DIABETIC KIDNEY DISEASE

Neuroblastoma suppressor of tumorigenicity 1 is a circulating protein associated with progression to end-stage kidney disease in diabetes

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Circulating proteins associated with transforming growth factor–β (TGF-β) signaling are implicated in the development of diabetic kidney disease (DKD). It remains to be comprehensively examined which of these proteins are involved in the pathogenesis of DKD and its progression to end-stage kidney disease (ESKD) in humans. Using the SOMAscan proteomic platform, we measured concentrations of 25 TGF-β signaling family proteins in four different cohorts composed in total of 754 Caucasian or Pima Indian individuals with type 1 or type 2 diabetes. Of these 25 circulating proteins, we identified neuroblastoma suppressor of tumorigenicity 1 (NBL1, aliases DAN and DAND1), a small secreted protein known to inhibit members of the bone morphogenic protein family, to be most strongly and independently associated with progression to ESKD during 10-year follow-up in all cohorts. The extent of damage to podocytes and other glomerular structures measured morphometrically in 105 research kidney biopsies correlated strongly with circulating NBL1 concentrations. Also, in vitro exposure to NBL1 induced apoptosis in podocytes. In conclusion, circulating NBL1 may be involved in the disease process underlying progression to ESKD, and its concentration in circulation may identify subjects with diabetes at increased risk of progression to ESKD.

INTRODUCTION

An increase in urine albumin excretion is an early clinical marker of progressive kidney disease in people with diabetes, but the decline in kidney function that characterizes this progression often occurs even in the absence of elevated albuminuria, suggesting that new markers that better reflect key mechanisms involved in diabetic kidney disease (DKD) may be needed. The transforming growth factor–β (TGF-β) superfamily of signaling proteins plays an important role in the development and progression of DKD (*1*–*10*). This superfamily consists of proteins active in intra- and extracellular compartments. In the latter, three subfamilies of proteins play a role in this setting: TGF-β subfamily, bone morphogenic proteins (BMP), and activins (*11*). Interaction among these proteins regulates intracellular TGF-β signaling and affects many processes including tissue differentiation during development and tissue repair in various diseases including DKD (*1*, *2*). TGF-β superfamily proteins have been investigated in the context of DKD, but mainly in in vitro and animal studies and without consideration for their extracellular versus intracellular interactions (*12*–*21*). We hypothesized that circulating proteins involved in the extracellular regulation of TGF-β signaling are associated with DKD progression.

Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and recent review (*11*, *22*), we could identify 38 extracellular and membrane proteins that impact on TGF-β signaling. Of the 38 proteins, 25 were measured on the first version of the SOMAscan proteomics platform; table S1 lists the proteins and related publications (*12*–*58*), and fig. S1 shows their possible interactions based on prior research.

The present study was conducted in patients participating in two follow-up studies: the Joslin Kidney Study and the Pima Indian Kidney Study. Subjects in these studies had baseline concentrations of circulating proteins (1129 proteins in first-stage and 564 in second-stage DKD) measured by the SOMAscan proteomics platform and were followed to ascertain progression to end-stage kidney disease (ESKD) during 10-year follow-up (*59*–*61*). The SOMAscan measurements were used to examine 25 prespecified circulating proteins modulating TGF-β signaling as predictors for progression to ESKD. This targeted approach was used in our recent studies (*62*–*64*). In this study, we identified increased concentration of neuroblastoma

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suppressor of tumorigenicity 1 (NBL1; aliases DAN and DAND1), a presumed inhibitor of BMP proteins, as a strong and independent predictor of progression to ESKD in all study cohorts (*65*–*71*). Furthermore, to explore possible mechanisms through which circulating NBL1 may damage the kidney, we used single-nucleus RNA sequencing (snRNA-seq) to examine *NBL1* expression in kidney cells, immunostaining to examine *NBL1* expression in kidney and 10 other tissues, and assessed the in vitro effects of NBL1 on apoptosis of kidney cells.

RESULTS

Study cohorts

Four independent cohorts of individuals with type 1 diabetes (T1D) and type 2 diabetes (T2D) at various DKD stages were followed to ascertain progression to ESKD. Clinical characteristics of the study cohorts are shown in Table 1. By design, the cohorts differed at baseline by race, diabetes type, age, sex, diabetes duration, glycemic control, albuminuria, and kidney function. Two cohorts recruited from the Joslin Kidney Study with late DKD at baseline, defined by albuminuria and impaired kidney function, were designated as the T1D discovery and the T2D replication cohorts. The late DKD T1D cohort was selected as the discovery cohort because it had the greatest statistical power to detect circulating proteins associated with risk of ESKD because of the large number of cases. Positive findings from this cohort were investigated in the T2D replication cohort and in two validation cohorts with early DKD. The first validation cohort included subjects with T1D with albuminuria and normal kidney function, recruited from the Joslin Kidney Study. The second validation cohort included subjects with T2D with either normoalbuminuria or albuminuria but normal kidney function, recruited from the Pima Indian Study. In the Joslin cohorts, 92% of subjects were of European ancestry. All subjects in the Pima cohort were American Indians. A subgroup of 105 Pima Indians had research kidney biopsies performed in conjunction with the baseline examination.

Of the 754 subjects included in the study cohorts, 227 progressed to ESKD during the first 10 years of follow-up. In combined cohorts, follow-up was 10 (median) and 7 to 12 (25 and 75%) years in subjects who did not develop ESKD, and 5 (median) and 3 to 7 (25%, 75%) years in subjects who progressed to ESKD (fig. S2).

Circulating TGF-β signaling proteins and risk of ESKD

Baseline concentrations of the 25 circulating TGF-β signaling proteins were evaluated in the discovery cohort for association with risk of ESKD. Seven of these proteins belonged to the TGF-β subfamily proteins, 13 to the BMP subfamily proteins, and 5 to the activin subfamily proteins. The names of these proteins and magnitude [hazard ratio (HR)] of their effects on the risk of ESKD in univariate Cox regression analysis are shown in Fig. 1. Six proteins associated with 10-year risk of ESKD were statistically significant after Bonferroni adjustment ($P < 0.0020$). These proteins included TGF-β receptor III (TGF-βRIII), anti-Muellerian hormone type-2 receptor (AMHR2), repulsive guidance molecule BMP coreceptor A (RGMA), repulsive guidance molecule BMP coreceptor B (RGMB), NBL1, and follistatin-like 3 (FSTL3). Of the six proteins associated with risk of ESKD in the discovery cohort, AMHR2 was not measured in the second stage of the SOMAscan screening because of technical reasons, and RGMA was not confirmed in the other cohorts (table S2).

Table 1. Clinical characteristics of subjects included in the study cohorts. DKD, diabetic kidney disease; late DKD, individuals with impaired kidney function; early DKD, individuals with normal kidney function; T1D, type 1 diabetes; T2D, type 2 diabetes; DM, diabetes mellitus; HbA1c, hemoglobin A1c; SBP, systolic blood pressure; DBP, diastolic blood pressure; ACR, urine albumin-to-creatinine ratio; eGFR, estimated glomerular filtration; ESKD, end-stage kidney disease (dialysis or kidney transplant).

Characteristics	Late DKD cohorts		Early DKD cohorts		
	Joslin	Joslin	Joslin	Pima	Pima
	T1D discovery cohort	T2D replication cohort	T1D validation cohort 1	T2D validation cohort 2	T2D cohort for auxiliary study*
N	219	144	238	153	105
Male/female (*n*)	106/113	94/50	131/107	43/110	29/76
Age (years)	45 ± 10	60 ± 6	39 ± 9	46 ± 9.8	45 ± 10
Diabetes duration (years)	30 ± 9	16 ± 9	26 ± 9	16 ± 6	15 ± 6
HbA_{1c} (DCCT, %)	8.8 ± 1.7	7.6 ± 1.6	9.0 ± 1.7	9.3 ± 2.3	9.2 ± 2.3
SBP (mmHg)	135 ± 20	140 ± 20	131 ± 18	124 ± 14	122 ± 13
DBP (mmHg)	77 ± 11	75 ± 11	78 ± 11	77 ± 9	77 ± 9
ACR (mg/g)¶	757 (214, 1792)	255 (56, 1100)	591 (250, 1195)	55 (13, 357)	40 (12, 124)
eGFR (ml/min/1.73 m^2)†	43 ± 11	49 ± 11	98 ± 21	150 ± 47	151 ± 47
During follow-up					
GFR slope (ml/min/year)¶	−3.8 (−7.5, −1.9)	−3.2 (−6.4, −0.9)	−3.1 (−7.7, −1.3)	−4.0 (−8.9, −1.3)	−4.4 (−10.3, −1.6)
New cases of ESKD within 10 years [*n* (%)]	108 (49%)	35 (24%)	50 (21%)	34 (22%)	15 (14%)

*Subgroup of T2D Pima validation cohort for kidney structural study. †In the Pima cohort, GFR (ml/min) was measured directly using urinary clearance of iothalamate. ¶Data are expressed as means ± SD or median (25% and 75%).

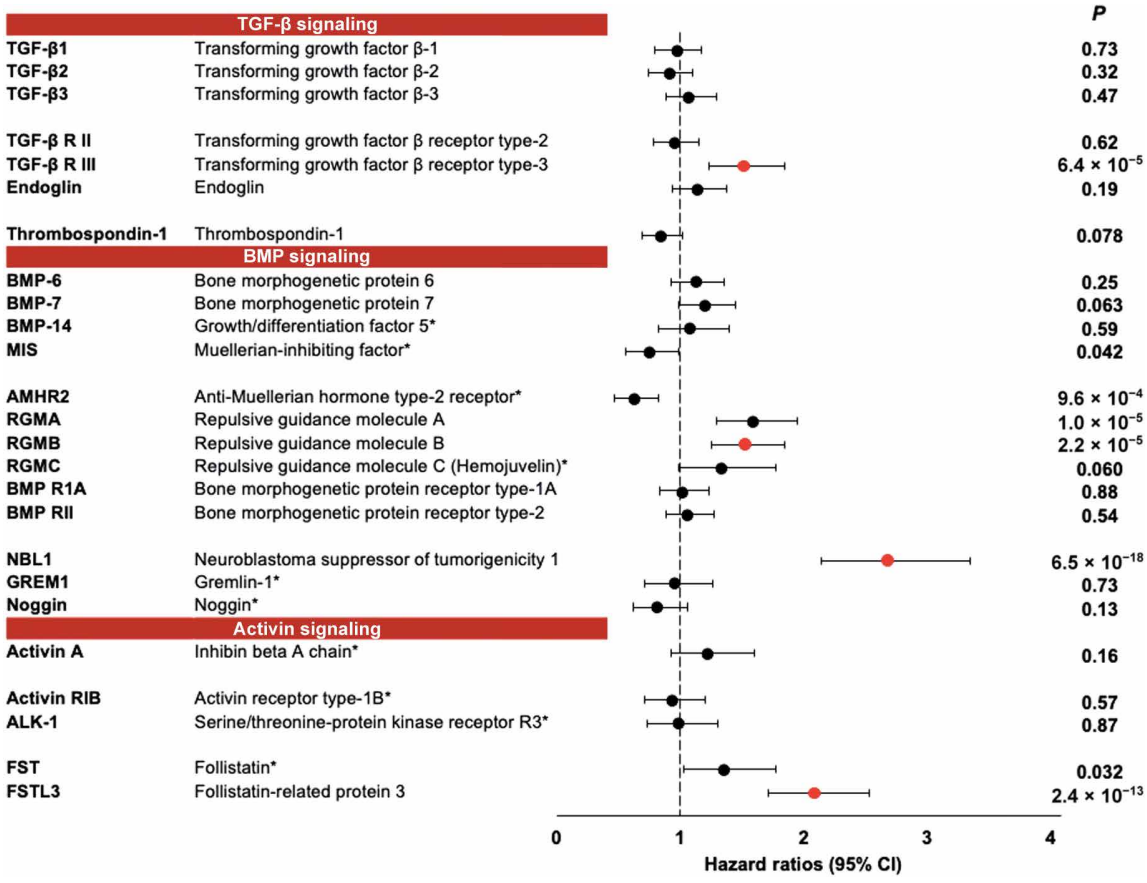

Fig. 1. Association of ESKD risk with baseline concentration of 25 circulating candidate proteins in the discovery cohort. Effect size (HR and 95% CI) in discovery cohort is presented per SD change in protein concentration. Proteins are grouped according to TGF-β, BMP, and activin signaling. Bonferroni correction for $n = 25$ independent tests (the number of examined proteins by the study design) yielded a threshold of $P < 2.0 \times 10^{-3}$. Bonferroni correction for $n = 1129$ independent tests (the number of proteins on SOMAscan) yielded a threshold of $P < 4.0 \times 10^{-5}$. Red dots show proteins that were replicated and validated in all three other study cohorts. See table S2. *Proteins which were measured only in 113 of 219 subjects in the discovery cohort in the first stage of SOMAscan screening. Anti-muellerian hormone type-2 receptor (AMHR2) was significant (Bonferroni corrected $P = 0.024$) in the first stage of SOMAscan screening but was not measured in the second stage because of technical reasons.

The four proteins confirmed in all cohorts had strong associations with risk of ESKD in models adjusted for clinical covariates (Fig. 2A). Because these proteins were highly intercorrelated (fig. S3), we examined which protein(s) had the greatest effect on the 10-year risk of ESKD using multivariable Cox model that included the four proteins and all clinical covariates. NBL1 was the only protein that had a strong and independent association with risk of ESKD in both late and early DKD (Fig. 2B). Figure 2C shows cumulative risk of ESKD according to duration of follow-up and quartiles of baseline concentration of NBL1. The cumulative risk of ESKD at 10 years in the late DKD cohorts was 85% in subjects in the highest NBL1 quartile and 12% in subjects in the lowest quartile. Subjects in the intermediate NBL1 quartiles had cumulative risks in between the two extremes. A similar pattern of risk was found in the early DKD cohorts; risk of ESKD was 51% in subjects in highest quartile versus 6.7% in the lowest quartile of NBL1.

Circulating NBL1 and risk of ESKD according to albuminuria

To determine whether the effect of circulating NBL1 on the risk of ESKD varied according to albuminuria, we performed a mediation analysis. As shown in Fig. 2D, 73% of the effect of NBL1 on risk of ESKD was independent of albuminuria, with a significant albuminuria-independent HR of 2.02 ($P < 0.0001$) for 10-year risk of ESKD per 1 SD increase of NBL1 in late and HR of 1.81 ($P < 0.0001$) in early DKD cohorts.

Circulating NBL1 and kidney structural lesions in early DKD

We next evaluated the correlation between circulating NBL1 and lesion severity in research kidney biopsies performed in 105 of the 153 individuals in the T2D Pima Indian cohort. The biopsies were performed within a median of 1.3 years of the examination in which the serum was obtained for SOMAscan measurements. Circulating NBL1 concentrations correlated negatively with podocyte number, fractional volume of podocyte cells per glomerulus, percent glomerular capillary fenestrated endothelium, and glomerular filtration surface density, and positively with mesangial fractional volume, glomerular basement membrane (GBM) width, and cortical interstitial fraction volume (fig. S4).

For comparison, correlations were evaluated between kidney structural lesions and circulating concentrations of several other proteins (Table 2). The correlations were absent for podocyte damage and weaker for other structural lesions for tumor necrosis factor

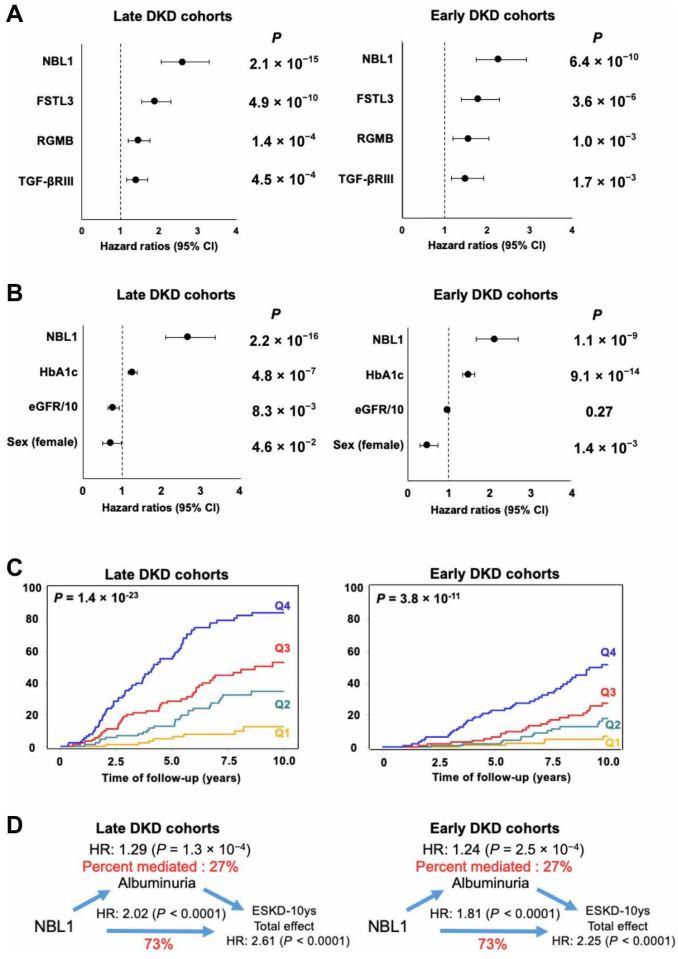

Fig. 2. Association of baseline circulating NBL1 with risk of ESKD during 10-year follow-up in late DKD (CKD stage 3) and early DKD (CKD stages 1 and 2) cohorts. (**A**) Risk of ESKD according to baseline circulating concentration of each of four confirmed proteins in late and early DKD cohorts. Results of Cox regression analysis are shown. Effect measures were expressed as HR and 95% CI per SD increase in protein concentration. Effect for each protein was adjusted for sex, duration of diabetes, HbA1c, systolic blood pressure, diastolic blood pressure, and baseline eGFR with stratification of type of diabetes. (**B**) Effect of baseline circulating concentration of NBL1 on risk of ESKD after adjustment for other candidate proteins and baseline clinical characteristics. Results of Cox regression analysis with backward elimination of covariates are shown. Factors considered were NBL1, FSLT3, RGMB, TGF-βRIII, sex, duration of diabetes, HbA1c, systolic blood pressure, diastolic blood pressure, and baseline eGFR. Results are shown for late and early DKD cohorts. Effect measures were expressed as HR and 95% CI per SD increase in NBL1 concentration, 1% increase in HbA1c, 10-ml change in baseline eGFR, and 1 for women and 0 for men. $P < 0.05$ was used to retain variables. (**C**) Cumulative incidence of ESKD according to quartiles of baseline circulating concentration of NBL1 in late and early DKD cohorts. Q1, first quartile; Q2, second quartile; Q3, third quartile; Q4, fourth quartile. (**D**) Mediation analysis of association between baseline circulating concentrations of NBL1 and risk of ESKD during 10-year follow-up according to baseline albuminuria (ACR) in late and early DKD cohorts. NBL1 concentration was considered as exposure, risk of ESKD was outcome, and ACR was mediator. In the analyses, we used Cox regression model for 10-year risk of ESKD adjusted for sex, duration of diabetes, HbA1c, systolic blood pressure, diastolic blood pressure, and baseline eGFR with stratification of type of diabetes. Effect measures were expressed as the HR per SD increase in NBL1 concentration. The effect of an NBL1 on ESKD (total effect) is split into a natural indirect effect (through ACR) and natural direct effect, which is independent from the ACR.

receptor 1 (TNFR1) than for NBL1, and none of the lesions were correlated with BMP-7 or TGF-β1 (*72*–*75*).

NBL1 expression in snRNA-seq of kidney cells

To further study the relationship between NBL1 and kidney structural lesions in DKD, we reanalyzed our previously published kidney cortex snRNA-seq libraries obtained from three healthy adults and three individuals with DKD (*76*). The individuals with DKD had estimated glomerular filtration rates (eGFRs) ranging from 56 to 85 ml/min/1.73 m^2, and two of the three had proteinuria and moderate interstitial fibrosis and glomerulosclerosis on biopsy. Kidney cell types showed minimal *NBL1* expression (Fig. 3B). In contrast, snRNA-seq detected a larger proportion of kidney cell types expressing *TNFRSF1A* (TNFR1) (Fig. 3C).

To validate these findings, we reanalyzed a previously published bulk RNA-seq dataset obtained from patients with DKD (*77*). This dataset consists of 37 kidney tissue samples from subjects with biopsy proven early DKD ($n = 6$), advanced DKD ($n = 22$), and healthy controls ($n = 9$). Differential expression analysis of the bulk RNA-seq dataset showed that subjects with advanced DKD had increased expression of *NBL1* [log fold change (FC) = 1.16, P adj. $=3.4 \times 10^{-10}$] compared to healthy controls. In contrast, subjects with early DKD had no increase in *NBL1* expression.

Bulk RNA-seq datasets are an aggregate measurement of the transcriptional changes in multiple cell types. Deconvolution of this bulk RNA-seq dataset showed an increase in the number of leukocytes in kidney specimens obtained from subjects with advanced DKD but not from early DKD or healthy controls (*78*). Thus, infiltration of leukocytes during progression of DKD may account for the increased expression of *NBL1* seen in the bulk RNA-seq of kidney specimens obtained from subjects with advanced DKD. Overall, the snRNA-seq and bulk RNA-seq data did not indicate that expression of *NBL1* in kidney preceded the development of DKD or that it is the source of elevated concentrations of NBL1 in circulation.

Immunostaining for NBL1 in kidney biopsy specimens

Immunohistochemical and immunofluorescence analyses were used to examine the distribution of NBL1 in healthy and DKD kidneys. DKD histological sections included glomeruli with mesangial thickening, glomerulosclerosis, interstitial fibrosis/inflammation, and tubular atrophy. In healthy kidney biopsies, only minimal NBL1 positivity was found in cytoplasmic and nuclear compartments of tubular epithelial and interstitial cells and moderate positivity in nuclei of podocytes, whereas in DKD kidney biopsies, proximal tubule epithelial cells were stained at high intensity (Fig. 4A).

To study the spatial localization of proteins in multiple cell types, additional immunofluorescence studies were used with cell-specific markers of macrophages and podocytes. NBL1 colocalized with CD68, a macrophage cell marker, in glomerular and tubulo-interstitial compartments of DKD kidney (Fig. 4B). In addition, NBL1 staining was observed in podocytes (Fig. 4C).

Immunostaining for NBL1 in other tissues

A comprehensive study of immunostaining of NBL1 in healthy human tissues was performed (fig. S5). NBL1 expression was strong in cells of the small and large intestines, with NBL1 detected in both cytoplasm and nuclei. A similar pattern was seen for NBL1 in prostate and testis. In contrast, only nuclear staining was evident in ovary tissue, as well as in skeletal, smooth, and heart muscles, although to

Table 2. Association between NBL1 and kidney structural lesions. Association between baseline serum concentrations of NBL1 and structural lesions observed in research kidney biopsies obtained from 105 subjects in the T2D Pima Indian validation cohort. Biopsies were obtained, on average, 1 year after baseline examination. For comparison, associations with three additional circulating proteins are shown: TNFR1, strong predictor of ESKD (*72*, *73*); BMP7 and TGF-β1, both proteins are not associated with risk of ESKD. Correlation was analyzed using Spearman rank correlation. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

		Circulating proteins			
		NBL1	TNFR1	BMP7	TGF-β1
Category of lesions	Measured lesion	Spearman correlation coefficients			
		r	*r*	*r*	*r*
Podocyte damage	Podocyte number per glomerulus (*N*)	**−0.26****	−0.08	−0.06	−0.01
	Fractional volume of podocyte cells per glomerulus	**−0.34*****	−0.16	0.17	−0.11
Mesangial expansion	Mesangial fractional volume (%)	**0.49*****	**0.31****	−0.07	−0.04
Glomerular filtration barrier disruption	Percent fenestrated endothelium (%)	**−0.52*****	**−0.52*****	−0.08	0.01
	Glomerular filtration surface density (μ^2/μ^3)	**−0.48*****	**−0.28****	−0.02	−0.01
	Glomerular basement membrane width (nm)	**0.41*****	**0.27****	0.06	−0.03
Kidney fibrosis	Cortical interstitial fractional volume (%)	**0.39*****	**0.25***	−0.03	0.13
	Global glomerular sclerosis (%)	0.17	0.12	0.07	−0.08

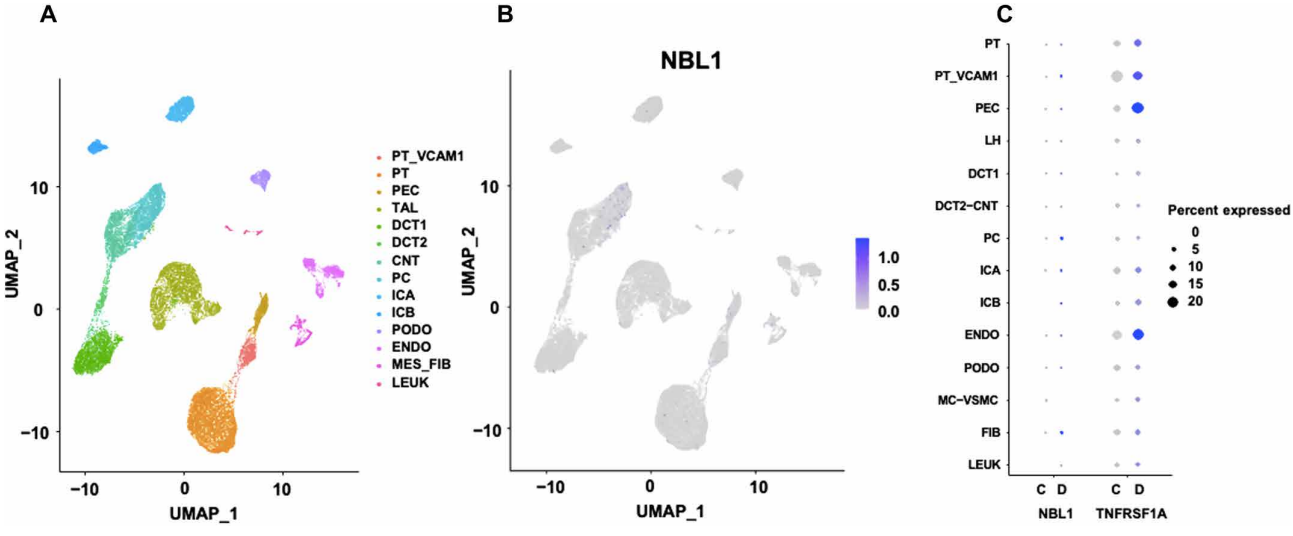

Fig. 3. snRNA-seq of kidney cortex. (**A**) UMAP of all kidney cell types identified in the aggregated dataset. PT_VCAM1, proximal tubule cells that express *VCAM1*; PT, proximal tubule; PEC, parietal epithelial cells; TAL, thick ascending limb; DCT1, early distal convoluted tubule; DCT2-CNT, late distal convoluted tubule and connecting tubule; PCs, principal cells; ICA, type A intercalated cells; ICB, type B intercalated cells; PODO, podocytes; ENDO, endothelial cells; MC-VSMC, mesangial and vascular smooth muscle cells; FIB, fibroblasts; LEUK, leukocytes. (**B**) *NBL1* expression in kidney cell types was obtained from individuals with diabetes. Scale represents normalized log fold change. (**C**) Relative expression was compared between control (C) and diabetic (D) cell types for each cell type in the aggregated snRNA-seq object using Seurat. See table S4.

a less extent in the latter. NBL1 staining was absent in mesenchymal tissues. On flow cytometry analysis, white blood cells revealed a high percentage of NBL1-positive cells, with T cells and monocytes the predominant subpopulations with the highest NBL1 expression (fig. S5B).

Effect of NBL1 on apoptosis of kidney cells in vitro

We examined the effect of circulating NBL1 on apoptosis of kidney cells in in vitro experiments. Human podocytes, mesangial cells, tubular cells, and umbilical vein endothelial cells were cultured for 48 hours with and without NBL1 at increasing concentrations

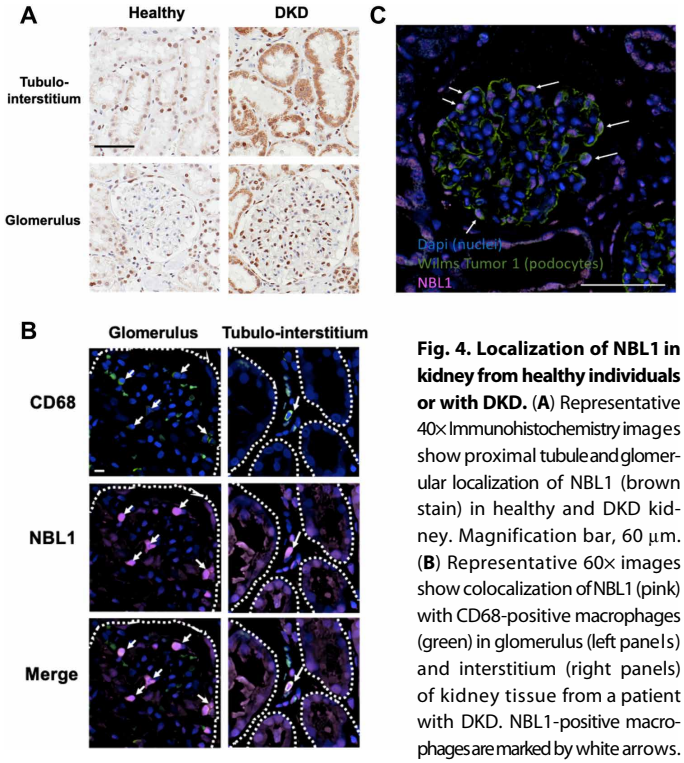

Fig. 4. Localization of NBL1 in kidney from healthy individuals or with DKD. (**A**) Representative 40× Immunohistochemistry images show proximal tubule and glomerular localization of NBL1 (brown stain) in healthy and DKD kidney. Magnification bar, 60 μm. (**B**) Representative 60× images show colocalization of NBL1 (pink) with CD68-positive macrophages (green) in glomerulus (left panels) and interstitium (right panels) of kidney tissue from a patient with DKD. NBL1-positive macrophages are marked by white arrows. Cell nuclei are stained with Dapi (blue). Glomerular Bowman's capsule (left panels) and tubules (right panels) are outlined with dashed white line. Magnification bar, 20 μm. (**C**) Representative 40× image showing glomerular colocalization of NBL1 (pink) and Wilms' tumor-1 (WT-1; green, podocyte cell–specific marker). Characteristic WT-1–positive podocytes in a diabetic kidney are labeled with arrows indicating colocalization of NBL1 signal in podocytes. Magnification bar, 60 μm.

(Fig. 5A). The culture media did not contain any of the circulating proteins that may modulate/regulate TGF-β signaling. The addition of NBL1 to culture media increased apoptosis/death of human podocytes and, to a lesser degree, mesangial and tubular cells, whereas cell death was undetectable in NBL1-cultured umbilical vein endothelial cells even at higher concentrations (Fig. 5, A and B). A transcriptome analysis performed in NBL1-treated human podocytes confirmed the up-regulation of several proapoptotic-related genes, including *BCL2A1*, *CASP2*, *TNFRSF10A*, and *FADD* (Fig. 5C).

Circulating NBL1 and clinical characteristics

To examine whether increased concentration of NBL1 in circulation was a consequence of impaired kidney function, we measured NBL1 urinary excretion and compared it with plasma NBL1 concentrations in specimens obtained at baseline from individuals who had fast progressive kidney decline (cases) versus those who had slow kidney decline (controls) during follow-up. From the T1D discovery cohort, we selected 29 cases and 31 controls. Similarly, we selected 26 cases and 26 controls from the T2D replication cohort (table S3). Baseline plasma and urine specimens were measured using the SOMAscan platform. FCs for NBL1 in plasma and urinary concentrations in cases over controls are shown in Fig. 6A. Cases with fast progressive kidney decline had higher plasma and urinary concentrations of NBL1 and TNFR1 at baseline examination than controls with slow kidney decline. Similar findings were observed in the T1D discovery and T2D replication cohorts (T1D: FC 2.2 with $P < 0.0001$ for plasma NBL1 and FC 1.8 with $P = 0.0014$ for urinary NBL1; T2D: FC 2.1 with $P < 0.0001$ for plasma NBL1 and FC 1.7 with $P = 0.046$ for urinary NBL1). This pattern indicates systemic overproduction that results in increased plasma and urine NBL1, rather than retention due to impaired kidney function. A similar pattern was seen for TNFR1.

To search for possible determinants of systemic overproduction of circulating NBL1, we examined correlations between plasma concentration of NBL1 and clinical characteristics of subjects in the T1D validation cohort with early DKD (Fig. 6B). No correlation of NBL1 was found with age, diabetes duration, body mass index (BMI), and HbA1c. However, there were moderate correlations of NBL1 with eGFR and albumin-to-creatinine ratio (ACR). A similar pattern was found between urinary NBL1 and clinical characteristics in subjects in the T1D and T2D with late DKD cohorts (Fig. 6B).

The highest concentration of circulating NBL1 was observed in individuals in the combined Joslin T1D cohorts, with the highest concentration of albuminuria (ACR, >300 mg/g) and the greatest impairment of kidney function (eGFR, <60 ml/min/1.73 m^2). The lowest concentration was observed in individuals in the same cohort with normoalbuminuria (ACR, <30 mg/g) and normal kidney function (eGFR, >120 ml/min/1.73 m^2) (Fig. 6C). The concentration of NBL1 in subjects with normoalbuminuria (ACR, <30 mg/g) and eGFR >60 ml/min/1.73 m^2 was almost twice as high as in non-diabetic subjects [1208 ± 452 relative fluorescent units (RFUs) versus 723 ± 150, $P = 1.1 \times 10^{-4}$). Similar concentrations of circulating NBL1 were found between men and women in all cohorts, although there were systematic differences among study cohorts (fig. S6).

Circulating NBL1 identifies subjects at risk of ESKD

To examine the performance of circulating NBL1 as a predictor of ESKD, four multivariable Cox regression models were developed using combined data from the four cohorts (γ = 754; Table 3). The first model included only clinical variables selected by Cox regression analysis with backward elimination. Of the baseline variables—sex, diabetes duration, HbA1c, systolic blood pressure (SBP), diastolic blood pressure (DBP), eGFR, and ACR—only three contributed significantly to 10-year ESKD risk: eGFR, ACR, and HbA1c. The second model added TNFR1, the third model added NBL1, and the fourth model added both TNFR1 and NBL1. In each of these additional models, the effects of all variables were highly statistically significant except for baseline eGFR. In the fourth model, HRs for both TNFR1 [HR, 1.76; 95% confidence interval (CI), 1.46 to 2.13; $P = 3.9 \times 10^{-9}$] and NBL1 (HR, 1.58; 95% CI, 1.30 to 1.92; $P = 4.4 \times 10^{-6}$) were highly statistically significant, suggesting that the effect of NBL1 was independent from clinical covariates and circulating concentrations of TNFR1.

The prognostic performance of these models in all cohorts (n = 754) is summarized in Table 3. The *C*-statistic for the clinical model was 0.797, which increased to 0.825 after adding TNFR1 and to 0.836 after adding TNFR1 and NBL1. Models with TNFR1 and NBL1 were characterized by improvements in the Uno's concordance statistic (model 3 versus model 1: $P = 6.4 \times 10^{-4}$; model 4 versus model 2: $P = 1.5 \times 10^{-2}$). Adding NBL1 to model 2 resulted in significant improvement of the net reclassification index (NRI; 0.076; $P < 0.001$) (Table 3). The Akaike information criterion (AIC) values also decreased in the more comprehensive models. All metrics demonstrated the added value of circulating NBL1 in predicting 10-year risk of ESKD.

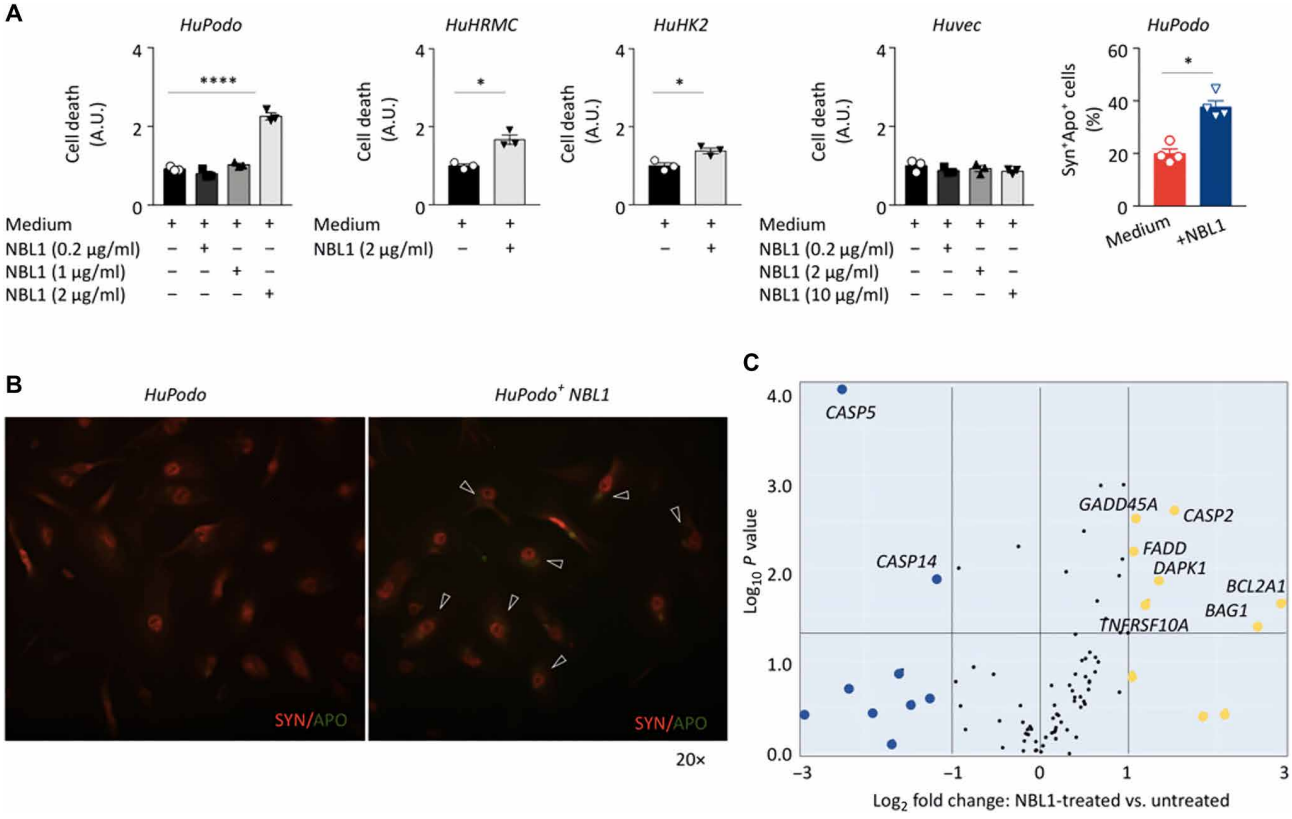

Fig. 5. Effect of NBL1 exposure on apoptosis of kidney cells. (**A**) Bar graph showing cell death analysis in human podocytes (HuPodo), human tubular (HuK2), mesangial (HuMRC) cell lines, and human umbilical vein endothelial cells (HUVECs) cultured with different doses of recombinant NBL1 and the count of Apoptag$^+$Synaptopodyn$^+$ HuPodo cells detected in the presence/absence of NBL1 by confocal analysis and presented as percentage of double-positive cells ($n = 4$). (**B**) Representative images of confocal analysis conducted on human podocytes cultured with NBL1 2 µg/ml or left untreated and stained with Apoptag and Synaptopodin. Merge pictures are presented. Magnification, ×20. (**C**) Transcriptome analysis of apoptosis-related genes in human podocytes cultured with NBL1 2 µg/ml or left untreated. Yellow dots represent up-regulated genes (fold regulation, >2); blue dots represent genes that are down-regulated (fold regulation < 2). The horizontal gray line indicates significance threshold $P < 0.05$. Three independent experiments were run in duplicates in (A). Data are presented as means ± SEM. *$P < 0.05$ and ****$P < 0.0001$ by two-sided t test or one-way ANOVA adjusted for multiple comparisons. A.U., arbitrary unit; Syn, synaptopodin; APO, apoptag.

We previously identified 17 circulating KRIS (kidney risk inflammatory signature) proteins associated with progression to ESKD in univariate analyses (*62*). Because the proteins were highly intercorrelated, only five of them had an independent effect on progression to ESKD in multivariable analysis, with TNFR1 having a dominant effect and the others [TNFRSF27, C-C motif chemokine 15 (CCL15), interleukin-17F (IL17F), and TNFSF15] contributing less. When these five KRIS proteins were added to model 4, NBL1, TNFR1, and TNFRSF27 remained statistically significant ($P = 1.3 \times 10^{-2}$, $P = 6.6 \times 10^{-6}$, and $P = 9.9 \times 10^{-3}$, respectively), with CCL15 and IL17F having only marginal significance ($P = 0.064$ and $P = 0.13$, respectively) (see model 5). The predictive power of this model increased slightly relative to model 4, but there was no statistically significant improvement of *C*-statistic or NRI.

DISCUSSION

Of the 25 circulating proteins that modulate TGF-β signaling in the extracellular space that we measured on the SOMAscan proteomic platform, circulating NBL1 (aliases DAN and DAND1) was robustly associated with high risk of progression to ESKD during the 10-year follow-up. This finding was validated in multiple cohorts comprising individuals with T1D and T2D, including Caucasians and Pima Indians. The mechanisms accounting for the observed association are unknown. However, we conjecture that circulating NBL1 may play an etiological role in progressive kidney function decline leading to ESKD in diabetes, and future work will need to discern its potential as a therapeutic target for reno-protective drugs. The concentration of circulating NBL1 can also potentially be used as a prognostic marker to identify subjects at increased risk of progression.

NBL1 is a 165–amino acid secretory protein originally identified as a tumor suppressor in neuroblastoma cell lines (*65*, *66*). Subsequently, it was demonstrated to have BMP inhibitory activity, especially for BMP-2 and BMP-7 (*67*–*71*). Since then, NBL1 has been investigated in numerous biological and in vitro assays as a BMP antagonist (*79*–*81*). NBL1 is one of the seven members of the differential screening-selected gene in neuroblastoma (DAN) family comprising a diverse group of BMP inhibitors (fig. S7). Two of them, Uterine sensitization-associated gene-1 (USAG-1) and Gremlin 1, contribute to kidney fibrosis in cellular and animal studies (*18*, *19*, *49*, *50*, *82*, *83*). However, we found no association between circulating Gremlin 1 concentration and progression to ESKD; USAG-1 was not measured on the SOMAscan platform.

Elevated concentrations of NBL1 in circulation and in urine were associated with risk of progression to ESKD. This suggests that

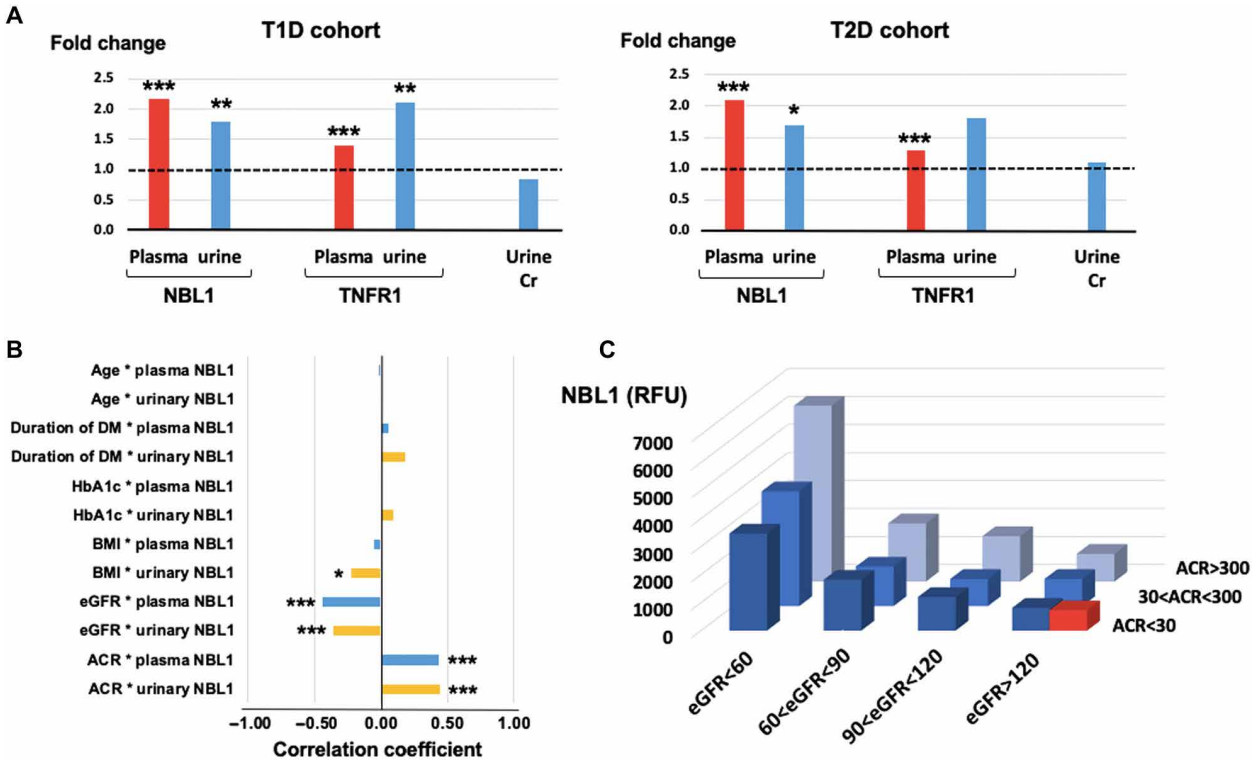

Fig. 6. Association of circulating NBL1 with clinical characteristics. (**A**) Fold changes in plasma concentrations of NBL1 and TNFR1 in cases versus controls in T1D discovery ($n = 219$) and T2D replication ($n = 144$) cohorts. Fold changes in urine concentrations of NBL1 and TNFR1 were normalized by urinary creatinine in cases versus controls in a nested case control study of T1D ($n = 60$) and T2D ($n = 52$) subjects selected from the Joslin late DKD cohorts. (**B**) Spearman rank correlation coefficient between baseline plasma NBL1 concentrations and clinical characteristics in Joslin T1D cohort with early DKD ($n = 238$), and urinary NBL1 concentrations and clinical characteristics in T1D ($n = 60$) and T2D ($n = 52$) subjects selected from the Joslin late DKD cohorts. (**C**) Mean circulating NBL1 concentrations in relative fluorescence units (RFUs) according to categories of baseline eGFR (ml/min/1.73m^2) and ACR (mg/g) in late and early combined Joslin T1D cohorts ($n = 457$) and healthy controls ($n = 79$). Red box shows mean RFU in healthy controls (see also fig. S6). There was no significant difference between concentrations of NBL1 in subjects with diabetes (ACR, <30 mg/g; eGFR, >120 ml/min/1.73 m^2) and in healthy controls (813 ± 393 versus 723 ± 150, $P > 0.05$).

overproduction, rather than impaired kidney excretion of NBL1, was responsible for the increased concentration of NBL1 in circulation and increased risk of ESKD. The organs/tissues responsible for such NBL1 overproduction are presently unknown. Our negative findings of snRNA-seq of kidney cells for NBL1 from healthy individuals and subjects with advanced DKD suggest that the kidney is an unlikely source of circulating NBL1. This inference is supported by the absence or minimal NBL1 immunostaining of kidney biopsies from healthy individuals. The NBL1 staining of tubules in kidney biopsies from subjects with DKD could be secondary to the development of DKD. We found strong NBL1 immunostaining in the intestine, which releases numerous factors into circulation, and could be a major source of NBL1 in circulation. NBL1 was also highly expressed in immune cells, such as monocytes and $CD4^+$ and $CD8^+$ T cells, suggesting another plausible nonkidney source of circulating NBL1.

Subjects with diabetes had higher concentrations of circulating NBL1 than healthy individuals. Through correlation analysis, we excluded the effect of HbA1c, age, and BMI on concentration of NBL1. However, correlations existed between the baseline concentration of NBL1 and baseline measurements of ACR and eGFR, and the results of mediation analyses and multivariable Cox regression showed that association between baseline circulating concentration of NBL1 and risk of ESKD was largely independent from the baseline ACR and eGFR.

Three possible mechanisms through which elevated concentrations of circulating NBL1 may affect progression to ESKD are outlined in fig. S8. In the first mechanism, NBL1 quenches BMPs and prevents BMP interaction with BMP receptors (BMP-Rs) thereby favoring profibrotic signaling pathways (*67*, *74*). In the second mechanism, NBL1 binds the BMP/BMP-R complex, thereby blocking its intracellular signaling and unlocking the TGF-β–mediated profibrotic action (*75*). The third mechanism postulates that circulating NBL1 damages podocytes through a yet unspecified process that is independent from the first and second mechanisms. This hypothesis is supported by our results. The extent of early structural kidney lesions in the Pima Indians was strongly correlated with concentration of circulating NBL1. The loss of podocytes and reduction in the fractional volume of podocyte cells per glomerulus were associated with elevated concentrations of circulating NBL1, but not with circulating TNFR1, another protein strongly associated with progression to ESKD. The results of in vitro experiments are also consistent with these findings as elevated concentrations of NBL1 in media had a toxic effect on podocytes leading to increased apoptosis. We did not observe an apoptotic effect of NBL1 on human umbilical vein endothelial cells (HUVECs). Because the media did not contain

Table 3. Predictive performance of the Cox regression models evaluating 10-year ESKD risk in combined four cohorts (*n* = 754). Cox regression models: model 1: HbA1c + ACR + baseline eGFR + cohort indicator; model 2: HbA1c + ACR + baseline eGFR + TNFR1 + cohort indicator; model 3: HbA1c + ACR + baseline eGFR + NBL1 + cohort indicator; model 4: HbA1c + ACR + baseline eGFR + TNFR1 + NBL1 + cohort indicator; Model 5: HbA1c + ACR + baseline eGFR + TNFR1 + NBL1 + 4 other KRIS proteins + cohort indicator. HR, hazard ratios; CI, confidence intervals; NRI, net reclassification improvement; AIC, Akaike information criterion.

	Model 1		Model 2		Model 3		Model 4		Model 5	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
eGFR/10	0.85 (0.79 to 0.92)	3.8×10^{-5}	0.95 (0.89 to 1.02)	0.15.	0.96 (0.89 to 1.03)	0.27.	1.00 (0.93 to 1.07)	0.94.	1.01 (0.95 to 1.09)	0.69
Log_2ACR	1.38 (1.29 to 1.47)	3.2×10^{-22}	1.30 (1.22 to 1.39)	4.2×10^{-16}	1.26 (1.18 to 1.35)	3.1×10^{-12}	1.26 (1.18 to 1.34)	8.2×10^{-12}	1.25 (1.17 to 1.34)	6.2×10^{-11}
HbA1c	1.23 (1.15 to 1.33)	1.4×10^{-8}	1.27 (1.18 to 1.36)	2.0×10^{-10}	1.27 (1.18 to 1.36)	1.0×10^{-10}	1.28 (1.19 to 1.37)	2.4×10^{-11}	1.27 (1.18 to 1.37)	8.4×10^{-11}
TNFR1			2.15 (1.83 to 2.52)	9.7×10^{-21}			1.76 (1.46 to 2.13)	3.9×10^{-9}	1.58 (1.29 to 1.93)	6.6×10^{-6}
NBL1					2.09 (1.76 to 2.48)	2.6×10^{-17}	1.58 (1.30 to 1.92)	4.4×10^{-6}	1.38 (1.07 to 1.78)	1.3×10^{-2}
TNFRSF27									1.25 (1.06 to 1.48)	9.9×10^{-3}
CCL15									1.17 (0.99 to 1.39)	6.4×10^{-2}
IL17F									1.15 (0.96 to 1.39)	0.13
TNFSF15									0.97 (0.80 to 1.18)	0.77
C-statistics	0.797 ± 0.012		0.825 ± 0.011		0.824 ± 0.011		0.836 ± 0.011		0.840 ± 0.013	
P* (vs model 1)	–		1.5×10^{-4}		6.4×10^{-4}		1.4×10^{-5}			
P* (vs model 2)	–		–		0.87		1.5×10^{-2}			
P* (vs model 4)									0.12	
***NRI* (vs model 1)†**	–		0.183 (0.124 to 0.242) ***		0.177 (0.121 to 0.234) ***		0.230 (0.168 to 0.292) ***			
***NRI* (vs model 2)†**	–		–		–		0.076 (0.032 to 0.120) ***			
***NRI* (vs model 4)†**									0.0016 (−0.052 to 0.055)	
−2 Log likelihood (2LL)	2567		2483		2495		2462		2447	
AI	2579		2497		2509		2478		2471	

*Uno's concordance statistics with two-sided *P* value. †Risk categories for ESKD during 10 years are defined by <5.0%, 5.0 to 9.9%, 10.0 to 19.9%, and 20% and more. ****P* for NRI <0.001.

any BMPs, the action of NBL1 on the podocytes could not be mediated through inhibition of BMPs and activation of TGF-β/Smad signaling.

Loss of podocytes is a mechanism of disease initiation and progression in many kidney diseases (*84*). In diabetes, podocytes are injured early in DKD (*85*–*87*), and this podocyte injury may precede the changes seen in the GBM and mesangial area by several years (*88*). Animal models show that enhancing podocyte injury during early disease promotes the development of mesangial expansion, interstitial fibrosis, and increased GBM thickness (*89*). All these lesions were strongly correlated with circulating concentrations of NBL1. Together, our findings suggest that circulating NBL1 causes early kidney structural lesions through apoptosis of kidney cells, especially podocytes, which in turn may contribute to progressive kidney function decline and progression to ESKD.

Our findings regarding circulating NBL1 suggest that this protein may be an accessible target for pharmaceutical modification of TGF-β signaling to slow DKD progression to ESKD. Inhibition of NBL1 is predicted to free BMP proteins to enable their interaction with and inhibition of TGF-β signaling, therefore diminishing

inflammation and fibrosis in kidney tissue and podocyte loss in DKD (*67*, *74*, *75*). A previous attempt to inhibit TGF-β1 directly with a therapeutic monoclonal antibody failed to slow eGFR loss, reduce albuminuria, or modulate DKD biomarkers of inflammation and disease, presumably because the antibody lacked access to TGF-β in kidney cells (*29*). NBL1 may be a more appropriate target in this pathway than TGF-β directly, because it is an upstream regulator of TGF-β activity and could be more accessible to therapeutic intervention. NBL1 knockdown mice are viable and have normal phenotypes, suggesting that inhibition of NBL1 might not lead to substantial off-target safety issues (*69*, *90*).

Independent of its role in the etiology of DKD, the concentration of NBL1 in circulation can be used as a prognostic marker for identifying individuals at risk of progression to ESKD. In our study, the prognostic performance of NBL1 in circulation was identical to the prognostic performance of circulating TNFR1. Measuring both proteins and combining the results with clinical markers such as HbA1c, eGFR, and ACR significantly increased the ability of a multimarker prognostic model to identify individuals at risk of progression to ESKD. However, for such a multimarker prognostic test to be useful in clinical practice, additional studies of large cohorts will be required to estimate the probabilities of ESKD during specific intervals of follow-up (for example, 5, 10, or 15 years).

Strengths of our study include prospective long-term follow-up in multiple cohorts, of different ethnicities, different types of diabetes, and different stages of DKD; use of gold standard definitions of DKD, that is, structural lesions assessed by quantitative morphometry in research kidney biopsies and 10-year risk of ESKD; unbiased and standardized measurements of key TGF-β–related signaling proteins; and robust results consistent in all cohorts. There are also some limitations. As described in table S2, in this study, we examined only 25 circulating proteins out of 38 considered as candidate proteins that regulate TGF-β signaling. The role of the 13 not examined proteins is unknown. Furthermore, the study is observational, and the causal relationship of NBL1 with kidney disease progression will need to be established through animal studies and clinical trials. In addition, because the study was conducted only for DKD in Caucasians and Pima Indians, we cannot assess the generalizability of these findings to Black subjects with diabetes or to individuals with other kidney diseases.

MATERIALS AND METHODS

Study design

Subjects were selected from participants of the Joslin Kidney Study and the Pima Indian Kidney Study. Subjects were followed for 7 to 12 years (25 and 75% in those who did not develop ESKD) to examine changes in kidney function and onset of ESKD. To identify circulating TGF-β signaling proteins associated with the development of ESKD, we selected four different cohorts: a discovery cohort (Joslin cohort with T1D CKD stage 3), a replication cohort (Joslin cohort with T2D CKD stage 3), and two validation cohorts [validation cohort 1 (Joslin cohort with T1D CKD stages 1 and 2) and validation cohort 2 (Pima cohort with T2D with CKD stages 1 and 2)]. The Joslin cohort with T2D CKD stage 3 was used to replicate the findings obtained in subjects with T1D CKD stage 3. The Joslin cohort with T1D CKD stages 1 and 2 and the Pima Indian cohort were used to further validate the findings in subjects with early-stage DKD and in those of different ethnicities. Clinical data, research measurements, and blood and urine specimens obtained at entry into the Joslin Kidney Study and Pima Indian Kidney Study and during follow-up examinations were used in the current research.

Joslin Kidney Study

The Joslin Diabetes Center Committee on Human Studies approved the Joslin Kidney Study, a longitudinal observational study that investigates the determinants and natural history of kidney function decline in T1D and T2D. About 2000 subjects with T1D and 1500 subjects with T2D were recruited into the study from among 20,000 subjects attending the Joslin Clinic between 1991 and 2009 (*62*, *91*). All the Joslin Kidney Study participants were queried every 2 years against the U.S. Renal Data System (USRDS) and the U.S. National Death Index (NDI) rosters to ascertain individuals who developed ESKD or died. The last inquiries were in 2017.

Discovery and replication cohorts with late DKD

We selected the T1D discovery (n = 219) and the T2D replication (n = 144) cohorts from participants enrolled in the Joslin Kidney Study between 1991 and 2006 with albuminuria and impaired kidney function (eGFR, 20 to 60 ml/min/1.73 m^2) at baseline. These subjects were monitored for 10 (median) years for changes in kidney function and ESKD onset (*62*, *91*). The late DKD T1D cohort was selected as the discovery cohort because it had the greatest statistical power to detect circulating proteins associated with risk of ESKD due to the large number of cases.

Validation cohort with early DKD

The T1D validation cohort (n = 238) included Joslin Kidney Study participants with albuminuria and eGFR of 60 to 170 ml/min/1.73 m^2 (median eGFR, 97) at enrollment (between 1991 and 2009). They were also followed for 7 to 15 years to monitor kidney function changes and for ascertainment of ESKD onset (*62*, *91*).

Healthy controls

The Joslin Kidney Study also examined parents of subjects with T1D. The group of nondiabetic parents of T1D subjects was derived from our genetic study on determinants of DKD in T1D. Parents had baseline examinations performed according to the same protocols as participants of the Joslin Kidney Study. Biospecimens obtained at examinations were stored at −85°C. For this study, 79 white nondiabetic parents aged 50 to 69 years at baseline examination were selected to be used as nondiabetic controls. Plasma specimens obtained at baseline examination underwent SOMAscan analysis.

Pima Indian Kidney Study

Pima Indians from the Gila River Indian Community in Arizona have a high prevalence of T2D and a high incidence of ESKD due to DKD (*92*). This population participated in a longitudinal study of diabetes and its complications for more than 40 years. Beginning in the late 1980s, informative subsets of subjects from this population were selected for more detailed longitudinal studies of DKD (*93*, *94*). A subset of 153 subjects from this kidney study cohort with baseline examinations between 1994 and 2007 was selected for our previous study on the role of circulating inflammatory proteins in ESKD development (*62*). For the current T2D validation cohort, we selected only individuals with a baseline GFR of 60 to 240 ml/min (median GFR, 150 ml/min). ESKD onset was ascertained in all participants through December 31, 2017. For the present study, we right censored at the 10-year of follow up. Within this time frame, 34 subjects developed ESKD. The study was approved by the Institutional Review Board of the National Institute of Diabetes and Digestive and Kidney Disease.

Two-stage study design to measure circulating proteins by SOMAscan platform

Because of the high cost of SOMAscan assays, we performed measurements in two stages. In the first stage, all 1129 plasma proteins available on the SOMAscan platform were measured in 113 (56 ESKD cases) subjects selected randomly from the 219 included in the T1D discovery (late DKD) Joslin cohort. At this stage, we identified 232 (20%) proteins "potentially" associated with risk of ESKD using univariate logistic regression analysis and liberal statistical threshold ($P < 0.01$). In the second stage, the 232 "potentially" associated proteins together with 332 (30%) proteins not associated with ESKD selected randomly from among all SOMAscan proteins were measured in individuals from the second half of the discovery cohort and in the three other cohorts. In total, 564 proteins were measured.

Statistical analyses

Clinical characteristics and summary of outcomes were expressed as counts and percentages (proportions) for categorical variables, means (SDs) for normally distributed continuous variables, and as medians (25 and 75%) for variables with skewed distributions. Effects of baseline proteins on the risk of ESKD were estimated using a Cox proportional hazards regression model and were expressed as HR and their CIs. We applied a Bonferroni correction for $n = 25$ independent tests (the number of examined proteins by the study design), which yielded a threshold of $P < 2.0 \times 10^{-3}$. This threshold for significance was applied to the results obtained in the discovery cohort. Nominal significance $P < 0.05$ was used to evaluate the findings obtained in the other study cohorts. Multivariable Cox regression analyses were performed to further examine the association of candidate proteins with the risk of ESKD. In these analyses, the effects of protein concentrations on risk of ESKD were expressed in terms of HRs per 1 SD increase for easier interpretability and uniform scaling. Models were adjusted for sex, duration of diabetes, HbA1c, SBP, DBP, and baseline eGFR (measured GFR in the Pima Indians) and stratified by cohort. ACR was not considered as a covariate because it was an outcome measure and a part of the disease presentation.

We selected the best predictors in Cox regression models, applying backward variable elimination at the 5% significance threshold in a combined analysis of the late and early DKD cohorts. Four candidate proteins, sex, duration of diabetes, HbA1c, SBP, DBP, and baseline eGFR were included in these models. We used the Kaplan-Meier method to estimate 10-year cumulative risk of ESKD and to plot survivor function estimates according to quartiles of baseline NBL1 concentrations.

To evaluate the effect of baseline concentration of circulating NBL1 on risk of progression to ESKD controlling for ACR as an intermediate outcome variable, we performed a mediation analysis. In our model of kidney disease progression, NBL1 was considered an upstream exposure whose effect was mediated by elevated ACR. In the analyses, we used Cox regression model for 10-year risk of ESKD adjusted for sex, duration of diabetes, HbA1c, SBP, DBP, and baseline eGFR with stratification of type of diabetes. Effect measures were expressed as the HR per SD increase in NBL1 concentration. The effect of NBL1 on ESKD (total effect) is split into an indirect effect (through ACR) and direct effect, which is independent from the ACR. SAS MEDIATE Macro was used for the analysis (*95*).

Correlations between candidate protein concentrations, and clinical factors, kidney structural lesions, and transcription in kidney were evaluated by Spearman rank correlation coefficients.

In the in vitro study, continuous variables are presented as means with standard errors. After testing for normal distribution (Shapiro-Wilk test), we used independent sample *t* tests or the Mann-Whitney test as appropriate to compare continuous variables. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Sidak post hoc test between the group of interest and all other groups were used. Two-tailed *P* values <0.05 were considered statistically significant.

Performance of prognostic models was compared with measures of calibration (AIC) and discrimination (NRI) in the dataset comprising the four cohorts. Considerations were inclusive for significant clinical covariates (sex, HbA1c, GFR or eGFR, and $\log_2$-transformed ACR), cohort indicator, five *KRIS* proteins, and NBL1. Fit of consecutive nested models was tested with likelihood ratio tests and by AIC values (the lower the better). Uno's *C*-statistic was calculated as proportions of pairs for subjects whose observed and predicted outcomes were concordant. In addition, the NRI methodology was applied for categorical risk estimates. Risk categories for ESKD during 10 years were defined by <5.0%, 5.0 to 9.9%, 10.0 to 19.9%, and 20% and more (*64*, *96*). Statistical analyses were performed with SAS version 9.4 (SAS Institute), R statistical software versions 3.2.4 and 4.0.2.

SUPPLEMENTARY MATERIALS

www.science.org/doi/10.1126/scitranslmed.abj2109
Materials and Methods
Figs. S1 to S9
Tables S1 to S4
MDAR Reproducibility Checklist
References (*97–106*)

View/request a protocol for this paper from *Bio-protocol*.

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Neuroblastoma suppressor of tumorigenicity 1 is a circulating protein associated with progression to end-stage kidney disease in diabetes

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Predicting kidney disease progression

Patients with diabetes are at risk of kidney complications. Kobayashi *et al.* surveyed 25 circulating proteins in patient cohorts of both type 1 and type 2 diabetes and report that circulating neuroblastoma suppressor of tumorigenicity 1 (NBL1) protein is associated with 10-year risk for progression to end-stage kidney disease across multiple cohorts. This association was backed up by analysis of biopsied renal tissue. NBL1 may thus provide a noninvasive risk predictor for advanced diabetic kidney disease.

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