Molecular Mechanism Underlying 1,25-Dihydroxyvitamin D Regulation of Nephrin Gene Expression*

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Dilip K. Deb†, Youli Wang‡, Zhongyi Zhang‡, Hongguang Nie§, Xueshi Huang§, Zhengwei Yuan§, Yunzi Chen‡, Qun Zhao§, and Yan Chun Li‡§

From the †Department of Medicine, Division of Biological Sciences, The University of Chicago, Chicago, Illinois 60637, the ‡Laboratory of Metabolic Disease Research and Drug Development, China Medical University, Shenyang, China, and the §Ministry of Health Key Laboratory of Congenital Malformation, Shengjing Hospital, China Medical University, Shenyang, China

Nephrin plays a key role in maintaining the structure of the slit diaphragm in the glomerular filtration barrier. Our previous studies have demonstrated potent renoprotective activity for 1,25-dihydroxyvitamin D (1,25(OH)2D3). Here we showed that in podocytes 1,25(OH)2D3 markedly stimulated nephrin mRNA and protein expression. ChIP scan of the 6-kb 5′ upstream region of the mouse nephrin gene identified several putative vitamin D response elements (VDREs), and EMSA confirmed that the VDRE at −312 (a DR4-type VDRE) could be bound by vitamin D receptor (VDR)/retinoid X receptor. Luciferase reporter assays of the proximal nephrin promoter fragment (−427 to +173) showed strong induction of luciferase activity upon 1,25(OH)2D3 treatment, and the induction was abolished by mutations within −312VDRE. ChIP assays showed that, upon 1,25(OH)2D3 activation, VDR bound to this VDRE leading to recruitment of DRIP205 and RNA polymerase II and histone 4 acetylation. Treatment of mice with a vitamin D analog induced nephrin mRNA and protein in the kidney, accompanied by increased VDR binding to the −312VDRE and histone 4 acetylation. 1,25(OH)2D3 reversed high glucose-induced nephrin reduction in podocytes, and vitamin D analogs prevented nephrin decline in both type 1 and 2 diabetic mice. Together these data demonstrate that 1,25(OH)2D3 stimulates nephrin expression in podocytes by acting on a VDRE in the proximal nephrin promoter. Nephrin up-regulation likely accounts for part of the renoprotective activity of vitamin D.

In the process of glomerular filtration in the kidney, proteins and other large molecules are kept from being filtered into the urine by the glomerular filtration barrier. The barrier has three layers: the fenestrated endothelium, the glomerular basement membrane, and the podocytes, whose foot processes interdigitate on the glomerular basement membrane and form the slit diaphragm that functions as the major size-selective and charge-selective barrier to protein leakage (1). The slit diaphragm is a highly specialized gap junction formed by several proteins. Nephrin is an important slit diaphragm protein produced by podocytes (2). This protein has a short intracellular domain, a transmembrane domain, and an extracellular domain with eight distal IgG-like motifs and one proximal fibronectin type III-like motif. Nephrin molecules interact with one another in a homophilic fashion, and it is thought nephrin molecules from adjacent foot processes interact in the middle of the slit to form a filtering structure (1). Genetic mutations of the nephrin gene result in congenital nephrotic syndrome of the Finnish type (or nephrotic syndrome type 1), characterized by massive proteinuria at or shortly after birth (3), and inactivation of the nephrin gene in the mouse causes massive proteinuria, absence of a slit diaphragm, and neonatal death (4). Nephrin levels are reduced in kidney disease such as diabetic nephropathy (5). Thus nephrin plays a key role in maintaining the integrity of the slit diaphragm.

Vitamin D deficiency is a prominent feature of chronic kidney disease (6). Vitamin D insufficiency is associated with increased prevalence of albuminuria in the general population (7). The anti-proteinuric activity of vitamin D analogs has been confirmed in a number of recent randomized clinical trials in diabetic patients with chronic kidney disease (8, 9). Studies with various animal models of kidney disease have well established the renoprotective property of vitamin D (10). For example, we reported that vitamin D receptor (VDR)−−null mice developed earlier and more robust albuminuria in diabetic state (11), and vitamin D analog therapy markedly reduced albuminuria and prevented podocyte loss in experimental models of type 1 and type 2 diabetes (5, 12, 13). Because VDR is highly inducible in podocytes (14), it is speculated that podocytes are main renoprotective target of vitamin D (15). One potential renoprotective mechanism of vitamin D is to regulate podocyte proteins involved in the formation of the slit diaphragm. In fact, nephrin expression is stimulated by 1,25-dihydroxyvitamin D (1,25(OH)2D3), the hormonal form of vitamin D, in cultured podocytes (16, 17). Thus nephrin may be an anti-proteinuric target of vitamin D in podocytes. The molecular mechanism whereby 1,25(OH)2D3 regulates nephrin expression, however, remains to be defined.

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1 To whom correspondence may be addressed: Ministry of Health Key Laboratory of Congenital Malformation, Shengjing Hospital, China Medical University, 36 Sanhao St., Heping District, Shenyang, China. Tel.: 0086-24-23261169; E-mail: cyan@medicine.bsd.uchicago.edu.
2 To whom correspondence may be addressed: Dept. of Medicine, The University of Chicago, 900 E. 57th St., KCB9-9110, Chicago, IL 60637. Tel.: 773-702-2477; Fax: 773-702-2281; E-mail: rezh2001@yahoo.com.
3 The abbreviations used are: VDR, vitamin D receptor; VDRE, vitamin D response element; 1,25(OH)2D3, 1,25-dihydroxyvitamin D; RXR, retinoid X receptor; DR, direct repeat; Dox, doxercalciferol; H4, histone 4.
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VDR, a member of the nuclear receptor superfamily, mediates the biological activities of 1,25(OH)2D3 (18). Once activated by 1,25(OH)2D3, VDR translocates into the nucleus and heterodimerizes with retinoid X receptor (RXR), and the VDR-RXR heterodimer binds to vitamin D response elements (VDREs) in the regulatory region of target genes to regulate gene expression. VDREs are typically composed of two hexameric half site direct repeats (DRs) separated by 3 or 4 nucleotides (DR3 or DR4), or exerted repeats spaced by 7–9 nucleotides (19–21). It is believed that VDR-RXR binding to VDRE leads to recruitment of co-activators and modification of the chromatin structure, allowing for RNA polymerase II entry to drive transcription (22). In this study, we explored the molecular mechanism whereby 1,25(OH)2D3 up-regulates nephrin gene expression in podocytes. Our data demonstrate that the up-regulation of nephrin by 1,25(OH)2D3 is mediated by a VDRE in the proximal nephrin gene promoter.

MATERIALS AND METHODS

Podocyte Culture—Immortalized podocyte line (23) was maintained in collagen-coated flasks at the permissive temperature (33 °C) in RPMI 1640 media supplemented with 10% FBS and 50 units/ml γ-interferon. The cells were differentiated at 37 °C without γ-interferon (non-permissive condition) in low glucose (5 mM) media for 2 weeks. The cells were synchronized in 1% FBS for 24 h before being cultured in low glucose or high glucose (30 mM) DMEM medium in the presence or absence of 1,25(OH)2D3 (20 nM).

RT-PCR—Total cellular RNAs were extracted using TRIzol reagents (Invitrogen). First strand cDNAs were synthesized using Moleney murine reverse transcriptase (Invitrogen) and hexanucleotide random primers. The cDNA template was amplified by PCR using specific primers. Quantitative RT-PCR was carried out using the LightCycler 480 real-time PCR system (Roche Applies Science). β2-Microglobulin or GAPDH was used as an internal control. The following primers were used in this study: nephrin primers: 5′-TACCCGTCGGAGCACAAA-3′ (forward) and 5′-GAGGATATCTCCGCCGAGCCAGGGAA-3′ (reverse). The PCR product was then cloned into the KpnI-NcoI sites of the pGL3-Luc vector (Promega, Madison, WI). Mutations in the mouse nephrin gene promoter were PCR-amplified using primers 5′-CGGGGTACCCGTCGGAGCACAAA-3′ (forward) and 5′-GAGGATATCTCCGCCGAGCCAGGGAA-3′ (reverse). The PCR product was then cloned into the KpnI-NcoI sites of the pGL3-Luc vector (Promega, Madison, WI). Mutations in the mouse nephrin gene promoter were PCR-amplified using primers 5′-CGGGGTACCCGTCGGAGCACAAA-3′ (forward) and 5′-GAGGATATCTCCGCCGAGCCAGGGAA-3′ (reverse). The PCR product was then cloned into the KpnI-NcoI sites of the pGL3-Luc vector (Promega, Madison, WI).

ChIP Assays—Podocytes in different treatments specified in each experiment were subject to ChIP assays according to previously published methods (25, 26), using antibodies specified in each experiment. PCR amplification was performed using primers specified in each experiment. Antibodies used in ChIP assays were purchased from the following sources: VDR and DRIP205 (Trap220) from Santa Cruz Biotechnology, RNA polymerase II from Millipore, and H4K8Ac and H4K16Ac from Cell Signaling.

ChIP Scan of Nephrin Gene Promoter—Putative VDREs within the 6-kb 5′ upstream region of the mouse nephrin gene were identified by in silico analyses (NUSIScan version 2.0). Seventeen pairs of PCR primers were designed to cover the 6-kb 5′ upstream sequence of the nephrin gene. The primer sequences are available upon request. Podocytes were treated with ethanol or 1,25(OH)2D3 (20 nM) for 15 h and then subject to ChIP assays using anti-VDR antibodies (Santa Cruz Biotechnology). The precipitated genomic DNA was amplified by PCR using these 17 pairs of primers. PCR products were resolved on 2% agarose gels and visualized with ethidium bromide staining.

Western Blot—Protein concentrations were determined using a Bio-Rad Protein Assay Kit. Proteins were separated by SDS-PAGE and transferred onto Immobilon membranes. Western blotting was carried out as described previously (24). The antibody against nephrin was obtained from Fitzgerald (Acton, MA).

ChIP Assays—Podocytes in different treatments or glomeruli freshly isolated from mice were subject to ChIP assays according to previously published methods (25, 26), using antibodies specified in each experiment. PCR amplification was performed using primers specified in each experiment. Antibodies used in ChIP assays were purchased from the following sources: VDR and DRIP205 (Trap220) from Santa Cruz Biotechnology, RNA polymerase II from Millipore, and H4K8Ac and H4K16Ac from Cell Signaling.

EMSA—Nuclear extracts were prepared from podocytes in different treatments specified in each experiment. EMSAs were performed according to previously published methods (25, 26), using 32P-labeled probes, together with cold competition probes or antibodies, as specified in each experiment.

Luciferase Reporter Assays—To construct reporter plasmids, a DNA fragment from −427 to +173 in the mouse nephrin gene promoter was PCR-amplified using primers 5′-CGGGGTACCCGTCGGAGCACAAA-3′ (forward) and 5′-GAGGATATCTCCGCCGAGCCAGGGAA-3′ (reverse). The PCR product was then cloned into the KpnI-NcoI sites of the pGL3-Luc vector (Promega, Madison, WI). Mutations in the −312VDR were achieved via PCR-based site-directed mutagenesis, and the mutated sequences were confirmed by DNA sequencing. HEK293 cells were transfected with the luciferase reporter plasmids using Lipofectamine 2000 (Invitrogen), with or without co-transfection with pcDNA3 or pcDNA-hVDR plasmid. After 24 h, the cells were treated with or without 1,25(OH)2D3. Luciferase activity was determined using the Luciferase Assay Systems (Promega) as described before (26). To confirm the role of VDR, in some experiments VDR were silenced using commercial VDR siRNA from Dharmacon before performing luciferase reporter assays.

Analysis of Glomeruli—Podocytes were treated daily by intraperitoneal injection for 7 days with vehicle (60% propylene glycol) or doxercalciferol (Dox, 200 ng/kg), a low calcemic vitamin D analog provided by Genzyme. Mice were then sacrificed, and glomeruli were purified from kidneys according to a previously published method with some modifications (27). Briefly, anesthetized mice were perfused with 0.25% (0.25 g/100 ml) iron oxide beads (Fisher Scientific) in PBS instead of deactivated M-450 Dynabeads. The perfused kidneys were cut into small pieces and ground on a 100-mesh sterile stainless sieve, and the bead-trapping glomeruli were collected with a magnetic bar. The experiments in which streptozotocin-induced diabetic mice or db/db mice were treated with vehicle or vitamin D analogs Dox or paricalcitol (provided by Abbott Laboratories) were described previously (12, 13). In the present study only kidney RNAs from these previous experiments were used for nephrin quantitation. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Chicago.

Statistical Analysis—Data values were presented as means ± S.E. Statistical comparisons were made using Student’s t test, with p ≤ 0.05 being considered significant.
RESULTS

We first assessed the effect of 1,25(OH)2D3 on nephrin expression in the podocyte culture. As shown in Fig. 1, 24-h treatment with 1,25(OH)2D3 markedly induced nephrin at mRNA (Fig. 1A) and protein (Fig. 1B) levels, and the stimulatory effect of 1,25(OH)2D3 on nephrin expression was dose-dependent (Fig. 1D). Interestingly, 1,25(OH)2D3 also dose dependently induced VDR levels in podocytes (Fig. 1C). These data suggest that VDR mediates the stimulatory effect of 1,25(OH)2D3 on nephrin. We speculated that the induction is mediated by VDREs in the nephrin gene promoter.

To test this hypothesis, we first carried out in silico analyses of the 6-kb 5′ upstream sequence of the mouse nephrin gene and identified 14 putative VDREs (Fig. 2A). We further employed a ChIP scan strategy to functionally confirm VDREs in this region. We designed 17 pairs of PCR primers to cover this 6-kb DNA fragment (Fig. 2A). Then ChIP assays were performed with anti-VDR antibodies in podocytes treated with ethanol or 1,25(OH)2D3 for 24 h, and the VDR antibody-precipitated genomic DNA was amplified by PCR using these 17 pairs of primers (Fig. 2B). As shown in Fig. 2, primer pairs 2, 3, and 9, which cover the putative VDREs at positions −312, −451, −1112, and −3155, respectively (Fig. 2A), amplified DNA fragments that were induced after 1,25(OH)2D3 treatment (Fig. 2B), suggesting that the putative VDREs at these positions might interact with VDR in podocytes. To validate the ability of these...
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FIGURE 3. Characterization of the VDRE by EMSA. A, competitive EMSA. Nuclear extracts isolated from 1,25(OH)₂D₃-stimulated podocytes were incubated with [³²P]-labeled −312VDRE probe (lane 3–7) or DR3VDRE probe (lane 2 and lanes 8–10), in the presence of 100-fold unlabeled (Cold) wild-type (WT) or mutant (Mut) −312VDRE probe as indicated. Note WT −312VDRE probe, but not Mut −312VDRE probe, was able to compete off both the −312VDRE and DR3VDRE probes. B, 1,25(OH)₂D₃-stimulated podocyte nuclear extracts were incubated with [³²P]-labeled DR3VDRE or −312VDRE probe in the presence (+) or absence (−) of anti-VDR antibodies as indicated. Note both probes produced a supershifted band as a result of antibody incubation. C, control reaction without nuclear extracts.

VDREs to interact with VDR, we performed competitive EMSAs (Fig. 2, C and D). Nuclear extracts prepared from 1,25(OH)₂D₃-treated podocytes were incubated with a [³²P]-labeled double-stranded DNA probe containing a well characterized DR3-type VDRE (DR3VDRE, 5'-AGCTTCAGGTCAAGGAGGTACAGCT-3'; the half sites are underlined) (28), in the presence of an excess amount (100× molar ratio) of one unlabeled probe each corresponding to a putative VDRE identified in the nephrin promoter (Fig. 2A). As shown in Fig. 2C, the interaction of nuclear proteins with the DR3VDRE probe could be competed off by itself (lane 2) and by probes 2-1 and 9 (lanes 4 and 9), respectively, but not by the other probes (Fig. 2C). In the presence of anti-VDR antibody, the DR3VDRE-proteins complex was supershifted (Fig. 2D, lane 2), validating the binding of VDR to the DR3VDRE. Again, this complex was competed off by probe 2-1 or 9, but not by probe 2-2. These data strongly suggest that the VDREs at −312 and −3155 interact with VDR.

In the following studies, we focused on the −312VDRE, as it is within the proximal nephrin gene promoter. This VDRE carries the sequence AGCTTCAGGTCAAGGAGGTACAGCT-3', that has two direct hexameric half site repeats separated by four nucleotides. Thus it is a DR4-type VDRE. To validate the function of this VDRE, we further studied it by EMSAs, together with the DR3VDRE. As shown in Fig. 3A, similar DNA-protein complex shift patterns were seen using [³²P]-labeled DR3VDRE probe or −312VDRE probe (5'-GCAGAATGAGTTCACACTGGGTC-CAAGC-3', i.e. probe 2-1) (lanes 2 and 3). The complex formed by either the −312VDRE or the DR3VDRE could be competed off by an excess amount of (100×) unlabeled −312VDRE probe (lanes 5 and 9) but not by its mutant form that carries a mutant sequence (AacTtACACTGccTcc) within the VDRE half sites (lanes 7 and 10). In the presence of anti-VDR antibodies, the DNA-protein complex was supershifted for both probes (Fig. 3B, lanes 2 and 4). These data confirm the VDR-binding property of the −312VDRE in the proximal nephrin promoter.

We then performed luciferase reporter assays to assess the activity of the −312VDRE in response to 1,25(OH)₂D₃ treatment. Luciferase reporter plasmids were generated in pGL3-Luc vector using a 5’ DNA fragment (from −427 to +173) within the proximal nephrin gene promoter that contains either the −312VDRE (WT-Luc) or its mutant sequence (Mut-Luc) (Fig. 4A). When HEK293 cells were co-transfected with the WT-Luc reporter and pcDNA3, luciferase activity was markedly induced (~2-fold) by 1,25(OH)₂D₃; co-transfection with pcDNA-hVDR resulted in further induction of luciferase activity (~5-fold) by 1,25(OH)₂D₃ (Fig. 4B). The induction of luciferase activity by 1,25(OH)₂D₃, however, was completely abolished using the Mut-Luc reporter construct, regardless of co-transfection with pcDNA3 or pcDNA-hVDR (Fig. 4B). Conversely, in HEK293 cells transfected with the WT-Luc reporter, 1,25(OH)₂D₃ induction of the luciferase activity was abolished when the endogenous VDR was knocked down by VDR-specific siRNA.
siRNA, but the induction of the activity was not affected in the control cells treated with scramble siRNA (Fig. 4, C and D). These functional data suggest that the −312VDRE mediates, at least in part, 1,25(OH)2D3 induction of nephrin expression, and this process is VDR-dependent.

We further used ChIP assays to assess the changes of DNA-protein interactions taking place on the −312VDRE in podocytes in response to 1,25(OH)2D3 treatment. As shown in Fig. 5, primers were designed to flank the −312VDRE site and the transcription start site for PCR detection of genomic DNA precipitated with various antibodies (Fig. 5A). Precipitated DNA was amplified using primer set 1 (VDR, DRIP205, H4K8Ac, and H4K16Ac) or primer set 2 (RNA Pol, Primer set 1 sequences are 5′-TGGGGAAGCAGCAGAA-3′ (forward) and 5′-CCTCCAGACCTAAATGCC-3′ (reverse), and set 2 sequences are 5′-AGGGGAGGGAGGGAGGAAGAGAA-3′ (forward) and 5′-TCCGCGAGCAGCAGGGAA-3′ (reverse). Inp, input; IgG, non-immune IgG control. C, quantitation of 1,25(OH)2D3-induced acetylation of H4 by ChIP assays. Podocytes were treated with EtOH or 1,25(OH)2D3 for 6, 12, and 24 h before ChIP assays with anti-H4K8Ac antibodies. ***, p < 0.001 versus corresponding EtOH.

To assess vitamin D regulation of nephrin in vivo, we treated mice with Dox for 5 days and analyzed the glomeruli. As shown in Fig. 6, Dox treatment markedly induced glomerular VDR and nephrin expression at mRNA and protein levels (Fig. 6, A–C). ChIP assays showed that Dox treatment also induced VDR binding and H4 acetylation on the −312VDRE in the glomeruli isolated from Dox-treated mice (Fig. 6, D and E). Conversely, in VDR-null mice, we detected significant reduction of baseline nephrin expression in the kidney compared with wild-type mice (Fig. 6, F and G).

We also assessed whether vitamin D was able to prevent nephrin down-regulation caused by hyperglycemia. In podocytes, high glucose markedly reduced nephrin mRNA expression; however, in the presence of 1,25(OH)2D3, high glucose-induced decline of nephrin was reversed (Fig. 7A). Similarly, ChIP assays showed that high glucose reduced VDR binding to the −312VDRE and H4 acetylation. **, p < 0.01 versus V; F and G, effect of VDR ablation on basal nephrin levels. Regular (F) and real-time RT-PCR (G) show marked reduction of nephren mRNA expression in VDR-null (VDRKO) mice relative to wild-type (WT) mice. ***, p < 0.001 versus WT; n = 3–5 mice in each group.

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FIGURE 5. DNA and protein interactions assessed by ChIP assays. A, schematic illustration of the −312VDRE in the nephrin gene promoter and the positions of PCR primers used for the ChIP assays. B, ChIP assays. Podocytes were treated with EtOH or 1,25(OH)2D3 (1,25VD) as indicated, and ChIP assays were performed using antibodies against VDR, DRIP205, RNA polymerase II (RNA Pol), H4K8Ac, or H4K16Ac as indicated. Precipitated DNA was amplified using primer set 1 (VDR, DRIP205, H4K8Ac, and H4K16Ac) or primer set 2 (RNA Pol). Primer set 1 sequences are 5′-TGGGGAAGCAGCAGAA-3′ (forward) and 5′-CCTCCAGACCTAAATGCC-3′ (reverse), and set 2 sequences are 5′-AGGGGAGGGAGGGAGGAAGAGAA-3′ (forward) and 5′-TCCGCGAGCAGCAGGGAA-3′ (reverse). Inp, input; IgG, non-immune IgG control. C, quantitation of 1,25(OH)2D3-induced acetylation of H4 by ChIP assays. Podocytes were treated with EtOH or 1,25(OH)2D3 for 6, 12, and 24 h before ChIP assays with anti-H4K8Ac antibodies. ***, p < 0.001 versus corresponding EtOH.

FIGURE 6. In vivo analysis of nephrin up-regulation in response to vitamin D analog treatment. Male BDA/2J mice were treated with vehicle (V) or doxercaliferol (Dox, intraperitoneal, 200 ng/kg) for 5 days. Glomeruli were isolated from the kidney for analyses. A, real-time RT-PCR quantitation shows VDR and nephrin up-regulated after Dox treatment; B, regular RT-PCR demonstrates up-regulation of nephrin by Dox treatment in isolated glomeruli; C, Western blot shows dramatic induction of nephrin protein by Dox treatment in glomerular lysates. D and E, ChIP analyses of glomeruli isolated from V- or Dox-treated mice, using antibodies against VDR (D) or H4K8Ac (E). The ChIP PCR primers flanking the −312VDRE is shown in Fig. 5A. Note Dox markedly increases VDR binding to the −312VDRE and H4 acetylation. **, p < 0.01 versus V; F and G, effect of VDR ablation on basal nephrin levels. Regular (F) and real-time RT-PCR (G) show marked reduction of nephrin mRNA expression in VDR-null (VDRKO) mice relative to wild-type (WT) mice. ***, p < 0.001 versus WT; n = 3–5 mice in each group.

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**FIGURE 7. Effects of vitamin D analogs on hyperglycemia-induced nephrin decline.** A and B, cultured podocytes were exposed to low glucose (LG) or high glucose (HG) in the presence or absence of 1,25(OH)2D3 (VD) for 24 h. Nephrin mRNA levels were quantified by real-time RT-PCR (A), and VDR recruitment to the −312VDRE in the nephrin promoter was assessed by ChIP assays (B). **, *p < 0.01;***, p < 0.001 versus corresponding control. C and D, streptozotocin (STZ)-induced diabetic mice and db/db mice were treated with vehicle (V) or a vitamin D analog (doxercalciferol for streptozotocin mice, and paricalcitol for db/db mice). Nephrin mRNA levels were assessed by regular RT-PCR (C) or real-time RT-PCR (D). **, *p < 0.01 versus the rest. n = 3–5 in each group. Diab, diabetes.

**DISCUSSION**

Albuminuria is a hallmark of kidney injury and thought to be the first step in the progression to proteinuria and renal failure (31, 32). Therefore, reduction of albuminuria is a major target for renoprotective therapy. Epidemiological data from the Third National Health and Nutrition Examination Survey have revealed a correlation between vitamin D insufficiency and increased prevalence of albuminuria in the U.S. adult population (7), suggesting an intrinsic anti-proteinuric activity for vitamin D. Furthermore, a number of recent prospective randomized clinical trials have demonstrated significant anti-proteinuric effects of vitamin D analogs in diabetes patients (8, 9). Given the importance of podocytes in the regulation of glomerular filtration, it is speculated that vitamin D targets podocytes to reduce proteinuria (15). The present study provides evidence to support this hypothesis.

Nephrin is synthesized by podocytes in the kidney that plays a key role in maintaining the structure and function of the slit diaphragm. Although vitamin D up-regulation of nephrin has been reported previously (16, 17), the molecular mechanism remains unknown. In the present study we demonstrated that 1,25(OH)2D3 up-regulates nephrin expression through a VDRE in the proximal promoter of the nephrin gene. By *in silico* analysis and ChIP scanning of the 6-kb 5′ upstream sequence of the nephrin gene we identified a number of putative VDREs in the nephrin gene promoter, and two of these VDREs at −312 and −3155 were validated by EMSAs. We further demonstrated that the −312VDRE, a DR4-type VDRE, was able to interact with VDR in vitro (by EMSAs) and in podocytes (by ChIP assays) and to functionally respond to 1,25(OH)2D3 stimulation (by luciferase reporter assays). Mutations in the half sites of this VDRE abolished these activities, providing compelling evidence for the functionality of this cis-regulatory element.

ChIP assay data showed that, in the presence of 1,25(OH)2D3, the interaction of VDR, DRIP205, and RNA polymerase II with the −312VDRE and the transcription start site was enhanced, so was the acetylation of H4 in this promoter region (Fig. 5). It is well known that VDR interacts with co-activators with the C-terminal AF2 domain. Liganded VDR can recruit SCR-1 and DRIP205, and it is believed that these two co-activators are recruited in a sequential and mutually exclusive manner. SCR-1, which has intrinsic histone acetyltransferase activity, is recruited earlier to remodel the chromatin, allowing the following recruitment of DRIP complex and the RNA polymerase II complex to promote gene transcription (22). Based on the ChIP data we speculate that these are probably the molecular events that take place around the −312VDRE and the proximal nephrin promoter in the presence of 1,25(OH)2D3 stimulation, leading to the up-regulation of nephrin. It is possible that other cis-regulatory elements, such as the putative −3155VDRE at the distal nephrin gene promoter, are also involved in VDR-mediated nephrin induction in podocytes. We did not pursue the −3155VDRE partly because we could not find a conserved VDRE core around that region. Further studies are required to clarify the potential role of the cis-elements, if any, around that region. In an early report nephrin expression was found to be maximally induced by a combination of 1,25(OH)2D3, all-trans-retinoic acid, and dexamethasone in cultured podocytes (17), while each of these hormones alone was able to induce nephrin to a lesser extent (16). Given the molecular mechanism underlying 1,25(OH)2D3 stimulation of nephrin that is elucidated in this study, the interplay between VDR, retinoic acid receptor, and glucocorticoid receptor in the regulation of nephrin would be an interesting and important subject.

We presented animal data to confirm vitamin D-induced up-regulation of nephrin *in vivo*. Treatment of mice with a vitamin D analog stimulated nephrin at both mRNA and protein levels in the glomeruli, and, as seen in *in vitro* podocyte culture, this is accompanied by an up-regulation of VDR, consistent with the idea that VDR mediates the biological effect of vitamin D on nephrin regulation. Importantly, by ChIP assays we also detected increased VDR binding to the −312VDRE and increased H4 acetylation in the proximal nephrin promoter in the glomeruli isolated from vitamin D analog-treated mice. These observations indicate that the molecular events detected in cultured podocytes likely occur in the mouse kidney. Furthermore, vitamin D analogs were able to prevent the decline of...
nephrin expression in both type 1 and 2 diabetic mouse models, together with alleviation of diabetic kidney damage in these mice (12, 13). The animal data have important physiological relevance with regard to the renoprotective mechanism of vitamin D and its analogs. In this regard, although a number of clinical studies have demonstrated potent anti-proteinuric activity for vitamin D and its analogs, there is little data in the literature regarding the relationship between vitamin D and nephrin in human kidneys. Thus it is important to confirm whether vitamin D up-regulates nephrin by a similar mechanism in humans.

In summary, in this study we presented evidence that a DR4-type VDRE in the proximal promoter of nephrin gene mediates, at least in part, the up-regulation of nephrin expression induced by 1,25(OH)2D3. Binding of this VDRE leads to chromatin remodeling and recruitment of DRIP complex and RNA polymerase II, resulting in increased transcription of the nephrin gene. This study unveils a molecular basis of nephrin up-regulation that likely accounts for part of the renoprotective mechanism of vitamin D and its analogs in vivo. This regulatory mechanism is worth being validated in future clinical studies with human patients.

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