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Identification of the vitamin D receptor in various cells of the mouse kidney

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The kidney is the major, if not sole, site for the production of 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the biologically active form of vitamin D that can stimulate calcium reabsorption in the kidney and may provide renoprotective benefits. The biological effects of $1,25(OH)_2D_3$ are mediated through a nuclear hormone receptor, known as the vitamin D receptor (VDR). It is well accepted that the VDR is present in the distal renal convoluted tubule cells; however, whether VDR is present in other kidney cell types is uncertain. Using a highly specific and sensitive anti-VDR antibody, we determined its distribution in the mouse kidney by immunohistochemistry. Our results show that the VDR is not only present in the distal but is also found in the proximal tubules, but at 24-fold lower levels. The VDR was also found in the macula densa of the juxtaglomerular apparatus, glomerular parietal epithelial cells, and podocytes. In contrast, the VDR is either very low or absent in interstitial fibroblasts, glomerular mesangial cells, and juxtaglomerular cells. Thus, identification of VDR in the proximal tubule, macula densa, and podocytes suggests that $1,25(OH)_2D_3$ plays a direct role in these cells under normal conditions.

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The primary functions of the kidney include waste removal, maintenance of homeostatic functions, such as calcium and phosphate and electrolyte regulation and acid–base balance.[1] Renal functions are performed within nephrons, the basic structural and functional units of the kidney. Each nephron is composed of a glomerulus and a number of structurally and functionally different renal tubules, including the proximal tubule, loop of Henle, and the distal tubule. Collecting ducts are not part of the nephron but connect with the distal tubule through connecting tubule, a part of the distal tubule. 1α, 25-Dihydroxyvitamin D_3 regulates calcium reabsorption in the distal tubules and collecting ducts of the kidney.[2–4] The role of 1α, 25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) in the active reabsorption of calcium in the distal tubule is important for the maintenance of serum calcium when calcium supply is low.[4]

The kidney is also a major site for vitamin D metabolism. 25-Hydroxyvitamin D_3 ($25(OH)D_3$) is converted into the biologically active $1,25(OH)_2D_3$, by 25-(OH)-vitamin D_3 1α-hydroxylase. 1α-Hydroxylase is a cytochrome P450 enzyme localized in both convoluted and straight proximal tubules.[5–7] Both $25(OH)D_3$ and $1,25(OH)_2D_3$ are metabolized by 25-$(OH)D_3$ 24-hydroxylase,[8] which is mainly localized in the proximal tubules,[7,9] resulting in their degradation. $1,25(OH)_2D_3$ can suppress 1α-hydroxylase gene transcription in the proximal tubule and activate 24-hydroxlase gene transcription. The actions of $1,25(OH)_2D_3$ in the regulation of 1α- and 24-hydroxylase genes are critical for maintaining the calcium homeostasis.[10]

Besides the regulation of calcium reabsorption and vitamin D hormone production, $1,25(OH)_2D_3$ can also suppress the production of renin.[11] Renin is a circulating proteinase produced by juxtaglomerular cells of the juxtaglomerular apparatus (JGA) in adult animals and human. This hormone involves the renin–angiotensin system and regulates blood pressure. Using renin gene promoter, which is restricted to the juxtaglomerular cells of the JGA in adult animals and human, Dr Li's group has showed that selective overexpression of human vitamin D receptor (VDR) in the juxtaglomerular cells either in the wild-type or the receptor knockout mice can significantly reduce the production of renin.[12] This inhibitory effect is independent of parathyroid hormone and calcium, suggesting a direct role of the receptor in the regulation of renin production. However, all groups

including ours using autoradiography, *in situ* hybridization, and immunohistochemical staining failed to detect *in vivo* the expression of VDR in the juxtaglomerular cells.[12–16]

In addition, several studies using animal models have shown that $1,25(OH)_2D_3$ exerts a renoprotective function in several chronic kidney diseases. The hormone seems to protect the podocytes and prevent proteinuria.[17–22] The cellular and molecular mechanisms of $1,25(OH)_2D_3$-mediated renoprotective actions remain largely unknown.

The actions of $1,25(OH)_2D_3$ are mediated through binding with its nuclear receptor, VDR. The receptor is a ligand-activated transcription factor that interacts with coregulators and transcriptional complexes to alter the rate of target gene transcription.[3,23] Ligand activation of the VDR promotes adequate calcium reabsorption in the distal renal tubules and collecting ducts by regulating the expression of several calcium transport proteins in the kidney.[3,24] The upregulation of calcium-binding proteins, such as calbindin D_{28k} (CaBP-D_{28k}) and D_{9k} (CaBP-D_{9k}),[25] and calcium transporters, such as TRVP5 and TRVP6,[26,27] are believed to have a role in calcium reabsorption in the distal renal tubules. Additionally, the genes involved in vitamin D metabolism in the kidney are known to respond to $1,25(OH)_2D_3$.[3] For example, the 24-hydroxylase gene transcription is activated by $1,25(OH)_2D_3$, while 1α-hydroxylase gene transcription is suppressed by $1,25(OH)_2D_3$.[10] Consequently, identification of VDR within renal structures is important for understanding the mechanism of $1,25(OH)_2D_3$ in these functions.

Currently, the segment-specific distribution of VDR is inconclusive. During the last three decades, multiple assays including autoradiography, polymerase chain reaction/quantitative polymerase chain reaction, *in situ* hybridization, and/or immunohistochemistry have been used to determine the distribution of VDR in the kidney. These studies demonstrate that VDR is present in distal renal tubules.[9,13,14,16,28–34] In the proximal tubules, there is evidence to support both the presence[9,14,29,30,34] as well as the absence of VDR expression.[16,28,32,33] More recently, immunohistochemical studies using a rabbit polyclonal antibody, C-20, showed the absence of VDR in proximal tubules of normal human kidneys.[28] In addition to the uncertainty regarding VDR expression within the proximal tubules, the presence of VDR in the JGA and glomerulus requires confirmative study, using a highly specific and sensitive method.

The antibody to VDR is essential for determination of the receptor in a target. We have previously characterized a set of VDR antibodies (nine antibodies) from different sources, including the widely used antibodies such as rat monoclonal 9A7 and rabbit polyclonal antibody C-20,[35] and successfully applied the selected antibody to address if the VDR is present in the muscle.[36] The sensitivity and specificity of these antibodies were carefully characterized by multiple immunological assays, such as immunoblotting, immunocytochemistry, and immunohistochemistry. Most importantly, we included samples from Demay VDR knockout mice, which have no native or truncated VDR. Using this negative control, we were able to learn the specificity of each antibody. In addition, comparing the intensity of the VDR band, we were able to learn the sensitivity of each antibody to the receptor. We concluded that the D-6 VDR antibody is highly specific and sensitive. Even more relevant to the questions regarding the expression of the receptor in certain segments of the kidney, the D-6 antibody selectively reacts with the VDR in whole-kidney lysates of wild-type mice. This antibody does not react with whole-renal lysate from the Demay VDR knockout mice. Thus, the D-6 antibody shows no nonspecific binding in any renal tissue. In contrast, other antibodies, such as C-20 and 9A7, bind to proteins in the lysates from the Demay mice.

Using the highly specific and sensitive antibody to VDR identified previously,[35] the presence of VDR in the proximal renal tubule has been clearly confirmed. In addition, the VDR is clearly found in the glomerular parietal epithelial cells, podocytes, and the macula densa of the JGA. In contrast, the interstitial fibroblasts, intraglomerular mesangial cells, and juxtaglomerular cells either do not contain VDR or have undetectable low levels.

RESULTS

VDR is present in mouse proximal tubules

To address if the VDR is present in the proximal renal tubules, glomeruli, and JGA, we used immunohistochemistry employing a well-characterized D-6 VDR antibody.[35] We first differentiated the proximal tubules from the distal tubules, and cortical and medullary collecting ducts. The kidney sections were stained with molecular markers, including E-cadherin and CaBP-D_{28k}, for identifying the distal tubules and collecting ducts. E-cadherin is highly expressed in the distal tubules and (cortical) collecting ducts, and is absent or expressed at a low level in the proximal tubules.[37] Therefore, the CaBP-D_{28k} and E-cadherin negative tubules denote the proximal tubules. As shown in Figure 1, the renal tubule with high levels of E-cadherin and CaBP-D_{28k} is the distal tubule, while the tubule with a high level of E-cadherin and with a low level of CaBP-D_{28k} is the cortical collecting duct or the medullary collecting duct. The tubules lacking both E-cadherin and CaBP-D_{28k} in the cortical region are the proximal tubules.

The spatial relationship between VDR and CaBP-D_{28k} can be used to identify the distal tubules and collecting ducts. The VDR and CaBP-D_{28k} costaining showed that VDR is specifically expressed in distal tubules in the renal cortex and medulla (Figure 2). The expression of VDR was very low in collecting ducts in the medulla. Analysis of the staining intensities of VDR and CaBP-D_{28k} indicated that their expression levels were highly correlated and colocalized (Figure 2).

Both VDR and E-cadherin were highly expressed and colocalized within the distal tubules (Figure 3). The cortical collecting ducts (strong E-cadherin-positive staining in the cortex) and medullary collecting ducts highly expressed

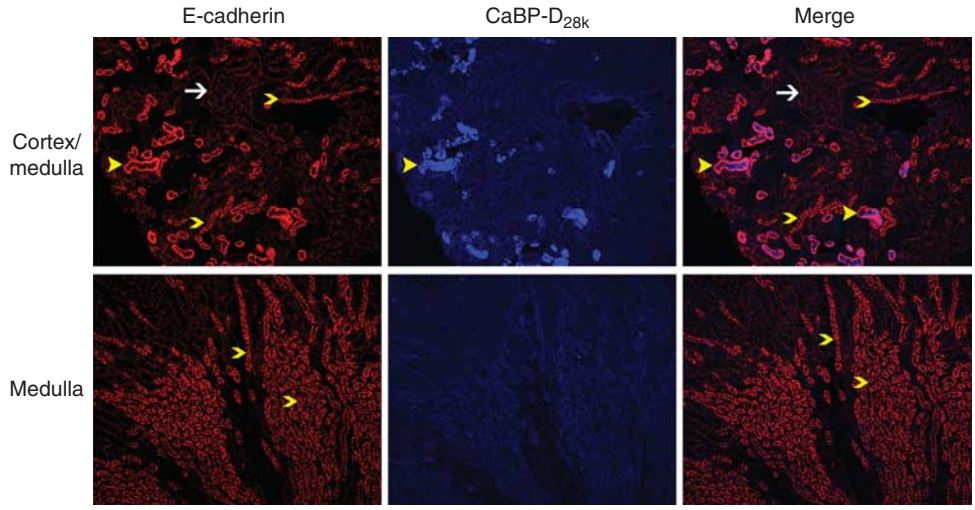

Figure 1 | Colocalization of E-cadherin (red) and CaBP-D_{28k} (blue) in mouse kidney (original magnification $\times$ 100). Distal renal tubule (yellow broad arrowhead) expressed the highest levels of CaBP-D_{28k} and E-cadherin (upper panel). Cortical collecting duct (yellow arrowhead) expressed E-cadherin with a level comparable to that in the collecting ducts (yellow arrowhead denoted) in the medulla, suggesting those were the cortical collecting ducts (upper and lower panels). Please note that the structures that exhibited the low levels of E-cadherin and CaBP-D_{28k} were the proximal renal tubules (white arrow denoted, upper panel).

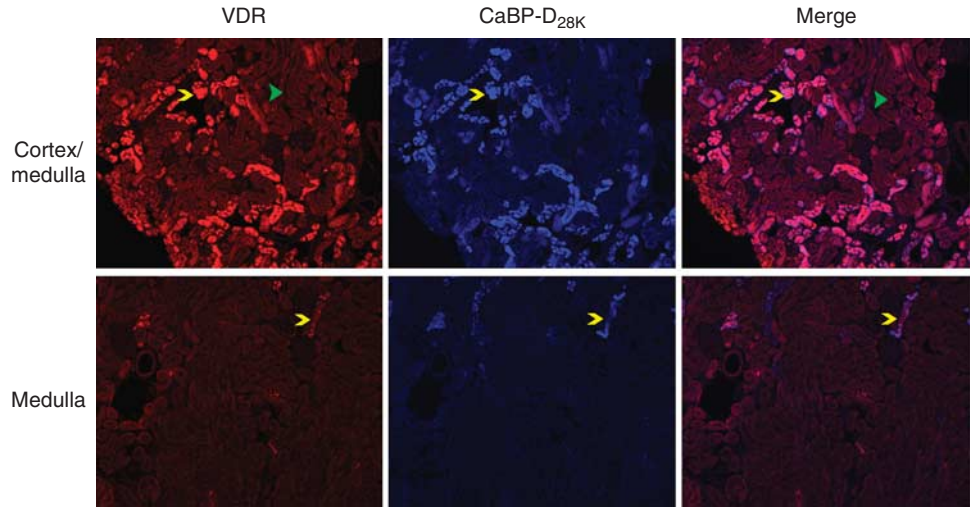

Figure 2 | Colocalization of vitamin D receptor (VDR) (red) and CaBP-D_{28k} (blue) in the cortex (upper panel) and the medulla (lower panel) of mouse kidney (original magnification $\times$ 100). The distal renal tubules (yellow arrowhead) contained the highest level of VDR and CaBP-D_{28k}. Collecting ducts (lower panel) in the medulla show a low level of CaBP-D_{28k}. Potential proximal renal tubules were denoted by green broad arrowheads. Lack of VDR signals in renal tubules such as the proximal tubules and collecting ducts might be attributable to the loss of antibody incurred with sequential washings required for double staining protocol (Materials and Methods section).

E-cadherin but expressed VDR at lower levels. In contrast, the proximal tubules were negative or extremely weak for the E-cadherin staining. Interestingly, these tubules were positive for VDR but its levels were low (Figure 3, middle and lower panels).

We found that double staining could alter the results of VDR antibody staining, such as losing weak immunosignals from the proximal tubules and collecting ducts. To avoid potential interference, we stained kidney sections using only VDR antibody to accurately assess VDR expression in kidney. We also used the kidney sections from Demay VDR knockout mice as proper negative controls to insure specific staining because these mice express neither native VDR nor truncated VDR protein.[38] The staining showed that VDR was present in the entire renal tubules located in the cortex and medulla of the wild-type mouse kidney (Figures 4a–g). The distal tubules expressed VDR at higher levels and were mainly located within the cortex of the kidney. Levels of VDR in the distal tubules varied from tubule to tubule. The collecting ducts in the medulla were weakly stained. Besides these tubules, the majority of the renal tubules in the cortex were weakly stained. These tubules were likely the proximal tubules and cortical collecting ducts. In contrast to the renal tubular epithelial cells, VDR staining was not detected in the renal

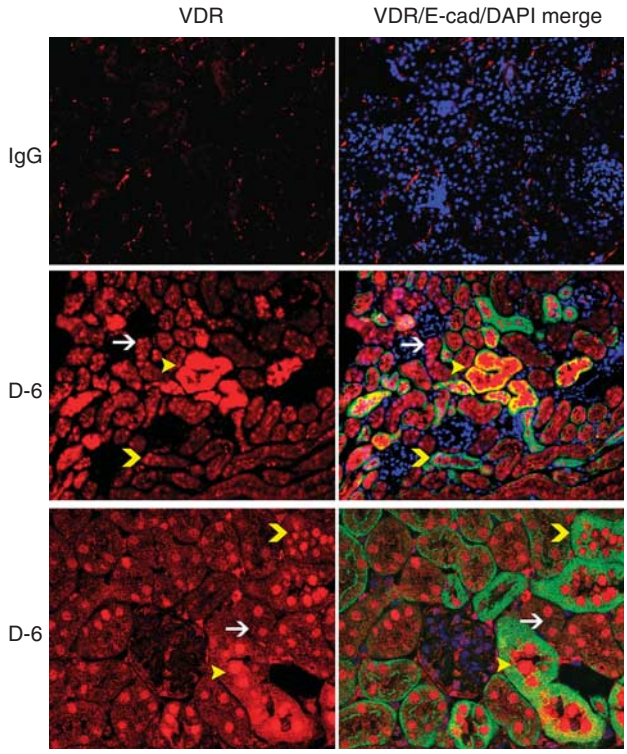

Figure 3 | Colocalization of vitamin D receptor (VDR) (red) and E-cadherin (green) in mouse kidney (original magnification × 100 or × 600). There was weak staining when mouse IgG isotypes were applied to the samples (upper panel). However, the staining was not found in the cell nucleus. The cell nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI) (blue). Distal renal tubule (yellow broad arrowhead) expressed the highest levels of VDR and E-cadherin (middle panel). Cortical collecting duct (yellow arrowhead) expressed E-cadherin and VDR with a relatively low level compared with the distal renal tubules (middle panel). Renal tubule lacking E-cadherin staining was the proximal renal tubule (white arrow). The lower panel (original magnification × 600) is confocal images of mouse renal tubules. Distal renal tubule was denoted by the yellow broad arrow, cortical collecting duct by the yellow arrowhead, and the proximal renal tubule by the white arrow.

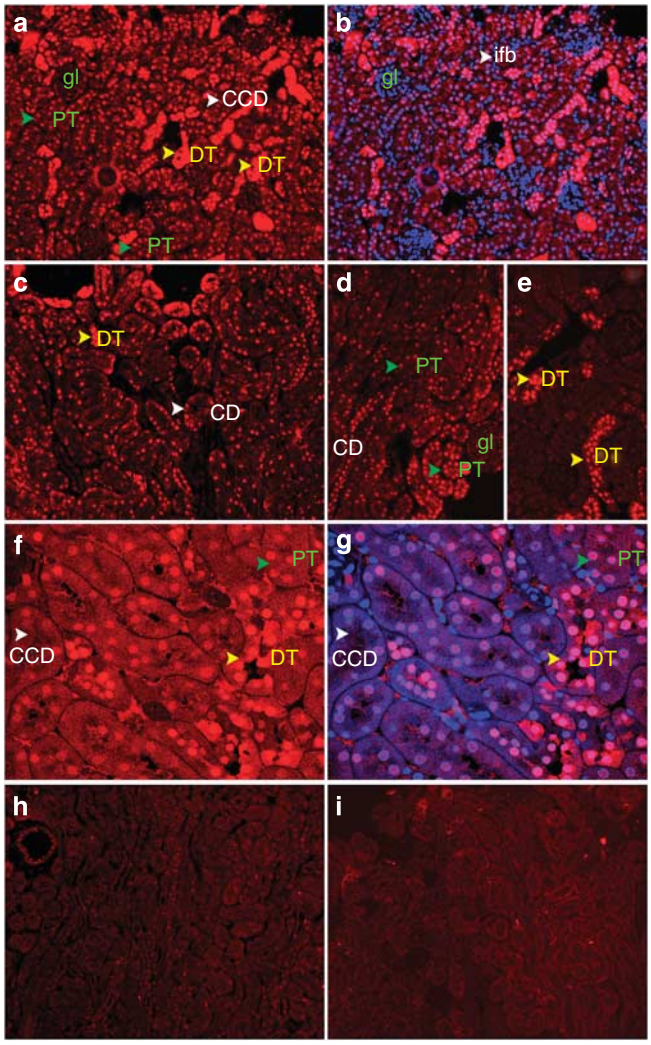

Figure 4 | The distribution of vitamin D receptor (VDR) in the mouse kidney (original magnification × 200 or × 600). (**a**) Distal renal tubules (DT, yellow broad arrowhead) of normal adult kidneys highly expressed VDR (red) but proximal renal tubules (PT, green arrowhead) and cortical collecting ducts (CCD, white broad arrowhead) expressed VDR with a low level. Glomerulus is denoted as gl. (**b**) VDR (red) was colocalized with 4′,6-diamidino-2-phenylindole (DAPI) (blue). This colocalization resulted in purple color in (**b**). Interstitial fibroblasts (ifb, white broad arrowhead) were located between the renal tubules, and their nuclei were stained by DAPI but not stained with the VDR antibody in (**a**). (**c**) Collecting ducts (CD, white broad arrowhead) in the medulla were positive for VDR. (**d**) Renal tubules with weak VDR staining in the cortex. The representative proximal tubules were denoted by the green broad arrowhead. (**e**) VDR was located in the distal tubule (DT, yellow broad arrowhead). The image was taken with the reduced exposure time compared with the image in (**a**). The images with less exposure showed that the VDR in the distal tubule was located in the nuclei of the tubule epithelial cells (**e**). (**f**, **g**) Confocal images of mouse kidney cortex sections stained for VDR and DAPI. The distal tubule was denoted with the yellow arrowhead and proximal tubule with the green arrowhead. (**h**) VDR antibody staining in the kidney sections of Demay VDR knockout mice served as negative controls. (**i**) Mouse isotype IgG did not generate significant immunostaining in the wild-type mouse kidney sections.

interstitial fibroblasts, located between renal tubules (Figures 4a, b, f, and g). There were also no significant signals in sections of the Demay VDR knockout kidneys (Figures 4h and i), and in the sections from wild-type mice stained only with the secondary antibody and mouse isotype IgG (Figure 3, upper panel). Together, these results demonstrate that the VDR immunosignals in wild-type mouse kidney sections were highly specific.

The relative contents of VDR in these segments were measured in a semiquantitative manner using Image J software to quantitate the intensity of immunostaining in the nuclei of the renal tubular epithelial cells as described in the Materials and Methods section. Our results showed that VDR expression in the distal tubules was 24-fold higher than the proximal tubules. Differences between distal and proximal tubules ranged between 4- and 69-fold (Table 1). These results demonstrated that the VDR expression was low

Table 1 | Relative levels of VDR expression in mouse renal distal tubule and proximal tubule

Type of tubules	Distal tubule	Proximal tubule	Average fold	Range
VDR intensity	7745 ± 5994	320 ± 121	24	4–69

Abbreviation: VDR, vitamin D receptor.
N=5 mice.

in proximal tubules and further indicated that the D-6 antibody was extremely sensitive and highly specific.

The VDR is highly expressed in distal tubules of the human kidney and is present in the proximal tubules and collecting ducts (Figure 5a and b). Parietal epithelial cells of glomerulus were also positive for VDR staining (Figures 5c and d). Noticeably, the VDR signal was not found in nuclei of the VDR-positive cells. In contrast, the hnRNP, a nuclear protein, which is normally localized in the nucleus, was found in the nuclei of human renal cells (Figure 5e), suggesting the nucleus–cytoplasm relocation of VDR in the human kidney. The reasons for the relocation were not clear. However, a number of factors, such as vitamin D status and tissue processing method, could alter the subcellular location of the receptor.[38,39]

VDR is present in mouse kidney glomerular podocytes

The glomerulus contains several cell types including glomerular capsule (parietal epithelial cells), parietal and visceral podocytes, mesangial cells, and endothelial cells. We showed that the VDR immunosignals were present within the glomerulus from the wild-type mice (Figures 6a–g), but not from the kidney samples from the Demay VDR knockout mice (Figure 6c). The expression of VDR seemed to be lower in podocytes than that in the proximal tubular cells. The VDR-positive cells located in the capsule and visceral areas of the glomerulus were likely parietal epithelial cells and podocytes. The parietal epithelial cells and podocytes have the same embryonic origin, but Wilms' tumor-1 (WT-1) is only present in podocytes.[40] Costaining of kidney sections with the antibodies to VDR and WT-1 showed that VDR colocalized with WT-1, thereby demonstrating that these were podocytes (Figures 6f and g). Our results also suggest that VDR is very low or not found in the other cell types such as intraglomerular mesangial and endothelial cells.

VDR is present in mouse kidney juxtaglomerular macula densa but is not found in granular cells

JGA is located at the vascular pole of each glomerulus.[41] We addressed if VDR was present in the juxtaglomerular cells and macula densa cells of JGA. Our results showed that the VDR was highly expressed in the macula densa (Figure 6a, b, d, and e). In contrast, the juxtaglomerular cells were not stained by the VDR antibody (Figures 6d and e). To specify the juxtaglomerular cells (smooth muscle origin), we first costained the glomeruli using antibodies to WT-1 and α-smooth muscle actin (αSMA), which is a marker for

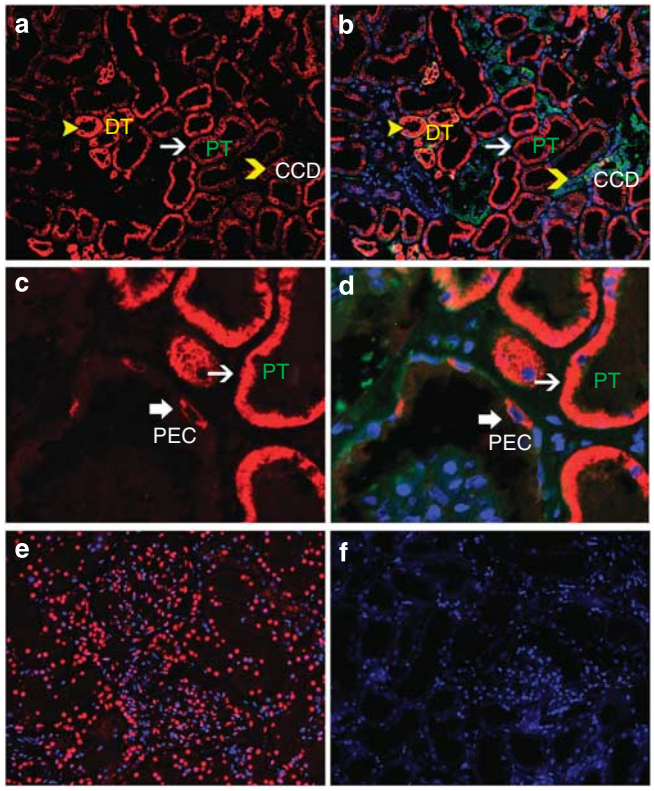

Figure 5 | Colocalization of vitamin D receptor (VDR) (red) and E-cadherin (green) in normal human kidney cortex (original magnification × 200). (**a**, **b**) Human kidney cortex sections were stained for VDR and E-cadherin. Distal renal tubule (yellow broad arrowhead) expressed the highest levels of VDR, E-cadherin, and 4′,6-diamidino-2-phenylindole (DAPI). Cortical collecting duct (yellow arrowhead) expressed E-cadherin and VDR with a relatively low level compared with the distal renal tubules. Renal tubule lacking E-cadherin staining was the proximal renal tubule (white arrow). (**c**, **d**) Digitally zoomed in images. Proximal tubule (PT, white arrows) had no E-cadherin but contained VDR. Parietal epithelial cells (PEC, white broad arrows) expressed VDR as well. Please note that the VDR immunosignals were localized at the cytoplasm of the renal cells. (**e**) Human kidney cortex sections were stained for hnRNP, a nuclear protein. Please note that hnRNP was localized at the nuclei of renal cells. (**f**) There was no staining when mouse IgG isotypes were applied to the samples, indicating the secondary antibody did not react with human IgG. The cell nuclei were stained by DAPI (blue).

juxtaglomerular cells (Figures 6h and i). The juxtaglomerular cells strongly expressed αSMA, while the intraglomerular mesangial cells and some interstitial fibroblasts expressed αSMA at lower levels. Importantly, the αSMA staining was not colocalized with WT-1 staining within the JGA. Therefore, the αSMA-positive intraglomerular mesangial and juxtaglomerular cells clearly lacked WT-1 (Figure 6i) as well as VDR (Figure 6g).

To further confirm the undetectable level of VDR in the juxtaglomerular cells, we costained the glomeruli using antibodies to VDR and renin (Figure 7). Renin is another specific marker for juxtaglomerular cells. The juxtaglomerular cells strongly expressed renin, while the intraglomerular mesangial cells and podocytes do not. After evaluating the

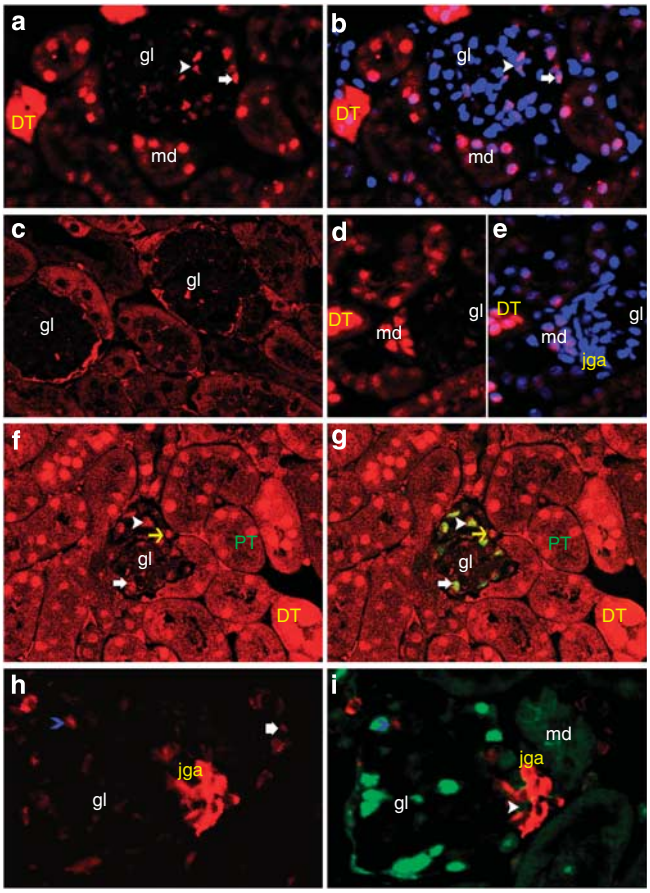

Figure 6 | Vitamin D receptor (VDR) in mouse glomerulus and juxtaglomerular apparatus (JGA) (original magnification × 200 or × 600; a–e and h–i were digitally enlarged). (**a**) VDR was present in the glomerular parietal (white broad arrow) and visceral (white broad arrowhead) podocytes, macula densa (md), and distal renal tubule (DT). The VDR staining was depicted by red fluorescent color. (**b**) Colocalization of VDR (red) and nucleus (4′,6-diamidino-2-phenylindole (DAPI) staining, blue) in mouse glomerulus. The VDR/DAPI merge resulted in purple color. (**c**) VDR staining in the glomerulus (gl) of Demay VDR knockout mouse kidney. (**d**) JGA of the wild-type mouse kidney. VDR was present in the cells of macula densa (md) and distal renal tubule (DT). (**e**) Colocalization of VDR (red) and DAPI (blue) staining in the JGA of the wild-type mouse kidney. VDR was not found in the juxtaglomerular cells (jga) but present in the macula densa (md) and DT. (**f**) A confocal image showed the VDR staining (red) in the glomerular parietal epithelial cells (yellow arrow) and parietal (white broad arrow) and visceral (white broad arrowhead) podocytes. (**g**) A confocal image showed the colocalization of Wilms' tumor-1 (green) and VDR (red) in the glomerulus. VDR/DAPI merge resulted in yellow color. (**h**) Staining of mouse kidney sections with antibody to α smooth muscle actin (αSMA, red). The juxtaglomerular cells (jga) strongly expressed αSMA whereas the intraglomerular mesangial cells (blue arrowhead) or interstitial fibroblasts (white broad arrow) expressed αSMA with low abundance. (**i**) The αSMA staining was not colocalized with the WT-1 staining (green) (white broad arrowhead).

immunostaining of the wild-type and Demay VDR knockout mouse samples by D-6 antibody and mouse isotype IgG (Figures 7a–h), we concluded that the D-6 antibody is highly specific. The immunohistochemical staining of the wild-type samples showed that the VDR is present in the renal tubular cells. The VDR was also found in some intraglomerular cells, which were likely the podocytes. Interestingly, some tubular cells and glomerular podocytes contained extremely low levels of VDR, showing a speckle-like staining in their nuclei (Figures 7e and f). In contrast, the VDR staining was clearly not found in the nuclei of juxtaglomerular cells, which were positive for the renin staining (Figures 7e–h).

DISCUSSION

Prior to the availability of antibodies to the VDR, a radiolabeled 1,25-$(OH)_2D_3$ was shown to localize in the nuclei of the distal tubule cells of the kidney but not in the proximal tubule. However, VDR protein and transcript were detected in proximal tubule cells by binding assay[31] or by RT-polymerase chain reaction/quantitative polymerase chain reaction,[9,30] and *in situ* hybridization.[29] Using rat monoclonal antibody 9A7 against chick VDR[14,29] or polyclonal antibodies against human VDR,[13] three groups have found VDR in the proximal renal tubules. However, these results differed from the findings of Liu *et al.*[28] in which a rabbit polyclonal antibody, C-20, failed to reveal VDR in human proximal tubules. These inconsistencies may be related to the sensitivity, specificity, and selectivity of the VDR antibodies. For example, rat monoclonal antibody 9A7 and rabbit polyclonal antibody C-20 not only bind to VDR but also nonspecifically to unknown proteins, especially in kidney tissues.[35,42]

When we used the highly specific and sensitive antibody D-6, VDR densely populated the distal tubules but also was found at low levels in the proximal tubules in both mouse (Figure 4) and human (Figure 5). The VDR levels in mouse proximal tubules are 24-fold lower than that in the distal tubules (Table 1). The low abundance of VDR in the proximal tubule by immunohistochemistry would require VDR antibodies with high sensitivity, a property not possessed by the C-20 antibody.

VDR was detected previously in renal glomerular podocytes using autoradiography.[16] VDR messenger RNA in laser-dissected glomerular segments was detected, but the cell types were not specified.[30] Kumar *et al.*[13] found that VDR is expressed only in the parietal epithelial cells of the glomerulus by immunohistochemistry using rabbit polyclonal antibody against human VDR. However, 1,25$(OH)_2D_3$ protects podocytes and prevents proteinuria in the animal models of chronic kidney disease.[17–19] Podocytes interact with glomerular basement membranes and form a tight interdigitating network of foot processes (pedicels) that control the filtration of proteins from the capillary lumen into Bowman's space. Podocyte injury in several chronic kidney diseases results in impaired ultrafiltration and proteinuria.[17,43] The identification of VDR in podocytes suggests that they are a target for 1,25$(OH)_2D_3$.

Transcription of the renin gene is suppressed by 1,25$(OH)_2D_3$, and VDR knockout mice exhibit hyperreninemia. Thus, the juxtaglomerular cells are proposed as the

target of $1,25(OH)_2D_3$.[11] This finding has not been confirmed in patients with hereditary vitamin D-resistance rickets type II (HVDRR).[44] HVDRR type II is caused by mutations of the VDR gene. This study showed that there was no significant increase in renin. Most significant is these patients do not have hypertension. Recent reviews also conclude that an association between vitamin D status and hypertension is still uncertain.[44–46] Another recent study failed to find any significant relation between plasma 25-hydroxyvitamin D and the RAS in 184 normotensive individuals.[47]

It is believed that JGA in the adult is a main site of renin production. The JGA is composed of two types of specialized cells, including the cells of macula densa and juxtaglomerular cells. If the vitamin D hormone has a direct effect on the renin production in these cells, the VDR must be present. Using an extremely sensitive autoradiographic technique, VDR was not detected in rat juxtaglomerular cells, while it was clearly found in the macula densa and glomerular prodocytes.[6] This was supported by Koike *et al.*[15] who failed to show the nuclear localization of a radioactive vitamin D analog in the juxtaglomerular cells while finding such localization clearly in podocytes and macula densa. Using the rat monoclonal antibody 9A7 and/or *in situ* hybridization, VDR was not detected in human,[14] rat, and pig[29] glomeruli including the juxtaglomerular cells while VDR was found in the renal tubular cells. Similarly, another group failed to detect the VDR in juxtaglomerular cells while finding it in podocytes and renal tubular cells.[13] VDR transgene expression in the JGA using immunohistochemical staining was reported.[12] However, they did not report VDR in the JGA of the wild type.

As the juxtaglomerular cells share origins with smooth muscle cells but not renal epithelial cells, parietal epithelial cells, and podocytes, the absence of VDR might not be unexpected because smooth muscle cells do not contain VDR.[32] However, our results do not exclude the possibility of expression of VDR in the juxtaglomerular cells during a disease process.

Interestingly, we demonstrated that VDR is present in macula densa (Figures 6a and d). The cells of macula densa

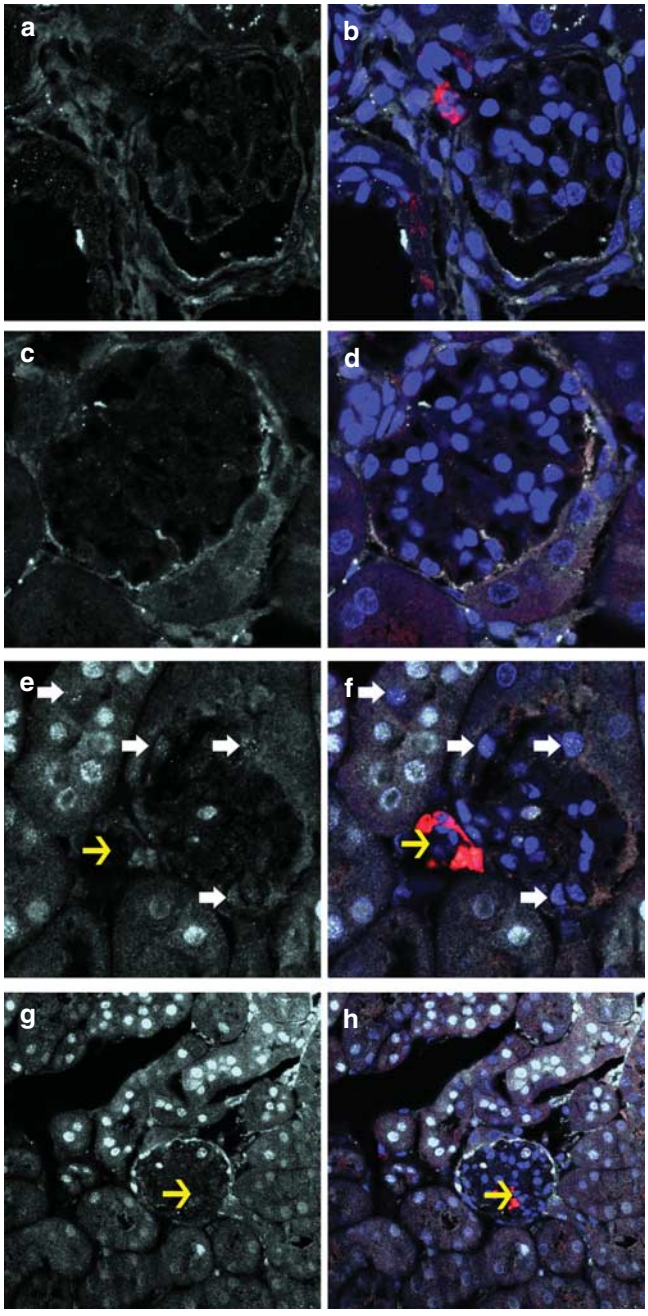

Figure 7 | Vitamin D receptor (VDR) in mouse glomerulus and juxtaglomerular apparatus (original magnification $\times$ 600; a–d were digitally enlarged to original magnification $\times$ 1200). The left panels (white color) are the VDR antibody staining while the right panels are the colocalization for VDR, renin (red), and 4′,6-diamidino-2-phenylindole (blue). (**a**, **b**) The VDR and renin staining in the glomerulus of Demay VDR knockout mouse kidney. The VDR staining (white color) is not seen in the nuclei of renal cells. The renin staining (red color) is selectively seen in the juxtaglomerular cells. Note that some white staining could be seen in the cytoplasm of the proximal renal tubular cells or the outside of renal cells. The VDR knockout kidney samples stained with only the secondary antibody showed a similar pattern (not shown), suggesting that it is most likely the staining for mouse endogenous IgG. The IgG could be detected in the proximal tubular cells because of protein and peptide reabsorption at this particular site. (**c**, **d**) Omission of the primary VDR and renin antibodies resulted in the absence of the primary antibody-specific staining in the nuclei of kidney samples from wild-type mice. (**e**, **f**) VDR and renin staining in the glomerulus of wild-type mouse kidney. The VDR staining (white color) is seen in the nuclei of renal tubular cells and glomerular podocytes. The cells with a low level of VDR are indicated by using white broad arrows; the renin staining (red color) is selectively seen in the juxtaglomerular cells. The VDR staining is not seen in the nuclei of juxtaglomerular cells (yellow arrow). (**g**, **h**) The VDR and renin staining in the glomerulus of wild-type mouse kidney. The VDR staining (white color) is seen in the nuclei of renal tubular cells and glomerular podocytes. The VDR staining is not seen in the nuclei of juxtaglomerular cells (yellow arrow). Note that the interstitial fibroblasts are not stained by either VDR or renin antibodies.

share the same origin with the distal tubule during the nephron development.[1] Our results not only confirm our previous finding of the presence of VDR in macula densa using autoradiography,[16] but also suggests the idea that $1,25(OH)_2D_3$ regulates renin production through the macula densa. It has been known that the cells of the macula densa are sensitive to the ionic content and water volume of the fluid in the tubule. If a low water volume is detected by these cells, they may signal renin secretion and production by juxtaglomerular cells. The VDR may serve to signal a suppression of renin secretion and production.

MATERIALS AND METHODS

Antibodies

Mouse anti-VDR (D-6), renin, anti-hnRNP A2B1 (DP313B), rabbit (anti-WT-1) (C-19), and goat anti-CaBP-D_{28k} antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-E-cadherin antibody was purchased from Cell Signaling Technology (Danvers, MA). Mouse anti-αSMA was purchased from Sigma (St Louis, MO). 4′,6-Diamidino-2-phenylindole, Alexa Fluor 594 goat anti-mouse-IgG, Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 594 donkey anti-mouse IgG, Alexa Fluor 647 donkey anti-mouse IgG, Alexa Fluor 594 donkey anti-rabbit IgG, Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 594 donkey anti-goat IgG, or Alexa Fluor 350 donkey anti-goat IgG secondary antibodies were purchased from Invitrogen (Carlsbad, CA).

Animals and maintenance

The C57BL/6 mice and Demay VDR knockout mice[48] were purchased from The Jackson Laboratory (West Grove, PA). The animals were maintained and scarified as described previously.[35] The 6–7-week-old wild-type and knockout female littermates were used for each experiment. Kidneys were collected and chemically fixed. Experimental protocols were reviewed and approved by the Animal Care and Use Committee (University of Wisconsin, Madison, WI).

Normal human kidney cortex sections

The paraffin-embedded human samples were purchased from US Biomax (Rockville, MD). The donor was a 38-year-old female.

The tissues were chemically fixed with formalin and were embedded with paraffin.

Immunohistochemistry

We employed a highly specific and extremely sensitive immunohistochemical assay using the anti-VDR antibody D-6, as described previously.[35] Images were captured using a fluorescence microscope (Nikon Inverted Microscope ECLIPSE TE2000-U) or confocal fluorescence microscope (Nikon A1R high-speed confocal microscope) (Nikon Instruments, Melville, NJ).

Double staining

For slides costained with VDR and CaBP-D_{28k}, following application of the VDR primary antibody, the sections were further treated with the second primary antibodies (CaBP-D_{28k}) at an optimal concentration (20 μg/ml) overnight at 4°C. For the slides costained with E-cadherin and CaBP-D_{28k}, the sections were treated with the rabbit anti-E-cadherin antibody (1:50 dilution) for 1 h at 37°C and then with goat anti-CaBP-D_{28k} antibody overnight. For the slides costained with VDR and E-cadherin, the sections were treated with the E-cadherin and VDR antibodies for 1 h at 37°C. For the slides costained with VDR and WT-1 antibodies, the sections were treated with the VDR and WT-1 (4 μg/ml) antibodies for 1 h at 37°C. For the slides costained with anti-αSMA (1:200 dilution) and WT1 antibodies, the sections were treated with the mouse anti-αSMA and rabbit anti-WT-1 antibodies for 1 h at 37°C. The secondary antibodies conjugated with an Alex flour fluorescent dye were used to visualize immunosignals as described previously.[35] Images were sequentially captured using a fluorescence microscope (Nikon Inverted Microscope ECLIPSE TE2000-U) or confocal fluorescence microscope (Nikon A1R high-speed confocal microscope) (Nikon Instruments).

Quantification of VDR staining in renal distal and proximal tubules using immunohistochemistry

Immunoreactivity was evaluated in a semiquantitative manner by analyzing nuclear staining intensity of renal tubular epithelial cells using the Image J Program (NIH, Bethesda, MD). As the VDR immunosignal strength in the distal tubules was strong but much weaker in the proximal tubules, we captured sample images with either short- or long-time exposures. Short-time exposures (400 ms) were used to measure high levels of immunostaining intensities in the segments such as the distal tubules, and long exposures (800 ms) were used for the weaker signals in the proximal tubules. The images with low exposure were used to quantify positive VDR staining in the distal tubules and the images with high exposure were used to quantify VDR staining within the proximal tubules. In order to compare the values of the distal tubules with the proximal tubules, the averaged values of 400 ms immunosignals in the distal tubule ($V_{dt/400}$) were proportionally converted to that obtained at 800 ms ($V_{dt/800}$). Thirty cells with intermediate intensity were used to determine ratios calculated by average intensity at 800 ms (V_{800}) divided by the intensity at 400 ms (V_{400}) ($Ratio_{800/400} = V_{800}/V_{400}$). The final values of VDR in the distal tubules were obtained by multiplying the value of VDR staining intensity at 400 ms with the ratio of the 800 ms to 400 ms ($V_{dt/800} = V_{dt/400} \times Ratio_{800/400}$). The calculated values represented the average of more than 50 cells from either the distal or proximal renal tubules.

DISCLOSURE

All the authors declared no competing interests.

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