Stromal cell–derived factor-1 is upregulated by dipeptidyl peptidase-4 inhibition and has protective roles in progressive diabetic nephropathy

Satoru Takashima[1,7], Hiroki Fujita[1,7], Hiromi Fujishima[1], Tatsunori Shimizu[1], Takehiro Sato[1], Tsukasa Morii[1], Katsushi Tsukiyama[2], Takuma Narita[1], Takamune Takahashi[3], Daniel J. Drucker[4,5], Yutaka Seino[6] and Yuichiro Yamada[1]

[1]Division of Endocrinology, Metabolism and Geriatric Medicine, Akita University Graduate School of Medicine, Akita, Japan; [2]Division of Metabolism and Clinical Nutrition Science, Akita University Graduate School of Medicine, Akita, Japan; [3]Division of Nephrology and Hypertension, Vanderbilt University Medical Center, Nashville, Tennessee, USA; [4]Department of Medicine, University of Toronto, Toronto, Ontario, Canada; [5]The Lunenfeld-Tanenbaum Research Institute, Mt. Sinai Hospital, Toronto, Ontario, Canada; and [6]Kansai Electric Power Medical Research Institute, Osaka, Japan

The role of stromal cell–derived factor-1 (SDF-1) in the pathogenesis of diabetic nephropathy and its modification by dipeptidyl peptidase-4 (DPP-4) inhibition are uncertain. Therefore, we studied this independent of glucagon-like peptide-1 receptor (GLP-1R) signaling using two Akita diabetic mouse models, the diabetic-resistant C57BL/6-Akita and diabetic-prone KK/Ta-Akita. Increased SDF-1 expression was found in glomerular podocytes and distal nephrons in the diabetic-prone mice, but not in kidneys from diabetic-resistant mice. The DPP-4 inhibitor linagliptin, but not the GLP-1R agonist liraglutide, further augmented renal SDF-1 expression in both *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ diabetic-prone mice. Along with upregulation of renal SDF-1 expression, the progression of albuminuria, glomerulosclerosis, periglomerular fibrosis, podocyte loss, and renal oxidative stress was suppressed in linagliptin-treated *Glp1r*$^{+/+}$ diabetic-prone mice. Linagliptin treatment increased urinary sodium excretion and attenuated the increase in glomerular filtration rate which reflects glomerular hypertension and hyperfiltration. In contrast, selective SDF-1 receptor blockade with AMD3100 reduced urinary sodium excretion and aggravated glomerular hypertension in the *Glp1r*$^{+/+}$ diabetic-prone mice. Thus, DPP-4 inhibition, independent of GLP-1R signaling, contributes to protection of the diabetic kidney through SDF-1–dependent antioxidative and antifibrotic effects and amelioration of adverse renal hemodynamics.

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Correspondence: *Hiroki Fujita, Division of Endocrinology, Metabolism and Geriatric Medicine, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan. E-mail: hirofuji@gipc.akita-u.ac.jp*

[7]These authors contributed equally to this work.

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Stromal cell–derived factor-1 (SDF-1), also known as CXCL12, is a CXC chemokine originally isolated from a murine bone marrow stromal cell line.[1] Six SDF-1 isoforms (SDF-1α, SDF-1β, SDF-1γ, SDF-1δ, SDF-1ε, and SDF-1ϕ) generated by alternative mRNA splicing have been identified so far.[2] SDF-1α is the predominant isoform and ubiquitously expressed in multiple organs, including bone marrow, primordial germ cells, and the central nervous system.[3–7] The SDF-1β isoform is distributed less abundantly than the SDF-1α isoform and found in several highly vascularized organs.[2] In contrast, the SDF-1γ isoform is primarily expressed in the heart.[8,9] The other isoforms have recently been detected in human pancreas.[2] The chemokine SDF-1 family exerts a variety of biological actions by binding its cognate receptors, CXC chemokine receptor 4 (CXCR4) and CXC chemokine receptor 7 (CXCR7).[10–12] Importantly, SDF-1 is a crucial mediator of cell and tissue repair under diverse conditions such as pancreatic β-cell apoptosis,[13] vascular occlusion,[14] and ischemic renal injury.[15,16] Moreover, a few experimental studies have reported that the genetic deletion of CXCR4 results in the gastrointestinal and renal vascular defects.[17,18] Thus, the SDF-1/CXCR4 signaling pathway appears to play a pivotal role in vascular and cell protection and in vascular development in multiple organs.

SDF-1 has been localized to glomerular podocytes and distal tubular cells in both humans and rodent kidney.[15,19,20] A recent experimental study of subtotally nephrectomized hypertensive rats reported glomerular endothelial nitric oxide synthase activation following SDF-1 administration and acceleration of renal functional decline and tubulointerstitial fibrosis after treatment with a CXCR4 antagonist.[20] These findings suggest a renoprotective function for the

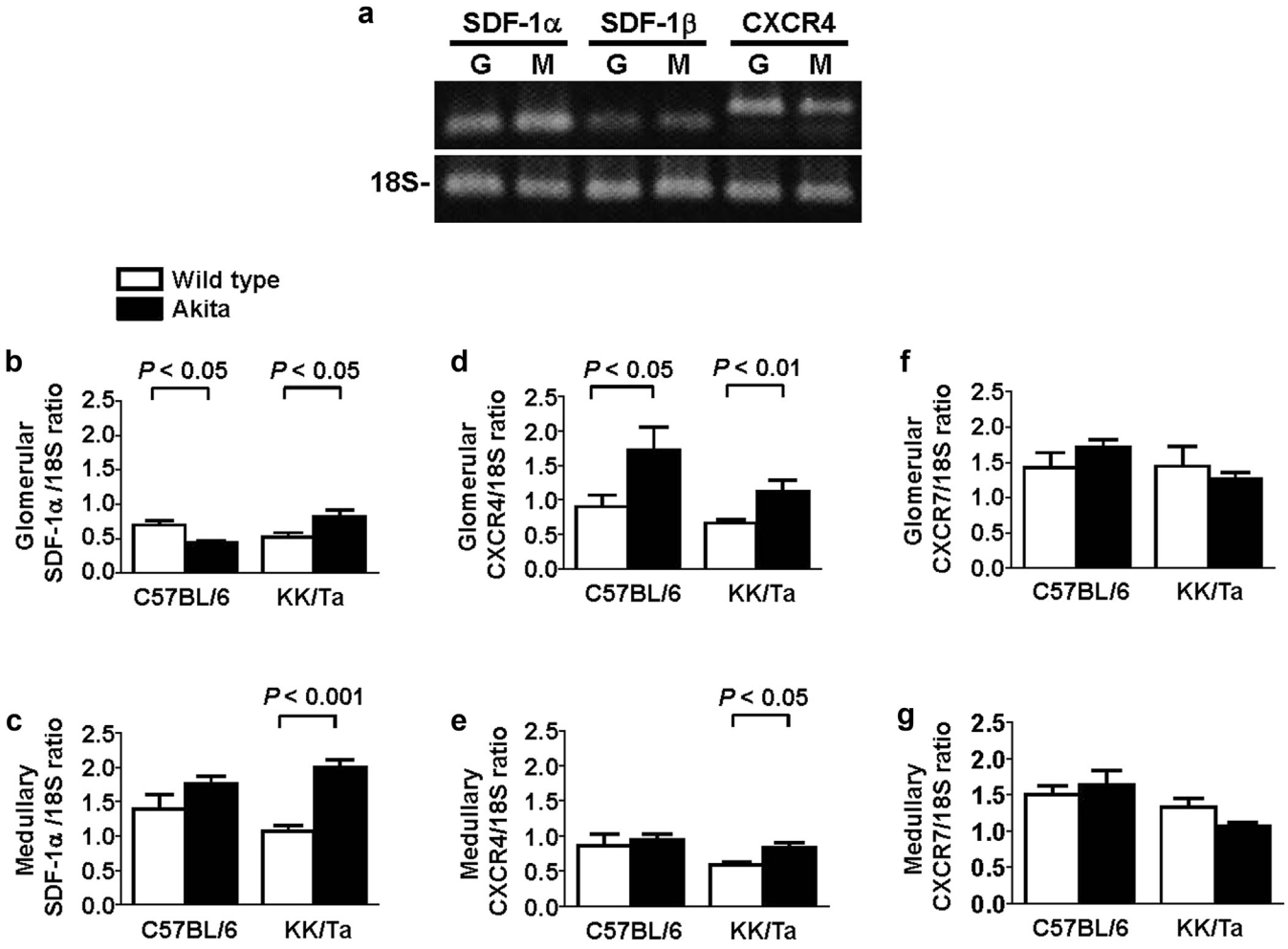

Figure 1 | Analysis of renal mRNA expression of stromal cell–derived factor-1α (SDF-1α), stromal cell–derived factor-1β (SDF-1β), CXC chemokine receptor 4 (CXCR4), and CXC chemokine receptor 7 (CXCR7) in C57BL/6-Akita and KK/Ta-Akita mice. Glomerular and medullary mRNA expression of SDF-1α, SDF-1β, CXCR4, and CXCR7 was analyzed in glomeruli and medullary tissues isolated from 15-week-old male mouse kidneys. Data were compared between nondiabetic wild-type and diabetic Akita mice in each mouse strain. (**a**) Electrophoresis images of reverse-transcriptase polymerase chain reaction products from KK/Ta-wild-type mice are shown in the upper panels. G and M indicate glomerulus and medullary tissue, respectively. (**b**–**g**) Lower panels show the results of quantitative reverse-transcriptase polymerase chain reaction analysis. $n = 5$ for C57BL/6–wild-type and KK/Ta–wild-type mouse groups. $n = 4$ for C57BL/6-Akita and KK/Ta-Akita mouse groups.

SDF-1/CXCR4 signaling pathway. In contrast, an experimental study of *db*/*db* diabetic mice has shown prevention of glomerulosclerosis, podocyte loss, and albuminuria following SDF-1 blockade.[19] Thus, the roles of SDF-1 in chronic experimental kidney disease induced by hypertension and diabetes remain uncertain.

SDF-1 is cleaved and inactivated by the dipeptidyl peptidase-4 (DPP-4) enzyme, which also cleaves incretin hormones such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP), leading to their inactivation.[21,22] Currently, DPP-4 inhibitors are widely used as antidiabetic agents, and their antidiabetic action is mainly mediated by an enhancement of active levels of GLP-1 and gastric inhibitory polypeptide. The importance of SDF-1α, also a well-known substrate of the DPP-4 enzyme,[21,23] for the therapeutic actions of DPP-4 inhibitors has been less well studied; however, treatment with DPP-4 inhibitors such as sitagliptin leads to elevated plasma total SDF-1α levels in patients with type 2 diabetes.[24] The DPP-4 enzyme is highly expressed in the kidney.[25] However, whether DPP-4 inhibition modulates the expression or activity of renal SDF-1 remains unclear. Moreover, the functional importance of SDF-1 in the diabetic kidney remains unclear.

Given evidence indicating the expression of SDF-1 in distal tubular cells, we hypothesized that SDF-1 may be involved in the regulation of urinary sodium excretion and renal hemodynamics. In addition, we speculated that DPP-4 inhibition may upregulate renal SDF-1 expression and modify renal phenotypes under chronic hyperglycemic conditions. To test these hypotheses and clarify the link of renal SDF-1 to the pathogenesis of diabetic nephropathy (DN), we first examined renal expression and localization of SDF-1 in two nonobese and hypoinsulinemic *Ins2*^{Akita} diabetic mouse models showing different susceptibility to the development and progression of DN, DN-resistant C57BL/6-*Ins2*^{Akita} (C57BL/6-Akita) and DN-prone KK/Ta-*Ins2*^{Akita} (KK/Ta-Akita).[26,27]

Next, we investigated the alteration of renal SDF-1 expression and its effects on renal phenotypes, including histology, hemodynamics, and biochemical markers following treatment with a DPP-4 inhibitor linagliptin in the DN-prone *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ KK/Ta-Akita mice.

RESULTS

Altered renal expression of SDF-1, CXCR4, and CXCR7 in diabetic mice with progressive nephropathy

As shown in Figure 1a, mRNA transcripts of SDF-1 and CXCR4 were detected in RNA from glomeruli and medullary tissues in normal mouse kidney. Furthermore, the quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of two major SDF-1 isoforms, SDF-1α and SDF-1β, revealed that SDF-1α is a predominant isoform in the mouse kidney whereas SDF-1β is expressed to a lesser extent in both glomerulus and renal medulla (Figure 1a).

We examined whether chronic hyperglycemia alters renal expression of SDF-1, CXCR4, and CXCR7 by using two Akita diabetic mouse models showing different susceptibility to the development and progression of DN. DN-prone KK/Ta-Akita and DN-resistant C57BL/6-Akita mice showed similar elevated levels of systolic blood pressure, serum lipids, blood urea nitrogen, plasma creatinine, and glomerular filtration rate (GFR) and renal hypertrophy as compared with their wild-type (WT) mice (Table 1). KK/Ta-Akita mice exhibited marked albuminuria whereas C57BL/6-Akita mice showed mild albuminuria (Table 1). In addition, urinary excretion levels of sodium and potassium were increased in both KK/Ta-Akita and C57BL/6-Akita mice (Table 1). Notably, the levels of both SDF-1α and CXCR4 mRNA expression were significantly elevated in glomeruli and medullary tissues of DN-prone KK/Ta-Akita mice as compared with in KK/Ta-WT mice (Figure 1b–e). In DN-resistant C57BL/6-Akita mice, only glomerular CXCR4 mRNA expression was significantly increased relative to that in C57BL/6-WT mice (Figure 1d).

Table 1 | Physiological and biochemical parameters in 15-week-old male C57BL/6–wild-type, C57BL/6-Akita, KK/Ta–wild-type, and KK/Ta-Akita mice

Parameter	C57BL/6–wild-type (n = 10)	C57BL/6-Akita (n = 7)	KK/Ta–wild-type (n = 6)	KK/Ta-Akita (n = 4)
BW (g)	25.9 ± 0.8	22.7 ± 0.6[b]	34.9 ± 1.5	21.7 ± 1.2[e]
SBP (mm Hg)	91 ± 2	106 ± 5[a]	111 ± 3	123 ± 4[d]
BG (mg/dl)	156 ± 9	345 ± 38[c]	184 ± 15	524 ± 49[e]
TC (mg/dl)	86 ± 4	77 ± 4	80 ± 3	168 ± 28[e]
TG (mg/dl)	81 ± 12	125 ± 7[a]	149 ± 10	220 ± 20[d]
BUN (mg/dl)	22.5 ± 1.1	40.3 ± 3.6[c]	19.6 ± 0.9	39.7 ± 2.3[e]
Cre (mg/dl)	0.17 ± 0.03	0.63 ± 0.08[b]	0.13 ± 0.02	0.73 ± 0.1[d]
ACR (μg/mg creatinine)	16.6 ± 0.8	76.9 ± 23.2[c]	82.6 ± 14.8	888.5 ± 120.0[e]
GFR (μl/min per g BW)	9.8 ± 0.4	16.2 ± 1.5[a]	11.2 ± 1.5	20.6 ± 1.2[d]
LKW/BW (g/kg)	5.7 ± 0.2	8.7 ± 0.3[c]	6.4 ± 0.2	11.5 ± 1.0[e]
Urinary sodium (mEq/mg creatinine)	0.38 ± 0.04	1.91 ± 0.46[c]	0.29 ± 0.04	1.04 ± 0.08[d]
Urinary potassium (mEq/mg creatinine)	0.30 ± 0.02	2.24 ± 0.53[c]	0.50 ± 0.03	1.50 ± 0.10[e]

ACR, urinary albumin-to-creatinine ratio; BG, blood glucose; BUN, blood urea nitrogen; BW, body weight; Cre, plasma creatinine; GFR, glomerular filtration rate; LKW, left kidney weight; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride. Values are means ± SEM.
[a]$P < 0.05$ versus C57BL/6–wild-type.
[b]$P < 0.01$ versus C57BL/6–wild-type.
[c]$P < 0.001$ versus C57BL/6–wild-type.
[d]$P < 0.05$ versus KK/Ta–wild-type.
[e]$P < 0.01$ versus KK/Ta–wild-type.

SDF-1 was localized by immunohistochemistry to glomerular podocytes, distal tubules, and collecting ducts, but not in proximal tubules or other glomerular regions (Figure 2). Consistent with the findings of mRNA expression, strong staining was observed in glomerular podocytes and distal tubules of DN-prone KK/Ta-Akita mice (Figure 2). Collectively, these findings suggest that SDF-1, mainly SDF-1α, and CXCR4 are upregulated in diabetic mice with progressive nephropathy.

Upregulation of renal SDF-1 by DPP-4 inhibition in *Glp1r*$^{+/+}$ KK/Ta-Akita mice

We investigated whether DPP-4 inhibition upregulates SDF-1 in the kidneys of DN-prone *Glp1r*$^{+/+}$ KK/Ta-Akita mice. Greater SDF-1 immunopositivity was observed in glomerular podocytes, distal tubules, and collecting ducts of *Glp1r*$^{+/+}$ KK/Ta-Akita mice treated with the DPP-4 inhibitor linagliptin compared with staining in the vehicle-treated control group. In contrast, levels of SDF-1 immunopositivity were not increased in kidneys from *Glp1r*$^{+/+}$ KK/Ta-Akita mice treated with the glucagon-like peptide-1 receptor (GLP-1R) agonist liraglutide (Figure 3a). Similarly, RT-PCR analysis indicated a significantly increased expression of glomerular and medullary SDF-1α mRNA in kidneys from linagliptin-treated *Glp1r*$^{+/+}$ KK/Ta-Akita mice, but not in renal tissue from liraglutide-treated *Glp1r*$^{+/+}$ KK/Ta-Akita mice (Figure 3b and c). No significant differences in glomerular and medullary CXCR4 mRNA expression were observed among the three treatment groups (Figure 3d and e). Glomerular CXCR7 mRNA expression was increased in linagliptin-treated *Glp1r*$^{+/+}$ KK/Ta-Akita mice (Figure 3f).

Effects of DPP-4 inhibition on renal function, histological changes, oxidative stress, and fibrogenic markers in DN-prone *Glp1r*$^{+/+}$ KK/Ta-Akita mice

As shown in Table 2, levels of plasma creatinine and GFR were significantly lower in linagliptin-treated *Glp1r*$^{+/+}$ KK/Ta-Akita mice than in the vehicle-treated control group. In addition, significantly increased urinary excretion of sodium and potassium was observed in linagliptin-treated *Glp1r*$^{+/+}$ KK/Ta-Akita mice but not in mice treated with liraglutide. Furthermore, albuminuria was significantly reduced after both linagliptin and liraglutide treatment relative to the baseline values (Figure 4b). Both the linagliptin- and liraglutide-treated groups exhibited reduced mesangial expansion and higher podocyte numbers compared with the vehicle-treated control group (Figure 4a, c, and d). Masson trichrome staining survey showed reduced periglomerular

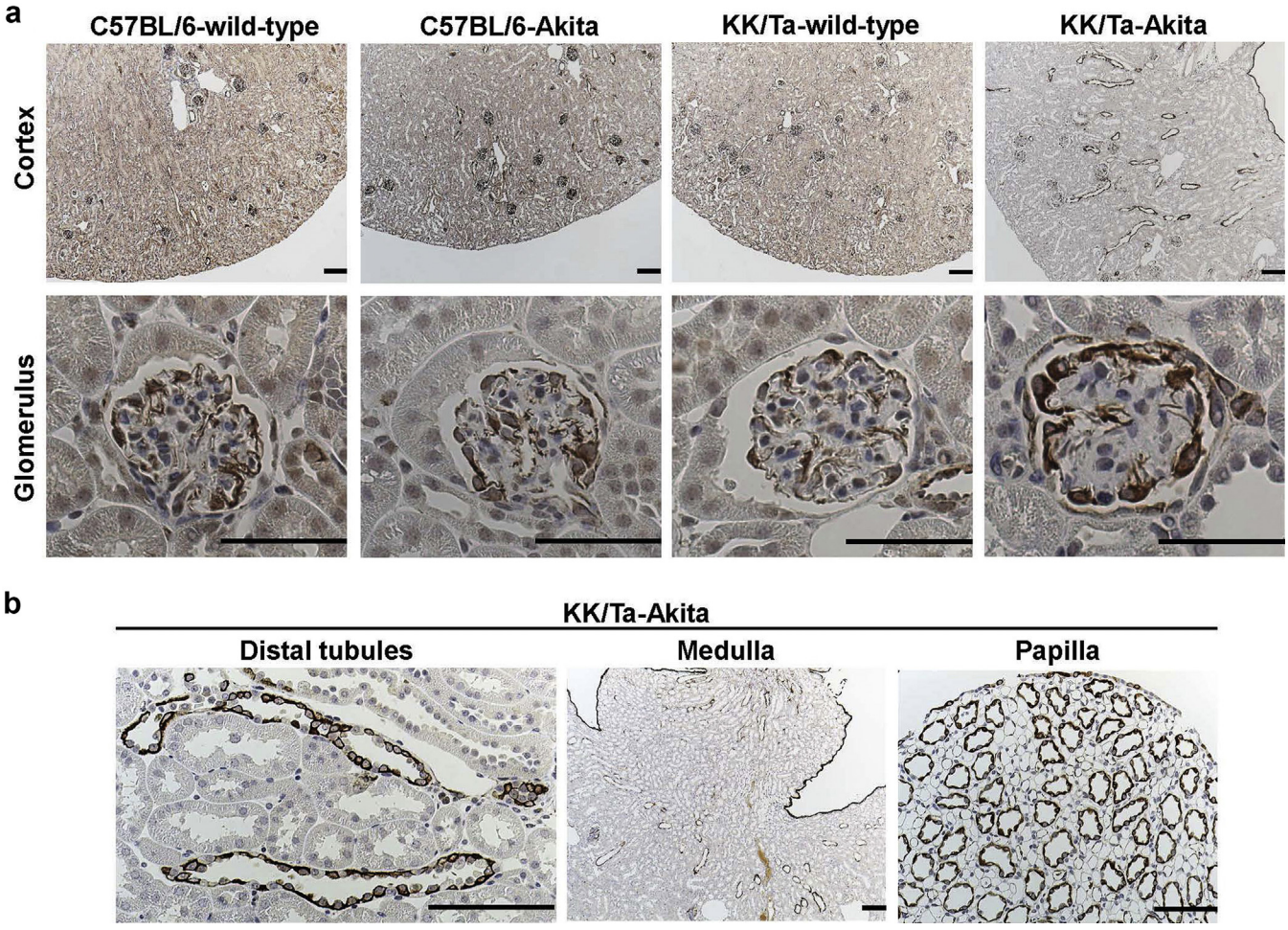

Figure 2 | Stromal cell–derived factor-1 immunohistochemistry in C57BL/6-Akita and KK/Ta-Akita mice. (**a**) Representative cortical and glomerular images of stromal cell–derived factor-1 immunohistochemistry in 15-week-old male mice. Bar = 100 μm for cortex images or 50 μm for glomerulus images. (**b**) Representative stromal cell–derived factor-1 immunohistochemistry images of distal tubules, medulla, and papilla in the kidney of KK/Ta-Akita mice. Bar = 50 μm for distal tubules and papilla images or 100 μm for medulla image.

fibrosis in both the linagliptin- and liraglutide-treated groups (Figure 4a).

The extent of renal oxidative stress, glomerular nitric oxide (NO), and renal fibrogenic marker expression was examined by malondialdehyde immunohistochemistry, fluorescence histochemistry, and diaminofluorescein-2 diacetate reaction. Relative intensity of staining for malondialdehyde, a lipid peroxidation marker, in distal tubules was reduced in both the linagliptin- and liraglutide-treated groups (Figure 5a). A reduction of glomerular superoxide levels and an elevation of glomerular nitric oxide levels were observed in both the linagliptin- and liraglutide-treated groups (Figure 5a–c). Moreover, the intensity of fluorescence signals for fibrogenic markers, thrombospondin-1 and fibronectin, was diminished in the linagliptin- and liraglutide-treated groups versus in the vehicle-treated control group (Figure 5a, d, and e).

Effects of DPP-4 inhibition on renal SDF-1 expression and urinary sodium excretion in *Glp1r*$^{-/-}$ KK/Ta-Akita mice

To elucidate a contribution for GLP-1 as a putative DPP-4 substrate acting on the kidney, we treated GLP-1R–deficient (*Glp1r*$^{-/-}$) KK/Ta-Akita mice with linagliptin. The linagliptin-treated *Glp1r*$^{-/-}$ KK/Ta-Akita mice exhibited strong immunohistochemical staining for SDF-1 in distal tubules (Figure 6a) and increased medullary SDF-1α expression in RT-PCR analysis (Figure 6c). Glomerular SDF-1α expression levels were similar between the vehicle- and linagliptin-treated *Glp1r*$^{-/-}$ KK/Ta-Akita mice (Figure 6a and b). Glomerular and medullary CXCR4 expression levels were not different between the two groups (Figure 6d and e). In contrast, both glomerular and medullary CXCR7 expression levels were increased in the linagliptin-treated *Glp1r*$^{-/-}$ KK/Ta-Akita mice (Figure 6f and g). Notably, linagliptin-treated mice showed higher urinary sodium excretion levels compared with the vehicle-treated group, whereas urinary potassium excretion levels were not significantly different between the groups (Table 3). Although reduction of albuminuria was not observed after treatment with linagliptin in *Glp1r*$^{-/-}$ KK/Ta-Akita mice (Figure 7b), linagliptin-treated mice showed lower mesangial expansion, higher podocyte numbers, and reduced malondialdehyde immunopositivity in distal tubules compared with the vehicle-treated control mice

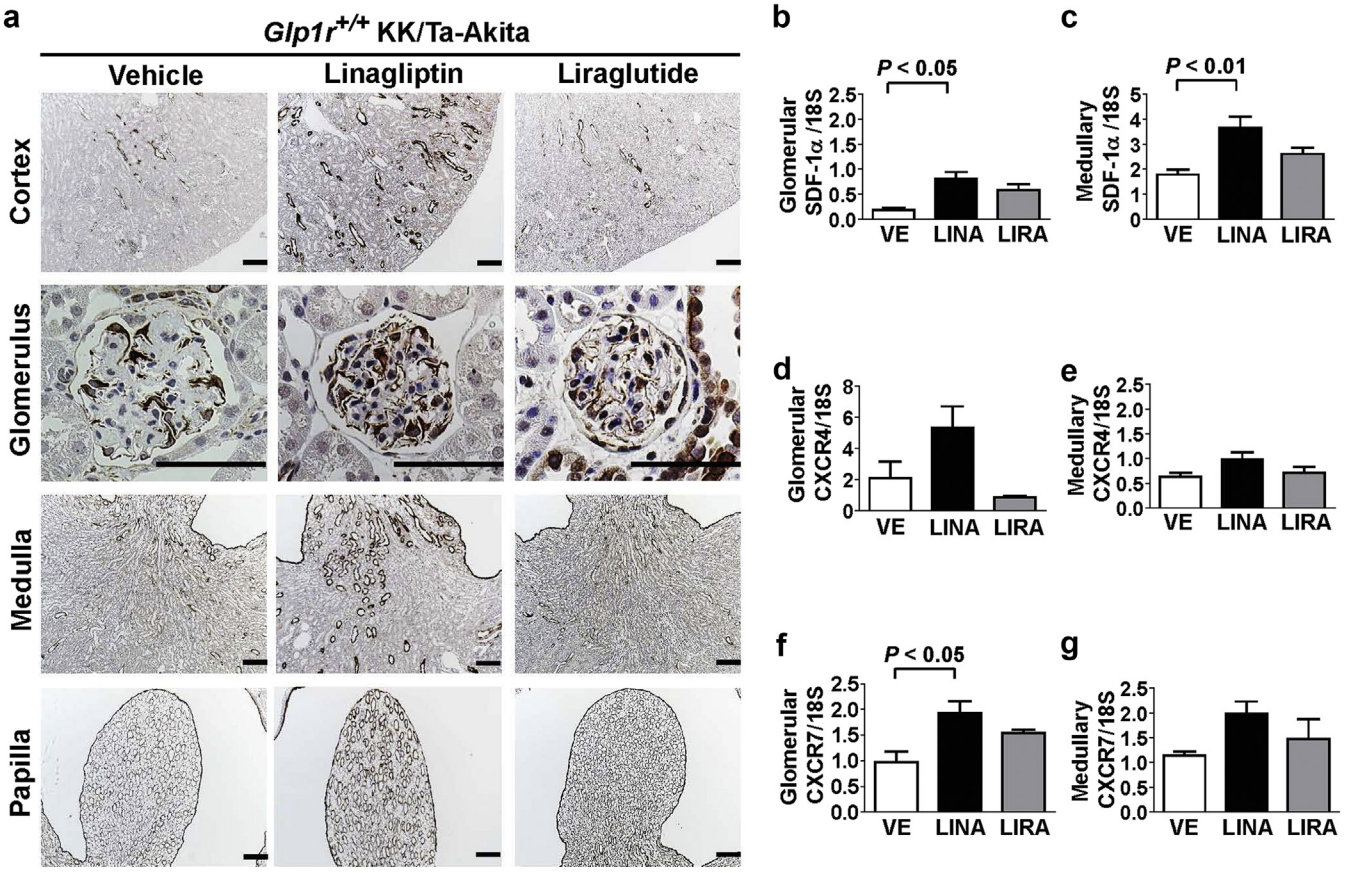

Figure 3 | Changes in renal expression of stromal cell–derived factor-1 (SDF-1), CXC chemokine receptor 4 (CXCR4), and CXC chemokine receptor 7 (CXCR7) in *Glp1r*$^{+/+}$ KK/Ta-Akita mice after treatment with linagliptin (LINA) and liraglutide (LIRA). Treatment with LINA (5 mg/kg/d) or LIRA (200 μg/kg/d) started at 6 weeks of age and ended at 12 weeks of age in male *Glp1r*$^{+/+}$ KK/Ta-Akita mice. (**a**) Representative images of renal SDF-1 immunohistochemistry. Bars = 150 μm for cortex, medulla, and papilla images or 50 μm for glomerulus images. (**b–g**) Right panels show the results of quantitative reverse-transcriptase polymerase chain reaction analysis of SDF-1α, CXCR4, and CXCR7. $n = 3$ for the vehicle (VE)-treated group, $n = 5$ for the LINA-treated group, and $n = 4$ for the LIRA-treated group in the glomerular analysis. $n = 5$ for the VE-treated group, $n = 5$ for the LINA-treated group, and $n = 4$ for the LIRA-treated group in the medullary analysis.

Table 2 | Physiological and biochemical parameters after 6-week treatment with linagliptin or liraglutide in male *Glp1r*$^{+/+}$ KK/Ta-Akita mice

Parameter	Vehicle ($n = 7$)	Linagliptin ($n = 7$)	Liraglutide ($n = 6$)
BW (g)	22.1 ± 0.4	22.6 ± 0.6	22.2 ± 0.6
SBP (mm Hg)	131 ± 5	132 ± 4	138 ± 11
BG (mg/dl)	589 ± 36	526 ± 47	440 ± 48
TC (mg/dl)	163 ± 13	148 ± 19	169 ± 9
TG (mg/dl)	147 ± 25	153 ± 19	215 ± 15
BUN (mg/dl)	43.9 ± 5.4	35.7 ± 1.8	39.9 ± 1.1
Cre (mg/dl)	0.69 ± 0.21	0.17 ± 0.04[a]	0.55 ± 0.09
GFR (μl/min per g BW)	13.5 ± 1.7	8.3 ± 1.5[a]	ND
LKW/BW (g/kg)	11.7 ± 0.4	13.8 ± 1.3	11.5 ± 0.2
Urinary sodium (mEq/mg creatinine)	0.98 ± 0.13	1.90 ± 0.27[a]	1.06 ± 0.24
Urinary potassium (mEq/mg creatinine)	1.42 ± 0.08	2.31 ± 0.31[a]	1.35 ± 0.21

BG, blood glucose; BUN, blood urea nitrogen; BW, body weight; Cre, plasma creatinine; GFR, glomerular filtration rate; LKW, left kidney weight; ND, not determined; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride.
Values are means ± SEM.
[a]$P < 0.05$ versus vehicle.

(Figure 7a, c, and d). In addition, both cortical and medullary cyclic adenosine monophosphate (cAMP) levels were elevated in the linagliptin-treated mice (Figure 7e and f). These observations demonstrate that many of the renal actions of linagliptin are independent of GLP-1R signaling and potentially linked to potentiation of SDF-1 activity in distal tubules.

Alterations of renal phenotypes, urinary sodium excretion, and renal hemodynamics by a SDF-1α receptor (CXCR4) antagonist

We next examined renal phenotypes, urinary sodium excretion, and renal hemodynamics following administration of the highly selective SDF-1α receptor (CXCR4) antagonist AMD3100 in *Glp1r*$^{+/+}$ KK/Ta-Akita mice with enhanced urinary sodium excretion and elevated GFR. Because high-dose and long-term treatment with AMD3100 was lethal for the KK/Ta-Akita mice (data not shown), we studied mice after short-term administration of AMD3100. Following a 10-day treatment with AMD3100, elevation of systolic blood pressure was observed (Table 4). Although both the vehicle- and AMD3100-treated groups exhibited an excessive urine output owing to osmotic diuresis, urine volume was similar between

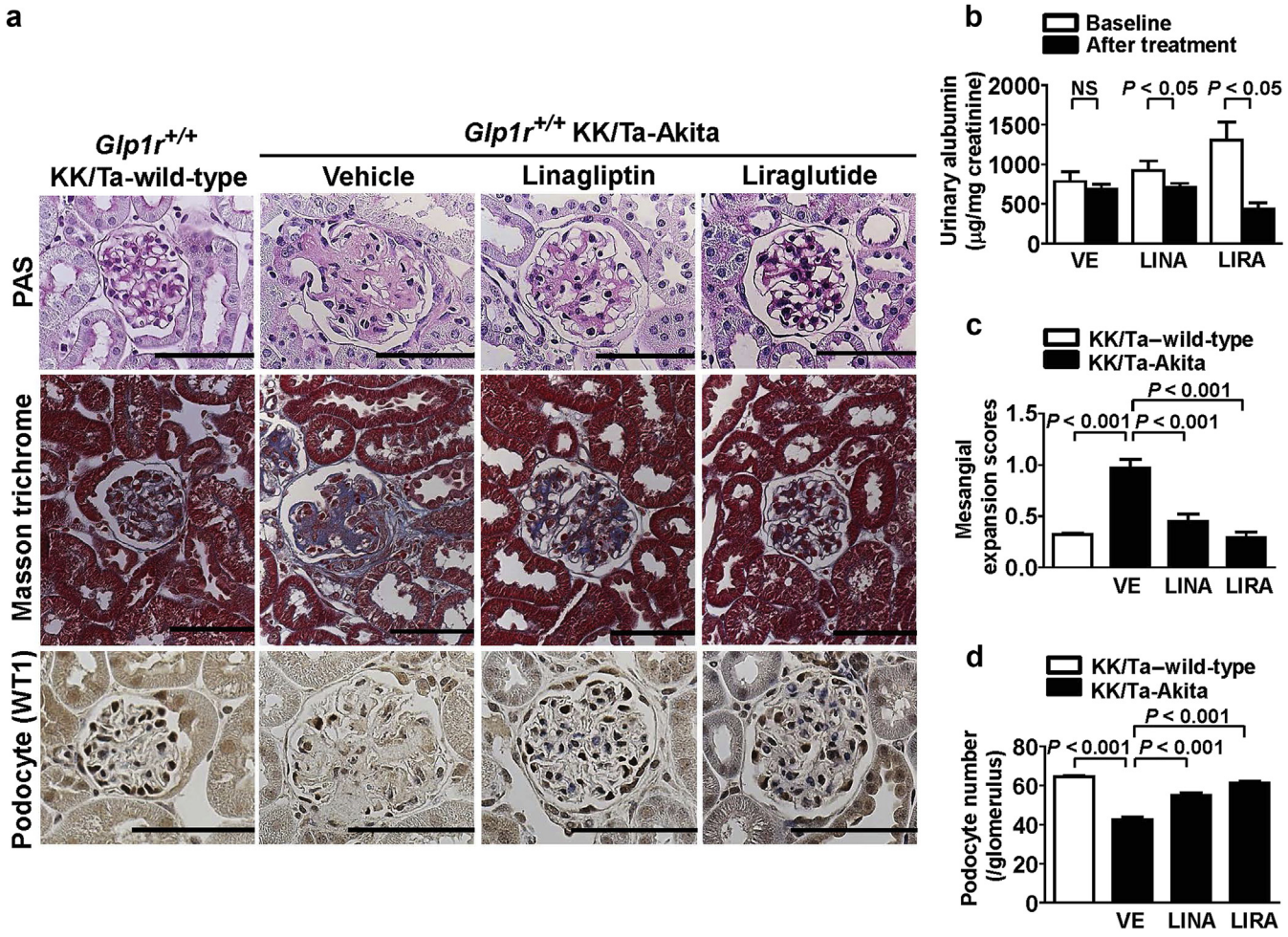

Figure 4 | Renal histopathology in *Glp1r*$^{+/+}$ KK/Ta-Akita mice after treatment with linagliptin (LINA) or liraglutide (LIRA). (**a**) Representative glomerulus images of periodic acid–Schiff (PAS), Masson trichrome, and WT1 staining. Bar = 50 μm for all images. (**b**) Changes in urinary albumin levels. $n = 7$ for the vehicle (VE)-treated group, $n = 7$ for the LINA-treated group, and $n = 6$ for the LIRA-treated group. NS, not significant. (**c**) Glomerular mesangial expansion scores. $n = 7$ for the age-matched male KK/Ta–wild-type mouse group, $n = 6$ for the VE-treated group, $n = 4$ for the LINA-treated group, and $n = 5$ for the LIRA-treated group. (**d**) The number of WT1-positive podocytes in glomeruli. $n = 7$ for the age-matched male KK/Ta–wild-type mouse group, $n = 6$ for the VE-treated group, $n = 4$ for the LINA-treated group, and $n = 5$ for the LIRA-treated group.

the two groups (Table 4). Albuminuria was not significantly changed during the treatment, whereas the AMD3100-treated mice showed higher mesangial expansion and lower podocyte numbers compared with the vehicle-treated control mice (Figure 8a–d). Interestingly, the *Glp1r*$^{+/+}$ KK/Ta-Akita mice showed a significant progressive reduction of urinary sodium excretion after AMD3100 administration (Figure 8e). Decreased urinary potassium excretion was also observed after 10 days in the AMD3100-treated mice (Figure 8f). In addition, the AMD3100-treated mice showed a markedly higher GFR compared with the vehicle-treated control group (Figure 8g). On the basis of these findings, it is conceivable that distal tubular SDF-1 enhances urinary sodium excretion and contributes to normalization of glomerular hypertension and hyperfiltration.

DISCUSSION

The present study demonstrates that SDF-1 and its cognate receptor CXCR4 are expressed in both cortical and medullary tissues of normal mouse kidneys. Through RT-PCR analysis, we identified SDF-1α as the predominant isoform in the kidney, whereas the SDF-1β isoform is expressed less abundantly relative to the SDF-1α isoform. The anti–SDF-1 antibody that we used in this study detects both SDF-1α and SDF-1β. On the basis of the aforesaid findings, the staining signals detected by SDF-1 immunohistochemistry predominantly reflect expression of SDF-1α.

KK/Ta-Akita mouse is a well-established model of progressive DN characterized by overt albuminuria, glomerular hypertension and hyperfiltration, and renal hypertrophy, as shown in Table 1. Advanced glomerulosclerosis also develops in this mouse.[26,28] In contrast, mild albuminuria and less extensive glomerular lesions develop in C57BL/6-Akita mice, which were initially generated in our facility, despite their exhibiting marked hyperglycemia.[26,27] KK/Ta-Akita mice with progressive DN, but not C57BL/6-Akita mice with mild DN, exhibited increased levels of expression of glomerular and medullary SDF-1α in RT-PCR analysis and greater intensity

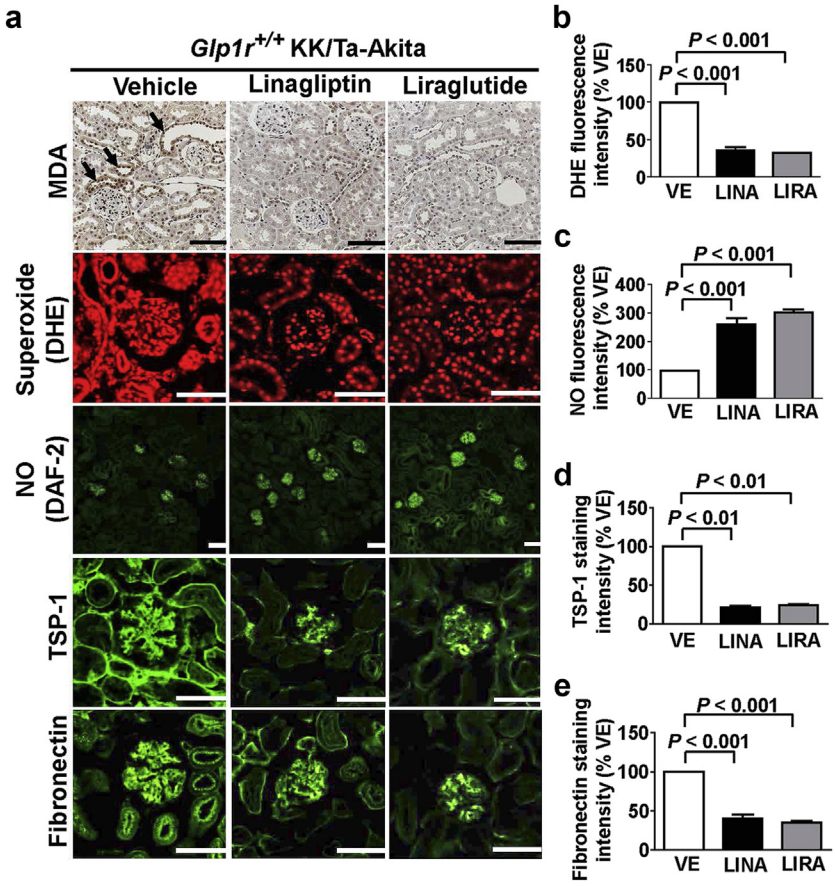

Figure 5 | Renal oxidative stress, nitric oxide (NO) level, inflammation, and fibrosis in *Glp1r*$^{+/+}$ KK/Ta-Akita mice after treatment with linagliptin (LINA) or liraglutide (LIRA). (**a**) Representative glomerular images of malondialdehyde (MDA) immunohistochemistry, dihydroethidium (DHE) staining, *in situ* NO detection by diaminofluorescein-2 diacetate (DAF-2DA) perfusion method, and thrombospondin-1 (TSP-1) and fibronectin immunohistochemistry. Arrows indicate distal tubules. Bar = 50 μm for all images. Semiquantified fluorescence intensity of (**b**) DHE staining, (**c**) NO, (**d**) TSP-1, and (**e**) fibronectin were determined as described in Materials and Methods. $n = 5$ for the vehicle (VE) -treated group, $n = 4$ for the LINA-treated group, and $n = 5$ for the LIRA-treated group in DHE staining and *in situ* NO detection. $n = 4$ for the VE-treated group, $n = 5$ for the LINA-treated group, and $n = 5$ for the LIRA-treated group in TSP-1 and fibronectin immunohistochemistry.

of SDF-1 immunohistochemical expression in glomerular podocytes, distal tubular cells, and collecting ducts. Experimental studies have reported that SDF-1 expression was increased in the ischemic injured kidney and heart of mice and rats, potentially contributing to the protection of damaged tissues.[15,16,29] Given several lines of evidence indicating that SDF-1 serves as a pivotal mediator of cell and tissue repair,[13–16] the SDF-1 upregulation observed in the KK-Akita mouse kidneys may represent a defense system against the progression of diabetic renal injury.

The DPP-4 enzyme is known to cleave not only GLP-1, which is regarded as its primary glucoregulatory substrate, but also multiple peptides including SDF-1α.[21–23] In the present study, we found that treatment with the DPP-4 inhibitor linagliptin caused further increases in expression of SDF-1α mRNA in glomeruli and medullary tissues; SDF-1 protein was also increased in glomerular podocytes, distal tubular cells, and collecting ducts in the *Glp1r*$^{+/+}$ KK/Ta-Akita mice. In contrast, significant renal SDF-1 upregulation was not observed in the *Glp1r*$^{+/+}$ KK/Ta-Akita mice treated with a GLP-1 receptor agonist liraglutide. Additionally, renal SDF-1 upregulation following linagliptin treatment was observed in *Glp1r*$^{-/-}$ KK/Ta-Akita mice. Thus, our current data suggest that DPP-4 inhibition upregulates SDF-1 expression in the diabetic kidneys in a GLP-1R-independent manner. Regarding the SDF-1 receptors CXCR4 and CXCR7, linagliptin treatment increased levels of expression of CXCR7 mRNA in glomeruli of the *Glp1r*$^{+/+}$ KK/Ta-Akita mice and glomeruli and medullary tissues of the *Glp1r*$^{-/-}$ KK/Ta-Akita mice. An experimental mouse study revealed an essential role for CXCR7 signaling in blood vessel protection and cardiac endothelial cell survival.[30] Therefore, renal CXCR7 upregulation following linagliptin treatment may partly contribute to protection of glomeruli as observed in the present study.

In parallel with renal SDF-1 upregulation, linagliptin treatment attenuated the progression of albuminuria, and the extent of glomerulosclerosis, periglomerular fibrosis, and podocyte loss in the KK/Ta-Akita mice. In addition, renal oxidative stress and glomerular and tubular fibrogenic changes were diminished following linagliptin treatment in the KK/Ta-Akita mice, as evidenced by reductions

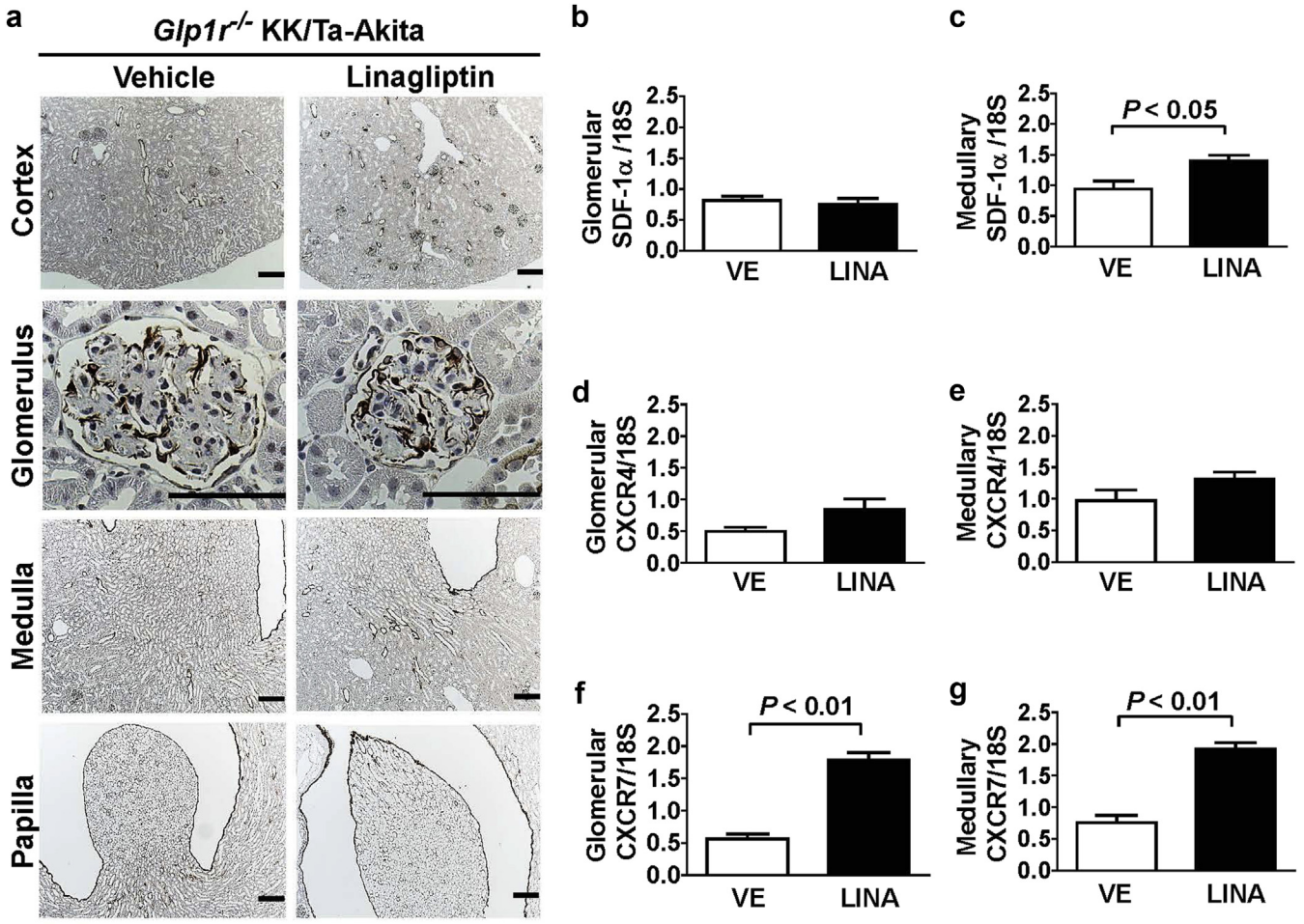

Figure 6 | Changes in renal expression of stromal cell–derived factor-1, CXC chemokine receptor 4 (CXCR4), and CXC chemokine receptor 7 (CXCR7) in *Glp1r*$^{-/-}$ KK/Ta-Akita mice after treatment with linagliptin (LINA). Treatment with linagliptin (5 mg/kg/d) started at 6 weeks of age and ended at 12 weeks of age in male *Glp1r*$^{-/-}$ KK/Ta-Akita mice. (**a**) Representative images of renal stromal cell–derived factor-1 immunohistochemistry. Bar = 150 μm for cortex, medulla, and papilla images or 50 μm for glomerulus images. (**b–g**) Right panels show the results of quantitative reverse-transcriptase polymerase chain reaction analysis of stromal cell–derived factor-1α, CXCR4, and CXCR7. $n = 5$ per group for the vehicle (VE)-treated group and the LINA-treated group.

of renal malondialdehyde immunopositivity, superoxide, thrombospondin-1, and fibronectin levels and elevation of glomerular nitric oxide levels. A previous study has shown that SDF-1 induces an elevation of cAMP and an activation of protein kinase A through increased CXCR4 receptor signaling in cultured neuron cells.[31] Furthermore, recent experiments have demonstrated that the production of nicotinamide adenine dinucleotide phosphate oxidase–dependent reactive oxygen species, including superoxide anion, is reduced by the activation of the cAMP–protein kinase A signaling pathway, resulting in attenuation of oxidative stress in colon cells, vascular smooth muscle cells, and renal tissues.[28,32–34] Additionally, reduction of renal oxidative stress leads to the subsequent inhibition of scavenging nitric oxide from glomerular endothelial cells.[27,28] It is well known that oxidative stress upregulates fibrogenic cytokines such as transforming growth factor-β1 and connective tissue growth factor in addition to thrombospondin-1, which is an endogenous activator of transforming growth factor–β1, leading to glomerulosclerosis and renal interstitial fibrosis.[35–37] The present study also indicated that renal cAMP levels were elevated

Table 3 | Physiological and biochemical parameters after 6-week treatment with linagliptin in male *Glp1r*$^{-/-}$ KK/Ta-Akita mice

Parameter	Vehicle ($n = 4$)	Linagliptin ($n = 5$)
BW (g)	19.3 ± 0.7	20.9 ± 0.5
BG (mg/dl)	311 ± 84	540 ± 81
TC (mg/dl)	174 ± 28	159 ± 16
TG (mg/dl)	296 ± 69	162 ± 13[a]
BUN (mg/dl)	42.4 ± 1.6	34.8 ± 1.9[a]
Cre (mg/dl)	0.58 ± 0.06	0.54 ± 0.11
LKW/BW (g/kg)	10.3 ± 0.8	10.6 ± 0.4
Urinary sodium (mEq/mg creatinine)	1.12 ± 0.08	2.07 ± 0.34[a]
Urinary potassium (mEq/mg creatinine)	1.83 ± 0.05	2.54 ± 0.37

BG, blood glucose; BUN, blood urea nitrogen; BW, body weight; Cre, plasma creatinine; LKW, left kidney weight; TC, total cholesterol; TG, triglyceride.
Values are means ± SEM.
[a]$P < 0.05$ versus vehicle.

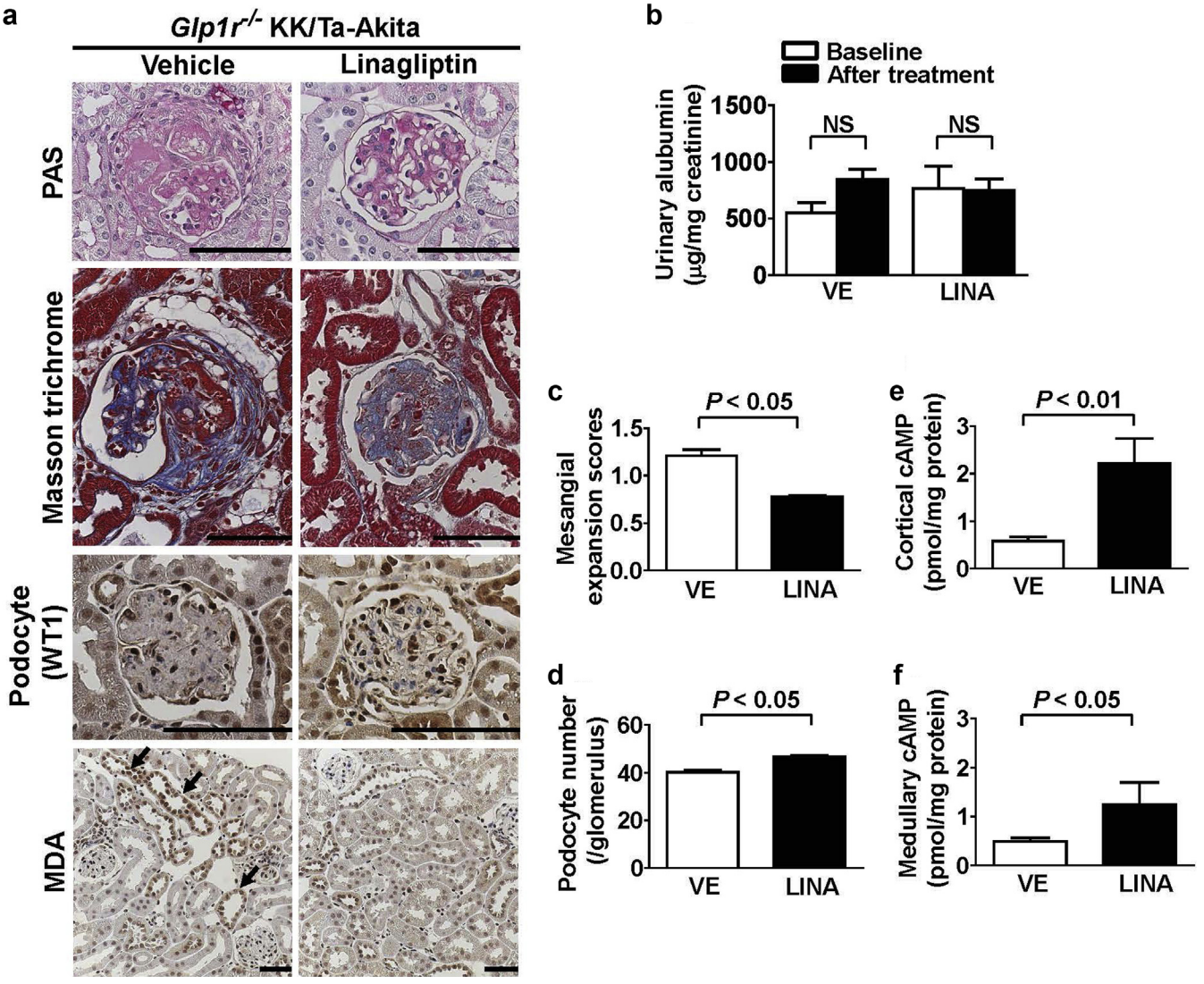

Figure 7 | Renal histopathology and changes in renal cyclic adenosine monophosphate (cAMP) levels in *Glp1r*$^{-/-}$ KK/Ta-Akita mice after treatment with linagliptin (LINA). (**a**) Representative glomerulus images of periodic acid–Schiff (PAS), Masson trichrome, and WT1 staining and malondialdehyde (MDA) immunohistochemistry. Arrows indicate distal tubules. Bar = 50 μm for all images. (**b**) Changes in urinary albumin levels. $n = 4$ for the vehicle (VE)-treated group and $n = 5$ for the LINA-treated group. NS, not significant. (**c**) Glomerular mesangial expansion scores. $n = 4$ for the VE-treated group and $n = 4$ for the LINA-treated group. (**d**) The number of WT1-positive podocytes in glomeruli. $n = 4$ for the VE-treated group and $n = 4$ for the LINA-treated group. (**e,f**) Renal cortical and medullary cAMP levels. $n = 6$ for the VE-treated group and $n = 6$ for the LINA-treated group.

in parallel with renal SDF-1 upregulation following DPP-4 inhibition even in *Glp1r*$^{-/-}$ KK/Ta-Akita mice. This observation would verify the presence of a SDF-1/cAMP signaling pathway independent of GLP-1R signaling in kidney. Hence, upregulation of renal SDF-1 expression by DPP-4 inhibition may contribute to preventing the progression of renal lesions, including interstitial fibrosis, possibly via the cAMP–protein kinase A pathway activation and the subsequent reduction of renal oxidative stress. Regarding the mechanism underlying the antifibrotic effects of linagliptin treatment in the kidney, recent experimental studies have shown that linagliptin restored levels of microRNA 29s, which are known to protect multiple organs from fibrotic injury, and suppressed endothelial levels of integrin β1 and phospho-integrin β1, which are associated with fibrosis progression, in the kidneys of streptozotocin-induced diabetic CD1 mice.[38,39] Thus, DPP-4 inhibition with linagliptin may induce antifibrotic effects in the diabetic kidney via several complementary mechanisms.

It is worth noting that SDF-1 expression was upregulated in podocytes of the KK/Ta-Akita mouse kidneys following DPP-4 inhibition. The podocyte and its foot processes function as a component of the glomerular filtration barrier involved in the restriction of passage of plasma protein including albumin.[40] A large body of evidence indicates that podocyte loss, including podocyte foot process effacement, contributes to the development of proteinuria

Table 4 | Physiological and biochemical parameters after 10-day treatment with AMD3100 in male *Glp1r*$^{+/+}$ KK/Ta-Akita mice

	Vehicle (n = 6)		AMD3100 (n = 6)	
Parameter	Baseline	10-day treatment	Baseline	10-day treatment
BW (g)	20.5 ± 0.6	20.8 ± 0.6	20.8 ± 0.3	20.4 ± 0.3
SBP (mm Hg)	122 ± 4	117 ± 8	110 ± 2	132 ± 2[a]
BG (mg/dl)	574 ± 38	497 ± 43	521 ± 41	572 ± 39
TC (mg/dl)	ND	162 ± 14	ND	167 ± 27
TG (mg/dl)	ND	226 ± 21	ND	211 ± 19
BUN (mg/dl)	ND	38.4 ± 3.5	ND	43.5 ± 4.2
Cre (mg/dl)	ND	0.55 ± 0.19	ND	0.72 ± 0.28
LKW/BW (g/kg)	ND	10.0 ± 0.2	ND	10.3 ± 0.2
Urine volume (ml/day)	26.8 ± 2.3	31.7 ± 1.4	26.1 ± 2.6	30.4 ± 1.6

BG, blood glucose; BUN, blood urea nitrogen; BW, body weight; Cre, plasma creatinine; LKW, left kidney weight; ND, not determined; SBP, systolic blood pressure; TC, total cholesterol; TG, triglyceride.
Values are means ± SEM.
[a]$P < 0.01$ versus AMD3100 baseline.

(albuminuria).[41–43] In the present study, linagliptin treatment significantly suppressed the progression of albuminuria and podocyte loss in the *Glp1r*$^{+/+}$ KK/Ta-Akita mice. In this regard, SDF-1 upregulation in podocytes by DPP-4 inhibition with linagliptin may work to protect the podocytes through the antioxidative effects as already mentioned, consequently suppressing an increase in albuminuria.

Another interesting and novel observation in the current study is the finding of SDF-1 upregulation in segments of the distal nephron following DPP-4 inhibition in both *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ KK/Ta-Akita mice. The distal convoluted tubule (DCT) mediates reabsorption of 5% to 10% of the filtered sodium load,[44] and this sodium reabsorption in the distal nephron is driven chiefly by two major pathways, thiazide-sensitive Na^+-Cl^- cotransporter in the early segment of DCT (DCT1) and the epithelial sodium channel in the late segment of DCT (DCT2) and connecting tubule.[45,46] In addition, the DCT2, the connecting tubule, and the collecting duct are known as an aldosterone-sensitive distal nephron that mediates sodium and potassium transport through aldosterone action.[47,48] In the current study, we observed a significant increase in urinary sodium excretion along with SDF-1 upregulation in the distal nephron following DPP-4 inhibition in both *Glp1r*$^{+/+}$ and *Glp1r*$^{-/-}$ KK/Ta-Akita mice. Furthermore, administration of the SDF-1α receptor (CXCR4) antagonist dramatically reduced urinary sodium excretion in the *Glp1r*$^{+/+}$ KK/Ta-Akita mice. These data provide evidence for involvement of SDF-1 in the distal nephron in regulating urinary sodium excretion, presumably via Na^+-Cl^- cotransporter or the epithelial sodium channel. Increased urinary sodium excretion in the distal nephron or systemic sodium retention could influence renal hemodynamics, as the inhibition of Na^+-Cl^- cotransporter by thiazide diuretics reduces blood pressure and GFR. Indeed, a high-salt diet has been shown to increase GFR in the hypertensive African American population[49] and subtotal nephrectomized rats.[50] As reported in our previous study, the KK/Ta-Akita mice exhibit an elevated GFR at 10 and 15 weeks of age, which is consistent with the development of glomerular hypertension and hyperfiltration.[26] Our data illustrate that this abnormality in renal hemodynamics is ameliorated by DPP-4 inhibition, resulting in renal SDF-1 upregulation and enhanced urinary sodium excretion. In addition, SDF-1α receptor (CXCR4) blockade further augments GFR in parallel with reduction of urinary sodium excretion. Regarding the role of CXCR4 in the diabetic renal tubules, a recent experimental study has shown that 4-week CXCR4 blockade with AMD3100 accelerated tubular epithelial cell death in the kidneys of streptozotocin-induced diabetic Sprague Dawley rats.[51] Thus, the amelioration of diabetes-induced renal hemodynamic change via renal SDF-1 upregulation and the protective role of SDF-1/CXCR4 signaling in renal tubules may be a possible mechanism through which DPP-4 inhibitor prevents the progression of DN.

Several experimental studies have reported that GLP-1R agonists increased urinary sodium excretion in salt-sensitive rats and mice.[52,53] However, our recent experiments demonstrated that *Glp1r* mRNA transcripts are not localized to renal tubules and collecting ducts in mice. On the other hand, in accordance with our current study, DPP-4 inhibition with sitagliptin enhanced urinary sodium excretion in spontaneously hypertensive rats.[54] Extending results of previous studies examining acute mechanisms regulating urinary sodium excretion in normoglycemic mice,[55] we found that chronic DPP-4 inhibition increased urinary sodium excretion even in diabetic *Glp1r*$^{-/-}$ KK/Ta-Akita mice. Therefore, the actions of DPP-4 inhibitors to increase urinary sodium excretion do not require GLP-1R signaling.

In conclusion, our findings, as summarized in Figure 9, suggest that renal SDF-1 upregulation by DPP-4 inhibition produces multiple protective actions on the diabetic kidney as follows: protection of glomerular podocyte against oxidative stress; enhancement of urinary sodium excretion and the consequent amelioration of glomerular hypertension and hyperfiltration. In the present study, we focused on the alteration of renal SDF-1 expression following DPP-4 inhibition and its effects on renal phenotypes in experimental DN models. As DPP-4 cleaves a large number of chemokines, neuropeptides, and peptide hormones besides SDF-1 and GLP-1, it seems likely that other DPP-4 substrates may be involved in renal protective effects of DPP-4 inhibition.

Finally, this study has several limitations. Whether linagliptin potentiates the CXCR4-dependent autoactivation of SDF-1 expression and activity in the kidney, as has been reported in pancreatic beta cell lines and islets,[56] was not directly addressed in our experiments. Furthermore, we were unable to determine whether linagliptin, via DPP-4 inhibition, potentiated the functional activity of renal SDF-1. Therefore, further studies are needed to elucidate the detailed molecular

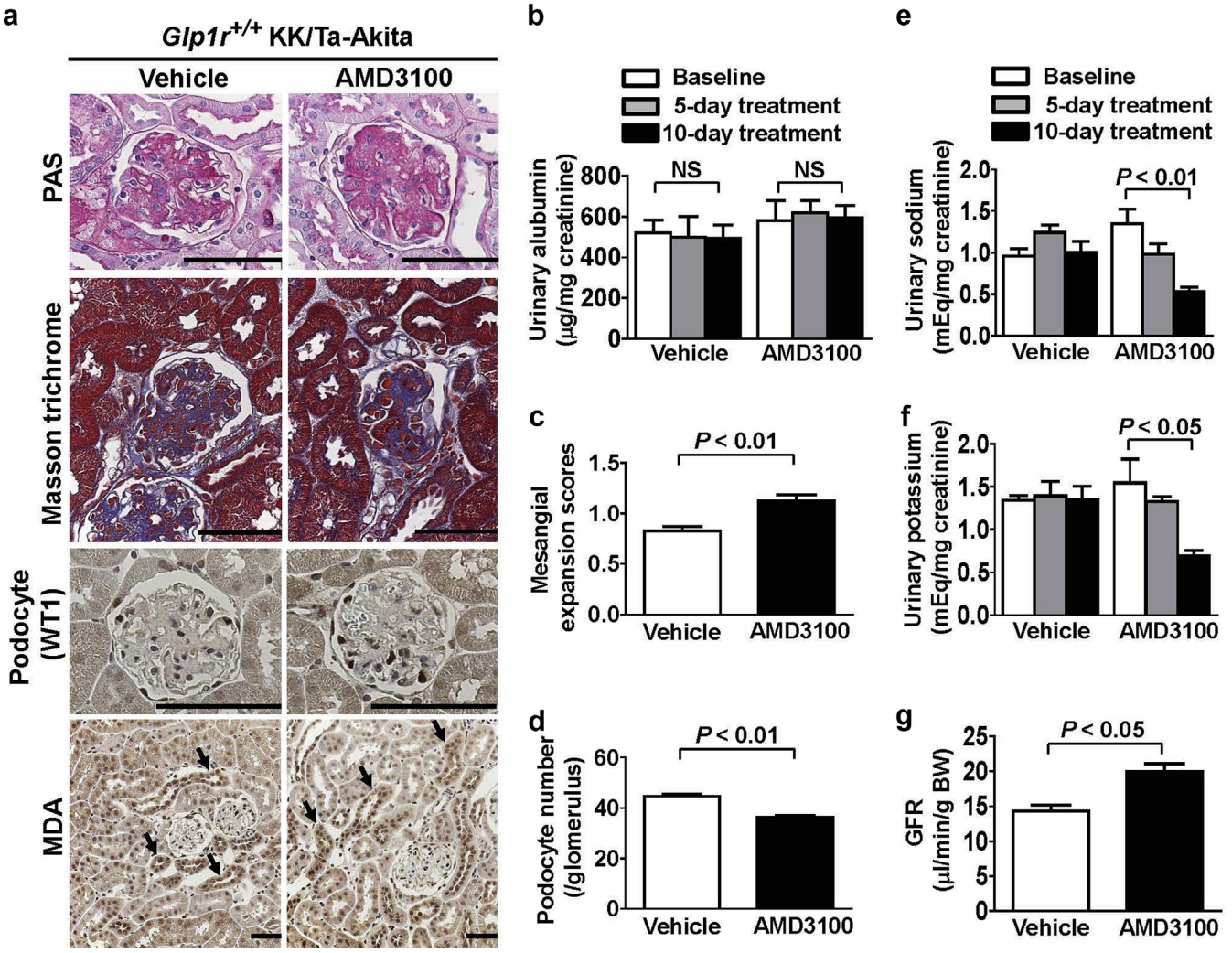

Figure 8 | Renal histopathology and changes in urinary sodium and potassium levels in *Glp1r*$^{+/+}$ KK/Ta-Akita mice after treatment with the stromal cell–derived factor-1α receptor antagonist AMD3100. AMD3100 (1 mg/kg/d) was intraperitoneally injected into male *Glp1r*$^{+/+}$ KK/Ta-Akita mice for 10 days. (**a**) Representative glomerulus images of periodic acid–Schiff (PAS), Masson trichrome, and WT1 staining and malondialdehyde (MDA) immunohistochemistry. Arrows indicate distal tubules. Bar = 50 μm for all images. (**b**) Changes in urinary albumin levels. $n = 6$ for vehicle and $n = 7$ for AMD3100. NS, not significant. (**c**) Glomerular mesangial expansion scores. $n = 7$ for vehicle and $n = 7$ for AMD3100. (**d**) The number of WT1-positive podocytes in glomeruli. $n = 7$ for vehicle and $n = 7$ for AMD3100. (**e,f**) Changes in urinary sodium and potassium levels during the AMD3100 administration. $n = 7$ for vehicle and $n = 7$ for AMD3100 in urinary sodium levels. $n = 6$ for vehicle and $n = 5$ for AMD3100 in urinary potassium levels. (**g**) Glomerular filtration rate (GFR) after 10-day administration of AMD3100. $n = 4$ for vehicle and $n = 5$ for AMD3100. BW, body weight.

mechanisms underlying the potential renal benefits of DPP-4 inhibition and SDF-1/CXCR4 signaling in DN.

MATERIALS AND METHODS

Experimental animals and treatment protocols

C57BL/6-Akita mice are available from SLC (Hamamatsu, Shizuoka, Japan). KK/Ta-Akita mice and *Glp1r*$^{-/-}$ C57BL/6-Akita mice were generated as described previously.[26,28] The generation of *Glp1r*$^{-/-}$ KK/Ta-Akita mice was started by crossing male *Glp1r*$^{-/-}$ C57BL/6-Akita mice to female *Glp1r*$^{+/+}$ KK/Ta-WT mice, followed by crossing male *Glp1r*$^{+/-}$ Akita mice in the F1-generation to female *Glp1r*$^{+/+}$ KK/Ta-WT mice. This backcrossing was repeated for 10 generations, and then male *Glp1r*$^{+/-}$ or *Glp1r*$^{-/-}$ Akita mice were repeatedly crossed to female *Glp1r*$^{+/-}$ or *Glp1r*$^{-/-}$ nondiabetic WT mice for more than 10 generations. Thus, *Glp1r*$^{-/-}$ Akita mice in the KK/Ta background were generated, and males were used in this study. To investigate the effects of DPP-4 inhibition in renal SDF-1α expression, the DPP-4 inhibitor linagliptin (5 mg/kg/d [Boehringer Ingelheim, Ingelheim am Rhein, Germany]) dissolved in a 0.5% carboxymethylcellulose-Na solution was given orally to 6-week-old male *Glp1r*$^{+/+}$ or *Glp1r*$^{-/-}$ KK/Ta-Akita mice for 6 weeks. A parallel group of control age-matched male *Glp1r*$^{+/+}$ or *Glp1r*$^{-/-}$ KK/Ta-Akita mice was orally administered an equivalent volume of 0.5% carboxymethylcellulose solution alone as the vehicle for 6 weeks. Another parallel group of age-matched male *Glp1r*$^{+/+}$ KK/Ta-Akita mice was subcutaneously injected with the GLP-1R agonist liraglutide (200 μg/kg/d [Novo Nordisk, Bagsvaerd, Denmark]) for 6 weeks, and they were compared with the group treated with linagliptin. To examine the effects of SDF-1α inhibition in urinary excretion of sodium and potassium, the SDF-1α receptor (CXCR4) antagonist AMD3100 (1 mg/kg/d [R&D Systems, Minneapolis,

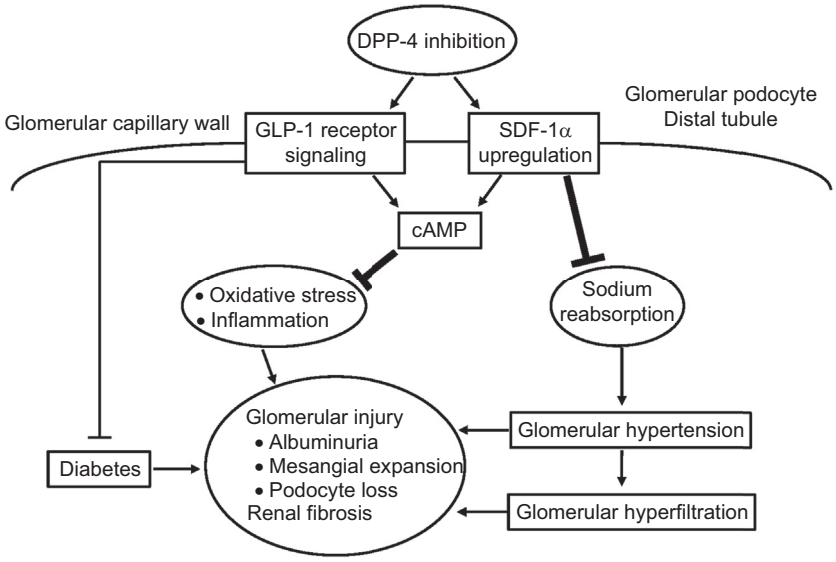

Figure 9 | Proposed mechanism of renal protection by dipeptidyl peptidase-4 (DPP-4) inhibition. DPP-4 inhibition upregulates stromal cell–derived factor-1α (SDF-1α) mainly in glomerular podocyte and distal tubule. Increased renal expression of SDF-1α contributes to the reduction of glomerular oxidative stress and inflammation and inhibits sodium reabsorption in distal tubules, leading to the amelioration of glomerular hypertension and hyperfiltration. DPP-4 inhibition may offer renal protective effects through the SDF-1α signaling pathway, irrespective of the glucagon-like peptide-1 (GLP-1) receptor signaling. cAMP, cyclic adenosine monophosphate.

MN]) was injected intraperitoneally for 10 days into 6-week-old male *Glp1r*$^{+/+}$ KK/Ta-Akita mice. The mice were allowed unrestricted access to standard rodent chow and water. Animal experiments were carried out in accordance with the Animal Welfare Guidelines of Akita University. All procedures were approved by the Committee on Animal Experimentation of Akita University.

Measurement of blood, urine, and physiological parameters

Blood glucose was measured after a 6-hour daytime fast. Urinary excretion of albumin, sodium, and potassium was determined by morning spot urine tests. Detailed protocols for measurement of blood, urine, and physiological parameters are described in Supplementary Material online.

Isolation of glomeruli and quantitative RT-PCR

Glomeruli were separated from renal cortex of male mice by the Dynabead perfusion method as reported previously, with some modifications.[57] The mRNA expression of SDF-1α, SDF-1β, CXCR4, and CXCR7 was examined by quantitative RT-PCR analysis using total RNA extracted from glomeruli and renal medullary tissues as described previously.[27]

Histologic analysis and immunohistochemistry

Detailed protocols for histologic analysis and immunohistochemistry are described in Supplementary Material online.

Measurement of renal cAMP levels

Kidney lysate was prepared using freshly removed renal cortical and medullary tissues as described previously.[26] Renal cAMP levels were determined using a DetectX Direct cAMP Immunoassay kit (Arbor Assays, Ann Arbor, MI), and expressed as renal cAMP-to-protein ratio.

Statistical analysis

All data are expressed as means ± SEM. Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA). Differences between two groups were determined by the Mann-Whitney test or Wilcoxon signed-rank test for nonnormally distributed data and by the Student *t* test or paired *t* test for normally distributed data. Differences between multiple groups were assessed by one-way analysis of variance followed by Bonferroni's multiple comparison test. $P < 0.05$ was considered statistically significant.

DISCLOSURE

HF received lecture fees from Boehringer Ingelheim. TN, YS, and YY received lecture fees from Boehringer Ingelheim, Eli Lilly, and Novo Nordisk. YS received an advisory fee from Novo Nordisk. DJD receives research support for cardiovascular studies from and is a consultant to Novo Nordisk. Both Boehringer Ingelheim and Novo Nordisk donated to the Division of Endocrinology, Metabolism and Geriatric Medicine, Akita University Graduate School of Medicine and were not directly related to this study. Sanwa Chemistry contributed to establishing the Division of Metabolism and Clinical Nutrition Science, Akita University Graduate School of Medicine. All the other authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary Material. Stromal cell–derived factor-1 is upregulated by dipeptidyl peptidase-4 inhibition and has protective roles in progressive diabetic nephropathy.
Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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Supplementary material for

Stromal cell–derived factor-1 is upregulated by dipeptidyl peptidase-4 inhibition and has protective roles in progressive diabetic nephropathy

Satoru Takashima, MD[1*]

Hiroki Fujita, MD, PhD[1*]

Hiromi Fujishima, MS[1]

Tatsunori Shimizu, MD[1]

Takehiro Sato, MD[1]

Tsukasa Morii, MD, PhD[1]

Katsushi Tsukiyama, MD, PhD[2]

Takuma Narita, MD, PhD[1]

Takamune Takahashi, MD, PhD[3]

Daniel J. Drucker, MD[4,5]

Yutaka Seino, MD, PhD[6]

Yuichiro Yamada, MD, PhD[1]

[1] Division of Endocrinology, Metabolism and Geriatric Medicine, Akita University Graduate School of Medicine, Akita, Japan

[2] Division of Metabolism and Clinical Nutrition Science, Akita University Graduate School of Medicine, Akita, Japan

[3] Division of Nephrology and Hypertension, Vanderbilt University Medical Center, Nashville, TN, USA

[4] Department of Medicine, University of Toronto, Toronto, Ontario, Canada

[5] The Lunenfeld-Tanenbaum Research Institute, Mt. Sinai Hospital, Toronto, Ontario, Canada

[6] Kansai Electric Power Medical Research Institute, Osaka, Japan

* These authors contributed equally to this work.

Correspondence: Hiroki Fujita, Division of Endocrinology, Metabolism and Geriatric Medicine, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan. Tel.: +81 18 884 6769; fax: +81 18 884 6449. E-mail: hirofuji@gipc.akita-u.ac.jp

Running headline: SDF-1, DPP-4 inhibition, and Diabetic Nephropathy

MATERIALS AND METHODS

Measurement of blood, urine and physiological parameters

Blood glucose was measured after a 6-hour daytime fast using Glutestmint (Sanwa Chemistry, Nagoya, Aichi, Japan). Blood urea nitrogen (BUN), plasma creatinine, plasma total cholesterol, plasma triglycerides, urinary sodium, and urinary potassium were measured by an autoanalyzer (Fuji Dry-Chem 800 and 5500, Fuji Film, Tokyo, Japan). Urinary albumin excretion was determined on morning spot urine as described previously.[1] Urinary excretion of sodium and potassium was expressed as a ratio to urinary creatinine. Systolic blood pressure was measured using a non-invasive tail cuff and pulse transducer system (BP-98A, Softron, Tokyo, Japan). GFR was determined by a single-bolus FITC-inulin injection and clearance method as described previously.[2]

Histologic analysis and immunohistochemistry

The kidneys were perfused via left ventricle with PBS followed by 4% paraformaldehyde in PBS, removed, and fixed in 4% paraformaldehyde in PBS for overnight at 4°C. Two μm-thick paraffin sections were stained with PAS and Masson trichrome, and used for immunohistochemistry. The degree of glomerular mesangial expansion was assessed using a semi-quantitative score as described previously.[3] Glomerular podocyte was stained by WT1 immunohistochemistry, and twenty cortical glomeruli were evaluated in each mouse. The podocyte number was calculated using the Weibel-Gomez method as reported previously.[4, 5] The immunohistochemistry for MDA, SDF-1, TSP-1, and fibronectin was performed using rabbit anti-MDA polyclonal antibody (1:100, Alpha Diagnostic, San Antonio, TX), mouse anti-SDF-1 monoclonal antibody (1:100, R & D Systems), mouse anti-TSP-1 monoclonal antibody (1:100; Invitrogen, Camarillo, CA), and

mouse anti-fibronectin monoclonal antibody (1:100; Thermo Scientific, Fremont, CA). The glomerular superoxide levels are assessed by dihydroethidium (DHE) histochemistry as described previously.[6] The NO production in the glomeruli was evaluated by the fluorescent intensity of the DAF-2DA reaction as reported previously.[7, 8] The fluorescent images were observed using confocal laser microscopy (LSM510; Carl Zeiss, Jena, Germany). The fluorescence intensity in twenty glomeruli in each mouse was semiquantified using Adobe Photoshop (version CS5; Adobe systems, San Jose, CA).

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