Linagliptin-mediated DPP-4 inhibition ameliorates kidney fibrosis in streptozotocin-induced diabetic mice by inhibiting endothelial-to-mesenchymal transition in a therapeutic regimen
Keizo Kanasaki1),##, Sen Shi1), Megumi Kanasaki1), Jinhua He1), Takako Nagai1), Yuka Nakamura2), Yasuhito Ishigaki2), Munehiro Kitada1), Swayam Prakash Srivastava1), Daisuke Koya1)##

Running title: Linagliptin inhibits diabetic kidney fibrosis
1) Division of Diabetology & Endocrinology, Kanazawa Medical University, Uchinada, Ishikawa, Japan 920-0293
2) Medical Research Institute, Kanazawa Medical University, Uchinada, Ishikawa, Japan 920-0293

Words count etc.: Abstract 232, Main text 4374, Main figure 6, table 0

## : Co-correspondence

Address correspondence to:

Keizo Kanasaki, MD, PhD
E-mail: kkanasak@kanazawa-med.ac.jp,
Or
Daisuke Koya
E-mail: koya0516@kanazawa-med.ac.jp,

Department of Diabetology & Endocrinology
Kanazawa Medical University
Uchinada, Ishikawa 920-0293, Japan
TEL: 81-76-286-2211(Ex3305)
FAX: 81-76-286-6927
Abstract
Kidney fibrosis is the final common of all progressive chronic kidney diseases, of which diabetic nephropathy is the leading cause. Endothelial-to-mesenchymal transition (EndMT) has emerged as one of the most important origins of matrix-producing fibroblasts. Dipeptidyl peptidase-4 (DPP-4) inhibitors have been introduced into the market as anti-diabetic drugs. Here, we found that the DPP-4 inhibitor linagliptin ameliorated kidney fibrosis in diabetic mice without altering their blood glucose levels associated with the inhibition of EndMT and the restoration of microRNA29s. Streptozotocin-induced diabetic CD-1 mice exhibited kidney fibrosis and strong immunoreactivity for DPP-4 after 24 weeks on the onset of diabetes. At 20 weeks after the onset of diabetes, mice were treated with linagliptin for 4 weeks. Linagliptin-treated diabetic mice exhibited a suppression of DPP-4 activity/protein expression and an amelioration of kidney fibrosis associated with the inhibition of EndMT. The therapeutic effects of linagliptin on diabetic kidneys were associated with the suppression of profibrotic programs, as assessed by mRNA microarray analysis. We found that the induction of DPP-4 observed in diabetic kidneys may be associated with suppressed levels of microRNA29s in diabetic mice; linagliptin restored microRNA29s and suppressed DPP-4 protein levels. Using cultured endothelial cells, we found that linagliptin inhibited TGFβ2-induced EndMT and such anti-EndMT effects of linagliptin were mediated through microRNA29s induction. These results indicate the possible novel pleiotropic action of linagliptin to restore normal kidney function in diabetic patients with renal impairment.
Introduction

Diabetic nephropathy is a leading cause of kidney disease, which progresses into end-stage renal disease, requiring kidney replacement therapy (1; 2). Glycemic control could be essential for therapies combatting diabetic nephropathy, although normalizing the blood glucose levels in such patients with appropriate monitoring is challenging (2). Therefore, to prevent/retard diabetic nephropathy, in addition to achieving proper glycemic control, strategies that are not directly related with blood glucose normalization are required.

Fibrosis in the kidney is the final common pathway of progressive kidney diseases and results in the destruction of the kidney structure and the deterioration of the kidney filtration function (3-8). Kidney fibrosis is caused by prolonged injury associated with the dysregulation of the normal wound healing process and an excess accumulation of extracellular matrix. Kidney fibroblasts play an important role in this fibrotic process, but the origin of the fibroblasts remains unclear and has become the focus of intense debate (2; 9). A significant heterogeneity of matrix-producing fibroblasts is thought to exist (2), and diverse origins for fibroblasts have been described, such as residential fibroblasts or pericytes, epithelial-to-mesenchymal transition, and endothelial-to-mesenchymal transition (EndMT) (2). Among these diverse origins of matrix-producing fibroblasts, EndMT seems to be an important origin of myofibroblasts or activated fibroblasts (9).

Dipeptidyl peptidase (DPP)-4 inhibitors enhance the activity of endogenous glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide (10), which have emerged as important prandial stimulators of insulin secretion and have many physiological actions (10; 11). Additionally, DPP-4 is distributed throughout the body
and cleaves numerous substrates other than incretin hormones(10). Aside from their glucose-lowering action, DPP-4 inhibitors are also associated with potentially protective organ effects due to their diverse, widely distributed, and pleiotropic action (12). Indeed, the kidney is where DPP-4 is expressed at the highest level per organ weight (13). Interestingly, DPP-4 has been associated with cell survival signaling and extracellular matrix remodelings (14-16).

Linagliptin, a new DPP-4 inhibitor, is mainly excreted via the bile; therefore, this drug can theoretically be prescribed for patients with renal dysfunction without adjusting the dosage (17; 18). In this study, we tested whether the DPP-4 inhibitor linagliptin could exert its therapeutic benefits in mice with kidney fibrosis associated with type 1 diabetes.

**Research Design and Methods**

**Reagents**

A rat polyclonal anti-mouse CD31 antibody was purchased from EMFRET Analytics GmbH & Co. KG (Eibelstadt, Germany). Mouse monoclonal anti-human CD31 and goat polyclonal anti-mouse DPP-4 antibodies (for tissue labeling) were purchased from R&D System (Minneapolis, MN). A rabbit polyclonal anti-αSMA antibody was purchased from Gene Tex (Irvine, CA). A rabbit polyclonal anti-SM22α antibody and a monoclonal antibody for VE-cadherin were obtained from Novus Biological (Littleton, CO). A rabbit polyclonal anti-eNOS antibody was purchased from Thermo Scientific (Waltham, MA). A goat polyclonal anti-DPP-4 antibody (for western blotting in human cells) and a rabbit polyclonal anti-GAPDH antibody were obtained from Sigma (St. Louis, MO). Fluorescence-, Alexa Fluor 647-, rhodamine-conjugated secondary
antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). A horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Cell Signaling Technology (Danvers, MA). TGF-β2 was purchased from PeproTech (Rocky Hill, NJ). Apoptosis was detected with an Annexin V assay kit, which was obtained from Clontech Laboratories (Mountain View, CA). DPP-4 activity was monitored by DPP-4 assay kit (BioVision, Milpitas, CA).

Animal experiments

Eight-week-old male CD-1 mice (Sankyo Lab Service, Tokyo, Japan) were utilized in all of the diabetic experiments. The mice were injected intraperitoneally with streptozotocin (STZ: 200 mg/kg). The induction of diabetes was confirmed as a blood glucose level > 16 mM 2 weeks after STZ injection. By 20 weeks after the induction of diabetes, the diabetic mice were divided into two groups (linagliptin [5 mg/kg BW/day in drinking water] and untreated). Dose of linagliptin was decided based on the dose dependent effects of this drug between 3-30 mg/kgBW/day (19). Linagliptin was diluted directly in drinking water. All mice were sacrificed 24 weeks after the induction of diabetes. Linagliptin was provided by Boehringer Ingelheim (Ingelheim, Germany), with a material transfer agreement (MTA). Blood pressure was monitored by the tail cuff method with BP-98A (Softron Co. Beijing, China).

EndMT detection in vivo

Frozen sections (5 µm) were used for the detection of in vivo EndMT. Cells undergoing EndMT were identified by double positive labeling with CD31-αSMA or CD31-FSP1.
The immunolabeled sections were analyzed by fluorescence microscopy (BioZero, Keyence, Osaka, Japan). For each mouse, 300x magnification pictures were obtained from 6 different areas, and quantification was performed.

**Morphological evaluation**

The glomerular surface area was calculated in 10 glomeruli per mouse using ImageJ software. To evaluate the mesangial matrix area (%), we utilized a point counting method. We analyzed 10 PAS-stained glomeruli from each mouse on a digital microscope screen grid containing 540 (27x20) points in Adobe Photoshop Element 6.0®. The number of grid points in the mesangial area (both matrix and cells) was divided by the total number of points in the glomerulus to obtain the percentage of relative mesangial matrix area in a given glomerulus. Masson’s trichrome-labeled sections were imaged and analyzed by ImageJ software, and fibrotic areas were quantified. In each mouse, 6 pictures (100x magnification) were evaluated.

**Immunohistochemistry**

Deparaffinized (2 min in xylene, four times; 1 min in 100% ethanol, twice; 30 s in 95% ethanol, 45 s in 70% ethanol, and 1 min in distilled water) mouse kidney sections were utilized for DPP-4 labeling. Immunohistochemistry was performed with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The DPP-4 primary antibody was diluted 1:100. In negative controls, the primary antibody was omitted and replaced with blocking solution.
In vitro EndMT

Human dermal microvascular endothelial cells (HMVECs, Lonza, Basel, Switzerland) cultured in EGM medium were used in this experiment. When the HMVECs on the adhesion reagent (Kurabo medical, Osaka, Japan) reached 70% confluence, 5 ng/ml recombinant human TGF-β2 for 48 h was placed in the experimental medium (HuMedia-MVG in serum-free RPMI at a 1:3 ratio) with or without linagliptin (100 nM) preincubation for 2 h. In control well, vehicle (DMSO) was added (3x10^-5 dilution of DMSO in final concentration). The protein lysate was harvested for western blot analysis.

Wound healing assay

The passaged HMVECs were placed in 6-well plates and cultured with EGM-2 until reaching 70%-80% confluence, and then the cells treated with TGF-β2 (2.5 ng/ml) in the presence or absence of linagliptin (100 nM) were incubated with a medium containing HuMedia MVG and RPMI1640 (1:3). At the same time, the control group was incubated in the same medium without TGF-β2 or linagliptin. In control well, vehicle (DMSO) was added (3x10^-5 dilution of DMSO in final concentration). Using a pipette tip at an angle of approximately 30°, each well received a straight scratch, simulating a wound. After 24 h and 48 h had passed, the number of cells that migrated into the wounded area was counted under a light microscope. Six different areas were evaluated in each group, and the experiment was repeated twice with similar results.

Cell migration Boyden chamber assay

The bottom side of the migration chamber (Cell Culture Insert, BD Falcon, San Jose,
California) was coated with Matrigel (BD), and 1000 HMVECs were passaged in the upper migration chamber. Twenty-four hours after passage, the medium was changed to the experimental medium (1:3 HuMedia-MVG: RPMI1640) in both the upper and the bottom wells. Subsequently, cells were exposed to TGF-β2 in the presence or absence of linagliptin (100 nM), while the control group was incubated with the same medium, lacking TGF-β2 and linagliptin. In control well, vehicle (DMSO) was added (3x10^{-5} dilution of DMSO in final concentration). After 48 h, the cells were washed with PBS, followed by fixation with formaldehyde (3.7% in PBS) at room temperature for 2 min. After washing twice with PBS, the cells were permeabilized with 100% methanol for 20 min at room temperature. Then, the cells were washed twice with PBS and stained with hematoxylin-eosin. After scraping off the non-migratory cells (upper well) with a cotton swab, the number of migrated cells was counted under a light microscope. Six different areas were evaluated in each group, and the experiment was repeated twice with similar results.

**Western blotting**

The protein lysates were denatured by boiling in sodium dodecyl sulfate (SDS) sample buffer at 100 °C for 5 minutes and then centrifuged (17,000 ×g for 10 minutes at 4 °C); subsequently, the supernatant was separated on SDS-polyacrylamide gels. Separated protein lysates were blotted onto PVDF membranes (Pall Corporation, Pensacola, FL) by the semidry method. After blocking with TBS-T (Tris-buffered saline containing 0.05% Tween 20) containing 5% non-fat dry milk or 5% BSA, the membranes were incubated with the primary antibodies of the target molecules (1:500 for all primary
antibodies) in TBST containing 5% BSA at 4 °C overnight. The membranes were washed three times and incubated with 1:2000 diluted HRP-conjugated secondary antibodies (Cell Signaling Technology) for 1 h at room temperature. The immunoreactive bands were visualized with an enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology, Rockford, IL) by ImageQuant LAS 400 (GE Healthcare Life Sciences, Uppsala, Sweden).

**mRNA array analysis**

The total RNA was isolated using a commercially available kit (RNaseasy Mini Kit; QIAGEN GmbH, Hilden, Germany). The concentration of RNA was quantified by photometry at 260/280 nm, and the quality of the RNA was determined by the ratio of the 18S/28S ribosomal band intensities in an ethidium bromide-containing 1% agarose gel after electrophoresis. The sense cDNA was prepared using an Ambion® WT Expression Kit (Ambion, Austin, TX), and target hybridizations were performed using a Mouse Gene 1.0 ST Array (Affymetrix, Santa Clara, CA), according to the manufacturer’s instructions. Hybridization was performed for 17 h at 45 °C in a GeneChip® Hybridization Oven 640 (Affymetrix). After washing and staining in a GeneChip® Fluidics Station 450, hybridized cDNAs were detected using the GeneChip® Scanner 3000. The digitalized image data were processed using the GeneChip® Operating Software (GCOS) version 1.4. Because replicate assays were not performed, the signal intensities of the selected genes that were up-regulated or down-regulated by at least two-fold compared with a control group were extracted using GeneSpring GX software package version 12.5 (Agilent Technologies, Santa Clara,
CA). After hierarchical clustering, the results were illustrated as a heat map. Ingenuity Pathway Analysis (IPA; Ingenuity Systems Inc., Redwood City, CA) was used to select the specific function-related genes.

**microRNA array analysis**

Total RNA was isolated using the miRNeasy Kit (Qiagen) following the manufacturer’s instructions. Quality-confirmed total RNA samples were assayed and qualified in duplicate using the microRNA microarray. The input for the Agilent microRNA labeling system was 100 ng total RNA. Dephosphorylated and denatured total RNA was labeled with cyanine 3-pCp and subsequently hybridized to the Agilent mouse microRNA microarray release version 15 using the microRNA Complete Labeling and Hyb Kit (Agilent Technologies, Inc., Santa Clara CA). Following hybridization for 20 h, the slides were washed with Gene Expression Wash Buffer Kit (Agilent) and measured using an Agilent Scanner G2565BA. Agilent Feature Extraction Software version 9.5.1 and GeneSpring GX software version 12.5 (Agilent) were used for data processing, analysis, and monitoring.

**microRNA isolation and qPCR**

Frozen kidney tissues (one each from a control, diabetic, and linagliptin-treated diabetic mouse; samples were kept on -70 °C) were first placed on the RNA later®-ICE (Life technologies) for 16 h at -20 °C before the subsequent homogenization process to avoid RNA degradation while extracting high-quality microRNA. microRNA was extracted
using miRNeasy Mini kit (Qiagen) according to the manufacturer’s instructions for homogenized samples. The complementary DNA was generated by a miScript II RT kit (Qiagen) using the hiSpec buffer method. microRNA expression was quantified using miScript SYBR Green PCR Kit (Qiagen) using 3 ng of complementary DNA. The primers to quantify Mm_miR-29a, Mm_miR-29b, and Mm_miR-29c were the miScript primer assays pre-designed by Qiagen. The mature microRNA sequences were 5’ UAGCACCAUCUGAAACGGGUUA for Mm_miR-29a, 5’ UAGCACCAUUGAAACUGGUUA for Mm_miR-29b, and 5’ UAGCACCAUUGAAACUCGGGUUA for Mm_miR-29c. All experiments were performed in triplicate, and Hs_RNU6-2_1 (Qiagen) was utilized as an internal control.

Transfection
For the transfection studies, HMVECs, which were maintained in EBM-2 medium supplemented with EGM™-2 (Lonza, USA), were passaged in 6-well plates with non-proliferative medium (HuMedia-MVG and RPMI at a ratio of 1:3). The HMVECs were transfected with 100 nM of antagomir® for miR-29a, miR-29c, amiR-29a+c (Fasmac, Japan), miR-29b inhibitor (Quiagen), or mimetics for miR29s (29a-3p: UAGCACCAUCUGAAACGGGUUA, 29b-3p: UAGCACCAUUGAAACUGGUU, 29c-3p: UAGCACCAUUGAAACUCGGGUUA) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

The cells were incubated for 6 h with Lipofectamine and the anti-microRNA complex in antibiotic-free medium, after which the medium was replaced with fresh medium before the cells were incubated for another 48 h. Upon the termination of the
incubation, the cells were scraped using RIPA buffer (with the addition of PMSF, sodium vanadate and protease inhibitor) to assess DPP-4 protein expression (Sigma Chemical Co., St. Louis, MO, USA) using the western blot technique.

**Luciferase assay**

For the luciferase assay to analyze the activity of 3’UTR in human DPP-4, we cloned the fragment of human DPP-4 3’UTR sequence by PCR using the primer set (Fw: ATAGAGCTCAATAGCTAGCAGCACAGCACACCAAC Rev: ATATCTAGAGTGTCATATGCCAGTGCGGTTTAGG) and BAC clone Human RP11 178A14 as template. Both purified PCR fragment and pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) were enzyme digested (Sac1 and Xba1), purified, and ligated (Thermo Fisher Scientific, Waltham, MA). Sequence of DPP-4 3’UTR was confirmed and amplified vector DNA (300ng/well in 12wells plate) was transfected into HMVEC cells. In the presence of control miRNA, mimetic, antagonim, or inhibitor of 29s (75nM in final concentration), transcriptional activity was evaluated by Dual-Luciferase® Reporter (DLR™) Assay System (Promega) in triplicate samples.

**Statistical analysis**

The data are expressed as the means ± s.e.m. The non-parametric Mann-Whitney U test was used to determine significance, which was defined as $P < 0.05$, if not specifically mentioned. GraphPad Prism software (Ver 5.0f) was used for the statistical analysis.
Results

Linagliptin restored the normal kidney structure of STZ-induced diabetic kidney fibrosis in CD-1 mice

We utilize a fibrotic diabetic kidney disease model, STZ-induced diabetes in male CD-1 mice. The immunohistochemistry analysis revealed robust DPP-4 immunolabeling in the glomerular basement membrane, tubules, and peritubular vascular cells in the kidney of STZ-induced diabetic CD-1 mice when compared with control CD-1 mice (Fig. 1 A, B). Such peritubular vascular cells were likely endothelial cells upon evaluation by immunofluorescence microscopy (Fig. 1 C, D, E). Western blot analysis using whole kidney revealed that, in the kidneys of diabetic mice, the DPP-4 protein levels were up-regulated compared with control kidneys (Fig. 1 F, G). As Sugimoto et al. elegantly showed (20), STZ-induced diabetic CD-1 mice exhibit kidney fibrosis, with both glomerulosclerosis and tubulointerstitial fibrosis occurring approximately 16 weeks after the onset of diabetes, (Supplementary Fig. 1), and at 24 weeks after the initiation of diabetes, diabetic mice exhibited severe fibrosis when compared with control mice (Fig. 2 A, B, D, E). Linagliptin-treated diabetic mice exhibited restored normal kidney structures (Fig. 2 C, F). Morphometric analysis of the kidneys revealed that diabetic mice displayed significantly enlarged glomeruli, mesangial expansion, and relative areas of Masson-trichrome-positive interstitial fibrosis (Fig. 2 G, H, I), while linagliptin restored normal kidney histology and normal architecture (Fig. 2 G, H, I). Urine albumin levels were elevated in STZ mice, and linagliptin treatment suppressed the trend of the urine albumin levels (Fig. 2J) as observed in human clinical trial(21). These antifibrotic effects of linagliptin in diabetic mice are associated with suppressed trend of TGFβ1 and DPP-4, and significant
suppression of TGFβ2 protein levels in kidney (Fig. 2 K-N). Linagliptin inhibited DPP-4 mRNA (Fig. 2O) and diabetes-enhanced DPP-4 activity in kidney and plasma of diabetic mice (Fig. 2 P and Q)

When compared with control mice, diabetic CD-1 mice exhibited lower blood pressure, lighter body weight, higher blood sugar, and increased kidney and liver weight (Fig. 3 A, B, C, D, E). Linagliptin treatment in STZ-induced diabetic CD-1 mice caused no alteration in blood pressure, body weight, blood sugar level, or organ weight (for kidney, liver, and heart) when compared with untreated diabetic mice (Fig. 3 A, B, C, D, E). The heart weight was lighter in all diabetic mice but showed insignificant changes in all groups analyzed (Fig. 3F).

The anti-fibrotic effects of linagliptin were associated with the inhibition of EndMT in diabetic kidneys

Kidney fibroblasts play essential roles in kidney fibrosis and originate from diverse sources (2; 9; 22). In our analysis, we analyzed EndMT, a recently described important source of kidney fibroblasts (2; 9; 23). When EndMT was analyzed by quantifying cells that co-expressed endothelial marker CD31 and a mesenchymal marker, either α-smooth muscle actin (SMA) or FSP1, diabetic CD-1 mice exhibited a significantly increased number of cells in the process of the EndMT when compared with control kidneys (Fig. 4 A, B, D, E, F, H). Linagliptin-treated mice exhibited significantly fewer cells undergoing EndMT in the kidney when compared to untreated diabetic mice (Fig. 4 C, D, G, H).

Linagliptin inhibited EndMT and apoptosis in cultured endothelial cells
In cultured endothelial cells, TGF-β2 induced the suppression of CD31 with concomitant upregulation of SM22α in HMVECs, suggesting that TGF-β2 induces EndMT (Fig. 5 A, B, C); TGF-β2-induced EndMT was inhibited by linagliptin pre-treatment (Fig. 5 A, B, C). Wound healing cell invasion assays revealed that TGF-β2 also induced the migration of fibroblast-like EndMT cells (Fig. 5 D, E, J, G, H), while linagliptin inhibited the invasion of those cells (Fig. 5 F, I, J). The Boyden chamber cell migration assay also revealed that linagliptin inhibited the endothelial cells’ transmigration through Matrigel (Fig. 5 K-N). When analyzing the molecular mechanisms of TGF-β2 induced EndMT effects, we found that linagliptin inhibited TGF-β2-induced smad3 phosphorylation in endothelial cells (Fig. 5O). Linagliptin inhibited TGF-β2-induced protein expression, mRNA expression, and activity of DPP-4 in endothelial cells (Fig. 5 P-R). Linagliptin and generic DPP-4 inhibitor KR62436 inhibited TGF-β2-induced endothelial cell apoptosis (Supplementary Fig. 2).

**Linagliptin inhibited profibrotic programming in the diabetic kidney**

A heat map indicated the differences among controls, diabetic mice, and diabetic mice treated with linagliptin (Supplementary Fig. 3A). Some groups of genes trended altered in the diabetic mouse and restored in the linagliptin-treated mouse (Supplementary Fig. 3A). To focus on fibrosis, we selected fibrosis-associated genes based in the Ingenuity Pathways Analysis database and plotted them in a scattered format (Supplementary Fig. 3B). Compared with controls, 13 genes were up-regulated, and 2 genes were down-regulated in the diabetic mouse (Supplementary Fig. 3C). However, the expression of these genes trended to be reversed into normal levels after
linagliptin treatment.

**Role of microRNA29 family suppression in the DPP-4 induction of diabetic kidney**

Finally, to identify the underlying mechanisms of how DPP-4 is increased in diabetic kidney, we analyzed the microRNA profiles of the animals and found that the microRNA 29 family tended to be suppressed in diabetic mice kidneys when compared with control kidneys (Fig. 6A: though no significant difference by Welch’s test yet). According to the prediction of microRNA targets by TargetScan (http://www.targetscan.org/vert_60/), DPP-4 may be regulated by the microRNA 29 family. Quantitative analysis revealed that microRNAs 29 a, b, and c were suppressed in the diabetic kidney when compared with control kidneys and linagliptin restored such diabetes-suppressed microRNA 29s (Fig. 6 B, C, D). Similarly TGF-β2-suppressed microRNA29s were restored by linagliptin in vitro analysis (Supplementary Fig. 4). When an antagomiR® for microRNA 29a was transfected into endothelial cells, DPP-4 protein levels were indeed increased (Fig. 6 E, F), whereas microRNA 29c antagomiR® transfection unchanged DPP-4 level (Fig. 6 E, F). The co-transfection of antagomiRs® for microRNAs 29a and 29c resulted in persistently higher DPP-4 protein levels in the endothelial cells compared with controls (Fig. 6 E, F). In addition, the inhibitor® for microRNA 29b induced DPP-4 protein expression in HMVECs (Fig. 6 G, H), suggesting that microRNAs, specifically the microRNA 29 family, regulate DPP-4 in the diabetic kidney. TGF-β2-enhanced DPP-4 protein expression was significantly suppressed by microRNA 29b mimetics and some trend of suppression was found in microRNA 29a,c mimetics-transfected cells (Fig. 6 I and J).
TGF-β2-induced EndMT and endothelial cell migration was largely inhibited by microRNA29s mimic transfection (Fig. 6 I, K-T). In contrast antagomiRs® and inhibitor ® for microRNA29s induced EndMT phenotype and migration (Supplementary Fig. 5). TGF-β2-stimulated luciferase activity of pmirGLO Dual-Luciferase miRNA Target Expression Vector containing 3’UTR fragment of DPP4, where microRNA 29 binding site was involved, were significantly suppressed by miR29 mimetics (Fig. 6U). In contrast, antagomiR® or inhibitor® of microRNA29s significantly induced DPP4 3’UTR luciferase activity (Fig. 6V).

Discussion

Both the inhibition of kidney fibrosis and the restoration of normal kidney structure are fundamental processes to research for developing therapies to combat progressive chronic kidney disease, including diabetic nephropathy. Although kidney fibroblasts have been implicated in the pathogenesis of kidney fibrosis, it would be challenging to inoculate only kidney fibroblasts as therapeutic targets. In our analysis, DPP-4 inhibition by linagliptin in the diabetic kidney seems to be a powerful therapy that inhibits the fibroblast-activating process.

We found that DPP-4 protein expression was increased in the whole kidney lysate, endothelial cells, tubules, and glomeruli of STZ-induced diabetic kidneys from CD-1 mice. Both the protein expression and activity levels of DPP-4 are higher in kidneys of high fat-fed, STZ-injected mice (24). Liu et al. reported that the administration of the DPP-4 inhibitor vildagliptin for 24 weeks prevented kidney damage in STZ-induced diabetic male Sprague-Dawley rats(25). In our analysis, we
utilized fibrotic STZ-induced diabetic CD-1 mice and tested whether intervention with linagliptin starting 20 weeks after the induction of diabetes could rescue fibrotic kidneys. We found that introducing this intervention to such fibrotic kidneys significantly ameliorated kidney fibrosis without altering physiological parameters, suggesting that increased DPP-4 in fibrotic diabetic kidneys is a therapeutically valuable target. Our microarray analysis clearly demonstrated that diabetic kidneys exhibited profibrotic signaling and that linagliptin restored a normal profile in the diabetic kidney.

We have not yet realized an entire series of DPP-4-mediated, kidney-damaging signals in our models, but we believe that signaling associated with endothelial cell survival and EndMT is involved. Linagliptin inhibited both the matrix- and fibroblast-generating pathways associated with EndMT and fibroblast-like EndMT cell invasion. Such enhanced DPP-4 activity is associated with matrix metalloproteinases, which are responsible for tissue remodeling (26). Furthermore, Takahashi et al. recently showed that DPP-4 inhibition by vildagliptin ameliorates heart failure and collagen deposition associated with inhibiting the TGF-β-Smad signaling pathway (27). We found that linagliptin inhibited TGF-β2-induced smad3 phosphorylation.

Our analysis reveals that the miR29 family is one of the potential molecular regulators of DPP-4 in the kidney and endothelial cells as well. The miR29 family protects organs from fibrotic damage (28-31); therefore, DPP-4 inhibition and the subsequent inhibition of fibroblast activation pathway may be involved in the anti-fibrotic effects of the miR29 family. DPP-4 has been implicated in fibrogenic pathologies (32-36). Regard with this, DPP-4 inhibition by linagliptin increased miR29s
both in vivo and in vitro. miR29s suppressions in either diabetic kidneys or TGF-β2-stimulated endothelial cells are associated with EndMT and induction of DPP-4. The inhibition of each microRNA29s can induce EndMT feature in HMVEC and migration; role of each microRNA29s in DPP-4 level regulation is somewhat complicated. Since microRNA29a inhibition results in strong induction of DPP-4 3’UTR-luciferase activity, in basal condition microRNA29a likely emerges main regulator of DPP-4 3’UTR; TGF-β2-induced DPP-4 protein level and cell migration were most efficiently inhibited by microRNA29b, suggesting that each microRNA29s could play distinct roles and corporate in the homeostasis of cells in context dependent manner. Also our data indicates TGF-β2/DPP-4/miR29s display cross-talk mechanisms in the onset of kidney fibrosis and linagliptin-mediated inhibition of DPP-4 would diminish pro-fibrotic signaling cross-talk in kidney. Among human fibrotic diseases, patients with hepatitis C viral infections also exhibit high DPP-4 activity; such DPP-4 activity could be a potential target for combatting such liver diseases (37). Further study is required to determine whether DPP-4 activity is greater in type 1 or type 2 diabetic kidney diseases, or in other fibrotic chronic and acute diseases, including kidney disease, in humans.

In our analysis, linagliptin inhibited kidney fibrosis and restored normal kidney structure. Even though convincing, there are several limitations. First, the concentration we used was very high (5mg/kgBW) when compared to that used in clinic (about 100µg/kgBW). Second, according to our data, we believe that DPP-4 inhibition by any of DPP-4 inhibitor can ameliorate kidney fibrosis. However it is not yet clear other DPP-4 inhibitor can efficiently suppress pro-fibrotic program in kidney due to difference in mechanisms/metabolism of each drugs. Third, we focused on EndMT as
the origin of activated fibroblasts. However such origins of fibroblasts in kidney fibrosis are still controversial and focus of intense debate (2; 9), and it could be possible that diverse origins of kidney fibroblast activating pathway (38) were affected by linagliptin. Finally analyze for distinct roles of each microRNA29s in the regulation of DPP-4 and kidney fibrosis would require further investigation.

Diabetic kidney disease represents a serious health problem worldwide and can develop into end-stage renal disease, which requires kidney replacement therapy. Progressive kidney fibrosis determines residual kidney function, and the restoration of the normal architecture to the fibrotic kidney would constitute a fundamental therapy. We reported here that the anti-fibrotic effects of linagliptin are beneficial for diabetic kidney disease, regardless of the blood sugar levels, via the suppression of the activated fibroblast-generating EndMT pathway at least in part. Linagliptin may be safe for use among kidney disease patients, given its ability to be exclusively eliminated via the bile; this specificity suggests that this DPP-4 inhibitor has potential utility for therapeutic use in combatting kidney fibrosis in diabetes.

Authors’ contribution
KK proposed the original idea and design of the experiments, supervised experiments, provided intellectual input, and wrote the manuscript. Sen S performed the quantification of the histological analysis and most of the in vitro analyses and DPP-4 activity measurement. Megumi K took care of the mice, performed the histological analysis, provided intellectual advice, and edited the manuscript. JH performed some of the animal experiments and some of the in vitro analysis. Munehiro K participated in
the discussions. TN performed some of the in vitro analysis regarding EndMT. Swayam S performed in vivo and in vitro qPCR for mRNA and miRNA, western blot, transfection, cloning DPP-4 3’UTR vector, and quantification. YN, and YI performed the microRNA and mRNA array analysis. DK provided intellectual input.

Acknowledgement

The authors declare no conflicts of interest related to this work. We thank Boehringer Ingelheim for providing the linagliptin with MTA. This work was partially supported by grants from the Japan Society for the Promotion of Science for M Kanasaki (24790329), M Kitada (24591218), TN (24659264), KK (23790381), DK (25282028, 25670414, and research grants from the Japan Research Foundation for Clinical Pharmacology to K.K. (2011), and Takeda visionally research grant to K.K. (2013). This work was partially supported by a Grant for Collaborative Research awarded to DK (C2011-4, C2012-1) and a Grant for Promoted Research awarded to KK (S2011-1, S2012-5) from Kanazawa Medical University. KK was also supported by several foundational grants, including grants from the Japan Research Foundation for Clinical Pharmacology, the Daiichi-Sankyo Foundation of Life Science, the Ono Medical Research Foundation, the NOVARTIS Foundation (Japan) for the Promotion of Science, the Takeda Science Foundation, and the Banyu Foundation. SP Srivastava is supported by the Japanese Government MEXT (Ministry of Education, Culture, Sports, Science, and Technology) Fellowship Program. S Shi and JH are supported by foreign scholar grants from Kanazawa Medical University. KK and DK received lecture fees from Boehringer Ingelheim and Eli Lilly. Both Boehringer Ingelheim and Eli Lilly donated to Kanazawa Medical University and were not directly associated with this project.
Drs. Sen Shi and Swayam Prakash Srivastava are guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

References

5. Risdon RA, Sloper JC, de Wardener HE: Relationship between renal function and histological changes found in renal biopsy specimens from patients with persistent glomerular nephritis. Lancet 1968 2:363


Figure legend

Figure 1 DPP-4 induction in diabetic kidneys. A, B: Immunohistochemical analysis of DPP-4 in control (A) or STZ-induced diabetic (B) CD-1 mice. Eight-week-old CD-1 mice received a single injection of STZ. The mice were sacrificed 24 weeks after the induction of diabetes. n=3 in each group, and representative pictures are shown. C-E: Immunofluorescence microscopy analysis for DPP-4 in diabetic mice. Frozen kidney samples were labeled with DPP-4 and endothelial marker CD31. Immunofluorescence analysis was performed by fluorescence microscopy. C: FITC-labeled DPP-4, D: Rhodamine-labeled CD31, E: Merged images are shown. The original magnification was 300x. Scale bar: 50 µm in each panel. Representative pictures from 3 mice are shown. F: Western blot analysis for DPP-4 in the kidney. The protein lysate (15 µg) was separated in polyacrylamide gels and transferred onto a PDVF membrane. Immunoreactive bands were analyzed by the ECL method. Representative data from 6 mice in each group are shown. G. Densitometric analysis of the DPP-4 Western blot analysis. n=6 in each groups were analyzed. Data are expressed as the means±s.e.m and are shown in the graph. Diabetic mice are designated as DM or D in the figure.

Figure 2 Inhibition of DPP-4 by linagliptin in diabetic kidneys is associated with the amelioration of kidney fibrosis. A-C: PAS staining for glomeruli. D-F: MTS staining in the indicated group of mice. Scale bar: 50 µm. G-I: A morphometric analysis of the kidney histology was performed as described in the methods section. Control, n=5; DM, n=7; and DM+linagliptin, n=5 were analyzed. J: Urine albumin excretion was analyzed by albumin-creatinine ratios. Control, n=5; DM, n=8; and DM+linagliptin, n=6 were analyzed. K. Western blot analysis for TGF-β1, -β2, and DPP-4. Representative blot from 4 independent experiments are shown. L-N. Densitometric analysis of indicated protein expression relative to actin levels are shown. n=4 in each groups were analyzed. O. qPCR analysis for DPP-4 in the kidney from 6 mice in each group. P, Q. DPP-4 activity measurements in kidney (P: control, n=3; DM, n=6, DM+linagliptin, n=6) and plasma (Q: all n=6) are analyzed. The graphs in the figure are expressed as the means±s.e.m. Diabetic mice are designated as DM in the figure.
Figure 3 Characteristics of animals. A) Systolic and diastolic blood pressure, B) body weight, C) blood sugar level, D) kidney weight (both right and left), E) liver weight, and F) heart weight are shown. Organ weights are shown as the weight per g BW of each mouse. Control, n=5; DM, n=5; and DM+linagliptin, n=3 were analyzed. Graphs are expressed as the means±s.e.m. Diabetic mice are designated as DM in the figure.

Figure 4 Linagliptin suppressed the EndMT in diabetic kidney. A-C: FSP1xCD31, E-G: αSMA-CD31, immunolabeling visualized by fluorescence microscopy. Arrows indicate cells undergoing EndMT. D, H: Quantification of FSP1-CD31 or αSMA-CD31 double-positive cells. The sections are labeled as described in the methods section, and the percentages of cells undergoing the EndMT were calculated among all DAPI-positive cells. Control, n=5; DM, n=5; and DM+linagliptin, n=3 were analyzed. The original magnification was 300x, and the scale bar on the picture indicates 50 μm. Diabetic mice are designated as DM in the figure. I: Western blot analysis using kidney samples. The protein lysate (15 μg) was separated in polyacrylamide gels and transferred onto a PVDF membrane. Immunoreactive bands were analyzed by the ECL method. Representative data from 4 mice in each group are shown. J-L: Densitometric analysis of the TGFβ1 (J), TGFβ2 (K), and DPPx4 (L) Western blot analysis normalized the level with actin. n=4 in each groups were analyzed. Data are expressed as the means±s.e.m in the graph. Diabetic mice are designated as DM in the figure.

Figure 5 In vitro EndMT was inhibited by linagliptin. A: Western blot analysis. HMVECs were incubated with 2.5 ng/ml of TGF-β2 in the presence or absence of linagliptin (200 ng/ml) for 48 h. Cell lysates were harvested with RIPA buffer and denatured; 15 μg of cell lysate was separated on the gel, transferred onto the PVDF membrane, and underwent chemiluminescence for the indicated antibody to detect immunoreactive bands. Representative results from 3 independent analyses are shown. B,C: Densitometric analysis of each protein’s expression, normalized as indicated in the figure. Data are shown as the means ± s.e.m. in the graph (n=3). D-I: Wound healing cell migration assay. EndMT cells at 80% confluence were incubated, scratched linearly, and subsequently stimulated with TGF-β2 in the presence or absence of linagliptin. Cell that migrated into the scratched wound were measured, and 5 different areas were
evaluated in 300x images. J: Quantification of the wound healing assay. K-M: Boyden chamber cell migration assays were performed as described in the methods section. Migrated cells in the bottom layer of the Boyden chamber were counted in 5 different areas, and quantification was performed (N). O: Smad3 phosphorylation was analyzed by western blot analysis. Densitometric analysis was also performed (n=3). P, Q: The protein (P) and mRNA (Q) expression in HMVEC cells analyzed by western blot analysis (n=4) or qPCR (n=5). R: DPP-4 activity in HMVEC cells (n=3). The graph in the figure is expressed as the means±s.e.m. Diabetic mice are designated as DM in the figure.

**Figure 6 microRNA29 alterations may be involved in the induction of DPP-4 in kidneys of diabetic mice.** A: microRNA array analysis of the microRNA 29 cluster. n=2 are used. B-D: qPCR analysis for microRNA 29 a-c. For microRNA 29a, control n=6, diabetes n=7, others n=4 or 5 in each group. Diabetic mice are designated as DM in the figure. E-H: microRNA inhibition experiments. Antagomir® for 29a or 29c or an inhibitor for microRNA 29b was transfected into HMVECs, and their DPP-4 protein levels were analyzed by western blots. Densitometric analyses (F, H) for DPP-4 protein levels were performed. In each experiment, relative values to the control cells were analyzed. n=3 in 29a, c experiment and n=4 in 29b experiment. I. HMVEC cells were stimulated with TGF-β2 in the presence of indicated microRNA mimetics transfection for 48 hours. Cells were harvested and analyzed with western blot analysis. J-N: Densitometric analysis was also performed and relative value against actin levels (n=3). O-S: migration assay using boyden chamber as Figure5. T: Migrated cells in the bottom layer of the Boyden chamber were counted in 6 different areas. U,V: DPP-4 3’UTR transcriptional activity measurement with pmirGLO Dual-Luciferase microRNA Target Expression Vector, Co-transfected with microRNA 29 mimetics in the presence of TGF-β2(U) or microRNA 29 antagomir®/inhibitor® (V). Experiments were performed twice, showing similar results. One-way ANOVA followed by Turkey test was used for statistics analysis. sc: scramble control microRNA. The graph in the figure is expressed as the means±s.e.m.
FIG1
157x136mm (300 x 300 DPI)
FIG4
162x62mm (300 x 300 DPI)
A. 

<table>
<thead>
<tr>
<th></th>
<th>TGF-β2</th>
<th>Linagliptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SM22α</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GAPDH</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

B. 

- CD31/GAPDH (Fold change)
- SM22α/GAPDH (Fold change)

C. 

- TGF-β2
- TGF-β2 + Linagliptin

D. Cont 24h
E. TGF-β2 24h
F. TGF-β2+linagliptin 24h

G. Cont 48h
H. TGF-β2 48h
I. TGF-β2+linagliptin 48h

J. Invaded cells (×100 field)

K. Cont 24h
L. TGF-β2 24h
M. TGF-β2+linagliptin 24h

N. Migrated cells (×100 field)

O. 

<table>
<thead>
<tr>
<th></th>
<th>TGF-β2</th>
<th>Linagliptin</th>
<th>p-Smad3</th>
<th>Actin</th>
</tr>
</thead>
</table>

P. 

<table>
<thead>
<tr>
<th></th>
<th>TGF-β2</th>
<th>Linagliptin</th>
<th>DPP-4</th>
<th>Actin</th>
</tr>
</thead>
</table>

Q. Fold change (DPP-4/18S)

R. DPP-4 activity (IU/10^6 cells)

FIG5
216x188mm (300 x 300 DPI)
FIG6
210x216mm (300 x 300 DPI)
Supplementary Figure 1 PAS and MTS staining at 16 weeks after the induction of diabetes in male CD-1 mice. A single injection of STZ was performed in 8-week–old male CD-1 mice, which were sacrificed 16 weeks after the induction of diabetes. A: PAS staining and B: MTS staining are shown. The original magnifications were 300x in (A) and 100x in (B). Scale bar: 50 µm in each panel.
Supplementary figure 2  TGF-β2-induced apoptosis in endothelial cells was suppressed with DPP-4 inhibitors. HMVECs were incubated with TGF-β2 (2.5 ng/ml) in the presence or absence of either linagliptin (A-D) or KR-62436 (E-H). Apoptosis was analyzed by AnnexinV labeling. The original magnification was 200x. Scale bar: 50 µm. D, H: Quantification of apoptotic cells per field. In each group, 6 fields were analyzed. The experiment was repeated twice and yielded the same data both times. Data are expressed as the means ± s.e.m. in the graph.
Supplementary Figure 3 mRNA microarray analysis. A. Heat map of the entire analyzed gene expression. Total RNA was isolated and used for synthesizing cDNA. cDNA was hybridized with Affymetrix Mouse Gene 1.0 ST Array. B. Ingenuity
Pathway Analysis was utilized to select a cluster of genes that were involved in profibrotic programs. C. The profibrotic genes restored by linagliptin treatments are listed. Red and blue bars indicate the genes that were reduced and induced, respectively, in the diabetic kidneys. The x-axis indicates the ratio of restoration, and 1.0 (or -1.0) means the complete restoration of genes that were reduced (or induced) in diabetic mice by linagliptin. n=2 and representative data are shown for each group.
Supplementary Figure 4 qPCR analysis for microRNA 29 family. microRNAs are isolated from HMVEC cells in the presence or absence of TGF-β2 (5ng/ml) with or without linagliptin. Cells are harvested after 48 hours stimulation. N=5 or 6 in each group.
Supplementary Figure 5 inhibition of microRNA 29 family induced EndMT. A. Cells were transfected with antagonir or inhibitor for microRNA 29s (75 nM in final concentration) and 48 hours after transfection, cells were harvested and isolated protein were analyzed by western blot analysis. B. Boyden chamber cell migration assays were performed as described in the methods section. C. Migrated cells in the bottom layer of the Boyden chamber were counted in 5 different areas, and quantification was performed. Experiment was performed twice showing similar results.