

The role of dipeptidyl peptidase 4 (DPP4) in the preservation of renal function: DPP4 involvement in hemoglobin expression

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Abstract

In a previous study, we demonstrated that dipeptidyl peptidase 4 (DPP4)-deficient rats were susceptible to reduced glomerular filtration rate as a result of streptozotocin (STZ)-induced diabetes. Therefore, we proposed that DPP4 might be responsible for the preservation of renal function. In this study, to verify the role of DPP4 in the preservation of renal function, we performed a microarray analysis of the kidneys of WT and DPP4-deficient rats after STZ treatment, and gene expression analysis using rat kidneys, human embryonic kidney 293 (HEK293) cells, and human renal cancer cells (Caki-1). The microarray analysis indicated that the expression levels of the transporter activity, heme-binding, and pheromone binding-related genes changed significantly. The results of gene expression analysis indicated that there were no significant differences in the expression levels of hemoglobin mRNA between the DPP4-deficient and WT rats; however, the expression levels of hemoglobin mRNA in the kidneys of DPP4-deficient rats tended to decrease when compared with those of both the non-STZ-treated and STZ-treated WT rats. The expression levels of hemoglobin in HEK293 and Caki-1 cells were significantly decreased when *DPP4* was knocked down by siRNA, were significantly increased by the addition of soluble human DPP4, and were also significantly increased by the addition of the DPP4 inhibitor, sitagliptin. The expression level of *DPP4* was also significantly increased by the addition of sitagliptin in both cell types. Our findings indicate that DPP4 regulates the expression of the hemoglobin genes, and might play a role in the preservation of renal function; however, the underlying mechanism of this preservation remains to be elucidated.

Key Words

- ▶ DPP4 deficient
- ▶ streptozotocin
- ▶ diabetes
- ▶ renal function
- ▶ hemoglobin

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Introduction

Diabetes is a major worldwide public health problem (Zimmet *et al.* 2001) that causes complications, such as retinopathy, nephropathy, neuropathy, dyslipidemia, and cardiovascular disease (Crofford 1995, Cannon 2008, Srinivasan *et al.* 2008). Recently, incretin mimetics, such

as glucagon-like peptide 1 (GLP1) agonists and dipeptidyl peptidase 4 (DPP4) inhibitors, have been used as anti-diabetic agents. DPP4 is present on the surface of various cell types, including kidney and liver cells, and in a soluble form in plasma (Mentlein 1999). DPP4 is a serine protease

that preferentially cleaves Xaa-Pro and Xaa-Ala dipeptides from the N-termini of polypeptides, such as glucose-dependent insulinotropic polypeptide and GLP1, which are members of the incretin system, and stimulate pancreatic insulin secretion, insulin biosynthesis, and proliferation of pancreatic β -cells, and inhibit food intake (Holst & Gromada 2004, Holst 2006). Therefore, GLP1 agonists and DPP4 inhibitors are considered to be useful as anti-diabetic agents that result in a low incidence of hypoglycemia and minimal weight gain (Nielsen 2005, Green *et al.* 2006, Hinnen *et al.* 2006, Kendall *et al.* 2006). DPP4 inhibitors have been shown to protect against myocardial injury, cardiac dysfunction (Chang *et al.* 2013), and asthma (Stephan *et al.* 2013), and also to reduce colon carcinogenesis (Femia *et al.* 2013) in animal models. However, the advantages and disadvantages of DPP4 inhibitors and the role of DPP4 in various tissues are not fully understood.

We previously showed that DPP4-deficient rats developed renal dysfunction when compared with WT rats following streptozotocin (STZ) treatment to induce diabetes. Creatinine clearance in DPP4-deficient rats after 42 days of STZ treatment was significantly lower than that in WT rats (Kirino *et al.* 2009). No significant difference in body weight or food intake was found between the WT and DPP4-deficient rats treated with or without STZ. F344/DuCrIj (DPP4-deficient) rats from Charles River Japan (Osaka, Japan), express *DPP4* mRNA but have reduced levels of the active protein because an abnormal isoform is translated. Thus, F344/DuCrIj rats lack DPP4 enzyme activity (Watanabe *et al.* 1987). Therefore, it has been suggested that DPP4 is related to the preservation of renal function. The goal of this study was to verify the role of DPP4 in the preservation of renal function by analyzing the genes expressed in the kidney tissues of WT and DPP4-deficient rats and from renal cell lines.

Materials and methods

Animals and tissue preparation

Four groups were used in this experiment, as previously reported (Kirino *et al.* 2009). Briefly, 8-week-old male F344/Jcl (WT) and F344/DuCrIj (DPP4-deficient) rats (140–160 g) were purchased from Clea Japan (Tokyo, Japan) and Charles River Japan respectively. At 9 weeks of age, the rats were randomly divided into the following four groups: non-STZ-treated, WT ($n=6$); STZ-treated, WT ($n=10$); non-STZ-treated, DPP4-deficient ($n=6$); and STZ-treated, DPP4-deficient ($n=10$) rats. In the

STZ-treated groups, experimental diabetes was induced by a single i.p. injection of STZ (30 mg/kg; Wako Pure Chemical, Osaka, Japan) in 0.05 M citrate buffer (pH 4.5) after an overnight fast. The rats were fed again following injections. The non-STZ-treated rats received an equivalent amount of citrate buffer. At 42 days after the STZ or vehicle treatment, the rats were placed under anesthesia with urethane (5 g/kg, i.p.; Sigma–Aldrich) and killed. The kidneys of each rat were collected, and the collected tissues were immediately cut into slices <5-mm thick, which were then stored in RNAlater RNA Stabilization Reagent (Qiagen) at -20°C . All animal care protocols and experiments were conducted in accordance to the guidelines of the animal use and care committee of the University of Tokushima.

Microarray analysis of gene expression

Total RNA was isolated from the kidneys of the rats using the RNeasy Lipid Tissue Mini Kit (Qiagen), according to the manufacturer's methods. The relative purity of the mRNA was examined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). First-strand cDNA was synthesized from 300 ng of total RNA using the GeneChip Whole Transcript (WT) cDNA Synthesis and Amplification Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's methods. The resultant cDNA was fragmented and end-labeled with the GeneChip WT terminal Labeling Kit (Affymetrix). Then, 5.5 μg of the fragmented and labeled DNA target were hybridized to the Affymetrix GeneChip Rat Gene 1.0 ST Array at 45°C for 17 h in a GeneChip Hybridization Oven 640 (Affymetrix), according to the manufacturer's recommendations. The abovementioned microarray chip contains 27 342 probe sets for known and unknown genes. The hybridized arrays were washed and stained in a GeneChip Fluidics Station 450 and scanned in a GeneChip Scanner 3000 7G (Affymetrix). CEL files were generated for each array. In each comparison experiment, the intensity data for the two chips were normalized by dye swap and flag treatments, and genes that demonstrated significantly different expression levels ($P<0.05$) between the WT and DPP4-deficient rats were categorized by gene ontology (GO) using the GeneSpring GX 10.0 Software (Silicon Genetics, Redwood City, CA, USA).

Quantitative PCR (qPCR)

cDNA was synthesized using 2 μg of total RNA and SuperScript III Reverse Transcriptase (Invitrogen)

according to the manufacturer's instructions. qPCR was carried out using SYBR Premix Ex Taq (Takara, Tokyo, Japan) in the AB 7500 real-time PCR system (Applied Biosystems) according to the following thermal cycling profile: initial denaturation at 95 °C for 10 s followed by 40 cycles of amplification (denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 34 s). Data were analyzed based on standard curve methods; i.e. we quantified the expression levels of various genes and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in each sample and normalized the expression level of each gene to that of *GAPDH*. The primer sets used are listed in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article.

Cell culture

Human embryonic kidney 293 (HEK293) cells and human renal cancer cells (Caki-1) were obtained from the Health Science Research Resources Bank (cell no. JCRB9068 and JCRB0801, respectively, Osaka, Japan), and cultured in DMEM (Sigma–Aldrich) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 mg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO₂ in air. Recombinant soluble human DPP4 (ATGen, Seongnam, South Korea) was added to the culture medium at a final concentration of 500 ng/ml. After 12, 24, and 48 h, the cells were harvested and used for qPCR. Total RNA was isolated from the cells using the RNeasy Plus Mini Kit (Qiagen), according to the manufacturer's instructions. Sitagliptin phosphate monohydrate (Sigma–Aldrich) dissolved in water was added to the culture medium at final concentrations of 10, 100, 1000, and 5000 µM. Forty-eight hours after the addition of sitagliptin, the cells were harvested and used for qPCR and the DPP4 activity assay. Proliferation was assessed using an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. After culturing in a 96-well plate, 20 µl of MTT solution (1 mg/ml) was added to each well, followed by further incubation at 37 °C for 4 h. The medium was removed, and 100 µl of DMSO was added to each well. Cell proliferation was measured at a wavelength of 550 nm.

Knockdown of DPP4

HEK293 and Caki-1 cells were plated into 60-mm dishes and cultured at 37 °C, before being transfected with siRNA against DPP4 (siDPP4-1, SI00030212; siDPP4-2, SI00030219; siDPP4-3, SI00030226; and siDPP4-5, SI03099642; Qiagen) or control siRNA (Silencer Negative Control #1 siRNA;

Ambion, Austin, TX, USA) at final concentrations of 5 nM, using the Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. After 48 h, the cells were harvested and used for qPCR.

DPP4 activity assay

To measure DPP4 activity, cells were harvested, washed with PBS, and solubilized in four volumes of homogenization buffer (50 mmol/l Tris–HCl (pH 7.5), 250 mmol/l sucrose, 5 mmol/l MgCl₂, 2 mmol/l ATP, 1 mmol/l dithiothreitol (DTT), and 0.5 mmol/l EDTA) containing 0.025% (w/v) digitonin. Then, the cells were disrupted by ultrasonication (20 pulses of 1 second). The cells were centrifuged, and the resultant supernatant was sampled. Protein concentrations were determined using the Bradford assay. DPP4 activity was determined from the cleavage rate of 7-amino-4-methylcoumarin (AMC) from the synthetic substrate *H*-glycyl-prolyl-AMC (Gly-Pro-AMC; Sigma–Aldrich), as previously described (Kirino *et al.* 2009). Briefly, 5 µl of sample was mixed with 35 µl of assay buffer (25 mmol/l HEPES, 140 mmol/l NaCl, 80 mmol/l MgCl₂, and 1% (w/v) BSA (pH 7.8)). After 5 min of pre-incubation at room temperature, the reaction was initiated by the addition of 40 µl of assay buffer containing 0.1 mmol/l of the substrate Gly-Pro-AMC. After incubation for 20 min, fluorescence was determined using a spectrofluorometer (Infinite M200 PRO, Tecan Japan Co. Ltd (Kawasaki, Japan); excitation: 380 nm/emission: 460 nm). The standard curve of free AMC was generated using 0–50 µmol/l solutions of AMC (Sigma–Aldrich). DPP4 activity was expressed as the amount of cleaved AMC.

Immunoblotting

The cells were washed with PBS, solubilized in four volumes of lysis buffer (50 mM Tris–HCl, pH 7.4, 1% Nonident P-40, 0.25% SDS, 150 mM NaCl, and 1 mM EDTA) with protease inhibitors, and ultrasonicated. The cells were centrifuged (15 000 g, 4 °C, 5 min) and the supernatant was used for further analysis. The protein concentrations were determined using the Bradford assay. The proteins were separated by SDS–PAGE (10%), and electrotransferred onto PVDF membrane (Hybond-P; GE Healthcare Bio-Sciences, Buckinghamshire, England). The membrane was then blocked with TBS-T buffer (20 mM Tris–HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween-20) with 5% Block Ace (DS Pharma Biomedical Co. Ltd, Osaka, Japan).

To detect DPP4, rabbit anti-human DPP4 (Santa Cruz Biotechnology, Inc.) and HRP-conjugated rabbit IgG antibodies were used as the primary and secondary antibodies respectively. The proteins were visualized using an ECL detection system (GE Healthcare Bio-Sciences) and the signals were detected using a luminescent image analyzer, LAS-3000 mini (Fujifilm Corp., Tokyo, Japan).

Statistical analyses

Data were compared using the unpaired *t*-test or one-way ANOVA followed by *post hoc* comparisons using Bonferroni's multiple comparison test. Statistical analyses were conducted using SPSS version 20.0 (SPSS, Inc.).

Results

Microarray and GO analysis in the rat kidneys

Microarray analysis using one randomly selected representative animal from each group was conducted to examine the differences between the gene expression in the kidneys of DPP4-deficient rats and WT rats after STZ treatment. In total, 71 genes were up- or down-regulated by at least twofold in the DPP4-deficient rats compared with the WT rats (Supplementary Tables 2 and 3, see section on supplementary data given at the end of this article). GO analysis was performed to categorize the genes according to biological process, molecular function, and cellular component. Our statistical filtering of the array analysis was based on at least a twofold change ($P < 0.05$)

in expression. GO analysis revealed that transporter activity, heme binding, and pheromone binding-related genes demonstrated significant changes in their expression (Table 1). Among those genes, hemoglobin genes (*Hbb* and *MGC72973*) were downregulated and the solute carrier family genes (*Slc5a3*, *Slc12a1*, and *Slco4a1*) were upregulated in the DPP4-deficient rats compared with the WT rats.

qPCR analysis of rat kidneys

To verify the results of the microarray analysis, qPCR was conducted for the 11 genes (*Hbb*, *MGC72973*, *Slc5a3*, *Slc12a1*, *Slco4a1*, *Obp3*, *Cyp2c* (*Cyp2c11*), *Cyp2c7* (*Cyp2c39*), *Ptgs1*, *Cyp24a1*, and *Cyp2c13*; Table 1) that were indicated in the GO analysis, using the kidney tissues of six non-STZ-treated WT, ten STZ-treated WT, six non-STZ-treated DPP4-deficient, and ten STZ-treated DPP4-deficient rats. Although there were no significant differences in the mRNA expression levels of *Hbb* between the DPP4-deficient and WT rats, the mRNA expression levels of *Hbb* in both the WT and DPP4-deficient rats treated with STZ were significantly decreased when compared with the WT rats not treated with STZ, and the mRNA expression levels of *Hbb* in the DPP4-deficient rats tended to decrease when compared with those in both the non-STZ-treated and STZ-treated WT rats (Fig. 1). Next, we carried out qPCR for other hemoglobin genes (*Hba1* and *Hba2*). Although there were no significant differences in the mRNA expression levels of *Hba1* between DPP4-deficient and WT rats, the mRNA expression levels of *Hba1*

Table 1 Gene ontology analysis of the genes whose expression differed by a factor of greater than twofold between the kidneys of DPP4-deficient and WT rats 42 days after treatment with STZ

Gene symbol	Gene name	Fold
Transporter activity	<i>P</i> value: 0.0005	
<i>Hbb</i>	Hemoglobin, beta	2.28 down
<i>MGC72973</i>	Beta-glo	2.94 down
<i>Slc5a3</i>	Solute carrier family 5 (inositol transporters), member 3	2.19 up
<i>Slc12a1</i>	Solute carrier family 12, member 1	2.10 up
<i>Slco4a1</i>	Solute carrier organic anion transporter family, member 4a1	2.06 up
<i>Obp3</i>	Alpha-2u globulin PGCL4	3.69 down
Heme binding	<i>p</i> value: 0.0055	
<i>Cyp2c</i>	Cytochrome P450, subfamily IIC	3.43 down
<i>Hbb</i>	Hemoglobin, beta	2.28 down
<i>MGC72973</i>	Beta-glo	2.94 down
<i>Cyp2c7</i>	Cytochrome P450, family 2, subfamily c, polypeptide 7	3.91 down
<i>Ptgs1</i>	Prostaglandin-endoperoxide synthase 1	2.62 up
<i>Cyp24a1</i>	Cytochrome P450, subfamily 24	2.50 up
<i>Cyp2c13</i>	Cytochrome P450 2c13	2.45 down
Pheromone binding	<i>P</i> value: 0.047	
<i>Obp3</i>	Alpha-2u globulin PGCL4	3.69 down

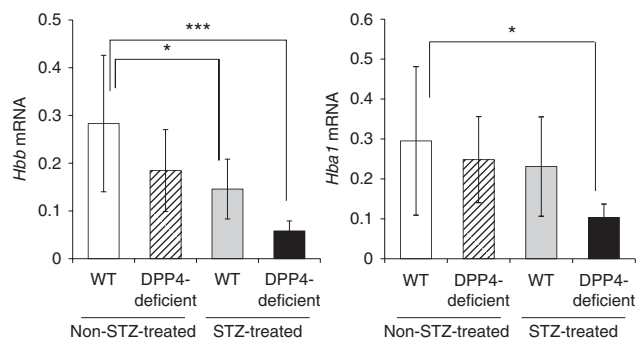


Figure 1

Changes in *Hbb* and *Hba1* mRNA expression levels in WT and DPP4-deficient rats at 42 days after the i.p. injection of STZ (30 mg/kg) or the vehicle. The mRNA expression levels were measured by qPCR. All mRNA signals were normalized to the signal for *Gapdh*. Data are shown as the mean \pm s.d. Non-STZ-treated, WT ($n=6$); STZ-treated, WT ($n=10$); non-STZ-treated, DPP4-deficient ($n=6$); and STZ-treated, DPP4-deficient ($n=10$). * $P<0.05$ and *** $P<0.001$; one-way ANOVA followed by *post hoc* comparisons using Bonferroni's multiple comparison test.

in the DPP4-deficient rats treated with STZ were significantly decreased when compared with the WT rats not treated with STZ, and the mRNA expression levels of *Hba1* in the DPP4-deficient rats also tended to decrease when compared with those in the both the non-STZ-treated and STZ-treated WT rats (Fig. 1). Several other genes could not be analyzed because their expression showed considerable variation between individuals.

Effect of DPP4 on mRNA expression of hemoglobin genes in HEK293 and Caki-1 cells

The results of the microarray and qPCR analyses indicated that DPP4 contributes to the regulation of hemoglobin gene expression. We then studied the effect of DPP4 siRNA knockdown and the effect of adding soluble human DPP4 on the expression levels of hemoglobin genes in HEK293 cells and human renal cancer cells (Caki-1). The hemoglobin molecule consists of two of each of two different types of polypeptide chains. Fetal hemoglobin (Hb F; $\alpha_2\gamma_2$) consists of two α -chains and two γ -chains. There are two identical α -globin genes, designated *HBA1* ($\alpha 1$) and *HBA2* ($\alpha 1$), and two identical γ -globin genes, designated *HBG1* ($A\gamma$) and *HBG2* ($G\gamma$). In postnatal hemoglobin (Hb A; $\alpha_2\beta_2$), the γ chains are gradually replaced by β chains, which are encoded by the β -globin gene, designated *HBB* (β) (Nussbaum *et al.* 2007). Therefore, we studied the expression of *HBA1*, *HBA2*, *HBG1*, and *HBG2* genes in HEK293 cells, and the expression of *HBA1*, *HBA2*, and *HBB* genes in Caki-1 cells. To identify the effect of siRNA

knockdown against human DPP4, we tested *DPP4* mRNA expression using four pre-designed siRNAs (siDPP4-1, siDPP4-2, siDPP4-3, and siDPP4-5). Among the pre-designed siRNAs, siDPP4-1 displayed the greatest knockdown of *DPP4* mRNA expression in the HEK293 and Caki-1 cells, as determined by RT-PCR (Figs 2a and 3a). Therefore, we used siDPP4-1 as the DPP4-specific siRNA. We achieved >70% knockdown of *DPP4* mRNA expression in each of the cell types, as determined by qPCR (Figs 2b and 3b). The expression levels of *HBA1*, *HBA2*, *HBG1*, and *HBG2* were significantly decreased by DPP4 siRNA knockdown in HEK293 cells (Fig. 2), and the expression levels of *HBA2* and *HBB* were significantly decreased by DPP4 siRNA knockdown in Caki-1 cells (Fig. 3). The *HBA1* gene could not be analyzed because the

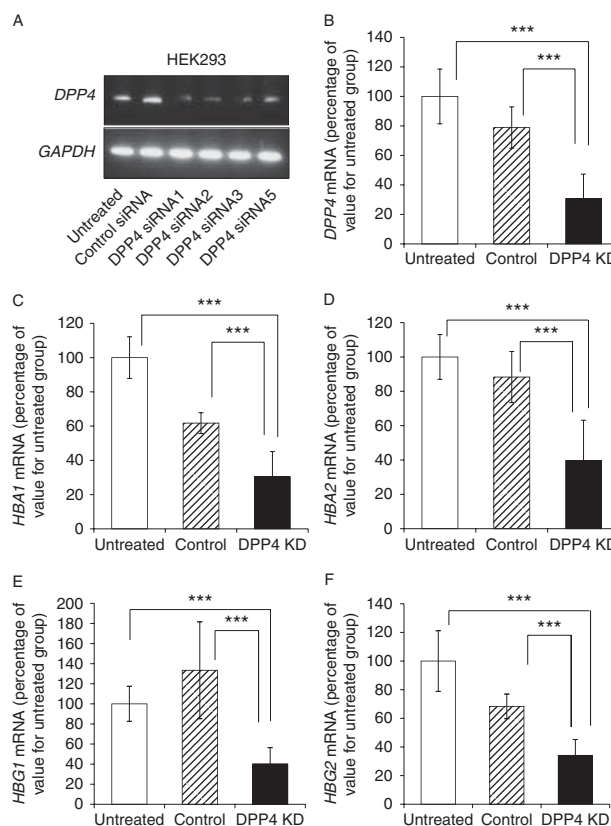
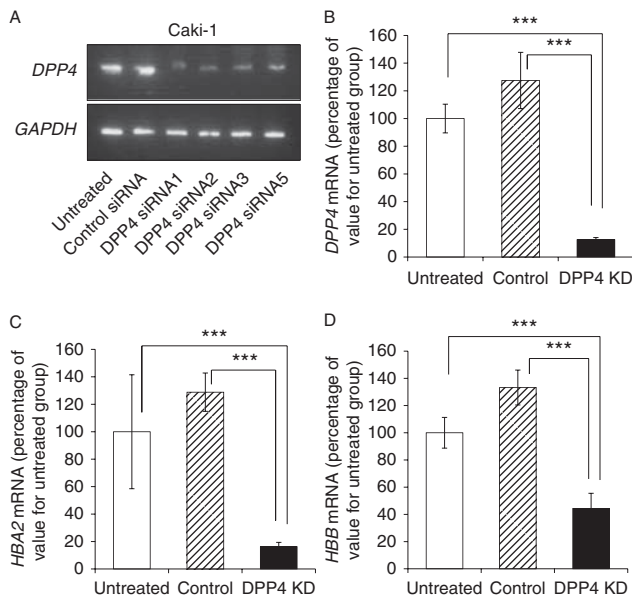


Figure 2

Changes in *DPP4* (B), *HBA1* (C), *HBA2* (D), *HBG1* (E), and *HBG2* (F) mRNA expression levels in HEK293 cells from the DPP4 siRNA knockdown using DPP4 siRNA1 (DPP4 KD) and control siRNA (Control). The levels of mRNA expression were measured by qPCR and compared with those for the untreated cells. mRNA signals were normalized to the signal for *GAPDH*. The experiments were repeated three times, and the data are shown as the mean \pm s.d. (A) RT-PCR analysis of *DPP4* mRNA expression by DPP4 knockdown using four pre-designed siRNAs and control siRNA. *** $P<0.001$; one-way ANOVA followed by *post hoc* comparisons using Bonferroni's multiple comparison test.

**Figure 3**

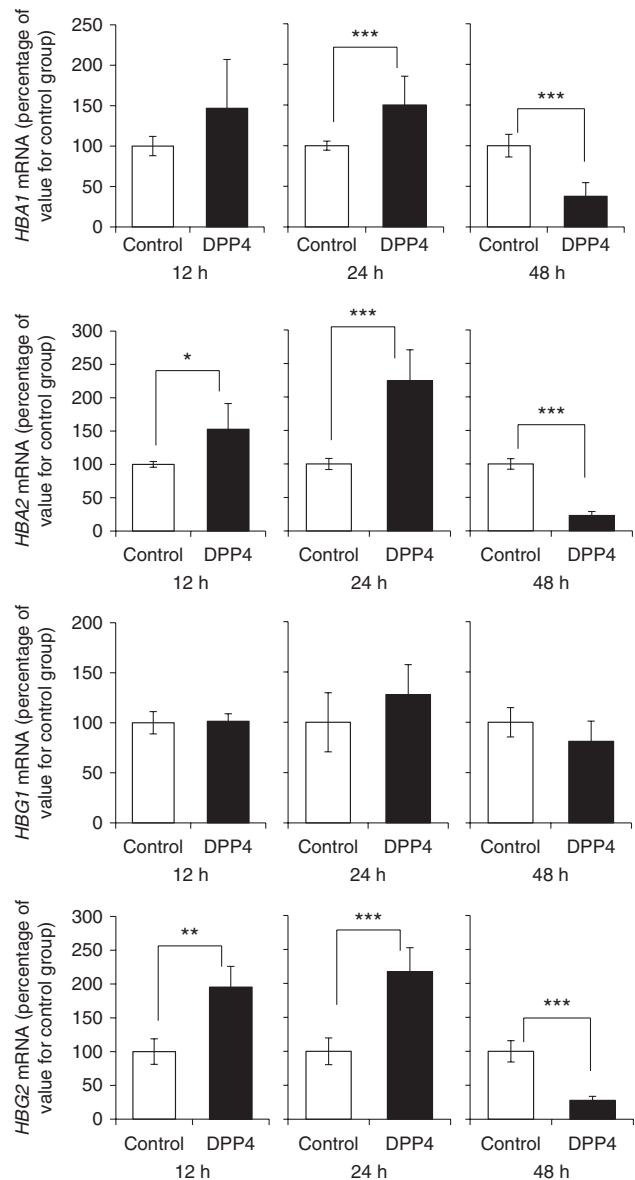
Changes in *DPP4* (B), *HBA2* (C), and *HBB* (D) mRNA expression levels in Caki-1 cells from the *DPP4* siRNA knockdown using *DPP4* siRNA1 (*DPP4* KD) and control siRNA (Control). The levels of mRNA expression were measured by qPCR and compared with those of the untreated cells. mRNA signals were normalized to the signal for *GAPDH*. The experiments were repeated three times, and the data are shown as the mean \pm s.d. (A) RT-PCR analysis of *Dpp4* mRNA expression by *DPP4* knockdown using four pre-designed siRNAs and control siRNA. *** $P < 0.001$; one-way ANOVA followed by *post hoc* comparisons using Bonferroni's multiple comparison test.

expression level was negligible. Next, we studied the effect of addition of soluble human *DPP4* on hemoglobin gene expression. The expression levels of *HBA1*, *HBA2*, and *HBB2* were significantly increased at 24 h, and subsequently they were significantly decreased at 48 h after the addition of soluble human *DPP4* in HEK293 cells. The expression levels of *HBB1* also tended to increase at 24 h after the addition of soluble human *DPP4* (Fig. 4). In the Caki-1 cells, the expression level of *HBA2* was significantly increased at 12 h, and subsequently it was decreased at 48 h after the addition of soluble human *DPP4*. The expression level of *HBB* was significantly increased at 48 h after the addition of soluble human *DPP4* (Fig. 5).

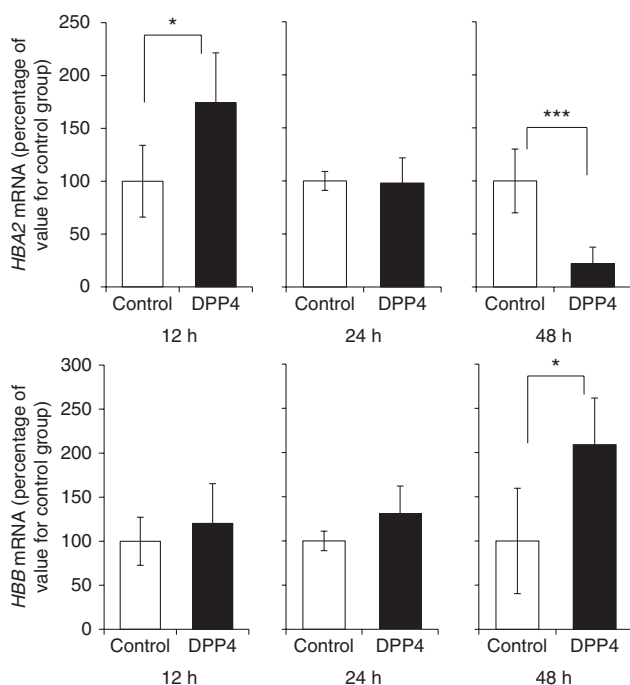
Effect of *DPP4* inhibitor on mRNA expression levels of hemoglobin genes in HEK293 and Caki-1 cells

Next, we studied the effect of a *DPP4* inhibitor on the expression of hemoglobin genes in HEK293 and Caki-1 cells. In this study, sitagliptin was used as the *DPP4* inhibitor. Sitagliptin is a highly selective *DPP4* inhibitor, and it is used as a monotherapy for glycemic control

in patients with type 2 diabetes. The expression levels of *HBA1*, *HBA2*, *HBB1*, and *HBB2* were significantly increased 48 h after the addition of sitagliptin at a final concentration of 1000 μ M in the HEK293 cells (Fig. 6). In the Caki-1 cells, the expression level of *HBA2* was significantly increased and that of *HBB* also tended to increase 48 h after the addition of sitagliptin (Fig. 7).

**Figure 4**

Changes in *HBA1*, *HBA2*, *HBB1*, and *HBB2* mRNA expression levels at 12, 24, and 48 h after the addition of soluble human *DPP4* (final concentration of 500 ng/ml) in HEK293 cells. The levels of mRNA expression were measured by qPCR and compared with those of the control group. mRNA signals were normalized to the signal for *GAPDH*. The experiments were repeated three times, and the data are shown as the mean \pm s.d. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; Student's *t*-test.

**Figure 5**

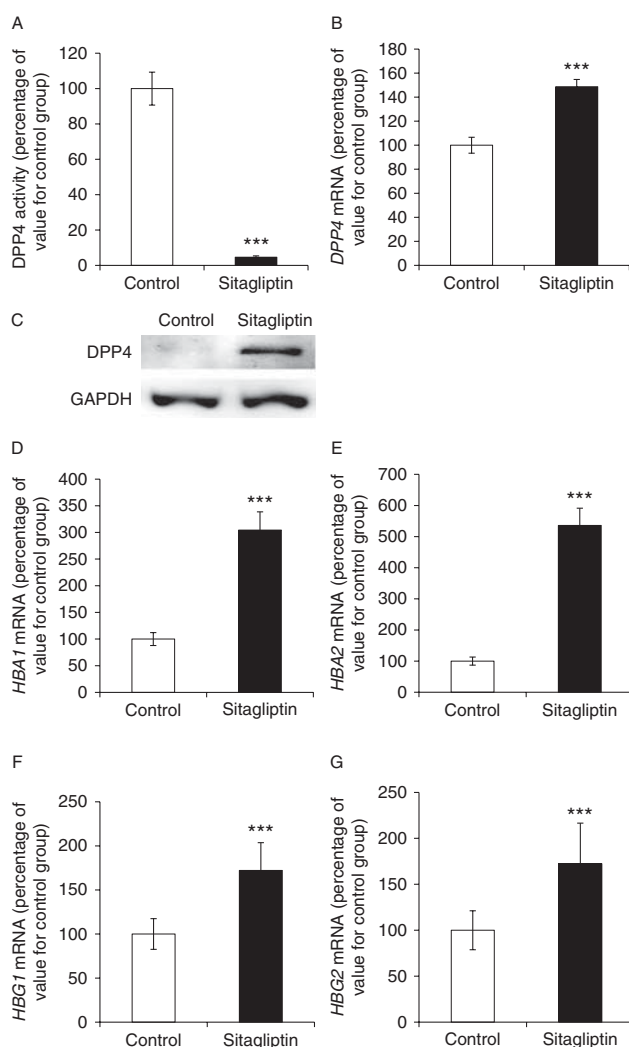
Changes in *HBA2* and *HBB* mRNA expression levels at 12, 24, and 48 h after the addition of soluble human DPP4 (final concentration of 500 ng/ml) in Caki-1 cells. The levels of mRNA expression were measured by qPCR and compared with those for the control group. mRNA signals were normalized to the signal for *Gapdh*. The experiments were repeated three times, and the data are shown as the mean \pm s.d. * $P < 0.05$ and *** $P < 0.001$; Student's *t*-test.

The expression level of *DPP4* mRNA was significantly increased 48 h after the addition of sitagliptin in both the HEK293 and Caki-1 cells (Figs 6b and 7b). The protein expression level of DPP4 was also increased (Figs 6c and 7c). This concentration of sitagliptin inhibited almost 100% of DPP4 activity (Figs 6a and 7a) and did not affect the cell viability of the HEK293 and Caki-1 cells when measured using the MTT assay (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

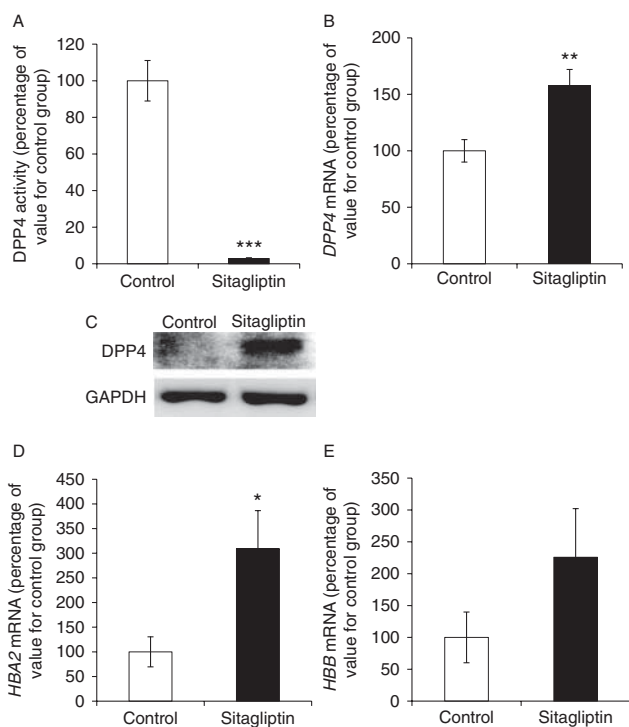
Discussion

GO analysis of the microarray results from the analysis of the rat kidneys indicated that hemoglobin genes and the alpha-2u globulin *PGCL4* gene (*Obp3*) were downregulated, and that the solute carrier family genes were upregulated in the DPP4-deficient rats when compared with the WT rats after STZ treatment. Previous reports have described reduced expression of alpha-2u globulin in the liver (Murty *et al.* 1986) and kidneys (Sharma & Tikoo 2013) of STZ-induced diabetic rats and in the renal cortex of a *Db/Db* mouse model of type 2 diabetes (Tilton *et al.* 2007).

It is also known that a number of chemicals induce alpha-2u globulin nephropathy (Swenberg *et al.* 1989, Borghoff *et al.* 1990). Our results indicate that the expression of *Obp3* was decreased by a feedback response with the accumulation of alpha-2u globulin in the kidney, because DPP4-deficient rats displayed renal dysfunction after STZ treatment, while WT rats did not. Solute carriers are membrane transport proteins that control the uptake and efflux of solutes (Schlessinger *et al.* 2010). Because the control of the solute uptake and efflux decreased due to renal dysfunction in the STZ-induced DPP4-deficient rats,

**Figure 6**

Changes in DPP4 activity (A), *DPP4* mRNA expression level (B), western immunoblots of DPP4 (C), and *HBA1* (D), *HBA2* (E), *HBG1* (F), and *HBG2* (G) mRNA expression levels 48 h after the addition of the DPP4 inhibitor, sitagliptin (final concentration of 1000 μ M), in HEK293 cells. The levels of mRNA expression were measured by qPCR and compared with those for the control group. mRNA signals were normalized to the signal for *GAPDH*. The experiments were repeated three times, and the data are shown as the mean \pm s.d. *** $P < 0.001$; Student's *t*-test.

**Figure 7**

Changes in DPP4 activity (A), *DPP4* mRNA expression level (B), western immunoblots of DPP4 (C), and *HBA2* (D), and *HBB* (E) mRNA expression levels 48 h after the addition of the DPP4 inhibitor, sitagliptin (final concentration of 1000 μ M), in Caki-1 cells. The levels of mRNA expression were measured by qPCR and compared with those for the control group. mRNA signals were normalized to the signal for *Gapdh*. The experiments were repeated three times, and the data are shown as the mean \pm s.d. * P <0.05, ** P <0.01, and *** P <0.001; Student's *t*-test.

the solute carrier family genes may have been upregulated to make up for this dysfunction. Among the genes that showed mRNA expression differences in microarray analysis of the kidneys between DPP4-deficient and WT rats after STZ treatment, only hemoglobin genes showed a significant mRNA expression difference among the four groups. The mRNA expression levels of hemoglobin genes in DPP4-deficient rats treated with STZ were significantly decreased relative to the levels in the control WT rats. Because the *Hbb* mRNA expression level in WT rats treated with STZ was significantly decreased when compared with those in the WT control rats, STZ treatment may decrease hemoglobin mRNA expression. It is suggested that the decrease in the hemoglobin mRNA level of DPP4-deficient rats treated with STZ was intensified by synergistic or additive effects of DPP4 deficiency and STZ treatment.

The mRNA expression levels of hemoglobin genes were downregulated by DPP4 siRNA knockdown, and upregulated by the addition of soluble DPP4 in HEK293 and Caki-1 cells. Therefore, it was suggested that DPP4

contributes to the regulation of hemoglobin gene expression. However, inhibition of DPP4 activity by the addition of the DPP4 inhibitor sitagliptin, resulted in significantly increased expression levels of hemoglobin genes in both cell types. Because the DPP4 expression levels were increased by the addition of the DPP4 inhibitor, it is considered that DPP4 was upregulated in response to the DPP4 inhibitor via an autoregulatory feedback mechanism linked to the increase in hemoglobin expression. Hence, it is suggested that in addition to the function of DPP4 in proteolytic activity, DPP4 also functions to contribute to the regulation of hemoglobin gene expression. DPP4 is a complex enzyme that is present on the surface of various types of cells, including kidney, liver, pancreas, and plasma cells, and is also present in a soluble form in the circulation (Mentlein 1999). DPP4 has been recognized to play an important role in the cleavage and inactivation of biologically active peptides (De Meester *et al.* 2000). In addition, DPP4, also known as CD26, is expressed on the CD4+ helper/memory T cell populations, as well as in a membrane-bound form, and can deliver a potent co-stimulatory T-cell activation signal. CD26 is the receptor for adenosine deaminase, an important cell surface immunoregulatory mechanism (Morimoto & Schlossman 1998). Recently, it has been reported that DPP4 has been identified as a functional receptor for human coronavirus–Erasmus Medical Center (hCoV–EMC; Raj *et al.* 2013), and was delineated as the molecular basis of interaction between Middle East respiratory syndrome CoV (MERS–CoV) and its receptor CD26 (Lu *et al.* 2013). Antibodies directed against DPP4 inhibited hCoV–EMC infection of primary human bronchial epithelial cells and Huh-7 cells; however, hCoV–EMC infection could not be blocked by the DPP4 inhibitors. Therefore, Raj *et al.* (2013) have proposed that the abundance of DPP4 receptors present on epithelial and endothelial tissues may be related to infection. In addition, Ikeda *et al.* (2013) have reported that the addition of soluble CD26 resulted in increased tumor necrosis factor alpha and interleukin 6 mRNA and protein expression, enhanced MAPK1/2 levels in the cytosol, and enhanced c-Fos, NF κ B p50, NF κ B p65, and CUX1 levels in the nuclei of THP1 cells and human monocytes. Our findings also indicated that soluble DPP4 enhanced the expression levels of hemoglobin genes. We consider that DPP4 does not affect transcription factors or epigenetics, because there is no nuclear transport signal in DPP4, and DPP4 does not localize in the nuclei of kidney cells (data not shown). Soluble DPP4, rather than the membrane-bound form, may indirectly contribute to the regulation

of hemoglobin gene expression in the cytosol. Further studies are required for the detailed analysis of the regulatory system by which DPP4 influences hemoglobin gene expression.

Hemoglobin is expressed by mesangial cells and it plays a cytoprotective role against oxidative insults (Nishi *et al.* 2008). In addition, the decrease in hemoglobin levels causes hypoxia. Oxidative stress and hypoxic stress induce endoplasmic reticulum (ER) stress (Ihara & Ikezaki 2010), which is involved in a wide range of renal pathophysiologicals (Kitamura 2008). Our findings indicate that soluble DPP4 enhances hemoglobin gene expression in the kidneys, and that hemoglobin protects renal function from diabetes by acting in a cytoprotective manner against oxidative, hypoxic, or ER stress.

In conclusion, we have shown for the first time, to our knowledge, that DPP4 contributes to the regulation of hemoglobin gene expression in the kidney. Although further studies are required, these findings indicate that enhanced hemoglobin expression with DPP4 expression play a key role in the preservation of renal function in diabetes.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-14-0016>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

The authors have made the following declaration about their contribution: Y S designed the study, collected and analyzed data, and wrote the manuscript. T K collected and analyzed data. A Y contributed to the discussion.

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References

Borghoff SJ, Short BG & Swenberg JA 1990 Biochemical mechanisms and pathobiology of $\alpha_2\mu$ -globulin nephropathy. *Annual Review of*

Pharmacology and Toxicology **30** 349–367. (doi:10.1146/annurev.pa.30.040190.002025)

- Cannon CP 2008 Mixed dyslipidemia, metabolic syndrome, diabetes mellitus, and cardiovascular disease: clinical implications. *American Journal of Cardiology* **102** 5L–9L. (doi:10.1016/j.amjcard.2008.09.067)
- Chang G, Zhang P, Ye L, Lu K, Wang Y, Duan Q, Zheng A, Qin S & Zhang D 2013 Protective effects of sitagliptin on myocardial injury and cardiac function in an ischemia/reperfusion rat model. *European Journal of Pharmacology* **718** 105–113. (doi:10.1016/j.ejphar.2013.09.007)
- Crofford OB 1995 Diabetes control and complications. *Annual Review of Medicine* **46** 267–279. (doi:10.1146/annurev.med.46.1.267)
- De Meester I, Durinx C, Bal G, Proost P, Struyf S, Goossens F, Augustyns K & Scharpé S 2000 Natural substrates of dipeptidyl peptidase IV. *Advances in Experimental Medicine and Biology* **477** 67–87. (doi:10.1007/0-306-46826-3_7)
- Femia AP, Raimondi L, Maglieri G, Lodovici M, Mannucci E & Caderni G 2013 Long-term treatment with Sitagliptin, a dipeptidyl peptidase-4 inhibitor, reduces colon carcinogenesis and reactive oxygen species in 1,2-dimethylhydrazine-induced rats. *International Journal of Cancer* **133** 2498–2503. (doi:10.1002/ijc.28260)
- Green BD, Flatt PR & Bailey CJ 2006 Dipeptidyl peptidase IV (DPP IV) inhibitors: a newly emerging drug class for the treatment of type 2 diabetes. *Diabetes & Vascular Disease Research* **3** 159–165. (doi:10.3132/dvdr.2006.024)
- Hinnen D, Nielsen LL, Waninger A & Kushner P 2006 Incretin mimetics and DPP-IV inhibitors: new paradigms for the treatment of type 2 diabetes. *Journal of the American Board of Family Medicine* **6** 612–620. (doi:10.3122/jabfm.19.6.612)
- Holst JJ 2006 Glucagon-like peptide-1: from extract to agent. The Claude Bernard Lecture, 2005. *Diabetologia* **49** 253–260. (doi:10.1007/s00125-005-0107-1)
- Holst JJ & Gromada J 2004 Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans. *American Journal of Physiology. Endocrinology and Metabolism* **287** E199–E206. (doi:10.1152/ajpendo.00545.2003)
- Ihara Y & Ikezaki M 2010 Oxidative stress and endoplasmic reticulum stress. *Nihon Rinsho. Japanese Journal of Clinical Medicine* **68** 49–52.
- Ikeda T, Kumagai E, Iwata S & Yamakawa A 2013 Soluble CD26/dipeptidyl peptidase IV enhances the transcription of IL-6 and TNF- α in THP-1 cells and monocytes. *PLoS ONE* **8** e66520. (doi:10.1371/journal.pone.0066520)
- Kendall DM, Kim D & Maggs D 2006 Incretin mimetics and dipeptidyl peptidase-IV inhibitors: a review of emerging therapies for type 2 diabetes. *Diabetes Technology & Therapeutics* **3** 385–396. (doi:10.1089/dia.2006.8.385)
- Kirino Y, Sato Y, Kamimoto T, Kawazoe K, Minakuchi KB & Nakahori Y 2009 Interrelationship of dipeptidyl peptidase IV (DPP4) with the development of diabetes, dyslipidemia and nephropathy: a streptozotocin-induced model using wild-type and DPP4-deficient rats. *Journal of Endocrinology* **200** 53–61. (doi:10.1677/JOE-08-0424)
- Kitamura M 2008 Endoplasmic reticulum stress in the kidney. *Clinical and Experimental Nephrology* **12** 317–325. (doi:10.1007/s10157-008-0060-7)
- Lu G, Hu Y, Wang Q, Qi J, Gao F, Li Y, Zhang Y, Zhang W, Yuan Y, Bao J *et al.* 2013 Molecular basis of binding between novel human coronavirus MERS-CoV and its receptor CD26. *Nature* **500** 227–231. (doi:10.1038/nature12328)
- Mentlein R 1999 Dipeptidyl-peptidase IV (CD26) – role in the inactivation of regulatory peptides. *Regulatory Peptides* **85** 9–24. (doi:10.1016/S0167-0115(99)00089-0)
- Morimoto C & Schlossman SF 1998 The structure and function of CD26 in the T-cell immune response. *Immunological Reviews* **161** 55–70. (doi:10.1111/j.1600-065X.1998.tb01571.x)
- Murty CV, Demyan WF, Chatterjee B & Roy AK 1986 Partial reversal of $\alpha_2\mu$ -globulin gene expression by thyroxine in the liver of diabetic rats. *Biochemistry* **25** 4376–4380. (doi:10.1021/bi00363a030)

- Nielsen LL 2005 Incretin mimetics and DPP-IV inhibitors for the treatment of type 2 diabetes. *Drug Discovery Today* **10** 703–710. (doi:10.1016/S1359-6446(05)03460-4)
- Nishi H, Inagi R, Kato H, Tanemoto M, Kojima I, Son D, Fujita T & Nangaku M 2008 Hemoglobin is expressed by mesangial cells and reduces oxidant stress. *Journal of the American Society of Nephrology* **19** 1500–1508. (doi:10.1681/ASN.2007101085)
- Nussbaum RL, McInnes RR & Willard HF 2007 Principle of molecular disease: lessons from the hemoglobinopathies. In *Thompson & Thompson Genetics in Medicine*, 6th edn, pp 181–202. Philadelphia, PA: W.B. Saunders Company.
- Raj VS, Mou H, Smits SL, Dekkers DH, Müller MA, Dijkman R, Muth D, Demmers JA, Zaki A, Fouchier RA *et al.* 2013 Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature* **495** 251–254. (doi:10.1038/nature12005)
- Schlessinger A, Matsson P, Shima JE, Pieper U, Yee SW, Kelly L, Apeltsin L, Stroud RM, Ferrin TE, Giacomini KM *et al.* 2010 Comparison of human solute carriers. *Protein Science* **19** 412–428. (doi:10.1002/pro.320)
- Sharma V & Tikoo K 2013 Stage-specific quantitative changes in renal and urinary proteome during the progression and development of streptozotocin-induced diabetic nephropathy in rats. *Molecular and Cellular Biochemistry* **388** 95–111. (doi:10.1007/s11010-013-1902-5)
- Srinivasan BT, Jarvis J, Khunti K & Davies MJ 2008 Recent advances in the management of type 2 diabetes mellitus: a review. *Postgraduate Medical Journal* **996** 524–531. (doi:10.1136/pgmj.2008.067918)
- Stephan M, Suhling H, Schade J, Wittlake M, Tasic T, Klemann C, Pabst R, Jurawitz MC, Raber KA, Hoymann HG *et al.* 2013 Effects of dipeptidyl peptidase-4 inhibition in an animal model of experimental asthma: a matter of dose, route, and time. *Physiological Reports* **1** e00095. (doi:10.1002/phy2.95)
- Swenberg JA, Short B, Borghoff S, Strasser J & Charbonneau M 1989 The comparative pathobiology of $\alpha_2\mu$ -globulin nephropathy. *Toxicology and Applied Pharmacology* **97** 35–46. (doi:10.1016/0041-008X(89)90053-7)
- Tilton RG, Haidacher SJ, Lejeune WS, Zhang X, Zhao Y, Kurosky A, Brasier AR & Denner L 2007 Diabetes-induced changes in the renal cortical proteome assessed with two-dimensional gel electrophoresis and mass spectrometry. *Proteomics* **7** 1729–1742. (doi:10.1002/pmic.200700017)
- Watanabe Y, Kojima T & Fujimoto Y 1987 Deficiency of membrane-bound dipeptidyl aminopeptidase IV in a certain rat strain. *Experientia* **43** 400–401. (doi:10.1007/BF01940426)
- Zimmet P, Alberti KG & Shaw J 2001 Global and societal implications of the diabetes epidemic. *Nature* **414** 782–787. (doi:10.1038/414782a)

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