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Original Article

Effect of stem cell leukemia gene transfer mediated by lentivirus on morphology of interstitial cell of Cajal in bladder under high glucose concentration

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Abstract: Purpose: This study is to investigate the effect of human stem cell leukemia (SCL) gene on the morphology of interstitial cells of Cajal (ICCs) in bladder under high glucose condition. Methods: Lentivirus vector expressing SCL was constructed upon the GV287-EGFP backbone. Lenti-SCL was packaged and the titer was calibrated. Bladder ICCs from guinea pigs were isolated, identified and were treated for 24 h with 5 mmol/L, 15 mmol/L or 25 mmol/L glucose, respectively. Then, cells were transduced with Lenti-SCL, Lenti-control or left untreated for 2, 3 and 5 days. The morphology and number of ICCs were analyzed by confocal fluorescence microscope observation. Results: Typical bladder ICCs were identified according to the morphology and specific c-Kit expression. High glucose (15 mmol/L and 25 mmol/L) impaired the morphology of ICCs. Lenti-SCL transduction successfully introduced SCL gene into ICCs. Lenti-SCL transduction gradually rescued the morphological damage induced by 15 mmol/L glucose. However, Lenti-SCL transduction didn't recover the morphological damage caused by 25 mmol/L glucose. In contrast, damaged ICCs in untreated and Lenti-control transduction groups were un-rescued. Additionally, Lenti-SCL transduction had no significant effect on cell number decrease induced by high glucose treatment. Conclusion: Human SCL is expressed in glucose-treated ICCs by Lenti-SCL transduction, resulting in the partial recovery of damaged cell morphology caused by high glucose concentration.

Keywords: Stem cell leukemia gene, lentiviral vectors, diabetic cystopathy, interstitial cells of Cajal

Introduction

Diabetic cystopathy (DCP), also named as diabetic neurogenic bladder or diabetic bladder dysfunction, occurs in 19-84% of diabetic patients [1]. DCP is hard to detect because of lack of obvious early symptoms [2]. DCP can not only lead to urinary tract infection, but also lead to renal dysfunction and even kidney failure, seriously affecting the quality of life of patients [3].

A kind of special interstitial cell, which looks like interstitial cells of Cajal (ICCs) morphologically, has been found in the bladder detrusor of guinea pigs, rats, humans and many other animals [4-7]. These cells are named as ICCs in bladder [8, 9]. Bladder detrusor spontaneously contracts, which is regulated by the bladder ICCs [10]. Studies revealed that ICC signal transduction receptor blockade or inhibition [11], mitochondrial ultrastructure damage, oxidative free radical formation, decrease of ion pump activity, reduction of nerve growth factor and abnormal glucose metabolism may cause DCP [12-16]. However, the underlying mechanism for DCP development is still not fully understood.

The c-Kit is a specific protein marker that could be detected on the membrane and in the cytoplasm of ICCs. The c-kit gene is located on human chromosome 4q12-13 and encodes this transmembrane tyrosine kinase receptor that belongs to type III tyrosine kinase [17]. In the urinary system, stem cell factor (SCF) is a kind of cell growth factor expressed by bladder smooth muscle cells and can regulate cell proliferation, differentiation and migration [18]. SCF binds to the c-Kit receptor to activate the SCF/c-Kit signaling pathways such as Ras, Raf, mitogen-activated protein kinase, phosphatidylinositol III kinase and etc. [19], thus regulating the expression of multiple genes including

c-Kit and mediating the growth, proliferation, differentiation and phenotype maintenance of ICCs [20]. The hyperglycemia induced by diabetes mellitus leads to the decrease of c-Kit signal molecules in ICCs, which impairs the function of SCF/c-KIT signal transduction [21]. Stem cell leukemia gene (SCL) regulates c-Kit expression mainly through the promoter of c-Kit and its binding to the c-Kit transcriptional regulation sites [22]. SCL can regulate the survival of SCF-dependent cells and CD34-positive cells by increasing c-Kit expression in a variety of stem cells, hematopoietic cells, and $CD34^+$ cells [23]. It has been reported that antisense SCL transfection in the $CD34^+$ TL-1 cells downregulated c-Kit expression and deprived its response to SCF, while overexpression of SCL upregulated c-Kit expression and rescued the response of c-Kit to SCF [24]. Therefore, we suppose that upregulation of c-Kit in ICCs through the introduction of exogenous SCL gene may promote ICC phenotypic recovery, and inhibit and reverse ICC damage, thereby treating DCP.

In this study, ICCs were exposure to high glucose culture conditions. Then, SCL gene was introduced into ICCs by lentiviral vector. The morphology of cells was observed to investigate whether SCL expression could recover the damage caused by high glucose.

Materials and methods

Animals

A total of 30 Hartley guinea pigs (6-10 month old and 350-450 g in weight) were provided by the experimental animal center of Xinjiang Medical University. They were kept in standard conditions with free access to food and water. All animal experiments were conducted according to the ethical guidelines of Xinjiang Medical University.

Lentiviral plasmid construction

SCL gene was amplified by polymerase chain reaction (PCR) method from GV287 plasmid containing SCL gene (Genechem, Shanghai, China). Primers used were SCL forward: 5'-GAGGATCCCCGGGTACCGGTCGCCACCATGACCGAGCGGCCGCCGAG-3' and SCL reverse: 5'-TCACCATGGTGGCGACCGGCCGAGGGCCGGCTCCATC-3'. The amplification product was cloned into GV287-EGFP plasmid by In-Fusion system (Clontech Laboratories, Inc., Mountain View, CA). The corrected constructed lentiviral plasmid expressing SCL (GV287-EGFP/SCL) was identified by sequencing.

Lentivirus packaging

The 293T cells were co-transfected with GV287-EGFP/SCL, pHelper1.0 and pHelper2.0 plasmids by lipofactamine 2000 (Thermo Fisher Scientific, San Jose, California, USA). After 8 h, medium was changed to complete medium and cultured for another 48-72 h. GV287-EGFP empty plasmid was used to transfect 293T cells as negative control. The supernatant was collected to obtain lentivirus and the titer was determined by GFP expression in 293T cells. The titer of lentivirus was 5×10^8 TU/mL.

In vitro culture of ICC in bladder

A total of 5 healthy guinea pigs were selected by random number method and were sacrificed by cervical dislocation. The bladders were dissected and cut into small pieces at 1 mm^3 and placed into 1.0 mg/mL trypsin (Sangon Biotech Co., Ltd., Shanghai, China) for 15 min at 37°C. The tissues were incubated in DMEM medium (containing 100 U/mL penicillin, 100 g/mL streptomycin and 1.0 mg/mL collagenase type V (Sigma Chemical Co., St. Louis, MO, USA)) for 90 min at 37°C with CO_2. After incubation, cells were filtrated and centrifuged at 350 g for 5 min. The supernatant containing collagenase was removed. Cells were then resuspended in 1% PBS and centrifuged again at 350 g for 5 min (three times). Finally, cells were resuspended in low glucose DMEM medium (containing 10% fetal bovine serum, 25 ng/mL SCF, 100 U/mL penicillin and 100 g/mL streptomycin) and seeded into 6-well culture plates with cover glasses. Cells were cultured at 37°C with CO_2. After 24 h, non-adherent smooth muscle cells were removed. After 72 h, the ICCs were identified with immunofluorescence analysis using antibody against c-Kit.

Immunofluorescence analysis

After 72 h culture of cells, cover glasses were taken out and washed with 1% PBS for three times. Cells were fixed in 100% acetone for 15 min, washed with 1% PBS for three times and treated with 0.03% H_2O_2 for 30 min to block endogenous peroxidase. After 1% PBS washing for three times, the cells were blocked in 1% FBS for 30 min. Then, primary antibody of rat anti-mouse monoclonal c-Kit antibody (diluted 1:100, Bioscience Co., Millipore, Billerica, MA, USA) was added and incubated in the dark at

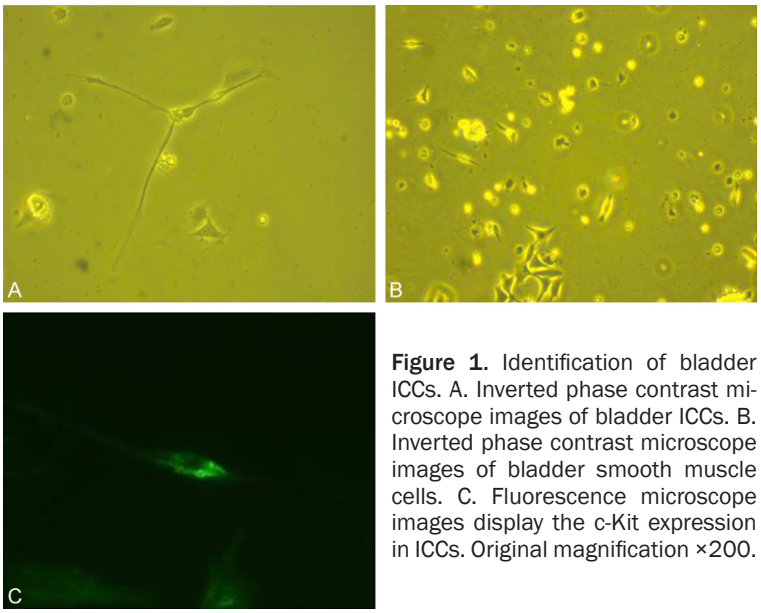

Figure 1. Identification of bladder ICCs. A. Inverted phase contrast microscope images of bladder ICCs. B. Inverted phase contrast microscope images of bladder smooth muscle cells. C. Fluorescence microscope images display the c-Kit expression in ICCs. Original magnification ×200.

room temperature for 2 h and then at 4°C for 48 h. After washing, the secondary antibody of rabbit anti-rat IgG antibody labeled with FITC (1:100, Sigma Co., St. Louis, MO, USA) was added and incubated at room temperature for 1 h. The slides were mounted and observed under confocal laser scanning microscopy (Leica TCSSP5, Germany).

Lentiviral transduction of ICCs in vivo

ICCs were seeded in 96-well plate and cultured under normal glucose (5 mmol/L) or high glucose condition (15 mmol/L and 25 mmol/L) for 24 h. Then, cells were transfected with empty lentivirus (Lenti-control) or lentivirus expressing SCL (Lenti-SCL) at the MOI=10 in the presence of 10 μg/mL polybrene, or left untreated. At 2 d, 3 d and 5 d after transduction, the morphology and GFP expression of cells were observed under laser scanning confocal microscopy (Leica TCSSP5, Germany) for consecutive 20 visual fields (×200), with the size of each field of 0.77 mm^2. Each experiment was repeated for four times independently. ICC number was counted by Photoshop software.

RNA extraction and real-time PCR

At 2 d, 3 d and 5 d after transduction, total RNAs were isolated using TRIzol® isolation reagent (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Total RNA was reverse transcribed using a Reverse Transcription Kit (Fermentas, Vilnius, Lithuania) into cDNA. The PCR procedure was as follows: pre-denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 1 min, and final extension at 72°C for 10 min. Primers used were as follows: SCL forward 5'-GAGGATCCCCGGTACCGGTCGCCACCATGCGAGCGGCCGCCGAG-3'; SCL reverse 5'-TCACCATGGTGGCGACCGACCGGCCGAGGGCCGGCTCCATC-3'; GAPDH forward 5'-ACCACAGTCCATGCCATCAC-3'; GAPDH reverse 5'-TCCACCACCCTGTTGCTGTA-3'. GAPDH was used as internal control. The relative expression levels were evaluated by a Gel Doc XR gel imaging and analysis system (BioRad, Hercules, CA, USA) after agarose gel electrophoresis.

Statistical analysis

Data analysis was carried out using the SPSS software 17.0 (IBM Corp, Chicago, IL) and expressed as mean ± SD. Differences between groups were evaluated using analysis of variance (ANOVA). $P<0.05$ was considered as significant.

Results

ICC culture and identification

The isolated bladder ICCs were identified according to both morphology and c-Kit expression. Smooth muscle cells and ICCs were separated based on the difference in adherent time. Under the inverted phase contrast microscopy, ICCs in the shape of long spindle or spindle can be observed. The cells had large nucleus, a few black glycogen particles in the cytoplasma and characteristic lateral branches extended from the center of the cell body to both sides (**Figure 1A**). The shape of these adherent cells was regular. In contrast, the non-adherent smooth muscle cells had different morphology in that the whole cell shape was flat, cell body was mostly ellipsoidal and no convex branches can be observed (**Figure 1B**). Therefore, ICCs with typical morphological characteristics had been separated.

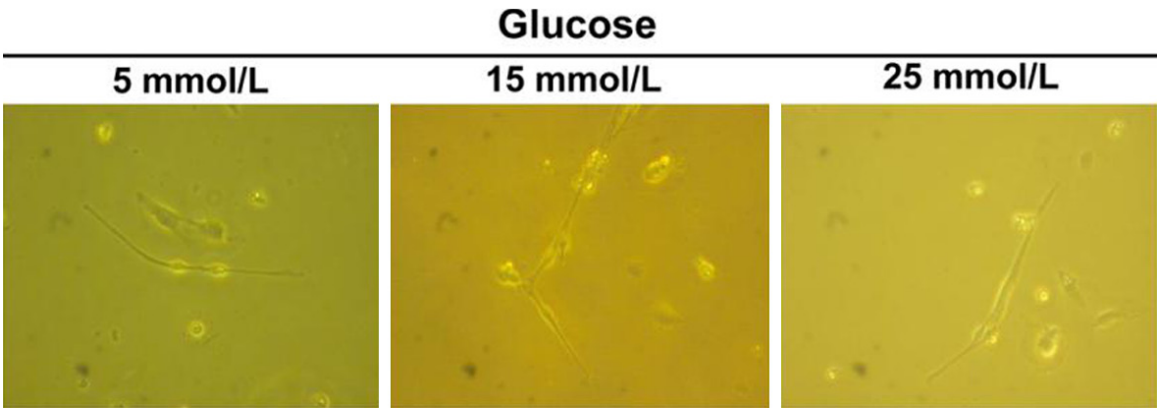

Figure 2. Morphology of ICCs after glucose treatment. ICCs were treated with 5 mmol/L, 15 mmol/L, or 25 mmol/L glucose for 24 h. Inverted phase contrast microscope images of cell morphology were shown. Original magnification ×200.

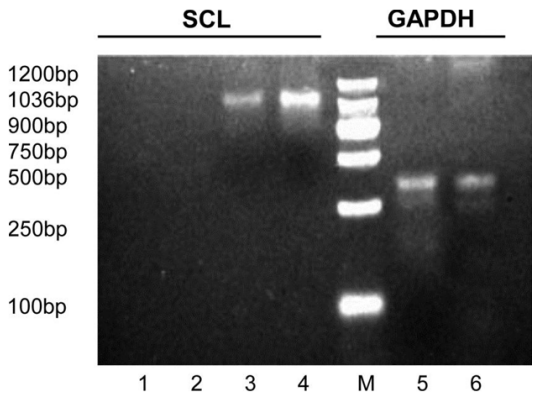

Figure 3. RT-PCR analysis of SCL mRNA expression. ICCs were transfected with Lenti-SCL, Lenti-control or left untreated. The expression of SCL and GAPDH mRNA was analyzed by RT-PCR at 3 d. Lane 1, Untreated; Lane 2, Lenti-control group; Lane 3 & 4, Lenti-SCL group; Lane 5 & 6, Lenti-control group.

Next the expression of ICC specific marker, c-Kit, was detected in the separated ICCs. As shown in **Figure 1C**, expression of c-Kit could be observed under confocal laser scanning microscopy, indicating that the adherent cells were indeed ICCs.

Effect of glucose concentration on ICCs

To investigate the effect of different concentration of glucose on the growth of ICCs, cells in each group were observed after 24 h of culture. The representative images were shown in **Figure 2**. Cells treated with 5 mmol/L glucose had similar morphology. These cells had characteristic spindle or long spindle shape of ICCs with two lateral branches in both sides. In the 15 mmol/L and 25 mmol/L glucose treated groups, some cells were in the shape of ellipsoidal and single branch from the body could be observed with the other side like comet. Moreover, some cells showed irregular shapes with single branch. These results show that 5 mmol/L glucose had no obvious effect on the morphology of ICCs, but 15 mmol/L and 25 mmol/L glucose impaired the morphology of ICCs.

Expression of SCL mRNA in ICCs

To investigate whether Lenti-SCL could introduce SCL gene into ICCs after transduction, expression of SCL mRNA was analyzed by real time PCR. As shown in **Figure 3**, the whole length of SCL gene (1036 bp) could be amplified in ICCs transduced with Lenti-SCL. No specific band was observed in Lenti-control transduced cells or untreated cells whereas GAPDH mRNA could be amplified in these cells. This result indicates that transduction with Lenti-SCL could successfully introduce SCL gene into bladder ICCs.

Effect of lentivirus transduction on cell morphology

To investigate the effect of Lentivirus transduction on cell morphology and number of bladder ICCs, GFP expression coded by the lentivirus vector was first observed by confocal laser scanning microscopy. As shown in **Figure 4**, no fluorescence could be observed in cells from each subgroups of the untreated and Lenti-control group. The morphology of cells was

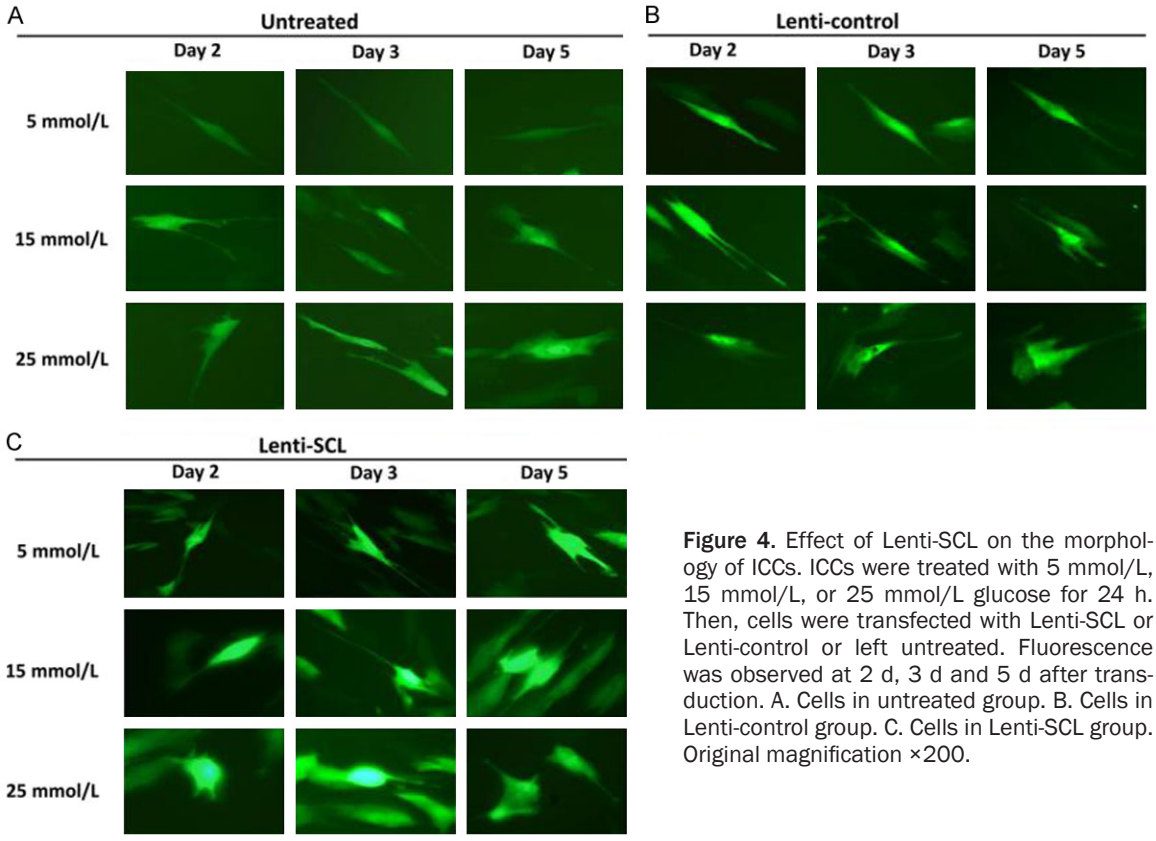

Figure 4. Effect of Lenti-SCL on the morphology of ICCs. ICCs were treated with 5 mmol/L, 15 mmol/L, or 25 mmol/L glucose for 24 h. Then, cells were transfected with Lenti-SCL or Lenti-control or left untreated. Fluorescence was observed at 2 d, 3 d and 5 d after transduction. A. Cells in untreated group. B. Cells in Lenti-control group. C. Cells in Lenti-SCL group. Original magnification ×200.

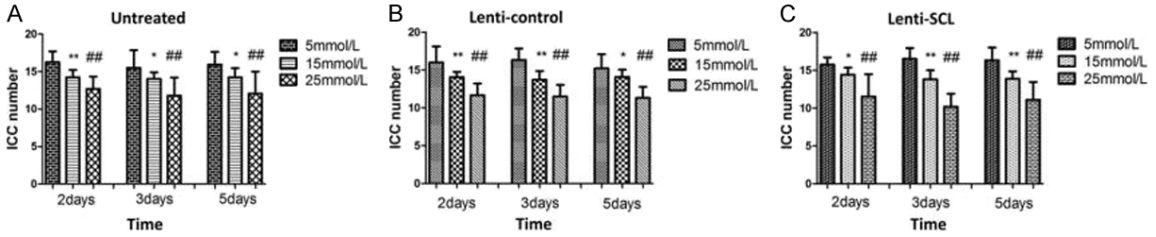

Figure 5. Effect of Lenti-SCL on the cell number of ICCs. ICCs were treated with 5 mmol/L, 15 mmol/L, or 25 mmol/L glucose for 24 h. Then, cells were transfected with Lenti-SCL or Lenti-control or left untreated. Cell number was calculated at 2 d, 3 d and 5 d after transduction. A. ICC cell number in untreated group. B. ICC cell number in Lenti-control group. C. ICC cell number in Lenti-SCL group. *P<0.05, **P<0.01, compared with 5 mmol/L glucose group; #P<0.05, ##P<0.01, compared with 15 mmol/L glucose group.

similar in all the 5 mmol/L glucose treated cells at 2 d, 3 d and 5 d, presented as long spindle or spindle shape with two lateral branches. The 15 mmol/L glucose treatment changed the shape of ICCs into ellipsoidal with single branch from the body and a comet-like side at 2 d and 3 d. At 5 d after transduction, 15 mmol/L glucose group cells became swollen with single branch, and the number of cells significantly decreased as compared with the 5 mmol/L glucose group (P<0.05; **Figure 5**). In the 25 mmol/L glucose groups, cells became irregular but with single branch at 2 d. At 3 d, these cells became strip-like shape and the single branch was shortened. At 5 d, the morphology of these ICC was almost lost, both the cells and nucleus were enlarged and no branches could be observed, and cell number was significantly decreased compared with the 15 mmol/L glucose group (P<0.01; **Figure 5**).

GFP expression was observed in cells after Lenti-SCL transduction. At 2 d, 3 d and 5 d, cells in the 5 mmol/L glucose group had typi-

cal long spindle or spindle shape, and the cells became shuttle-like with 2-4 lateral branches (**Figure 4C**). The shape of cells treated with 15 mmol/L glucose changed from ellipsoidal to spindle, and the branches changed from single to two-side at 2 d and 3 d (**Figure 4C**). At 5 d, typical ICC morphology was observed with bilateral branches, and the cell number was significantly decreased compared with the 5 mmol/L glucose treatment subgroup ($P<0.05$; **Figure 5C**). At 2 d and 3 d after transduction in the 25 mmol/L glucose treated cells, the swollen round ICCs gradually became ellipsoidal, and single lateral branches could be observed (**Figure 4C**). At 5 d the shape of cells was still irregular and no obvious changes could be seen compared with former time points (**Figure 4C**). Additionally, cell number was significantly less than that in 15 mmol/L glucose group (**Figure 5C**).

Taken together, in cells transfected with Lenti-SCL, GFP expression was observed. Moreover, lentivirus mediated SCL expression in ICCs gradually rescued the morphological changes caused by 15 mmol/L glucose. However, SCL over-expression didn't recover the morphological changes induced by 25 mmol/L glucose. In contrast, ICCs in untreated and Lenti-control transduction groups were damaged by high glucose treatment. Additionally, Lenti-SCL transduction had no significant effect on cell number decrease imposed by 15 mmol/L or 25 mmol/L glucose treatment.

Discussion

It has been demonstrated that abnormalities in ICC number and functions can cause bladder dysfunction, and thus promote the occurrence and development of DCP [25]. In particular, the bladder detrusor has spontaneous excitability and contractility independent of neurological factors, and ICCs are able to control this myogenic excitatory and contractile [26]. In addition, ICCs are able to pace bladder activity and conduct nerve information [27]. Spontaneous action potentials of bladder ICCs can be detected by patch clamp technique, and detrusor contraction is significantly inhibited if the action potential is blocked [28].

Specific interaction of c-Kit receptor in ICCs with its ligand SCF activates SCF/c-Kit signaling pathway, which is indispensable for the maintenance of ICC physiological characteristics and phenotype [22, 25, 29]. SCL is a critical regulator upstream of c-Kit, regulating c-Kit expression through binding to its promoter. Over-expression of SCL enhances c-Kit expression, promoting the recovery of ICC function and phenotype in DCP [30]. Therefore, intact SCF/c-Kit signaling pathway is of great importance for the function and phenotype of ICCs. Based on these findings, we suppose that expression of human SCL gene in guanine pig ICCs that are exposed to high glucose condition would rescue the damage of ICC phenotype, which may provide new idea for the gene therapy of DCP.

Gene delivery is the process of introducing target genes into cells to express these genes, sometimes by integrating these genes into host genome [31]. With the development of gene engineering a variety of gene vectors, such as plasmid vectors, phage vectors, adenovirus vectors and lentivirus vectors were used to deliver genes [32, 33]. In this study, we used lentivirus as the vector to introduce SCL gene. Lentivirus came from human immunodeficiency virus 1, and it has high efficiency of transduction and integration. Exogenous genes could be stably expressed by lentivirus for a long term. Moreover, lentivirus has high biosafety since the packaged virus particles cannot synthesize their own proteins due to the lack of viral genes. Therefore it is an ideal vector for *in vivo* and *in vitro* experiments [34-37]. In a previous study, Yu et al. successfully expressed SCL gene in guinea pig bladder ICCs with high efficiency by lentivirus transduction, and found that the transduction efficiency was best (more than 80%) when MOI=10 [38]. Hence, MOI=10 was used in this study.

To simulate high glucose environment, the control of glucose concentration is critical. Overdose glucose may result in cell death, covering the phenotype of SCL gene expression. Here, we chose three glucose concentrations of 5 mmol/L, 15 mmol/L and 25 mmol/L to treat ICCs, and after glucose treatment, cells were transduced with lenti-SCL. We found that as the normal physiological concentration, 5 mmol/L glucose had no obvious effect on the typical phenotype of ICCs, whereas SCL expression changed the morphology of ICC into shuttle-like shape. The 15 mmol/L glucose damaged the morphology of ICCs to some extent, which could be completely rescued by

SCL gene introduction. The 25 mmol/L glucose severely damaged ICC morphology to the extent that the typical phenotype of ICCs was lost, and introduction of SCL gene partially recovered such damage. Additionally, cell number was reduced with the increase of glucose concentration, which could not be ameliorated by SCL gene. It may be due to that the proliferation ability of ICCs during embryo development is lost in adults [39]. It has been reported that no obvious proliferation can be observed in bladder ICCs from adult guinea pigs after one week culture under normal or high glucose concentration [40].

Therefore, lenti-SCL transduction in high glucose treated ICCs could recover or ameliorated its morphology, which was dependent on the concentration of glucose. The recovery effect was reduced with more serious cell damage. In this study, cells were treated with glucose for 24 h followed by lenti-SCL transduction. Whether lenti-SCL transduction has similar effect in cells treated by glucose for 48 h, 72 h and longer time needs further investigation. Moreover, since *in vitro* experiments in this study cannot fully reflect the effect of SCL gene under *in vivo* conditions, these results needs validation in lenti-SCL transduced bladders.

Taken together, by transduction of lenti-SCL into glucose treated bladder ICCs, we successfully introduced SCL genes into these cells. Expression of SCL completely or partially recovered the morphological damage imposed by high glucose treatment, but not the reduced cell number, suggesting that lenti-SCL could be used for gene therapy of DCP.

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Disclosure of conflict of interest

None.

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