RESEARCH ARTICLE

RUNNING HEAD: Vasohibin-2 peptide vaccine for diabetic nephropathy

Preventive effects of vasohibin-2-targeting peptide vaccine for diabetic nephropathy

Yuri Nakashima,[1] Katsuyuki Tanabe,[1] Tomoyo Mifune,[1] Takato Nakadoi,[1] Hiroki Hayashi,[2] Hironori Nakagami,[2] Yasufumi Sato,[3] and Jun Wada[1]

[1] Department of Nephrology, Rheumatology, Endocrinology and Metabolism, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan
[2] Department of Health Development and Medicine, Osaka University Graduate School of Medicine, Osaka, Japan
[3] New Industry Creation Hatchery Center, Tohoku University, Sendai, Japan

Correspondence: *Katsuyuki Tanabe (tanabek@okayama-u.ac.jp).*

ABSTRACT

Diabetic nephropathy remains the leading cause of end-stage kidney disease in many countries, and additional therapeutic targets are needed to prevent its development and progression. Some angiogenic factors are involved in the pathogenesis of diabetic nephropathy. Vasohibin-2 (VASH2) is a novel proangiogenic factor, and our previous study showed that glomerular damage is inhibited in diabetic *Vash2* homozygous knockout mice. Therefore, we established a VASH2-targeting peptide vaccine as a tool for anti-VASH2 therapy in diabetic nephropathy. In this study, the preventive effects of the VASH2-targeting peptide vaccine against glomerular injury were examined in a streptozotocin (STZ)-induced diabetic mouse model. The mice were subcutaneously injected with the vaccine at two doses 2 weeks apart and then intraperitoneally injected with 50 mg/kg STZ for 5 consecutive days. Glomerular injury was evaluated 20 weeks after the first vaccination. Treatment with the VASH2-targeting peptide vaccine successfully induced circulating anti-VASH2 antibody without inflammation in major organs. Although the vaccination did not affect blood glucose levels, it significantly prevented hyperglycemia-induced increases in urinary albumin excretion and glomerular volume. The vaccination did not affect increased VASH2 expression but significantly inhibited renal angiopoietin-2 (Angpt2) expression in the diabetic mice. Furthermore, it significantly prevented glomerular macrophage infiltration. The preventive effects of vaccination on glomerular injury were also confirmed in db/db mice. Taken together, the results of this study suggest that the VASH2-targeting peptide vaccine may prevent diabetic glomerular injury in mice by inhibiting Angpt2-mediated microinflammation.

NEW & NOTEWORTHY

This study demonstrated preventive effects of VASH2-targeting peptide vaccine therapy on albuminuria and glomerular microinflammation in STZ-induced diabetic mouse model by inhibiting renal Angpt2

expression. The vaccination was also effective in db/db mice. The results highlight the importance of VASH2 in the pathogenesis of early-stage diabetic nephropathy and the practicability of anti-VASH2 strategy as a vaccine therapy.

Keywords: Vasohibin-2; peptide vaccine; diabetic nephropathy; albuminuria; macrophages

INTRODUCTION

Diabetic nephropathy is a serious microvascular complication of persistent hyperglycemia and develops in approximately 40% of patients with diabetes(1). It remains the leading cause of end-stage kidney disease in most countries despite the availability of novel anti-diabetic agents in the past years. Recent studies have demonstrated that sodium–glucose transport protein-2 inhibitors, in addition to inhibiting renin–angiotensin–aldosterone system, successfully delay the progression of diabetic renal injury(2, 3). However, once the disease progresses to an advanced stage, it is difficult to reverse, highlighting the importance of early prevention of nephropathy in patients with diabetes.

Various angiogenic factors mediate the development and progression of diabetic nephropathy, making them candidate targets for the early prevention of nephropathy(4). Vascular endothelial growth factor (VEGF) is a potent proangiogenic factor involved in the formation of early-phase diabetic glomerular lesions. Specific anti-VEGF strategies can prevent albuminuria and glomerular lesions in diabetic animal models. However, anti-VEGF antibody treatment induces glomerular endothelial injury with massive proteinuria in patients with advanced cancer(5). Therefore, recent studies have focused on alternative angiogenic signaling pathways in diabetic nephropathy. Activation of angiopoietin-1 (Angpt1)-Tie2 receptor signaling(6) and inhibition of leucine-rich α2-glycoprotein-1-mediated proangiogenic transforming growth factor (TGF)-β signaling(7) prevent glomerular injury in diabetic animal models. Furthermore, diabetic glomerular damage and albuminuria are ameliorated in vasohibin-2 (VASH2)-deficient mice(8). VASH2 was originally identified as a homolog of the antiangiogenic factor vasohibin-1 (VASH1). However, VASH2 exhibits proangiogenic activity by preventing the termination of capillary sprouting(9). While normally differentiated cells express VASH2 at extremely low levels, cellular dedifferentiation such as cancer transformation upregulates VASH2 protein expression. In tumor tissues, VASH2 expression is associated with tumor growth and angiogenesis(10, 11). Renal VASH2 expression is upregulated in animal models of diabetic nephropathy and acute kidney injury(8, 12). Notably, *Vash2* homozygous knockout mice show no obvious phenotypes in contrast to VEGF(8). Thus, anti-VASH2 therapy may be a promising strategy for treating diseases related to abnormal angiogenesis.

Peptide vaccines have many potential benefits in preventing chronic diseases. In particular, vaccines can be mass-produced at a low cost and require infrequent administration, resulting in a substantial reduction in the healthcare financial burden for chronic diseases. However, vaccines against chronic diseases need to target self-antigens and not exogenous pathogens; hence, they must overcome immune tolerance systems and avoid autoimmune injury(13). Peptide-based vaccines targeting self-antigens induce the production of therapeutic neutralizing antibodies without cytotoxic T cell activation. For example, peptide vaccines that target angiotensin II, dipeptidyl peptidase-4, and proprotein convertase subtilisin/kexin-9 effectively and safely prevent hypertension, hyperglycemia, and hyperlipidemia in experimental animal models(14-16). Considering the anti-tumor effects of anti-VASH2

neutralizing monoclonal antibody(17), we established a novel peptide vaccine targeting the VASH2 N-terminal epitope as a tool for anti-VASH2 therapy(18).

We examined the preventive effects of the VASH2-targeting peptide vaccine against the progression of glomerular injury in a streptozotocin (STZ)-induced diabetic mouse model and obese db/db diabetic mouse model. The vaccination efficiently induced the production of circulating anti-VASH2 antibody and suppressed albuminuria and glomerular microinflammation in diabetic mice. Our results suggest the VASH2-targeting peptide vaccine as a promising preventive strategy against early-stage diabetic nephropathy.

MATERIALS AND METHODS

Preparation of VASH2 peptide vaccine

As the VASH2 epitope, the N-terminal 1–11 amino acid sequence that is conserved between mice and humans was selected, and the peptide was synthesized by Peptide Institute Inc. (Osaka, Japan), as previously described(18). Briefly, the VASH2 peptide was conjugated to keyhole limpet hemocyanin (KLH) as a carrier protein using the glutaraldehyde method, and the synthetic peptides were purified using reverse-phase high-performance liquid chromatography (>98% purity). The peptide vaccine was dissolved in saline with ultrasonication and mixed with an equal volume of complete (for first injection)/incomplete (for second injection) Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA) before immunization(19).

Animal experiment

C57BL/6J mice and db/db mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). The mice were fed standard pellet laboratory chow and provided with water *ad libitum* at the Department of Animal Resources of the Advanced Science Research Center at Okayama University (Okayama, Japan). Six-week-old male C57BL/6J mice were subcutaneously injected VASH2-targeting peptide vaccine mixed with Freund's adjuvant (50 µg of the synthetic peptide in 100 µL) at weeks 0 and 2. Two weeks later, diabetes was induced through the intraperitoneal injection of 50 mg/kg STZ (Sigma-Aldrich) dissolved in 0.1 M citrate buffer (pH 4.5) for 5 consecutive days as previously described(20). Non-diabetic control mice were only injected with citrate buffer. At week 8, STZ-treated mice without hyperglycemia (defined as a non-fasting blood glucose concentration >280 mg/dL) were excluded from subsequent experiments. Blood glucose levels at week 8 in mice included in this study are presented in Supplemental Table. Less than 10 µL of blood samples were collected from the tail vein every 4 weeks to determine VASH2 antibody titer. At week 16, 24 h urine samples were collected from the metabolic cages. At week 20, blood samples were collected from the inferior vena cava, and the kidneys and other organs were harvested. Finally, the subgroups included 6 non-diabetic mice without vaccination (NDM-V), 7 non-diabetic mice with vaccination (NDM+V), 9 diabetic mice without vaccination (DM-V), and 9 diabetic mice with vaccination (DM+V). Six-week-old male db/db mice received the VASh2-targeting peptide vaccine at weeks 0 and 2 in the same manner as described above. Vaccinated (db/db+V; n = 6) and unvaccinated (db/db-V; n = 6) mice were also compared. Urine samples were collected at weeks 2, 9, and 15, and the kidneys were harvested at week 16. The experimental protocols were approved by the Animal Care and Use Committee of Okayama University (approval no. OKU-2020598 and OKU-2020592).

Enzyme-linked immunosorbent assay

The VASH2 antibody titer in the serum was determined using enzyme-linked immunosorbent assay (ELISA) as previously described(21). Briefly, mouse VASH2 peptide (Peptide Institute Inc.) was coated on 96-well Immuno Plates (Thermo Fisher Scientific, Waltham, MA, USA). After the wells were blocked with phosphate buffered saline (PBS) containing 5% skim milk for 2 h, serum samples diluted 100-fold to 312,500-fold were placed on the wells and then incubated overnight at 4°C. The wells were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (GE Healthcare, Buckinghamshire, UK) for 3 h at room temperature. HRP-conjugated anti-mouse IgG1, IgG2a, IgG2b, and IgG2c antibodies (all purchased from Abcam, Cambridge, UK) were used to determine the antibody titers of IgG subclasses. After washing, the wells were incubated with 3,3',5,5'-tetramethyl benzidine (Sigma-Aldrich) as a chromogenic substrate for 30 min, and the reaction was terminated with 0.5 N sulfuric acid. The absorbance of each well was measured at 450 nm using an iMARK microplate reader (Biorad, Hercules, CA, USA). The antibody titer is expressed as dilution ratio with half-maximal binding.

Blood and urine examination and blood pressure measurement

Blood glucose levels in the venous blood samples were measured using a glucometer (Glutest Neo; Sanwa Kagaku, Nagoya, Japan). Serum and urine creatinine, urine albumin, blood urea nitrogen (BUN), and hemoglobin A1c (HbA1c) levels were measured by Oriental Yeast Co., Ltd. (Tokyo, Japan). Urine albumin excretion was normalized to urine creatinine concentration. Arterial blood pressure was measured using a programmable sphygmomanometer (BP-98A; Softron, Tokyo, Japan).

Histology and Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (4 μm thickness) were stained with periodic acid-Schiff (PAS) and Masson's trichrome for the kidneys and hematoxylin and eosin (HE) for the other organs. Thirty glomerular images were obtained at 200× magnification from each PAS-stained kidney section, and the areas surrounded by glomerular capillary tufts were measured using cellSens imaging software (Olympus, Tokyo, Japan) to determine the mean glomerular cross-sectional tuft area (G_A). The mean glomerular volume (G_V) was calculated as $G_V = \beta/k \times (G_A)^{3/2}$, where $\beta = 1.38$ is the shape coefficient for spheres, and $k = 1.1$ is the size distribution coefficient(22, 23). Mesangial and interstitial areas were quantified as aniline blue-stained areas inside and outside the glomeruli, respectively, on sections stained with Masson's trichrome (200× magnification) using cellSens imaging software. These areas were expressed as the percentage of blue-stained areas in the glomerular tuft and extra-glomerular field areas, respectively.

Paraffin-embedded kidney sections were deparaffinized and treated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase activity. Subsequently, sections were incubated overnight at 4°C with rat anti-Mac-2 (lectin galactoside-binding soluble 3) antibody (Cedarlane, Burlington, Ontario, Canada) or goat anti-tumor necrosis factor (TNF)-α antibody (R&D Systems, Minneapolis, MN, USA). After being washed with PBS, the sections were incubated with the immune-peroxidase polymer Histofine Simple Stain Mouse MAX PO (Rat) or Mouse MAX PO (G) (Nichirei Bioscience, Tokyo, Japan). ImmPACT DAB (Vector Laboratories, Burlingame, CA, USA) was used as the chromogen. Nuclei were counterstained with hematoxylin. The number of Mac-2-positive cells was counted at 400× magnification in at least 30 glomerular images per section(24).

Electron microscopy

Each kidney cortical tissue was fixed with 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, and then embedded in EPON epoxy resin. Ultra-thin sections were imaged using a transmission electron microscope (H-7650; Hitachi, Tokyo, Japan) at the Central Research Laboratory of Okayama University Medical School. In the magnified images, the widths of slit diaphragms, defined as the distances between neighboring foot processes, were measured using ImageJ software as previously described(23).

Immunoblotting

Recombinant mouse VASH2/small vasohibin-binding protein (SVBP) was provided by New Industry Creation Hatchery Center, Tohoku University (Sendai, Japan). Total protein was extracted from whole kidney tissue using RIPA lysis buffer (Santa Cruz Biotechnology, Dallas, TX, USA). The recombinant and extracted proteins were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and transferred onto nitrocellulose membranes using the iBlot2 dry blotting system (Thermo Fisher Scientific). Serum samples from vaccinated mice were diluted 1:250. Anti-mouse intercellular adhesion molecule-1 (ICAM-1; Proteintech, Rosemont, IL, USA), anti-angpt2 (Abcam), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Merck Millipore, Burlington, MA, USA) primary antibodies were used. Peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Cell Signaling Technology, Danvers, MA, USA) was used as secondary antibodies. Enzyme activity was detected using the ECL Western blotting detection kit (GE Healthcare), and images were obtained using ImageQuant LAS 4000 (GE Healthcare). The density of the bands was determined using ImageJ software.

Real-time polymerase chain reaction

RNA extraction from renal cortical tissues and cDNA preparation were performed using the RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA) and SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) in accordance with the manufacturers' protocols. The cDNA was added to Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) using specific oligonucleotide primers, as shown in Table 1. Quantitative real-time polymerase chain reaction (PCR) was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems). The amount of PCR product was normalized to *Actb* mRNA levels.

Statistical analysis

All values are expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with *post-hoc* comparisons using Tukey's test was employed for intergroup comparisons and *t*-test was employed for two-group comparisons using JMP version 17 software (SAS Institute Inc, Cary, NC, USA). Statistical significance was considered at $P < 0.05$.

RESULTS

Induction of circulating anti-VASH2 antibody by treatment with the VASH2-targeting peptide vaccine

The VASH2 peptide vaccine was subcutaneously injected twice at an interval of 2 weeks. As shown in Figure 1*A*, the vaccination successfully induced serum anti-VASH2 antibody titers >1:10,000. The VASH2 antibody titer peaked at 8 and 12 weeks after the first injection in the non-diabetic and diabetic mice, respectively. In both non-diabetic and diabetic mice, an average VASH2 antibody titer of >1:10,000 was maintained for 20 weeks. Immunoblot assay results demonstrated that the VASH2 antibody induced in the blood by the vaccination can directly bind to mouse VASH2 protein (Fig. 1*B*). Furthermore, IgG

subclass analysis showed that the anti-VASH2 antibody in the blood was predominantly IgG1 rather than IgG2 in both non-diabetic and diabetic mice (Fig. 1, *C* and *D*), suggesting that the vaccination caused a Th2-dominant immune response. The vaccine-induced immune response did not cause recognizable inflammatory infiltration in any major organs, including the kidney, heart, lung, liver, pancreas, or aorta (Fig. 2, *A*–*F*). No TNF-α-positive cells were observed in these major organs compared with in the positive control of normal spleen (Supplemental Fig. S1).

Effects of VASH2-targeting peptide vaccine on diabetic glomerular alterations

Compared with the non-diabetic mice, the STZ-treated diabetic mice showed significantly lower body weight and significantly higher blood glucose and HbA1c levels (Fig. 3, *A*–*C*). Notably, the vaccination did not affect the body weights, blood glucose levels, and HbA1c levels in both non-diabetic and diabetic mice (Fig. 3, *A*–*C*). Furthermore, no significant differences in systolic blood pressure were found among the four groups (NDM-V; 119.2 ± 5.6, NDM+V; 117.8 ± 5.5, DM-V; 119.6 ± 10.0, DM+V; 123.9 ± 11.1 mmHg). BUN and serum creatinine levels were significantly higher in the diabetic mice than in the non-diabetic mice. The vaccination decreased these renal parameters in the diabetic mice, but the effect was not statistically significant (Fig. 3, *D* and *E*). By contrast, hyperglycemia markedly increased urinary albumin excretion, and the vaccination significantly decreased albuminuria in the diabetic mice (Fig. 3*F*).

In terms of glomerular histology, the diabetic mice showed greater glomerular volume, which manifested as glomerular hypertrophy, than in the non-diabetic mice. The vaccination significantly prevented glomerular hypertrophy in the diabetic mice (Fig. 4, *A* and *B*). Moreover, electron microscopy revealed that the vaccination improved the shortened length of the slit diaphragm between neighboring foot processes in the diabetic mice, but the effect was not statistically significant (Fig. 4, *C* and *D*). In the diabetic mice, both the glomerular mesangial area and interstitial fibrous areas on kidney sections stained with Masson's trichrome were increased, and vaccination significantly prevented the diabetes-induced expansion of mesangial and interstitial areas (Supplemental Fig. S2).

Altered renal expressions of angiogenesis-related factors by VASH2-targeting peptide vaccine

Renal *Vash2* mRNA expression was significantly higher in the diabetic mice than in the non-diabetic mice (Fig. 5*A*), whereas *Vash1* mRNA expression was lower in the diabetic mice than in the non-diabetic mice (Fig. 5*B*). The vaccination did not affect renal VASH1 and VASH2 expression in both non-diabetic and diabetic mice (Fig. 5, *A* and *B*). SVBP serves as a secretory chaperone that contributes to the activity of VASH1 and VASH2 on angiogenesis(25). No differences in *Svbp* mRNA expression were fund among the four groups (Fig. 5*C*). Consistent with previous reports(5), the present findings showed that renal VEGF expression was significantly higher in the diabetic mice than in the non-diabetic mice, but vaccination did not significantly affect this parameter (Fig. 5*D*). Furthermore, renal Angpt1 expression was significantly lower in the diabetic mice than in the non-diabetic mice, but the vaccination did not significantly affect this parameter (Fig. 5*E*). By contrast, renal angiopoietin-2 (Angpt2) expression was higher in the diabetic mice than in the non-diabetic mice, and the vaccination significantly prevented the increased renal Angpt2 expression in the diabetic mice (Fig. 5*F*). Increased protein levels of Angpt2 induced by hyperglycemia were also prevented by vaccination (Supplemental Fig. S3).

Inhibitory effects of VASH2-targeting peptide vaccine on glomerular microinflammation

Similar to Angpt2 expression, glomerular Mac-2-positive macrophage infiltration was also higher in the diabetic mice than in the non-diabetic mice. The vaccination significantly reduced the number of glomerular Mac-2-positive cells in the diabetic mice (Fig. 6, *A* and *B*). Moreover, it significantly prevented the diabetes-induced increase in renal ICAM-1 expression (Fig. 6*C*). These results indicate that circulating anti-VASH2 can suppress Angpt2-related endothelial destabilization and hyperpermeability, thereby inhibiting glomerular microinflammation.

Renal effects of VASH2-targeting peptide vaccine in type 2 diabetic db/db mice

Finally, the beneficial effects of VASH2-targeting vaccine observed in the STZ-induced diabetic model were examined in type 2 diabetic obese db/db mice. In the vaccinated db/db mice, the VASH2 antibody titer increased to an average of 1:14,820 at week 16. No significant differences in body weight or HbA1c levels were observed between the non-vaccinated and vaccinated db/db mice (Fig. 7, *A* and *B*). In addition, no significant differences were observed in BUN, serum creatinine, or creatinine clearance levels between the two groups (Fig. 7, *C*–*E*). Urinary albumin excretion was significantly increased in non-vaccinated db/db mice at weeks 15 and 21 than in those at week 2, and vaccination prevented the increase in urinary albumin excretion at weeks 15 and 21 (Fig. 7*F*). Furthermore, glomerular hypertrophy and mesangial expansion were significantly ameliorated by vaccination of the db/db mice (Supplemental Fig. S4). Thus, the preventive effects of VASH2-targeting vaccine against diabetic glomerular injury were confirmed in a type 2 diabetic mouse model.

DISCUSSION

In the present study, subcutaneous injection of the VASH2-targeting peptide vaccine successfully induced circulating anti-VASH2 antibody without organ inflammation and prevented albuminuria in both STZ-induced type 1 diabetic mice and type 2 diabetic db/db mice.

Vaccines have long been used as a preventive strategy against various infectious diseases. Recently, this strategy has been applied to prevent and/or treat chronic diseases such as hypertension by targeting pathogenic self-antigens. Vaccines targeting self-antigens must overcome the intrinsic immune tolerance system to induce the production of effective neutralizing antibodies. VASH2-targeting peptide vaccine utilizes KLH as a carrier protein containing a strong T cell epitope. In the present study, although the VASH2-targering peptide vaccine markedly elevated the serum anti-VASH2 antibody titer in both non-diabetic and diabetic mice, the elevation of the antibody titer was obviously delayed at week 8 in the diabetic mice compared with the non-diabetic mice. This delay might be attributed to acute hyperglycemia in the diabetic mice. Another potential cause was STZ injection, considering a previous report that the agent is directly toxic for T cells(26). No delay has been observed in diabetic db/db mice treated with a KLH-based prorenin peptide vaccine(27). However, a high anti-VASH2 antibody titer was maintained until the end of the study in both groups. Adjuvants strongly affect immune responses to vaccination(28). Freund's adjuvant, a water-in-oil emulsion, commonly induces a Th1 rather than Th2 immune response. In the present study, VASH2-targeting peptide vaccine with Freund's adjuvant induced a Th1 response. Although the Th1 immune response may be associated with autoimmune disorders, VASH2-targeting peptide vaccine did not induce obvious inflammation in major organs. Similar to the results of a previous report(16), the findings of the present study proved the favorite safety and practicality of the KLH-based peptide vaccine.

In contrast to its homolog VASH1, VASH2 was originally identified as a proangiogenic factor. VASH2 is secreted by cancer cells and promotes tumor angiogenesis. In the skin flap model, preventing the termination of angiogenesis at the sprouting front has been determined to be a potential mechanism of the proangiogenic action of VASH2. Unfortunately, the detailed mechanisms by which VASH2 affects the vascular endothelium have not been determined, and the candidate receptor(s) for extracellular VASH2 in endothelial cells have not yet been identified. Abnormal angiogenesis and various angiogenic factors are involved in the pathogenesis of diabetic nephropathy(4). In the present study, increased renal VASH2 expression was associated with increased urinary albumin excretion and glomerular hypertrophy in the diabetic mice, similar to the previously reported adverse effects of increased VEGF(5). However, the anti-VASH2 antibody induced by the VASH2-targeting peptide vaccine did not affect renal VASH2 expression, suggesting that the anti-VASH2 antibody only inhibited the action of circulating or extracellular VASH2. The vaccination also exerted no significant effects on the renal expressions of VASH1, VEGF, and Angpt1 in the diabetic mice. Notably, the vaccination significantly prevented the diabetes-induced upregulation of renal Angpt2 expression. Angpt2 antagonizes the action of Angpt1 on the Tie2 receptor and destabilizes endothelial cells by enhancing their sensitivity to VEGF(29). Its expression level in normal kidneys is extremely low, whereas Angpt2 expression is upregulated in kidney disease. Indeed, Angpt2 mRNA levels are elevated in glomeruli isolated from patients with diabetes(30). Restored Angpt1 expression and Tie2 activity reduce albuminuria in diabetic mice(6, 30). Thus, Angpt2 is considered an aggravating factor in diabetic nephropathy. Furthermore, Angpt2 expression is involved in the regulation of inflammation-related signaling in various inflammatory diseases such as inflammatory bowel disease(31) and associated with vascular endothelial inflammation in diabetes mellitus(32). Specifically, it upregulates ICAM1 expression in endothelial cells(33), and accelerates inflammatory infiltration. Recent evidence has suggested that Angpt2 inhibitors can block macrophage infiltration in a renal fibrosis model(34). Considering that Angpt2 is mainly released from endothelial cells, we speculate that the anti-VASH2 antibody may prevent Angpt2 expression in glomerular endothelial cells, leading to the suppression of ICAM1 expression and macrophage infiltration.

Although the VASH2-targeting peptide vaccine prevented albuminuria and glomerular microinflammation in the diabetic mice, these beneficial effects appeared modest compared with those previously observed in diabetic *Vash2* homozygous knockout mice(8). One possible reason for this difference is that a higher anti-VASH2 antibody titer is required to completely prevent the pathogenic effects of VASH2 in diabetes. Another reason may be related to the intracellular effects of VASH2, in addition to its proangiogenic properties. Overexpression of VASH2 causes epithelial–to–mesenchymal transition in cancer cells(35) by promoting TGF-β signaling(36). In diabetic *Vash2* knockout mice, reduced mesangial matrix expansion can be mediated by inhibiting the activation of TGF-β signaling related to VASH2 upregulation in mesangial cells(8). Furthermore, recent studies have proposed α-tubulin detyrosination as a novel biological role of VASH2 and VASH1(37, 38). Detyrosination is the posttranslational modification in the C-terminus of α-tubulin, and the tyrosination/detyrosination cycle can alter the structure and function of microtubules(39). VASH2 increases detyrosinated α-tubulin in cardiomyocytes, leading to impaired cellular contractility(40). Thus far, the association of α-tubulin detyrosination and diabetic nephropathy has not been established. Theoretically, anti-VASH2 antibody induced by the vaccination could block the proangiogenic effect of extracellular VASH2 but not inhibit its intracellular actions, including TGF-β signal activation and α-tubulin detyrosination. The results of the present study demonstrated that the VASH2-targeting peptide vaccine significantly prevented

albuminuria and glomerular microinflammation by inhibiting the pathogenic effects of extracellular VASH2.

This study had some limitations. First, this study revealed the preventive but not therapeutic effects of the VASH2-targeting peptide vaccine. In the study on db/db mice, mild albuminuria was observed at the time of the second vaccination, suggesting that this peptide vaccine may have a therapeutic effect on albuminuria in diabetic mice. However, appropriate evaluation of the therapeutic effects requires vaccination after urinary albumin excretion reaches its peak level. A disadvantage of vaccine therapy is the lack of immediate effects. Indeed, the peak anti-VASH2 antibody titer was reached 12 weeks after the first vaccination in the diabetic model. Thus, a substantially longer experimental period is required to demonstrate the therapeutic effects, which are initiated after increased urine albumin excretion, of the VASH2-targeting peptide vaccine. Long-term hyperglycemia causes marked polyuria in conjugation with bladder dysfunction in diabetic mice(41), leading to the development of hydronephrosis. Thus, this animal model may not clearly demonstrate the therapeutic effects of the vaccine. Second, we could not determine whether the VASH2-targeting peptide vaccine lowered the blood concentration of VASH2. In contrast to VASH1, which is constitutively expressed in endothelial cells, VASH2 is barely expressed in differentiated cells. Therefore, the blood concentration of VASH2 is extremely low, except in patients with poorly differentiated malignant tumors. Although only one clinical study has determined plasma VASH2 levels in patients with esophageal cancer(42), the blood concentration of VASH2 is difficult to accurately measure in mice because standardized methods are lacking. Furthermore, the main source(s) of circulating VASH2 has not been determined in normal subjects without malignancy. Finally, the mechanisms by which VASH2 regulates Angpt2 expression in endothelial cells remains unclear. As the receptor and downstream signaling of VASH2 in endothelial cells have not been identified, clarifying the detailed mechanisms involved in *in vitro* experiments is currently challenging. This issue is should be addressed in future studies.

In conclusion, treatment with the VASH2-targeting peptide vaccine prevented albuminuria and glomerular microinflammation in murine models of early-stage diabetic nephropathy possibly by inhibiting Angpt2 and ICAM-1 expression. Although pathogenic roles of VASH2 warrant further investigation, the anti-VASH2 strategy developed in this study may still be used to treat other renal diseases involving Angpt2-mediated microinflammation.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL DATA

Supplemental Figs. S1–S5 and Table: DOI: 10.17632/9wmyr7222r.2

ACKNOWLEDGMENTS

We thank Dr. Yasuhiro Suzuki for providing technical assistance and the recombinant mouse VASH2 protein.

GRANTS

This work was supported by JSPS KAKENHI Grant JP21K08278 (2021−2023, to K.T.).

DISCLOSURES

The Department of Health Development and Medicine at Osaka University is an endowed department supported by AnGes, Daicel, and FunPep. H. Nakagami is a scientific advisor and stockholder of FunPep. J. Wada received speaker honoraria from Astra Zeneca, Bayer, Boehringer Ingelheim, Daiichi Sankyo, Kyowa Kirin, Novo Nordisk, and Mitsubishi Tanabe, and received grant support from Bayer, Chugai, Kyowa Kirin, Otsuka, Shionogi, Sumitomo, and Mitsubishi Tanabe.

AUTHOR CONTRIBUTIONS

K.T. conceived and designed research; Y.N, K.T., T.M., T.N. and H.H. performed experiments; Y.N. and K.T. analyzed data; Y.N., K.T. and J.W. interpreted results of experiments; Y.N. and K.T. prepared figures; K.T. drafted manuscript; H.H., H.N., Y.S. and J.W. edited and revised manuscript; all authors approved final version of manuscript.

REFERENCES

1. **Umanath K, and Lewis JB**. Update on Diabetic Nephropathy: Core Curriculum 2018. *Am J Kidney Dis* 71: 884-895, 2018.
2. **DeFronzo RA, Reeves WB, and Awad AS**. Pathophysiology of diabetic kidney disease: impact of SGLT2 inhibitors. *Nat Rev Nephrol* 17: 319-334, 2021.
3. Impact of diabetes on the effects of sodium glucose co-transporter-2 inhibitors on kidney outcomes: collaborative meta-analysis of large placebo-controlled trials. *Lancet* 400: 1788-1801, 2022.
4. **Tanabe K, Wada J, and Sato Y**. Targeting angiogenesis and lymphangiogenesis in kidney disease. *Nat Rev Nephrol* 16: 289-303, 2020.
5. **Tanabe K, Maeshima Y, Sato Y, and Wada J**. Antiangiogenic Therapy for Diabetic Nephropathy. *Biomed Res Int* 2017: 5724069, 2017.
6. **Carota IA, Kenig-Kozlovsky Y, Onay T, Scott R, Thomson BR, Souma T, Bartlett CS, Li Y, Procissi D, Ramirez V, Yamaguchi S, Tarjus A, Tanna CE, Li C, Eremina V, Vestweber D, Oladipupo SS, Breyer MD, and Quaggin SE**. Targeting VE-PTP phosphatase protects the kidney from diabetic injury. *J Exp Med* 216: 936-949, 2019.
7. **Hong Q, Zhang L, Fu J, Verghese DA, Chauhan K, Nadkarni GN, Li Z, Ju W, Kretzler M, Cai GY, Chen XM, D'Agati VD, Coca SG, Schlondorff D, He JC, and Lee K**. LRG1 Promotes Diabetic Kidney Disease Progression by Enhancing TGF-β-Induced Angiogenesis. *J Am Soc Nephrol* 30: 546-562, 2019.
8. **Masuda K, Tanabe K, Ujike H, Hinamoto N, Miyake H, Tanimura S, Sugiyama H, Sato Y, Maeshima Y, and Wada J**. Deletion of pro-angiogenic factor vasohibin-2 ameliorates glomerular alterations in a mouse diabetic nephropathy model. *PloS one* 13: e0195779, 2018.
9. **Kimura H, Miyashita H, Suzuki Y, Kobayashi M, Watanabe K, Sonoda H, Ohta H, Fujiwara T, Shimosegawa T, and Sato Y**. Distinctive localization and opposed roles of vasohibin-1 and vasohibin-2 in the regulation of angiogenesis. *Blood* 113: 4810-4818, 2009.
10. **Iida-Norita R, Kawamura M, Suzuki Y, Hamada S, Masamune A, Furukawa T, and Sato Y**. Vasohibin-2 plays an essential role in metastasis of pancreatic ductal adenocarcinoma. *Cancer science* 110: 2296-2308, 2019.
11. **Horie S, Suzuki Y, Yamamoto T, Obika S, Mohri K, Kiyota C, Ren Q, Warashina S, Wada Y, Watanabe Y, Mukai H, and Sato Y**. Novel strategy of liver cancer treatment with modified antisense oligonucleotides targeting human vasohibin-2. *Cancer science* 114: 3740-3749, 2023.
12. **Miyake H, Tanabe K, Tanimura S, Nakashima Y, Morioka T, Masuda K, Sugiyama H, Sato Y, and Wada J**. Genetic Deletion of Vasohibin-2 Exacerbates Ischemia-Reperfusion-Induced Acute Kidney Injury. *International journal of molecular sciences* 21: 2020.

13. **Nakagami H, Hayashi H, Shimamura M, Rakugi H, and Morishita R**. Therapeutic vaccine for chronic diseases after the COVID-19 Era. *Hypertension research : official journal of the Japanese Society of Hypertension* 44: 1047-1053, 2021.
14. **Nakagami F, Koriyama H, Nakagami H, Osako MK, Shimamura M, Kyutoku M, Miyake T, Katsuya T, Rakugi H, and Morishita R**. Decrease in blood pressure and regression of cardiovascular complications by angiotensin II vaccine in mice. *PloS one* 8: e60493, 2013.
15. **Pang Z, Nakagami H, Osako MK, Koriyama H, Nakagami F, Tomioka H, Shimamura M, Kurinami H, Takami Y, Morishita R, and Rakugi H**. Therapeutic vaccine against DPP4 improves glucose metabolism in mice. *Proceedings of the National Academy of Sciences of the United States of America* 111: E1256-1263, 2014.
16. **Kawakami R, Nozato Y, Nakagami H, Ikeda Y, Shimamura M, Yoshida S, Sun J, Kawano T, Takami Y, Noma T, Rakugi H, Minamino T, and Morishita R**. Development of vaccine for dyslipidemia targeted to a proprotein convertase subtilisin/kexin type 9 (PCSK9) epitope in mice. *PloS one* 13: e0191895, 2018.
17. **Koyanagi T, Suzuki Y, Komori K, Saga Y, Matsubara S, Fujiwara H, and Sato Y**. Targeting human vasohibin-2 by a neutralizing monoclonal antibody for anti-cancer treatment. *Cancer science* 108: 512-519, 2017.
18. **Lee ES, Suzuki Y, Tomioka H, Nakagami H, and Sato Y**. Development of a Novel and Simple Anti-Metastatic Cancer Treatment Targeting Vasohibin-2. *Tohoku J Exp Med* 2023.
19. **Fukami H, Morinaga J, Nakagami H, Hayashi H, Okadome Y, Matsunaga E, Kadomatsu T, Horiguchi H, Sato M, Sugizaki T, Kuwabara T, Miyata K, Mukoyama M, Morishita R, and Oike Y**. Vaccine targeting ANGPTL3 ameliorates dyslipidemia and associated diseases in mouse models of obese dyslipidemia and familial hypercholesterolemia. *Cell reports Medicine* 2: 100446, 2021.
20. **Tanabe K, Lanaspa MA, Kitagawa W, Rivard CJ, Miyazaki M, Klawitter J, Schreiner GF, Saleem MA, Mathieson PW, Makino H, Johnson RJ, and Nakagawa T**. Nicorandil as a novel therapy for advanced diabetic nephropathy in the eNOS-deficient mouse. *American journal of physiology Renal physiology* 302: F1151-1160, 2012.
21. **Hayashi H, Sun J, Yanagida Y, Yoshida S, Baba S, Tenma A, Toyoura M, Kawabata S, Ehara T, Asaki R, Sakaguchi M, Tomioka H, Shimamura M, Morishita R, Rakugi H, Tomita T, and Nakagami H**. Peptide-based vaccine targeting IL17A attenuates experimental spondyloarthritis in HLA-B27 transgenic rats. *RMD open* 9: 2023.
22. **Nasu T, Maeshima Y, Kinomura M, Hirokoshi-Kawahara K, Tanabe K, Sugiyama H, Sonoda H, Sato Y, and Makino H**. Vasohibin-1, a negative feedback regulator of angiogenesis, ameliorates renal alterations in a mouse model of diabetic nephropathy. *Diabetes* 58: 2365-2375, 2009.
23. **Mifune T, Tanabe K, Nakashima Y, Tanimura S, Sugiyama H, Sato Y, and Wada J**. Vasohibin-1 has α-tubulin detyrosinating activity in glomerular podocytes. *Biochemical and biophysical research communications* 599: 93-99, 2022.
24. **Tanimura S, Tanabe K, Miyake H, Masuda K, Tsushida K, Morioka T, Sugiyama H, Sato Y, and Wada J**. Renal tubular injury exacerbated by vasohibin-1 deficiency in a murine cisplatin-induced acute kidney injury model. *American journal of physiology Renal physiology* 317: F264-f274, 2019.
25. **Suzuki Y, Kobayashi M, Miyashita H, Ohta H, Sonoda H, and Sato Y**. Isolation of a small vasohibin-binding protein (SVBP) and its role in vasohibin secretion. *Journal of cell science* 123: 3094-3101, 2010.
26. **Muller YD, Golshayan D, Ehirchiou D, Wyss JC, Giovannoni L, Meier R, Serre-Beinier V, Puga Yung G, Morel P, Bühler LH, and Seebach JD**. Immunosuppressive effects of streptozotocin-induced diabetes result in absolute lymphopenia and a relative increase of T regulatory cells. *Diabetes* 60: 2331-2340, 2011.

27. **Yokota H, Hayashi H, Hanaguri J, Yamagami S, Kushiyama A, Nakagami H, and Nagaoka T**. Effect of prorenin peptide vaccine on the early phase of diabetic retinopathy in a murine model of type 2 diabetes. *PloS one* 17: e0262568, 2022.
28. **Pulendran B, P SA, and O'Hagan DT**. Emerging concepts in the science of vaccine adjuvants. *Nat Rev Drug Discov* 20: 454-475, 2021.
29. **Saharinen P, Eklund L, and Alitalo K**. Therapeutic targeting of the angiopoietin-TIE pathway. *Nat Rev Drug Discov* 16: 635-661, 2017.
30. **Dessapt-Baradez C, Woolf AS, White KE, Pan J, Huang JL, Hayward AA, Price KL, Kolatsi-Joannou M, Locatelli M, Diennet M, Webster Z, Smillie SJ, Nair V, Kretzler M, Cohen CD, Long DA, and Gnudi L**. Targeted glomerular angiopoietin-1 therapy for early diabetic kidney disease. *J Am Soc Nephrol* 25: 33-42, 2014.
31. **Scholz A, Plate KH, and Reiss Y**. Angiopoietin-2: a multifaceted cytokine that functions in both angiogenesis and inflammation. *Ann N Y Acad Sci* 1347: 45-51, 2015.
32. **Chandel S, Sathis A, Dhar M, Giri H, Nathan AA, Samawar SKR, Gupta A, Gopal J, Harish R, Mohan V, and Dixit M**. Hyperinsulinemia promotes endothelial inflammation via increased expression and release of Angiopoietin-2. *Atherosclerosis* 307: 1-10, 2020.
33. **Chen JX, Zeng H, Reese J, Aschner JL, and Meyrick B**. Overexpression of angiopoietin-2 impairs myocardial angiogenesis and exacerbates cardiac fibrosis in the diabetic db/db mouse model. *Am J Physiol Heart Circ Physiol* 302: H1003-1012, 2012.
34. **Chang FC, Liu CH, Luo AJ, Tao-Min Huang T, Tsai MH, Chen YJ, Lai CF, Chiang CK, Lin TH, Chiang WC, Chen YM, Chu TS, and Lin SL**. Angiopoietin-2 inhibition attenuates kidney fibrosis by hindering chemokine C-C motif ligand 2 expression and apoptosis of endothelial cells. *Kidney Int* 102: 780-797, 2022.
35. **Xue X, Zhang Y, Zhi Q, Tu M, Xu Y, Sun J, Wei J, Lu Z, Miao Y, and Gao W**. MiR200-upregulated Vasohibin 2 promotes the malignant transformation of tumors by inducing epithelial-mesenchymal transition in hepatocellular carcinoma. *Cell Commun Signal* 12: 62, 2014.
36. **Norita R, Suzuki Y, Furutani Y, Takahashi K, Yoshimatsu Y, Podyma-Inoue KA, Watabe T, and Sato Y**. Vasohibin-2 is required for epithelial-mesenchymal transition of ovarian cancer cells by modulating transforming growth factor-β signaling. *Cancer science* 108: 419-426, 2017.
37. **Aillaud C, Bosc C, Peris L, Bosson A, Heemeryck P, Van Dijk J, Le Friec J, Boulan B, Vossier F, Sanman LE, Syed S, Amara N, Couté Y, Lafanechère L, Denarier E, Delphin C, Pelletier L, Humbert S, Bogyo M, Andrieux A, Rogowski K, and Moutin MJ**. Vasohibins/SVBP are tubulin carboxypeptidases (TCPs) that regulate neuron differentiation. *Science* 358: 1448-1453, 2017.
38. **Nieuwenhuis J, Adamopoulos A, Bleijerveld OB, Mazouzi A, Stickel E, Celie P, Altelaar M, Knipscheer P, Perrakis A, Blomen VA, and Brummelkamp TR**. Vasohibins encode tubulin detyrosinating activity. *Science* 358: 1453-1456, 2017.
39. **Sanyal C, Pietsch N, Ramirez Rios S, Peris L, Carrier L, and Moutin MJ**. The detyrosination/re-tyrosination cycle of tubulin and its role and dysfunction in neurons and cardiomyocytes. *Semin Cell Dev Biol* 137: 46-62, 2023.
40. **Yu X, Chen X, Amrute-Nayak M, Allgeyer E, Zhao A, Chenoweth H, Clement M, Harrison J, Doreth C, Sirinakis G, Krieg T, Zhou H, Huang H, Tokuraku K, Johnston DS, Mallat Z, and Li X**. MARK4 controls ischaemic heart failure through microtubule detyrosination. *Nature* 594: 560-565, 2021.
41. **Kim AK, Hamadani C, Zeidel ML, and Hill WG**. Urological complications of obesity and diabetes in males and females of three mouse models: temporal manifestations. *American journal of physiology Renal physiology* 318: F160-f174, 2020.
42. **Yamamoto M, Ozawa S, Ninomiya Y, Koyanagi K, Oguma J, Kazuno A, Hara H, Yatabe K, Kajiwara H, Nakamura N, and Sato Y**. Plasma vasohibin-1 and vasohibin-2 are useful biomarkers in

patients with esophageal squamous cell carcinoma. *Esophagus : official journal of the Japan Esophageal Society* 17: 289-297, 2020.

FIGURE LEGENDS

Figure 1. Induction of anti-VASH2 antibody by subcutaneous injection of the VASH2-targeting peptide vaccine. ***A***: Serum anti-VASH2 antibody titer determined by ELISA in both non-diabetic and diabetic mice treated with the VASH2-targeting peptide vaccine. Antibody titer was expressed as dilution ratio with half-maximal binding. ***B***: Immunoblot for VASH2 showing the direct binding of serum anti-VASH2 antibody with mouse full-length VASH2 protein. Recombinant mouse VASH2/SVBP protein (10, 20, and 30 μg) was loaded, and diluted serum (1:250) from a vaccinated mouse was used as primary antibody. Arrow indicates VASH2/SVBP complex (61 kDa), and arrowhead indicates VASH2 alone (41 kDa). ***C***, ***D***: IgG subclasses of anti-VASH2 antibody induced by the vaccination in non-diabetic (***C***) and diabetic (***D***) mice. n = 6 for non-diabetic mice with vaccination (NDM+V) and n = 9 for diabetic mice with vaccination (DM+V). Each column shows the mean ± SD.

Figure 2. Representative images of major organs from NDM-V and NDM+V groups. All organs were harvested at week 20. ***A***: Kidney, ***B***: Heart, ***C***: Lung, ***D***: Liver, ***E***: Pancreas, ***F***: Aorta. Kidney tissues were stained by PAS and the other tissues were stained by HE (original magnification: 100×). No apparent inflammatory infiltration was observed in all organs.

Figure 3. Preventive effect of the VASH2-targetng peptide vaccine on urinary albumin excretion in diabetic mice independent of blood glucose levels. ***A***–***C***: Body weight loss (***A***), hyperglycemia (***B***), and increased HbA1c level (***C***) were confirmed in diabetic mice compared with non-diabetic mice. The vaccination did not affect these parameters. ***D***, ***E***: Blood urea nitrogen (***D***) and serum creatinine (***E***) levels were significantly higher in diabetic mice than in non-diabetic mice. Differences in the renal function parameters between diabetic mice with and without the vaccination did not reach statistical significance. ***F***: Urinary albumin excretion was markedly increased in diabetic mice, and the vaccination significantly prevented hyperglycemia-induced albuminuria. n = 6 for NDM-V, 7 for NDM+V, 9 for DM-V, and 9 for DM+V. $^{*}P < 0.01$ versus NDM-V, $^{\#}P < 0.05$ versus DM-V. Each column shows the mean ± SD.

Figure 4. Effects of the VASH2-targeting peptide vaccine on histological and ultrastructural alterations in the glomeruli. ***A***: Representative light microscopic images of glomeruli from non-diabetic mice with or without vaccination and diabetic mice with or without vaccination (PAS staining). Scale bars indicate 50 μm. ***B***: Diabetes-induced increase in glomerular volume was significantly prevented by treatment with the VASH2-targeting peptide vaccine. ***C***: Representative transmission electron microscopic images of glomerular capillary tufts from non-diabetic mice with or without vaccination and diabetic mice with or without vaccination. Scale bars indicate 1 μm. ***D***: Decrease in slit diaphragm width observed in diabetic mice was prevented by treatment with the VASH2-targeting peptide vaccine (not significant). n = 6 for NDM-V, 7 for NDM+V, 9 for DM-V, and 9 for DM+V. $^{*}P < 0.01$ versus NDM-V, $^{\#}P < 0.05$ versus DM-V. Each column shows the mean ± SD.

Figure 5. Effects of the VASH2-targeting peptide vaccine on the mRNA expressions of angiogenesis-related factors in the kidney. ***A***–***C***: Vash2 mRNA expression (***A***) increased whereas *Vash1* mRNA expression (***B***) decreased in diabetic mice. Treatment with the VASH2-targeting peptide vaccine did not

affect *Vash2* and *Vash1* expression, and no difference in *Svbp* expression (***C***) was found among the four groups. ***D***: *Vegfa* mRNA expression was increased in diabetic mice, and treatment with the VASH2-targeting peptide vaccine had no effect on *Vegfa* expression. ***E***, ***F***: Angpt1 mRNA expression (***E***) decreased whereas Angpt2 mRNA expression (***F***) increased in diabetic mice. Treatment with the VASH2-targeting peptide vaccine significantly prevented Angpt2 upregulation by hyperglycemia. n = 6 for NDM-V, 7 for NDM+V, 9 for DM-V, and 9 for DM+V. *$P < 0.01$ versus NDM-V, $^{\#}P < 0.05$ versus DM-V. Each column shows the mean ± SD.

Figure 6. Preventive effects of the VASH2-targeting peptide vaccine on glomerular microinflammation. ***A***: Representative glomerular images of immunohistochemistry for Mac-2 in non-diabetic mice with or without vaccination and diabetic mice with or without vaccination (original magnification, 400×). Arrows indicate Mac-2-positive cells. In the negative control (NC) images, the primary antibody was replaced with normal rat IgG. ***B***: Diabetes-induced increase in the infiltration of glomerular Mac-2-positive macrophages was significantly prevented by treatment with the VASH2-targeting peptide vaccine. ***C***: Immunoblot for ICAM-1 and GAPDH. Each lane was loaded with 40 μg protein. Densitometry of the immunoblot normalized to GAPDH. n = 6 for NDM-V, 7 for NDM+V, 9 for DM-V, and 9 for DM+V. *$P < 0.01$ versus NDM-V, $^{\#}P < 0.05$ versus DM-V. Each column shows the mean ± SD.

Figure 7. Preventive effect of the VASH2-targeting peptide vaccine on urinary albumin excretion in db/db mice. ***A***, ***B***: The vaccination did not affect body weight (***A***) and HbA1c (***B***) in db/db mice. ***C***–***E***: No significant differences were observed in BUN (***C***), serum creatinine (***D***), and creatinine clearance levels (***E***) between db/db mice with and without the vaccination. ***F***: Urinary albumin excretion was significantly increased in the db/db-V group but was not in the db/db+V group in the period of weeks 2 to 21. n = 6 for each group. *$P < 0.05$ versus week 2. Each column shows the mean ± SD.

TABLES

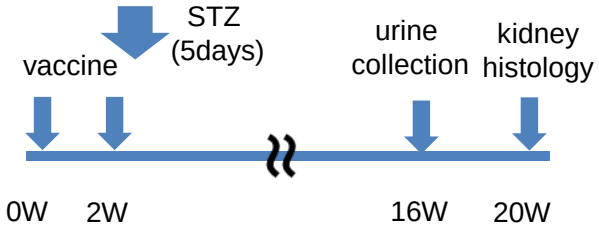
Table 1. Primer sequences for real-time PCR

Gene	Forward	Reverse
Vash2	GGCTAAGCCTTCAATTCCCC	CCCATTGGTGAGATAGATGCC
Vash1	CTGTCATGCTAGCCACCCATC	CCTCAGTACCCAGTCTCTAGGCTTC
Svbp	CAGAAATCTGCCCAGCAGGAG	CGGCTGCATCTGCTTACAGAAC
Vegfa	AACCCATTCCTGGCCCTGA	GATCCACAAAGCATGCCATGTC
Angpt1	CCGAGCCTACTCACAGTACGACAG	TGAAATCGGCACCGTGTAAGA
Angpt2	TGGTGGGCACAGGTTATCATC	CATTCACCAACATGGCGCTTA
Actb	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA

Vasohibin-2-targeting peptide vaccine against diabetic nephropathy

METHODS

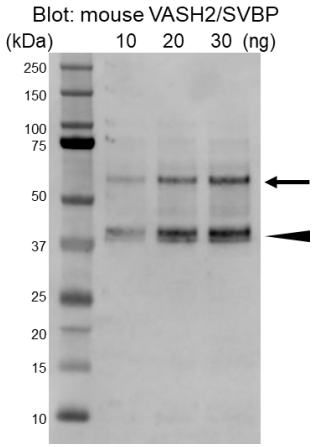
- Vasohibin-2 (VASH2)-targeting peptide vaccine was subcutaneously injected at two doses 2 weeks apart
- Diabetes was induced by intraperitoneal injection of streptozotocin (STZ; 50mg/kg) for 5 consecutive days



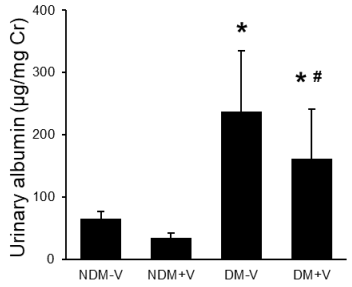
<study group>
1.non-diabetic without vaccine (NDM-V; n=7)
2.non-diabetic with vaccine (NDM+V; n=8)
3.Diabetic without vaccine (DM-V; n=9)
4.Diabetic with vaccine (DM+V; n=9)

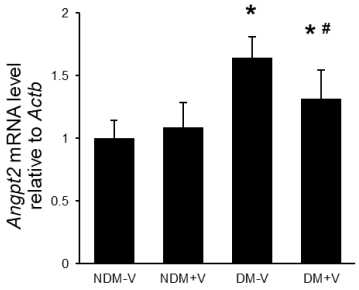
OUTCOME Prevention of diabetes-induced glomerular damage by treatment with VASH-2-targeting peptide vaccine

- VASH2-targeting peptide vaccine successfully induced circulating anti-VASH2 antibody

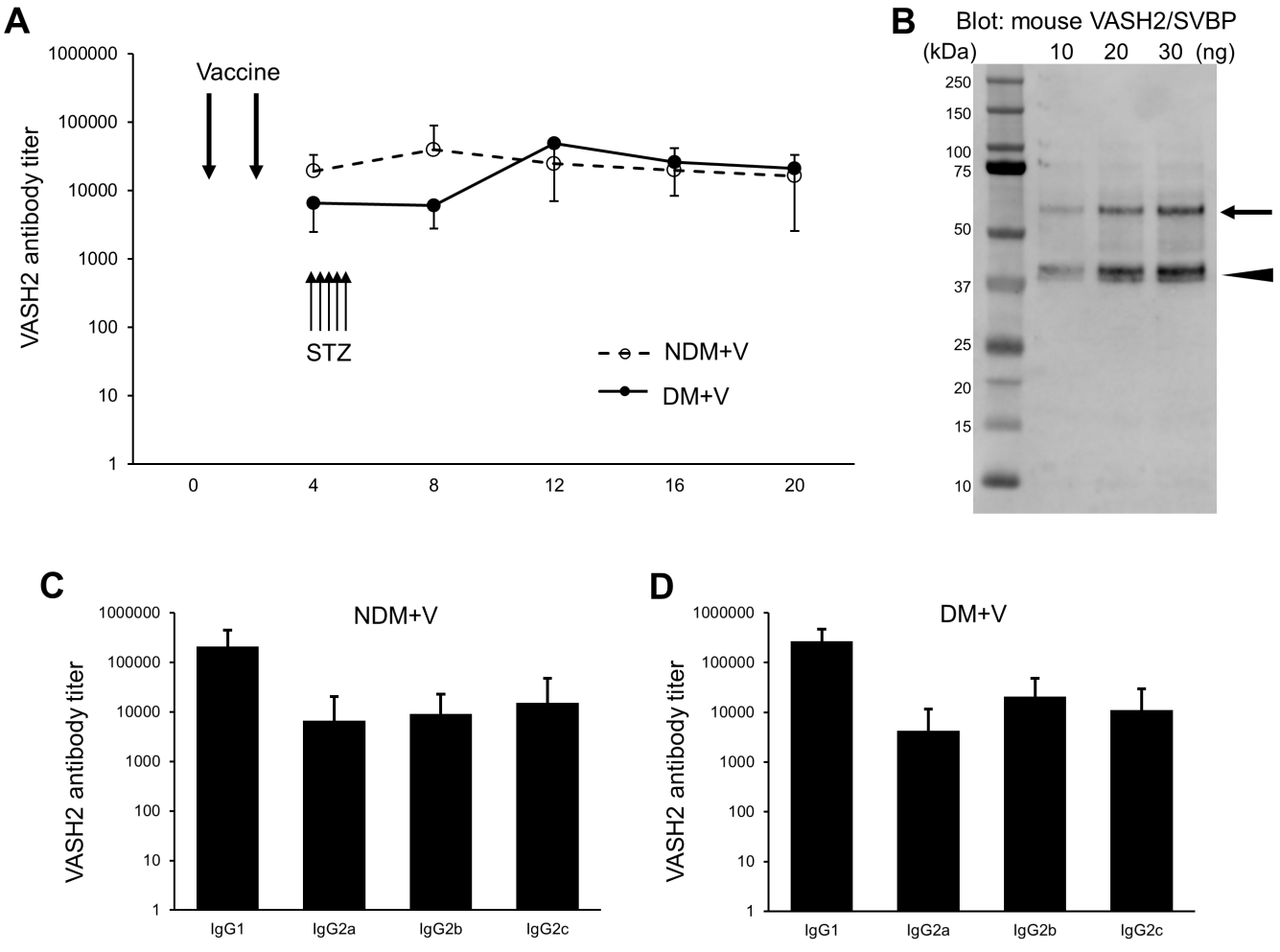


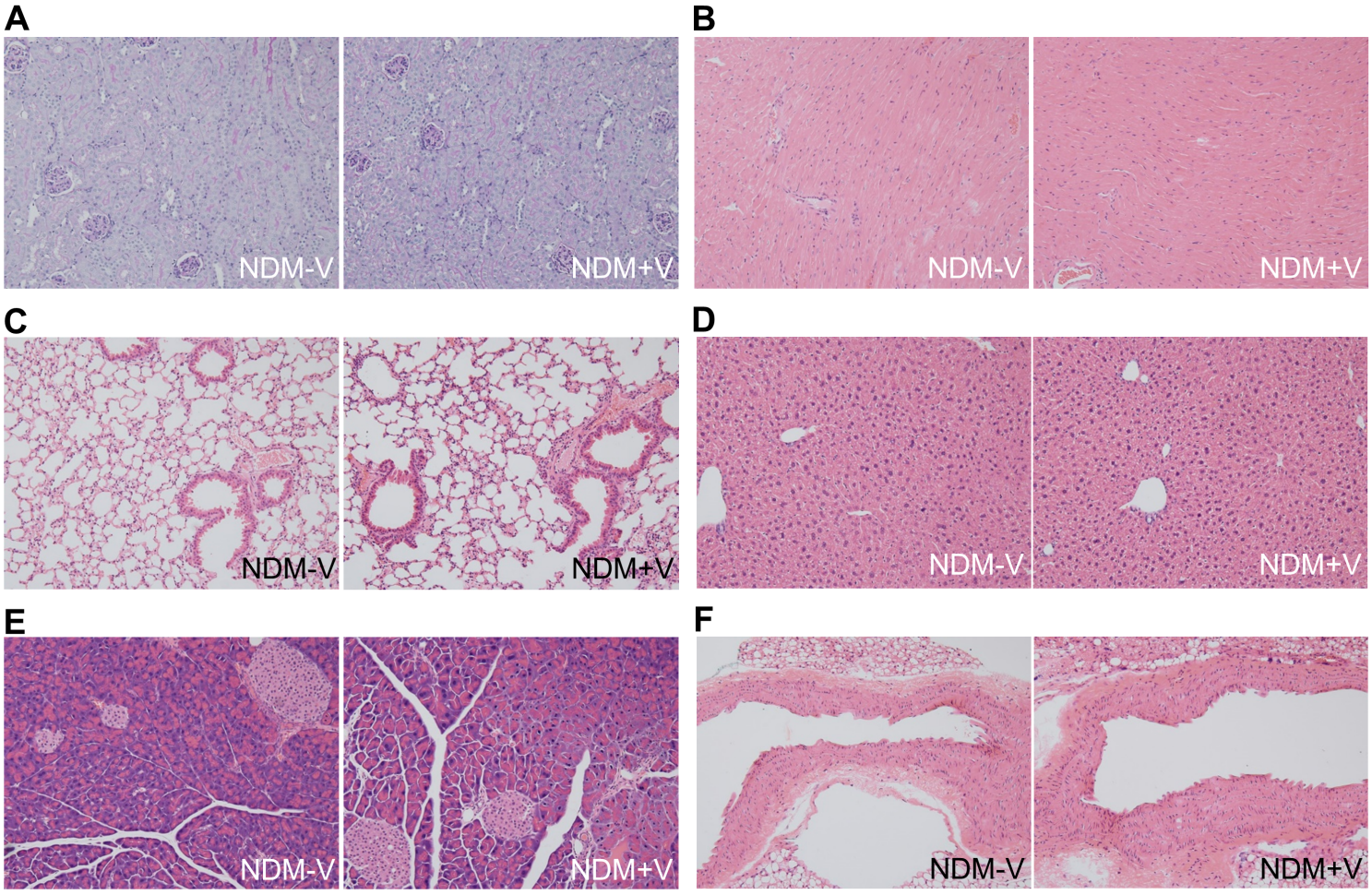
- The vaccination significantly reduced albuminuria in diabetic mice
- Induction of anti-VASH2 antibody significantly prevented renal angiopoietin-2 (Angpt2) expression in diabetic mice

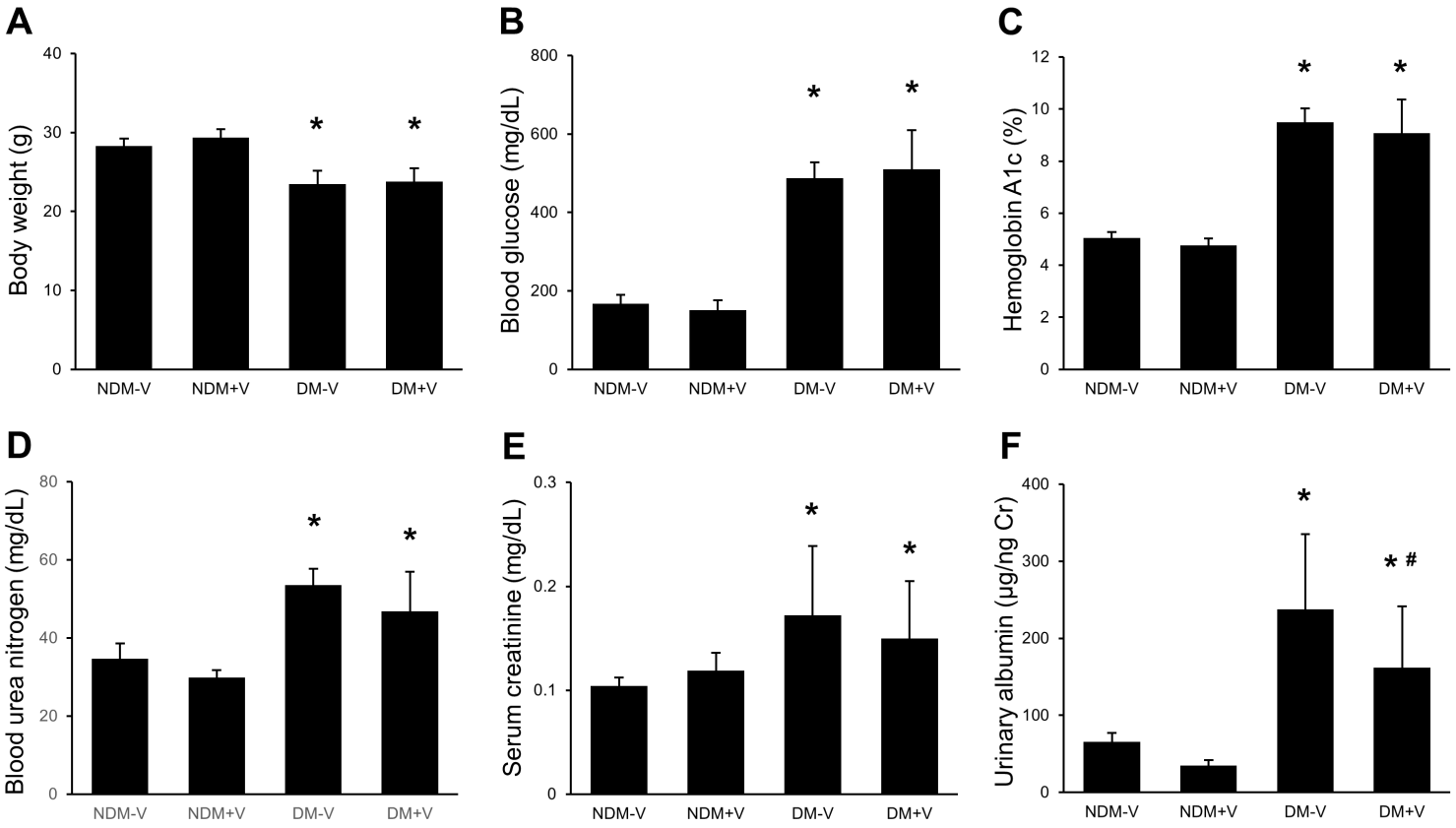


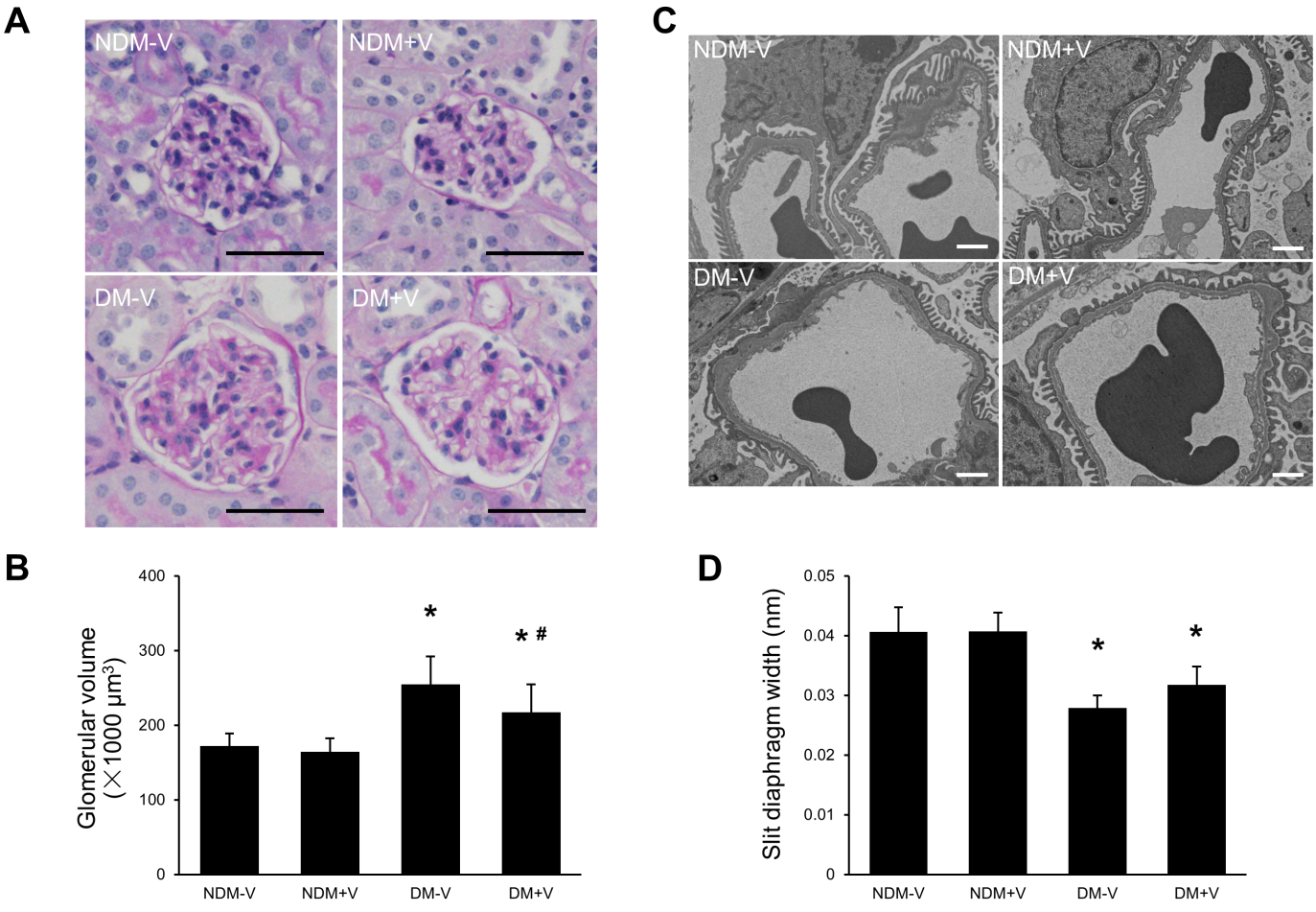


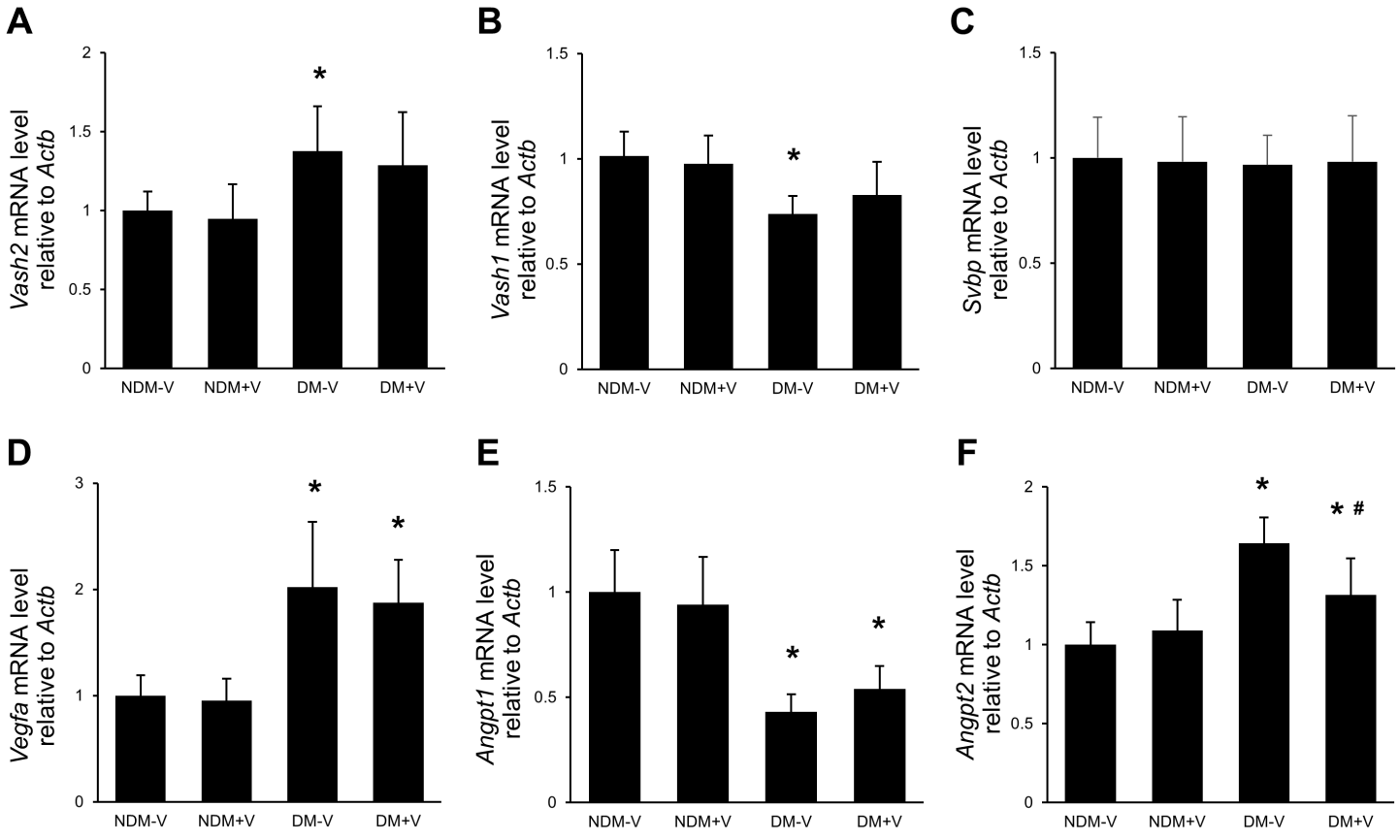
CONCLUSION VASH2-targeting peptide vaccine is an useful tool for preventive strategy against early-stage diabetic nephropathy

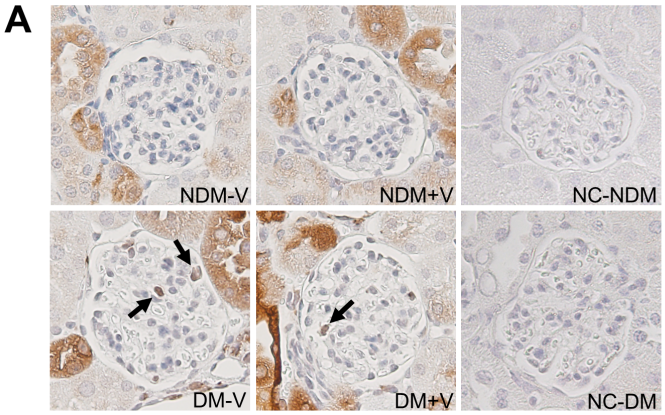

A
VASH2 antibody titer
1000000
100000
10000
1000
100
10
1
Vaccine
STZ
NDM+V
DM+V
0
4
8
12
16
20
B
Blot: mouse VASH2/SVBP
(kDa)
10
20
30
(ng)
250
150
100
75
50
37
25
20
15
10
C
NDM+V
VASH2 antibody titer
IgG1
IgG2a
IgG2b
IgG2c
D
DM+V
VASH2 antibody titer
IgG1
IgG2a
IgG2b
IgG2c

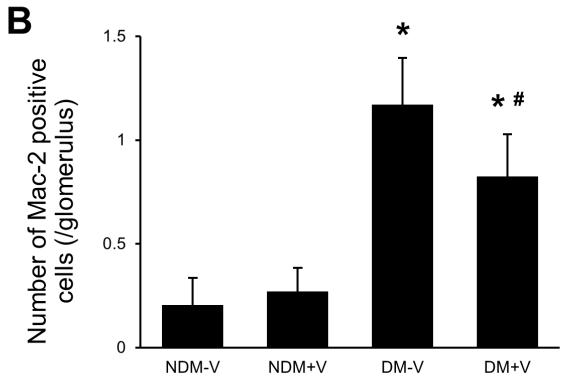

A
NDM-V
NDM+V
B
NDM-V
NDM+V
C
NDM-V
NDM+V
D
NDM-V
NDM+V
E
NDM-V
NDM+V
F
NDM-V
NDM+V

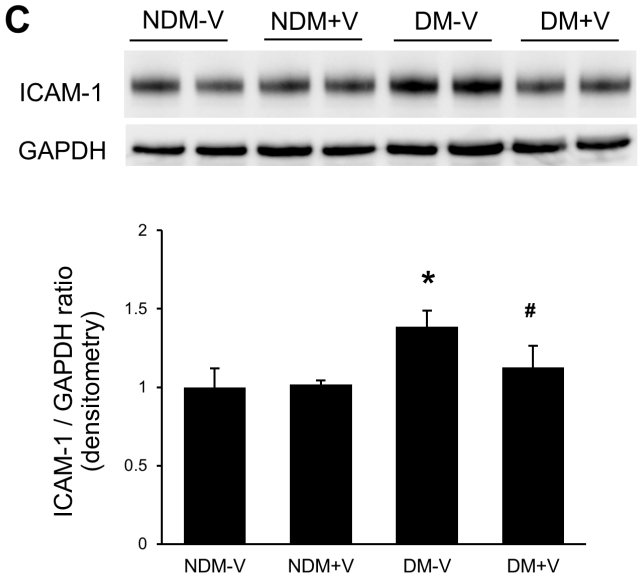

A
Body weight (g)
40
30
20
10
0
NDM-V
NDM+V
DM-V
DM+V
*
*
B
Blood glucose (mg/dL)
800
600
400
200
0
NDM-V
NDM+V
DM-V
DM+V
*
*
C
Hemoglobin A1c (%)
12
10
8
6
4
2
0
NDM-V
NDM+V
DM-V
DM+V
*
*
D
Blood urea nitrogen (mg/dL)
80
60
40
20
0
NDM-V
NDM+V
DM-V
DM+V
*
*
E
Serum creatinine (mg/dL)
0.3
0.2
0.1
0
NDM-V
NDM+V
DM-V
DM+V
*
*
F
Urinary albumin (μg/ng Cr)
400
300
200
100
0
NDM-V
NDM+V
DM-V
DM+V
*
* #

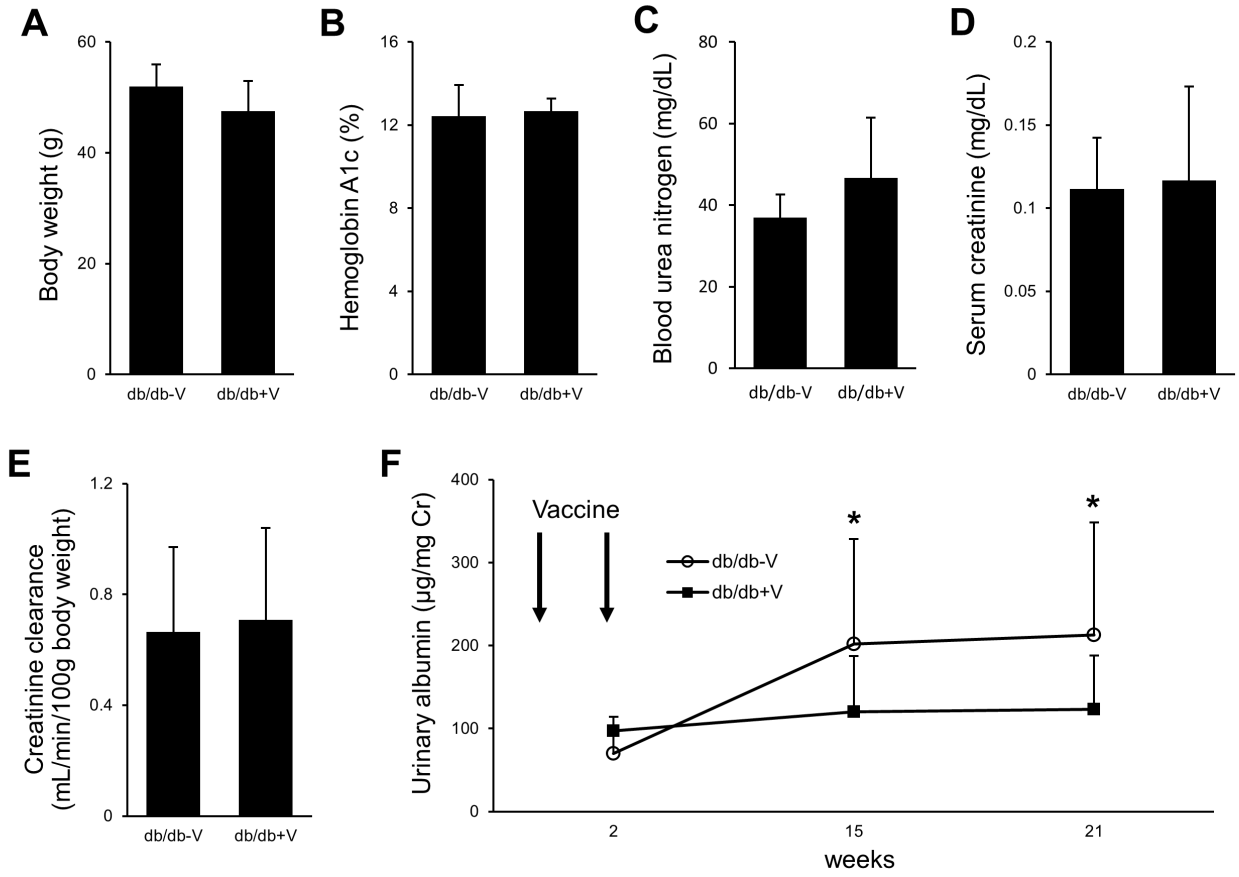

A
NDM-V
NDM+V
DM-V
DM+V
C
NDM-V
NDM+V
DM-V
DM+V
B
Glomerular volume
(×1000 μm³)
400
300
200
100
0
*
* #
NDM-V
NDM+V
DM-V
DM+V
D
Slit diaphragm width (nm)
0.05
0.04
0.03
0.02
0.01
0
*
*
NDM-V
NDM+V
DM-V
DM+V


A
Vash2 mRNA level relative to Actb
2
1.5
1
0.5
0
*
NDM-V
NDM+V
DM-V
DM+V
B
Vash1 mRNA level relative to Actb
1.5
1
0.5
0
*
NDM-V
NDM+V
DM-V
DM+V
C
Svbp mRNA level relative to Actb
1.5
1
0.5
0
NDM-V
NDM+V
DM-V
DM+V
D
Vegfa mRNA level relative to Actb
3
2
1
0
*
*
NDM-V
NDM+V
DM-V
DM+V
E
Angpt1 mRNA level relative to Actb
1.5
1
0.5
0
*
*
NDM-V
NDM+V
DM-V
DM+V
F
Angpt2 mRNA level relative to Actb
2
1.5
1
0.5
0
*
* #
NDM-V
NDM+V
DM-V
DM+V


A
NDM-V
NDM+V
NC-NDM
DM-V
DM+V
NC-DM


B
Number of Mac-2 positive cells (/glomerulus)
1.5
1
0.5
0
*
* #
NDM-V
NDM+V
DM-V
DM+V


C
NDM-V
NDM+V
DM-V
DM+V
ICAM-1
GAPDH
ICAM-1 / GAPDH ratio (densitometry)
2
1.5
1
0.5
0
*
#
NDM-V
NDM+V
DM-V
DM+V


A
Body weight (g)
60
40
20
0
db/db-V
db/db+V
B
Hemoglobin A1c (%)
16
12
8
4
0
db/db-V
db/db+V
C
Blood urea nitrogen (mg/dL)
80
60
40
20
0
db/db-V
db/db+V
D
Serum creatinine (mg/dL)
0.2
0.15
0.1
0.05
0
db/db-V
db/db+V
E
Creatinine clearance
(mL/min/100g body weight)
1.2
0.8
0.4
0
db/db-V
db/db+V
F
Urinary albumin (μg/mg Cr)
400
300
200
100
0
Vaccine
db/db-V
db/db+V
*
*
2
15
21
weeks