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RESEARCH ARTICLE

# Mesenchymal stem cells derived from the kidney can ameliorate diabetic nephropathy through the TGF-β/Smad signaling pathway

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## Abstract

Diabetic nephropathy (DN) has been introduced as one of the main microvascular complications in diabetic patients, the most common cause of end-stage renal disease (ESRD). Based on the therapeutic potential of mesenchymal stem cells in tissue repair, we aimed to test the hypothesis that kidney stem cells (KSCs) might be effective in the kidney regeneration process. Stem cells from rat kidney were separated, and the surface stem cell markers were determined by flow cytometry analysis. Thirty-two Sprague Dawley rats were divided into four groups (control, control that received kidney stem cells, diabetic, diabetic treated with stem cells). To establish diabetic, model STZ (streptozotocin) (60 mg/kg) was used. The KSCs were injected into experimental groups via tail vein ($2 \times 10^6$ cells/rat). In order to determine the impact of stem cells on the function and structure of the kidney, biochemical and histological parameters were measured. Further, the expression of miRNA-29a, miR-192, IL-1β, and TGF-β was determined through the real-time PCR technique. Phosphorylation of Smad2/3 was evaluated by using the standard western blotting. The KSCs significantly reduced blood nitrogen (BUN), serum creatinine (Scr), and 24-h urinary proteins in DN ($P < 0.05$). IL-1β and TGF-β significantly increased in the kidney of diabetic rats. In addition, the expression of miR-29a is significantly increased, whereas miR-192 decreased after treatment with KSCs ($P < 0.05$). Diabetic rats showed an increased level of phosphorylation of both Smad2 and Smad3 ($P < 0.05$). Periodic acid-Schiff (PAS) staining showed improved histopathological changes in the presence of KSCs. Stem cells derived from adult rat kidney may be an option for treating the early DN to improve the functions and structure of kidneys in rats with DN.

**Keywords** Cell therapy · Diabetic nephropathy · Kidney stem cells · TGF-β-Smad signaling

## Introduction

It was estimated that global diabetes was around 9.3% in 2019. It is projected that the global diabetes prevalence will increase to 10.2% by 2030 and 10.9% by 2045 (Saeedi et al. 2019). In the mentioned study, high-quality data were extracted from 255 sources and scored by analytical hierarchy process (AHP).

Diabetic nephropathy (DN) has been introduced as one of the main microvascular complications in diabetic patients. In addition, diabetes is still the critical cause of chronic kidney disease, which involves nearly 50% of all end-stage renal diseases (ESRD) worldwide (Kanwar et al. 2011; Wang et al. 2014). Macrophages are major players in triggering renal injuries in a variety of kidney diseases, including DN. In fact, the accumulation of these cells in the interstitial tissue and glomeruli of kidneys can lead to both functional and

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structural disturbances in kidneys (Nguyen et al. 2006; Chow et al. 2004a; Cao et al. 2010).

The inflammatory reactions are mediated through paracrine and autocrine pathways triggered by macrophage-released inflammatory markers, and cytokines such as interleukin-6 (IL-6), IL-1β, and tumor necrosis factor alpha (TNF-α) exacerbate renal injuries in these pathological conditions (Prockop and Oh 2012; Chow et al. 2004b).

Some studies have demonstrated that transforming growth factor-β1 (TGF-β1) has an effective role in DN development (Wang et al. 2005; Huang et al. 2002). Once TGF-β1 binds to its receptor, two essential downstream mediators, namely Smad2 and Smad3, are activated for exerting biological activity, including extracellular matrix (ECM) generations. Previous studies have already focused on the impact of TGF-β1/Smad3 signaling pathway on mediating renal fibrosis (Oujo et al. 2014; Zhao et al. 2015). Recently, miRNAs have been suggested as potential diagnostic or prognostic biomarkers in various renal diseases and pathologies, including chronic kidney allograft rejection, DN, as well as ischemic acute kidney injury. Some of these miRNAs include miR-192, miR-205, miR429, miR-141, miR-200b, and miR-200a (Kantharidis et al. 2011). Among these, miR-192 has been shown to modulate collagen type 1 α-2 (COL1A2) expression via regulating TGF-β signaling and suppressing E-box repressors zinc finger E-box–binding home box, ZEB1 and ZEB2 (Kato et al. 2007). Through these mechanisms, miRNA has been suggested to play an important role in matrix formation and DN pathogenesis.

On the other hand, TGF-$\beta$1 has been suggested to regulate miRNA biology and expression. The upregulation of TGF-$\beta$1 has been linked to the activation of Smd3, triggering its binding to miR-29, which suppresses the expression of this miRNA. This observation indicates that TGF-$\beta$1–induced fibrogenesis is at least partially mediated through the function of the miR-29 family as important downstream effectors (Wang et al. 2012). Wang et al. have reported miR29c, miR-29b, and miR-29a are reduced in the early and late phases of kidney fibrosis in the context of a variety of renal diseases (Wang et al. 2015).

However, a few options in managing chronic kidney disease (CKD) have been recommended (Wang et al. 2012). Using sodium-glucose cotransporter-2 (SGLT2) inhibitors (Baud et al. 1988), controlling the blood glucose use of an angiotensin-converting enzyme inhibitor (ACE-I), or use of an angiotensin receptor blocker (ARB) might be effective in reducing the risk of diabetic nephropathy development (De Zeeuw et al. 2004).

Moreover, diabetes is frequently diagnosed too late due to asymptomatic initial phases. Kidneys with severe damages can propel kidney fibrosis and ESRD. In this stage, dialysis or kidney transplantation is needed. In case of early treatment, it may retard ESRD development. Therefore, the initial-phase identification, as well as appropriate therapy, is essential.

Researchers assume that stem cell–based treatments are accompanied by considerable benefits observed in numerous cases with a broad range of diseases and injuries. As a result of the efficiency and safeness of mesenchymal stem cells (MSCs), experts in this field have utilized the allogeneic MSCs for treating acute kidney injuries, cardiomyopathy patients, and clinical trials, who faced greater risks of post-operative chronic kidney injuries (Tögel and Westenfelder 2012). Additionally, autologous MSCs have been easily harvested, expanded, and differentiated in culture and were free of immune rejection (Lee et al. 2010), implying their promising application in clinical treatment (Lee et al. 2006).

Numerous studies have proved the existence of various stem cells in the kidney (Gheisari et al. 2009; Gupta et al. 2006; Chen et al. 2008). Furthermore, their advantageous impacts on acute kidney injury have been shown via histological and functional assessments in acute kidney diseases (Aggarwal et al. 2016). Nonetheless, there are obscure points related to the interactions between kidney stem cells (KSCs) and Smad expression in diabetic nephropathy (DN). Previous studies have reported that MSCs may regenerate tissue through paracrine action of diverse cytokines to decrease the undesirable reactions of kidney cells and improve endogenous repair of kidney tissues in various experimental models, like DN (Bruno et al. 2009; van Koppen et al. 2012; Eliopoulos et al. 2010; Park et al. 2012). The present study focused on isolation and characterization of adult rat KSCs and evaluated their impact on TGF-β/Smad pathway, miR-192, miR-29a expression, and histological changes in the DN model.

## Materials and methods

### *In vivo* study

#### Animal model and groups

Thirty-two male Sprague Dawley rats weighing 200–250 g from the animal house of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, were purchased. The rats were maintained under the standard housing conditions (12:00 h dark/12:00 h light, 24 ± 2 °C temperature), and they accessed water and food freely.

#### Diabetes induction

A single-intraperitoneal administration of streptozotocin (STZ) (60 mg/kg, Sigma Aldrich, St. Louis, USA) (Sanyanusin et al. 1996) was dissolved in fresh citrate buffer (pH of 6.0) and then was injected to induce diabetes. The

control group was administered with the same content of citrate buffer. Diabetes induction was verified 3 days following the STZ administration. Rats with blood glucose levels ≥ 350 mg/dl under fasting conditions were included in this study.

### Experimental design

After diabetes induction, rats were divided randomly into 4 experimental groups ($n = 8$), including: Control group (Ctrl) that received normal saline, control that received KSCs (ctrl-cell), diabetic nephropathy group (DN) that was injected with normal saline, the diabetic group which was treated with kidney stem cells (DN-cell). The stem cells at a concentration of $2 \times 10^6$ cells/rat (Wang et al. 2013) were given once through the tail vein route after 3 weeks of diabetic induction following a single injection of cyclosporine A (10 mg/ kg, subcutaneously) (Latifpour et al. 2011). After 2 weeks of KSC injection, the animals in various groups were sacrificed for obtaining the samples of blood and kidney.

### Biochemical analysis

At the end of the experiment, animals were maintained in metabolic cages, and the urine was collected. The BCA kit was used to measure 24-h urinary protein excretion (UPE). (De Zeeuw et al. 2004, Jin J et al. 2019, Ebrahim et al. 2018) . After that, animals were sacrificed under ketamine (80 mg/kg) and xylazine (8 mg/kg) anesthesia. The blood samples were collected from the left ventricle and centrifuged at 3500 rpm for 5 min, and levels of serum creatinine (Scr) and blood nitrogen (BUN) were measured.

### The histological evaluation of kidney tissue

Obtained left kidneys were washed by PBS, fixed in buffer formalin 10%, and embedded in paraffin, then tissue Sects. (1–4-μm thickness) were prepared, and periodic acid-Schiff (PAS) staining was done. Three sections from each kidney with 10 fields for each slide were assessed for semiquantitative analysis by light microscopy (Olympus CX31) without knowledge of the experimental protocols. The percentage of glomerulosclerosis (% GS) was determined to evaluate segmental hyalinization in glomeruli (Lv et al. 2014).

### RNA extraction and RT-qPCR

The right kidneys were rinsed, snap-frozen in liquid nitrogen, and then kept at − 80 °C for molecular assessment. Total RNA and miRNA were extracted from frozen samples using RNA extraction kit (Roche, Cat. No; 05,080,576,001, Germany) following the manufacturer's instructions. The purity of the extracted RNA was assessed using spectrophotometry (NanoDrop Thermo Scientific S.N: D015).

Real-time quantification of miRNAs was performed with the miScript II RT Kit (BONmiR, BON209001, Iran) according to the manufacturer's protocol. For mRNA analysis, single-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA with a high-capacity cDNA archive kit (RevertAid First Strand cDNA, Thermo Scientific), according to the standard protocol. A total of 1μL of cDNA was amplified by real-time PCR. GAPDH and U6 were used as housekeeping reference genes. Each sample was tested in triplicate. Fold change expression was determined as $2^{-\Delta\Delta CT}$ and normalized to the controls. Primers were designed by Metabion company (Germany). Primers used in this study are shown in Table 1.

### Western blot

The samples were lysed in radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology; Dallas, 116 TX, USA). Bradford protein assay (Cat No. P0068, Beyotime, China) was performed to determine protein concentration. The same amounts of proteins (40 μg) were exposed to sodium dodecyl sulfate/poly-acrylamide gel electrophoresis (SDS/PAGE) (12% gels). After the electrophoresis, the proteins were transported onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA) in a running buffer. Then, nonspecific sites were blocked with 5% (w/v) non-fat dried skimmed milk powder in TBST (2-M Tris–HCl buffer with a pH of 7.6, 0.05 M NaCl, and 0.05% Tween 20) at 37 °C for 60 min. Subsequently, the membranes were incubated at 4 °C overnight with the primary antibodies including anti-Smad3 (ab63403, Abcam: USA), anti-Smad2 (ab63576, Abcam, USA), phosphorylated Smad3 (ab40854, Abcam, USA), and phosphorylated Smad2 (ab53100, Abcam: USA). After that, the membranes were washed four times in Tris buffer saline and Tween 20 (TBST). Then,

**Table 1** The primer sequences in qRT-PCR assay

| Genes | Primers sequences (5′ 3′) |
|---|---|
| MiR-192 | Forward: CGC CTG ACC TAT GAA<br>Reverse: ACT TAT GTT TTT GCC GTT T |
| MiR-29a | Forward: TAGTAGGCGATAGCACC<br>Reverse: ACT TAT GTT TTT GCC GTT T |
| U6 | Forward: ACT TAT GTT TTT GCC GTT<br>Reverse: ACT TAT GTT TTT GCC GTT T |
| TGF-β | Forward: CAT TGC TGT CCC GTG CAG A<br>Reverse: AGG TAA CGC CAG GAA TTG TTG CTA |
| IL-1β | Forward: CTACCTATGTCTTGCCCGTGGAG<br>Reverse: GGGAACAT CACACACTAGCAGGTC |
| GAPDH | Forward: ACAAGATGGTGAAGGTCGGTG<br>Reverse: AGAAGGCAGCCCTGGTAACC |

horseradish-peroxidase–conjugated secondary antibodies (ab6721, Abcam, USA) were used to incubate the membrane for 1 h. Finally, enhanced chemiluminescence (ECL) was used for visualizing the protein bands and analyzed by ImageJ software.

## *In vitro* study

### Isolation of kidney stem cells (KSCs) from adult rats

For isolation of KSCs, the kidneys were harvested, and renal capsule and renal fat were separated. Then, the kidneys were minced and incubated in collagenase I (0.3%, Sigma, USA) at 37 °C for 25 min. The dispersed cells were collected by centrifugation (1500 rpm, 5 min). Isolated cells were disseminated in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO-BRL; Grand Island; NY, USA) containing F12 10% (GIBCO-BRL), streptomycin (1000 U), penicillin (100U) (GIBCO-BRL), and 20% fetal bovine serum (FBS) (GIBCO-BRL). The debris and nonadherent cells were removed after 24 h. Then, a fresh medium was added to the adherent cells. The cultured media were kept at a temperature of 37 °C with 5% $CO_2$.

### Evaluation of differentiation capability

The cells were cultured in the 6-well plates using DMEM culture medium containing 20% FBS. In order to differentiate into osteocytes, 10-mM beta-glycero-phosphate (Merck, Darmstadt, Germany), $10^{-7}$ M dexamethasone (Sigma), and 50-g/ml ascorbic acid 2-phosphate were utilized for treating the cells for 23 days. The presence of calcium deposits was evaluated by staining with Alizarin Red S (Sigma) at 4 °C for 10 min after fixing the cells by paraformaldehyde 4% (Sigma). Adipocyte differentiation was done in the presence of 0.5-mM isobutylmethylxanthine (Sigma) and $10^{-7}$ M dexamethasone (Sigma). Accumulation of the lipid droplets in vacuoles was assayed by the Oil Red O staining after 21 days. Every 3–4 days, culture media in each well was changed (Feng et al. 2018).

### Flow cytometry assay

To evaluate surface markers of KSCs, flow cytometry analysis was conducted. Briefly, $10^5$ cells/ml KSCs were incubated with anti-rat antibody in dilution (1:100) against $PAX_2$, $OCT_4$, and CD133 as positive markers and CD45 as a negative marker. Finally, FlowJo CellQuest software and FACS Calibur (Becton Dickinson) machine were used to examine the cells.

### Cell labeling

CM-Dil cell labeling solution (50 µg/ml, Invitrogen Life Technologies) was used overnight to label the KSCs. Cells were centrifuged and were rinsed two times in a serum-free medium. After that, cell pellets were suspended in phosphate-buffered saline (PBS) and were injected through the tail vein. After 2 weeks, the kidney tissue was evaluated by a fluorescence microscope for detecting and tracking the cells.

### Statistical analysis

In all statistical analyses, the data were expressed as mean ± SE. Data were evaluated by the Kolmogorov–Smirnov test. Moreover, one-way analysis of variance (ANOVA) and Tukey's test were used to determine significance among groups. $P$ values $< 0.05$ were considered statistically significant.

## Results

### Isolation and differentiation of rat kidney stem cells

About 3–5 days after the culture of KSCs, colonies detected adherent fibroblast-like morphology. The successive cycles of trypsinization, seeding, and culture for more than 2 weeks in vitro were done for expanding the adherent cells (Fig. 1A, B). In vitro, adipogenic, and osteogenic differentiation of KSCs were verified by special stains and morphological alterations (Fig. 1C, D). The cultured cells proliferate and form a monolayer of ~ 80% confluence after 1 month (Fig. 1).

### Flow cytometry analysis

Flow cytometry analysis demonstrated positive stem cells for CD133 (75.9%), $OCT_4$ (83%), $PAX_2$ (86%), and negative stem cells for CD45(24.2%) (Fig. 2).

### Cell labeling

The KSCs 24 h after labeling by CM-Dil fluorescent dye (Fig. 3A) were identified in kidney tissue approved the cells placed into the kidney tissue (Fig. 3B–D).

### Effect of kidney stem cells on renal function

Biochemical analysis indicated the increased level of 24-h UPE, Scr, BUN, and blood glucose in diabetic nephropathy animals in comparison with normal control rats. These changes ameliorated remarkably after KSCs significantly

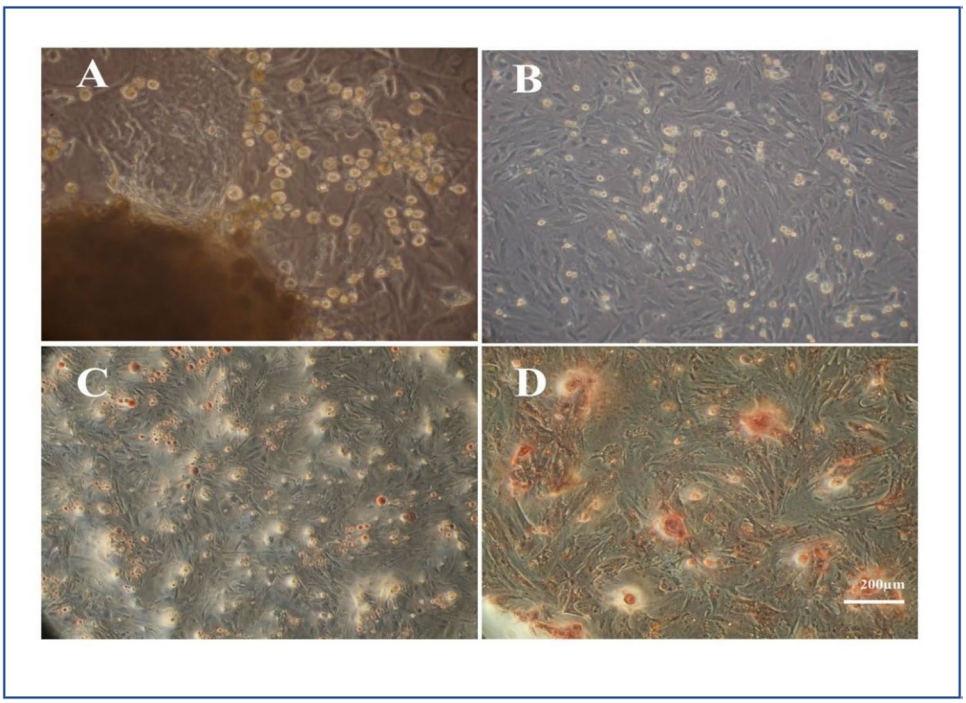


**Fig.1** Seeding and evaluation of isolated cells from KSCs. Adherent cells before passages of cultured cells (**A**). Adherent cells in passage three (**B**). Adipogenic differentiation by Oil Red staining (**C**). Osteogenic differentiation by Alizarin Red staining (**D**)

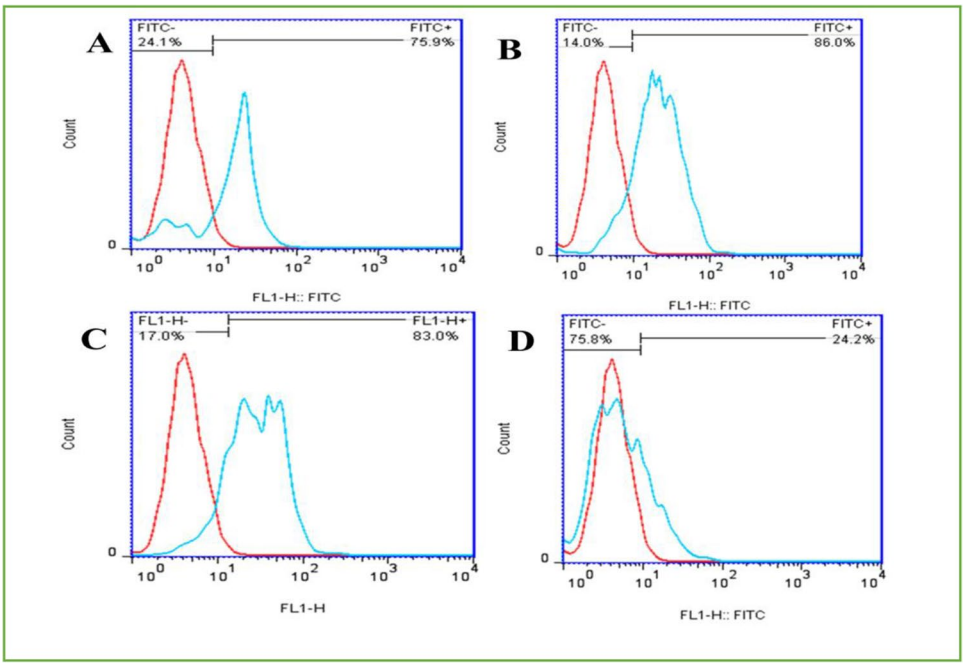


**Fig.2** Cells were stained with the *CD133*, $PAX_2$, $OCT_4$, and *CD45* antibodies and analyzed by flow cytometry. The expression levels of *CD133* (**A**), $PAX_2$ (**B**), $OCT_4$ (**C**), and *CD45* (**D**) of KSCs are presented as a histogram

except for blood glucose. Biochemical parameters did not show a difference between control and control-cell groups (Fig. 4A–C).

## Kidney histological changes

After PAS staining, mesangial expansion and glomerular structure abnormality were seen in the DN group (Fig. 5C) compared to those in the control group (Fig. 5A). In KSC treatment group, these pathological changes declined considerably in comparison with DN group (Fig. 5D). No difference was observed between the control-cell and control groups (Fig. 5B). Furthermore, these results were reported quantitatively (Fig. 5E).

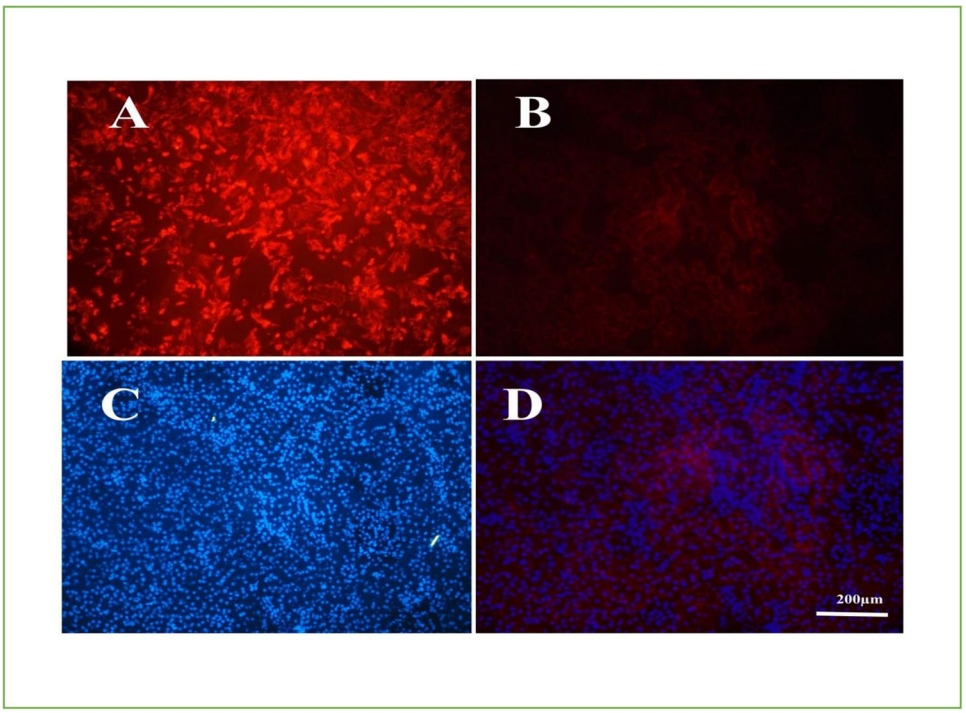


**Fig.3** Detection of kidney stem cells labeled by CM-Dil fluorescent in vitro. **A** The microscopic picture showed that KSCs distributed in kidney tissue after 2 weeks with red fluorescent (**B**) nuclei were stained by DAPI with blue fluorescence (**C**) and merged (**D**)

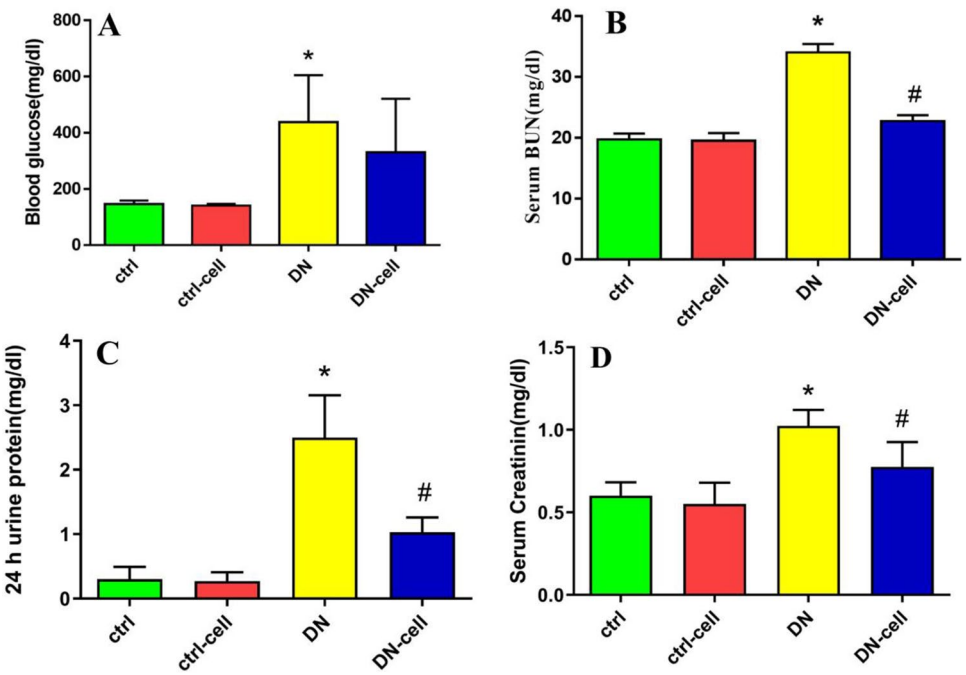


**Fig.4** Effect of KSCs on biochemical parameters: **A** blood glucose, **B** BUN of serum, **C** 24-h UPE, and **D** Scr in four groups of rats. ctrl, normal group; ctrl-cell, normal group that received KSCs; DN-cell, diabetic nephropathy treated with KSCs; DN, diabetic nephropathy. $*P < 0.05$ compared to ctrl group, $\#P < 0.05$ compared to DN group. Data were expressed as mean ± SE

## Real-time PCR expression analysis

As shown in Fig. 6 (A, B), TGF-β and IL-1β expression increased noticeably in the DN group. Based on the qRT-PCR results, the miR-192 expression enhanced considerably, but miR-29a expression declined significantly following DN. KSC administration decreased TGF-β, IL-1β, and miR-192 levels and increased miR-29a expression significantly. Comparing Ctrl and Ctrl-cell, the results were identical (Fig. 6C, D).

## Western blot analysis

Analysis of western blot showed that phosphorylation of Smad2 and Smad3 were declined considerably in the DN-cell group in comparison to the DN group (Fig. 7A, B).

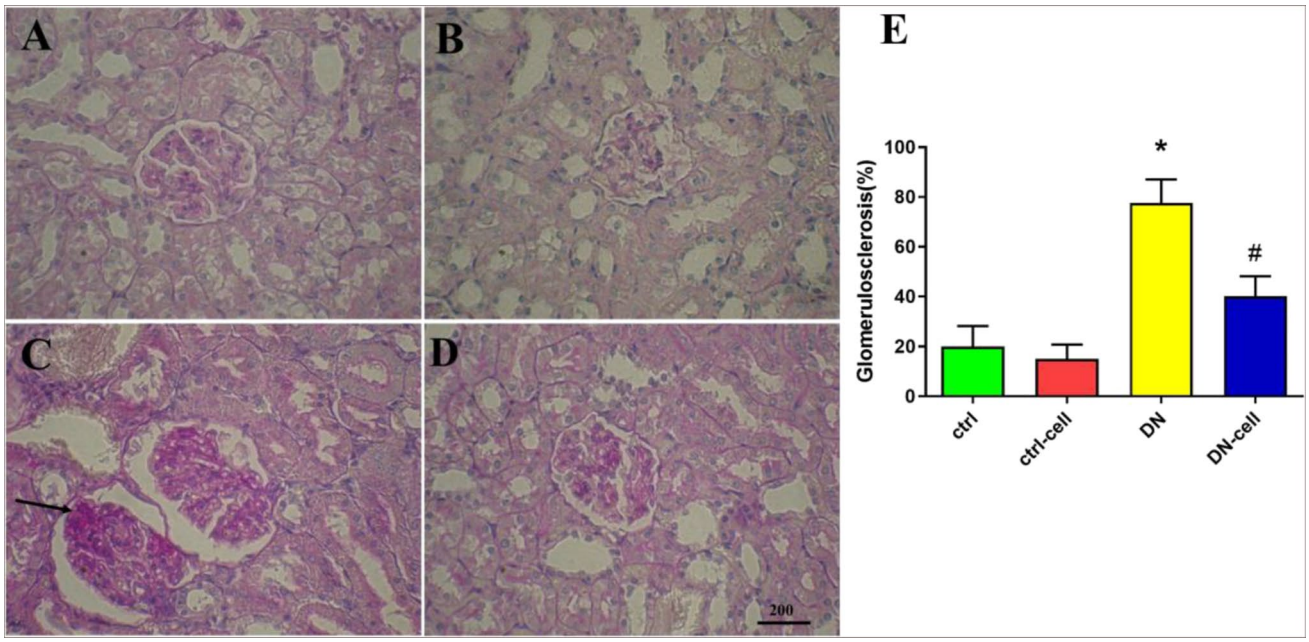


**Fig.5** Effect of KSCs on kidney histopathological changes: Light microscopy examination of tissue sections stained by PAS staining in different groups. Control (**A**), ctrl-cell (**B**), DN (**C**), DN-cell (**D**) (black arrow: GS), and semiquantitative analysis of GS (**E**) evaluated by the percentage of glomeruli exhibiting sclerotic lesions (%GS). ctrl, normal group; ctrl-cell, normal group that received KSCs; DN-cell, diabetic nephropathy treated with KSCs; DN, diabetic nephropathy. $*P<0.05$ compared to ctrl group, $\#P<0.05$ compared to DN group. Data were expressed as mean ± SE

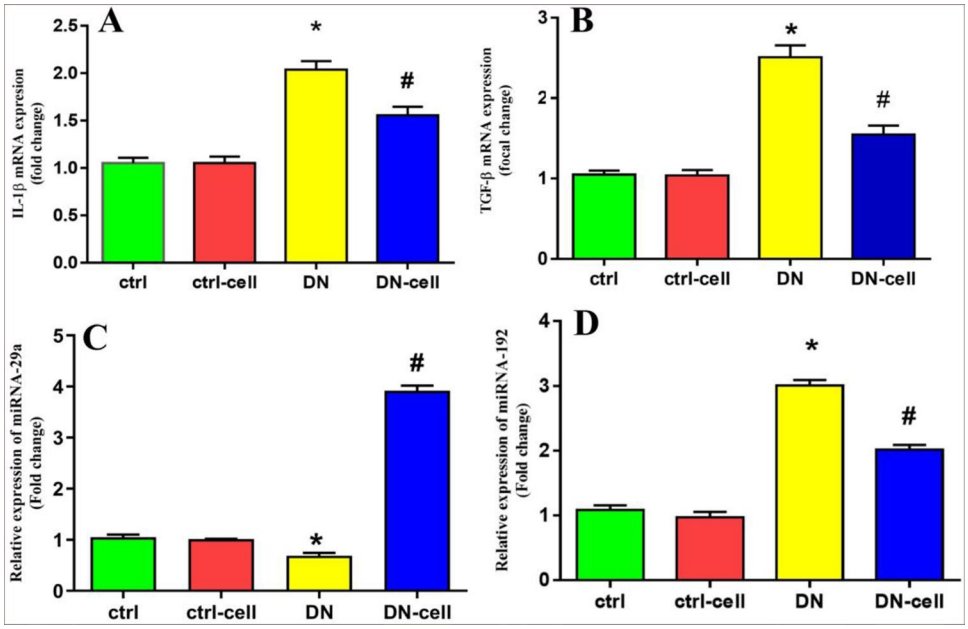


**Fig.6** Effects of KSCs on the expression of IL-1β, TGF-β, miRNA-192, and miRNA-29a in the kidney tissue of different groups. Analysis of the qRT-PCR results showed that IL-1β and TGF-β are significantly increased. In addition, the expression of miR-29a is significantly decreased while miR-192 increased in rats with DN. ctrl, normal group; ctrl-cell, normal group that received KSCs; DN-cell, diabetic nephropathy treated with KSCs; DN, diabetic nephropathy. $*P<0.05$ compared to ctrl group, $\#P<0.05$ compared to DN group. Data were expressed as mean ± SE

The KSCs suppressed the phosphorylation of Smad2 and Smad3 in DN. The results were similar for the control and control-cell groups. The quantitative analysis by ImageJ software is shown in Fig. 7(C, D).

## Discussion

The presence of progenitor or stem cells in a tissue maintains the cell replacement. In fact, activated stem cells may

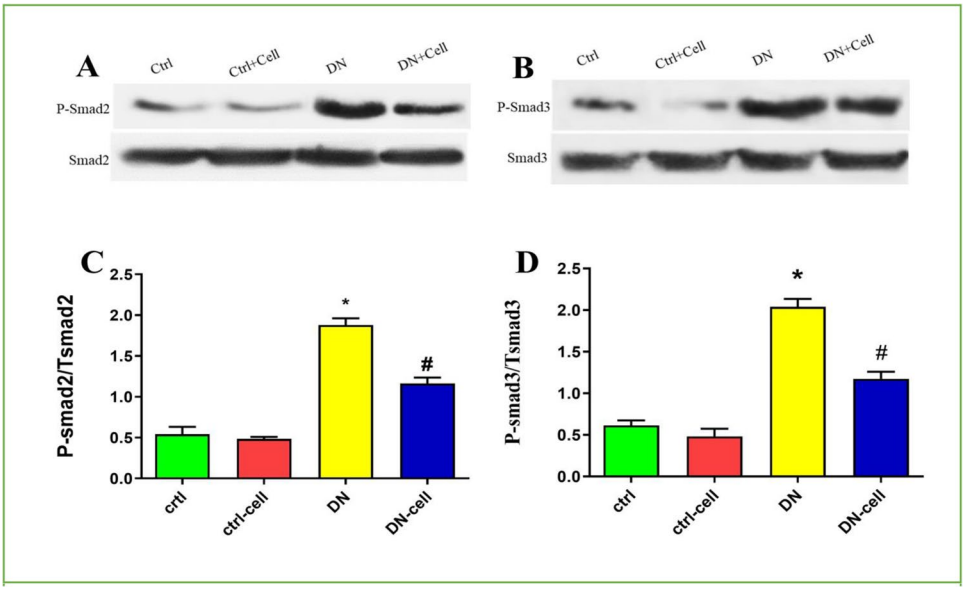


**Fig.7** Effects of KSCs on the phosphorylation of Smad2 and Smad3 in the kidney tissue of different groups (**A**, **B**). The western blotting was performed three times. Quantitative data of protein levels were expressed as the intensity ratio of phosphorylation of target proteins $Smad_2$ (**C**) and $Smad_3$ (**D**) ctrl, normal group; ctrl-cell, normal group that received KSCs; DN-cell, diabetic nephropathy treated with KSCs; DN, diabetic nephropathy. $*P < 0.05$ compared to ctrl group, $\#P < 0.05$ compared to DN group. Data were expressed as mean ± SE

divide the expansion of the cellular pool, which differentiates along a specific cell lineage for making tissue (Blanpain et al. 2007; Gurtner et al. 2008). Even though the action of cell-based therapy against DN has been demonstrated for years, the type of cells with the best therapeutic impact still remains obscure. The present work aimed to find suitable KSCs that are actively engaged in kidney regeneration after DN. The obtained results indicate that KSCs with spindle morphology express stemness markers, such as $PAX_2$, $OCT_4$, and CD133 but not CD45. Expressing these markers in the isolated KSCs reflects their contribution to proliferation, survival, and self-renewal (Maeshima et al. 2003). In addition, previous investigations provided the protective effect of CD133 + -expressing cells isolated from adult human kidneys on improving kidney function in AKI (Bussolati and Camussi 2015). Herein, we showed the capability of KSCs to differentiate into the cells that do not usually present in the kidney, including adipocytes and osteocytes. In agreement with our results, Gheisari et al. (2009) suggested that KSCs can differentiate into osteocyte and adipocyte (Gheisari et al. 2009).

Our results indicated severe hyperglycemia, proteinuria, enhanced Scr, and glomerular pathological alteration 3 weeks following STZ injection to animals. Consistent with these results, Liu et al. (2011) reported that 21 days after STZ injection, the animals showed all the characteristics of early DN (Liu et al. 2011). Using KSCs, all the abovementioned parameters improved except for hyperglycemia. Moreover, previous investigations have reflected the mediation of reversal STZ-induced complications after MSC injection, even high blood glucose levels (Ende et al. 2004). Mobilization capability of endogenous progenitor cells with the ability of mitigation to injuries and participation in repair have been illustrated completely (Shi et al. 1998; Orlic et al. 2001). It has been reported that stem cells may automatically transfer to the damaged kidney via automatic homing and differentiate to intended cells in order to improve and normalize tubular and glomerular damages. Finally, injured kidney cells were repaired, and their functions were restored (Volarevic et al. 2011). In agreement with our study, Ezquer et al. (2009) showed that administration of MSC, as one of the cell therapy strategies, would contribute to the prevention of renal diseases caused by diabetes even if the patient is still with the hyperglycemia condition (Ezquer et al. 2009). Similar studies have indicated that an increased level of glucose damages podocytes and induces apoptosis in them, but BMSCs in combination with miR-124a can reduce apoptosis in podocytes and increase their proliferation (Li and Siragy 2014; Sun et al. 2018).

The stage of DN is associated with proinflammatory cytokine as well as fibrosis signaling pathway (Bai et al. 2019). Synthesis and biological activity of TGF-β1 increase under hyperglycemia (Xinli et al. 2014) , which can stimulate the transformation of fibroblasts into myofibroblasts (α-smooth muscle actin–positive cells; α-SMA), and catalyze the expression of collagen type III (Col III) and fibronectin (FN) with subsequent induction of renal fibrosis, degradation of kidney function, and renal damage (Gómez and Velarde 2018, Hill et al. 2000). Moreover, upregulation of TGF-β1 induces epithelial-to-mesenchymal transition (EMT) in proximal tubules, collecting duct cells, glomerular podocytes, and glomerular parietal epithelium (Chang et al. 2016) . We found that TGF-β and IL-1β were overexpressed in the DN group compared to those in the control group. In accordance with these observations, Li et al. (2018) reported that the mRNA and/or

protein levels of Il-6, TNF-α, and IL-1β, as the proinflammatory cytokines released by activated macrophages, were significantly elevated while FN and TGF-β fibrosis markers decreased in the MSC group (Li et al. 2018). TGF-β upregulates many of the genes involved in fibrosis, and it also regulates EMT; thus, a blockade of TGF-β signaling in the experimental models prevents the progression of kidney diseases (Isaka 2018) .

According to our results, KSC treatment reduced the tissue levels of TGF-β and IL-1β proinflammatory cytokines. Researchers believe that the role of MSCs in ameliorating DN is mainly mediated by the paracrine effect rather than the differentiation mechanism (Ezquer et al. 2008; Oliveira-Sales et al. 2013). In the same line, studies in vitro and in vivo suggest that MSCs could inhibit the upregulation of TGF-β expression stimulated by high glucose in mesangial cells by the secretion of trophic factors (Lv et al. 2014). They are able to antagonize TGF-β1 (Motazed et al. 2008) (Fig. 8).

As mentioned previously, DN pathogenesis has been proposed to be related to certain miRNAs (Wu et al. 2014). In the context of renal fibrosis, expression levels of miR-192 increased significantly in glomeruli isolated from diabetic mice (Kato et al. 2007). TGF-β increased Col1a2 mRNA in mouse mesangial cells via miR-192, and this finding could be related to kidney dysfunction under pathological conditions such as diabetes (Kato et al. 2007). Our results showed parallel increases in TGF-β and miR-192 in diabetic rats, suggesting TGF-β–induced miR-192 is responsible for kidney damage. Such miRNAs attached to 3′UTR of the reno-protective genes resulting in their diminished expression. Accordingly, the upregulated miRNAs participate in DN pathogenesis. On the contrary, downregulated miRNAs showed renal protective impacts (Petrillo et al. 2017).

In diabetes, miR-29a/b seems to be a major factor in kidney protection through inducing anti-fibrotic and antiapoptotic effects, and its suppression has been related to progressive renal damage (Lin et al. 2014a, b; Wang et al. 2012). High glucose increases the expression of histone deacetylase 4 (HDAC4), which causes deacetylation of nephrin (Lin et al. 2014a, b). Expression of HDAC4 is translationally attenuated by miR-29a (Winbanks et al. 2011). However, collagen IV was reported as the target of the miR 29a. Thus, miR-29a regulates collagen expression (Du et al.2010). In accordance with these findings, suppression of miR-29a via the high glucose/TGF-β pathway has been observed to

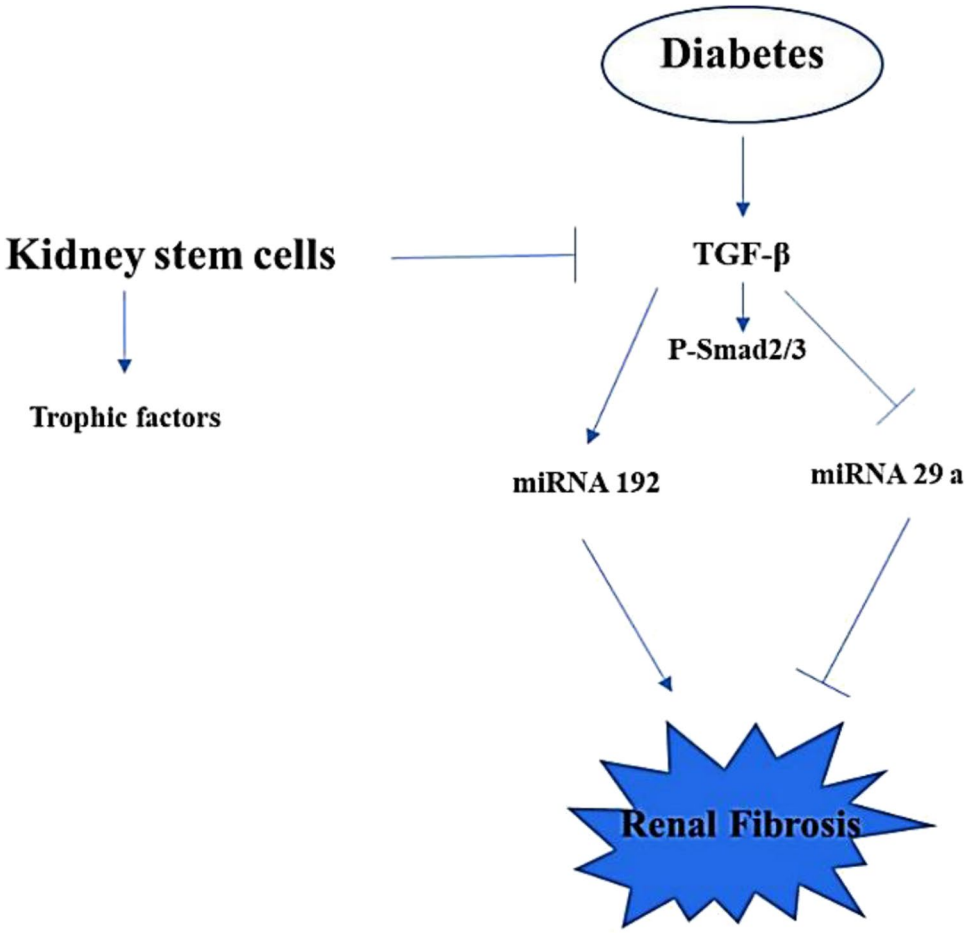


**Fig.8** A model for the role of TGF-β/Smad2 and Smad3 signaling in diabetic animals. High expression of TGF-β can downregulate miR-29a and upregulate the miR192. Activation of Smad2/3 induced by TGF-β results in renal damage. KSCs can suppress TGF-β/Smad signaling by trophic factor probably

increase proximal tubule cell damage secondary to excessive collagen deposition (Du et al. 2010).

Following KSC administration, the expression of miRNA-29a was found to be significantly elevated, whereas that of miR-192 considerably decreased in the renal tissues of DN rats. Our observations demonstrated that KSCs could prevent DN-induced renal fibrosis in part through activating miR-29a which in turn could suppress the TGF-β1/Smad signaling pathway. In line with this observation, Mao et al. noted that ASI could alleviate DN-related renal damage via suppressing miR-192 and then renal fibrosis (through the modulation of the TGF-β1/Smad/miR-192 signaling pathway), as well as reducing mesangial proliferation (Mao et al. 2019). The specific mechanism of the KSCs on miRNAs is not clear yet. Inhibition of TGF-β by paracrine/endocrine factors secreted from KSCs (Fleig and Humphreys 2014) can affect miR-192 and miRNA29-a indirectly. So, the modulation of miRNAs through various molecular mechanisms could be promising strategies for treating DN in the future (Fig. 8).

In an attempt to elucidate the mechanisms of healing action in the kidney by KSC administration, we examined Smad2 and Smad3 phosphorylation. TGF-β binds to its receptor, TGF-β receptor IIβ, which activates the TGF-β receptor I (TβRI) kinase. Activated TβRI then phosphorylates receptor-regulated Smads (R-Smads), Smad2, and Smad3 (Benigni et al. 2003). According to the analysis, phosphorylation of Smad2 and Smad3 increased during diabetes, suggesting that activation of Smad signaling is involved in diabetic-induced kidney injuries (Chen et al.2003). Smad proteins had a correlation to kidney fibrosis, and it was demonstrated that in the case of stimulation of renal tubular epithelial cells using the exogenous TGF-β, Smad2 might be phosphorylated and transported into the nucleus. Furthermore, kidney tubular epithelial cells secreting collagen (I, III, and IV types), as well as α-SMA protein expression, may be induced (Yin et al. 2014; Chen et al. 2014). Although Smad2 and Smad3 were activated in this study, no fibrosis was found in the kidney, which documents the early stage of DN. Based on our findings, the DN group, which received stem cells, showed downregulated Psmad2 and Psmad3 compared to the DN group. Treatment with stem cells suppressed fibrosis progression by preventing Smad2 and Smad3 activation. This could occur through the prevention of nuclear translocation in glomerular and tubular cells (Sierra-Mondragon et al. 2019). Treatment with stem cells suppressed fibrosis progression by preventing Smad2 and Smad3 activation. This could occur through the prevention of nuclear translocation in glomerular and tubular cells (Sierra-Mondragon et al. 2019). Regulation of renal TGFβ-Smad signaling is a potentially promising approach for alleviating renal fibrosis (Fig. 8).

This study presented a technique to separate adult stem cells from rat kidneys and subsequently transplant them into DN rats. It was found that transplanted stem cells ameliorate DN model. To approve the role of these stem cells in regeneration cells in DN model, the presence and location of stem cells in the diseased location were examined and approved. Although $CD133^+$ cells isolated from human kidney biopsies can be effective in improving renal function and ameliorating tissue damage significantly, these cells do not engraft in the kidney, instead of being entrapped in the lungs where they rapidly undergo cell death. These findings indicate that the therapeutic effects of the cells are mediated by secreted factors, possibly released by the dying cells (Rangel et al. 2018) . Our findings showed that KSCs could ameliorate functions of the kidney and regenerate the kidney tissues in DN rats, likely through their role in molecular pathways. The possible mechanisms underlying these effects involved the transient inhibition of the TGF-$\beta$ expression and the triggering of a proregenerative microenvironment which includes the production of protective trophic factors and the reduction of both the oxidative stress damage and the proinflammatory response in the kidney. Additionally, MSCs contribute to podocyte regeneration by trophic factor secretion when administered in an animal model of Alport syndrome (Prodromidi et al.2006).

Another population of kidney stem cells that expresses the specific marker of those cells (CD133 + CD24 + CD106 + cells and CD133 + CD24 + CD106 − cells) have high proliferative potential and differentiate into podocytes and tubular lineages, respectively (Angelotti et al. 2012). C-$kit^+$ progenitor/stem cells can contribute to kidney regeneration through autophagy activation and intracellular homeostasis. However, paracrine mechanisms could also account for kidney regeneration (Gomes et al. 2018). Moreover, MSCs secreted a number of factors, including HGF, basic fibroblast growth factor (bFGF), and insulin-like growth factor-1(IGF-I), which may have contributed to the amelioration of kidney damage via their antiapoptotic, mitogenic, and other cytokine actions (Togel et al.2007). Although KSCs can inhibit diabetic nephropathy development, the approach for obtaining a sufficient amount of KSCs is technically difficult because most tissues are obtained from partial nephrectomy of renal cancer patients. Alternatively, human urine is a noninvasive source of renal stem cells with regeneration potential (Rahman et al. 2020). Altogether, adult kidney stem cells may present a new candidate for therapeutic practice and preventing diabetic nephropathy.

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**Data availability** The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Declarations

**Ethics approval** The Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.ABHC.REC.1399.016) approved all experimental protocols. All procedures were done in accordance with the ethics committee of Ahvaz Jundishapur University of Medical Sciences (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

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**Competing interests** The authors declare no competing interests.

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