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Disruption of branched-chain amino acid homeostasis promotes the progression of DKD via enhancing inflammation and fibrosis-associated epithelial-mesenchymal transition

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ABSTRACT

Background and aims: The disrupted homeostasis of branched-chain amino acids (BCAAs, including leucine, isoleucine, and valine) has been strongly correlated with diabetes with a potential causal role. However, the relationship between BCAAs and diabetic kidney disease (DKD) remains to be established. Here, we show that the elevated BCAAs from BCAAs homeostatic disruption promote DKD progression unexpectedly as an independent risk factor.

Methods and results: Similar to other tissues, the suppressed BCAAs catabolic gene expression and elevated BCAAs abundance were detected in the kidneys of type 2 diabetic mice and individuals with DKD. Genetic and nutritional studies demonstrated that the elevated BCAAs from systemic disruption of BCAAs homeostasis promoted the progression of DKD. Of note, the elevated BCAAs promoted DKD progression without exacerbating diabetes in the animal models of type 2 DKD. Mechanistic studies demonstrated that the elevated BCAAs promoted fibrosis-associated epithelial-mesenchymal transition (EMT) by enhancing the activation of proinflammatory macrophages through mTOR signaling. Furthermore, pharmacological enhancement of systemic BCAAs catabolism using small molecule inhibitor attenuated type 2 DKD. Finally, the elevated BCAAs also promoted DKD progression in type 1 diabetic mice without exacerbating diabetes.

Conclusion: BCAA homeostatic disruption serves as an independent risk factor for DKD and restoring BCAA homeostasis pharmacologically or dietarily represents a promising therapeutic strategy to ameliorate the progression of DKD.

1. Introduction

Diabetic kidney disease (DKD) is the most common microvascular complication in individuals with diabetes and the leading cause of end-stage renal disease worldwide [1,2]. Despite high clinical demand and significant efforts, treatment options for DKD remain limited [3]. While hyperglycemia is a major player, it does not account fully for the development of DKD, suggesting other risk factors may be required as a "second strike" to initiate and/or accelerate the progression of DKD [4,5].

BCAAs are a group of essential amino acids with similar side chain structures [6]. In recent years, BCAAs have been tightly linked with diabetes [7–9]. The levels of BCAAs are elevated in the blood of people with type 1 diabetes mellitus (T1DM) or type 2 diabetes mellitus (T2DM) [10,11]. BCAAs are now recognized as one strong biomarker of a range of metabolic diseases and cardiovascular diseases [12–15]. It has been suggested that disrupted BCAA homeostasis plays a critical role in the development of diabetes. Insulin resistance can occur via dysfunctional BCAA catabolism or BCAA levels acting as signaling molecules hampering the insulin signaling pathways [16,17]. Accordingly,

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disrupted BCAA homeostasis may affect the development of diabetic complications like kidney disease and retinopathy, which remains to be investigated.

In addition to building proteins, BCAAs also act as key nutrition signals or metabolic regulators for glucose homeostasis, immune response, and intestinal development [18,19]. Hence, BCAA homeostasis is tightly controlled. Alterations in systemic BCAAs are an outcome of their effective catabolism and dietary intake [20]. As the rate-limiting enzyme of BCAA catabolism, the activity of branched-chain α-keto acid dehydrogenase (BCKD) is tightly regulated by the reversible phosphorylation of the E1α subunit. BCKD kinase (BCKDK) phosphorylates the BCKD E1α subunit and inhibits BCKD, whereas protein phosphatase 2Cm (PP2Cm) dephosphorylates the BCKD E1α subunit and activates BCKD [20]. BCAA catabolism is active in the kidney [20]. The oxidative flux of BCAAs is higher in the kidney than in the other tissues, except for the heart and brown fat [21,22]. A study by Piret et al. suggested that BCAA catabolic gene expression is reduced in diverse acute kidney injuries in mice and humans. Past studies have shown an altered BCAA profile in AKI and chronic kidney disease (CKD), suggesting a correlation between BCAA metabolic homeostasis and kidney disease [23–25].

In recent years, several clinical studies have validated the effectiveness of low protein diet (LPD) on chronic kidney disease [26–28]. DKD is one of the most common etiologies of CKD. Studies found a strong association between low-protein intake and the decline in GFR and proteinuria in the individuals with DKD [29–33]. The beneficial effects of LPD might be attributed to the reduced consumption of specific essential amino acids. In a prospective study, Zhu et al. suggest that high levels of valine and isoleucine may be one of the risk factors for the development of DKD [34]. The potential causal relationship between BCAAs and DKD remains to be investigated.

In the current study, we found that the elevated BCAAs promoted renal inflammation and fibrosis and thus the progression of DKD without exacerbating diabetes. Importantly, restoring BCAA homeostasis via a pharmacological inhibitor attenuated DKD progression. These findings suggest that the systemic disruption of BCAA homeostasis is an independent risk factor for DKD and BCAA catabolic pathway can serve as a potential therapeutic target for the disease.

2. Methods

2.1. Animals

Pp2cm germ-line knockout mice (*Pp2cm*$^{-/-}$) and control mice (*Pp2cm*$^{+/+}$) were generated and maintained as previously described [35]. All mice were on a C57BL6/J genetic background. Type 1 diabetes in mice was induced by intraperitoneal injection of streptozotocin (STZ, 120 mg/kg). Type 2 diabetes was induced by a combination of STZ (35 mg/kg) and high-fat diet (HFD) [36]. Diabetes was successfully induced when blood glucose levels reached 16.7 mM for 3 consecutive days. *db/db* mice with the nephropathy-susceptible BKS background (*BKS-db/db mice*) were used as a model of type 2 diabetic kidney disease [37–39], with db/m mice as the control group. *db/db* mice were used for the investigation of 3,6-dichlorobenzo[b]thiophene-2-carboxylic acid (BT2), dietary intervention and other studies. Mice were purchased from GemPharmatech Co Ltd, Jiangsu, China. Male mice were used in the studies. Animals were housed and bred in SPF facility. The experiments started after 2 weeks of animal acclimatization to the facilities environment. Mice were matched for age, and body weight. During experiments, one researcher was aware of treatment allocation but others were blinded.

BT2 was dissolved in DMSO and diluted in 5 % DMSO, 10 % Cremophor EL and 85 % 0.1 mol/L sodium bicarbonate (pH 9.0) for delivery. From 10 weeks of age, mice were treated with BT2 (40 mg/kg/day) or an equivalent volume of vehicle daily at 5:00 pm by intragastric administration for 10 weeks. An isocaloric normal protein diet (NPD, 20 % protein; M19091702) and a low-protein diet (LPD) (6 % protein; M19091701) were purchased from BIOPIKE, Beijing, China (Table. S3) [40]. 10-week-old *db/db* mice were fed the NPD or LPD for 10 weeks. The LPD + BCAA group was gavaged daily with BCAAs (1.5 mg/g bw/day, Leu:Ile:Val = 1.5:0.8:1) additionally after 4 weeks of LPD administration for 6 weeks [41].

Animals were randomly used for experiments. All behavior experiments were performed in a blinded and randomized fashion. No animals or data points were excluded. Quantifications were performed in a blinded fashion. Measurements were taken from distinct samples for each experiment. For each test, the experimental unit was an individual animal. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field. Data distribution was assumed to be normal, but this was not formally tested. Tissue samples from kidneys were rapidly harvested under anesthesia and frozen in liquid nitrogen and stored at −80 °C until processed. All animal experiments were approved by the Ethics Committee of Tianjin Medical University Chu Hsien-I Memorial Hospital (Tianjin, China) (DXBYY-IACUC-2023001).

2.2. Participants

Participants admitted to the Tianjin Medical University Chu Hsien-I Memorial Hospital were enrolled into this study. The following groups were defined according to the albuminuria category classified by the Kidney Disease: Improving Global Outcomes (KDIGO) Diabetes Work Group [42]: healthy participants with negative urine routine results and no abnormal renal biochemistry; Early DKD individuals with microalbuminuria ($30 \leq$ UACR < 300 mg/g, and eGFR ≥ 60 ml/min per 1.73 m^2). Participants with renal impairment caused by other diseases such as polycystic kidney disease were excluded in study. Participants with other endocrine diseases (except diabetes mellitus) were also excluded. A total of 25 healthy participants and 25 early DKD individuals were selected in terms of matched gender, age. The clinical characteristics of all participants are shown in Table S1. The experiments were approved by the Ethics Committee of Tianjin Medical University Chu Hsien-I Memorial Hospital (Tianjin, China) (DXBYYkMEC2021–14).

2.3. Statistics analysis

Data were analyzed using GraphPad Prism 8.0.1 software. Student's *t*-test was used to compare two groups. One-way ANOWA with Tukey analysis was used for comparisons across multiple groups. All data are expressed as the mean ± SD. p values <0.05 were considered statistically significant.

2.4. Supplementary methods

Detailed materials and methods are described in the Supplementary Materials and Methods sections.

3. Results

3.1. BCAA homeostatic disruption was a metabolic signature of type 2 DKD

The *db/db* mouse is an established model to study kidney injury in T2DM [38,43]. Compared to the *db/m* mice, the *db/db* mice showed glomerular basement membrane thickening, matrix accumulation, inflammatory cell infiltration, tubular damage, and interstitial fibrosis in the kidney, together with impaired kidney function. (Fig. S1A-H). We performed RNA-sequencing analysis of kidney tissues from *db/db* mice to identify overrepresented biological pathways. Interestingly, the pathway of valine, leucine and isoleucine degradation was significantly enriched (Fig. 1A, S1I). Examination of specific gene expression revealed a systemic downregulation of the BCAA catabolic pathway in the kidneys of *db/db* mice (Fig. 1B). We verified the changes in key

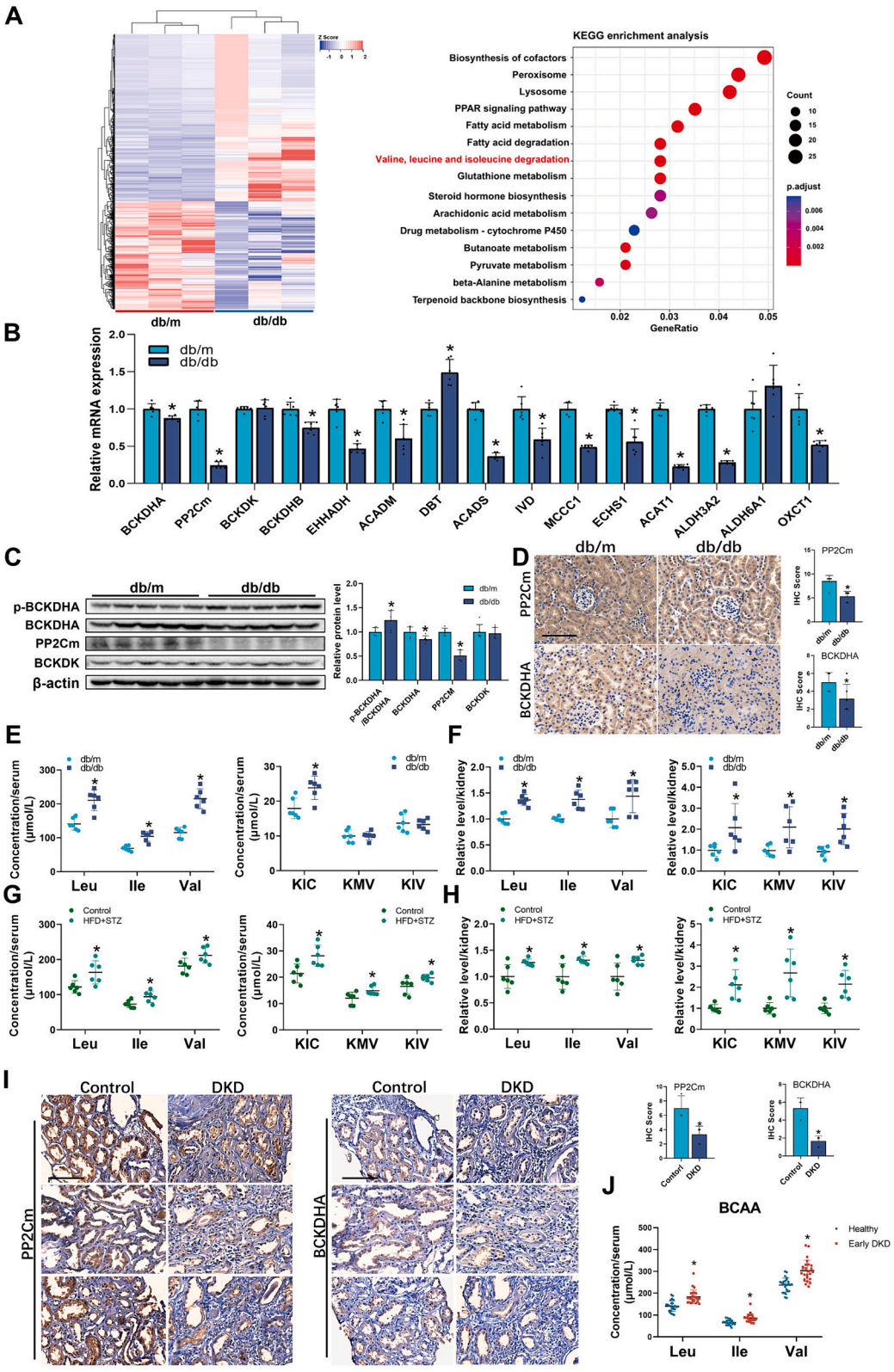

Fig. 1. BCAA homeostasis is disrupted in diabetic kidney disease.

(A-F) Results of *db/db* mice. (A) Heatmap of the differentially expressed genes and KEGG pathway analysis for the downregulated genes. (B) Quantitative PCR results of BCAA catabolic genes in the kidney, normalized to β-actin. (C) Representative blots and a quantitation graph of p-BCKDHA (Ser293), BCKDHA, PP2Cm and BCKDK protein expression in the kidney. (D) Representative micrographs showing kidney PP2Cm and BCKDHA IHC staining (scale bar = 100 μm), with IHC scores shown on the right. (*E*-F) Serum concentrations (E) and relative renal abundances (F) of BCAAs (Leu, Ile, and Val) and BCKAs (KIC, KMV, and KIV) in *db/db* mice. (G-H) Serum concentrations (G) and relative renal abundances (H) of BCAAs (Leu, Ile, and Val) and BCKAs (KIC, KMV, and KIV) in HFD + STZ mice. Male mice were deprived of food for 6 h (8:00 am to 2:00 pm) before being sacrificed. (I) Representative micrographs showing kidney PP2Cm and BCKDHA IHC staining (scale bar = 100 μm) in human, with IHC scores shown on the right. ($n = 3$) (J) Serum concentrations of BCAAs (Leu, Ile, and Val) in human ($n = 25$). All the data are presented as the means ± SD; (A-H) $n = 6$ per group. *$P < 0.05$. KIC, α-ketoisocaproic acid; KIV, α-ketoisovaleric acid; KMV, α-keto-b-methylvaleric acid.

BCAA catabolic genes at the protein level, revealing reduced expression of BCKDHA and PP2Cm but not BCKDK. Moreover, elevated BCKDHA phosphorylation was observed, indicating reduced BCKDH activity (Fig. 1C-D). LC–MS analysis confirmed the accumulation of BCAAs and BCKAs in the serum and kidneys of *db/db* mice (Fig. 1E-F). We also detected elevated BCAAs and BCKAs levels in the serum and kidney of mice with type 2 diabetes induced by a combination of HFD feeding and low-dose STZ injection (Fig. 1G-H). Thus, similar to other tissues (skeletal muscle, adipose tissue, and liver) [21,44], the BCAAs catabolic gene expression was suppressed and BCAAs abundance was elevated in the kidneys of type 2 diabetic mice, which could contribute to the systemic BCAA metabolic perturbation.

We next explored the BCAA dysregulation in human with type 2 DKD (T2DKD). The expression of PP2Cm and BCKDHA in human kidney was decreased in DKD group compared to that of control group (Fig. 1I). Moreover, we collected serum from healthy participants and individuals with early DKD (Table. S1). Serum levels of BCAAs were significantly increased in early DKD group compared to those in healthy group (Fig. 1J). Collectively, these data demonstrated that the BCAAs homeostatic disruption was a metabolic signature of type 2 DKD.

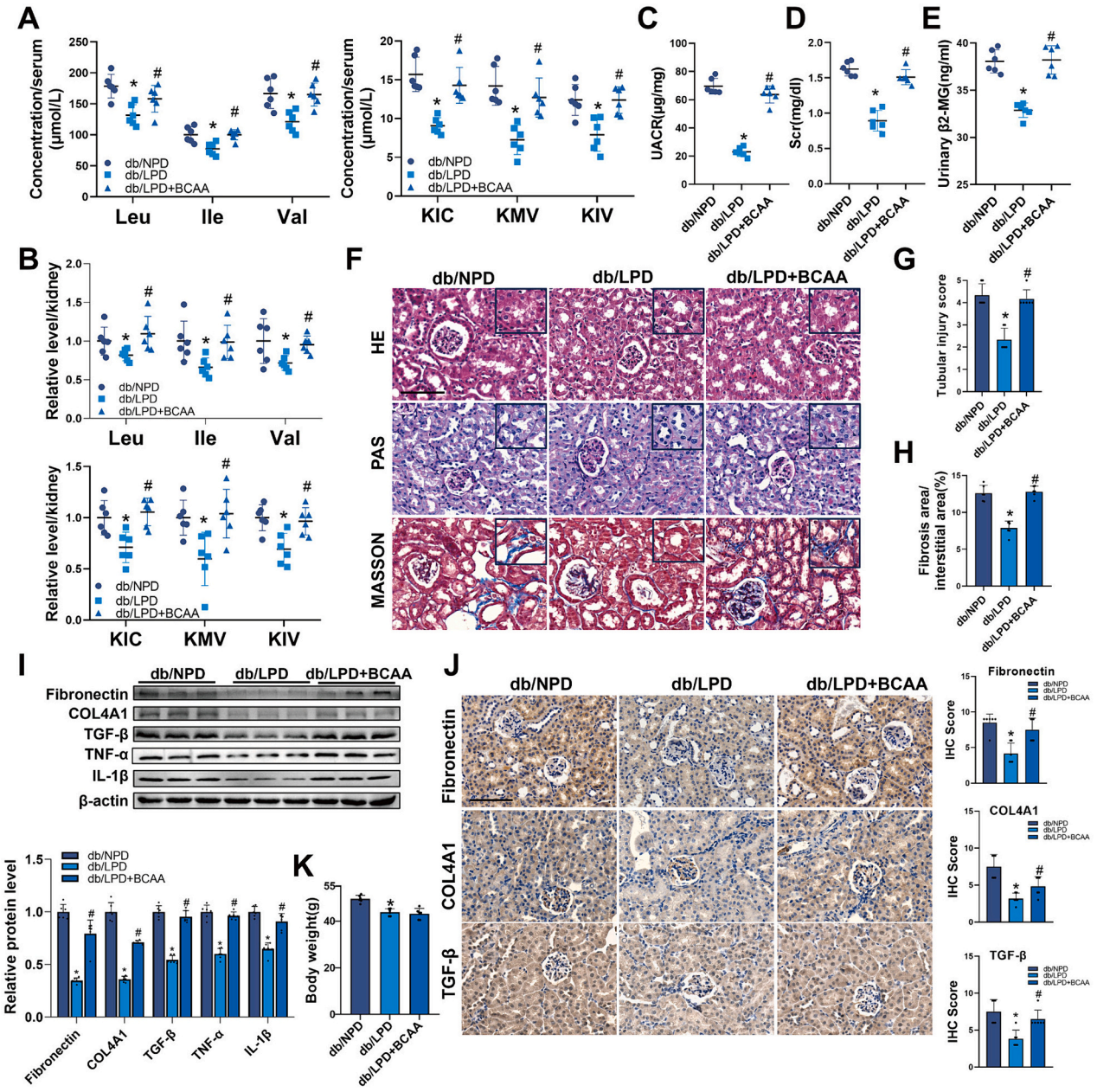

Fig. 2. Altering BCAA intake affects the development of DKD in *db/db* mice.
(A-B) Serum concentrations (A) and relative renal abundances (B) of BCAAs (Leu, Ile, and Val) and BCKAs (KIC, KMV, and KIV). (C) Urinary albumin-to-creatinine ratio (UACR). (D) Serum creatinine (Scr). (E) Urinary β2-Microglobulin (β2-MG). (F) Representative images of kidney sections stained with HE, PAS, and Masson's trichrome. Original magnification = 400. Scale bar = 100 μm. A magnification is shown in the box. (G) Quantitative analysis of tubular damage, on the basis of HE staining. (H) Quantitative analysis of the fibrosis area, on the basis of Masson's trichrome staining. (I) Representative blots and a quantitation graph of Fibronectin, COL4A1, TGF-β, TNF-α, and IL-1β protein expression in the kidney. (J) Representative micrographs showing kidney Fibronectin, COL4A1, and TGF-β IHC staining (scale bar = 100 μm), with IHC scores shown on the right. (K) Body weight. Male mice were deprived of food for 6 h (8:00 am to 2:00 pm) before being sacrificed. All the data are presented as the means ± SD; n = 6 per group. *P < 0.05, db/NPD vs. db/LPD; #P < 0.05, db/LPD vs. db/LPD + BCAA; KIC, α-ketoisocaproic acid; KIV, α-ketoisovaleric acid; KMV, α-keto-b-methylvaleric acid.

3.2. Elevated BCAAs were a pathogenic factor for T2DKD

We next assessed the impacts of elevated BCAAs on T2DKD. An isocaloric low protein diet (LPD, 6 % protein by weight) was used to reduce BCAA intake in *db/db* mice. The LPD reduced serum and renal abundances of BCAAs and BCKAs compared to the normal protein diet (NPD, 20 % protein by weight) (Fig. 2A-B). Importantly, the LPD significantly improved renal function (Fig. 2C-E). Furthermore, BCAA gavage increased serum and renal BCAA and BCKA abundances and significantly exacerbated renal function in *db/db* mice fed the LPD (Fig. 2A-E). Histopathological examination showed attenuation of kidney damage in LPD mice, while BCAA supplementation showed the opposite effect (Fig. 2F-H). Moreover, the levels of fibrosis-associated factors (Fibronectin, COL4A1, and TGF-β), and inflammatory factors (TNF-α and IL-1β) were markedly attenuated by the LPD but exacerbated by BCAA supplementation (Fig. 2I-J). Of note, while the LPD reduced serum levels of numerous amino acids, BCAA supplementation only reversed the reduction in BCAAs. LPD feeding caused a slight weight loss in *db/db* mice, while supplementation with BCAAs did not affect body weight (Fig. 2K). No significant renal changes in healthy mice supplemented with BCAAs were observed (Fig. S2A—D). Collectively, these results suggested that elevated BCAAs were a pathogenic factor for DKD in *db/db* mice.

3.3. Defective BCAA catabolism promoted T2DKD without exacerbating diabetes

We further investigated the impacts of impaired BCAA catabolism on DKD by utilizing *Pp2cm* knockout ($Pp2cm^{-/-}$) mice in which BCKD activity was inhibited due to elevated BCKDHA phosphorylation (Fig. 3A). $Pp2cm^{-/-}$ mice showed impaired BCAA catabolism and elevated BCAA and BCKA levels in serum and kidney compared to controls (Fig. 3B-C). Under basal conditions, no significant change in renal function or morphology was observed in $Pp2cm^{-/-}$ mice (Fig. S3A—B). However, after T2DM was induced by HFD and STZ, $Pp2cm^{-/-}$ mice showed hypertrophic kidneys (Fig. 3D). The elevated UACR and serum creatinine (Scr) levels in diabetic $Pp2cm^{-/-}$ mice indicated impaired glomerular function, while the elevated β2-MG levels indicated worse tubular function (Fig. 3E-G). Urinary glucose level was not affected (Fig. 2H). In addition, we observed increased glomerular size, glomerular basement membrane thickening, matrix accumulation, tubulointerstitial injury, tubular atrophy and inflammatory cell infiltration in the kidneys of diabetic $Pp2cm^{-/-}$ mice (Fig. 3I-J). Interstitial fibrosis is a major cause of decreased renal function, which was significantly increased in diabetic $Pp2cm^{-/-}$ mice (Fig. 3I, K), accompanied by elevated expression of fibrosis-related factors (Fibronectin, COL4A1, and TGF-β) (Fig. 2L-M).

Under basal conditions, $Pp2cm^{-/-}$ mice showed slightly lower body weight and enhanced glucose tolerance (Fig. S3C—D), consistent with previous observations [45]. After T2DM was induced, the body weight remained lower in $Pp2cm^{-/-}$ mice compared to that of control mice (Fig. 3N). Of note, glucose tolerance was comparable in diabetic $Pp2cm^{-/-}$ and control mice, whereas insulin sensitivity was protected in diabetic $Pp2cm^{-/-}$ mice (Fig. 3O-P). Together, these data showed that BCAA catabolic defect promoted DKD without exacerbating diabetes.

3.4. BCAA catabolic defects promoted EMT and inflammation in diabetic kidneys

Interstitial fibrosis is an important component of DKD progression, of which EMT is a major driver [46,47]. We therefore estimated the levels of EMT markers in the kidneys of diabetic $Pp2cm^{-/-}$ mice. The expression of ZO-1, E-cadherin (epithelial marker) was reduced, and vimentin, α-SMA (mesenchymal marker) expression was increased in the kidneys of $Pp2cm^{-/-}$ mice (Fig. 4A-B). Given that the inflammatory response is the major contributor to the development of renal interstitial fibrosis and macrophages are the main cellular players in chronic inflammation, we assessed the extent of inflammatory cell infiltration in the kidneys of diabetic $Pp2cm^{-/-}$ mice. F4/80-positive macrophage infiltration and the expression of proinflammatory factors, including IL-6, TNF-α, and iNOS were significantly increased in the kidneys of $Pp2cm^{-/-}$ mice (Fig. 4C-E). These results suggested that defective BCAA catabolism promoted renal inflammation and EMT in the diabetic kidney.

3.5. BCAA boosted the activation of proinflammatory macrophages via mTOR

We next explored the impacts of different concentrations of BCAAs on inflammatory response of macrophage. The concentration of BCAAs in the serum of fasted healthy people and mice is ~50–200 μM (Fig. 1E, G,J), which can increase with ~2 folds after meal [40,42,48]. Under diabetic condition, the BCAA concentrations can increase with ~1.5 folds (Fig. 1E,G,J). Macrophages were exposed to LPS, with or without different concentrations of BCAAs in culture medium for 24 h. We found that BCAAs enhanced LPS-induced expression of inflammatory factors in macrophages in a dose-dependent manner (Fig. 5A). 3,6-Dichlorobenzo[*b*]thiophene-2-carboxylic acid (BT2) is a potent inhibitor of BCKDK that enhances BCAA catabolism by phosphorylating BCKDHA (Fig. 5B). BT2 inhibited LPS + BCAA-induced inflammation (Fig. 5C). These results suggested that high concentrations of BCAAs boosted the activation of proinflammatory macrophages.

The mammalian target of rapamycin (mTOR) pathway plays an important role in inflammatory responses [49–51]. BCAAs, especially leucine, is a potent activator of the mTORC1 pathway [52,53]. We then investigated whether BCAAs boosted macrophage activation through mTOR signaling pathway. As expected, BCAAs enhanced the activity of mTORC1 in macrophages (Fig. 5D). Importantly, the mTOR inhibitor rapamycin abolished the production of proinflammatory factors stimulated by BCAAs (Fig. 5E). These data suggested that BCAAs enhanced the proinflammatory responses in macrophages in an mTOR-dependent manner.

3.6. BCAA-boosted macrophages promoted EMT in renal tubular epithelial cells

Given the tight relationship between inflammation and EMT [54], it was of interest to determine whether BCAA-stimulated macrophages promoted EMT in renal tubular epithelial cells. After macrophages were stimulated with or without LPS (100 ng/mL) in presence or absence of BCAAs for 24 h, fresh DMEM medium was applied for all groups for 12 h and collected as conditioned media (CM), designated as Con-CM, BCAA-CM, Con+LPS-CM, and BCAA+LPS-CM. The conditioned media were then applied to HK2 cells for 24 h. The morphology of HK2 cells cultured in Con+LPS CM changed from epithelial-like to mesenchymal-like appearance (Fig. 5F). Moreover, decreased expression of epithelial markers (ZO-1, E-cadherin) and increased expression of mesenchymal markers (Vimentin, α-SMA) and fibrosis-related factors were detected in HK2 cells cultured with Con+LPS CM, compared to those with Con-CM (Fig. 5G-I). These results suggested that the conditioned medium from proinflammatory macrophages induced EMT of HK2 cells. More importantly, compared to HK2 cells in Con+LPS CM, the changes of morphology and marker gene expression were significantly enhanced in HK2 cells cultured in BCAA+LPS CM (Fig. 5F-I). Together, these results demonstrated that BCAA-boosted proinflammatory macrophages promoted EMT of HK2 cells.

To understand the molecular properties of the EMT-stimulating factor(s) secreted by macrophages, we heat-inactivated the conditioned medium to denature the protein components. The EMT stimulatory effect from BCAA-boosted proinflammatory macrophages was ablated after heat inactivation (Fig. 5F-I), suggesting that BCAA promote macrophage activation and thus EMT in epithelial cells through secreted protein(s).

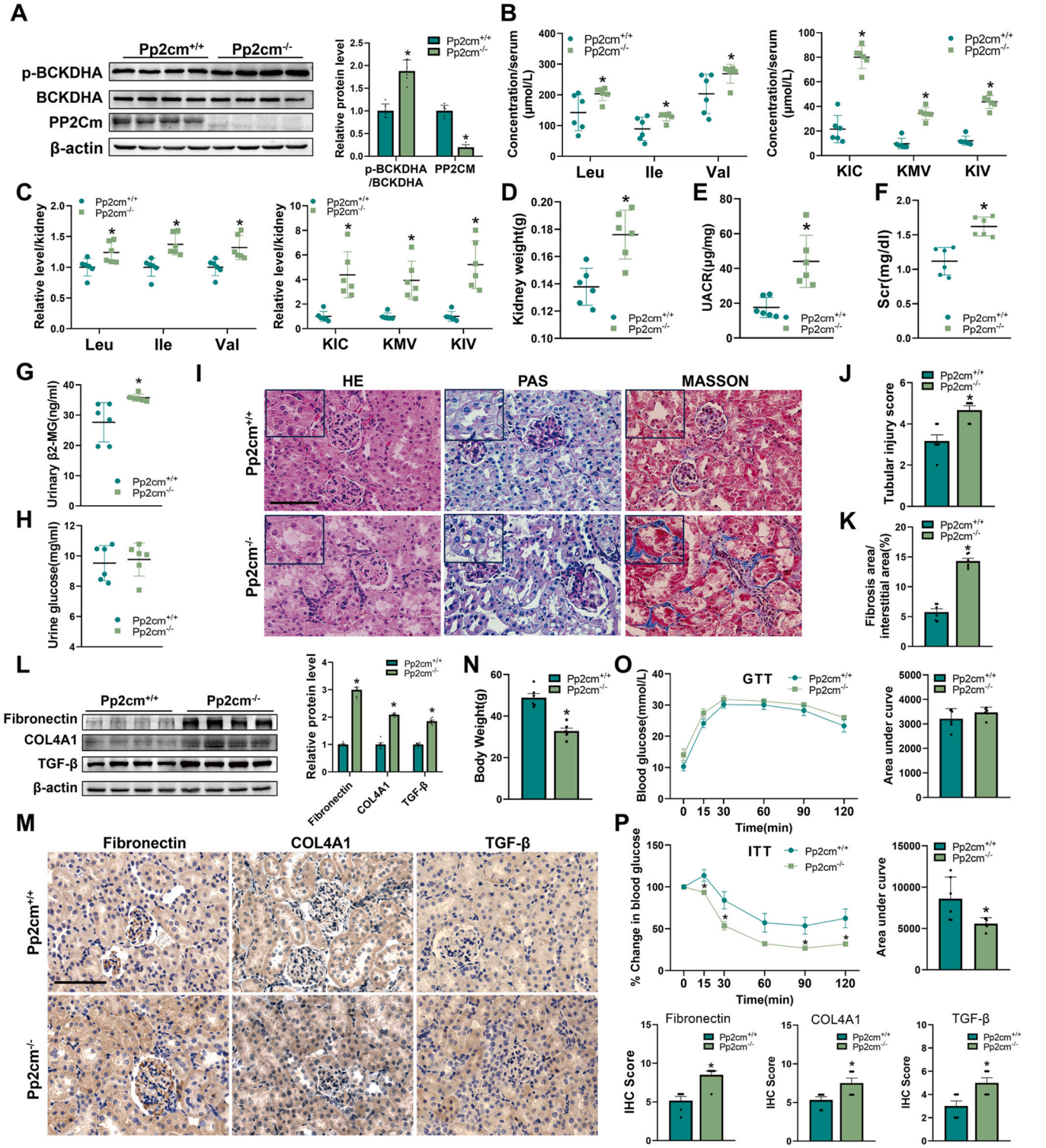

Fig. 3. BCAA catabolic defects promote DKD progression.

(A) Representative blots and a quantitation graph of p-BCKDHA (Ser293), BCKDHA, and PP2Cm protein expression in the kidney. (B—C) Serum concentrations (B) and relative renal abundances (C) of BCAAs (Leu, Ile, and Val) and BCKAs (KIC, KMV, and KIV). (D) Kidney weight (KW). (E) Urinary albumin-to-creatinine ratio (UACR). (F) Serum creatinine (Scr). (G) Urinary β2-Microglobulin (β2-MG). (H) Urine glucose concentrations. (I) Representative images of kidney sections stained with HE, PAS, and Masson's trichrome. Original magnification = 400. Scale bar = 100 μm. A magnification is shown in the box. (J) Quantitative analysis of tubular damage, on the basis of HE staining. (K) Quantitative analysis of the fibrosis area, on the basis of Masson's trichrome staining. (L) Representative blots and a quantitation graph of Fibronectin, COL4A1 and TGF-β protein expression in the kidney. (M) Representative micrographs showing kidney Fibronectin, COL4A1, and TGF-β IHC staining (scale bar = 100 μm), with IHC scores shown on the right. (N) Body weight. (O) Blood glucose levels (mmol/L) following IPGTT and the area under the curve (AUC) analysis. (P) Insulin tolerance test (ITT) with blood glucose represented as the percentage decrease from baseline and the area under the curve (AUC) analysis. Male mice were deprived of food for 6 h (8:00 am to 2:00 pm) before being sacrificed. All the data are presented as the means ± SD; $n = 6$ per group. *$P < 0.05$. KIC, α-ketoisocaproic acid; KIV, α-ketoisovaleric acid; KMV, α-keto-b-methylvaleric acid.

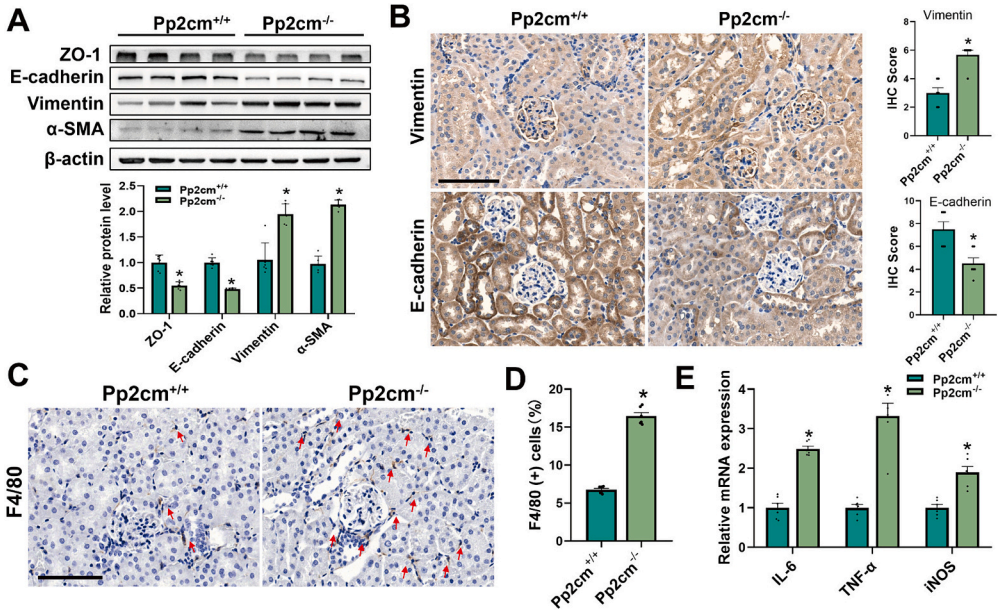

Fig. 4. Renal inflammation and EMT were exacerbated in mice with BCAA catabolic deficiency.
(A) Representative blots and a quantitation graph of ZO-1, E-cadherin, α-SMA and Vimentin protein expression in the kidney. (B) Representative micrographs showing kidney E-cadherin, Vimentin IHC staining (scale bar = 100 μm), with IHC scores shown on the right. (C) Representative micrographs showing F4/80 IHC staining (scale bar = 100 μm). Arrows indicates F4/80 -positive macrophage. (D) Quantification of F4/80-positive macrophages. (E) Quantitative PCR analysis of IL-6, TNF-α and iNOS genes in kidney, normalized to β-actin. Male mice were deprived of food for 6 h (8:00 am to 2:00 pm) before being sacrificed. All the data are presented as the means ± SD; n = 6 per group. *P < 0.05.

3.7. Pharmacological restoration of BCAA homeostasis attenuated T2DKD in diabetic mice

Given the critical role of defective BCAA catabolism in the pathogenesis of DKD, pharmacological restoration of BCAA homeostasis may attenuate DKD. BT2-treated *db/db* mice showed a significant reduction in the phosphorylation of BCKDHA as well as the serum and renal BCAA and BCKA abundances (Fig. 6A-C). Reduction of BCAAs and BCKAs by BT2 administration did not affect body weight, kidney weight, and blood or urinary glucose level (Fig. S4A—F). Notably, UACR, Scr, and β2-MG were significantly decreased in BT2-treated mice compared to control mice, indicating improved renal function (Fig. 6D-F). Furthermore, renal injury and interstitial fibrosis were improved after BT2 treatment (Fig. 6G-I). Similarly, there was a reduction in the expression of fibrosis-related factors in BT2-treated mice (Fig. 6J-K). BT2 also attenuated inflammation and EMT in the kidneys of *db/db* mice (Fig. 6J, L-O). Macrophage infiltration and proinflammatory cytokine release were reduced in the BT2-treated group. As expected, the expression level of E-cadherin was increased, while the expression of Vimentin was decreased. Taken together, these results showed that pharmacological restoration of BCAA homeostasis attenuated DKD in diabetic mice.

3.8. Elevated BCAAs promoted T1DKD progression without exacerbating diabetes

In addition to T2DM, BCAA levels are also elevated in animals with T1DM [55–57]. We detected higher levels of BCAAs and BCKAs in the serum and kidney of wildtype mice with T1DM (Fig. 7A-B), which was further elevated in *Pp2cm*$^{-/-}$ mice with T1DM (Fig. 7C-D). Importantly, the progression of DKD was exacerbated in *Pp2cm*$^{-/-}$ mice compared with that in wildtype control, as shown by worse kidney function and structural damage (Fig. 7E-J). The elevated expression of fibrosis-associated factors, inflammatory factors, and EMT markers were detected in diabetic *Pp2cm*$^{-/-}$ mice compared with those in diabetic wildtype mice (Fig. 7K-L). Of note, wildtype and *Pp2cm*$^{-/-}$diabetic mice showed similar glucose tolerance and insulin tolerance (Fig. 7M-N). Together, these results suggested that the elevated BCAAs, as an independent risk factor, promoted DKD in mice with T1DM. Furthermore, LPD feeding reduced the abundances of BCAAs and BCKAs in the serum and kidney (Fig. 7C-D), protected kidney function and ameliorated kidney structural damage (Fig. 7E-J), and attenuated marker expression (Fig. 7K-J) in both wildtype and *Pp2cm*$^{-/-}$ diabetic mice.

4. Discussion

In the present study, we showed that the disruption of BCAA homeostasis was a metabolic signature of both T1DKD and T2DKD. Reducing BCAA levels with a low-protein (and therefore BCAAs) diet attenuated DKD in *db/db* mice, while BCAA supplementation promoted DKD. *Pp2cm*$^{-/-}$ mice with genetic BCAA catabolic defect and accumulated BCAAs exhibited exaggerated T1DKD and T2DKD. Importantly, the elevated BCAAs promoted DKD progression without exacerbating diabetes. Mechanistically, the elevated BCAA promoted EMT by enhancing the activation of proinflammatory macrophages. Finally, pharmacological restoration of BCAA homeostasis in *db/db* mice with a small molecule inhibitor attenuated DKD. Taken together, the disruption of BCAA homeostasis is an independent risk factor for DKD and BCAA catabolic pathway represents a potential therapeutic target for DKD.

It has been shown that kidney is an important metabolic organ for BCAA catabolism [6,22]. DKD individuals reportedly have reduced BCAA catabolic products in urine, suggesting a possible impairment in BCAA catabolic flux in kidney [58–60]. Our study revealed that BCAA catabolic genes were downregulated in the kidney of DKD individuals and *db/db* mice, indicating impaired renal BCAA catabolism. The elevated BCAA abundances in kidney of diabetic mouse concurred with the impaired catabolism. On the other hand, the increase of renal BCAA abundances occurred concurrently with the elevation of blood BCAA levels. Skeletal muscle, adipose tissue, and liver have also been recognized as major contributors to systemic BCAA homeostasis [21,44]. Thus, the change in renal BCAAs could be a consequence of systemic BCAA metabolic dys-regulation. It remains to be further investigated whether the impaired renal BCAA catabolism contributes to systemic

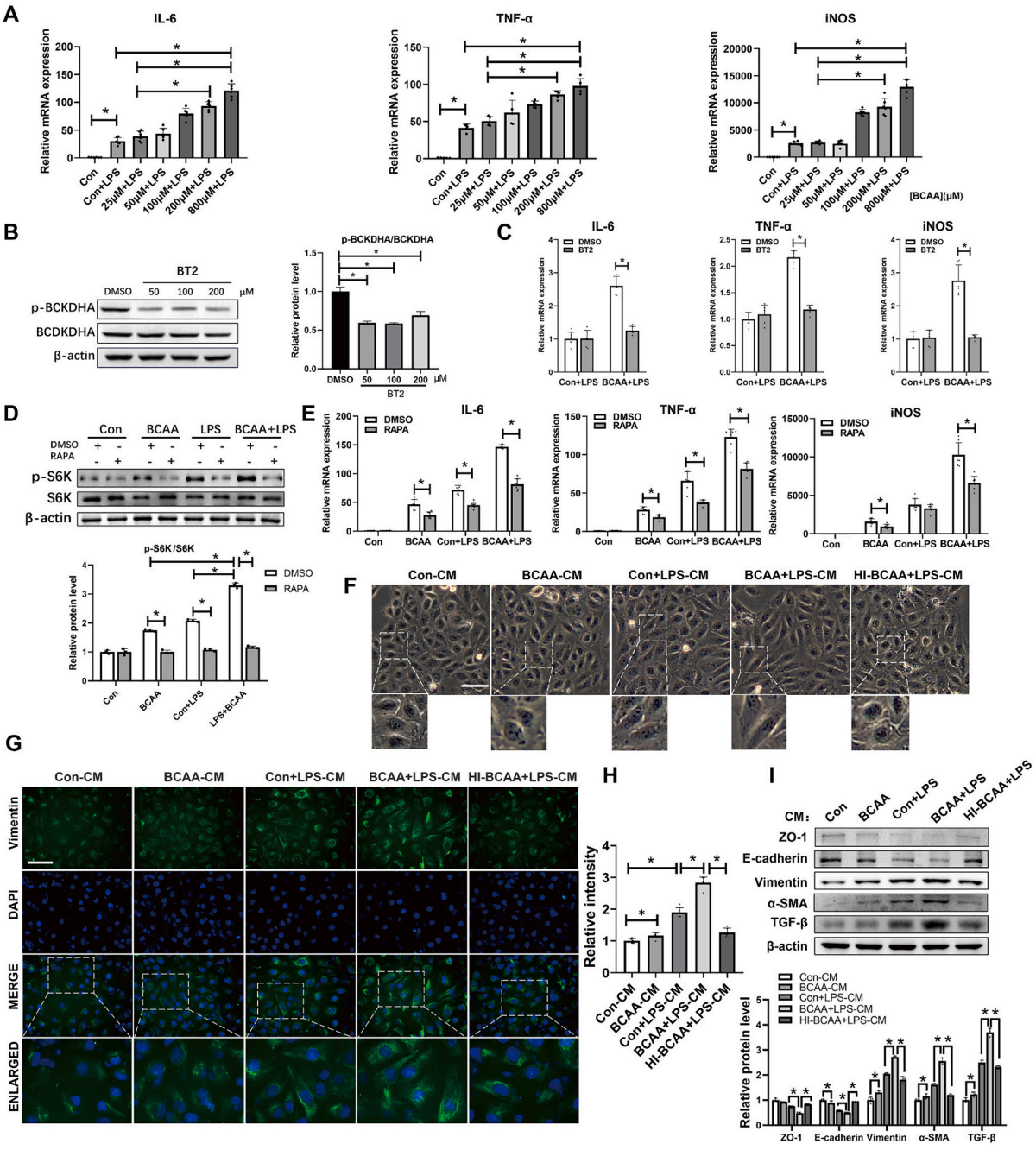

Fig. 5. BCAA-enhanced macrophage M1 polarization promoted EMT in HK2 cells.
(A) Quantitative PCR analysis of IL-6, TNF-α and iNOS gene expression levels in the macrophages, normalized to β-actin. (B—C) Macrophages were preincubated with BT2(50 μmol/L) for 1 h. (B) Representative blots and a quantitation graph of p-BCKDHA and BCKDHA protein expression in the macrophages. (C) Quantitative PCR analysis of IL-6, TNF-α and iNOS gene expression levels in the macrophages, normalized to β-actin. (D-E) Macrophages were preincubated with rapamycin (100 nmol/L) for 1 h. (D) Representative blots and a quantitation graph of S6K and p-S6K protein expression in the macrophages. (E) Quantitative PCR analysis of IL-6, TNF-α and iNOS gene expression levels in the macrophages, normalized to β-actin. (F) Representative images show the effect of the conditional medium (CM) from different treated macrophages on the HK2 cell morphology (scale bar = 50 μm). (G) Immunofluorescent staining of Vimentin in HK2 cells cultured with CM from different treated macrophages (scale bar = 50 μm). (H) Quantification of fluorescence intensity (I) Representative blots and a quantitation graph of ZO-1, E-cadherin, Vimentin, α-SMA and TGF-β protein expression in the HK2 cells. Macrophages were obtained from the peritoneal cavity of mice. LPS: 100 ng/mL; Con:0 μm BCAA; BCAA:800 μm BCAA. Con-CM: CM from 0 μm BCAA-treated macrophages. BCAA-CM: CM from 800 μm BCAA-treated macrophages. Con+LPS-CM: CM from LPS- and 0 μm BCAA-treated macrophages. BCAA+LPS-CM: CM from LPS- and 800 μm BCAA-treated macrophages. HI-BCAA+LPS-CM: heat-inactivated CM from LPS- and 800 μm BCAA-treated macrophages. All the data are presented as the means ± SD; $^{*}P < 0.05$.

BCAA changes and promotes the DKD progression.

Previous studies have shown a connection between BCAAs and obesity-associated insulin resistance (IR). IR is involved in the progression of DKD [40,61–64]. Thus, BCAA-promoted IR might contribute to the progression of DKD. However, in both T1DM and T2DM models in the current study, DKD was promoted by defective BCAA catabolism without exacerbated diabetes *in Pp2cm*$^{-/-}$ mice. Furthermore, BT2 treatment improved DKD without affecting glucose tolerance and IR in *db/db* mice. Together, these data suggest that the impact of BCAAs on DKD is independent of IR and diabetic status in diabetic mouse.

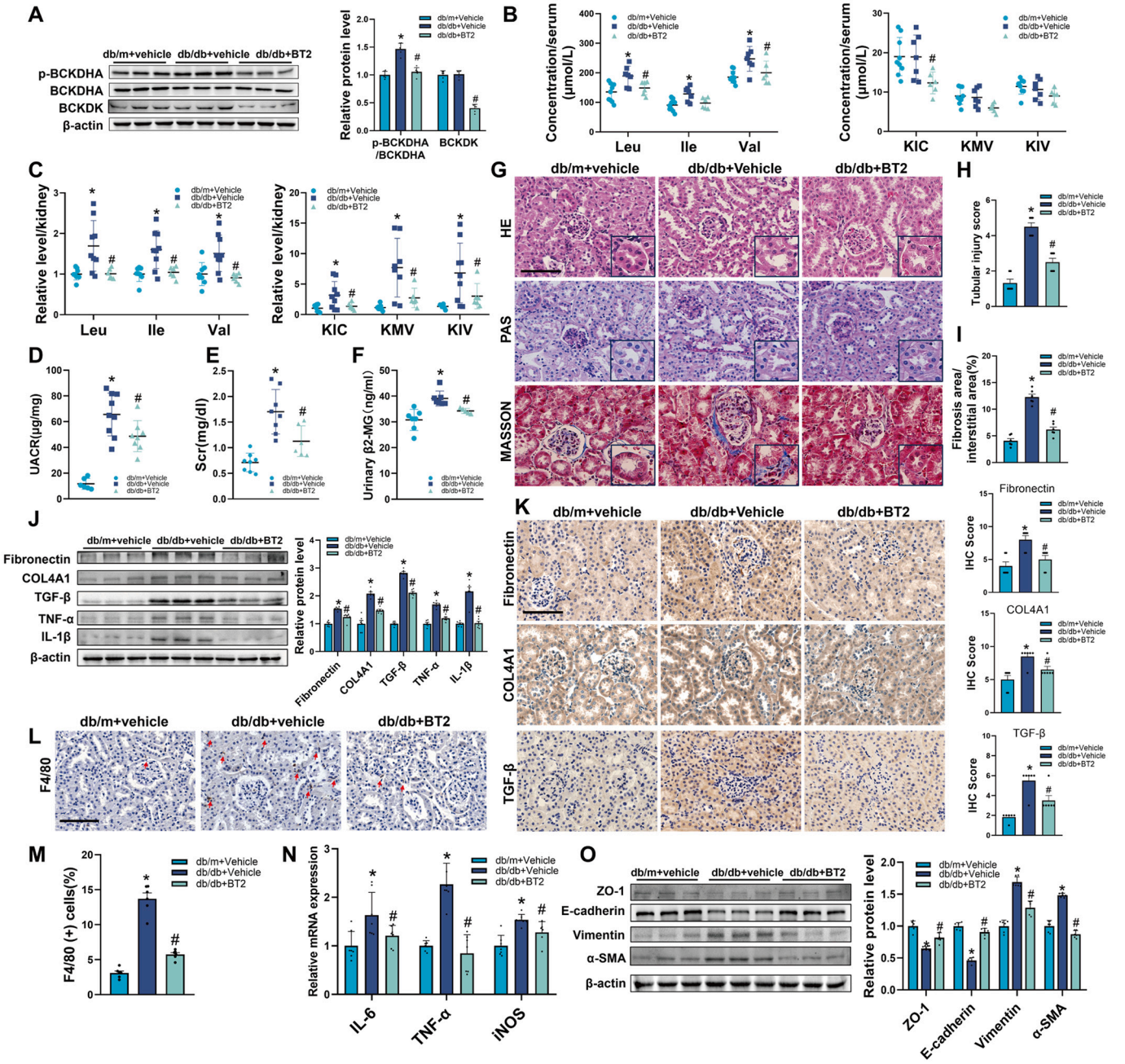

Fig. 6. The BCKDK inhibitor BT2 promoted BCAA catabolism and attenuated DKD in *db/db* mice.
(A) Representative blots and a quantitative graph of p-BCKDHA (Ser293), BCKDHA, and BCKDK protein expression in the kidney. (B—C) Serum concentrations (B) and relative renal abundances(C) of BCAAs (Leu, Ile, and Val) and BCKAs (KIC, KMV, and KIV). (D) Urinary albumin-to-creatinine ratio (UACR). (E) Serum creatinine (Scr). (F) Urinary β2-Microglobulin (β2-MG). (G) Representative images of kidney sections stained with HE, PAS, and Masson's trichrome. Original magnification = 400. Scale bar = 100 μm. A magnification is shown in the box. (H) Quantitative analysis of tubular damage, on the basis of HE staining. (I) Quantitative analysis of the fibrosis area, on the basis of Masson's trichrome staining. (J) Representative blots and a quantitation graph of Fibronectin, COL4A1, TGF-β TNF-α, and IL-1β protein expression in the kidney. (K) Representative micrographs showing kidney Fibronectin, COL4A1, and TGF-β IHC staining (scale bar = 100 μm), with IHC scores shown on the right. (L) Representative micrographs showing F4/80 IHC staining (scale bar = 100 μm). (M) Quantification of F4/80-positive macrophages. (N) Quantitative PCR analysis of IL-6, TNF-α and iNOS gene expression levels in the kidney, normalized to β-actin. (O) Representative blots and a quantitation graph of ZO-1, E-cadherin Vimentin, and α-SMA protein expression in the kidney. Mice were treated with BT2 (40 mg/kg/day) or an equivalent volume of vehicle daily at 5:00 pm by intragastric administration for 10 weeks. Male mice were deprived of food for 6 h (8:00 am to 2:00 pm) before being sacrificed. All the data are presented as the means ± SD; $n = 6$–10 per group. *P < 0.05, db/m + vehicle vs. *db/db* + vehicle. #P < 0.05, *db/db* + vehicle vs. *db/db* + BT2; KIC, α-ketoisocaproic acid; KIV, α-ketoisovaleric acid; KMV, α-keto-b-methylvaleric acid.

Disrupted BCAA homeostasis serves as an independent risk factor for the progression of DKD.

The discrepancy of BCAAs' impacts on diabetes between the current observations and those in previous studies may be attributed to different animal models. Leptin-deficient *ob/ob* mice and leptin receptor-deficient db/db mice are commonly used mice models mimicking the conditions of obesity and type 2 diabetes. However, while both *ob/ob* and *db/db* mice gain weight and develop massive obesity similarly, *db/db* mice are more diabetic than *ob/ob* mice, exhibiting higher blood glucose and more severe diabetic complications. They show differences in glucose

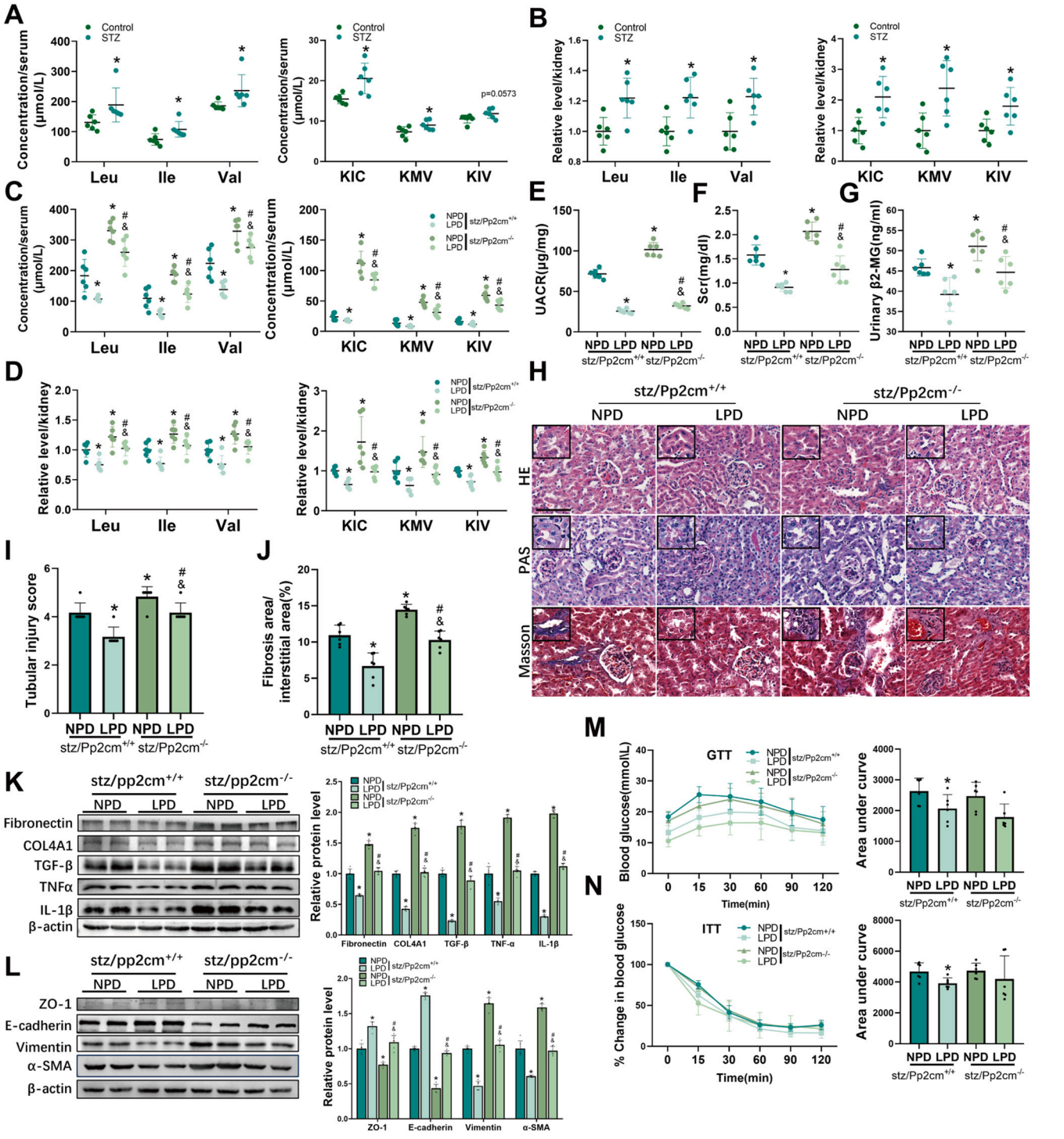

Fig. 7. Elevated BCAAs promoted DKD progression in Type 1 diabetes.

Type 1 diabetes was induced by injection of STZ. (A-B) Serum concentrations (A) and relative renal abundances (B) of BCAAs (Leu, Ile, and Val) and BCKAs (KIC, KMV, and KIV) in diabetic wildtype mice. (C-M) Results of wildtype and *Pp2cm*$^{-/-}$ mice on NPD or LPD. (C—D) Serum concentrations (C) and relative renal abundances (D) of BCAAs (Leu, Ile, and Val) and BCKAs (KIC, KMV, and KIV). (E) Urinary albumin-to-creatinine ratio (UACR). (F) Serum creatinine (Scr). (G) Urinary β2-Microglobulin (β2-MG). (H) Representative images of kidney sections stained with HE, PAS, and Masson's trichrome. Original magnification = 400. Scale bar = 100 μm. A magnification is shown in the box. (I) Quantitative analysis of tubular damage, on the basis of HE staining. (J) Quantitative analysis of the fibrosis area, on the basis of Masson's trichrome staining. (K) Representative blots and a quantitation graph of Fibronectin, COL4A1, TGF-β, TNF-α, and IL-1β protein expression in the kidney. (L) Representative blots and a quantitation graph of ZO-1, E-cadherin, Vimentin, and α-SMA protein expression in the kidney. (M) Blood glucose levels (mmol/L) following IPGTT and the area under the curve (AUC) analysis. (N) Insulin tolerance test (ITT) with blood glucose represented as the percentage decrease from baseline and the area under the curve (AUC) analysis. Male mice were deprived of food for 6 h (8:00 am to 2:00 pm) before being sacrificed. All the data are presented as the means ± SD; n = 6 per group. *P < 0.05, vs. stz/*Pp2cm*$^{+/+}$+NPD. #P < 0.05 , stz/*Pp2cm*$^{-/-}$ + NPD vs. stz/*Pp2cm*$^{-/-}$ + LPD. &P < 0.05, stz/*Pp2cm*$^{+/+}$+LPD vs. stz/*Pp2cm*$^{-/-}$ + LPD; KIC, α-ketoisocaproic acid; KIV, α-ketoisovaleric acid; KMV, α-keto-b-methylvaleric acid.

metabolism, glucose-induced insulin secretion, inflammatory profiles, among others. These differences could be linked to specific inflammatory tones, serum LPS concentration, bile acid metabolism, short-chain fatty acid profile, and gut microbiota composition [65]. These differences could attribute to the distinct responses to BT2 in *db/db* mice and *ob/ob* mice.

Renal interstitial inflammation and fibrosis are prominent features of DKD [66–69]. Inflammation plays a central role in the initiation and progression of renal injury [70,71]. Persistent inflammation contributes to the progression of fibrosis in chronic kidney disease [72]. We found that the elevated BCAAs significantly increased inflammation and fibrosis in the kidneys of diabetic mice as well as the macrophage activity and EMT in vitro, suggesting that the disrupted BCAA homeostasis promotes DKD by enhancing inflammation. The occurrence of diabetes is often accompanied with chronic inflammation. Elevated BCAAs may amplify the inflammatory response to promote the progression of DKD. This may help explain why the negative impact of BCAA on kidney function occurs in the diabetic state but not in healthy mice.

In addition to the inflammation-EMT mechanism revealed in the current study, other physiological process may be involved in the BCAAs-promoted DKD. For example, a recent study shows that the renal ablation of glucagon receptor impairs kidney functions and causes widespread fibrosis in aged mice, demonstrating a critical role of glucagon in kidney health and disease [73]. Meanwhile, another study shows that disordered BCAAs metabolism is associated with hypersecretion of glucagon in diabetic mice and BCAAs can increase plasma glucagon levels [74]. Therefore, it is possible that glucagon may play a role in BCAAs-promoted DKD, which warrants further investigation.

Activation of mTORC1 has been associated with the progression of chronic kidney disease (CKD) caused by diabetes and other factors [49,75–77]. Inhibition of mTOR by rapamycin ameliorates interstitial inflammation, fibrosis, and loss of renal function in CKD. In the current study, BCAA boosted the activation of macrophages and the release of proinflammatory factors through mTORC1 pathway, which promoted EMT in epithelial cells. EMT plays a critical role in interstitial fibrosis [46,78,79]. These results provide a potential mechanism underlying mTOR activation in DKD and support the pathogenic role for mTOR signaling in DKD.

Disrupted BCAA homeostasis and elevated BCAA levels can be restored via dietary and pharmacological approaches. Our data showed that BT2 corrected the defect in BCAA catabolism and attenuated DKD in *db/db* mice. Furthermore, a low-protein diet reduced dietary BCAA intake, leading to decreased BCAA abundances and alleviated progression of DKD. Thus, BCAA homeostasis provides a promising therapeutic target for DKD.

In conclusion, the current study provides new insights into the metabolic mechanisms underlying the progression of DKD by revealing a critical role of BCAA homeostasis. The elevated BCAAs are an independent risk factor for DKD, highlighting a nutritional approach that reduces dietary BCAA intake to alleviate DKD. Furthermore, BCKDK may serve as a promising pharmacological target for DKD. In addition, the impact of dietary BCAA intake on DKD progression provides a mechanistic basis for previous report showing that protein restriction improves renal function in DKD in human [31,32,80] , shedding light on the benefits of a low-protein diet in chronic kidney disease individuals.

CRediT authorship contribution statement

Xiaoqing Deng: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Chao Tang:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Ting Li:** Writing – review & editing, Methodology, Investigation. **Xiaoyu Li:** Writing – review & editing, Resources. **Yajin Liu:** Methodology, Data curation. **Xuejiao Zhang:** Writing – original draft, Supervision, Methodology. **Bei Sun:** Writing – original draft, Methodology, Investigation. **Haipeng Sun:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Liming Chen:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data associated with this study are available from the corresponding author upon reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2024.156037.

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