

Original Research

Distinct Temporal Modulation of Mesenchymal Stem Cells and Empagliflozin on Renal c-Kit Cells in Diabetic Kidney Disease

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Abstract

Background: Diabetic kidney disease (DKD) remains a leading cause of chronic kidney disease worldwide despite current standard therapies. c-Kit⁺ renal progenitor cells have been implicated in kidney repair and regeneration. We investigated whether mesenchymal stem cells (MSCs) and the sodium-glucose cotransporter-2 inhibitor empagliflozin are associated with differential modulation of intrarenal c-Kit⁺ cell expression in a preclinical model of DKD using BTBR^{ob/ob} mice. **Methods:** These BTBR^{ob/ob} mice were allocated into three groups: untreated DKD, MSC-treated DKD, and empagliflozin-treated DKD, and were compared with BTBR wild-type (WT) controls. BTBR^{ob/ob} mice received empagliflozin formulated in chow ad libitum at 25 mg/kg (Jardiance®, Boehringer Ingelheim, Ingelheim am Rhein, RP, Germany) starting at 4–5 weeks of age. MSCs (1 × 10⁶ cells per dose) were administered intraperitoneally at 8 and 10 weeks of age. Animals were evaluated at 10, 14–15, and 18–20 weeks. Renal c-Kit gene expression was quantified by quantitative real-time PCR (qPCR), and protein expression was assessed by immunohistochemistry in the cortex, medulla, and glomeruli. Statistical analyses included mixed-effects models and two-way analysis of variance (ANOVA). **Results:** c-Kit expression was significantly influenced by time and treatment in both cortex and medulla. In the renal cortex, MSC therapy was associated with a transient increase in c-Kit gene expression at 14–15 weeks, followed by a reduction at 18–20 weeks. Empagliflozin predominantly modulated cortical c-Kit expression at later time points. Immunohistochemistry demonstrated higher cortical c-Kit protein expression in MSC-treated animals compared with untreated DKD mice. In the medulla, MSC-treated mice exhibited increased c-Kit expression at 14–15 weeks, which declined at 18–20 weeks. Empagliflozin had a modest effect on c-Kit expression and was associated with infrequent glomerular immunoreactivity at 14–15 weeks. **Conclusions:** MSC therapy and empagliflozin are associated with distinct temporal patterns of c-Kit expression in DKD. MSC treatment showed early modulation of c-Kit expression, whereas empagliflozin exhibited more delayed effects. These findings suggest that c-Kit⁺ cells are dynamically regulated during DKD progression. Given the absence of functional and mechanistic analyses, these results should be considered hypothesis-generating and warrant further investigation.

Keywords: diabetic nephropathies; diabetes mellitus, type 2; obesity; stem cells; obese mice; hyperglycemic mice

1. Introduction

The global prevalence of diabetes mellitus (DM) is projected to increase by approximately 45% between 2024 and 2050, affecting more than 800 million people worldwide [1]. Diabetic kidney disease (DKD), the leading cause of chronic kidney disease (CKD), affects nearly one in three individuals with DM [2]. Moreover, 30–40% of patients with DKD will ultimately require dialysis or kidney transplantation. Importantly, a substantial proportion of individuals with DKD will die from cardio- or cerebrovascular events before initiating kidney replacement therapy [3].

In addition to lifestyle modifications, current DKD management relies on four major pharmacological pillars: blockade of the renin-angiotensin system, sodium-glucose

co-transporter-2 (SGLT2) inhibitors, mineralocorticoid receptor antagonists, and glucagon-like peptide-1 receptor agonists [4]. Although these therapies significantly slow disease progression, a considerable residual risk of kidney function decline remains.

In this context, identifying additional therapeutic strategies capable of further attenuating DKD progression remains a critical unmet need. One promising approach is cell-based therapy using mesenchymal stem cells (MSCs) [5]. MSCs are multipotent cells found in various tissues, including bone marrow, adipose tissue, and umbilical cord blood, and are characterized by specific surface markers, plastic adherence, and a fibroblast-like morphology [6]. Their biological effects are primarily mediated



through paracrine mechanisms, largely driven by the release of extracellular vesicles [7]. In preclinical DKD and type 2 DM (T2DM) models, MSC therapy modulates multiple dysregulated signaling pathways, reducing inflammation, oxidative stress, apoptosis, fibrosis, and cellular senescence, while promoting angiogenesis and cellular proliferation, thereby improving both functional and structural renal and pancreatic outcomes [8,9,10]. In humans with DKD, MSC therapy has been shown to be safe and to contribute to preservation of kidney function [11,12].

Concurrently, increasing attention has been directed toward the investigation of putative resident kidney stem/progenitor cell populations as potential therapeutic targets [13,14]. In the context of kidney development and repair, c-Kit identifies a distinct population of progenitor cells. c-Kit⁺ cells delineate a previously unrecognized progenitor compartment extending from the aorta-gonad-mesonephros region to the developing mouse kidney and are molecularly distinct from the Foxd1-expressing stromal lineage [15]. These cells are highly expressed in the ureteric bud, where they interact with their ligand, stem cell factor (SCF), which is expressed in the metanephric mesenchyme [16].

Although c-Kit signaling does not appear to be essential for *in vivo* cell survival, *ex vivo* studies suggest that these cells become dependent on SCF signaling under defined conditions, likely reflecting the loss of redundant survival pathways present *in vivo*. Exogenous SCF has been shown to expand intrarenal c-Kit⁺ cells, increase angioblast abundance, and accelerate nephron development, whereas c-Kit antagonism promotes apoptosis of interstitial c-Kit⁺ cells, reduces Flk-1⁺ angioblasts, impairs ureteric bud branching, and ultimately decreases nephron number [15]. Additionally, the Kit/SCF pathway plays a role in postnatal growth of epithelial, endothelial, and endocrine cells [17]. Collectively, these findings indicate that c-Kit signaling modulates progenitor cell dynamics and nephrogenesis in a context-dependent manner, with potential implications for renal development and regenerative strategies.

In human kidneys, c-Kit⁺ cells have been identified in both the cortex and medulla of deceased donors with acute tubular injury [18]. In healthy individuals, c-Kit⁺ cells have been observed in proximal and distal tubules [19,20]. In renal cancer, c-Kit expression shows strong cytoplasmic immunoreactivity in papillary renal cell carcinomas and membranous staining in chromophobe renal cell carcinomas, the latter originating from the intercalated cells of the collecting duct [20]. However, other studies have failed to detect c-Kit⁺ cells in renal tissue [21], highlighting ongoing uncertainty regarding their distribution and functional role.

Our group has previously characterized a kidney-derived stem/progenitor cell population expressing the c-Kit receptor (CD117), demonstrating clonogenicity, multipotency, self-renewal capacity, and regenerative potential

in models of ischemia-reperfusion injury in models of acute ischemia-reperfusion injury [22] and nephrotic syndrome [23].

The aim of the present study was to evaluate whether bone marrow-derived MSCs and empagliflozin, a SGLT2 inhibitor, modulate intrarenal c-Kit⁺ cell expression in a preclinical model of T2DM, obesity, and DKD using BTBR^{ob/ob} (Black and Tan, Brachyury) mice, compared with untreated diabetic and lean control mice. We hypothesized that MSC therapy would be associated with early modulation of the intrarenal c-Kit⁺ progenitor compartment, whereas empagliflozin would influence this population indirectly through metabolic and hemodynamic mechanisms.

2. Material and Methods

2.1 BTBR^{ob/ob} Mice

To evaluate the effects of the proposed therapeutic strategies, BTBR^{ob/ob} mice (BTBR.Cg-Lepob/WiscJ; #004824, The Jackson Laboratory, Bar Harbor, ME, USA) were used. The animals were randomly assigned to the intervention or control groups. No animals were excluded from the study after randomization, and all allocated animals were included in the final analyses. This strain is widely regarded as one of the most robust experimental models of progressive DKD. Due to leptin deficiency, BTBR^{ob/ob} mice develop severe hyperphagia, resulting in obesity and early-onset diabetes [24]. The sustained hyperglycemic state, combined with marked insulin resistance, leads to progressive structural and functional renal alterations that closely recapitulate human DKD, as described by The Jackson Laboratory (<https://www.jax.org/strain/004824>).

Briefly, BTBR^{ob/ob} mice exhibit a rapid and progressive metabolic phenotype that closely recapitulates key features of human type 2 diabetes. Additional details regarding the phenotype are available on The Jackson Laboratory website (<https://www.jax.org/jax-mice-and-services/strain-data-sheet-pages/phenotype-information-004824>). As early as 4–6 weeks of age, these animals develop obesity driven by hyperphagia, followed by persistent hyperglycemia and impaired glucose tolerance between 6 and 8 weeks of age [24,25,26,27]. This is accompanied by marked insulin resistance and dyslipidemia, including elevated triglyceride and cholesterol levels. Between 8 and 12 weeks, the metabolic syndrome becomes fully established, with systemic inflammation and oxidative stress contributing to disease progression. This early and robust metabolic derangement makes the BTBR^{ob/ob} strain particularly suitable for studying interventions targeting the initial phases of diabetic complications.

From approximately 12–16 weeks onward, these mice develop progressive diabetic kidney disease, characterized by glomerular hypertrophy, mesangial matrix expansion, and the onset of albuminuria. By 16–20 weeks, the renal

phenotype advances to severe nephropathy, including diffuse glomerulosclerosis, arteriolar hyalinosis, and tubulointerstitial injury, closely resembling advanced human DKD [24,25,28]. Importantly, the rapid and predictable temporal progression of renal injury in this model provides a valuable framework for investigating stage-specific therapeutic effects. This feature is particularly relevant for evaluating interventions with distinct temporal dynamics, such as early regenerative approaches versus later metabolic and hemodynamic modulation.

All experimental procedures were approved by the institutional Animal Care and Use Committee and were conducted in accordance with ethical guidelines. The study was registered under the Ethics Committee on the Use of Animals of Hospital Israelita Albert Einstein, São Paulo, SP, Brazil (protocol number 4900-21), in accordance with ARRIVE guidelines.

The biological material used for the present quantitative real-time PCR (qPCR) and immunohistochemical analyses was obtained from previously published experimental studies conducted by our group. Specifically, kidney samples from BTBR^{ob/ob} and wild-type BTBR mice treated with empagliflozin and MSCs, as well as their respective control groups, were derived from the cohorts previously described in references [29,30,31]. The original studies reported the metabolic, functional, and histopathological outcomes of these interventions, whereas the current investigation was specifically designed to evaluate renal c-Kit expression at the gene and protein levels using archived biological specimens from these previously characterized experimental cohorts.

2.2 Experimental Design and Groups

Wild-type BTBR mice (BTBR WT), healthy and disease-free, were used as a reference lean control group. An additional control group consisted of untreated BTBR^{ob/ob} mice (obese and T2DM), representing the natural progression of DKD. A total of four experimental groups were established, each comprising six male mice (n = 6 per group, biological replicates) (Fig. 1):

1. Wild-type BTBR mice without treatment (WT; lean control);
2. BTBR^{ob/ob} mice without treatment (obese, T2DM, and DKD control);
3. BTBR^{ob/ob} mice treated with mesenchymal stem cells (MSCs);
4. BTBR^{ob/ob} mice treated with a SGLT2 inhibitor (empagliflozin).

The sample size was limited by the intrinsic biological constraints of the BTBR^{ob/ob} model, including reproductive difficulty, increased fragility, and higher mortality during longitudinal follow-up.

2.3 Treatment With SGLT2 Inhibitor and MSCs

Empagliflozin was administered via diet. At 4–5 weeks of age (weaning), animals assigned to the SGLT2 inhibitor group were housed in cages with ad libitum access to chow (RHOSTER, Araçoiaba da Serra, SP, Brazil) supplemented with empagliflozin at a concentration of 25 mg/kg and free access to drinking water, as previously described by our group [29]. Untreated groups received standard chow (NUVILAB CR-1 Irradiated) and water ad libitum.

MSCs were obtained from male 6–8-week-old mT/mG mice (#007576; JAX Laboratories, Bar Harbor, ME, USA). These cells were extracted from femoral/tibial bone marrow (BM) flush, followed by purification in culture through passages in DMEM/F12 1:1 Ham (Invitrogen, Carlsbad, CA, USA) supplemented with 20% FBS (#F063—Cultilab, Campinas, SP, Brazil) and 1% PS (#15140122—Gibco, Thermo Fisher Scientific, San Diego, CA USA). We characterized MSCs at passages 5–6 and injected these cells at passages 7–10. Incubation conditions: 37°C, 5% CO₂, and humidified atmosphere. Cells were expanded, purified, and characterized according to established criteria, including fibroblast-like morphology, immunophenotyping by flow cytometry, and tri-lineage differentiation potential (osteogenic, chondrogenic, and adipogenic) [30]. Consistent with published data, MSCs expressed CD29, CD44, CD90, CD105, and Stem cell antigen-1 (Sca-1), while lacking expression of CD31 (endothelial marker) and the hematopoietic markers CD45 and CD34. Cell viability was considered acceptable when exceeding 80%, as determined by Trypan Blue exclusion. Mycoplasma testing was performed routinely every six months in accordance with Good Laboratory Practice (GLP) requirements in our laboratory. Furthermore, the specific mesenchymal stem cell samples used in this study were tested for mycoplasma contamination, and all results were negative.

BTBR^{ob/ob} mice assigned to MSC treatment received intraperitoneal injections of 1×10^6 cells at 8 and 10 weeks of age, targeting an early therapeutic window prior to the expected peak of proteinuria, which typically occurs between 12 and 14 weeks of age in this model [24].

Importantly, empagliflozin was administered early (from 4–5 weeks of age), targeting metabolic and hemodynamic pathways, whereas MSCs were administered at 8 and 10 weeks, targeting a regenerative window prior to peak renal injury. This design allowed us to investigate whether these therapies exert distinct temporal effects on c-Kit⁺ cell dynamics, which represents the central hypothesis of our study.

Animals were euthanized by an overdose of 2.5–5% isoflurane (Cristália, Itapira, SP, Brazil), followed by cervical dislocation.

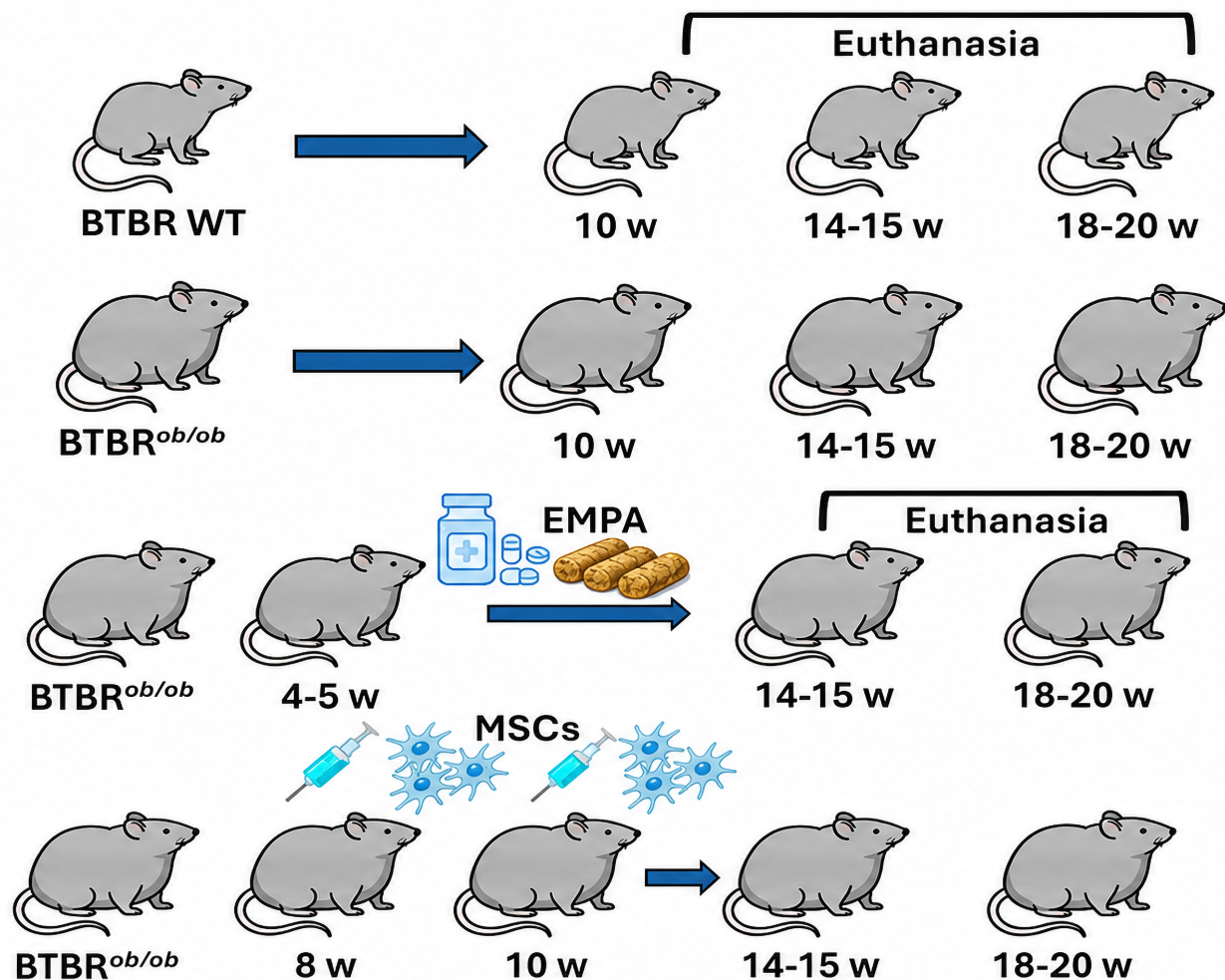


Fig. 1. Schematic representation of the treatment protocols. BTBR wild-type (WT) and untreated BTBR^{ob/ob} mice were euthanized at three different time points: 10 weeks (w), 14–15 weeks, and 18–20 weeks of age. Empagliflozin (Empa)-treated BTBR^{ob/ob} mice were followed from 4–5 weeks of age until euthanasia at 14–15 and 18–20 weeks. In mesenchymal stem cell (MSC)-treated BTBR^{ob/ob} mice, injections were administered at the 8th and 10th weeks of age, and euthanasia was performed at 14–15 and 18–20 weeks.

2.4 Quantitative Real-Time PCR (qPCR)

To further quantify renal *c-Kit* gene expression, quantitative real-time PCR was performed. Renal tissue samples were preserved in RNeasy lysis solution and stored at -80°C until processing. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Hilden, NRW, Germany), and RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA samples were standardized to $10\text{ ng}/\mu\text{L}$ and reverse-transcribed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit.

qPCR reactions were conducted using TaqMan™ Gene Expression Master Mix on a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Waltham, MO, USA). Beta-glucuronidase (GUSB; Mm01197698_m1) was used as the endogenous reference gene, and *c-Kit* (Kit; Mm00445212_m1) as the target gene. Relative gene ex-

pression was calculated using the comparative threshold cycle method ($\Delta\Delta\text{CT}$), with results expressed as fold change relative to the wild-type control group.

2.5 Immunohistochemistry

At 10, 14, and 20 weeks of age, animals from each experimental group were euthanized, and kidney tissues were harvested. Samples were fixed in formalin, embedded in paraffin, and sectioned at a thickness of $3.5\text{ }\mu\text{m}$. Immunohistochemical staining was performed using the EnVision FLEX High pH system (Agilent Technologies, Santa Clara, CA, USA). Following deparaffinization, rehydration, and antigen retrieval, endogenous peroxidase activity was blocked. Sections were incubated overnight at 4°C with a rabbit polyclonal anti-*c-Kit* antibody (Abcam, ab5506, Cambridge, MA, USA) at a dilution of 1:200.

After incubation with the appropriate secondary antibody, immunoreactivity was visualized using diaminobenzidine (DAB) as the chromogen, resulting in brown staining in c-Kit⁺ areas. Both qualitative and quantitative analyses were performed under light microscopy (magnification 20×) using CellSens software (version 1.16, Olympus, Shinjuku, Tokyo, Japan). To calculate the c-Kit⁺ fraction area, we analyzed six distinct images per animal. For the assessment of c-Kit⁺ glomeruli, at least 30 glomeruli were counted per animal.

2.6 Statistical Analysis

Data obtained by qPCR and immunohistochemistry were analyzed using two-way analysis of variance (two-way ANOVA) or mixed-effects models, according to data structure and the presence or absence of missing values. Two-way ANOVA was restricted to complete datasets, whereas mixed-effects models were applied when missing data were present, preserving the robustness of the inferences without compromising analytical quality. Normality was assessed using the Shapiro-Wilk test. When normal distribution was not observed, data were transformed to meet the assumptions of parametric models.

Specifically, qPCR data from the renal medulla showed a non-normal distribution and therefore underwent logarithmic transformation using the Box-Cox formula with $\lambda = -0.5$. This transformation was applied to normalize data distribution and enable the use of statistical tests with greater power. After transformation, data were analyzed using a mixed-effects model, considering time, treatment, and their interaction as fixed effects. Intragroup multiple comparisons over time were performed using Tukey's test, while intergroup comparisons at each time point were conducted using Dunnett's test. For qPCR analyses in the renal cortex, a mixed-effects model with the same factors was applied, and multiple comparisons followed the same testing strategy.

For immunohistochemical analysis of the renal cortex, a two-factor repeated-measures ANOVA (time and treatment) was used, preceded by normality assessment with the Shapiro-Wilk test. Tukey's test was applied for both intra- and intergroup multiple comparisons. Immunohistochemical data from the renal medulla were analyzed using a mixed-effects model with time and treatment as factors, following confirmation of normality by the Shapiro-Wilk test. Tukey's (intragroup) and Dunnett's (intergroup) tests were employed for multiple comparisons.

Similarly, data related to glomerular quantification were analyzed using a mixed-effects model with time and treatment as factors, with normality assessed by the Shapiro-Wilk test. Intra- and intergroup multiple comparisons were performed using Tukey's test.

We performed two analyses: (i) comparisons at the 10-week point among MSC-treated, empagliflozin-treated, and untreated BTBR^{ob/ob} and lean mice; and (ii) longitudi-

nal comparisons between 14–15 and 18–20 weeks to evaluate the effects of MSC and empagliflozin exposure in BTBR^{ob/ob} mice relative to untreated diabetic and lean controls.

All statistical analyses were conducted using GraphPad Prism software, version 8 (GraphPad Software, La Jolla, CA, USA), adopting a significance level of 5% ($p < 0.05$) for all comparisons.

3. Results

3.1 qPCR Analysis Results

3.1.1 Quantitative Analysis of the Renal Cortex

The mixed-effects analysis revealed a statistically significant effect of time on the expression of c-Kit-positive cells ($p < 0.0001$), indicating a temporal modulation of this cell population in the kidney. Likewise, significant differences were observed among the treatment groups ($p < 0.0001$), as well as a significant interaction between time and treatment ($p = 0.0394$).

Intragroup analyses performed using Tukey's test with BTBR WT as the reference group demonstrated relevant temporal changes in specific groups (Fig. 2A). The untreated BTBR^{ob/ob} group showed a significant reduction in expression between 10 and 20 weeks ($p = 0.0184$), with a similar trend observed between 14–15 and 18–20 weeks ($p = 0.0735$). In contrast, the MSC-treated BTBR^{ob/ob} group exhibited a significant increase from week 10 to week 14 ($p = 0.0233$), followed by a significant decrease between weeks 14–15 and 18–20 ($p = 0.0388$), indicating a dynamic behavior of this cellular population. In the empagliflozin-treated BTBR^{ob/ob} group, although the difference between weeks 10 and 18–20 did not reach statistical significance ($p = 0.0782$), the narrow confidence interval and the p -value close to 0.05 suggest a possible downward trend over time, further supported by the significant reduction observed between weeks 14–15 and 18–20 ($p = 0.0296$).

For intergroup comparisons, a statistically significant difference was observed at week 10 between BTBR WT and BTBR^{ob/ob} mice ($p = 0.0409$). At weeks 14–15, significant differences were found between BTBR WT and empagliflozin-treated BTBR^{ob/ob} mice ($p = 0.025$), as well as between BTBR WT and MSC-treated BTBR^{ob/ob} mice ($p = 0.0025$). At weeks 18–20, a significant difference was observed between BTBR WT and MSC-treated BTBR^{ob/ob} mice ($p = 0.0305$).

When excluding the 10-week time point and comparing all groups between 14–15 and 18–20 weeks using mixed-effects analysis, significant effects of time ($p < 0.0001$), treatment ($p = 0.0003$), and interaction between time and treatment ($p = 0.0085$) were observed (Fig. 2B). Tukey post hoc analyses indicated that all BTBR^{ob/ob} groups exhibited higher *c-Kit* expression compared with WT mice at 14–15 weeks ($p = 0.0078$ for untreated BTBR^{ob/ob}; $p < 0.0001$ for MSC- and empagliflozin-treated groups), but not at 18–20 weeks. Intragroup analyses demonstrated a sig-

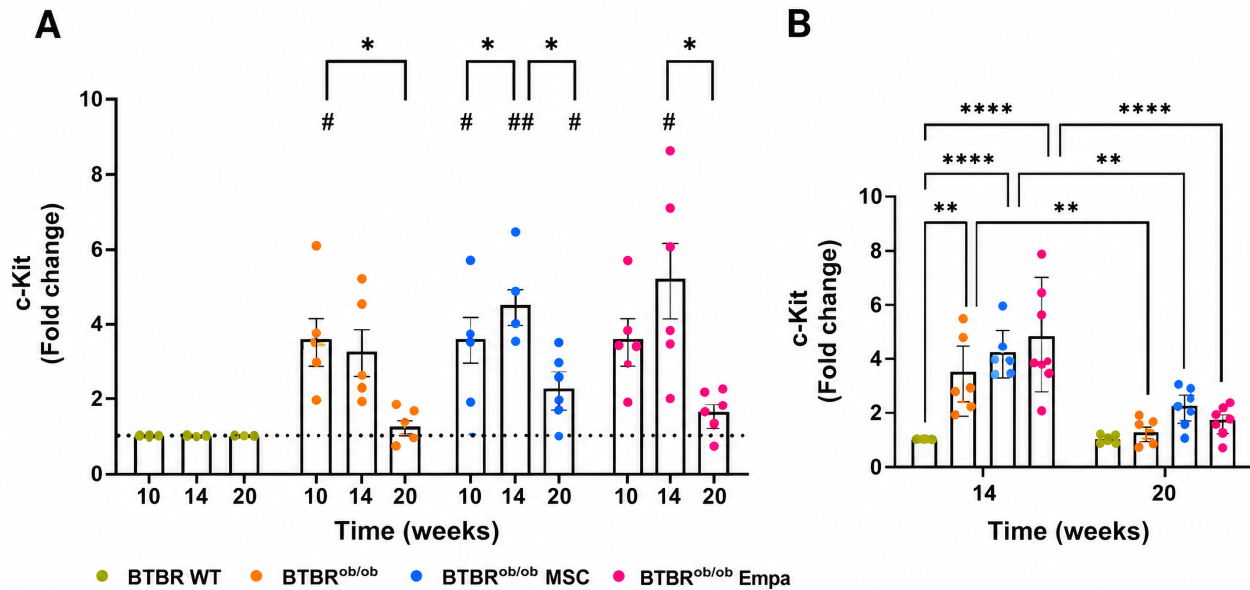


Fig. 2. *c-Kit* gene expression in the renal cortex of BTBR mice across experimental groups and time points. (A) Relative *c-Kit* expression (fold change) in BTBR wild-type (WT), untreated BTBR^{ob/ob}, mesenchymal stem cell (MSC)-treated BTBR^{ob/ob}, and empagliflozin (Empa)-treated BTBR^{ob/ob} mice at 10, 14–15, and 18–20 weeks of age. **p* < 0.05 indicates significant intragroup differences; #*p* < 0.05, ##*p* < 0.01 indicate significant differences compared with the BTBR WT group at the indicated time points. (B) Relative *c-Kit* expression (fold change) in BTBR wild-type (WT), untreated BTBR^{ob/ob}, MSC-treated BTBR^{ob/ob}, and empagliflozin-treated BTBR^{ob/ob} mice at 14–15 and 18–20 weeks of age. At 14–15 weeks, *c-Kit* expression was significantly increased in BTBR^{ob/ob} mice compared with BTBR WT, and this increase was further modulated by MSC and Empa treatments. At 18–20 weeks, *c-Kit* expression was reduced compared with 14–15 weeks across groups. *p* values are indicated as follows: ***p* < 0.01; *****p* < 0.0001. Data are presented as mean ± SEM (Standard Error of the Mean) with individual data points shown. The dotted line represents baseline expression (fold change = 1). *n* = 5–6 animals per group.

nificant decrease in *c-Kit* expression from 14–15 to 18–20 weeks in untreated BTBR^{ob/ob} mice (*p* = 0.0065), MSC-treated BTBR^{ob/ob} mice (*p* = 0.0028), and empagliflozin-treated BTBR^{ob/ob} mice (*p* < 0.0001).

Overall, these findings indicate distinct temporal patterns of *c-Kit*⁺ cell expression among the experimental groups, with the most pronounced differences emerging at the late stage of disease progression, particularly at 18–20 weeks.

3.1.2 Quantitative Analysis of the Renal Medulla

Renal medullary data initially did not follow a normal distribution, requiring Box-Cox transformation ($\lambda = -0.5$) to achieve normality. After transformation, a mixed-effects model was applied, considering time and treatment as fixed factors. The analysis demonstrated a significant effect of time (*p* = 0.0002), treatment (*p* = 0.0002), and the interaction between time and treatment (*p* = 0.001). Subsequent multiple comparisons were performed to further characterize intra- and intergroup differences over time (Fig. 3A).

In the intragroup analyses, no statistically significant differences were observed across time points. However, a trend toward reduced expression between weeks 14–15 and 18–20 was noted in the MSC-treated BTBR^{ob/ob} group (*p*

= 0.0975), as well as a trend toward increased expression between weeks 10 and 14–15 in the empagliflozin-treated BTBR^{ob/ob} group (*p* = 0.0648).

In the intergroup comparisons at 14–15 weeks, both the MSC-treated BTBR^{ob/ob} group (*p* = 0.0412) and the empagliflozin-treated BTBR^{ob/ob} group (*p* = 0.011) exhibited significantly higher values compared with BTBR WT. At 18–20 weeks, no statistically significant differences were detected among the groups.

When excluding the 10-week time point and comparing all groups between 14–15 and 18–20 weeks using mixed-effects analysis, significant effects of time (*p* = 0.0003), treatment (*p* = 0.0005), time × treatment interaction (*p* = 0.0009) were observed (Fig. 3B). Tukey post hoc analyses showed that the MSC-treated BTBR^{ob/ob} group exhibited significantly higher *c-Kit* expression at 14–15 weeks compared with untreated BTBR^{ob/ob} mice (*p* = 0.0009), empagliflozin-treated BTBR^{ob/ob} mice (*p* = 0.0007), and BTBR WT mice (*p* < 0.0001). This effect was not observed at 18–20 weeks. From 14–15 to 18–20 weeks, *c-Kit* expression was significantly downregulated in the MSC-treated BTBR^{ob/ob} group (*p* < 0.0001), whereas expression remained low in the other groups.

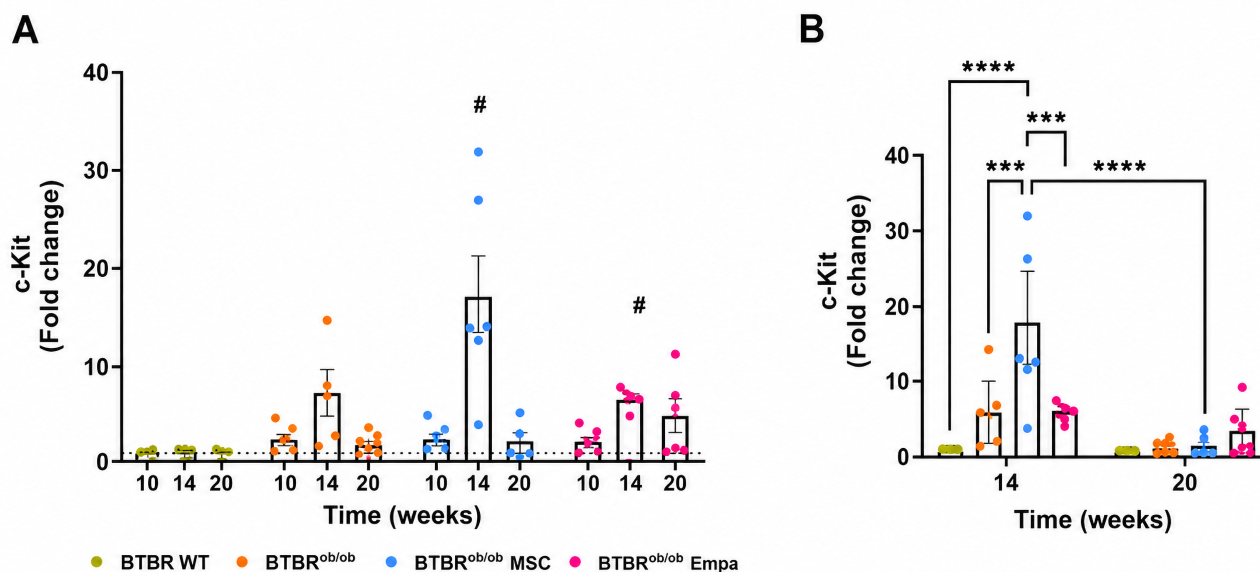


Fig. 3. *c-Kit* gene expression in the renal medulla of BTBR mice across experimental groups and time points. (A) Relative *c-Kit* expression (fold change) was evaluated in BTBR wild-type (WT), untreated BTBR^{ob/ob}, mesenchymal stem cell (MSC)-treated BTBR^{ob/ob}, and empagliflozin (Empa)-treated BTBR^{ob/ob} mice at 10, 14–15, and 18–20 weeks of age. A marked increase in *c-Kit* expression was observed at 14–15 weeks in the MSC-treated group, followed by a reduction at 18–20 weeks. Empagliflozin treatment also showed increased expression at 14–15 weeks compared with earlier time points. #*p* < 0.05 indicates intergroup significant differences compared with the BTBR WT at the indicated time points. (B) Relative *c-Kit* expression (fold change) was measured in BTBR wild-type (WT), untreated BTBR^{ob/ob}, MSC-treated BTBR^{ob/ob}, and Empa-treated BTBR^{ob/ob} mice at 14–15 and 18–20 weeks of age. At 14–15 weeks, *c-Kit* expression was significantly increased in MSC-treated BTBR^{ob/ob} mice compared with BTBR WT, untreated BTBR^{ob/ob}, and Empa-treated BTBR^{ob/ob} mice. At 18–20 weeks, *c-Kit* expression was markedly reduced across groups compared with 14–15 weeks. Significance is indicated as ****p* < 0.001 and *****p* < 0.0001. Data are presented as mean ± SEM with individual data points displayed. The dotted line indicates baseline expression (fold change = 1). *n* = 5–6 animals per group.

Similar findings have also been reported when cortical and medullary data were analyzed collectively (Supplementary Fig. 1A,B).

3.2 Immunohistochemistry (IHC) Results

3.2.1 Quantitative Analysis of Renal Cortical Tubules by IHC

Quantitative immunohistochemical analysis of the renal cortex was performed using a two-factor repeated-measures ANOVA (time and treatment). The model revealed a significant effect only for the treatment factor (*p* < 0.0001), while no significant effects were observed for time (*p* = 0.9118) or for the interaction between time and treatment (*p* = 0.4085).

In intragroup analyses, no statistically significant temporal differences were detected across all experimental groups (Fig. 4A). In the BTBR WT group, a non-significant trend toward reduced c-Kit-positive cell expression between 10 and 18–20 weeks was observed (*p* = 0.0665). Similarly, the untreated BTBR^{ob/ob} MSC-treated, BTBR^{ob/ob}, and empagliflozin-treated BTBR^{ob/ob} groups did not show relevant changes over time.

Intergroup comparisons revealed no significant differences at 10 or 14–15 weeks. However, at 18–20 weeks, both the untreated BTBR^{ob/ob} group and the empagliflozin-treated BTBR^{ob/ob} group exhibited significantly higher c-Kit expression compared with BTBR WT (*p* = 0.0315 and *p* = 0.0006, respectively). The MSC-treated BTBR^{ob/ob} group did not differ significantly from the healthy control at this time point. No other significant intergroup differences were identified.

When excluding the 10-week time point and comparing all groups between 14–15 and 18–20 weeks using two-way ANOVA, a significant main effect for treatment was observed (*p* < 0.0001) (Fig. 4B). At 14–15 weeks, MSC-treated BTBR^{ob/ob} mice exhibited higher c-Kit expression compared with untreated BTBR^{ob/ob} mice (*p* = 0.0136) and BTBR WT mice (*p* = 0.0041). This difference was sustained at 18–20 weeks (*p* = 0.0409 and *p* = 0.0008, respectively). In contrast, empagliflozin-treated BTBR^{ob/ob} mice showed no significant differences in c-Kit expression compared with untreated, MSC-treated or WT controls at either time point.

Representative immunohistochemical images of c-Kit expression in the renal cortex are shown in Fig. 4C. c-

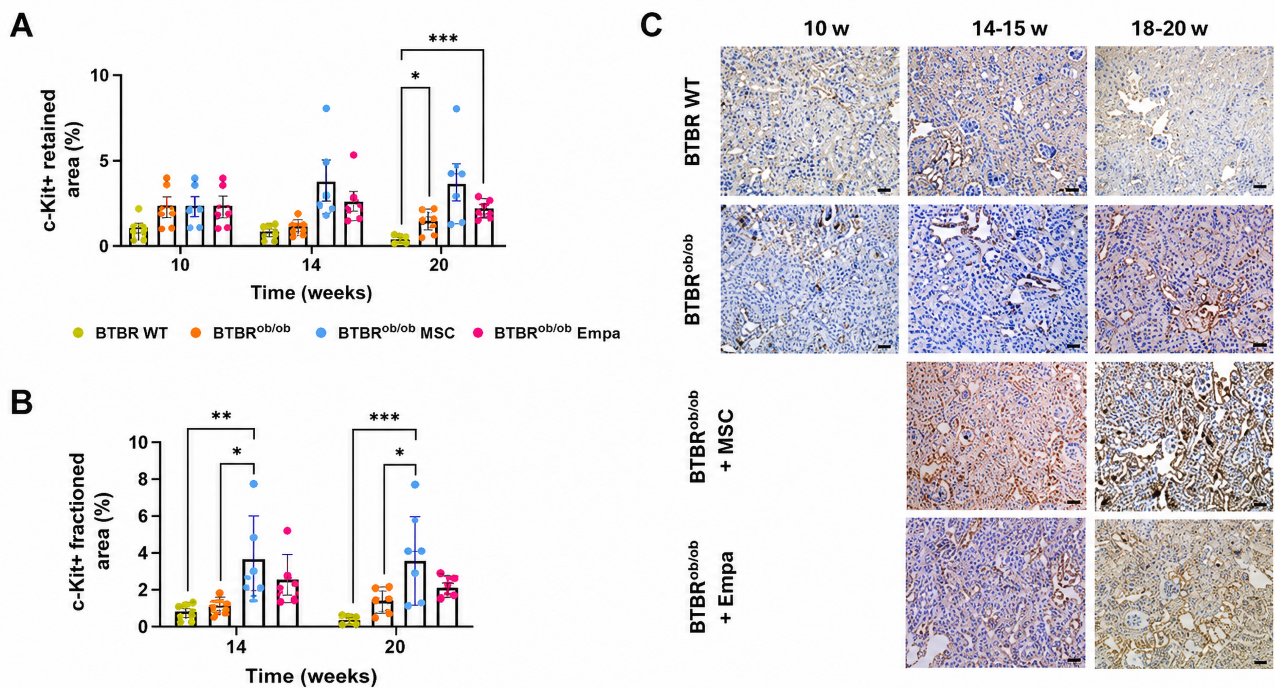


Fig. 4. Quantitative and representative immunohistochemical analysis of c-Kit expression in the renal cortex of BTBR mice. (A) Quantification of c-Kit⁺ fractional area (%) in the renal cortex of BTBR wild-type (WT), untreated BTBR^{ob/ob}, mesenchymal stem cell (MSC)-treated BTBR^{ob/ob}, and empagliflozin (Empa)-treated BTBR^{ob/ob} mice at 10, 14–15, and 18–20 weeks of age. (B) Quantitative comparisons at 14–15 and 18–20 weeks highlighting intergroup differences. (C) Representative immunohistochemistry (IHC) images of renal cortical sections from each experimental group at 10, 14–15, and 18–20 weeks. Brown staining indicates c-Kit⁺ cells. Scale bars represent 20 μ m. Data are presented as mean $\pm$ SEM with individual data points shown. Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by appropriate post hoc testing. Significance is indicated as * p < 0.05, ** p < 0.01, and *** p < 0.001. n = 6 animals per group.

Kit immunoreactivity was predominantly localized to distal tubules and a subset of collecting ducts, with no detectable expression in proximal tubules.

3.2.2 Quantitative Analysis of Renal Medullary Tubules by IHC

Quantitative immunohistochemical analysis of the renal medulla was performed using a mixed-effects model including time and treatment as factors. A significant effect was observed for treatment (p = 0.0061), whereas no significant effects were detected for time (p = 0.2934) or for the time and treatment interaction (p = 0.2683).

In intragroup comparisons, no statistically significant differences were observed across time points in any of the experimental groups (Fig. 5A). This included BTBR WT, untreated BTBR^{ob/ob}, MSC-treated BTBR^{ob/ob}, and empagliflozin-treated BTBR^{ob/ob} groups.

Intergroup comparisons using Dunnett's test revealed a statistically significant difference only at week 20 between BTBR WT and the untreated BTBR^{ob/ob} group, with higher c-Kit expression observed in diseased untreated animals (p = 0.0419). At the same time point, no signif-

icant differences were found between treated BTBR^{ob/ob} groups and controls. Although not statistically significant, higher c-Kit expression was observed in all diseased groups compared with BTBR WT at 14–15 weeks, with p values of 0.1102, 0.0834, and 0.0905 for untreated, MSC-treated BTBR^{ob/ob}, and empagliflozin-treated BTBR^{ob/ob} groups, respectively.

When excluding the 10-week time point and comparing all groups between 14–15 and 18–20 weeks using mixed-effects analysis, a significant main effect of treatment was observed (p = 0.0085) (Fig. 5B). However, post hoc analyses did not reveal any significant differences between groups.

Representative immunohistochemical images of c-Kit expression in the renal medulla are shown in Fig. 5C.

Similar findings have also been reported when cortical and medullary data were analyzed collectively (Supplementary Fig. 2A,B).

3.2.3 Quantitative Analysis of Glomeruli by IHC

Immunohistochemical analysis allowed the identification of c-Kit⁺ cells within glomerular structures. Accord-

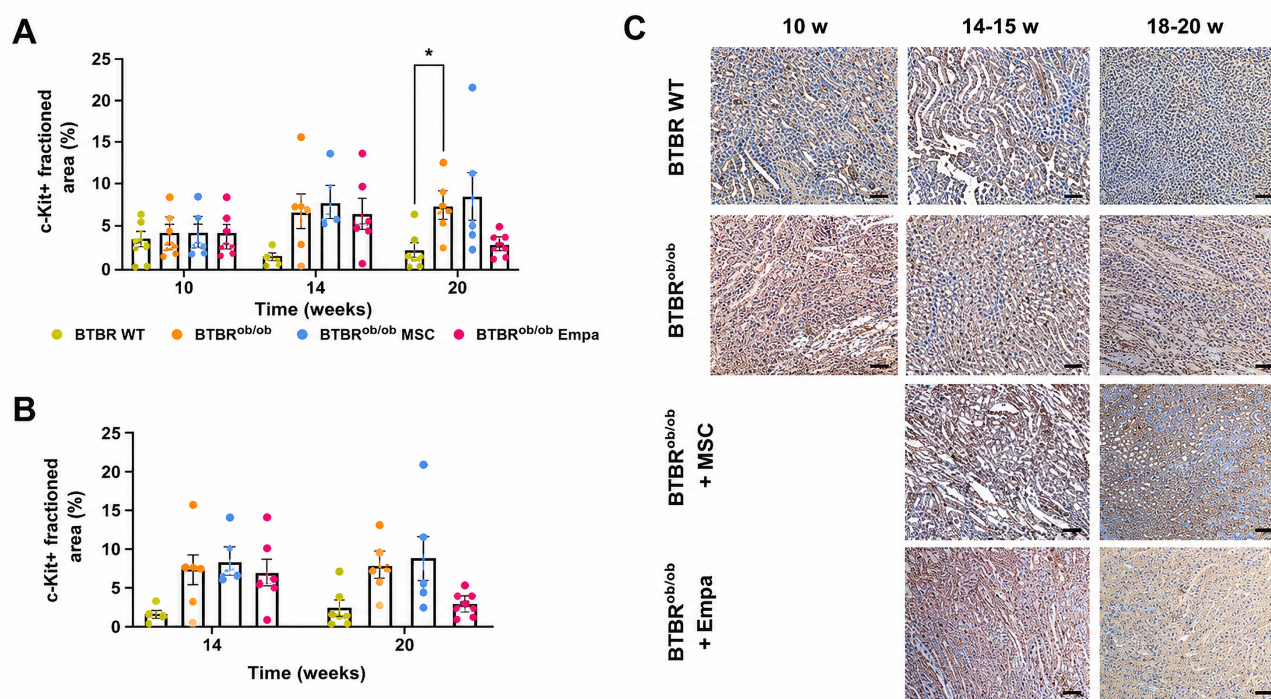


Fig. 5. Immunohistochemical quantification and representative images of c-Kit expression in the renal medulla of BTBR mice. (A) Quantification of c-Kit⁺ fractional area (%) in the renal medulla of BTBR wild-type (WT), untreated BTBR^{ob/ob}, mesenchymal stem cell (MSC)-treated BTBR^{ob/ob}, and empagliflozin (Empa)-treated BTBR^{ob/ob} mice at 10, 14–15, and 18–20 weeks of age. (B) Quantitative comparison at 14–15 and 18–20 weeks. (C) Representative IHC images of renal medulla sections from each experimental group at 10, 14–15, and 18–20 weeks. Brown staining indicates c-Kit⁺ cells within the medullary tubular compartment. Scale bars represent 20 μ m. Data are presented as mean $\pm$ SEM with individual data points shown. Statistical analysis was performed using mixed-effects model followed by appropriate post hoc testing. * $p < 0.05$ indicates statistically significant differences between the indicated groups. $n = 4$ –6 animals per group.

ingly, the proportion of positive glomeruli relative to the total number of analyzed glomeruli was quantified and expressed as a percentage.

Statistical analysis using a mixed-effects model showed no significant effects of time ($p = 0.5318$), treatment ($p = 0.1838$), or time and treatment interaction ($p = 0.8121$) (Fig. 6A). In intragroup analyses, no statistically significant temporal differences were detected in any of the experimental groups, with the proportion of positive glomeruli remaining stable over time.

In intergroup comparisons, a single statistically significant difference was identified at 14 weeks, where the empagliflozin-treated BTBR^{ob/ob} group exhibited a higher proportion of c-Kit⁺ glomeruli compared with the BTBR WT group ($p = 0.0362$). No other significant differences were observed at any of the evaluated time points.

When excluding the 10-week time point and comparing all groups between 14–15 and 18–20 weeks, no significant differences were observed among groups (Fig. 6B).

Representative immunohistochemical images of c-Kit expression in the glomeruli are shown in Fig. 6C.

4. Discussion

In this study, we evaluated the temporal modulation of c-Kit expression in the BTBR^{ob/ob} murine model of DKD, T2DM, and obesity following treatment with MSCs or empagliflozin, compared with untreated diabetic and obese mice as well as lean controls. Our findings demonstrate that c-Kit expression is dynamically regulated over time and is differentially influenced by therapeutic intervention. Specifically, MSC treatment was associated with an early and transient increase in c-Kit gene expression, whereas empagliflozin exerted a more modest and delayed modulatory effect. At the protein level, treatment, particularly MSC therapy, was associated with sustained differences in cortical c-Kit expression.

The localization of c-Kit⁺ cells predominantly in distal tubular segments and collecting ducts is consistent with previous reports suggesting that these cells may represent a distinct renal epithelial or progenitor-like population. Their absence in proximal tubules further supports the concept of compartment-specific distribution within the kidney. The detection of c-Kit⁺ cells within glomeruli, particularly in empagliflozin-treated animals at 14–15 weeks, was an un-

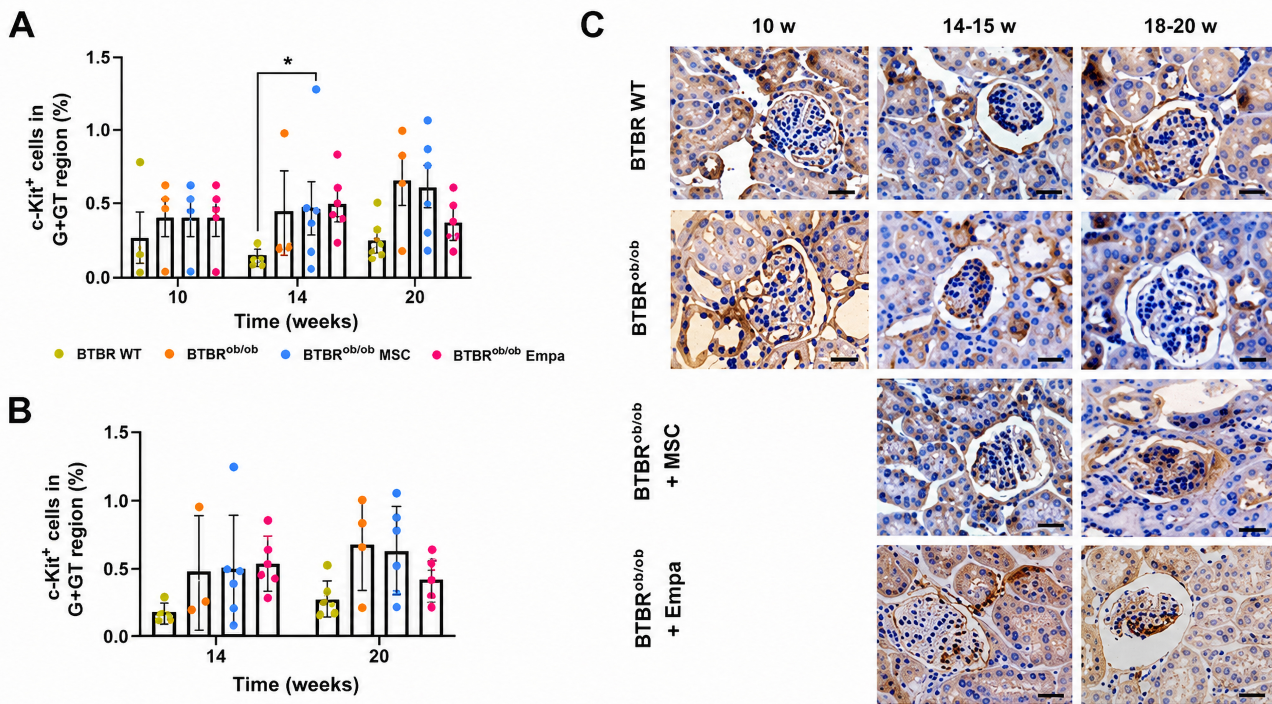


Fig. 6. Quantitative and representative immunohistochemical analysis of glomerular c-Kit⁺ cells in BTBR mice. (A) Quantification of c-Kit⁺ cells in the glomerular tuft (G+/GT region, %) in BTBR wild-type (WT), untreated BTBR^{ob/ob}, mesenchymal stem cell (MSC)-treated BTBR^{ob/ob}, and empagliflozin (Empa)-treated BTBR^{ob/ob} mice at 10, 14–15, and 18–20 weeks of age. (B) Quantitative comparison at 14–15 and 18–20 weeks. (C) Representative immunohistochemistry (IHC) images of glomeruli from each experimental group at 10, 14–15, and 18–20 weeks. Brown staining indicates c-Kit⁺ cells within the glomerular compartment. Scale bars represent 10 μ m. Data are presented as mean $\pm$ SEM with individual data points shown. Statistical analysis was performed using mixed-effects model followed by appropriate post hoc testing. * $p < 0.05$ indicates statistically significant differences between the indicated groups. $n = 4$ –6 animals per group.

expected finding and warrants further investigation, as this compartment has not been consistently associated with c-Kit expression in prior studies.

The biological role of c-Kit⁺ cells in the adult kidney remains incompletely understood [32]. Although there is ongoing debate regarding the identity and abundance of renal cell populations, particularly given the presence of multiple progenitor pools and differentiation pathways [33], single-cell transcriptomic analyses have identified c-Kit expression in intercalated cells of the collecting duct in mammalian kidneys [34]. In addition, healthy kidneys harbor resident immune cells, including lymphocytes and macrophages [35]. During disease progression, c-Kit expression has also been observed in mast cells [36] and may contribute to fibrotic processes in chronic kidney disease [37]. In this context, targeting the c-Kit/SCF pathway has been shown to reduce the expression of inflammatory and fibrotic markers in preclinical models of interstitial fibrosis and chronic kidney disease [38].

Conversely, experimental evidence suggests that c-Kit signaling may contribute to renal repair processes, although its role appears to be highly dependent on both the biolog-

ical context and the specific cell-type. Our group previously demonstrated that c-Kit⁺ cells isolated from neonatal rat kidneys, when delivered intra-aortically to adult rats subjected to ischemia-reperfusion injury, resulted in improved renal function, increased tubular cell proliferation, reduced kidney injury scores, and multicompartmental engraftment [22]. Consistently, in ischemia-reperfusion models, c-Kit/SCF signaling has been shown to promote epithelial cell survival through autocrine mechanisms [39].

In a puromycin-induced nephrotic syndrome model, the administration of c-Kit⁺ cells was associated with modulation of autophagy and attenuation of podocyte foot process effacement [23]. Moreover, when combined with MSCs therapy, c-Kit⁺ cells improved renal function and blood pressure, while reducing ventricular stiffness in a swine model of CKD induced by 5/6 embolization-mediated nephrectomy and heart failure [40]. In feline models, the administration of kidney-derived c-Kit⁺ cells has also been demonstrated to be safe in CKD settings [41].

Taken together, these findings support the potential translational relevance of c-Kit⁺ cells and suggest that their identification and characterization in the human kidney may

represent a promising avenue for the development of cell-based therapeutic strategies [18].

In our study, we observed modulation of c-Kit⁺ cells in a DKD model, a condition with high global prevalence as a leading cause of CKD. DKD is a progressive disorder characterized by glomerulosclerosis, arteriolar hyalinosis, and tubulointerstitial fibrosis [42]. However, in its early stages, such as those observed in the BTBR^{ob/ob} model, DKD is marked by increased glomerular volume and tubular epithelial hypertrophy. Our previous studies have demonstrated increased expression of E-cadherin and podocyte markers in BTBR^{ob/ob} mice [29,31], as well as elevated expression of SGLT2 in proximal tubules [31]. These findings may partially explain the detection of c-Kit⁺ cells in BTBR^{ob/ob} mice, particularly in the renal medulla, compared with lean controls.

In BTBR^{ob/ob} mice treated with MSC or empagliflozin, prior work from our group also demonstrated improvements in albuminuria and morphological parameters, including attenuation of mesangial matrix expansion, preservation of podocyte number, and reductions in oxidative stress and apoptosis [29,30]. In this context, the modulation of c-Kit expression observed in the present study may be associated with these broader therapeutic effects. However, as functional or lineage-specific analyses were not performed, these findings should be interpreted as associative rather than causal.

MSCs can be isolated from multiple sources and administered through different routes, doses, and treatment regimens in DKD, with consistent functional, structural, and molecular benefits reported in preclinical models [8,43]. Although intravenous, intra-arterial, and intrarenal delivery routes have been extensively explored [44], we selected the intraperitoneal route based on prior evidence demonstrating effective homing of MSCs to inflamed renal tissue via systemic circulation [45]. While less targeted than intra-arterial delivery route, this approach provides a practical balance between feasibility and therapeutic relevance. Notably, intraperitoneal transplantation administration has been associated with prolonged MSC survival and renal protection primarily through paracrine mechanisms, even in the absence of direct renal engraftment [29,30,46].

MSCs may modulate c-Kit expression through indirect effects on key signaling pathways and biological processes, as well as through potential direct interactions with c-Kit⁺ cells. Experimental evidence from co-culture systems, particularly in cardiac models, suggests that MSCs can promote c-Kit⁺ cell proliferation, migration, and differentiation, at least in part via modulation of the c-Kit/SCF signaling pathway [47,48].

A critical determinant of stem cell therapy efficacy in diabetes is glycemic control, as persistent hyperglycemia may attenuate therapeutic benefits, as previously observed in individuals with poorly controlled type 1 diabetes mellitus [49]. In line with these observations, our previous

study using MSC monotherapy (administered at 8 and 10 weeks of age) in BTBR^{ob/ob} mice demonstrated efficacy up to 14 weeks despite ongoing hyperglycemia and weight gain, which may explain the lack of sustained effects at 20 weeks [30]. In the present study, although hyperglycemia persisted throughout the protocol, MSC-treated BTBR^{ob/ob} mice exhibited increased c-Kit⁺ cell expression, particularly in the renal cortex, up to 20 weeks. These findings suggest that early MSC exposure may exert lasting effects on cellular dynamics, independent of metabolic control. However, this interpretation remains speculative and requires further investigation. Although MSC monotherapy has been reported to improve glucose control in both pre-clinical [8] and clinical studies [50], inconsistent efficacy has also been observed in these settings [30,51].

Empagliflozin, a sodium-glucose cotransporter 2 (SGLT2) inhibitor, exerts multiple renal effects by reducing sodium and glucose reabsorption in the proximal tubule, thereby promoting natriuresis, glycosuria, and osmotic diuresis [52]. It also inhibits sodium-hydrogen exchanger 3 (NHE3), further enhancing natriuresis [53]. These effects translate into important hemodynamic changes, including reduced intraglomerular pressure via modulation of afferent and efferent arteriolar tone [54]. In addition, SGLT2 inhibitors attenuate inflammation, oxidative stress, and fibrosis, while preserving podocyte number and reducing mesangial matrix expansion [28,29]. They have also been shown to restore autophagic flux [55], and reduce apoptosis [56] and cellular senescence [57], ultimately slowing CKD progression [58] and improving cardiovascular outcomes [59]. Therefore, empagliflozin may indirectly modulate c-Kit⁺ cell dynamics by targeting these signaling pathways and biological processes, thereby contributing to a microenvironment that may be more favorable for progenitor cell maintenance.

In our previous study, empagliflozin achieved time-dependent glycemic control but did not significantly affect weight gain [29]. Its effects on c-Kit expression were more evident at later time points, particularly in the renal cortex. These findings may reflect the delayed impact of metabolic and hemodynamic modulation on renal cellular dynamics [60]. We also observed low-frequency c-Kit immunoreactivity within glomeruli, particularly in empagliflozin-treated animals at 14-15 weeks. As this finding has not been consistently reported in the literature [19,20], it warrants further investigation. Podocyte regeneration remains a subject of debate, as parietal epithelial cells appear to have limited capacity to generate new podocytes in diseased kidneys [61]. Nevertheless, recent evidence indicates that targeting pathways such as GSK3 [62] and the renin [63,64] may promote podocyte formation.

SGLT2 inhibitors may further confer renoprotection by improving renal morphology, as evidenced by cortical enlargement detected by computed tomography in patients with diabetes [65]. Such structural changes may, at least in

part, contribute to the modulation of c-Kit expression observed in our study.

5. Limitations

This study has several limitations that should be acknowledged. First, the sample size was relatively small, reflecting the inherent biological constraints of the BTBR^{ob/ob} model, including reduced breeding efficiency, increased fragility, and higher mortality during longitudinal follow-up. In addition, a priori sample size calculation was not performed, which may limit statistical power and generalizability. To mitigate these limitations, we employed statistical approaches appropriate for small and partially incomplete datasets, including mixed-effects models, and present individual data points to ensure transparency. Nevertheless, the findings should be interpreted as exploratory and require validation in larger cohorts.

Second, the study was limited to the assessment of c-Kit expression at the gene and protein levels, without functional characterization of c-Kit⁺ cells or direct correlation with histopathological and functional outcomes at the individual-animal level. Therefore, no conclusions can be drawn regarding the biological activity, lineage, or direct contribution of these cells to renal repair. Although previous studies from our group reported metabolic, functional, and histopathological improvements following empagliflozin and MSC therapy in the BTBR^{ob/ob} model [29,30,31], the current study was conducted as a retrospective analysis focused exclusively on renal c-Kit expression using archived biological specimens obtained from these previously characterized cohorts. Because c-Kit analyses were not prospectively planned alongside the original functional assessments, direct animal-by-animal correlation analyses could not be performed. In addition, the remaining biological material was limited, as several RNA samples and paraffin-embedded tissue blocks had already been exhausted during previous and current investigations, preventing further histological and molecular correlation studies.

Third, mechanistic pathways, including SCF/c-Kit signaling and downstream inflammatory, oxidative, or fibrotic mediators, were not directly evaluated. Fourth, lineage-tracing or loss-of-function approaches were not performed, precluding assessment of causality.

Finally, a combined treatment group receiving MSCs and empagliflozin was not included, which would be important to explore potential additive or synergistic effects. These aspects represent important directions for future research.

6. Conclusions

In conclusion, this study demonstrates that MSC therapy and empagliflozin are associated with distinct temporal patterns of c-Kit expression in a preclinical model of DKD. MSC treatment was associated with an early and dynamic

modulation of c-Kit expression, whereas empagliflozin exhibited more delayed effects, particularly in the renal cortex. These findings indicate that c-Kit⁺ cells are dynamically regulated during DKD progression and may reflect changes in renal cellular responses to different therapeutic interventions.

Given the absence of functional and mechanistic analyses, these results should be interpreted as associative and hypothesis-generating. Further studies are required to determine the functional role of c-Kit⁺ cells, elucidate underlying signaling pathways, and evaluate whether targeting this cellular population may have therapeutic relevance. Additionally, investigation of combination strategies integrating regenerative and pharmacological approaches may provide new insights into optimizing treatment for DKD.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author, ÉBR.

Author Contributions

Conceptualization, ÉBR; data curation, GEJ; RMCB; CSS; SB; MLF; formal analysis, GEJ; ÉBR; funding acquisition, ÉBR; investigation, GEJ; RMCB; CSS; SB; MLF; SAG; and ÉBR; project administration, GEJ and ÉBR; validation, GEJ and ÉBR; writing-original draft, GEJ; SAG; and ÉBR; writing-review and editing, GEJ; SAG; and ÉBR; final approval: ÉBR. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental procedures were approved by the institutional Animal Care and Use Committee and were conducted in accordance with ethical guidelines. The study was registered under the Ethics Committee on the Use of Animals of Hospital Israelita Albert Einstein, São Paulo, SP, Brazil (protocol number 4900-21), in accordance with ARRIVE guidelines. The international governing standards are in accordance with the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) and the 3Rs framework.

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Conflicts of Interest

The authors declare no conflicts of interest.

Declaration of AI and AI-Assisted Technologies in the Writing Process

The AI tool was used exclusively to enhance the quality of written English and did not contribute to the generation of scientific data, interpretation of results, study design, or formulation of conclusions. For the graphical abstract: original figure created by the authors using PowerPoint, with refinements assisted by ChatGPT 5.0. After using this tool, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/FBL51501>.

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