β in αSMA-positive glomeruli in wild-type (C) compared to Podocin-rtTA/Tet-O-Vegf164 mice (D). (G) The percentage of the phosphorylated PDGF receptor β-positive glomeruli among all αSMA-positive glomeruli in the dual transgenic and wild-type mice, as presented in Fig. 1 (D). *$p < 0.05$. Magnification: ×200 (C-F)

Figure1

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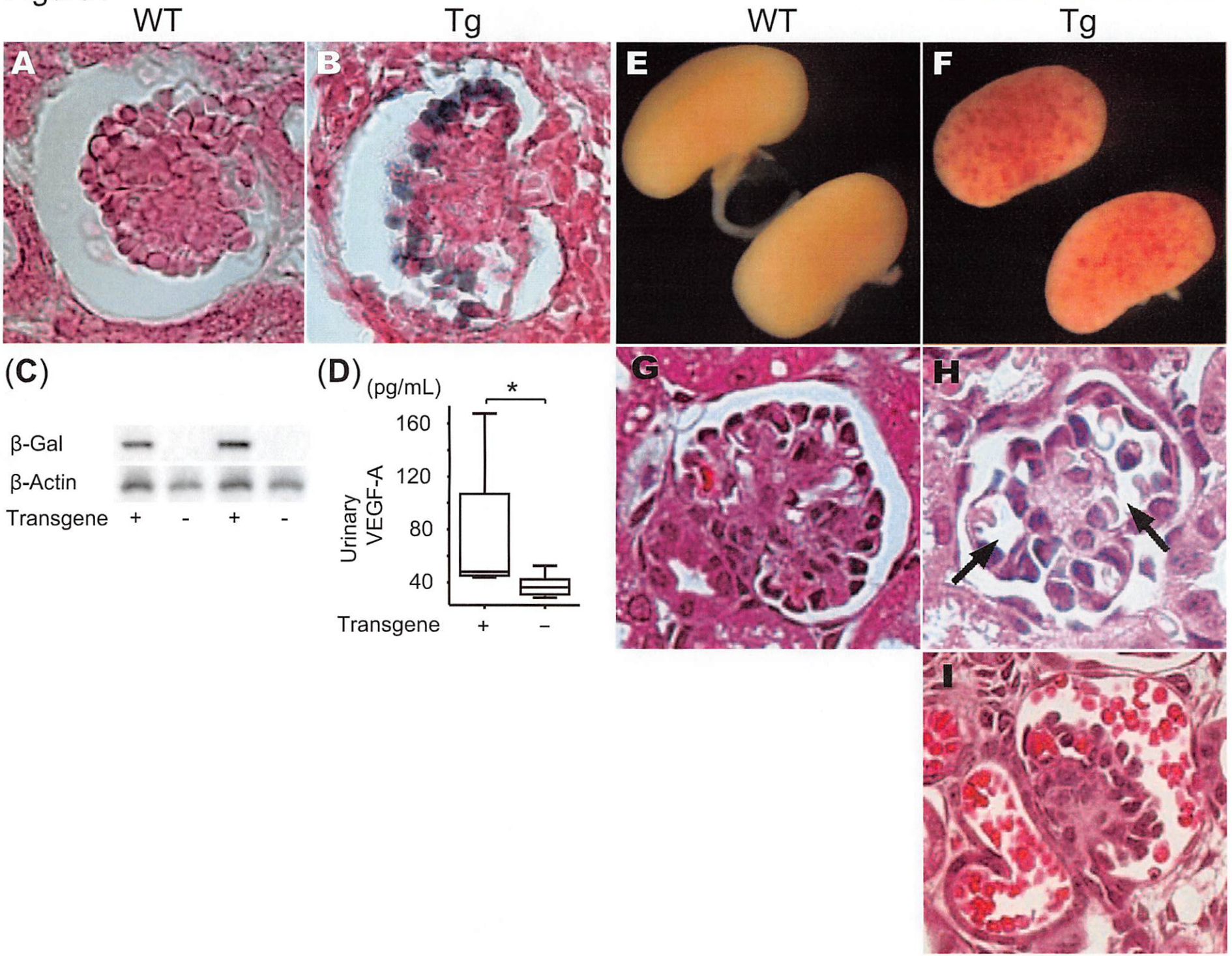

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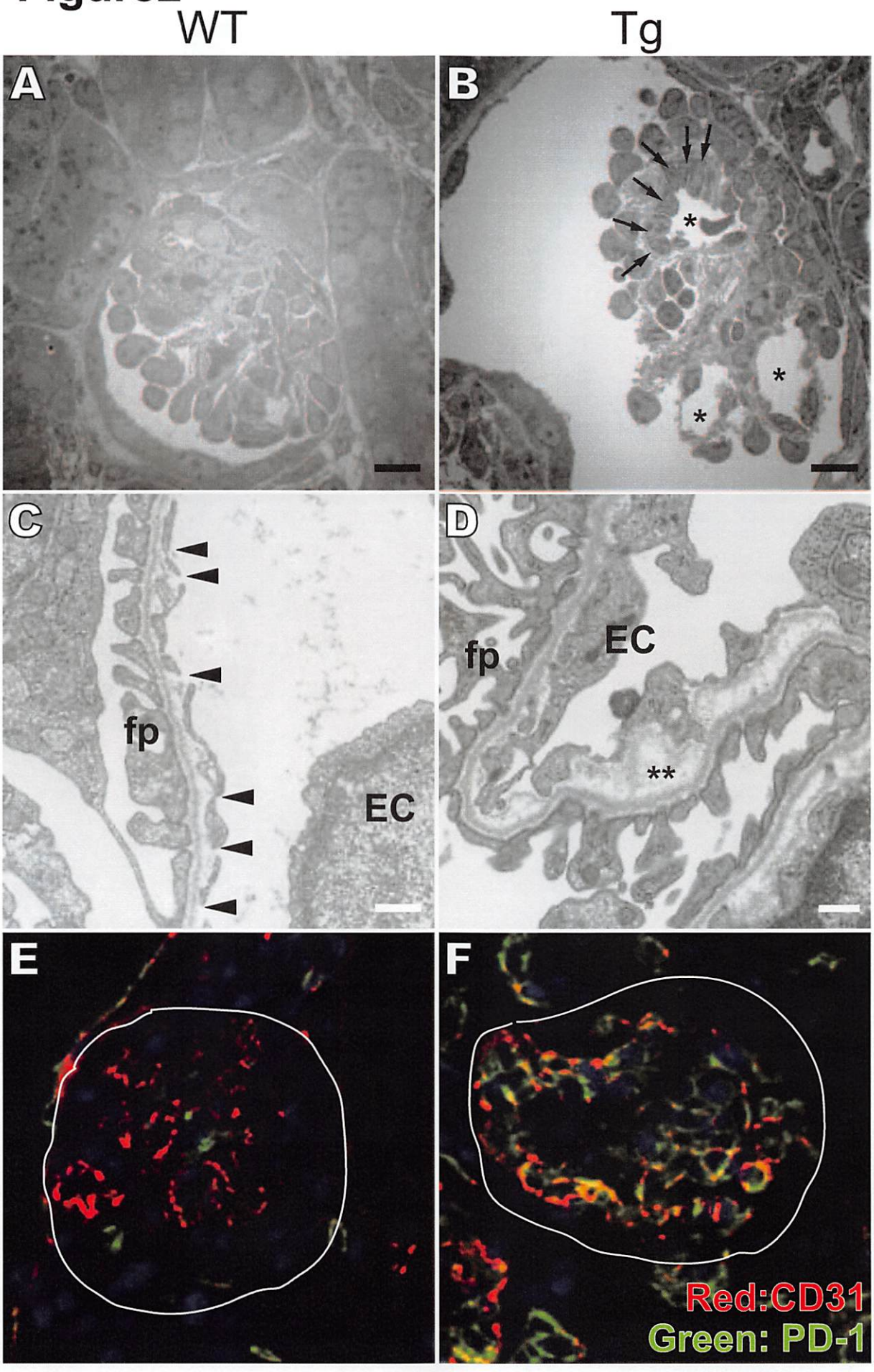

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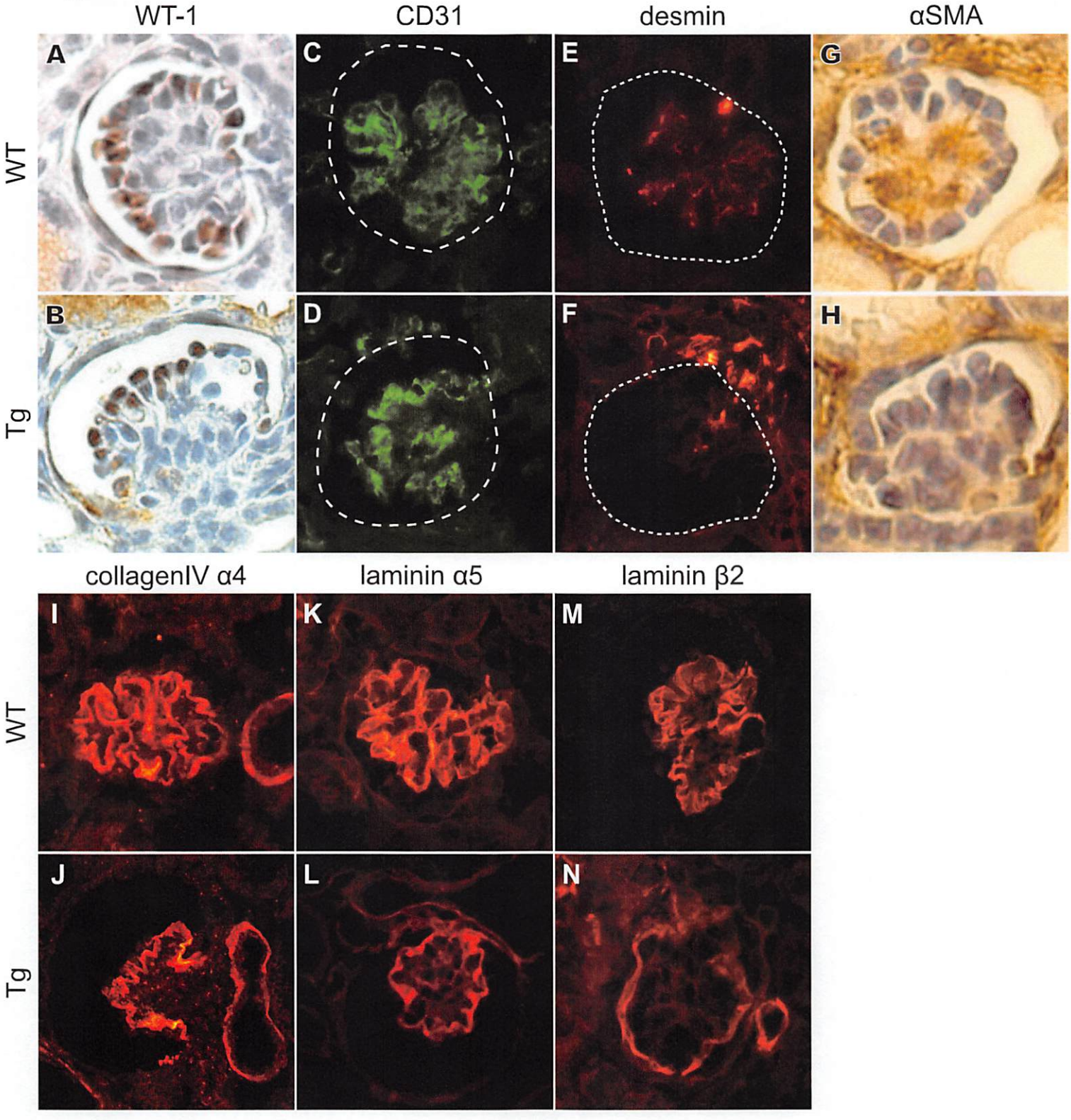

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