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Role of the retinoic acid receptor- α in HIV-associated nephropathy

Krishna K. Ratnam^{1,9}, Xiaobei Feng^{2,9}, Peter Y. Chuang¹, Vikram Verma¹, Ting-Chi Lu¹, Jinshan Wang³, Yuanmeng Jin², Eduardo F. Farias⁴, Joseph L. Napoli³, Nan Chen², Lewis Kaufman¹, Tomoko Takano⁵, Vivette D. D'Agati⁶, Paul E. Klotman¹, and John C. He^{1,7,8}

¹Division of Nephrology, Mount Sinai School of Medicine, New York, New York, USA

²RuiJin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

³Department of Nutritional Science and Toxicology, University of California, Berkeley, Berkeley, California, USA

⁴Division of Hematology, Department of Medicine, Mount Sinai School of Medicine, New York, New York, USA

⁵Division of Nephrology, McGill University Health Centre, Montreal, Quebec, Canada

⁶Department of Pathology, Columbia University, New York, New York, USA

⁷Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, New York, USA

⁸James J. Peters VA Medical Center, New York, New York, USA

Abstract

All-*trans* retinoic acid protects against the development of HIV-associated nephropathy (HIVAN) in HIV-1 transgenic mice (Tg26). *In vitro*, all-*trans* retinoic acid inhibits HIV-induced podocyte proliferation and restores podocyte differentiation markers by activating its receptor- α (RAR α). Here, we report that Am580, a water-soluble RAR α -specific agonist, attenuated proteinuria, glomerulosclerosis, and podocyte proliferation, and restored podocyte differentiation markers in kidneys of Tg26 mice. Furthermore, RAR α -/- Tg26 mice developed more severe kidney and podocyte injury than did RAR α +/- Tg26 mice. Am580 failed to ameliorate kidney injury in RAR α -/- Tg26 mice, confirming our hypothesis that Am580 acts through RAR α . Although the expression of RAR α -target genes was suppressed in the kidneys of Tg26 mice and of patients with HIVAN, the expression of RAR α in the kidney was not different between patients with HIVAN and minimal change disease. However, the tissue levels of retinoic acid were reduced in the kidney cortex and isolated glomeruli of Tg26 mice. Consistent with this, the expression of two key enzymes in the retinoic acid synthetic pathway, retinol dehydrogenase type 1 and 9, and the overall enzymatic activity for retinoic acid synthesis were significantly reduced in the glomeruli of Tg26 mice. Thus, a defect in the endogenous synthesis of retinoic acid contributes to loss of the protection by retinoic acid in HIVAN. Hence, RAR α agonists may be potential agents for the treatment of HIVAN.

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Correspondence: John C. He, Department of Medicine/Nephrology, One, Gustave L Levy Place, Box 1243, Mount Sinai School of Medicine, New York, New York 10029, USA. cijiang.he@mssm.edu.

⁹These authors contributed equally to this work.

DISCLOSURE

All the authors declared no competing interests.

Keywords

HIV; kidney disease; podocytes; proteinuria; retinoic acid receptor

Retinoic acids (RAs) are metabolites of vitamin A that have multiple cellular functions including inhibition of proliferation, induction of cell differentiation, regulation of apoptosis, and inhibition of inflammation.¹ Vitamin A (retinol) is converted into retinal by retinol dehydrogenases (RDHs), and retinal is converted into RA by retinal dehydrogenases.² RA concentration is also regulated by catabolism via Cyp26.³ The kidney is a major organ of RA synthesis.^{4,5} During kidney development, RA affects tubulogenesis and nephron number.⁶ In addition to its established benefits for the treatment of acute promyelocytic leukemia, RA has been found to provide protection in multiple experimental models of kidney disease including mesangioproliferative glomerulonephritis, puromycin-induced nephrosis, lupus nephritis, and diabetic nephropathy.^{5,7–11}

Human immunodeficiency virus (HIV)-associated kidney disease (HIVAN) remains a leading cause of end-stage renal disease among young African Americans.¹² The unique feature of collapsing focal segmental glomerulosclerosis in HIVAN is characterized by podocyte proliferation and dedifferentiation.^{13–15} HIV-infected podocytes express increased levels of cyclin A, E, and D, and reduced levels of p27 and p57.^{15,16} Podocytes also lose differentiation markers including synaptopodin, nephrin, and Wilms tumor 1 (WT-1).^{13,14} Local HIV-1 infection is responsible for podocyte injury.¹⁷ Transgenic mice expressing HIV-1 genes in podocytes develop a kidney disease identical to HIVAN.^{18,19} HIV infection of cultured podocytes causes proliferation and dedifferentiation via Nef-induced Src–Stat3 (signal transducer and activator of transcription 3) and MAPK (mitogen-activated protein kinase) 1 and 2 pathways.²⁰ Although a combination antiretroviral regimen improves patient survival and ameliorates kidney disease,²¹ additional treatments for HIVAN are still lacking, particularly for patients who do not respond to antiretroviral treatment.

Recently, we found that all-*trans* retinoic acid (ATRA) reduces proteinuria and glomerulosclerosis in the HIV-1 transgenic mouse, Tg26, which is the most robust animal model of human HIVAN.²² We also found that ATRA inhibits HIV-induced cell proliferation and restores differentiation markers in HIV-infected podocytes through activation of cyclic adenosine monophosphate production.²² ATRA also inhibits MAPK phosphorylation through activation of MKP1 (MAPK phosphatase 1) in podocytes.²³ In addition, we find that Am580, a specific retinoic acid receptor- α (RAR α) agonist, stimulates cyclic adenosine monophosphate production, inhibits HIV-induced podocyte proliferation, and promotes podocyte differentiation *in vitro*, similar to what we had observed with ATRA.²² Furthermore, the effects of ATRA on podocytes are reduced by a specific RAR α antagonist (Ro-41-5253). These data suggest that RAR α is required for the antiproliferative and differentiation effects of ATRA on HIV-infected podocytes.

As both the animal and *in vitro* studies strongly suggest a beneficial role of ATRA in kidney disease, a phase II clinical study has been approved for the use of ATRA to treat patients with steroid-resistant minimal change disease, focal segmental glomerulosclerosis, and HIVAN (ClinicalTrials.gov Identifier NCT00098020). ATRA has a significant side-effect profile in patients.²⁴ Previous studies suggest that the side effects of RA are mediated mostly through RAR γ . Am580, a specific RAR α agonist, causes less side effects than ATRA.^{25,26} In this study, we found that Am580 protects Tg26 against renal injury, similar to ATRA. Using RAR α knockout mice we determined that the Am580 acts through a RAR α -dependent pathway to confer protection against podocyte injury and kidney disease.

Tissue levels of RA and the expressions of key enzymes in the RA synthetic pathway (RDH1 and RDH9) are reduced in the kidneys of Tg26, suggesting that HIV-associated reduction in RA synthesis contributes to the development of HIVAN.

RESULTS

Am580 reduced proteinuria and glomerulosclerosis in Tg26

As our previous study suggests that RAR α has a key role in mediating the protective effects of RA on podocytes,²² we wanted to determine whether Am580, a specific agonist for RAR α , improves kidney disease similar to what we had observed with ATRA. Both Tg26 and their littermates ($n = 20$) were treated daily with Am580-containing chow (0.3 mg/kg/day) or control chow (prepared similarly without Am580) from the age of 4 weeks, and were killed at the age of 12 weeks. We found that Am580 prevented kidney hypertrophy and preserved renal function in Tg26 compared with mice treated with the control chow (Table 1). Also, Am580 significantly diminished proteinuria and improved glomerulosclerosis, podocyte hypertrophy, and tubular injury in Tg26 compared with the mice treated with the control chow (Table 2 and Figure 1). Our previous *in vitro* studies suggest that ATRA inhibits cell proliferation and restores differentiation in HIV-infected podocytes through activation of RAR α .²² Therefore, we used orally administered Am580 to determine if a RAR α agonist would have a similar effect on the renal phenotype *in vivo*. By immunostaining, we found that Am580 restored the expression of synaptopodin, a podocyte differentiation marker, in the kidneys of Tg26 (Figure 2a). By co-immunostaining for both Ki67 and nestin, we found that Am580 also inhibited HIV-induced podocyte proliferation (Figure 2b). Nestin was used for podocyte colocalization because studies by others and our group have shown that the expression of nestin is preserved in diseased kidneys.^{27,28} To further confirm these findings, we examined the mRNA levels for synaptopodin, nephrin, WT-1, Ki67, and cyclin E in glomeruli isolated from these mice by real-time PCR. We found that expression of podocyte differentiation markers were preserved in glomeruli isolated from Tg26 that received Am580 (Figure 3a). Also, proliferation markers including Ki67 and cyclin E were suppressed in glomeruli isolated from Tg26 that received Am580 (Figure 3b). Changes in markers of podocyte differentiation and proliferation were also confirmed by western blot analysis (Figure 3c). These findings suggest that Am580 prevents podocyte injury *in vivo* similar to what we observed previously *in vitro* using ATRA.²²

Knockout of RAR α in Tg26 aggravated proteinuria, glomerulosclerosis, and podocyte injury

To further assess the critical role of RAR α in HIVAN, we hypothesized that knockout of RAR α in the Tg26 background would aggravate kidney injury. RAR α ^{-/-} mice were crossed with Tg26 as described in the Materials and Methods to generate RAR α ^{+/+} Tg26, RAR α ^{+/-} Tg26, RAR α ^{-/-} Tg26, and their littermates (wild type (WT)) as controls. Mice from the same litter were used for comparison. We found that RAR α ^{-/-} mice developed more severe proteinuria and kidney damage compared with RAR α ^{+/-} Tg26 and RAR α ^{+/+} Tg26 (Table 3 and Figure 4). By real-time PCR, we found that the expression of podocyte differentiation markers was further suppressed in glomeruli isolated from RAR α ^{-/-} Tg26 mice compared with RAR α ^{+/+} Tg26 and RAR α ^{+/-} Tg26 (Figure 5a). The expression of proliferation markers (Ki67 and cyclin E) was further increased in glomeruli isolated from RAR α ^{-/-} Tg26 compared with RAR α ^{+/-} and RAR α ^{-/-} mice (Figure 5b). These changes could also be the result of differences in the expression of the HIV transgene. To eliminate this possibility, we measured the expression of *nef*, one of the important regulatory genes in the pNL4-3 deletion mutant used to generate the transgenic mice. We found that the expression of HIV-1 *nef* was not different among RAR α ^{-/-} Tg26, RAR α ^{+/+} Tg26, and RAR α ^{+/-} Tg26 mice (Figure 5c). In addition, the changes in the expression of markers of podocyte

proliferation and differentiation were confirmed at protein levels by western blot (Figure 5d). Taken together, these data suggest that knockout of *RAR α* caused more severe podocyte injury and kidney damage in Tg26, and *RAR α* likely acts as an endogenous protective pathway to prevent the development and the progression of kidney disease.

Am580 failed to improve proteinuria and glomerulosclerosis in Tg26

Next, to establish whether the beneficial effect of Am580 is mediated through agonism of *RAR α* , we evaluated the impact of Am580 on the development of kidney disease in Tg26 mice lacking *RAR α* . *RAR α* ^{-/-} Tg26, *RAR α* ^{+/-} Tg26, and *RAR α* ^{+/+} Tg26 mice were fed with Am580 or control diet from 4 weeks of age and killed at 12 weeks of age as described in the Materials and Methods. We found that whereas Am580 significantly reduced proteinuria and kidney damage in *RAR α* ^{+/+} Tg26 and *RAR α* ^{+/-} Tg26, it failed to improve proteinuria, glomerulosclerosis, and tubular injury in *RAR α* ^{-/-} Tg26 (Table 4). These data further confirm a critical role of *RAR α* in mediating the protective effects of Am580 in Tg26.

Expression of *RAR α* and *RAR β* in kidneys of Tg26 mice and patients with HIVAN

As our studies suggest a critical role of *RAR α* in mediating the protective effects of retinoids in the HIVAN model, we speculated that the basal expression of *RAR α* between Tg26 and WT kidneys could be different. However, the expression of *RAR α* was not different between Tg26 and WT kidneys by immunostaining (Figure 6a). Kidneys from *RAR α* ^{-/-} mice were used as the negative control. In contrast, the expression of *RAR β* , which is a target gene of *RAR α* , was significantly downregulated in kidneys of Tg26 and in kidneys of *RAR α* ^{-/-} mice (Figure 6a). Similarly, the expression of *RAR α* was not different between kidneys from patients with HIVAN and patients with minimal change disease, and the expression of *RAR β* was significantly downregulated in kidneys from patients with HIVAN (Figure 6b). These findings were confirmed by real-time PCR showing that mRNA levels of *RAR α* were not different between Tg26 and WT kidneys, whereas *RAR α* expression was suppressed in Tg26 kidneys (Figure 6c). Furthermore, we found that *RARRES1* (retinoic acid receptor responder protein 1) and *RORA* (retinoid-related orphan receptor- α) were also significantly reduced in glomeruli of Tg26 (Figure 6c).

Retinoid synthesis in Tg26 kidneys

We were surprised to find that *RAR α* expression was not different in Tg26 kidneys, and hence we hypothesized that endogenous RA synthesis might be impaired instead. We measured kidney tissue levels of retinol and RA and found that whereas retinol was not different in kidney cortices between WT and Tg26 mice, RA levels were significantly reduced in Tg26 kidneys compared with WT kidneys (Figure 7a). As we are interested in RA synthesis in podocytes, we also measured retinol and RA levels in isolated glomeruli. Similar to the level of RA in kidney cortices, RA expression is suppressed in isolated glomeruli of Tg26 compared with WT mice, whereas retinol levels did not differ between these animals (Figure 7b). The suppression of RA in isolated glomeruli was more pronounced than in kidney cortices. Interestingly, the amount of RA per gram of tissue was much higher in isolated glomeruli than in kidney cortices. To determine whether the reduction of RA synthesis in Tg26 was tissue specific, we also compared the levels of retinol and RA in the liver of Tg26 and WT mice. We found that both retinol and RA levels were similar in the liver of WT and Tg26 mice (Figure 7c). This suggests that the impairment of RA synthesis in kidneys of Tg26 is tissue specific. In addition, we determined the rate of RA synthesis in isolated glomeruli by measuring the conversion rate of retinol to RA in the presence of glomerular lysates from either Tg26 or WT mice. We confirmed that glomerular lysates of Tg26, when compared with lysates of WT, had a reduced rate of retinol to RA conversion, which suggests that synthesis of RA is lower in Tg26 (Figure 7d).

To further investigate which enzyme(s) involved in the synthesis of RA is responsible for the lower RA level in Tg26 glomeruli, we analyzed the expression of genes responsible for RA metabolism. We performed gene expression microarray analysis on glomeruli isolated from Tg26 and WT kidneys and generated an expression profile of genes related to retinoid metabolism. We found that two key enzymes of RA synthesis, RDH1 and RDH9, were significantly downregulated in the kidneys of Tg26, whereas there was a significant increase in the expression of Cyp26A1, which is an enzyme responsible for the degradation of RA. These findings were further confirmed by real-time PCR (Figure 7e). Using immunostaining, we confirmed that RDH1 expression is also suppressed in the kidney of patients with HIVAN (Figure 7f). The expression of cellular retinol binding protein was not different between Tg26 and WT mice (Figure 7e). The expression of other enzymes in the RA synthesis pathway, including retinal dehydrogenase 1, 2, and 4, was not suppressed in Tg26 glomeruli (data not shown). Using real-time PCR, we confirmed that there was no difference in the mRNA level of RDH1, RDH9, and Cyp26A1 in the liver of Tg26 and WT mice (data not shown). Taken together, these data indicate that endogenous synthesis of RA is likely impaired in diseased kidneys, leading to the loss of endogenous protective effects of RAs.

DISCUSSION

Although the incidence of HIVAN decreased in the highly active antiretroviral therapy era, its prevalence is increasing because of the aging of patients.^{21,29} Furthermore, highly active antiretroviral therapy is only partially protective and many patients with HIVAN ultimately progress to end-stage renal disease.³⁰ HIVAN remains the leading cause of end-stage renal disease among young African Americans¹² on highly active antiretroviral therapy. Therefore, it is of paramount importance to identify additional treatment options for patients with HIVAN.

A large amount of evidence suggests that RA ameliorates kidney injury in many animal models of kidney disease including HIVAN. Unfortunately, treatment strategies using RA are limited because of significant side effects from ATRA. Our current study suggests that Am580, a RAR α agonist, improves kidney disease in an animal model of HIVAN (Tg26) similar to ATRA. Am580 can be given orally. It has significantly less side effects than ATRA because it targets only RAR α . In addition, it is believed that the side effects of ATRA are mediated mostly by RAR γ .²⁶ Our data suggest that Am580 could be a potentially important drug in the treatment of patients with HIVAN without causing significant side effects. Recent studies suggest that Am580 inhibits tumor oncogenesis in animal models with mammary tumor through inhibition of proliferation and induction of differentiation of tumor cells.³¹ No significant side effects were reported in these animals treated with Am580.

Our studies using RAR α -null mice further confirm the critical role of RAR α in kidney disease. Knockout of RAR α causes the loss of an endogenous protective pathway and leads to a more aggressive kidney disease. The role of RAR α in kidney disease was further supported by the lack of protection by Am580 in RAR α -/- Tg26 mice. These studies strongly suggest that RAR α is a potential therapeutic target to treat patients with HIVAN. Further drug screening of RAR α agonists should be encouraged as an alternative or adjunctive form of therapy for patients with HIVAN.

How RA improves kidney disease remains unclear. It has been shown that RA has both anti-inflammatory and antifibrosis effects.^{5,32} Recent studies suggest that RA can also induce podocyte differentiation and maintain nephrin expression in cultured podocytes.³³ We found that ATRA inhibits HIV-induced podocyte proliferation and restores differentiation markers

in HIV-infected podocytes via activation of RAR α .²² Our current findings show that activation of RAR α *in vivo* by a specific agonist protects podocytes from injury, whereas knockdown of RAR α aggravates podocyte injury and disease severity. Taken together, this indicates that RAR α is a key molecule mediating the protective effects of RA in podocytes.

Interestingly, we found that RAR α -mediated target genes, including RAR β and RARRES1, were inhibited in kidneys of Tg26. This suggests that the RAR α -mediated protection is impaired in Tg26 kidneys. However, the expression of RAR α in kidneys did not differ between Tg26 and the WT littermates. The kidney is known to be a major organ for RA acid synthesis. In this study, we measured the level of retinol and RA in the renal cortex, isolated glomeruli, and liver of Tg26 and WT mice. Interestingly, we found that the level of RA was significantly suppressed in the Tg26 kidney cortex and even more suppressed in the isolated glomeruli, whereas the level of retinol remained unchanged. These findings suggest that RA synthesis is severely impaired in the glomeruli of Tg26. The level of retinol and RA in the liver was not different between Tg26 and WT. This suggests that the suppression of RA in Tg26 is likely not a generalized phenomenon. The measurement of key synthetic enzymes involved in RA metabolism in the kidney revealed that both RDH1 and RDH9 are severely suppressed in the kidneys of Tg26, whereas Cy26a, a RA acid catalytic enzyme, is increased in Tg26. Although we showed that the general enzymatic activity of RA synthesis is markedly reduced in Tg26 glomeruli, we were unable to measure the enzymatic activity of RDH1 and RDH9 because of the lack of isoform-specific antibodies. Our findings suggest that the restoration of normal RA synthesis in kidneys could be a potential therapeutic approach. The reasons for the differential regulation of RDH1, RDH9, and Cy26a in Tg26 and WT kidneys remain to be determined. Finally, Am580 might be particularly effective in treating Tg26 mice or individuals with HIVAN because Am580 is resistant to degradation by Cyp26a,^{34,35} which we have found to be upregulated in HIVAN.

In conclusion, our findings demonstrate that endogenous RA synthesis is impaired in diseased kidneys. RAR α -mediated signaling acts as an endogenous protective pathway to slow the progression of kidney disease, and selective activation of the RAR α receptor may be a promising treatment option for patients with HIV-related renal diseases.

MATERIALS AND METHODS

Generation of RAR α ^{-/-} and RAR α ^{+/-} Tg26 mice

RAR α ^{-/-} mice were provided by Dr Pierre Chambon, (Strasbourg, France) as described.³⁶ Derivation of a HIV-1 transgenic mouse line (Tg26) that bears a defective HIV-1 provirus lacking gag-pol (Tg26) has been described.³⁷ Both RAR α ^{-/-} and Tg26 mice are in the same FVB/N background. RAR α ^{-/-} female mice were crossed with male Tg26^{+/-} (heterozygote) mice to generate RAR α ^{+/-} Tg26^{+/-} mice. Then, we crossed male RAR α ^{+/-} Tg26^{+/-} mice with female RAR α ^{+/-} Tg26^{+/-} mice to generate RAR α ^{+/+} Tg26^{+/-}, RAR α ^{+/-} Tg26^{+/-}, RAR α ^{-/-} Tg26^{+/-} mice, and their corresponding littermates without the Tg26 gene. Mice generated from the same litter of Tg26 mice were used in the studies. Genotyping by tail prep and PCR were performed at 2 weeks of age as described.²⁸

Treatment of animals with Am580

Tg26, RAR α ^{+/-} Tg26, RAR α ^{-/-} Tg26, and their corresponding littermates ($n = 20$ per group including 10 male and 10 female mice in each group) were fed Am580 at a concentration of 0.3 mg/kg/day, and the control foods, which were prepared similarly without Am580. Am580 was a gift from Dr K Shudo (Research Foundation ITSUU Laboratory, Molecular and Functional Bioscience, Japan). The mice were fed these diets everyday from the age of 4 weeks to 12 weeks. Based on the daily measurement of food intake, the average food intake

ranged from 3.5 to 4.5 g/day per mouse. Unrestricted water was provided throughout the duration of the experiment. The mice were killed at 12 weeks of age for blood, urine, and tissue collection by exposure to carbon monoxide. Body and kidney weight were recorded. All animal studies were performed according to the protocols approved by the institutional animal care and use committee at the Mount Sinai School of Medicine.

Measurement of blood urea nitrogen, urine protein, and creatinine

Mouse serum was collected for measurement of blood urea nitrogen in the laboratory of Mount Sinai Hospital, New York using a urease-based assay (Vitros BUN/UREA slides detected using Vitros 5,1 FS Instrument, Ortho-Clinical Diagnostics, Rochester, NY). Urine albumin was quantified by enzyme-linked immunosorbent assay using a kit from Bethyl Laboratory (Houston, TX). Urine creatinine levels were measured in the same samples using QuantiChrom Creatinine Assay Kit (DICT-500; BioAssay Systems, Hayward, CA) according to the manufacturer's instruction. The urine albumin excretion rate was expressed as the ratio of albumin to creatinine.

Quantitative histopathology

Mice were perfused with phosphate-buffered saline containing 4% paraformaldehyde, and kidneys were further fixed in 4% paraformaldehyde for 2 h. Kidney tissue was embedded in paraffin by American Histolabs (Gaithersburg, MD) and 3 μ m thick sections were stained with periodic acid-Schiff. Renal histological abnormalities were scored by a renal pathologist (VD'A) as previously described.³¹ The three parameters scored included the percentage of glomeruli exhibiting collapse and segmental or global sclerosis, the percentage of total renal parenchyma occupied by tubular casts and/or microcysts, and the severity of podocyte hypertrophy and hyperplasia (graded on a semiquantitative scale (0–3+)).³⁸ For the latter parameter, podocyte hypertrophy and hyperplasia was graded as 0 (absent), 1+ (involving 1–25% of all glomeruli sampled), 2+ (involving 26–50% of glomeruli), and 3+ (involving >50% of glomeruli) as described.³⁹ An average of 50 glomeruli was sampled per animal.

Isolation of glomeruli from mice for western blot and real-time PCR

Mouse glomeruli were isolated as described.⁴⁰ Briefly, animals were perfused with Hank's buffered salt solution containing 2.5 mg/ml iron oxide and 1% bovine serum albumin. At the end of perfusion, kidneys were removed, decapsulated, minced into 1-mm³ pieces, and digested in Hank's buffered salt solution containing 1 mg/ml collagenase A and 100 U/ml deoxyribonuclease I. Digested tissue was then passed through 100 μ m cell strainer and collected by centrifugation. The pellet was resuspended in 2ml of Hank's buffered salt solution and glomeruli were collected using a magnet. The purity of glomeruli was verified under microscopy and by western blot analysis for podocyte-specific markers including synaptopodin and WT-1.

Immunohistochemistry

Kidney sections from these mice and from kidney biopsies were prepared as described.²⁰ Human kidney biopsies from patients with HIVAN and non-HIV patients with minimal change disease were collected at Columbia University under a protocol approved by its institutional review board. These biopsy samples were from five patients with HIVAN and five patients with minimal change disease, which was confirmed by a renal pathologist. Immunostaining was performed using anti-synaptopodin (gifts of Dr Peter Mundel), anti-RAR α , anti-RAR β , and anti-RDH1 (all retinoid-related antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA), and then followed by biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) as described. After staining, slides were

mounted in Aqua Poly/Mount (Polysciences, Warrington, PA) and photographed under an Olympus BX60 microscope (Olympus Optical, Center Valley, PA) with a digital camera. For colocalization studies, sections were incubated with anti-Ki67 antibodies (Dako, Carpinteria, CA) together with anti-neslin antibody (Santa Cruz Biotechnology) at room temperature for 1 h. After washing, sections were incubated with a fluorophore-linked secondary antibody (Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 568 anti-mouse IgG from Invitrogen, Carlsbad, CA). After mounting, slides were examined by fluorescence microscopy.

Real-time PCR

Total RNA was isolated from kidney glomeruli of mice using TRIzol (Invitrogen). Real-time PCR was performed with a Roche Light-cycler (Roche Applied Science, Indianapolis, IN) and Qiagen QuantiTect SYBR green PCR kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Predesigned primer sets were obtained from Qiagen (GeneGlobe) for synaptopodin, nephrin, WT-1, cyclin E, Ki67, RAR α , RAR β , RORE, Cyp26A, RDH1, and RDH9. The following sequences were used for the tubulin primers: 5'-TGCCTTTGTGCACTGGTATG-3' and 5'-CTGGAGCAGTTTG ACGACAC-3'. Light cycler analysis software was used to determine crossing points using the second derivative method. Data were normalized to housekeeping genes (*tubulin*) and presented as fold increase compared with RNA isolated from WT animals using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Glomerular lysates were subjected to western blot analysis using the following specific antibodies including: rabbit anti-synaptopodin (a gift from Dr Peter Mundel, University of Miami), rabbit anti-nephrin (a gift from Dr Tomoko Takano, McGill University Health Centre), anti-Ki67 (Abcam, Cambridge, MA), anti-neslin (Santa Cruz Biotechnology), anti-cyclin E (EMD Chemicals, Gibbstown, NJ), anti-WT-1 (Santa Cruz Biotechnology), and anti- β -actin antibodies (Sigma-Aldrich, St Louis, MO).

Measurement of tissue retinoids by mass spectrometry as described

Retinol and ATRA levels were determined as described.^{41,42} Briefly, kidney tissue was homogenized in 0.9% NaCl. KOH/ethanol (0.025 N) was added to the homogenates, followed by extraction with 10 ml of hexane. The top (hexane) layer containing retinol was removed and evaporated under nitrogen and then resolved by reversed-phase high performance liquid chromatography and quantified by their ultraviolet absorbance at 325 nm. The bottom layer was then extracted with 4N HCl and followed by 10ml of hexane. The resulting top layer containing ATRA was evaporated and resolved by a liquid chromatography/tandem mass spectrometry.

Glomerular enzymatic activity for RA acid synthesis

Glomeruli were isolated from Tg26 and little-matched WT mice ($n = 5$) by the graded sieving method. The purity of isolated glomeruli was verified under microscope.

Glomeruli were homogenized by motor-driven homogenizer in the presence of a buffer containing 10% sucrose (w/w), 10 mmol/l Tris-HCl, 1 mmol/l EDTA, 1.5 mmol/l dithiothreitol, pH = 7.4, and protease inhibitors. Protein concentration was determined by the bicinchoninic acid method.

The enzymatic activity for RA acid synthesis was determined by measuring the conversion of retinol (10 μ M) to RA in the presence of glomerular lysates (30 μ g) from either WT or Tg26 mice. The reaction occurred in a buffer containing 50 mmol/l HEPES, pH = 8.0, 150 mmol/l KCl, 1 mmol/l EDTA, 2 mmol/l dithiothreitol, NAD + 4 mmol/l, and NADP + 2

mmol/l at 37 °C for 30 min and was stop by placing assay tubes into liquid nitrogen, and RA acid levels were measured as described above. The enzymatic activity for RA synthesis was expressed as pmol of RA generation per mg of glomerular lysates per min of incubation (pmol/mg/min).

Statistical analysis

Data were expressed as mean±s.d.. The unpaired *t*-test was used to analyze data between two groups. Statistical significance will be considered when $P < 0.05$.

Acknowledgments

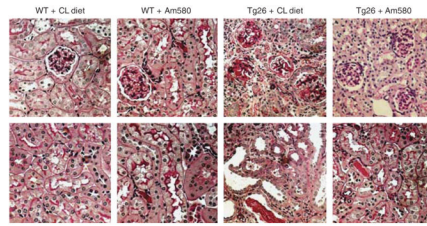
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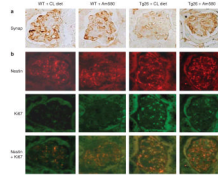
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**Figure 1. Kidney histology**

Wild-type (WT) and Tg26 mice were treated with either vehicle or Am580 as described in the Materials and Methods. The representative pictures of kidney histology (hematoxylin and eosin (H&E) staining) of these mice are shown. Top and bottom panels are representative fields showing glomerular and tubulointerstitial area, respectively (original magnification $\times 200$).

**Figure 2. Immunostaining**

Kidney sections from mice treated with either vehicle or Am580 were used for immunohistochemistry of **(a)** synaptopodin (Synap), a podocyte differentiation marker, and for **(b)** immunofluorescence of Nestin and Ki67. Nestin is a podocyte-specific marker. Ki67 is a marker for proliferating cells. The representative pictures are shown ($n = 5$).

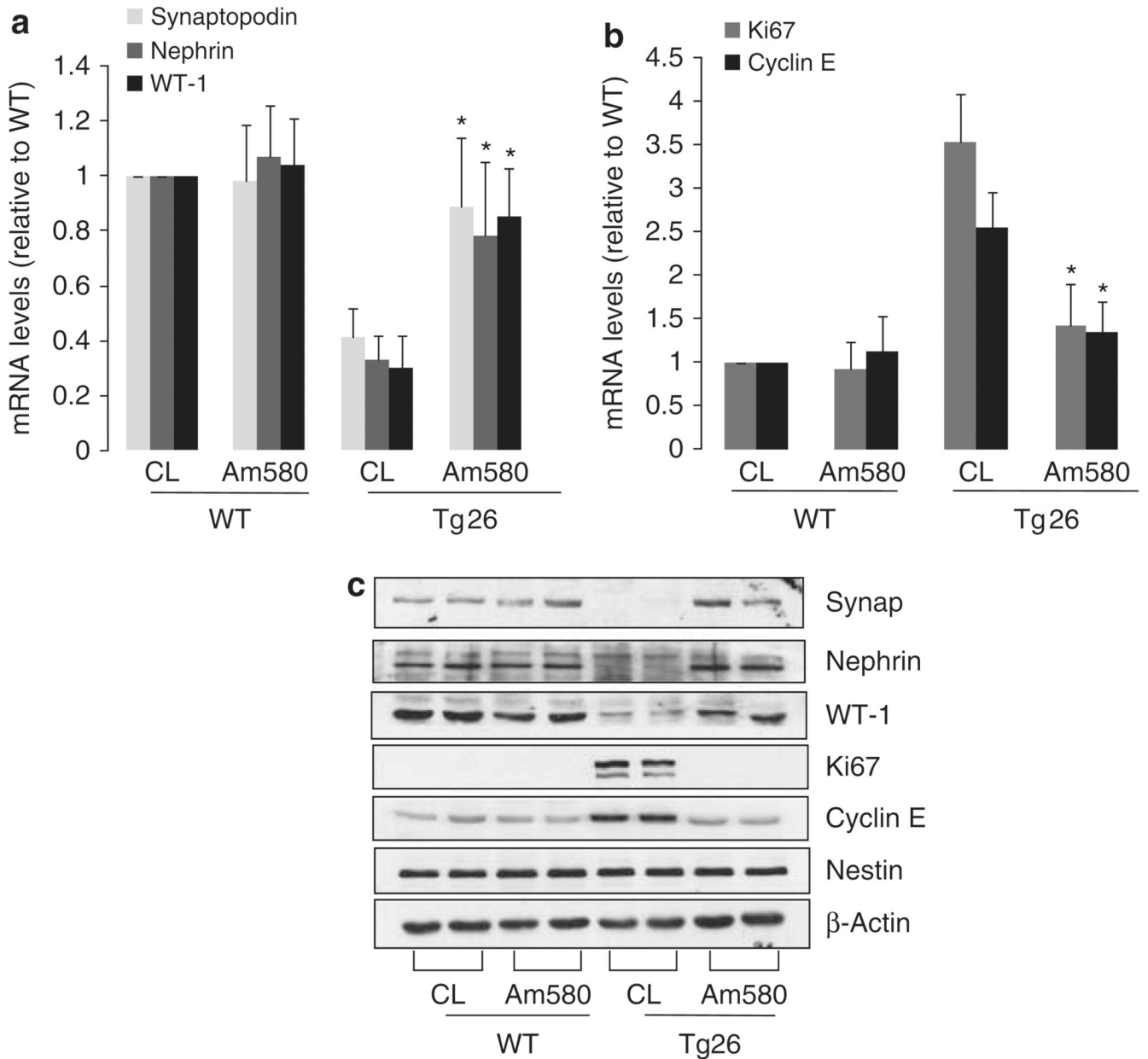
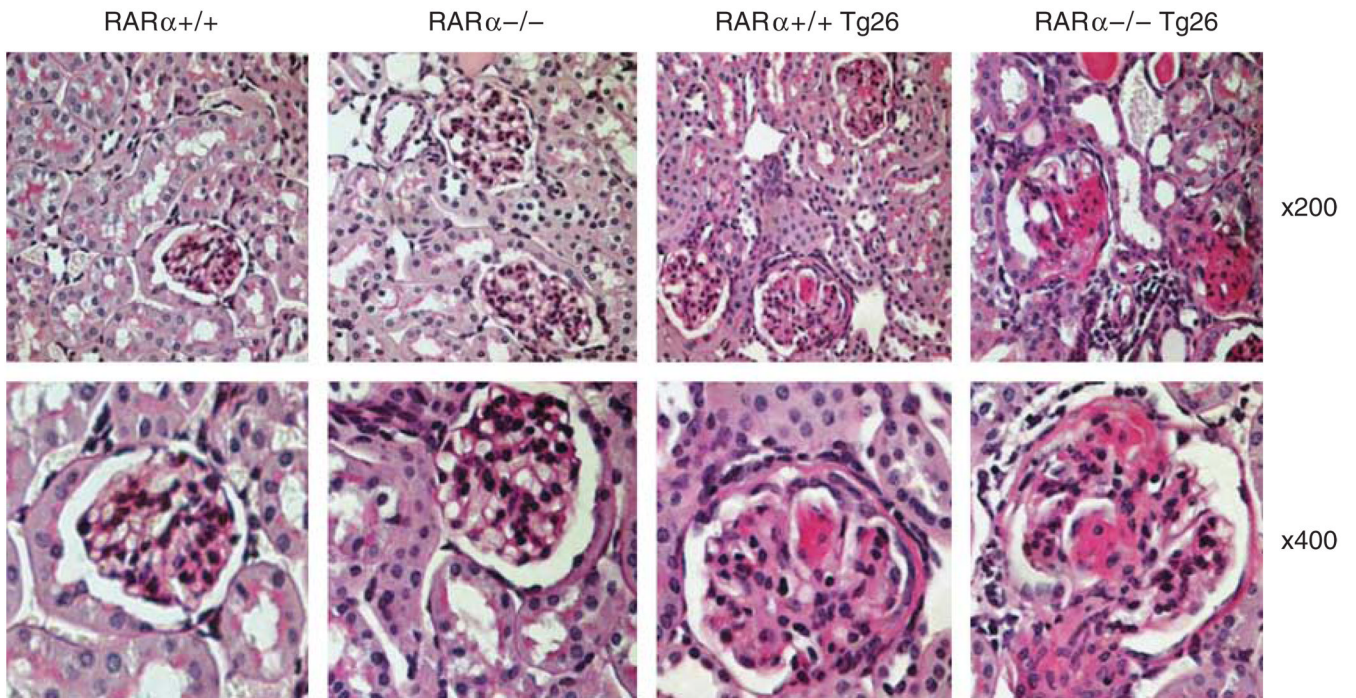


Figure 3. Real-time PCR

Glomeruli were isolated from mice treated with either control vehicle (CL) or Am580 as described. Total RNA was isolated from these glomeruli for real-time PCR analysis of genes associated with (a) podocyte differentiation (synaptopodin, nephrin, and WT-1) and (b) podocyte proliferation (Ki67 and cyclin E). The ratio of tested genes to tubulin expression was calculated. The ratio of treated mice to control wild type (WT) is shown, $n = 10$, $*P < 0.01$. (c) Protein lysates from these glomeruli were used for western blot analysis for the above podocyte differentiation and proliferation markers. The representative blots of at least three independent experiments are shown.

**Figure 4. Kidney histology**

$RAR\alpha^{+/+}$, $RAR\alpha^{-/-}$, $RAR\alpha^{+/+}$ Tg26, and $RAR\alpha^{-/-}$ Tg26 mice were killed at the age of 12 weeks for histology analysis. The representative fields of hematoxylin and eosin (H&E) staining are shown. Original magnification (top panel) $\times 200$; (low panel) $\times 400$.

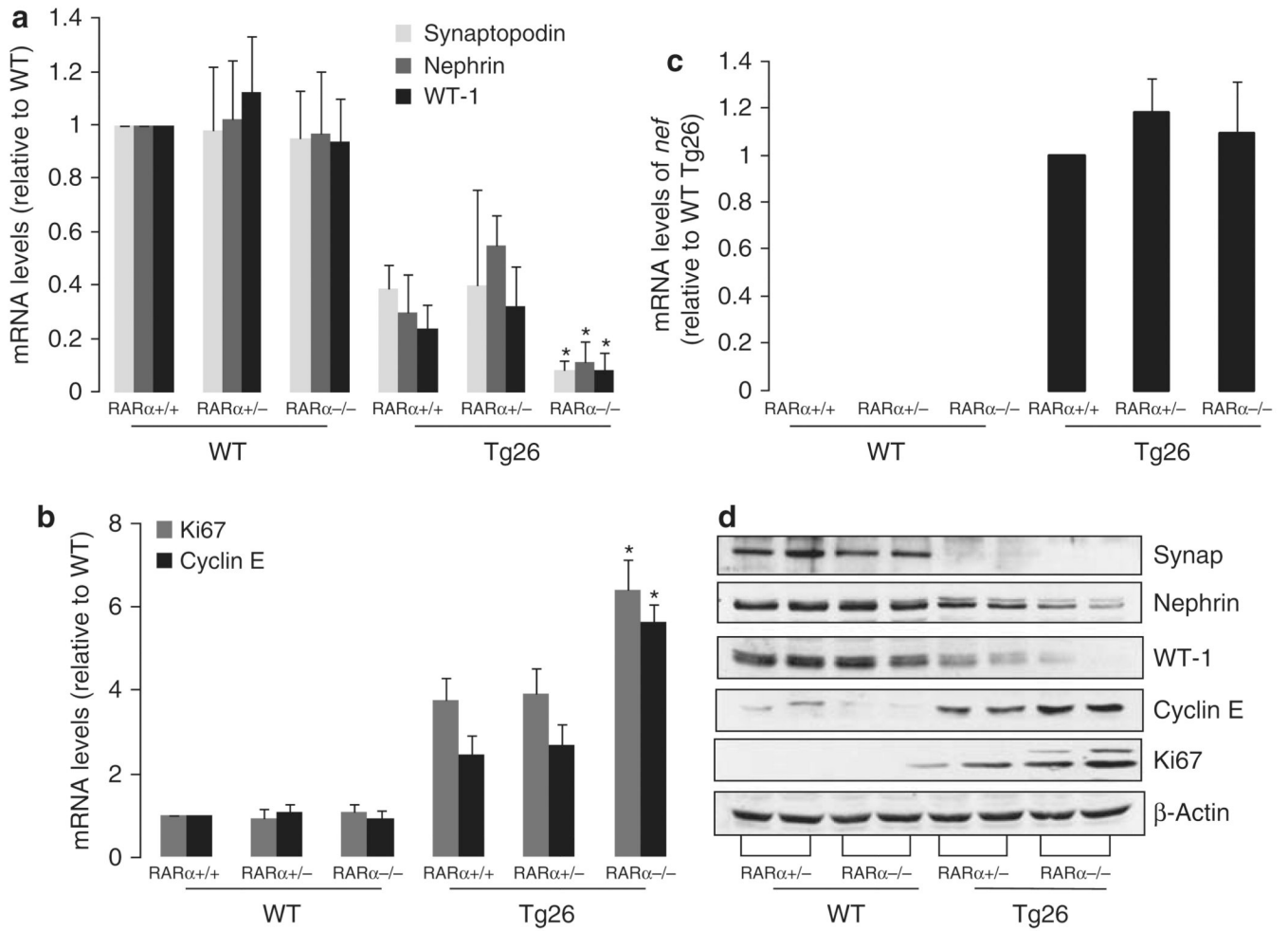


Figure 5. Real-time PCR

Glomeruli were isolated from $RAR\alpha^{+/+}$, $RAR\alpha^{-/-}$, $RAR\alpha^{+/+}$ Tg26, and $RAR\alpha^{-/-}$ Tg26 mice at 12 weeks of age. Total RNA was isolated from glomeruli for real-time PCR analysis of genes indicative of (a) podocyte differentiation (synaptopodin, nephrin, and WT-1) and (b) proliferation (Ki67 and cyclin E). The ratio of tested genes to tubulin gene was calculated. The ratio of treated mice to wild type (WT) was shown, $n = 10$, $*P < 0.01$. (c) Real-time PCR was also performed to verify the expression of HIV-1 *nef* gene in the glomeruli of these mice. (d) Glomerular lysates were used for western blot analysis of the above podocyte differentiation and proliferation markers. The representative blots of at least three independent experiments are shown.

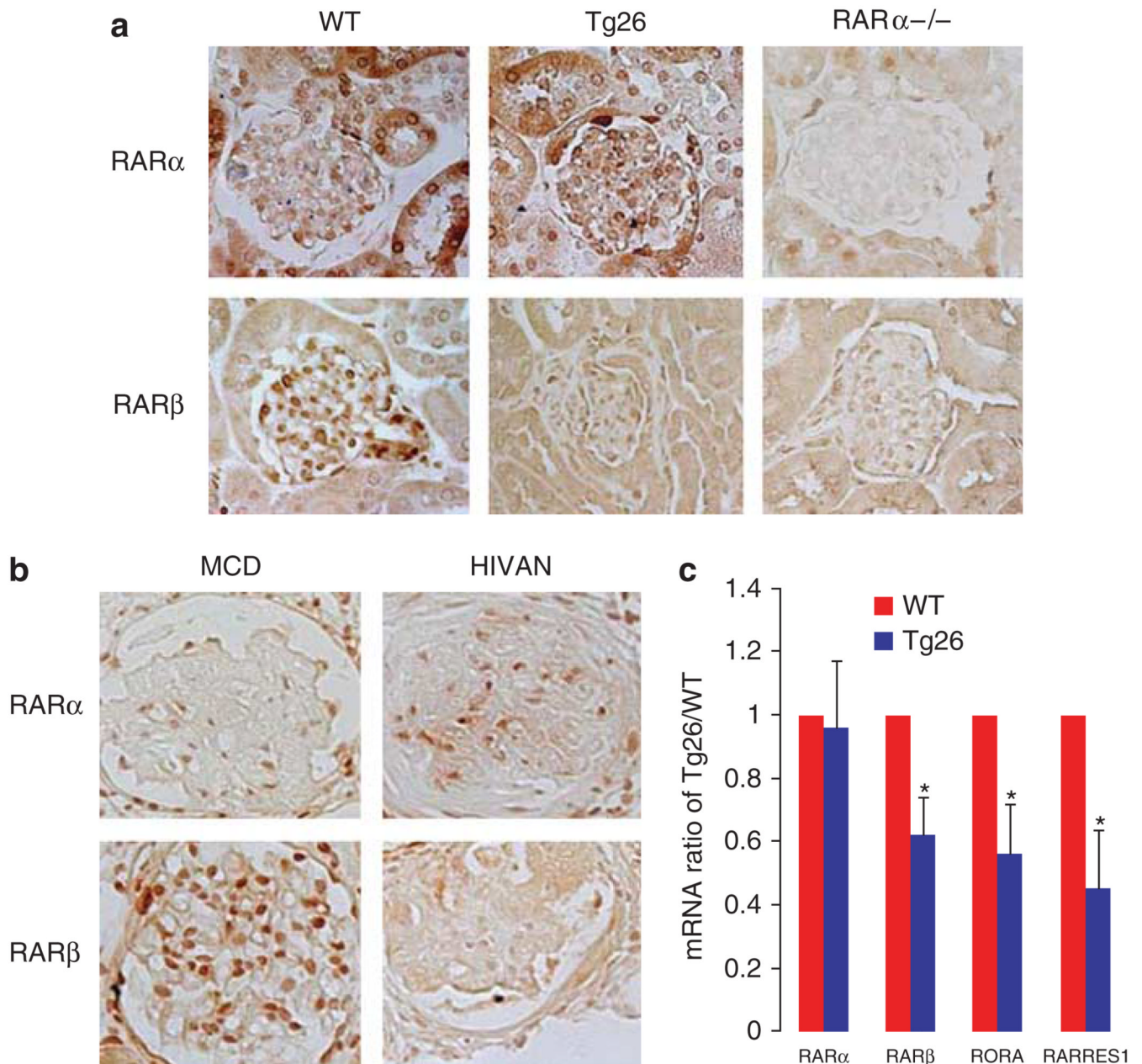


Figure 6. Immunostaining for retinoic acid receptor (RAR) α and RAR β

(a) Immunostaining was performed in kidneys of wild-type (WT), Tg26, and RAR α -/- mice using anti-RAR α and anti-RAR β antibodies (Santa Cruz Biotechnology). (b) Immunostaining was also performed in kidneys from patients with human immunodeficiency virus (HIV)-associated kidney disease (HIVAN) and with minimal change disease (MCD) using anti-human RAR α and RAR β antibodies (Santa Cruz Biotechnology). The representative pictures are shown here ($n = 5$). (c) Total RNA was isolated from glomeruli of Tg26 and WT littermates. Real-time PCR was performed for RAR α , RAR β , RORA (retinoid-related orphan receptor- α), and RARRES1 (retinoic acid receptor responder protein 1). Fold change of gene expression in Tg26 relative to WT is shown here. * $P < 0.01$, $n = 10$.

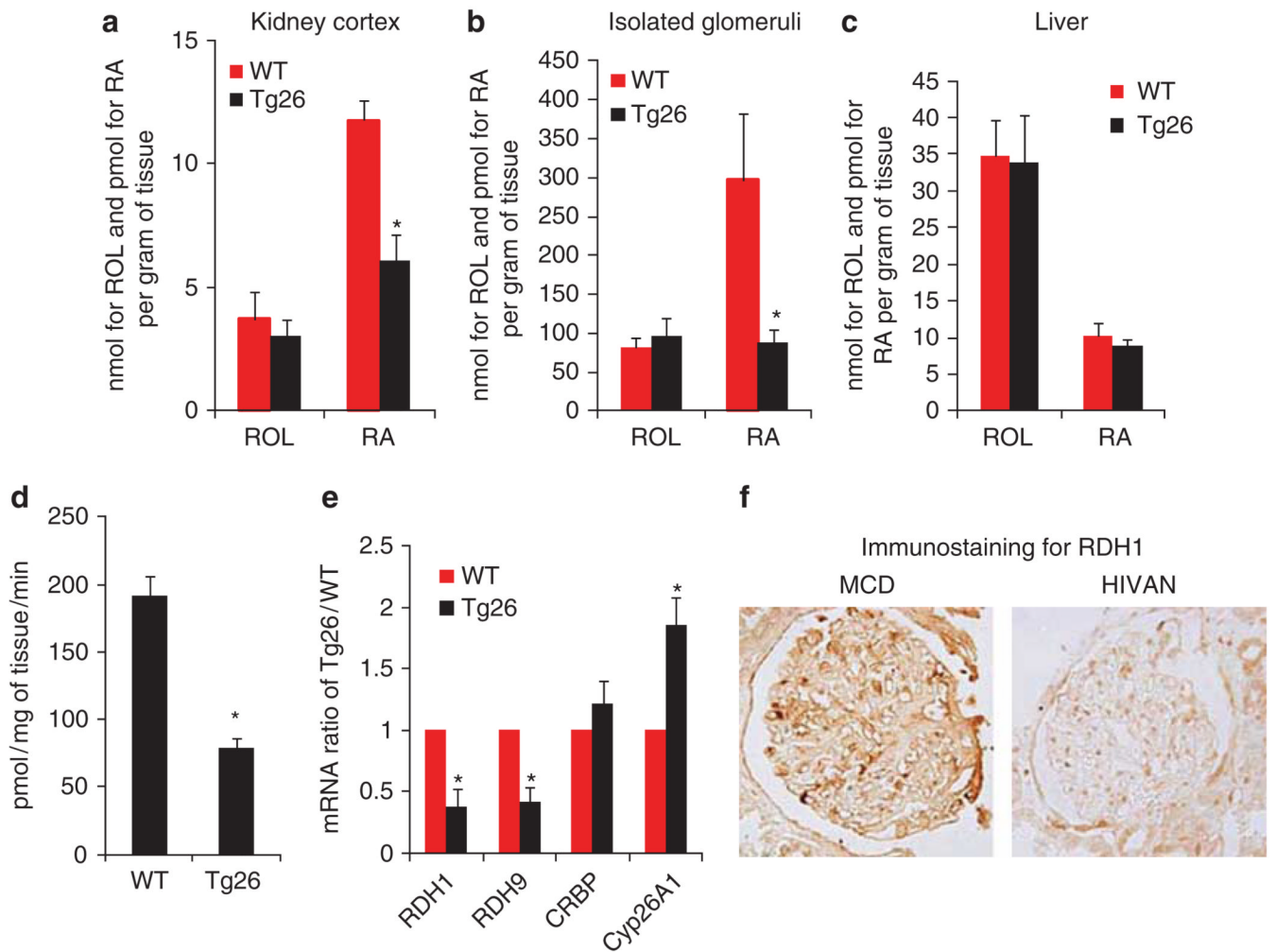


Figure 7. Tissue levels of retinoids

Retinol (ROL) and retinoic acid (RA) levels were measured in (a) kidney cortices, (b) isolated glomeruli, and (c) livers of Tg26 and wild-type (WT) mice as described in the Materials and Methods ($n = 5$, $*P < 0.01$). (d) The enzymatic activity of RA synthesis was also determined in isolated glomeruli by measuring the conversion of retinol to RA, $n = 5$, $*P < 0.001$. (e) Total RNA was isolated from glomeruli of Tg26 and WT littermates. Real-time PCR were performed for retinol dehydrogenase (RDH)1, RDH9, and Cyp26A1. Fold change of gene expression in Tg26 relative to WT is shown here, $*P < 0.01$, $n = 10$. (f) Immunostaining of RDH1 was performed in kidneys from patients with human immunodeficiency virus (HIV)-associated kidney disease (HIVAN) and minimal change disease (MCD). The representative pictures of five individual patients are shown here.

Table 1

Am580 prevents kidney hypertrophy and improves renal function in Tg26

	BW (g)	KW (mg)	KW/BW (mg/g)	BUN	Urine Pro/Cr ratio
WT+control diet	25.3±4.0	222±51	8.7±1.3	0.21±2.32	0.15±0.12
WT+Am580	24.8±3.7	220±43	8.8±1.4	0.22±1.89	0.18±0.61
Tg26+control diet	25.4±4.2	235±46	9.3±1.0	0.76±1.67	32.4±6.5
Tg26+Am580	25.1±3.4	225±63*	8.8±1.6*	0.25±0.22*	3.5±1.5*

Abbreviations: BUN, blood urea nitrogen; BW, body weight; Cr, creatinine; KW, kidney weight; Pro, protein; WT, wild type.

Tg26 and WT littermates were treated with either control diet or Am580 for a total of 8 weeks as described in the Materials and Methods. When mice were killed at the age of 12 weeks, BW and KW were recorded and their ratio (KW/BW) was calculated. BUN and urinary protein and creatinine levels were measured. Urine Pro/Cr ratio was calculated, $n=20$.

* $P<0.01$ compared with Tg26+control diet group.

Table 2

Am580 improves kidney injury in Tg26

	GS index	Podocyte hypertrophy	Tubular casts/cysts
WT+control diet	0	0	0
WT+Am580	0	0	0
Tg26+control diet	19.5±3.2	1.9±0.9	10.2±2.5
Tg26+Am580	2.7±1.1*	0*	1.5±1.2*

Kidney histological analysis was performed in wild type (WT) and Tg26 treated with either control diet or Am580. Glomerulosclerosis (GS) index, podocyte hypertrophy, and tubular casts/cysts were quantified as described in the Materials and Methods, $n=20$.

* $P<0.001$ compared with Tg26+control diet.

Table 3Knockout of RAR α aggravates kidney injury in Tg26

	Urine Pro/Cr ratio	GS index	Podocyte hypertrophy	Tubular casts/cysts
RAR α +/+	0.12 \pm 0.10	0	0	0
RAR α +/+ Tg26	33.4 \pm 15.6	18.5 \pm 11.1	1.2 \pm 0.8	9.4 \pm 5.6
RAR α +/-	0.21 \pm 0.22	0	0	0
RAR α +/- Tg26	35.1 \pm 19.5	21.7 \pm 7.8	1.3 \pm 0.5	8.4 \pm 3.9
RAR α -/-	0.29 \pm 0.32	0	0	0
RAR α -/- Tg26	140 \pm 89*	45.5 \pm 22.9*	2.3 \pm 0.8*	23.9 \pm 14.5*

Abbreviations: Cr, creatinine; GS, glomerulosclerosis; Pro, protein; RAR, retinoic acid receptor.

A comparison of proteinuria (urine Pro/Cr ratio), GS index, podocyte hypertrophy, and tubular casts/cysts was performed between RAR α +/+, RAR α +/+ Tg26, RAR α +/-, RAR α +/- Tg26, RAR α -/-, and RAR α -/- Tg26 mice, $n=20$.

* $P<0.001$ compared with the Tg26 group.

Table 4Am580 failed to improve kidney disease in RAR $\alpha^{-/-}$ Tg26

	Urine Pro/Cr ratio	GS index	Podocyte hypertrophy	Tubular casts/cysts
RAR $\alpha^{+/+}$ Tg26 control diet	31.2 \pm 7.3	16.5 \pm 3.1	1.2 \pm 0.8	9.4 \pm 5.6
RAR $\alpha^{+/+}$ Tg26 Am580	2.6 \pm 1.6*	1.7 \pm 1.4*	0*	2.5 \pm 1.4*
RAR $\alpha^{+/-}$ Tg26 control diet	33.1 \pm 10.5	12.7 \pm 7.4	1.3 \pm 0.5	6.9 \pm 1.3
RAR $\alpha^{+/-}$ Tg26 Am 580	3.4.1 \pm 1.5*	1.9 \pm 1.2*	0*	1.9 \pm 0.9*
RAR $\alpha^{-/-}$ Tg26 control diet	123.2 \pm 22.3	41.2 \pm 12.3	3.5 \pm 1.2	21.6 \pm 7.2
RAR $\alpha^{-/-}$ Tg26 Am580	110 \pm 11.2	38.3 \pm 9.2	3.3 \pm 0.9	19.7 \pm 6.4

Abbreviations: Cr, creatinine; GS, glomerulosclerosis; Pro, protein; RAR, retinoic acid receptor.

RAR $\alpha^{+/+}$ Tg26, RAR $\alpha^{+/-}$ Tg26, and RAR $\alpha^{-/-}$ Tg26 mice were treated with either control diet or Am580 from 4 to 12 weeks of age. Then, mice were killed for analysis of proteinuria and histology as summarized here, $n=20$.

* $P<0.001$ compared with RAR $\alpha^{+/+}$ Tg26 control diet.