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RESEARCH PAPER

# SGLT2 inhibition promotes glomerular repopulation by cells of renin lineage in experimental kidney disease

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## Abstract

**Aim:** Sodium glucose co-transporter-2 (SGLT2) inhibitors stimulate renal excretion of sodium and glucose and exert renal protective effects in patients with (non-)diabetic chronic kidney disease (CKD) and may as well protect against acute kidney injury (AKI). The mechanism behind this kidney protective effect remains unclear. Juxtaglomerular cells of renin lineage (CoRL) have been demonstrated to function as progenitors for multiple adult glomerular cell types in kidney disease. This study assesses the impact of SGLT2 inhibition on the repopulation of glomerular cells by CoRL and examines their phenotypic commitment.

**Methods:** Experiments were performed in Ren1cre-tdTomato lineage-trace mice. Either 5/6 nephrectomy (5/6NX) modeling CKD or bilateral ischaemia reperfusion injury (bIRI) mimicking AKI was applied, while the SGLT2 inhibitor empagliflozin (10 mg/kg) was administered daily via oral gavage for 14 days.

**Results:** Both 5/6NX and bIRI-induced kidney injury increased the number of glomerular CoRL-derived cells. SGLT2 inhibition improved kidney function after 5/6NX, indicated by decreased blood creatinine and urea levels, but not after bIRI. In line with this, empagliflozin in 5/6NX animals resulted in less glomerulosclerosis, while it did not affect histopathological features in bIRI. Treatment with empagliflozin resulted in an increase in the number of CoRL-derived glomerular cells in both 5/6NX and bIRI conditions. Interestingly, SGLT2 inhibition led to more CoRL-derived podocytes in 5/6NX animals, whereas empagliflozin-treated bIRI mice presented with increased levels of parietal epithelial and mesangial cells derived from CoRL.

**Conclusion:** We conclude that SGLT2 inhibition by empagliflozin promotes CoRL-mediated glomerular repopulation with selective CoRL-derived cell types depending on the type of experimental kidney injury. These findings suggest a previously unidentified mechanism that could contribute to the renoprotective effect of SGLT2 inhibitors.

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KEYWORDS
cells of renin lineage, kidney repair, progenitor, RAAS, regeneration, SGLT2 inhibition

## 1 | INTRODUCTION

Sodium glucose cotransporter 2 (SGLT2) inhibitors were originally developed as oral anti-hyperglycemia agents as they prevent glucose reabsorption at the renal proximal tubules.[1] Following animal studies[2,3] and, later, clinical trials in patients with type 1 and 2 diabetes, a remarkable renoprotective effect was observed in both the short- and long-term.[4,5] Compared to placebo, SGLT2 inhibition reduces albuminuria, diminishes major decline in glomerular filtration rate, leads to fewer clinically relevant renal events and even shows lower numbers of renal replacement therapies needed in subjects. Strikingly, more recent reports on clinical trials including non-diabetic CKD participants report consistent renal benefits provided by SGLT2 inhibition.[6,7] SGLT2 inhibitors are also considered as potential drugs in the prevention of acute kidney injury (AKI). However, no clear outcome has been established as some randomized trials using SGLT2 inhibitors described lower AKI incidence, while other post-marketing reports on SGLT2 inhibitory treatment warn for potential AKI risk, particularly in patients suffering from diabetes type 2.[8,9] Additional investigations are now essential to elucidate the relevant mechanism behind the kidney-related advantage of SGLT2 inhibitory treatment and in defining for which patient group these drugs are advisable.

For a long time, it has been the consensus that humans assemble their final pool of nephrons before birth,[10] offering major limitations for glomerular recovery after kidney damage-induced cell depletion. Podocytes for illustration, a fundamental cell type in the glomerulus providing vascular barrier function, tend to have a quiescent phenotype; they display a terminally arrested cell cycle and therefore fail to proliferate even after injury.[11] These observations led to a quest as to whether glomerular cells could be replenished from other innate sources. Interestingly, in recent years, some existing cell populations with adult progenitor propensity within the kidney have been reported including parietal epithelial cells (PEC)[12] and cells of the renin lineage (CoRL).[13,14]

CoRL are eminent during nephrogenesis where they determine the construction and branching of the kidney's arterioles and subsequently differentiate primarily into a smooth muscle cell-like mural cell population.[15,16] In the developed kidney only a limited number of these cells reside in the walls of the afferent arterioles in the juxtaglomerular apparatus (JGA) serving to express and secrete renin. Renin is a proteolytic, rate-limiting enzyme crucial for the activation of the renin-angiotensin-aldosterone-system (RAAS), a cascade modulating the blood pressure and electrolyte balance.[17] Several rodent studies have demonstrated that in times of experimental renal disease, but also aging, a subset of the renin-producing juxtaglomerular CoRL show remarkable plasticity and can proliferate and migrate inward the glomerulus to replenish various cell types like mesangial cells, podocytes, and parietal epithelial cells.[18–20]

SGLT2 inhibition affects tubuloglomerular feedback and electrolyte balance in patients by altering the reuptake of sodium and glucose.[21,22] Considering the stimulatory effect of sodium and volume depletion on renin release, we hypothesized that SGLT2 inhibition could also have an enhancing effect on the CoRL-induced endogenous glomerular repair. To address this question, we examined SGLT2 inhibition versus vehicle treatment in a 5/6 nephrectomy (5/6NX) CKD mouse model in comparison to a renal ischaemia–reperfusion injury mouse model.

## 2 | RESULTS

### 2.1 | 5/6 Nephrectomy and ischaemia–reperfusion injury increase glomerular CoRL-derived cell number

First, we determined whether CoRL-mediated glomerular cell repopulation could be observed in a 5/6NX induced Ren1cre-tdTomato mouse model for CKD, coherently with observations previously made by others.[14] Figure 1A illustrates the experimental setup, and Figure 1C the corresponding blood urea values observed as an indication of induced kidney injury. 5/6NX resulted in over 7-fold increased numbers of CoRL-derived cells in the glomerulus, while in healthy controls CoRL were almost exclusively found outside of the glomerular tuft in the juxtaglomerular apparatus (JGA) (Figure 1D,E, $p < 0.001$). Next, we aimed to assess whether also a non-glomerular injury model such as the acute kidney injury model bilateral ischaemia–reperfusion injury (bIRI) would affect CoRL migration. Surprisingly, the histological evaluation showed an eminent similar increment of tomato-positive cells in the glomeruli of bIRI-treated animals, in contrast to virtually no CoRL within glomeruli at baseline ($p < 0.001$).

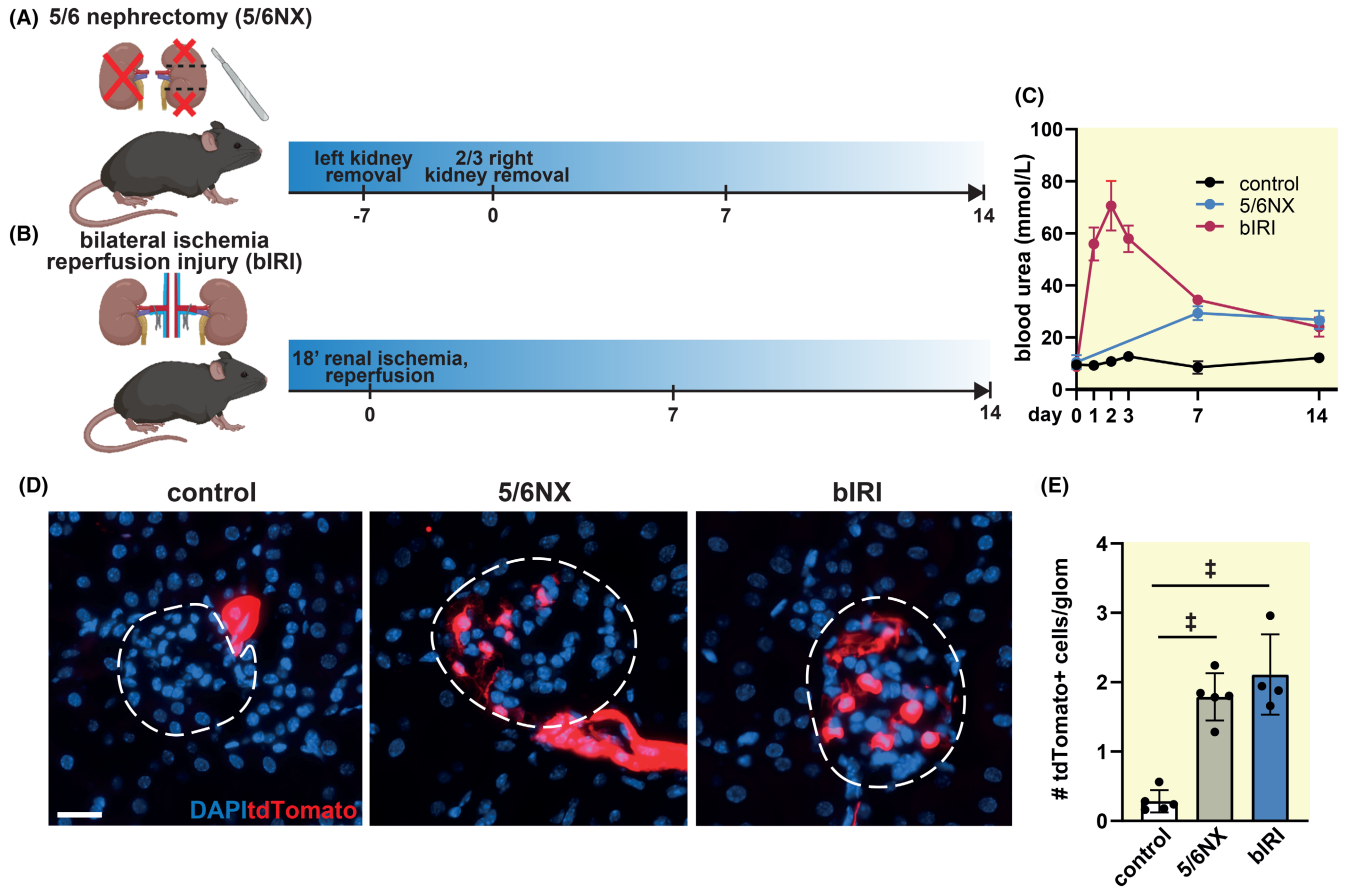


FIGURE 1 Different kidney injury mouse models lead to an increase in glomerular CoRL-derived cells. (A) Experimental setup of kidney injury models 5/6 nephrectomy (5/6NX) and (B) bilateral ischaemia reperfusion injury (bIRI). (C) Blood urea values as a measurement of kidney function of kidney-injured mice during 14 day experimental time span. (D) Representative pictures of tdTomato CoRL-signal within glomeruli (dashed lines) in different kidney injury models, bar = 25 μm, and corresponding quantification (E). Data are represented as mean ± SD, $n = 4–5$, $^{‡}p < 0.001$.

## 2.2 | SGLT2 inhibition preserves kidney function in the 5/6NX model but not in bIRI

To examine the effects of SGLT2 inhibition on CoRL-mediated glomerular repopulation, we next performed the 5/6NX and bIRI models in the Ren1cre-tdTomato mouse strain while administering the SGLT2 inhibitor empagliflozin or a vehicle control for two weeks (Figure 2A,B). We assessed the body weight of the animals during the complete experiment and observed a trend of reduced weight loss after surgery upon SGLT2 inhibition (Figure 2C). Although not statistically significant, it is suggestive of improved recovery related to empagliflozin intake.

Evaluation of urinary glucose levels on day 14 demonstrated inhibited renal glucose reuptake, indicating an effective dosage of the SGLT2 inhibitor in both models (Figure 2D). To assess kidney function and blood glucose levels, tail vein blood samples were collected at different time points post-surgery. As expected, blood glucose levels slightly increased throughout the experimental period in both kidney injury mouse models. However, in line with non-diabetic patients using empagliflozin,[23] blood glucose did not change upon empagliflozin or vehicle treatment in both the 5/6NX and bIRI groups (Figure 2E).

Day 14 plasma creatinine levels in 5/6 nephrectomized animals treated with SGLT2 inhibitor were lower compared to vehicle controls (Figure 2F, $p < 0.05$), while empagliflozin did not have an effect on plasma creatinine in the ischaemia–reperfusion injury AKI model. Additionally, we observed an increase from baseline in blood urea levels as a result of the kidney injury in both models (Figure 2G). In bilateral ischaemia reperfusion injury, empagliflozin treatment did not affect blood urea levels within the 14-day time span of the experiment (18.4 ± 3.1 vs. 19.4 ± 2.7 mmol/L on day 14). In mice that underwent a 5/6NX, however, empagliflozin intake resulted in lower urea levels (16.8 ± 2.18 mmol/L) compared to vehicle-treated littermates (20.4 ± 3.11 mmol/L) at day 14 ($p < 0.05$). Taken together, these data suggest ameliorated kidney function recovery facilitated by SGLT2 inhibition.

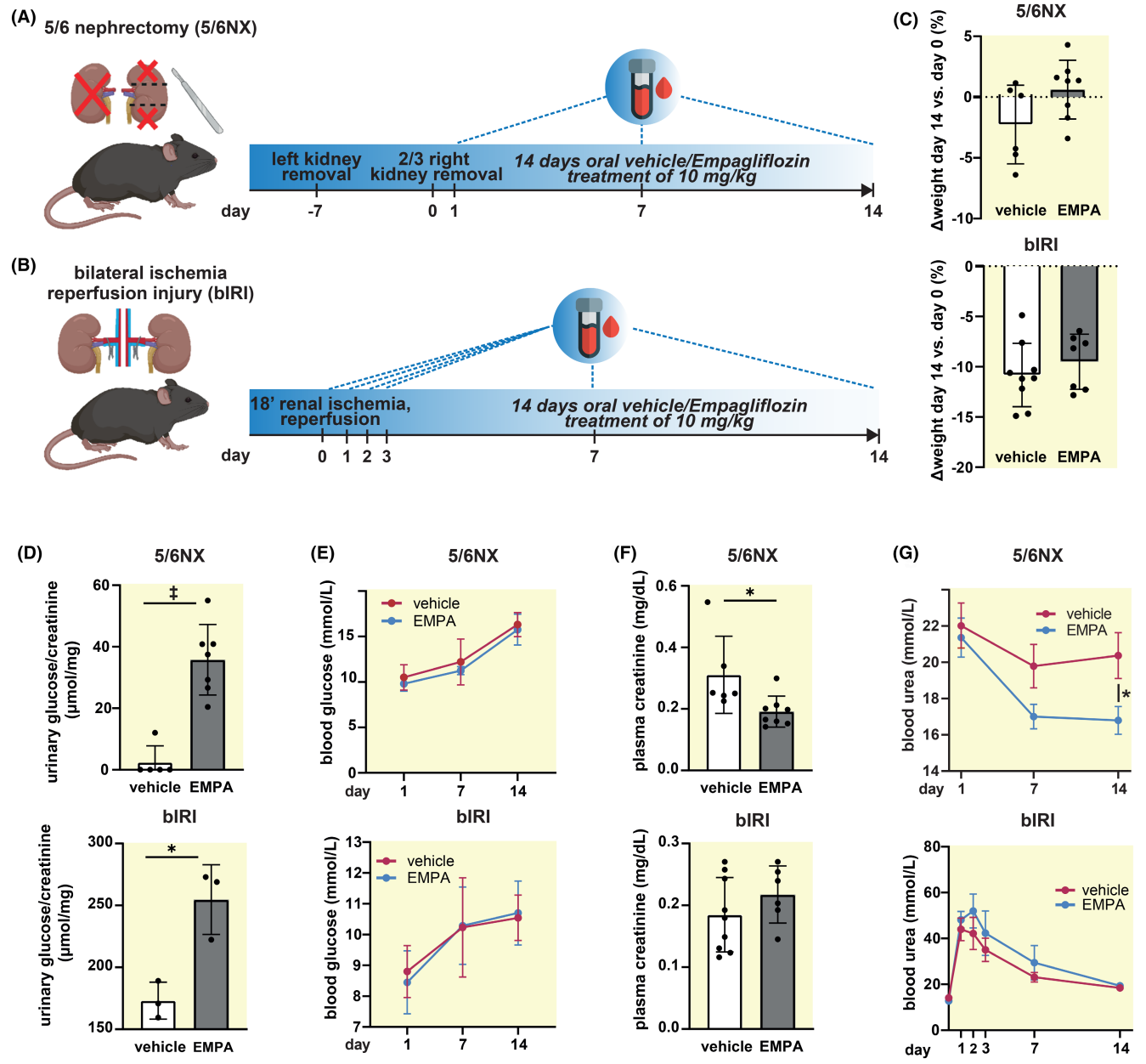


FIGURE 2 Homeostasis measurements on influence of SGLT2 inhibition in different experimental kidney injury models. (A) Experimental setup of kidney injury models 5/6NX and (B) bIRI with a 14 day treatment. (C) Weight changes during experimental window in 5/6NX and bIRI mice with control (vehicle) and SGLT2 inhibitory treatment (EMPA). (D) Day 14 urinary glucose measurements in 5/6NX and bIRI exposed animals with and without SGLT2 inhibitory treatment shows effective dosage of empagliflozin. (E) Blood glucose levels in 5/6NX and bIRI mice with and without SGLT2 inhibitory treatment. (F) Day 14 plasma creatinine values and (G) blood urea values as a measurement of kidney function in 5/6NX and bIRI mice with control or SGLT2 inhibitory treatment. Data are represented as mean ± SD, $n = 6–9$, $^*p < 0.05$.

## 2.3 | Decreased glomerulosclerosis upon SGLT2 inhibition in 5/6NX mouse model

Two weeks after the start of daily SGLT2 inhibitory treatment, kidneys of all mice were harvested to analyze histopathological tissue injury. As expected after subtotal nephrectomy, all animals in the 5/6NX group showed severe tissue injury hallmarks like glomerular hypertrophy (Figure 3A,D, as compared to mean healthy control glomerular size: 1752 µm$^2$) and inflammatory cell infiltration (Figure 3C,G), that was unaffected by empagliflozin. Remarkably, glomerulosclerosis was reduced in SGLT2 inhibitor-treated 5/6 nephrectomized animals, as indicated by a 50% decrease in glomerulosclerosis score compared to vehicle controls ($p < 0.05$, Figure 3E) In line with this, total kidney Picro Sirius

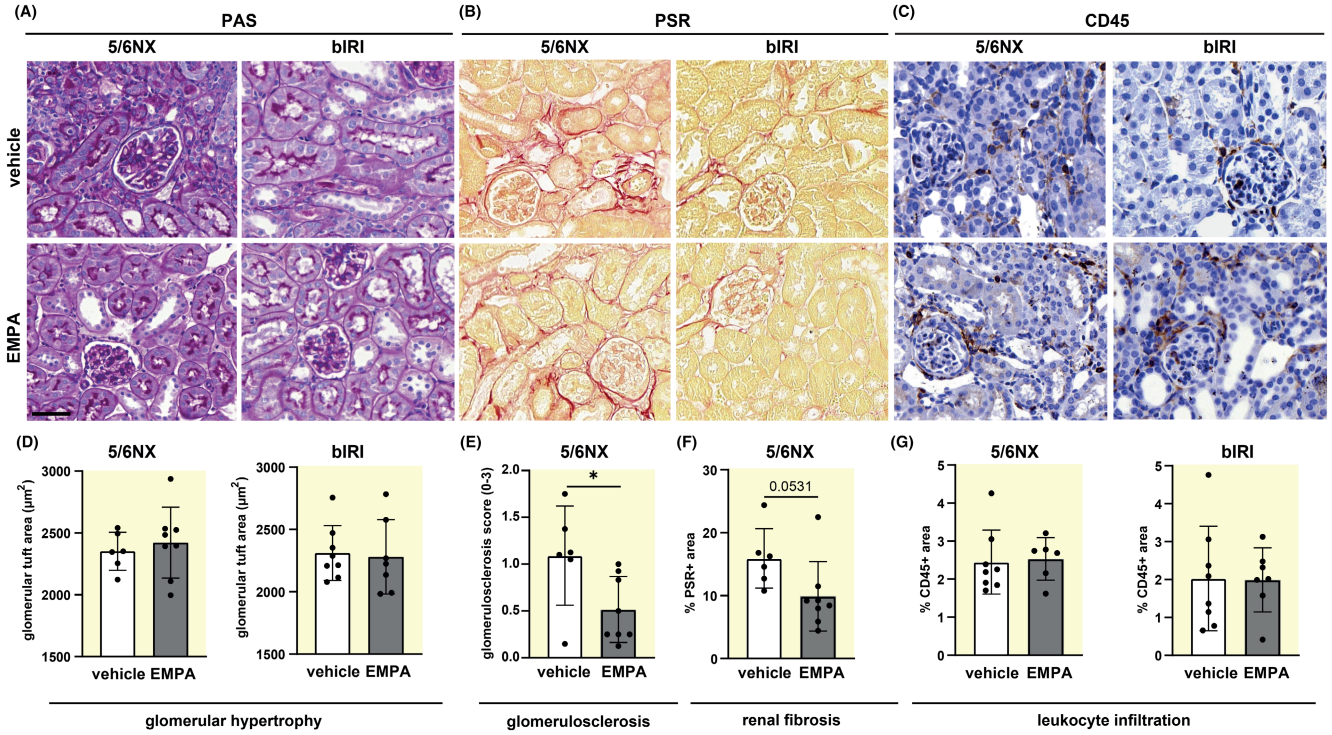


**FIGURE 3** Injury assessments of 5/6NX and bIRI-exposed mice upon SGLT2 inhibitory treatment. (A) Representative Periodic Acid-Shiff (PAS) images indicate glomerular hypertrophy as quantified in (D) in both 5/6NX and bIRI which is not further affected by SGLT2 inhibition (EMPA). (B) Picro Sirius Red (PSR) stainings for collagen indicate less renal fibrosis in 5/6NX upon SGLT2 inhibition. (C) CD45 stainings for leukocyte infiltration analysis. (E) semi-quantitative glomerulosclerosis score based on PAS images, in which 0 means <10% of glomeruli affected, 1: 10%–25% affected, 2: 26%–50% affected and >50% of glomeruli affected. (F) Quantification of (B). (G) Quantification of (C). Bar = 100 µm, data are represented as mean ± SD, $n = 6–8$ *$p < 0.05$.

Red (PSR) staining, indicative of interstitial fibrosis, showed a strong trend toward decreased renal fibrosis upon SGLT2 inhibition in the 5/6NX group ($p = 0.0531$, Figure 3F).

Consistent with kidney function data that did not change dependent on SGLT2 inhibition (Figure 2F,G), we also did not observe any empagliflozin-mediated effects on kidney fibrosis in the bIRI group (Figure S1A), nor any changes in renal tubular damage by scoring tubular injury hallmarks like tubular remodeling, necrotic casts, tubular dilatation, loss of brush boarders, and fibroblast infiltration (Figure S1B).

## 2.4 | SGLT2 inhibition increases number of glomerular CoRL-derived cells in both CKD and AKI mouse models

Next, we sought to determine the effect of SGLT2 inhibition on the capacity of CoRL to populate injured glomeruli. To that end, histological analysis of endogenous tdTomato signal within glomeruli was performed on both kidney disease mouse models with either vehicle or empagliflozin treatment (Figure 4A). In 5/6NX animals receiving control treatment, on average, 1.392 ± 0.473 cells per glomerulus were from CoRL origin. Strikingly, SGLT2 inhibition led to an increased number of CoRL-derived intraglomerular cells, measured by over twice as much tdTomato-positive glomerular cells compared to vehicle control glomeruli (3.458 ± 0.879, Figure 4B, $p < 0.001$). In order to establish the effect of SGLT2 inhibition on CoRL in a model for AKI, we performed the same analysis in bIRI-exposed mice. After vehicle treatment, 1.613 ± 0.349 cells per glomerulus presented with tdTomato signal. Surprisingly, empagliflozin treatment in the bIRI group led to an increment of tdTomato-positive glomerular cells as well (3.558 ± 0.6821 cells per glomerulus, Figure 4B, $p < 0.0001$). These data demonstrate that glomerular repopulation by CoRL is stimulated as a consequence of SGLT2 inhibition, not only in glomerular-injury-based CKD but also in a mouse ischaemia–reperfusion model that relates to AKI.

To discern the nature of these intraglomerular CoRL-derived cells, we conducted co-stainings for tdTomato and renin protein. We observed strict colocalization of renin and tdTomato in the JGA, with no evidence of colocalization within glomeruli, ruling out de novo expression of the renin gene. Figure 4C illustrates a representative

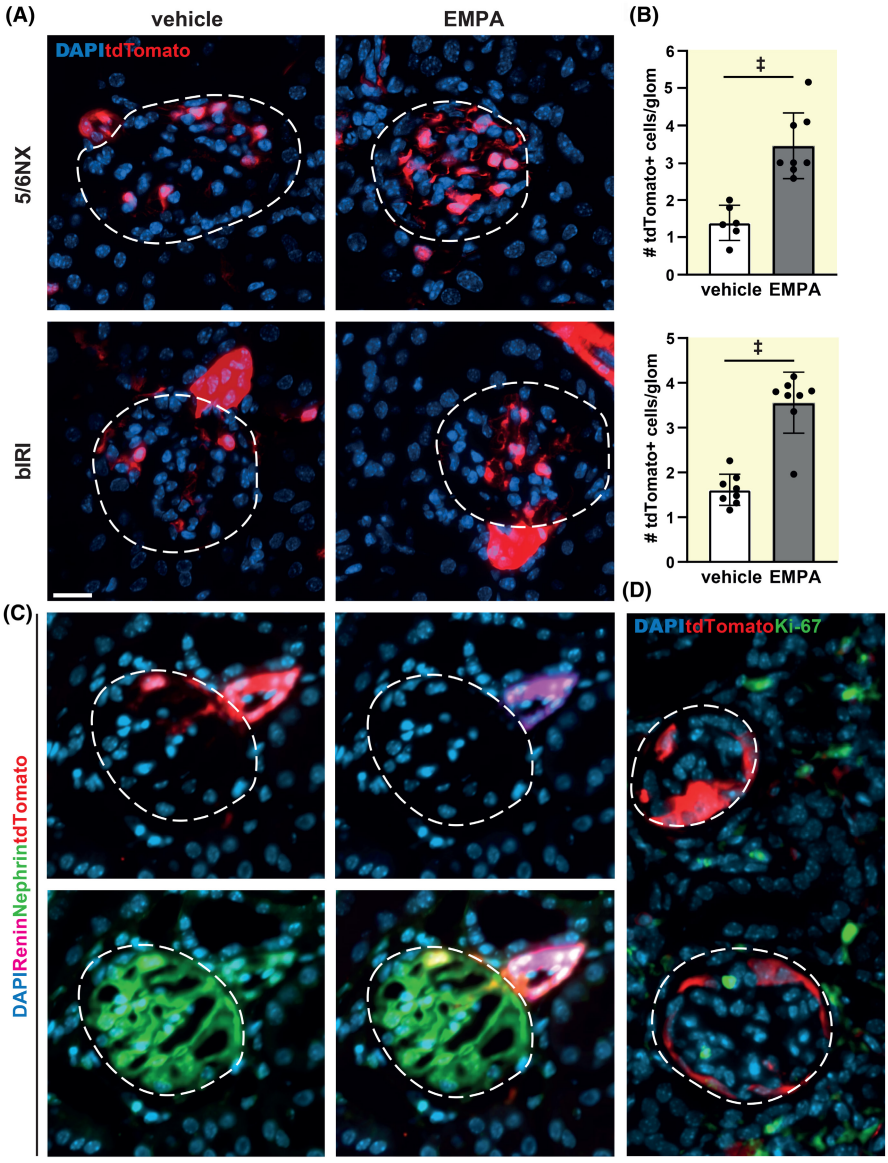


**FIGURE 4** Increased glomerular CoRL-derived cell number upon SGLT2 inhibition in 5/6NX and bIRI mouse models. (A) Representative images of tdTomato positive CoRL found within glomeruli (dashed lines), bar = 20 μm, and (B) corresponding quantification. (C) Representative picture of co-stainings for tdTomato (red), nephrin (green), and renin (magenta), showing that intraglomerular CoRL do not express renin. (D) Representative image of co-stainings for tdTomato (red) and Ki-67 (green), indicating that intraglomerular CoRL are not proliferating. Data are represented as mean ± SD, $n = 6–9$, $^{‡}p < 0.001$.

example in which a CoRL-derived podocyte (tdTomato- and nephrin-positive) is negative for renin signal. Similarly, we performed stainings for the cell proliferation marker Ki-67, which inside glomeruli never co-localized with the CoRL-tdTomato signal (Figure 4D). This suggests that the presence of these CoRL-derived cells is more likely due to migration, rather than the proliferation of the limited pre-existing CoRL-derived cells initially present within glomerular tufts at baseline.

## 2.5 | SGLT2 inhibition decreases plasma renin concentration in an AKI mouse model

In steady state, CoRL are mainly responsible for producing and secreting renin. Data on how SGLT2 inhibition affects plasma renin concentration (PRC) have been inconsistent. While some studies in non-diabetic mice report elevated PRC after empagliflozin treatment,[3] no clear RAAS activity changes in CKD (models) have been reported on the longer term.[24] It has been shown by us and others that CoRL lose their renin expression upon the migration from the JGA to the glomerulus.[20] As we observed increased migration of CoRL, we consequently sought to investigate whether this change in function of CoRL coincided with altered renin levels. Both 5/6NX and bIRI injury models showed a vast decline in PRC compared to healthy controls (4460 ± 1156 ng AngI/mL/h). Empagliflozin treatment inhibiting SGLT2 in the 5/6NX animals did not alter PRC levels any further (Figure S2A). Remarkably, bIRI mice treated with empagliflozin (1539 ± 197 ng AngI/mL/h) showed an even further decreased PRC of 1.5-fold ($p < 0.01$) compared to vehicle controls (2302 ± 673 ng AngI/mL/h) (Figure S2B), indicating reduced RAAS activity after ischaemic injury upon SGLT2 inhibition.

## 2.6 | Selective glomerular cell type repopulation by CoRL upon SGLT2 inhibition in CKD and AKI injury models

Since we observed elevated numbers of CoRL in glomeruli of diseased animals treated with SGLT2 inhibitors compared to vehicle treated littermates, we next investigated the cell fate of these CoRL. Several studies have shown the ability of CoRL to give rise to podocytes. Kidney sections of all mice were first analyzed for tdTomato colocalization with protein markers specific to podocytes. Immunofluorescent signal of podocin and Wilms tumor protein (WT1) coincided with tdTomato CoRL-lineage trace reporter label in both control and empagliflozin-treated mice, suggesting that CoRL in both of these kidney injury mouse models possess the capability to indeed differentiate toward a podocyte-like phenotype (Figure 5A). Although not significant, we observed a trend toward increased podocin-positive area in 5/6NX animals upon SGLT2 inhibition (Figure S3A). Interestingly, analysis of the number of these cells that actually derived from CoRL, indicated that 5/6NX animals showed an over 2-fold increase ($p < 0.001$) in CoRL-derived podocytes when treated with empagliflozin ($1.998 \pm 0.548$ cells per glomerulus) compared to vehicle controls ($0.904 \pm 0.250$, Figure 5B).

Compensating for podocyte's large nuclear size in these relatively thin histological sections, we next used a method proposed by Venkatareddy and colleagues[25] to estimate CoRL-derived podocyte density. Also after correcting for the potential counting overestimation in 2D sections, SGLT2 inhibition in 5/6NX led to an increase of podocyte density derived from CoRL compared to vehicle control, while it did not act on CoRL-derived podocyte count or density in the bIRI model (Figure 5B,C).

In addition to podocytes, it has been previously reported that CoRL can replenish mesangial cells and PEC. Therefore, we next determined whether SGLT2 inhibition affected the amount of these CoRL-derived cells in 5/6NX and bIRI-exposed animals. Surprisingly, in bIRI we found an increase in the area covered (Figure S4) and number of CoRL-derived PECs in SGLT2 inhibitor treated animals ($0.214 \pm 0.106$) compared to vehicle treated counterparts ($0.087 \pm 0.048$ CoRL PECs per glomerulus, $p < 0.01$, Figure 6A,B). Moreover, empagliflozin administration in bIRI model animals also led to an over 2-fold increase ($p < 0.0001$) of tdTomato-α-integrin8 double positive glomerular cells (Figure 6C,D, $1.209 \pm 0.244$ vs. $0.478 \pm 0.145$ cells per glomerulus). In contrast to this, SGLT2 inhibition did not alter the levels of CoRL-derived PECS and mesangial cells in the

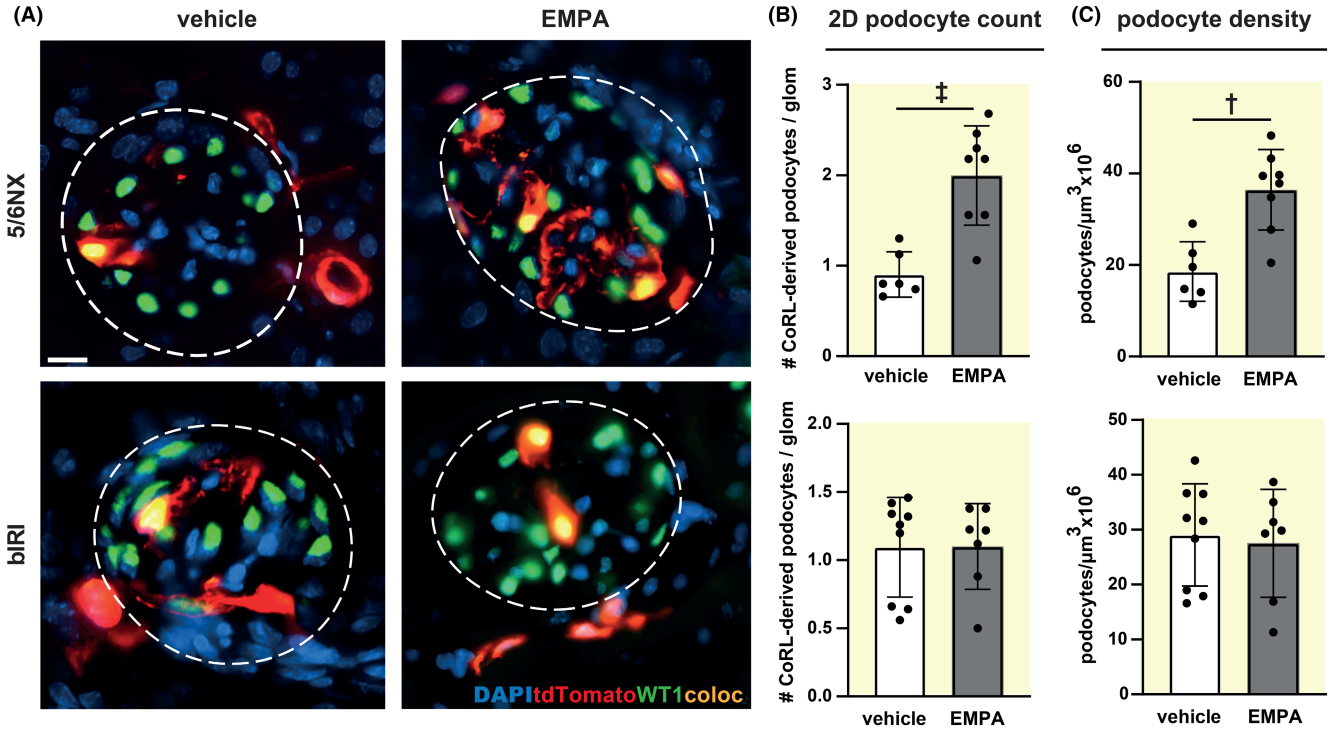


FIGURE 5 SGLT2 inhibition increases CoRL-derived podocyte number and density in 5/6 nephrectomized animals, but not in bIRI. (A) Representative images of stainings for WT1 (green) and tdTomato labeled CoRL (red). Colocalization is displayed in yellow and indicates these podocytes are derived from CoRL. Dashed lines indicate glomeruli, bar = 20 µm. (B) Quantification of CoRL-derived podocytes in 2D sections in both disease models with and without SGLT2 inhibition. (C) Corresponding estimated CoRL-derived podocyte densities. Data are represented as mean ± SD, $n = 6–9$, $^{\dagger}p < 0.01$, $^{\ddagger}p < 0.001$.

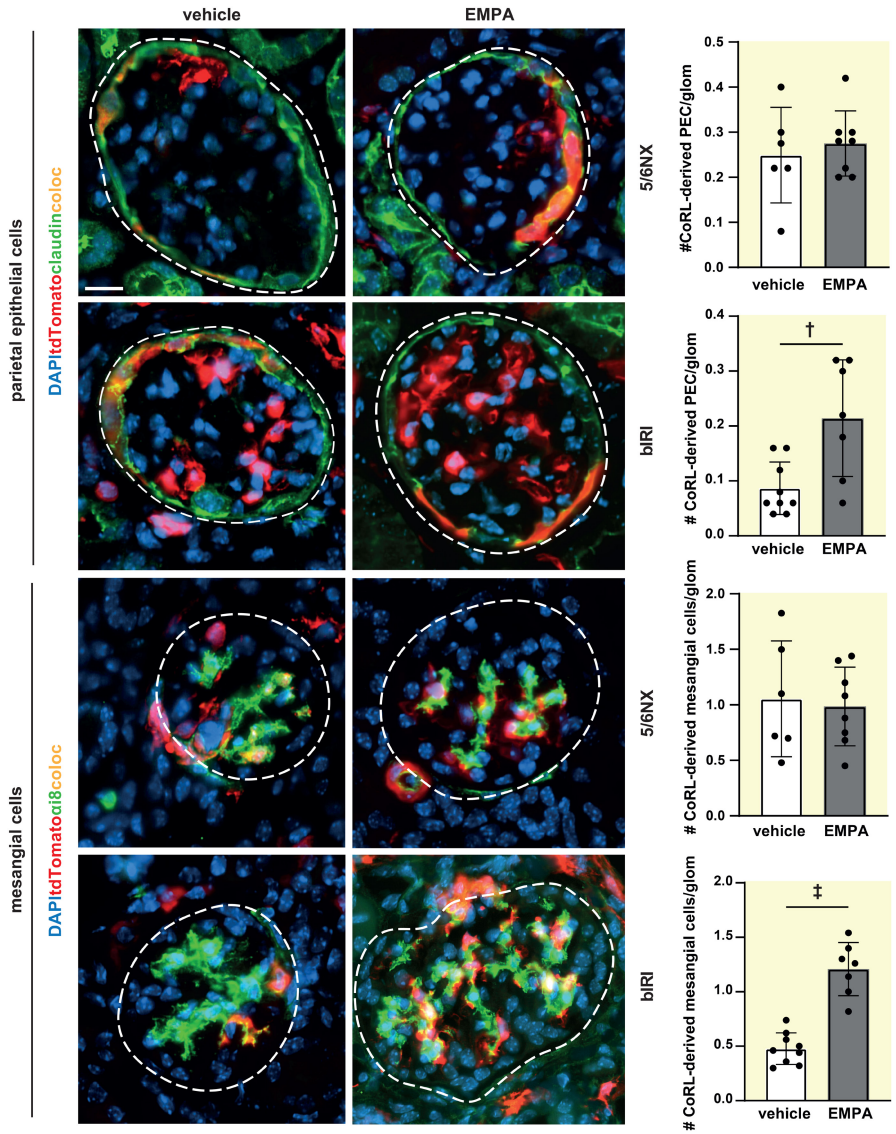


**FIGURE 6** Repopulation of different glomerular cell types alters upon SGLT2 inhibition depending on injury type. (A) Representative images of the stainings for parietal epithelial cells (PEC) (green) and tdTomato labeled CoRL (red). Colocalization is displayed in yellow and suggests the PEC is CoRL-derived and (B) corresponding quantification. (C) Representative images of stainings for mesangial cells (green) and tdTomato labeled CoRL (red). Yellow staining indicates colocalization, suggesting the particular mesangial cell derived of CoRL. (D) Quantification of (C). Dashed lines indicate glomeruli, bar = 20 μm. Data are represented as mean ± SD, $n = 6–9$, $^{\dagger}p < 0.01$, $^{\ddagger}p < 0.0001$.

5/6NX-exposed mice. This difference between these two models suggest that CoRL selectively replenish different glomerular cell types upon SGLT2 inhibition, depending on the type of kidney injury.

## 3 | DISCUSSION

Here, we describe that SGLT2 inhibition protects against kidney injury in a murine CKD model and significantly increases the number of CoRL-derived glomerular cells in murine 5/6NX and bIRI models. In agreement with previous work from other groups, we demonstrated that CoRL are able to replenish at least three different glomerular cell types: parietal epithelial cells, mesangial cells and podocytes.[18–20] Additionally, a new observation in our study is that different types of kidney injury lead to selective glomerular cell type replenishment by CoRL under the influence of SGLT2 inhibition. While in the 5/6NX model SGLT2 inhibition specifically causes an increase in CoRL-derived podocytes, empagliflozin intake resulted in an increase in CoRL-derived PECs and mesangial cells in the bIRI model. This injury-dependent glomerular cell type renewal is coherent with the concept that the podocyte niche is the main Achilles heel for disease progression in CKD,[26] while in AKI mouse models, no injury-induced podocyte loss is found.[27] Taken together, our data support a role for CoRL-mediated glomerular repopulation in explaining the beneficial effect of SGLT2 inhibitory treatment in kidney disease.

The ability of CoRL to (trans)differentiate toward glomerular cells upon injury is a naturally occurring process, indicating the existence of an intrinsic renal regenerative capacity.[28] Notwithstanding, end stage kidney disease prevalence incontrovertibly illustrates that the intrinsic regenerative potential of the kidney is usually not potent enough to counteract the progressive functional loss that most patients at risk for CKD have to deal with. For

that reason, the aim of the present study was to discover whether this mechanisms of intrinsic kidney glomerular regeneration could be augmented via SGLT2 inhibition.

The majority of glucose reuptake takes place via SGLT2 in the renal proximal tubule. Inhibiting these transporters forces glucose and sodium to pass distally, where SGLT1 can partially compensate for the loss of reabsorption. Nonetheless, sufficient SGLT2 inhibition will consequently lead to natriuresis and high sodium levels sensed by the macula densa, after which a decrease in renin secretion may be expected.[29] However, it also triggers a fall in intraglomerular capillary hydrostatic pressure and osmotic diuresis, shifting tubuloglomerular cross talk, eventually resulting in increased renin levels.[30,31] In our bIRI model, animals showed reduced plasma renin levels upon SGLT2 inhibition, possibly due to natriuresis or the impact of IRI. On the contrary, we demonstrated no changes in PRC upon empagliflozin exposure in a CKD mouse model. As 5/6NX is a reduced-mass CKD model, little tissue and thus low numbers of renin producing cells remain present compared to healthy controls.[32] Therefore, regardless of treatment, PRC is generally low, potentially leaving a limited window for further treatment-induced differences in activity. Foregoing studies regarding the effect of SGLT2 inhibitory treatment on diabetic nephropathy by Wang et al.[33] suggested that activation of tubuloglomerular feedback by adenosine receptor normalization led to less glomerular injury. Consequently, we speculate that, in a similar manner, tubuloglomerular feedback alterations stimulate the CoRL to initiate their migration and (trans) differentiation trail upon SGLT2 blockage. Studies alike ours, in which the effects of specific RAAS inhibitors—also altering tubuloglomerular feedback—were examined on CoRL-mediated glomerular repopulation during experimental podocyte depletion, observed similar increased glomerular CoRL number upon treatment.[34] Combining RAAS- and SGLT2-inhibitory therapy has recently been proposed to substantially increase kidney failure-free survival in CKD patients.[35] It would be of great interest to see the effect of such duplex treatment on CoRL-mediated renal repair. As several macula densa-mediated mechanisms involving, e.g., nNOS, COX-2 and Wnt signaling are shared with other (progenitor) cells and tissue types during development and repair, macula densa cells, considered as chief cells of tubuloglomerular feedback, now also have been suggested to be the nephron's central command driving endogenous tissue remodeling under influence of organ-specific physiological signals like body-fluid and salt levels by others.[36] As such, these macula densa cells may be the key drivers of CoRL-mediated regeneration. Meanwhile, it has been argued that SGLT2 inhibitory treatment is not able to affect tubuloglomerular feedback in advanced CKD, but rather acts on pleiotropic mechanisms like local metabolism and favorable effects on vascular function.[37]

While we found that empagliflozin significantly increases the CoRL-driven regenerative migration in mouse models for both CKD and AKI, a direct effect on improved kidney function upon SGLT2 inhibition was solely observed in the 5/6NX induced CKD group. Whereas glomerulosclerosis after bIRI does occur,[38] post-ischaemic injury is mainly characterized by tubular and vascular damage, a feature that we do not expect to improve upon glomerular cell replenishment. It is therefore surprising to observe increased numbers of CoRL-derived cells in this injury model. We hypothesize that the intraglomerular migration of CoRL upon IRI might be activated by the ability of tubular injury to over-stimulate the macula densa, resulting in abnormal tubuloglomerular cross talk, maladaptively increasing glomerular feedback. Such a dysfunctional tubuloglomerular response is previously described by Lim et al.,[39] as a potential essential mechanism in the clinical progression from AKI to CKD.

Despite the increased CoRL-derived cells observed in glomeruli, renal function did not change upon SGLT2 inhibition in bIRI exposed animals in our study setup. There have been several rodent studies reporting improved renal function and morphology after ischaemic injury comparing SGLT2 inhibitory treatment with placebo.[40–43] However, the majority of these start treatment well ahead of injury induction and terminate the experiment already after 24–48 h of reperfusion time. Similarly to when we pharmacologically induced SGLT2 blockade, genetically altered SGLT2 knock out mice also show no beneficial, neither detrimental outcomes upon bIRI compared to their wildtype littermates.[44] These results combined with the current study are indicative of disease-modifying effects of lower SGLT2 activity in preventing the (early) detrimental effects of ischaemia–reperfusion, but specific timing and minimal preservation of SGLT2 might be essential to obtain the effective treatment conditions. Alternatively, SGLT2 inhibitors might induce unidentified off-target effects.

Within the scope of this study, we cannot validate whether the increased replenishment of CoRL-derived glomerular cells after bIRI is an advantageous process. Intuitively, increased glomerular cell replenishment seems favorable, but the main concern here is that the newly recruited mesangial cells could be involved in an alternative fibrotic response. Activated mesangial cells in pathology are known for secreting an excess of extracellular matrix and inflammatory cytokines as a maladaptive healing strategy.[45] Alternatively, SGLT2 inhibition might impact ischaemic reperfusion injured kidneys in a more pleiotropic fashion,[46] hence it would be of interest to follow empagliflozin-treated AKI animals for a longer time

and study renal biology beyond CoRL migration. This research line would especially be pivotal since recent studies describe that in contrast to other SGLT2 inhibitors, particularly empagliflozin is a promising treatment for acute kidney injury as well,[47] but long-term reports are lacking.

In order to spatially follow CoRL, we made use of the Ren1cre-tdTomato lineage trace mouse line. We therefore cannot exclude that the reporter label derives from other cells that possibly originate from the renin lineage, or mirrors de novo expression of the renin gene. Nonetheless, we and others demonstrate that renin expression in these cells is restricted to the JGA.[20] Few tdTomato positive, but renin-negative, cells were found in the glomerulus at baseline; these most probably reflect CoRL-derived mesangial cells and a naturally occurring turn-over of damaged glomerular cells. Thus, with the spatial information we obtain in this study setup, we believe our findings indeed reflect CoRL-mediated glomerular repopulation.

We were able to detect different glomerular cell types derived from the renin lineage upon injury in our study. Podocytes and CoRL originate from different embryonic lineages during normal renal development, but do share the expression of certain transcription factors at different time frames,[13,48] making it particularly interesting to delineate the exact (trans)differentiation sequence they experience upon injury. Successive studies into (single cell) transcriptomics of CoRL in a similar study set up might teach us more about the crucial pathway that governs this phenotypic switch and how this is potentially influenced by SGLT2 inhibition.

# 4 | MATERIALS AND METHODS

## 4.1 | Animals

8–12-week-old male C57BL/6J Ren1c-Cre[49] x Rs-tdTomato-R (JAX®, The Jackson Laboratory) renin-lineage trace reporter mice were used in all experiments (for baseline experiments $n = 4–5$ per group, for experiments including treatment $n = 6–9$ per group. For treatment experiments, animal inclusion at start was 8–9 animals per group. In 5/6 nephrectomy, two animals died during surgeries, before start of the treatment. In bIRI one animal dropped out due to a technical error related to sutures). Mice were kept in accordance with the Experiments on Animals Act (Wod, revision 2014, the Netherlands) and EU directive no. 2010/63/EU. Animals were housed at 20–22°C in individually ventilated cages, humidity controlled (55%) with free access to food and water and a light/dark cycle of daytime (06:30–18:00) and nighttime (18:00–06:30). Animal experiments were approved by the Ethical Committee on Animal Care and Experimentation of the Leiden University Medical Center (permit no. AVD1160020171145).

## 4.2 | 5/6 nephrectomy surgeries (5/6NX)

In short, mice underwent a two-step surgical process. First, left kidneys were fully removed through a flank incision. Seven days later, the right kidney was exposed and the upper and lower poles were surgically removed and staunched with styptic gelatin sponge. Fourteen days after the second surgery, mice were killed by exsanguination, and kidneys, urine, and blood were harvested.

## 4.3 | Bilateral ischaemia–reperfusion surgery (bIRI)

bIRI surgery was performed as previously described, with minor changes.[50] In short, mice were temperature regulated using a rectal probe set at 36.7°C. Renal arteries and veins were exposed through laparotomy with median incisions on both sides. Arteries and veins of both kidneys were a-traumatically clamped for 18 min, after which clamps were removed and renal reperfusion took place. Ischaemia of 18 min provided the optimal balance between severe injury and sufficient recovery. After 14 days, mice were killed by exsanguination and kidneys, urine and blood were harvested.

All surgeries and sacrifices were performed under isoflurane anesthesia, and efforts for pain minimization were made by administering buprenorphine 0.1 mg/kg subcutaneously.

## 4.4 | SGLT2 inhibition treatment

In experiments containing SGLT2 inhibitory treatment, animals were administered 10 mg/kg bodyweight of SGLT2 inhibitor empagliflozin daily (2.5 mg/mL in 0.5% hydroxyethyl cellulose) by oral gavage for the duration of the study. This concentration is used in several preclinical studies and is demonstrated to not act on SGLT1.[51] Empagliflozin was a kind gift of Boehringer-Ingelheim (Ingelheim, Germany). Control animals received oral gavages of similar volumes with 0.5% hydroxyethyl cellulose solely.

## 4.5 | Biochemical analysis

All mice underwent serial blood collections via tail vein puncture for blood urea level and blood glucose

assessment during the timespan of the study. Whole blood urea was measured using the Reflotron Plus (Roche Diagnostics), and blood glucose levels were determined in whole blood with a glucometer (Accu-chek, Roche Diagnostics). Endpoint plasma was collected by centrifuging whole blood in K2EDTA buffered tubes at 4000 rpm for 10 min. Plasma Renin Concentration (PRC) was determined by quantifying Angiotensin I generation (ng AngI/mL/h) in the presence of an excess of sheep angiotensinogen measured by radioimmunity.[52] Urine was collected from the bladder during sacrifice. Urinary glucose was measured with a glucometer (Accu-chek, Roche Diagnostics). Plasma and urinary creatinine was determined by an enzymatic assay kit (80 350, Crystal Chem).

## 4.6 | Morphological assessment

Harvested kidney tissues were fixed in 4% formaldehyde overnight and embedded in paraffin. Sections were cut at a thickness of 4 µm. After dewaxing and rehydrating, sections were treated with appropriate staining protocol for either Periodic Acid-Shiff (PAS) or Picro Sirus Red (PSR). After dehydration, xylene clearing and mounting, sections were imaged using 3D Histech Pannoramic MIDI Scanner (Sysmex).

For PAS staining, slides were incubated in 1% Periodic Acid for 30 min followed by a 45 min incubation with Shiff's reagent (Sigma). Nuclei were counterstained with Hematoxylin for 1 min. Kidney sections of 5/6 nephrectomized animals were qualitatively evaluated by an experienced pathologist in a blind fashion and scored from 0 to 3 for glomerulosclerosis using a standardized method previously described,[53] in which 0 means <10% of glomeruli affected, 1: 10%–25% affected, 2: 26%–50% affected and >50% of glomeruli affected. For bIRI animals, whole kidney sections were scored clockwise and at least 12 fields of view in a 20× magnification were examined for each section. Tubulointerstitial damage was scored in a semiquantitative fashion with scores ranging from 0 to 3 in which 0 represents a normal morphology, 1: mild injury, 2: moderate injury, and 3: severe injury. Injury score was based on different features like loss of brush border, tubular dilation, leukocyte infiltration, fibrosis, and necrotic tubular cast, as previously described.[54]

PSR staining for collagen deposition was performed by an 1 h incubation of sections in 0.1% Sirius Red solution, followed by 2 rinses in 1% acidic acid. PSR staining was quantified over the entire kidney section using HistoQuant software (3D HISTECH, Hungary).

## 4.7 | Immunostainings

Kidney tissue was harvested, decapsulated, and fixed in 4% paraformaldehyde for 2–4 h, before overnight incubation in a 30% sucrose solution. The next day, tissue was mounted in optimum cutting temperature formulation, snap frozen, and stored at −80°C. Tissue was sectioned using a cryotome at 4 µm thickness. For analysis of endogenous tdTomato signal, slides were incubated with DAPI (1:10.000) and mounted with ProLong gold before imaging. Before primary antibody co-staining, heat-mediated antigen retrieval and antigen blocking in 1% BSA was performed. Sections were incubated with primary antibodies overnight at 4°C (rabbit α-podocin (1:1000 AB50339 Abcam), goat α-integrin α8 (1:200, AF4076, R&D), rabbit α-claudin 1 (1:200, RB-9209, ThermoScientific), goat α-RFP(tdTomato) (1:500, AB1140, Origene), rabbit α-RFP(tdTomato) (1:500, LS-C60076, Isbio)), rat α-CD45 (1:25, 550 539, BD Bioscience), mouse α-Ki-67 (1:200, 550 609, BD Bioscience), sheep α-nephrin (2 ug/mL, AF4269, R&D Systems), and corresponding Alexa-secondary antibodies raised in donkey (1:250, Invitrogen) or at room temperature for 2 h. Complete sections were imaged using 3D Histech Pannoramic MIDI Scanner (Sysmex).

## 4.8 | Evaluation of histochemical data

All slides were digitalized, and at least 50 glomeruli per tissue section were annotated in CaseViewer Software (3DHISTECH, Hungary). For the automated quantification of all stainings used in the current study, marker specific masks were developed in HistoQuant software (3DHISTECH, Hungary). Additionally, for CoRL-derived cell quantifications, individual cell were counted manually within the same annotations to determine cell number rather than area covered by these cells. Podocyte density and related correction factors were determined using methods previously described.[25]

## 4.9 | Statistics

Power of experiments was set at 80%. All data were analyzed using Graphpad Prism. Data normality and equal variances were tested by Shapiro-Willk analysis. When normally distributed, data were compared with regular ANOVA with post-hoc Tukey test or Student's *t*-test. When not normally distributed or containing ordinal datapoints, Mann–Whitney U was performed instead. $p < 0.05$ was considered as statistically significant.

## 5 | CONCLUSION

In conclusion, our study demonstrates a novel mechanism via which SGLT2 inhibition could impact on intrinsic regenerative mechanisms by CoRL-induced glomerular repopulation in CKD. Additional investigations in the mechanism behind these effects in different settings are of great interest and directed to explore further mechanistic insights and therapeutic opportunities of SGLT2 inhibition.

## AUTHOR CONTRIBUTIONS

**Loïs A. K. van der Pluijm:** Conceptualization; investigation; writing – original draft; methodology; visualization; writing – review and editing; formal analysis. **Angela Koudijs:** Investigation; methodology; writing – review and editing. **Wendy Stam:** Investigation; writing – review and editing. **Joris J. T. H. Roelofs:** Investigation; writing – review and editing. **A. H. Jan Danser:** Investigation; writing – review and editing. **Joris I. Rotmans:** Writing – review and editing. **Kenneth W. Gross:** Writing – review and editing; supervision; resources. **Michael P. Pieper:** Resources. **Anton Jan van Zonneveld:** Writing – review and editing; supervision. **Roel Bijkerk:** Conceptualization; funding acquisition; writing – original draft; methodology; writing – review and editing; visualization; project administration; supervision.

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## CONFLICT OF INTEREST STATEMENT

M.P.P. is employed by Boehringer Ingelheim.

## DATA AVAILABILITY STATEMENT

Derived data supporting the findings of this study are available from the corresponding author RB on request.

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