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Original Articles

The demonstration of αKlotho deficiency in human chronic kidney disease with a novel synthetic antibody

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ABSTRACT

Background. αKlotho is the prototypic member of the Klotho family and is most highly expressed in the kidney. αKlotho has pleiotropic biologic effects, and in the kidney, its actions include regulation of ion transport, cytoprotection, anti-oxidation and anti-fibrosis. In rodent models of chronic kidney disease (CKD), αKlotho deficiency has been shown to be an early biomarker as well as a pathogenic factor. The database for αKlotho in human CKD remains controversial even after years of study.

Methods. We used a synthetic antibody library to identify a high-affinity human antigen-binding fragment that recognizes human, rat and mouse αKlotho primarily in its native, rather than denatured, form.

Results. Using an immunoprecipitation–immunoblot (IP-IB) assay, we measured both serum and urinary levels of full-length soluble αKlotho in humans and established that human CKD is associated with αKlotho deficiency in serum and urine. αKlotho levels were detectably lower in early CKD preceding disturbances in other parameters of mineral metabolism and progressively declined with CKD stages. We also found that exogenously added αKlotho is inherently unstable in the CKD milieu suggesting that decreased production may not be the sole reason for αKlotho deficiency.

Conclusion. Synthetic antibody libraries harbor tremendous potential for a variety of biomedical and clinical applications. Using such a reagent, we furnish data in support of αKlotho deficiency in human CKD, and we set the foundation for the development of diagnostic and therapeutic applications of anti-αKlotho antibodies.

Keywords: assay, chronic kidney disease, klotho, synthetic antibodies

INTRODUCTION

αKlotho was identified as an anti-aging substance [1, 2] but has diverse effects including regulating ion transport, Wnt and insulin signaling, renin-angiotensin system, stem cells, carcinogenesis, fibrosis and oxidative stress. The highest level of expression of αKlotho is in the kidney [1, 3, 4]. In addition to its transmembrane form, which is a co-receptor for fibroblast growth factor (FGF) 23 [5–7], αKlotho is released into the circulation, urine and cerebrospinal fluid as an endocrine substance [3, 8, 9] generated by transcript splicing into a truncated peptide [10] or proteolytic release by secretases [11, 12]. A

substantial portion of the circulating αKlotho is nephrogenic in origin [13], posing the questions of whether kidney disease begets systemic αKlotho deficiency and whether there are dire downstream consequences of this deficiency. The phenotypic similarities between genetic αKlotho ablation and chronic kidney disease (CKD) support the notion that αKlotho deficiency is pathogenic [1, 14].

Reduced renal αKlotho transcript or protein levels [8, 15–21] and serum αKlotho concentration [8, 17] were demonstrated in rodent CKD from nephron reduction surgery, ischemia reperfusion injury, immune complex glomerulonephritis, polygenic or hormonal hypertension, metabolic syndrome and diabetes [8, 15–21]. The convergence suggests that αKlotho deficiency is a generic consequence of nephron loss. αKlotho reduction is potentially a sensitive and early biomarker of CKD and prognostic of CKD complications [22]. Restoration of αKlotho in experimental CKD in rodents ameliorates the kidney disease and extra-renal complications [8, 19, 20], which expands the role of αKlotho beyond biomarker and transforms the landscape presenting αKlotho replacement as a potential therapy. αKlotho deficiency has also been documented in acute kidney injury (AKI) in both rodents and humans [23]. αKlotho can potentially serve as an early biomarker for AKI [24]. In addition, αKlotho was shown in rodents to prevent AKI from nephrotoxin [25], ischemia reperfusion [23] and ureteral obstruction [26], the most common causes of human AKI.

αKlotho measurement and replacement are pivotal advancements in kidney disease from diagnostic, prognostic and therapeutic points of view. However, one must first establish whether the preclinical findings are pertinent to human CKD. Low αKlotho transcript and protein have been described in human kidney from nephrectomy samples of end-stage kidneys and biopsies from patients with CKD [18, 27], but tissue levels have limited clinical utility. Studies using an immune-based assay have shown widely disparate results in terms of absolute values of serum αKlotho concentration and direction of change (increased, decreased or no change) with CKD and age [18, 28–49]. These discrepancies have thwarted progress and incapacitated our ability to determine whether the rodent data can be translated into meaningful human application.

In addition to CKD, AKI from a variety of causes is also associated with rapid and dramatic decrease of αKlotho in the kidney [23, 26, 50–53] and serum [23] in rodents and in urine in humans [23]. There is no data on human serum αKlotho in AKI to date. There is a dire need for an early, sensitive and specific marker for AKI in humans [54]. An accurate and reproducible assay is absolutely pivotal to generate such databases.

Generating antibodies to conserved proteins is challenging, as animal immunization and antibody production are subject to protection against auto-immunity. Synthetic antibody technology offers a powerful alternative because it is applied under defined *in vitro* conditions, uses antibody libraries that are not subjected to tolerance selection to remove self-reactive antibodies and yields antibodies with high affinities and specificities [55–59]. Within an optimized antibody framework, sequence diversity is introduced into the complementary determining regions (CDRs) by combinatorial mutagenesis. These libraries are coupled with phage display, with each phage particle displaying a unique antigen-binding fragment (Fab) on its surface while carrying the encoding DNA internally, thus achieving direct phenotype–genotype relations. Fab-displaying phages that bind to an antigen of interest are enriched using binding selections with purified antigens on solid support. The CDRs of binding phage clones are identified by DNA sequencing, and the Fab proteins are purified from bacteria or converted to the full-length IgG in mammalian cells.

Driven by the need for highly specific anti-αKlotho antibodies to develop reliable high-throughput diagnostic assays, we screened a synthetic antibody library and generated a Fab with high affinity for human and rodent αKlotho. We characterized this novel antibody, sb106, using recombinant proteins, cultured cells and body fluids and tissues from humans and rodents. We accurately and precisely quantified αKlotho levels in serum and urine in human and rodents using immunoprecipitation and immunoblot (IP-IB), and we demonstrated that both serum and urine αKlotho are dramatically reduced in early human CKD. While IP-IB is too cumbersome for large a sample size, our work lays the foundation to eventually produce high-throughput assays that enable large-scale population-based human studies that are direly needed. Finally, we strengthen a growing body of data supporting the hypothesis that CKD is a state of αKlotho deficiency that is worthy of exploration in humans.

MATERIALS AND METHODS

Preparation of the binary αKlotho–FGFR1c complex

The ligand-binding domain of human fibroblast growth factor receptor (FGFR)1c (D142 to R365) was expressed in *Escherichia coli*, refolded *in vitro* from inclusion bodies, and purified by published methods [60, 61]. The extracellular domain of murine αKlotho (A35 to K982) was expressed in human embryonic kidney (HEK)293 cells with a C-terminal FLAG tag, and the binary complex of the αKlotho ectodomain and the FGFR1c ligand-binding domain was prepared as described [5].

Isolation and characterization of sb106

Sb106 was isolated from a synthetic human Fab phage-displayed library (Library F) [62]. Binding selections, phage ELISAs and Fab protein purification were performed as described [55, 63, 64]. Briefly, phage from Library F were cycled through rounds of panning with the binary complex of αKlotho extracellular domain and FGFR1c ligand-binding domain on 96-well Maxisorp Immunoplates (Fisher Scientific, Nepean, ON, Canada) as the capture target. After five rounds of selection, phage were produced from individual clones grown in a 96-well format and phage ELISAs were performed to detect specific binding clones. Clones with positive binding were subjected to DNA sequencing. A competitive binding ELISA was performed by pre-incubating sb106 phage with

serial dilutions of soluble human αKlotho (50–0.0005 nM × 1 h) prior to binding to an ELISA plate coated with human αKlotho. The genes encoding for variable heavy- and light-chain domains of sb106 were cloned into vectors designed for production of light chain or IgG1 heavy chain, respectively, and sb106-IgG was expressed from 293F cells (Invivogen, San Diego, CA, USA). Fab and IgG proteins were affinity-purified on Protein A affinity columns (GE Healthcare, Mississauga, ON, Canada).

αKlotho assays

The ELISA was performed as per the manufacturer's protocol (Immuno-Biological Laboratory, Japan). For the IP-IB assay, 50 µL of serum or urine were diluted with KRH buffer [25 mM HEPES–NaOH (pH 7.4), 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.3 mM CaCl2, 1.3 mM KH2PO4] to a final volume of 0.5 mL and incubated with 2 µg of sb106-Fab (overnight at 4°C). Sepharose beads (50 µL) conjugated with anti-FLAG antibody (50% v/v Flag-Tag M2 beads, Sigma–Aldrich A2220l) were added, incubated (4°C × 2 h), washed (×3, KRH —500 µL per tube × 3; 22°C) and pelleted (5000 g for 30 s). The immune complex was eluted with 2× SDS sample loading buffer (50 µL; 100°C × 3 min; 4°C × 3 min; spun) and removed to a separate siliconized tube. Twenty micrograms of the sample was fractionated by SDS–PAGE, transferred to nitrocellulose membranes, blocked (5% milk, 1 h, 22°C) and incubated with a primary anti-αKlotho antibody (KM2076, 3.1 mg/mL, 1:5000 dilution) and diluent (Dako#S3022, Carpinteria, CA, USA) overnight (4°C, rocker). The membrane was washed (×3, Tris-buffered saline with 0.1% Tween; TBS–T), exposed to ECL™ anti-rat IgG (NA935, GE Healthcare in 5% milk/TBS–T × 1 h) and washed (×3 TBS–T). For chemiluminescence, the membrane was covered with SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific, Rockford, IL, USA) and exposed for 30–90 s. The 130-kD bands were scanned, and density was compared with internal control samples of known amounts of Klotho using Adobe Photoshop CS4. The supplement contains the entire bench protocol in detail.

RESULTS

Identification of an anti-αKlotho synthetic Fab

After rounds of biopanning of a phage-displayed synthetic Fab library on recombinant αKlotho ectodomain complexed with the ligand-binding domain of FGFR1c, we identified several binding phages. Clone sb106 (Figure 1A) was chosen for further characterization based on binding specificity. In phage ELISA (Figure 1B), sb106 phage bound to both human and mouse αKlotho, demonstrating cross-species reactivity, and to either αKlotho alone or in complex with FGFR1c, indicating that its epitope is not obscured by co-receptor complex formation. Sb106 phage did not bind to FGFR1c alone, neutravidin (NAV) or bovine serum albumin (BSA). Sb106 binds to human αKlotho with affinity in the single-digit nanomolar range (IC_{50} = 1.7 nM, Figure 1C). Sb106-Fab also binds with high affinity to the binary αKlotho–FGFR1c complex immobilized on a biosensor chip, and it does not interfere with ternary complex formation between FGF23, αKlotho and FGFR1c (Supplementary Figure 1).

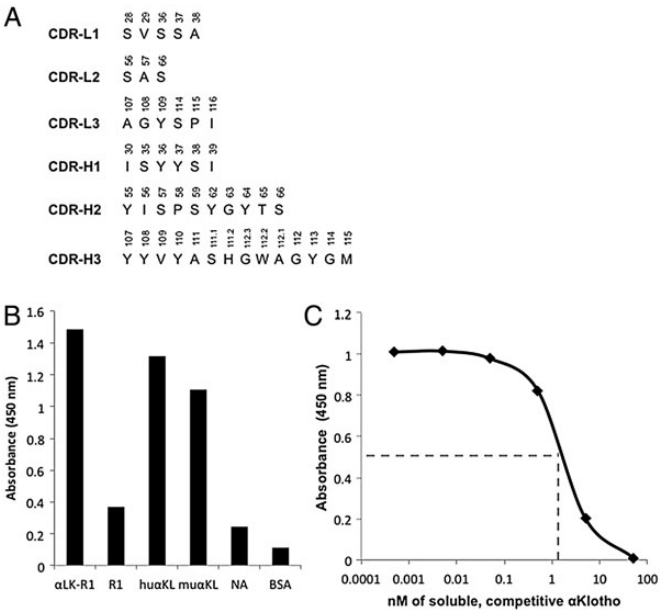

FIGURE 1: Sequence, specificity and affinity of sb106 (**A**) Sequences of the CDRs of sb106 in the IMGT numbering scheme [69]. (**B**) Specificity of sb106 by phage ELISA. Sb106 phage were assayed for binding to the following immobilized proteins: human αKlotho (Hu αKL), mouse αKlotho (Mu αKL), complex of the extracellular domain of mouse αKlotho and the ligand-binding domain of human FGFR1c (Mu αKL:FGFR1c), ligand-binding domain of human FGFR1c (FGFR1c), neutravidin (NAV), and bovine serum albumin (BSA). (**C**) Estimation of the affinity of sb106 for αKlotho by competitive phage ELISA. Sb106 phage were pre-incubated with serial dilutions of human αKlotho (*x*-axis), prior to capture with immobilized antigen and detection by a colorimetric assay (*y*-axis).

Characterization of the anti-αKlotho Fab sb106

Using the unique CDR sequences of sb106 (Figure 1A), both Fab and full-length IgG proteins were produced. sb106 was highly reactive against αKlotho under native conditions. Immunoblot signals under denaturing conditions against mouse, rat and human kidney tissue were weak, but in samples from transgenic mice overexpressing αKlotho [2], sb106-Fab detected a band corresponding to the full-length extracellular domain of αKlotho (Figure 2A). In cultured cells, sb106-Fab cannot detect αKlotho in immunoblots under denaturing conditions with lysates from normal rat kidney cells expressing small amounts of native αKlotho but can detect the antigen in HEK293 cell lysates overexpressing αKlotho (Figure 2B). In immunohistochemistry with freshly frozen unfixed rat parathyroid tissue (and other tissues known to express αKlotho, data not shown), sb106-IgG detected αKlotho but the same tissue was negative when fixed (Figure 2C), compatible with the fact that sb106 binds only to the native αKlotho (Figure 1B). In immunocytochemistry of freshly fixed cells,

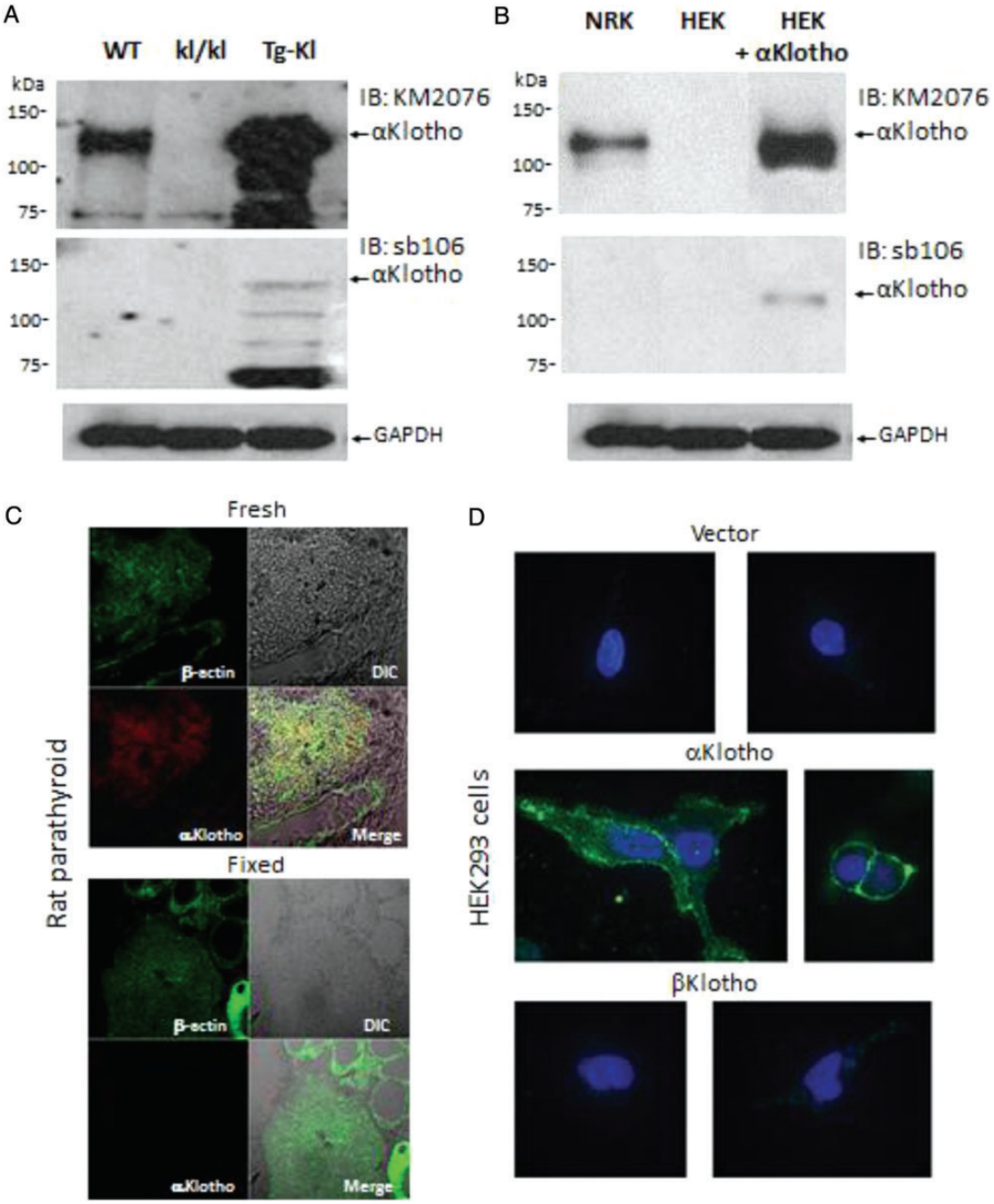

FIGURE 2: Characterization of sb106-Fab by immunoblot, immunohistochemistry and immunocytochemistry (**A**) Immunoblot of kidney lysate from wild-type (WT) mice, homozygous αKlotho hypomorphic mice (*kl/kl*) and transgenic αKlotho-overexpressing mice (Tg-Kl), using the monoclonal antibody KM2076 or the sb106-Fab. GAPDH: glyceraldehyde phosphate dehydrogenase. (**B**) Immunoblot of lysates from normal rat kidney (NRK) cells, human embryonic kidney (HEK) cells and HEK cells transfected with a plasmid for over-expression of αKlotho, using the monoclonal antibody KM2076 or the sb106-Fab. (**C**) Fresh or fixed rat parathyroid tissue probed with phalloidin for β-actin (green) or sb106-IgG (red). (**D**) HEK293 cells transfected with empty vector or vector for over-expression for αKlotho or βKlotho, stained with sb106-Fab (green) and DAPI (blue).

there was unequivocal staining in HEK293 cells heterologously overexpressing αKlotho but not in cells overexpressing βKlotho (Figure 2D). Even in cells overexpressing αKlotho, prolonged fixation greatly diminished or abolished the staining with sb106 (data not shown). In sum, sb106 reacts specifically with native human, rat and mouse αKlotho but not with denatured αKlotho.

Immunoprecipitation of αKlotho

We tested the ability of sb106-Fab to precipitate soluble αKlotho using a sequential IP-IB assay. Sb106-Fab pulled-down αKlotho from total cell lysates and conditioned cell culture medium and from αKlotho-overexpressing cells (Figure 3A). We compared the sb106-Fab pull-down with that of an anti-FLAG antibody using soluble αKlotho with a

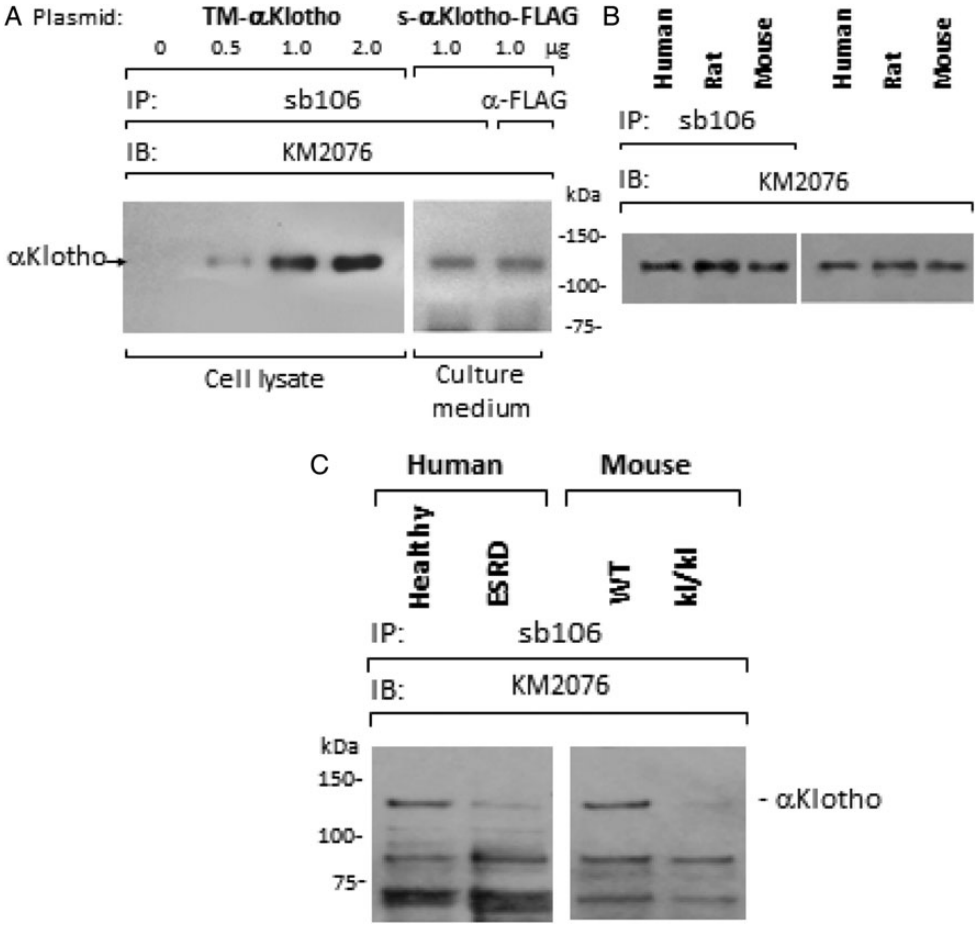

FIGURE 3: Characterization of sb106-Fab by immunoprecipitation. (**A**) HEK293 cells were transfected with empty vector or varying quantities (μg/dish) of vector for expression of transmembrane full-length αKlotho (TM-αKlotho) or soluble extracellular domain of αKlotho with a C-terminal FLAG epitope (s- αKlotho-FLAG). Cell lysates or cell culture medium was immunoprecipitated (IP) with either sb106-Fab or anti-FLAG MAb. Immunocomplexes were resolved by SDS–PAGE and immunoblotted (IB) with monoclonal anti-αKlotho antibody KM2076. (**B**) Urine from rat, mouse or human was immunoprecipitated with sb106-Fab, resolved by SDS–PAGE and immunoblotted (IB) with KM2076 (left three lanes). Size-selected urine (100-kDa cutoff) was directly subjected to SDS–PAGE and immunoblotted (right three lanes). (**C**) Sera from a healthy volunteer (healthy), a patient with end-stage renal disease (ESRD), WT mice and homozygous Klotho hypomorphic mice with systemic Klotho deficiency (*kl/kl*) were subjected to immunoprecipitation by sb106-Fab and immunoblot by KM2076.

C-terminal FLAG tag in HEK293 cells. Sb106-Fab and anti-FLAG precipitated proteins with the exact same electrophoretic mobilities.

Sb106-Fab precipitated an ~130-kDa protein from human, mouse and rat sera that reacted with the anti-αKlotho antibody KM2076 (Figure 3B). Immunoprecipitation from urine also showed an ~130-kDa band (Figure 3B). To further support the authenticity of the IP-IB band by sb106, we examined the intensity of this band in human sera from a normal individual versus a patient with CKD stage 5, and sera from a wild-type mouse versus a homozygous αKlotho hypomorph (Figure 3C). Only the ~130-kDa band (Figure 3C) was reduced in human advanced CKD and was absent in the αKlotho-deficient mice (*kl/kl*) [1]. The mobility of the full-length soluble αKlotho and transmembrane αKlotho is identical on SDS–PAGE, and there is no reagent that recognizes the short intramembranous region so one cannot distinguish the two species by IP-IB. However, the probability of a transmembrane protein circulating in the blood is extremely low.

αKlotho levels in human CKD

We evaluated whether the IP-IB method can reliably determine serum αKlotho levels from a single-center database of CKD patients. We spiked in known amounts of recombinant human αKlotho to test the linearity of the assay as well as the extrapolated *y*-intercept. IP-IB was performed with sera from a normal healthy volunteer and a patient with stage 5 CKD spiked with a range of different concentrations of recombinant αKlotho (Figure 4A). There was graded increase in signal with the incrementally inoculated exogenous αKlotho. The serum from the CKD patient also showed increases in signal with increasing exogenous αKlotho but, at any given concentration of αKlotho, the signal intensity was lower than the normal serum.

The serum from the healthy volunteer gave the same signal in the absence or presence of a protease inhibitor cocktail, whereas serum from the CKD patient displayed an increase in measured αKlotho levels with protease inhibition (Figure 4B). This suggests that while endogenous αKlotho exists in a stable

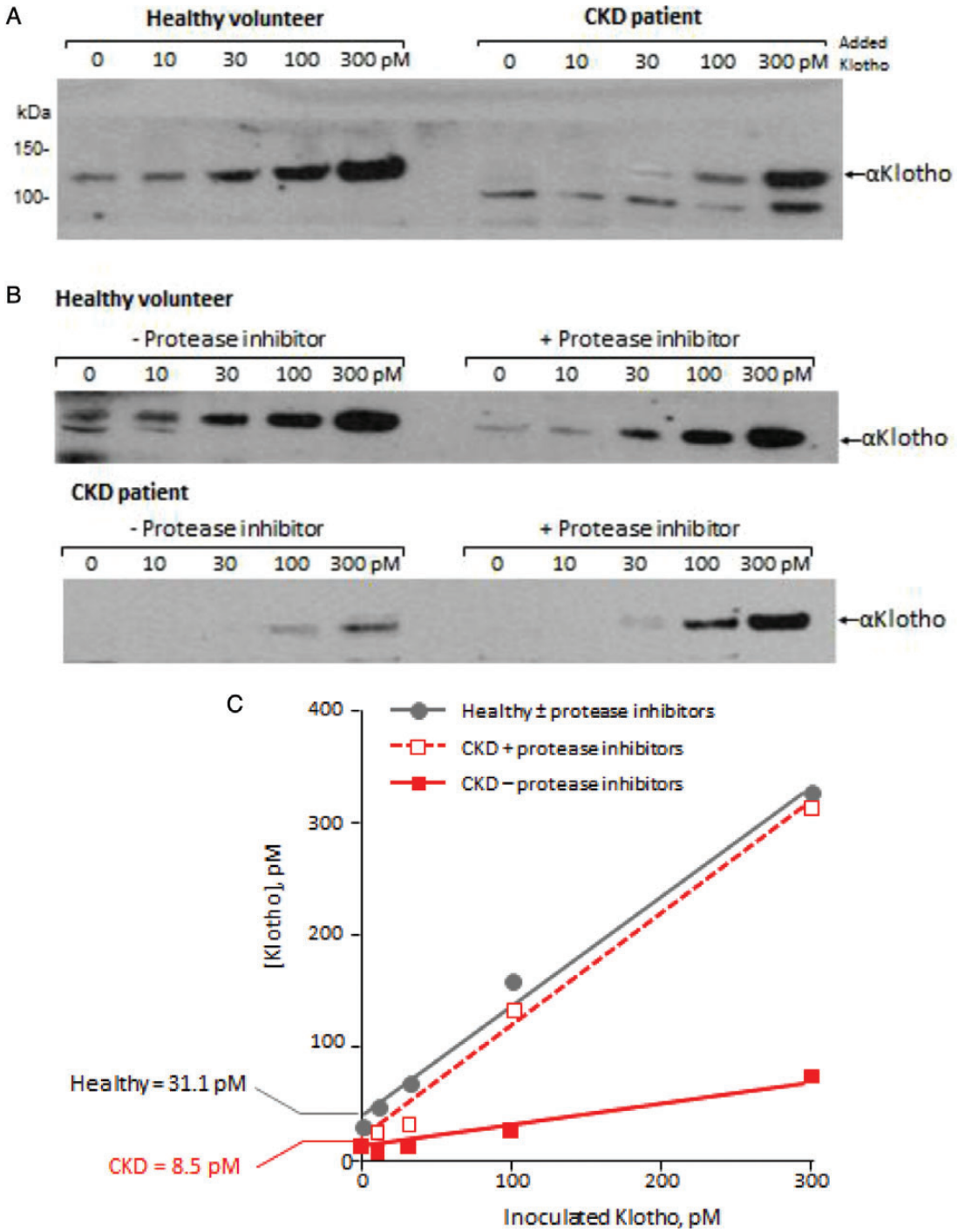

FIGURE 4: Validation of IP-IB assay using human serum spiked with recombinant αKlotho. (**A**) Known amounts of soluble human αKlotho ectodomain were added to sera from a healthy volunteer or an anuric dialysis patient (CKD patient). αKlotho was measured in the sera using the IP-IB assay. (**B**) Similar experiment as in (A) except comparisons was made where protease inhibitors (AEBSF 0.1 mM, aprotinin 0.3 μM, bestatin 10 μM, E-64 1 μM, leupeptin 50 μM, pepstatin A 1 μM) were either included or excluded from the IP. (**C**) αKlotho levels determined by IP-IB (*y*-axis) were plotted against the added recombinant αKlotho (*x*-axis) in the four conditions described earlier. Extrapolation to zero spiking shows the level of endogenous αKlotho in the serum treated with protease inhibitors. Only one line is shown for healthy serum with or without protease inhibitors as the results were indistinguishable.

steady state in uremia, exogenously added αKlotho may undergo proteolysis in uremic but not in normal serum. A quantitative summary of the spiking experiment is shown in Figure 4C. Both healthy and CKD sera showed linear responses to αKlotho inoculation, but the signal from CKD sera has a lower slope. When protease inhibitors were included, the slope of the CKD line approached that of the healthy subject without affecting its intercept. Extrapolation to zero inoculation showed that the serum from the normal individual had 31.1 pM αKlotho whereas that from the CKD patient had 8.5 pM αKlotho. Similar

extrapolations were obtained from a number of subjects with normal renal function or CKD (data not shown).

The constituents of our CKD clinic closely resemble the national profile of CKD where diabetes and hypertension predominate (Table 1). Despite the scatter, there is a clear progressive decline of αKlotho with stages of CKD (Figure 5A). The decrease in serum αKlotho occurred early in CKD and preceded high FGF23, high PTH and hyperphosphatemia (Table 1). We directly compared the IP-IB assay with a commercial αKlotho ELISA kit using the same samples (Figure 5B). Overall, there is correlation between the two, but there is separation on both sides of the line of identity. In fresh samples, the ELISA shows higher values than IP-IB (gray diamonds to the left of the line of identity, Figure 5B), but in samples that have been through one or more cycles of freeze-thaw, the ELISA values are much lower (black diamonds to the right of the line of identity, Figure 5B). When the exact same samples were tested by the two methods before and after repeated freeze-thaw, the IP-IB assay gave more stable results while the ELISA values dropped (Figure 5C).

We previously described low urinary αKlotho in human CKD patients by directly immunoblotting urine [8]. The IP-IB assay with sb106-Fab showed dramatic reduction of urinary αKlotho in CKD patients (Figure 6A). In contradistinction from serum, the ELISA yielded more comparable values to the IP-IB assay in the urine, but the magnitude of decrease in αKlotho concentration is more dramatic when detected by the IP-IB assay than by ELISA (Figure 6B). These results unequivocally showed that human CKD is a state of αKlotho deficiency in both serum and urine.

DISCUSSION

Impact of synthetic antibody technology on basic and clinical research

Antibody-based reagents are invaluable tools for both research and clinical applications for detection of proteins, protein isolation and purification, and numerous downstream applications. We developed a synthetic antibody to enhance our understanding of the role of αKlotho in CKD. The ability to consistently and reliably detect αKlotho in samples from both humans and rodents is of significant value. The commercial reagents available are limiting; there are no antibodies for specifically detecting native, folded αKlotho protein. Moreover, the commercial ELISA kit for αKlotho detection yields highly variable results, and a description of its components is not publicly available.

Synthetic antibodies with designed antigen-binding sites are fine-tuned and tailored for molecular recognition of vast repertoires of targets. Coupled with *in vitro* phage display, selections are performed in the absence of tolerance mechanisms that eliminate self-reactive antibodies. Selections with our library yielded sb106, an antibody with exquisite specificity for native human, mouse and rat αKlotho, making it an ideal affinity reagent for the study of αKlotho levels in the sera and urine of multiple species.

Table 1. Characteristics of human subjects.

Subject	*n*	Age years	Gender M/F	PCr [mg/dL (μM)]	Serum Pi [mg/dL (mM)]	Serum PTH [pg/mL (pM)]	FGF23 (pg/mL)	25(OH)vitamin D ng/mL (nM)	Etiology of CKD (number of subjects)
Healthy	34	50 ± 17	14/20	0.8 ± 0.2 (70 ± 18)	3.6 ± 0.6 (1.2 ± 0.2)	59 ± 25 (5.9 ± 2.5)	30 ± 10	32 ± 10 (80 ± 25)	
CKD1	10	43 ± 10	7/3	0.8 ± 0.1 (70 ± 9)	3.9 ± 0.5 (1.3 ± 0.2)	47 ± 19 (4.7 ± 1.9)	61 ± 23	26 ± 7 (65 ± 18)	None
CKD2	11	50 ± 22	4/7	1.1 ± 0.2 (97 ± 18)	3.6 ± 0.5 (1.2 ± 0.2)	56 ± 22 (5.6 ± 2.2)	70 ± 27	21 ± 13 (53 ± 33)	DM (1), HTN (3), GN (7)
CKD3	10	57 ± 17	5/5	1.7 ± 0.4 (150 ± 35)[a]	3.2 ± 0.8 (1.0 ± 0.3)	86 ± 51 (8.6 ± 5.1)	79 ± 18[a]	25 ± 8 (63 ± 20)	DM (2), HTN (4), GN (4), RK (3)
CKD4	14	62 ± 13	8/6	2.7 ± 0.6 (238 ± 53)[a]	3.5 ± 0.9 (1.1 ± 0.3)	202 ± 101 (20.2 ± 10.1)[a]	204 ± 173[a]	21 ± 8 (53 ± 20)[a]	DM (3), HTN (7), GN (3), IN (1)
CKD5	11	62 ± 12	5/6	4.7 ± 2.0 (413 ± 18)[a]	5.1 ± 3.5 (1.6 ± 1.1)[a]	223 ± 188 (22.3 ± 18.8)[a]	580 ± 427[a]	21 ± 9 (53 ± 23)[a]	DM (4), HTN (10), GN (3), RK (1)
Dialysis	14	50 ± 12	6/8	11.9 ± 15.6 (1047 ± 1372)[a]	4.8 ± 1.7 (1.5 ± 0.5)[a]	500 ± 650 (50.0 ± 65.0)[a]	760 ± 286[a]	26 ± 8 (65 ± 20)	DM (7), HTN (7), GN (2)
									DM (7), HTN (10), GN (5), PKD (1)

n, number of subjects; PCr, plasma creatinine; serum Pi, serum inorganic phosphate; PTH, parathyroid hormone; FGF23, fibroblast growth factor 23; DM, diabetes mellitus; HTN, hypertension; GN, glomerulonephritis; RK, remnant kidney; IN, interstitial nephritis; PKD, polycystic kidney disease. Some patients carried more than one diagnoses. Results are shown as mean ± standard deviation.

[a]$P < 0.05$ compared with healthy volunteers by ANOVA.

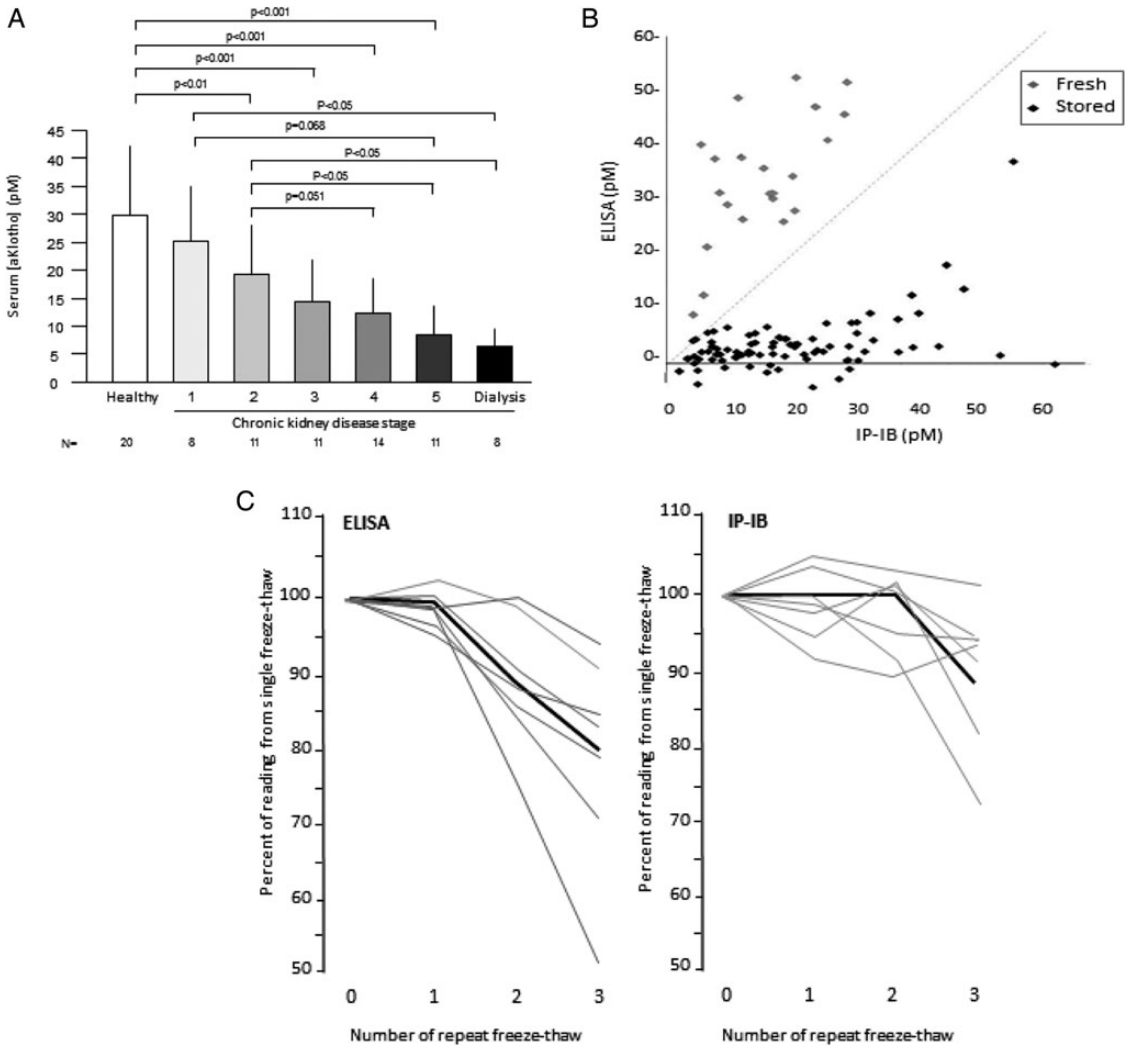

FIGURE 5: IP-IB assay of serum αKlotho in humans with chronic kidney disease. (**A**) αKlotho was measured by the IP-IB assay in human sera from normal healthy volunteers and patients from a CKD clinic and dialysis unit using the conventional numerical staging using recombinant αKlotho as a calibration curve. Bars and error bars denote means and standard deviations. The data were analyzed by ANOVA followed by Student–Newman–Keuls test for pairwise multiple comparisons. P-values achieving statistical significance between groups are indicated above the brackets. The number of subjects in each group is indicated at the bottom. (**B**) The concentrations of αKlotho in a large variety of human sera were determined either by IP-IB (*x*-axis) or by a commercial ELISA (*y*-axis) in the same samples. The dotted line represents identity. The black diamonds represent sera that have been through one or more freeze-thaw cycles (stored), and the gray diamonds represent sera thawed only once (fresh). (**C**) Sera from human subjects were assayed by IP-IB and ELISA. The same sera were subjected to the indicated cycles of repeated freeze-thaw and then assayed. Results for each sample were expressed as a percentage of the reading from the same sample thawed only once. The heavy lines denote the mean of the different subjects.

Biologic and clinical implications of αKlotho deficiency in CKD

In addition to its role in mineral metabolism, soluble αKlotho circulates in many bodily fluids and has multiple 'house-keeping' functions that maintain cellular integrity throughout the body. Although the mechanism of action of soluble αKlotho remains poorly understood, the biologic impact of αKlotho deficiency is unequivocally shown [65]. αKlotho transcripts are present in multiple organs, but the kidney by far has the highest expression [1]. CKD is a state of multiple metabolic derangements and is a complex syndrome from the accumulation of under-excreted endogenous and exogenous toxins as well as deficiency in substances normally responsible for health maintenance.

There is unequivocal evidence in experimental animals that both AKI and CKD are states of systemic αKlotho deficiency. Not only is this a sensitive biomarker, restoration of αKlotho ameliorates renal dysfunction. Independent from its renoprotective effects, αKlotho also reduces extra-renal complications in CKD [8, 66]. The preclinical data suggest that αKlotho can have significant diagnostic, prognostic and therapeutic value in human AKI and CKD, yet a robust database is not currently available.

Validation of the IP-IB assay and comparison with the commercial ELISA

In addition to retrospective or cross-sectional data, population-based multicenter prospective human studies are required

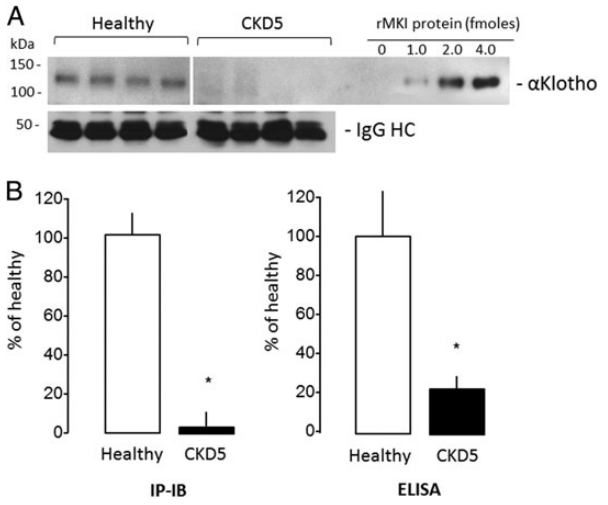

FIGURE 6: Human urinary αKlotho levels. αKlotho was measured in the urine of healthy volunteers or patients with chronic kidney disease stage 5 (CKD5). (**A**) A representative IP-IB assay using recombinant murine αKlotho (rMKl) as a calibration with four subjects in each group under steady state conditions. Equal amounts of urine creatinine were used for IP-IB. (**B**) Summary of the data from the IP-IB assay and the commercial ELISA. Bars and error bars represent mean and standard deviation from eight subjects in each group. The mean of the healthy volunteers was set as a reference of 100%.

to determine the diagnostic and prognostic value of αKlotho. Interventional studies also need monitoring of serum αKlotho levels. A specific reproducible high-throughput assay is essential for the field to move forward.

Currently available commercial assays have no consistent correlation between them [35, 67]. Studies in healthy humans and CKD patients based on one ELISA [47] have yielded baffling results. The absolute levels of αKlotho in normal and CKD ranged from 0.4 [36] to over 2000 pg/mL [30] with most readings in the hundreds [37, 39, 44, 47–49, 67]. Based on this assay, αKlotho levels have been described to be low [37, 41, 43, 46–48], no relationship to [29, 30, 39, 40, 42] or increased [33, 36] with decreasing glomerular filtration rate. Likewise, αKlotho levels have been reported as not changed or decreased with age [31, 42, 47, 48]. This renders the interpretation of human αKlotho data difficult, and the collective data derived from different centers have no value. There is also no published data on the reagents, namely, the antibodies that form the foundation of this assay.

We generated a high-affinity synthetic antibody that recognizes αKlotho in its native conformation (Figures 1–3). Sb106-Fab or IgG pulls down αKlotho from cell lysate, culture medium, serum and urine. Additional bands may be shorter fragments of αKlotho, but the intensity of these bands did not decrease in the *kl/kl* mice, which argue against this possibility. We have limited our analysis to the ∼130-kDa band, which is unequivocally full-length soluble αKlotho, something that the ELISA cannot achieve.

The linearity of the spiking experiment indicates that the inoculated αKlotho is detected (Figure 4). An unexpected finding was that exogenously added recombinant αKlotho is proteolytically degraded in uremic serum whereas no such phenomenon was observed in normal sera. This challenges the view that the low αKlotho in kidney disease stems solely from decreased production and opens up additional mechanisms and new avenues for investigation. In addition to uncovering new mechanisms of αKlotho deficiency in CKD, this may have significant implications in terms of recombinant αKlotho replacement as some strategy has to be devised to stabilize exogenously administered recombinant αKlotho.

There is graded reduction in serum αKlotho with advancing CKD (Figure 5A). The broad range of values within each group is not due to assay variations but is in fact biologic. The coefficient of variation of the IP-IB assay was 4% for serum and 7% for urine (data not shown). There are many factors that can affect αKlotho levels, and they are certainly not controlled in this study. The IP-IB assay also showed low urinary αKlotho in advanced CKD (Figure 6). In fact, the reduction in urinary αKlotho is more dramatic than that in serum and may represent a more sensitive marker for CKD.

Both IP-IB and the commercial ELISA detected the low urine αKlotho in CKD, although the absolute levels of αKlotho are higher with the ELISA assay and the percent reduction is not the same as with the IP-IB assay. With drastic reduction in urinary αKlotho levels in CKD, the two assays yielded the same conclusion but with quantitative differences. The situation in serum is different. Although there is overall positive correlation, the comparison of the two assays segregated into two groups (Figure 5B). The fresh samples showed higher readings for the ELISA whereas the stored samples yielded very low levels with the ELISA. The ELISA may be measuring αKlotho and other cross-reacting proteins in fresh samples. While the IP-IB assay did lose some efficacy with repeated freeze-thaw, this is a much more serious problem with the ELISA. Since most clinical sample banking involves freezing and sometimes the samples were thawed for measurement of other parameters, this may explain some of the large variations in the current literature where freeze-thaw of banked samples is common.

Another advantage of the IP-IB assay is that it detects αKlotho in both humans and rodents equally well, whereas the use of the currently available ELISA in rodent can potentially be problematic as it detects very high circulating αKlotho levels in rats with CKD, which is a well-documented state of pan-αKlotho deficiency [68]. It should be emphasized that the IP-IB is still very much a tool for laboratory animal and smaller-scale human metabolic studies. The labor intensity and the fact that IB is less quantitative than ELISA do not render this format of the αKlotho assay ideal for large population-based epidemiologic work. These antibodies, along with proper sample handling, can be optimized for a high-throughput format and will be powerful tools not only for animal studies but also for large-scale clinical studies.

SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

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