５．田中智洋
βKlotho による栄養代謝制御の全貌解明を目指して
第 22 回糖尿病セミナーUP-TO-DATE（岡山）、2012/9/13（招請講演）

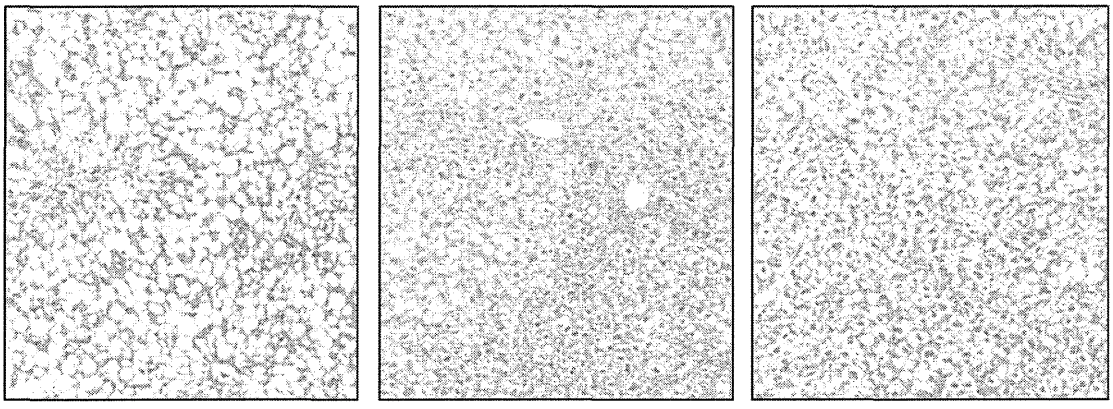
E．　知的財産権の出願・登録状況

なし

（参考図）

高脂肪食負荷NASHモデルにおける効果（脂肪化）

6週齢雄性C57BL/6Jマウスに5週間高脂肪食(HFD)を給餌
治療群は小柴胡湯0.4g/kg/day、UDCA 35mg/kg/day相当を混餌投与



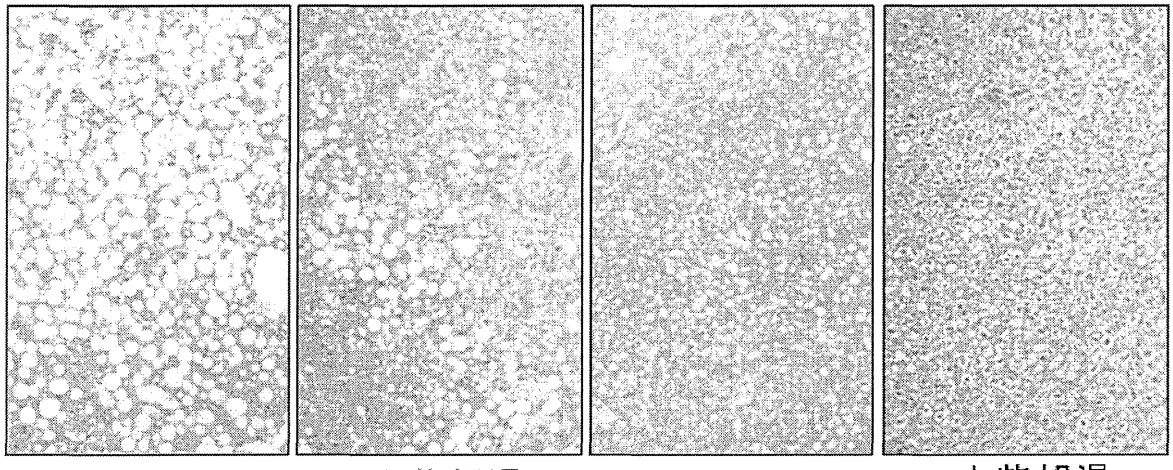
HFD (60%kcal fat)　　+ 小柴胡湯　　+ ウルソデオキシコール酸 (UDCA)

小柴胡湯、熊胆剤の主成分UDCAの投与で肝脂肪化の改善を認めた

(H&E x100)

メチオニン・コリン欠乏食NASHモデルにおける効果（脂肪化）

6週齢雄性C57BL/6Jマウスに4週間メチオニン・コリン欠乏食(MCD)を給餌
治療群は小柴胡湯0.4g/kg/day、UDCA 35mg/kg/day相当を混餌投与



MCD (ad lib.)　　+ 小柴胡湯　　+ UDCA　　+ 小柴胡湯・UDCA併用

小柴胡湯、熊胆剤の主成分UDCAの投与で肝脂肪化の改善を認めた

(H&E x100)

図1

PPARγリガンド投与の効果

白色脂肪組織

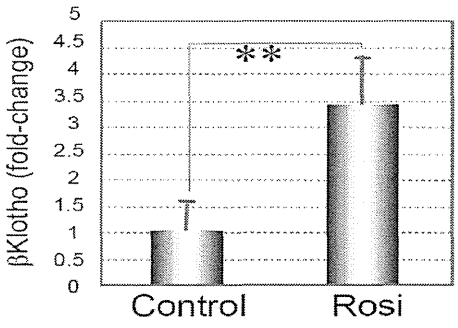


図2

Fastingの効果

肝臓

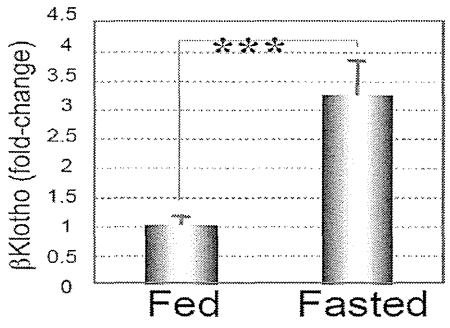
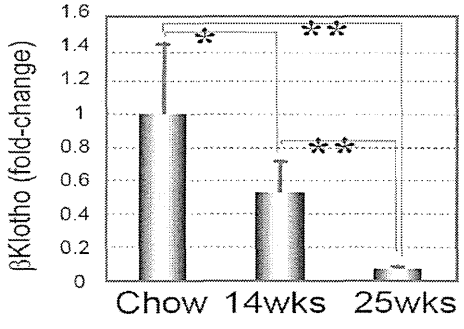


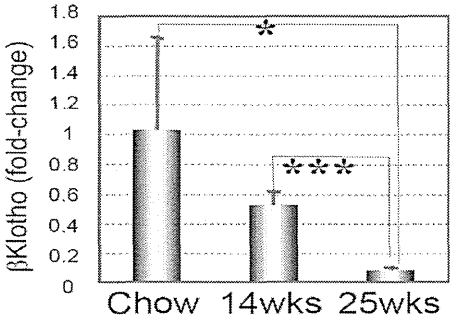
図3

High fat-diet投与の効果

白色脂肪組織



褐色脂肪組織



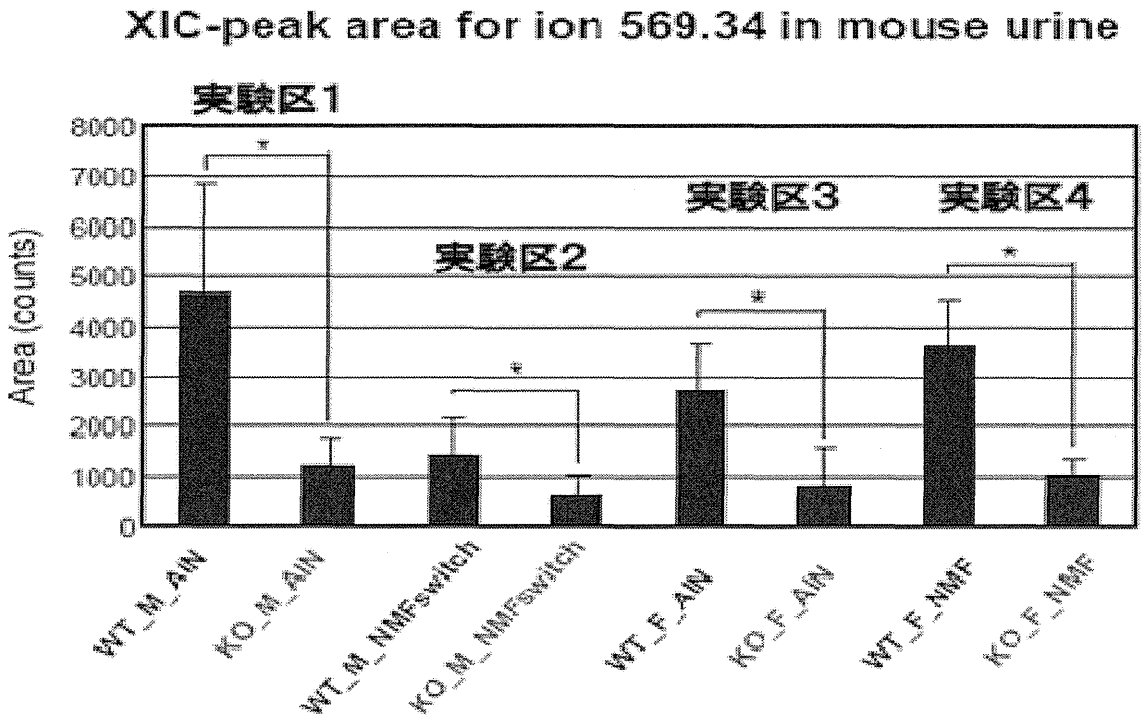


図1　βKlotho ノックアウトマウス尿中で減少する未知化合物（イオン 569）

縦軸はイオン 569 に相当する分子量（569.34）で抽出したクロマトグラム（Extracted ion chromatogram(XIC)）から算出した、ピークエリアを表す。飼育コロニーの餌（AIN または NMF）や性別（M または F）により区分した４つの実験区のいずれにおいても、イオン 569 は野生型マウス（WT）に比べて、βKlotho ノックアウトマウス（KO）において有意に減少する。エラーバーは標準誤差、アスタリスクは $P<0.05$ を示す。

（参考図）

βKlothoの発現量に大きな変化が認められる組織と飼育条件の同定

肝臓

Fasting

白色脂肪組織

High fat-diet投与

PPARγリガンド投与

褐色脂肪組織

High fat-diet投与

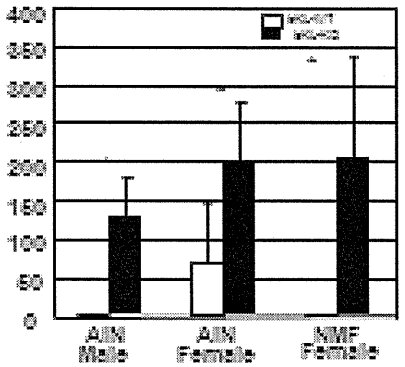
PPARγリガンド投与

βKlothoノックアウトマウスで発現量が恒常的に変化する分子の発見

肝臓

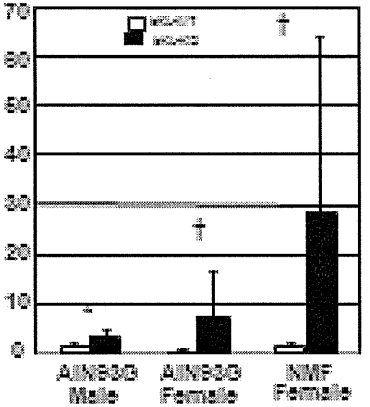
L-Pgds

リポカリン型プロスタグランジンD2合成酵素



Corin

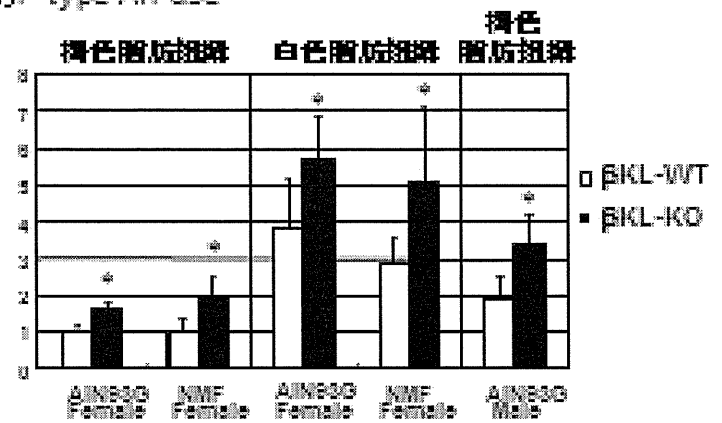
Pro-ANP（ナトリウム利尿ペプチド）変換酵素



脂肪組織

Atp10d

機能不明のP-type ATPase



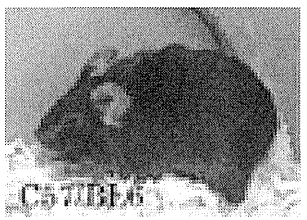
当研究室で立ち上げたメタボローム解析システム

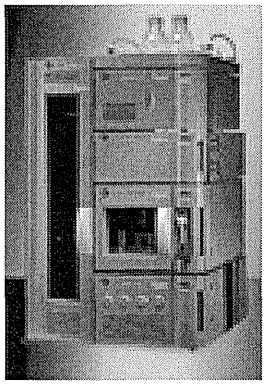
Liquid column-chromatography

ACQUITY UPLC
(Waters)

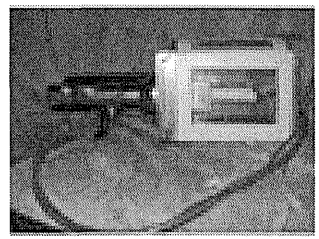
Biological fluid

from mutant mice





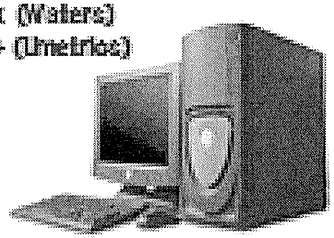
Ionization

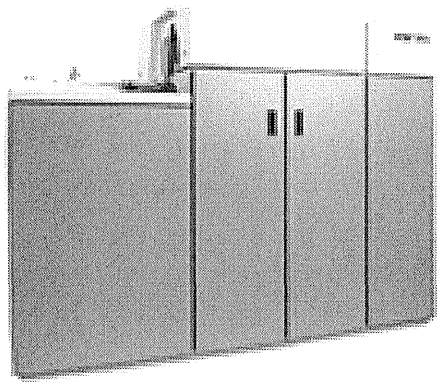


Turbo Ion Spray (ESI)
(Applied Biosystems)

Data analysis

Analyst (Applied Biosystems)
MassLynx (Waters)
SIMCA-P+ (Umetrics)





Mass-spectrometry

Q-Star XL
(Applied Biosystems)

βKlothoノックアウトマウス尿中で有意に減少する分子(イオン569)の発見

4つの実験区を設定し、全実験区で共通する変化に注目

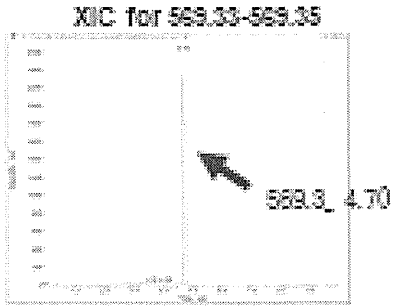
実験区1) 繁殖用NMF (天然原料含有餌)を投与した、雄マウス
WT-Male (WT-M-NMF)
KO-Male (WT-M-NMF)

実験区2) 繁殖用NMF (天然原料含有餌)を投与した、雌マウス
WT-Female (WT-F-NMF)
KO-Female (WT-F-NMF)

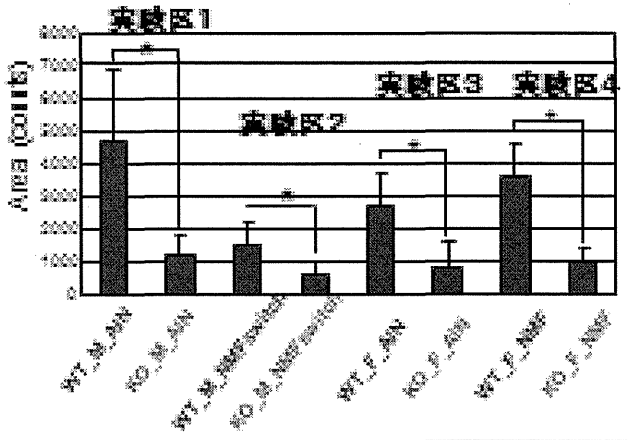
実験区3) 精製餌 (AIN−93G)を投与した、雄マウス
WT-Male (WT-M-AIN)
KO-Male (WT-M-AIN)

実験区4) 精製餌 (AIN−93G)を投与した、雌マウス
WT-Female (WT-F-AIN)
KO-Female (WT-F-AIN)

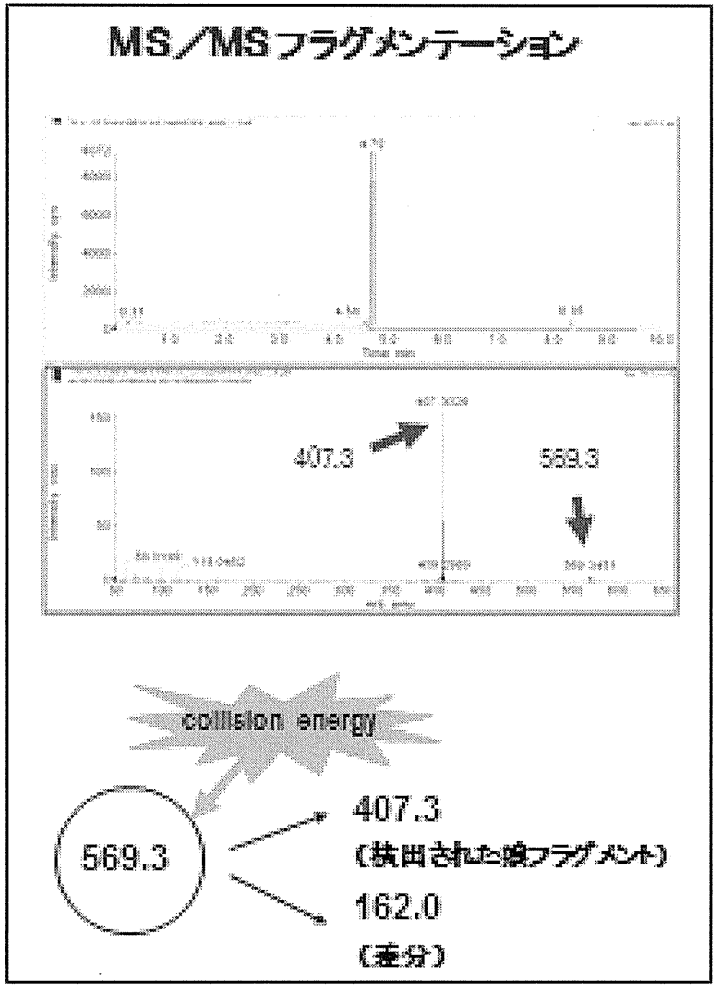
569.3_4.70 (分子量(m/z)_カラム保持時間(min)) という分子をβKlothoノックアウトマウス尿中で常に減少するマーカーとして同定した



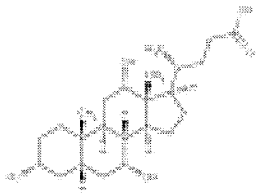
XIC-peak area for Ion 569.34 in mouse urine



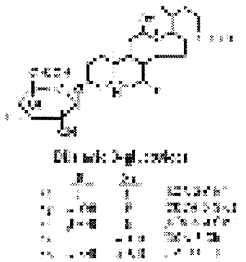
イオン569の構造同定



コール酸
407.3



コール酸グルコシド
569.3



研究成果の刊行に関する一覧表

書籍

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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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田中智洋、益崎裕章、細田公則、中尾一和	メタボリックシンドロームのマスターレギュレーターとしてのPPARγ	日本臨牀	68	203-209	2010
田中智洋、鍋島陽一	「寿命関連因子と糖代謝」Klothoから考える生体恒常性と老化	内分泌糖尿病科	29	561-569	2009
伊藤慎二	βKlotho の同定とその機能	内分泌糖尿病科	26	562-573	2008

Decreased renal α-Klotho expression in early diabetic nephropathy in humans and mice and its possible role in urinary calcium excretion

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Hypercalciuria is one of the early manifestations of diabetic nephropathy. We explored here the role of α-Klotho, a protein expressed predominantly in distal convoluted tubules that has a role in calcium reabsorption. We studied 31 patients with early diabetic nephropathy and compared them with 31 patients with IgA nephropathy and 7 with minimal change disease. Renal α-Klotho expression was significantly lower and urinary calcium excretion (UCa/UCr) significantly higher in diabetic nephropathy than in IgA nephropathy or minimal change disease. Multiple regression analyses indicated that α-Klotho mRNA was inversely correlated with calcium excretion. We next measured these parameters in a mouse model of streptozotocin (STZ)-induced diabetic nephropathy, characterized by glomerular hyperfiltration, as seen in early diabetic nephropathy. We also confirmed a reduction of renal α-Klotho mRNA down to almost 50% and enhanced calcium excretion in mice with STZ-induced diabetic nephropathy in comparison with nondiabetic mice. Hypercalciuria was exacerbated in heterozygous α-Klotho knockout mice in comparison with wild-type mice, each with STZ-induced diabetic nephropathy. Thus, α-Klotho expression was decreased in distal convoluted tubules in diabetic nephropathy in humans and mice. Renal loss of α-Klotho may affect urinary calcium excretion in early diabetic nephropathy.

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KEYWORDS: diabetic nephropathy; hypercalciuria; hypoxia

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Hypercalciuria is one of the early findings of uncontrolled diabetes mellitus in both humans and experimental animal models.[1–4] Hypercalciuria is essentially associated with a negative calcium balance hallmarked by renal calcium loss in diabetes mellitus,[1,2] which has been proposed to contribute to the increased risk of bone fracture.[5,6] Although osmotic diuresis and increased dietary calcium intake secondary to hyperphagia may have a role in the hypercalciuria seen in diabetic patients,[2] control of osmotic diuresis with appropriate insulin therapy only partially corrects hypercalciuria.[7] Furthermore, increased dietary calcium intake may not increase the calcium load on the kidney, because calcium absorption in the gut is also decreased in the early stages of streptozotocin (STZ)-induced diabetes.[8,9] Thus, there may be impairment in the renal handling of calcium in diabetes mellitus. Although ultrafiltered calcium is reabsorbed in the proximal tubules and the distal convoluted tubules (DCTs),[10] previous studies have not revealed any decreases in calcium reabsorption in the proximal tubules in diabetic models.[7,11,12] Thus, DCTs may contribute to the impairment in calcium transport,[7] the underlying molecular mechanisms of which remain unclear.

α-Klotho (α-KL) mutant mice were originally used to model disorders associated with human aging, because the phenotype of the mutant animals includes arteriosclerosis, ectopic calcification (including vascular calcification), emphysema, and osteoporosis.[13] The *α-KL* gene is predominantly expressed in the parathyroid glands, the DCTs in the kidney, and the choroid plexus in the brain,[14,15] all of which have an important role in calcium–phosphorus homeostasis. Recently, evidence has accumulated showing that α-KL regulates (1) parathyroid hormone (PTH) release in the parathyroid gland,[14] (2) the production of $1,25(OH)_2$ vitamin D_3 by negatively regulating the expression of 1α-hydroxylase, which encodes a rate-limiting enzyme of active vitamin D synthesis,[16,17] and (3) transepithelial calcium transport in the

DCTs via activation of the transient receptor potential vanilloid 5 (TRPV5) channel.[18] In fact, mice lacking α-KL display diminished renal calcium reabsorption, resulting in severe hypercalciuria.[19]

In this study, we showed that renal α-KL expression levels were decreased in patients with early diabetic nephropathy (DN) and in the STZ-induced mouse model of diabetes, and that renal α-KL expression levels were inversely correlated with urinary calcium augmentation in patients with DN and a mouse model of DN. Furthermore, we confirmed the exaggerated urinary excretion of calcium in STZ-induced DN in heterozygous α-KL knockout mice (α-KL$^{+/-}$ mice). Thus, we propose that renal α-KL expression levels are partially involved in hypercalciuria in early DN.

RESULTS

Elevation of urinary calcium excretion (UCa/UCr) in patients with DN

Urinary calcium excretion (UCa/UCr), which was normalized to the urinary creatinine, was significantly higher in patients with DN than in those with either IgA nephropathy (IgAN) or minimal change disease (MCD) (DN, 0.081 ± 0.044; IgAN, 0.037 ± 0.021; MCD, 0.039 ± 0.022; $P<0.001$; Figure 1a). Serum calcium concentrations were similar among the three patient groups (Table 1). On considering the population subset with an estimated glomerular filtration rate (eGFR) greater than 60 ml/min per 1.73 m^2, UCa/UCr levels in DN were highly elevated relative to those seen in

Table 1 | Clinical characteristics of study population

	DN	IgAN	MCD
Number of patients	74	90	26
Age (years)	64 ± 10†	40 ± 16	35 ± 16
Serum creatinine (mg/dl)	0.95 ± 0.48	0.85 ± 0.31	0.82 ± 0.22
eGFR (ml/min per 1.73 m^2)[a]	74.4 ± 24.9	79.2 ± 23.9	87.2 ± 22.5‡
Corrected serum calcium (mg/dl)	9.4 ± 0.4	9.3 ± 0.3	9.5 ± 0.4

Abbreviations: DN, diabetic nephropathy; eGFR, estimated glomerular filtration rate; IgAN, IgA nephropathy; MCD, minimal change disease.
Clinical parameters are presented as means ± s.d.
[a]eGFR was calculated by means of the creatinine-based Modification of Diet in Renal Disease Study Equation. $^{\dagger}P<0.05$ vs. IgAN or MCD. $^{\ddagger}P<0.05$ vs. DN.

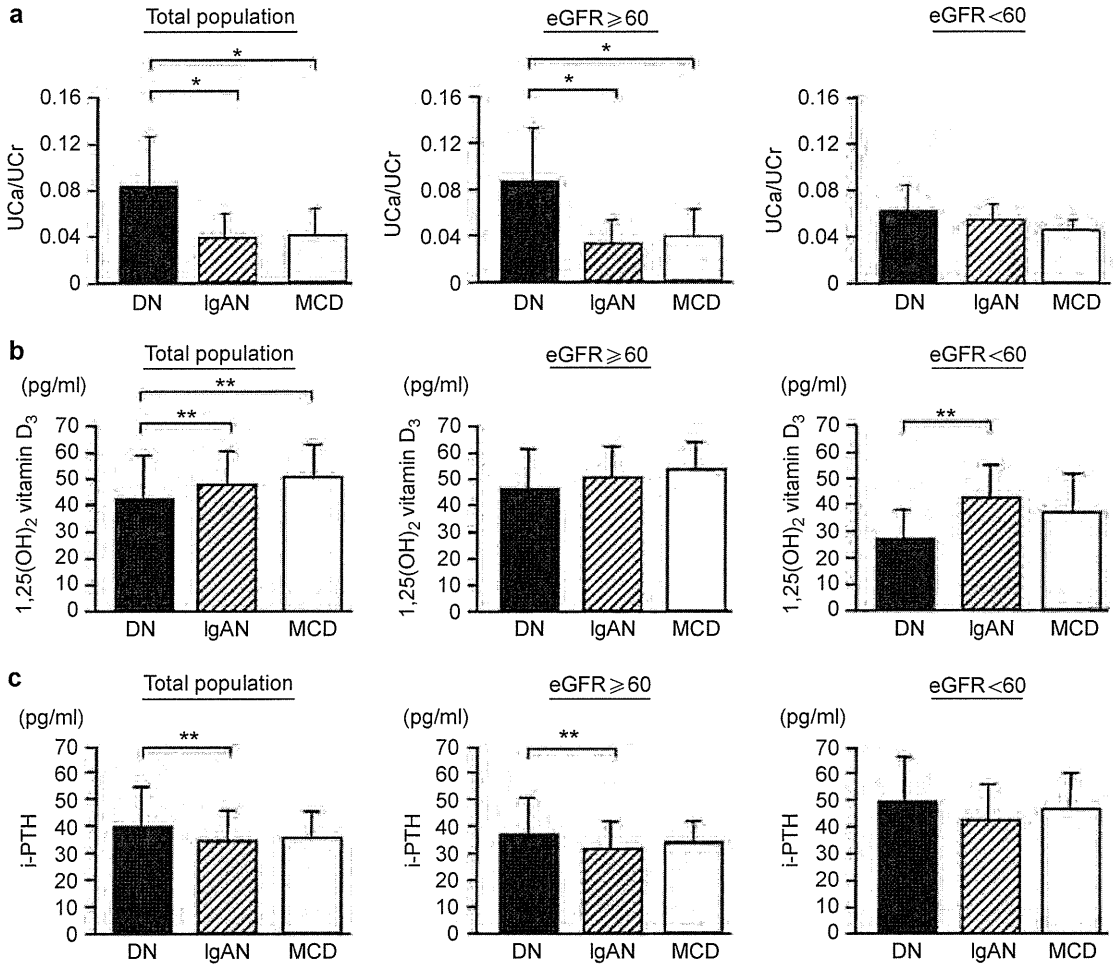

Figure 1 | Elevation of urinary calcium excretion (UCa/UCr) in patients with diabetic nephropathy (DN). (**a**) UCa/UCr, (**b**) serum 1,25(OH)$_2$ vitamin D$_3$ concentration, and (**c**) serum intact parathyroid hormone (i-PTH) concentration were measured in patients with DN (black bars), IgA nephropathy (IgAN) (striped bars), or minimal change disease (MCD) (white bars). Data are shown as means ± s.e.m. for each group. Kruskal–Wallis analysis of variance by ranks with Bonferroni adjustment was used to compare groups. *$P<0.001$; **$P<0.01$.

IgAN and MCD (DN, 0.086 ± 0.047; IgAN, 0.032 ± 0.020; MCD, 0.038 ± 0.024; $P<0.001$; Figure 1a). In patients with an eGFR under 60 ml/min per 1.73 m^2, however, there was no significant difference in UCa/UCr levels between DN, IgAN, and MCD (DN, 0.062 ± 0.021; IgAN, 0.054 ± 0.013; MCD, 0.045 ± 0.009; $P=0.265$; Figure 1a). Serum levels of $1,25(OH)_2$ vitamin D_3 were significantly lower in DN than in IgAN and MCD (DN, 41.69 ± 16.1 pg/ml; IgAN, 48.18 ± 12.51 pg/ml; MCD, 50.2 ± 12.6 pg/ml; $P=0.004$; Figure 1b). In the population with an eGFR greater than 60 ml/min per 1.73 m^2, however, serum $1,25(OH)_2$ vitamin D_3 levels were lower in DN, as compared with IgAN and MCD, but this decrease was not significant (DN, 45.86 ± 14.88 pg/ml; IgAN, 50.00 ± 11.87 pg/ml; MCD, 52.61 ± 10.95 pg/ml; $P=0.069$; Figure 1b). Serum intact PTH (i-PTH) values were significantly increased in DN in comparison with those in IgAN for both the total study population (DN, 39.2 ± 15.19 pg/ml; IgAN, 33.68 ± 11.79 pg/ml; MCD, 35.23 ± 9.96 pg/ml; $P=0.027$, Figure 1c) and the population subset with an eGFR greater than 60 ml/min per 1.73 m^2 (DN, 36.53 ± 13.65 pg/ml; IgAN, 31.16 ± 10.20 pg/ml; MCD, 33.09 ± 7.99 pg/ml; $P=0.032$; Figure 1c).

Reduction in α-KL expression in the early stages of human DN

In immunohistological examination, although α-KL reactivity was detectable exclusively in DCTs in patients with MCD and IgAN (Figure 2b and c), the reactivity was significantly reduced in samples from DN patients (Figure 2a). α-KL reactivity was also detected in the proximal convoluted tubules, although the reactivity in proximal convoluted tubules was weaker than that in DCTs. α-KL immunoreactivity in proximal convoluted tubules was similar to that in DN, IgAN, and MCD. Renal α-KL mRNA expression levels, quantitatively measured by real-time PCR, were significantly correlated with eGFR ($r=0.353$, $P=0.0034$; Figure 2e), suggesting that renal α-KL mRNA expression levels were decreased with the advance of renal failure. Notably, looking at the population with an eGFR greater than 60 ml/min per 1.73 m^2, renal α-KL mRNA expression levels were markedly decreased in the presence of DN in comparison with IgAN and MCD (DN, 0.45 ± 0.27 AU; IgAN, 1.07 ± 0.45 AU; MCD, 1.29 ± 0.58 AU; $P=0.0004$; Figure 2f). However, in the population subset with an eGFR under 60 ml/min per 1.73 m^2, renal α-KL mRNA expression levels were similar to those in DN, IgAN, and MCD (Figure 2f).

Next, we used the same renal biopsy specimens to investigate the degree of renal tubulointerstitial damage progression in the DN, IgAN, and MCD groups. Neither the average numbers of CD31-positive peritubular capillaries (PTCs) nor the percentage of type I collagen–positive areas per single field in DN were significantly different from those seen in IgAN or MCD when the eGFR was greater than

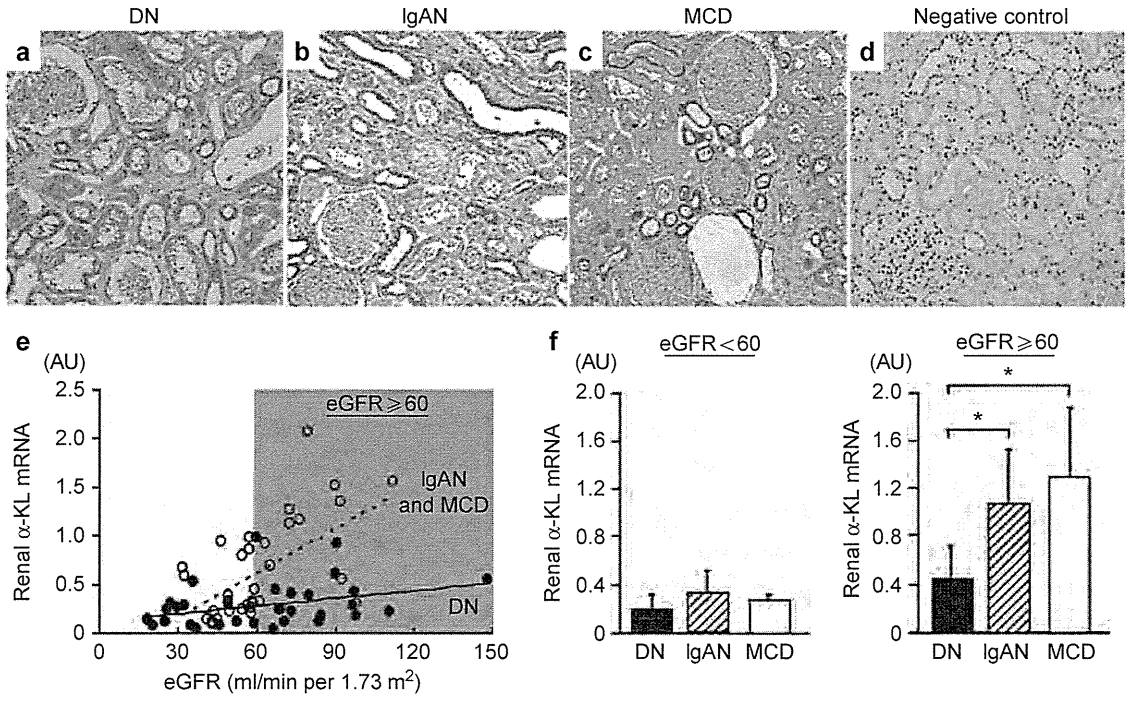

Figure 2 | Reduction in α-Klotho (α-KL) expression in human early diabetic nephropathy (DN). (**a–d**) Representative immunoperoxidase staining for α-KL protein in renal biopsy sections from human patients with DN, IgA nephropathy (IgAN), or minimal change disease (MCD), and negative control (MCD treated with rat immunoglobulins instead of rat anti-α-KL antibody). Original magnification, × 100. (**e**) Correlation of renal α-KL mRNA expression levels with estimated glomerular filtration rate (eGFR) in patients with DN (closed circles) and IgAN and MCD (open circles). (**f**) Renal α-KL mRNA expression levels in DN (black bars), IgAN (striped bars), and MCD (white bars). Data are shown as means ± s.e.m. for each group. Kruskal–Wallis analysis of variance by ranks with Bonferroni adjustment was used to compare groups. *$P<0.01$.

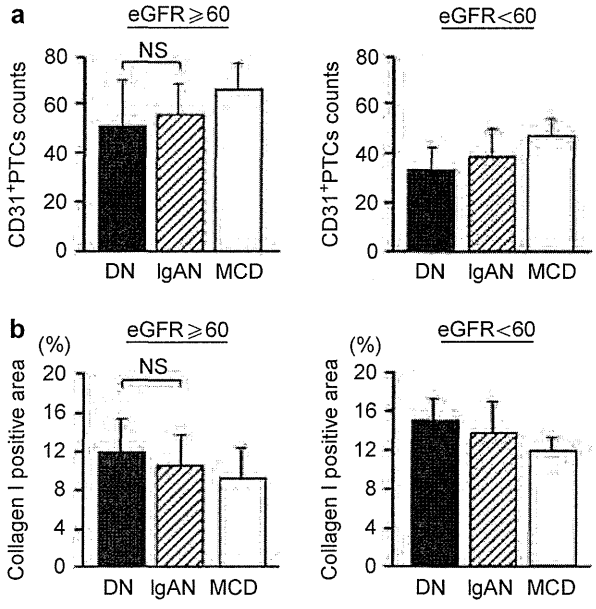

Figure 3 | The degree of renal tubulointerstitial damage progression in the diabetic nephropathy (DN), IgA nephropathy (IgAN), and minimal change disease (MCD). (**a**) The average number of CD31-positive peritubular capillaries (PTCs) and (**b**) the average proportion of type I collagen-positive areas per field in renal biopsy specimens from patients with DN (black bars), IgAN (striped bars), and MCD (white bars). Data are shown as means ± s.e.m. for each group. Kruskal–Wallis analysis of variance by ranks with Bonferroni adjustment was used to compare groups.

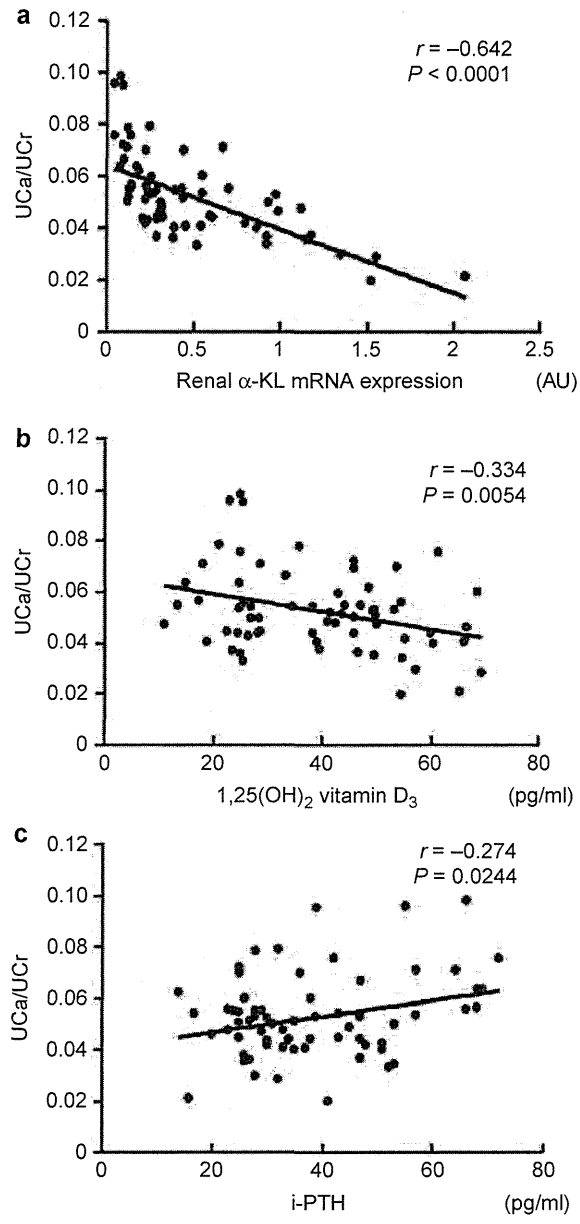

Figure 4 | Correlation of renal α-Klotho (α-KL) mRNA expression levels with urinary calcium excretion (UCa/UCr). (**a**) UCa/UCr with renal α-KL mRNA expression indices ($r=-0.642$, $P<0.0001$), (**b**) serum $1,25(OH)_2$ vitamin D_3 concentrations ($r=-0.334$, $P=0.0054$), and (**c**) serum intact-parathyroid hormone (i-PTH) concentrations ($r=0.274$, $P=0.0244$) in patients who had undergone renal biopsy. Correlations were evaluated using the Pearson correlation coefficient.

60 ml/min per 1.73 m^2 (CD31-positive PTC numbers: DN, 50.6 ± 18.9; IgAN, 55.2 ± 12.8; MCD, 66.8 ± 10.6; $P=0.174$; Figure 3a; Type I collagen positive areas: DN, 11.7 ± 3.5%, IgAN 10.4 ± 3.2%, MCD 8.7 ± 3.2%; $P=0.234$; Figure 3b).

Correlation of renal α-KL mRNA expression levels with UCa/Ucr

An examination of three clinical parameters governing calcium metabolism—levels of renal α-KL expression, serum $1,25(OH)_2$ vitamin D_3, and serum i-PTH—showed that levels of renal α-KL mRNA expression ($r=-0.642$, $P<0.0001$), serum $1,25(OH)_2$ vitamin D_3 ($r=-0.334$ $P=0.0054$), and serum i-PTH ($r=0.274$, $P=0.0244$) correlated significantly with UCa/UCr across all patients with DN, IgAN, and MCD, who had undergone renal biopsy (Figure 4). Multiple regression analyses revealed that renal α-KL expression levels were significantly and inversely correlated with UCa/UCr ($\beta=10.644$, $P<0.0001$) as an independent variable in order of importance ($R^2=0.375$, $P<0.0001$), but serum $1,25(OH)_2$ vitamin D_3, serum i-PTH, serum calcium, eGFR, and age were not (Table 2), among all patients who had undergone renal biopsy. In patients with an eGFR greater than 60 ml/min per 1.73 m^2, univariate analysis showed that renal α-KL mRNA expression levels correlated significantly with only UCa/UCr ($r=-0.755$, $P<0.0001$; data not shown).

Development of DN in STZ-treated mice

In this human study, we revealed a significant decrease in renal α-KL expression levels and an increase in UCa/UCr in DN with an eGFR greater than 60 ml/min per 1.73 m^2. Therefore, to confirm that renal α-KL expression levels were decreased in early DN, and that this decrease was related to elevations in UCa/UCr, we used the STZ-induced mouse model of diabetes. In this model mouse, urinary albumin to creatinine ratios were increased at 2, 4, 6, and 8 weeks after

Table 2 | Multiple regression analysis[a] for urinary calcium excretion (UCa/UCr)

Independent variable	$\beta^{\dagger}$	*P*-value
Renal α-Klotho expression levels	−0.644	<0.0001
Serum 1,25$(OH)_2$ vitamin D_3	0.048	0.7131
Serum intact-PTH	0.151	0.1952
eGFR	0.05	0.7207
Corrected serum calcium	−0.035	0.7268
Age	0.047	0.6961

Abbreviations: eGFR, estimated glomerular filtration rate; PTH, parathyroid hormone; UCa/UCr, urinary calcium (mg/dl)/urinary creatinine (mg/dl).
[a]Adjusted coefficient of determination (R^2); R^2=0.375, $P<0.0001$.
†Standard partial regression coefficient.

the establishment of diabetes (Supplementary Figure S1 online). This diabetic mouse model did not show apparent histopathological findings characteristic with DN at 8 weeks after the establishment of diabetes, indicating to be a useful model for early DN (Supplementary Figure S2 online).

Decrease in renal α-KL expression levels and elevation of UCa/UCr in STZ-induced diabetic mice

In STZ-induced diabetic mice, renal α-KL mRNA expression levels were maintained until 4 weeks after the establishment of diabetes mellitus, but they decreased significantly to about 70% of the levels of nondiabetic control mice at 6 weeks and to about 50% at 8 weeks ($P<0.001$, Figure 5c). This decrease was also confirmed at the protein level by immunohistological examination and western blotting (Figure 5a and b). UCa/UCr in diabetic mice was slightly but significantly increased beginning 2 weeks after the establishment of diabetes mellitus, and it was further elevated at 6 and 8 weeks ($P<0.001$; Figure 5d). Interestingly, the levels of UCa/UCr in diabetic mice at 6 weeks significantly increased to about one and a half times as much as those in diabetic mice at 4 weeks, and these levels at 8 weeks showed a further significant increase to about two times as much as those at 4 weeks (Figure 5d). Thus, the reduction of renal α-KL expression in STZ-induced diabetic mice may be related to further enhancement of UCa/UCr levels.

Exaggeration of UCa/UCr in STZ-induced diabetic α-KL$^{+/-}$ mice

To confirm whether or not the approximately 50% reduction in renal α-KL expression resulted in an elevation in UCa/UCr, we measured this excretion in α-KL$^{+/-}$ mice. There were no significant differences in UCa/UCr levels between α-KL$^{+/-}$ and α-KL$^{+/+}$ mice (Figure 6a). Next, we examined STZ-induced DN in α-KL$^{+/-}$ mice. To avoid the DN-related decline of renal α-KL expression, we examined UCa/UCr beginning 2 weeks after the establishment of diabetes mellitus in both diabetic α-KL$^{+/+}$ and α-KL$^{+/-}$ mice. During the first 2 weeks, although renal α-KL expression levels were similar in diabetic and nondiabetic mice of both genotypes (Figure 6b and c), UCa/UCr levels in diabetic α-KL$^{+/-}$ mice increased to about one and a half times as much as those in

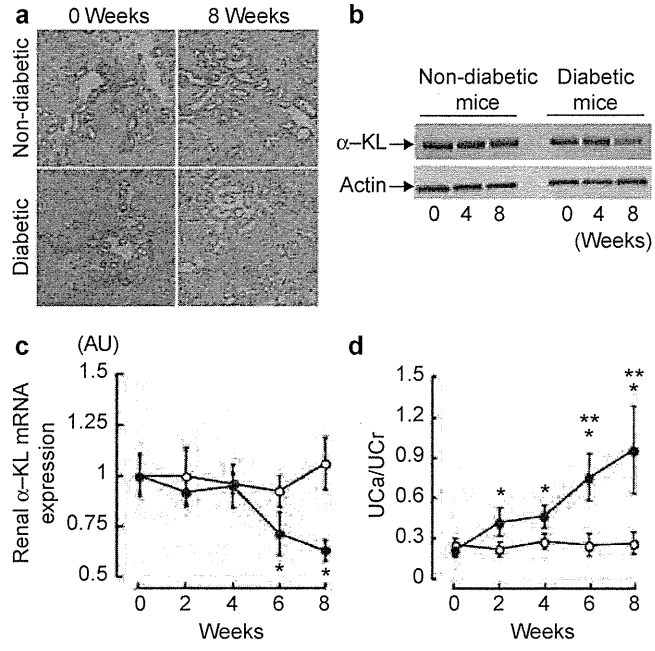

Figure 5 | Decrease in renal α-Klotho (α-KL) expression levels and elevation of urinary calcium excretion (UCa/UCr) in streptozotocin (STZ)-induced diabetic mice. (**a**) Representative immunoperoxidase staining for α-KL in kidney specimens, and (**b**) western blotting to detect α-KL of the kidney from the indicated mice. (**c**) Time course of renal α-KL mRNA expression levels and (**d**) time course of the urinary calcium (mg/dl) to creatinine (mg/dl) ratio in diabetic (closed circles) and nondiabetic (open circles) mice. Original magnification, × 100. Student's *t*-test was used to compare groups. *$P<0.001$, vs. nondiabetic mice; **$P<0.05$, vs. STZ-treated mice 4 weeks after the establishment of diabetes.

diabetic α-KL$^{+/+}$ mice ($P<0.001$; Figure 6a). Thus, when diabetes was induced, the 50% reduction in renal α-KL expression may be related to the greater increases in UCa/UCr levels in α-KL$^{+/-}$ mice than those in α-KL$^{+/+}$ mice.

The effect of α-KL expression levels on the activity of TRPV5

TRPV5 is predominantly involved in renal calcium handling. TRPV5 colocalizes with α-KL in DCTs, and its activity is stimulated by α-KL.[18] Therefore, to investigate the mechanism by which the reduction of renal α-KL expression is related to the increase of urinary calcium excretion, we studied the effect of α-KL expression on TRPV5 activity. First, we verified that the level of renal TRPV5 expression was not significantly lower in DN with an eGFR greater than 60 ml/min per 1.73 m^2 than in IgAN or MCD, and also not lower in STZ-induced diabetic mice at 8 weeks than nondiabetic mice (Figure 7a–d), indicating that there is no significant association between the levels of α-KL expression and those of TRPV5 expression in the kidney. Next, to clarify whether or not TRPV5 channel activity is associated with the level of α-KL expression, we transfected both TRPV5 and various amounts of adenoviral vectors carrying the *α-KL* gene into human embryonic kidney 293 (HEK293) cells, and then

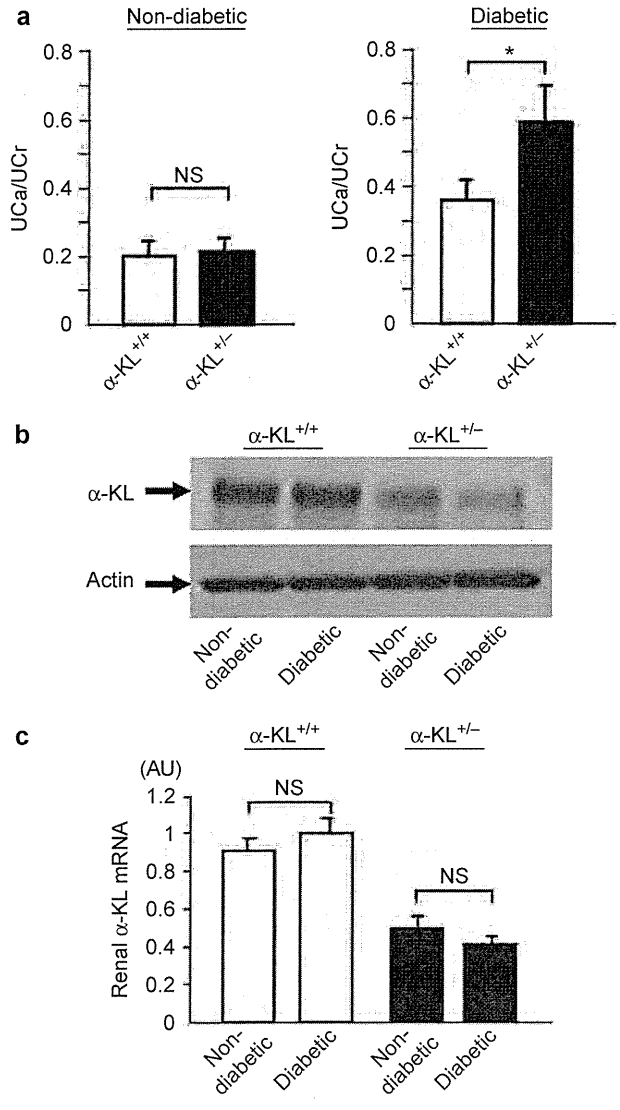

Figure 6 | Exaggeration of urinary calcium excretion (UCa/UCr) in streptozotocin (STZ)-induced diabetic α-Klotho (α-KL)$^{+/-}$ mice. (**a**) UCa/UCr in diabetic and nondiabetic mice of α-KL$^{+/-}$ (black bars) and α-KL$^{+/+}$ (white bars) strains. (**b**) Western blotting for α-KL in kidney specimens from the indicated mice, and (**c**) renal α-KL mRNA expression indices in diabetic and nondiabetic mice of α-KL$^{+/-}$ (black bars) and α-KL$^{+/+}$ (white bars) strains. Student's *t*-test was used to compare groups. *$P<0.001$.

analyzed calcium uptake in the transfectants. In this *in vitro* experiment, we showed that, in HEK293 transfected with both *TRPV5* and *α-KL* gene, calcium uptake was significantly increased in proportion to the α-KL expression (Figure 7e). In this experiment, HEK293 cells transfected with both *TRPV5* and *Lac Z* gene served as the control. We also found that calcium uptake did not significantly increase in HEK293 cells in the absence of TRPV5, even when α-KL expression was elevated (Figure 7e).

DISCUSSION

This study demonstrated, for the first time, that renal α-KL expression levels in patients with DN were markedly decreased in comparison with those in patients with either IgAN or MCD with an eGFR greater than 60 ml/min per 1.73 m^2. We confirmed that UCa/UCr in patients with DN was markedly increased in comparison with patients with IgAN or MCD, and renal α-KL expression levels correlated significantly with UCa/UCr in humans. In the STZ-induced mouse model of diabetes, we also observed that the decreases in renal α-KL expression levels correlated with greater enhancement of UCa/UCr. Moreover, we confirmed the exacerbated renal calcium loss in KL$^{+/-}$ mice with STZ-induced DN. These findings indicate for the first time a significant correlation between renal α-KL loss and hypercalciuria in early DN.

Our study revealed that renal α-KL expression levels were significantly decreased in patients of DN with an eGFR greater than 60 ml/min per 1.73 m^2. Although eGFR is grossly related to the degree of renal tubulointerstitial damage, eGFR may not always be correlated with renal tubulointerstitial damage because of the development of glomerular hyperfiltration in the early stages of DN.[20,21] To exclude the possibility that increased tubulointerstitial damage was responsible for the observed decreases in renal α-KL expression in DN, we immunohistologically investigated PTC counts and the degree of interstitial fibrosis.[22,23] In the population of patients with an eGFR greater than 60 ml/min per 1.73 m^2, we did not observe any significant histological differences in tubulointerstitial damage among DN, IgAN, and MCD specimens. In addition to human examination, we confirmed the reduced expression of α-KL mRNA in the kidneys of STZ-induced diabetic mice, in which apparent glomerular and tubulointerstitial injury were not detected. These results suggest that a reduction of renal α-KL expression in the early stages of DN is a characteristic finding of DN.

Hypercalciuria is also an early finding characteristic of patients with DN,[1] and renal tubular calcium excretion is indicated to be increased in STZ-induced diabetes mellitus, despite exhibiting normal plasma calcium concentrations.[2–4] The precise mechanism of hypercalciuria in DN, however, remains unclear. In the present study, multiple regression analyses clearly showed that the renal expression of α-KL mRNA is an independent determinant of UCa/UCr, and the reduction of renal α-KL expression in STZ-induced diabetic mice was shown to enhance the diabetes-related increase of UCa/UCr levels. Thus, it is possible that the decline of α-KL mRNA in DCTs is responsible for the increase of UCa/UCr observed in DN. Given the evidence that α-KL$^{-/-}$ mice showed a tremendous increase in UCa/UCr,[19] this scenario is plausible but not evident, because the reduction of α-KL mRNA levels was at most 50% in the diabetic patients and mouse model of diabetes.

To confirm the scenario, we investigated UCa/UCr levels in α-KL$^{+/-}$ mice. Half reduction of α-KL expression *per se* did not lead to hypercalciuria in α-KL$^{+/-}$ mice. However, when we induced diabetes by STZ injection in α-KL$^{+/-}$ and α-KL$^{+/+}$ mice, diabetes-induced increment of UCa/UCr levels was significantly larger in α-KL$^{+/-}$ mice than in