IGF-1-induced Muscle-Derived Stem Cells as a Potential Treatment of Stress Urinary Incontinence in Female Rats

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Purpose: Stress urinary incontinence (SUI) is prevalent among elderly women. This study aimed to discuss the potential of muscle-derived stem cells (MDSCs)-based therapy in treating SUI by exploring the effect of Insulin-like growth factor-1 (IGF-1) on transplanted MDSC and urethral sphincter function.

Materials and Methods: Bilaterally pudendal nerve-transected (PNT) female rats were divided into four groups: sham, PNT+ phosphate buffered solution (PBS) injection, PNT+IGF-1/MDSCs and PNT+ green fluorescent protein (GFP)/MDSCs. IGF-1 was expressed in MDSCs by lentiviral vector. Viable MDSCs were detected by laser scanning confocal microscopy (LSCM). The expression of Myosin heavy chain (MyHC), vascular endothelial growth factor (VEGF), VEGF receptor 2 (VEGFR-2), microvessel density (MID) and urethral resistance function were assessed.

Results: IGF-1 promoted the survival and differentiation of MDSCs. IGF-1-expressing MDSCs facilitated local angiogenesis and muscle fiber regeneration, and alleviated symptoms of SUI.

Conclusion: IGF-1-expressing MDSCs may be used as a novel treatment for patients with SUI.

Keywords: insulin-like growth factor-1; muscle-derived stem cells; myogenic differentiation; pro-survival; stress urinary incontinence

INTRODUCTION

Urinary incontinence (UI) describes the loss of bladder control, often leading to the accidental leakage of urine. UI is especially common among elderly women, with over 200 million cases worldwide[1]. The most common subtype of UI, stress urinary incontinence (SUI) refers to urine loss triggered by physical activities, such as coughing and exercise. The pathophysiology of SUI has been associated with the apoptosis of urethral sphincter cells in aging women. Risk factors for SUI also include obesity, nerve injuries, pregnancy and childbirth. To maintain urinary continence, the pudendal nerves that regulate the external urethral sphincter are stimulated, resulting in urethral contraction. Currently, transurethral bulking agent injection is a widely used minimally invasive procedure to increase urethral resistance. However, such operation can only relieve the symptoms and does not completely eradicate the UI condition, and the injection of materials (bulking agents) into the tissues surrounding the urethra can lead to serious complications.

The advancement in tissue engineering suggests a new potential in using muscle-derived stem cells (MDSCs)-based therapy to regenerate and restore the urethral function in SUI patients[2,3]. During myogenesis, myoblasts, or skeletal muscle precursors proliferate and differentiate, followed by an increase in the expression of muscle-specific genes. Subsequently, myoblasts fuse into multinucleate myofibers, forming new skeletal muscle[4]. In adults, cells capable of becoming myoblasts remain inactive as satellite cells until injury, during which they will proliferate and fuse to repair the damaged muscle[4]. Thus, the regeneration of skeletal muscle tissue is dependent on the number of satellite cells, which declines with aging[5]. On the other hand, MDSCs can serve as precursors of the satellite cells in adults, aiding in muscle repair[6]. However, it remains unknown whether MDSCs would go through myogenic differentiation in SUI patients after implantation.

IGF-1, a peptide growth hormone and an anti-aging factor, can alleviate the age-related muscle atrophy while facilitating cell proliferation in adult rats after muscle damage[7]. On the cellular level, IGF-1 plays dual roles in inhibiting apoptosis and promoting differentiation of transplanted myoblast cells[8,9]. Another study showed that IGF-1 could promote proliferation and myogenic differentiation of urethral rhabdosphincter satellite cells[10]. Therefore, IGF-1 may be able to mediate the growth of skeletal muscle substitutes and the restoration of muscle function in vivo[11]. Herein, we hypothesized that IGF-1 could promote better viability, growth and differentiation of MDSCs, aiding in the restoration of urethral sphincter function in SUI patients. To test our hypothesis, we used bilateral pudendal nerve-transected female rats as the model for SUI to investigate the effects of IGF-1 on MDSCs.

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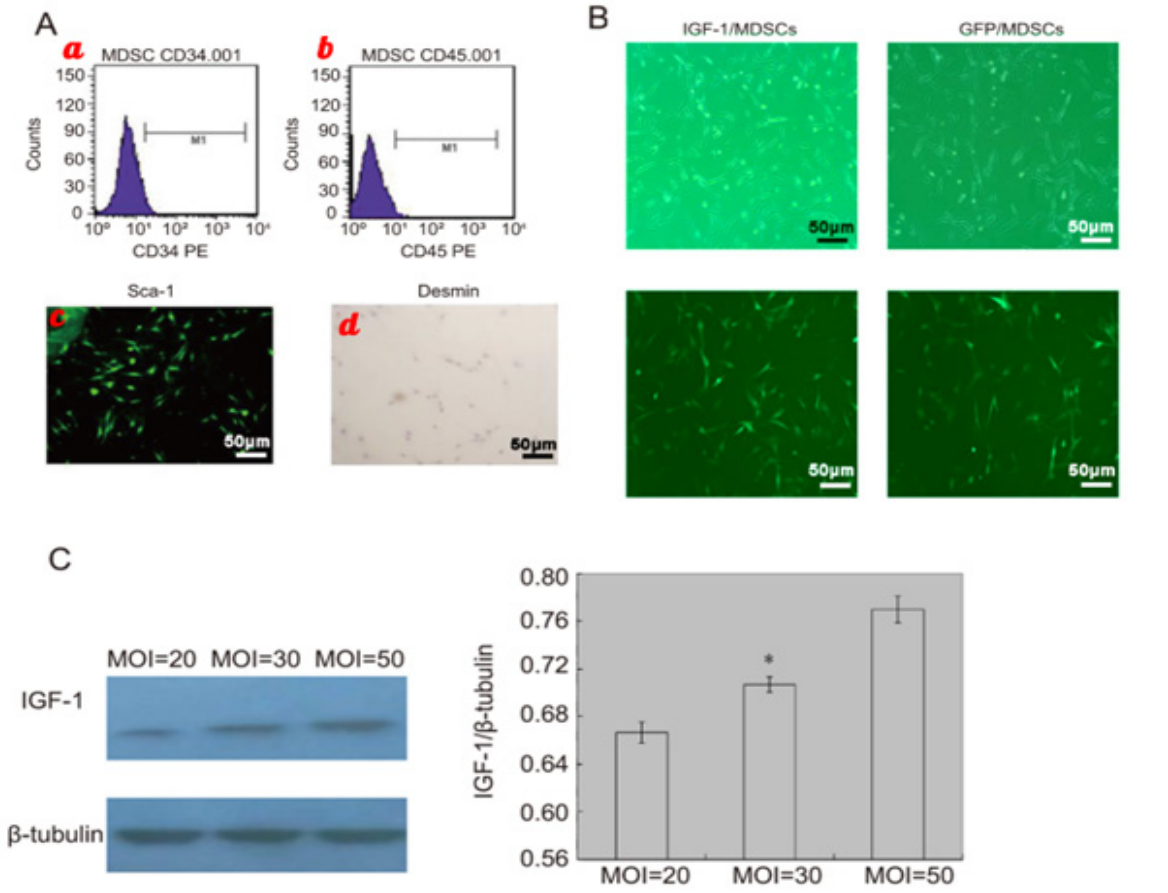

Figure 1. Purification and lentiviral transduction of MDSCs. **(A)** P3-MDSCs were isolated from the hind gastrocnemius and examined for CD34 and CD45 by flow cytometry, and immunostaining for Sca-1 and desmin by immunofluorescence and immunocytochemistry, respectively. **(B)** Similar morphological changes were observed in GFP/MDSCs and IGF-1/MDSCs, shown in the upper panels; more than 90% of MDSCs were infected by lentivirus vector as indicated by GFP fluorescence in the lower panels. **(C)** the expression of IGF-1 was dependent on the levels of MOI. The error bar representing mean and 95% CI. *indicates significant difference as compared to other groups ($p < 0.05$).

MATERIALS AND METHODS

Experimental animals and model establishment

The animal study was approved by our Hospital Institutional Animal Care and Use Committee. Sixty 4-week-old female rats were randomly assigned to four groups: sham (S), PNT+PBS injection (P), PNT+GFP/MDSCs injection (M) and PNT+IGF-1/MDSCs injection (I). All but sham rats received bilateral pudendal nerve transection (PNT) (12). Briefly, the rats were anesthetized by chloral hydrate [0.3 mL/100g, intraperitonal (i.p.)]. Following a bilateral dorsal longitudinal incision, the pudendal nerve was isolated with operating microscope. The pudendal nerve was transected in a 2cm segment on both sides of the vertebral column. Sham rats were put through the same surgical procedures with the exception of pudendal nerve transection.

Animal treatment

Two weeks post-PNT/sham, transplantation was performed. Each rat was anesthetized and urethra was isolated. An injection was made on each side of the 1/3 upper middle urethra (0.5cm to the bladder, 3 and 9 o'clock) with a micro-syringe. Sham rats received the same surgical intervention without injection. Group P was microinjected with 50 μL 1xPBS. Group M was injected with 50 μL GFP/MDSCs (1 x 106 /50 μL PBS). Group I was injected with 50 μL IGF-1/MDSCs (1 x 106 /50 μL PBS). Each group was then subdivided into 1-week (N=5), 2-week (N=5) and 4-week (N=5) subgroups randomly.

Lentiviral vector construction and titer determination

Rat Igf-1 cDNA (GENECHEM, Shanghai, China) was cloned into GFP-carrying IGF-1 recombinant lentiviral vector (pGC-FU) (GENECHEM, Shanghai, China), confirmed by sequencing (invitrogen) (**Supplementary Table 1**). Lentiviral titer was determined by real-time fluorescence quantitative PCR (RT-qPCR). Briefly, 293T cells (1 x 105 cells/500 μL/well) were passaged in 24-well TC plate every 48 hours. Lentiviral extract was serial-diluted in DMEM/F12 medium (10% FBS) and mixed with 293T. The infected cells were incubated at 37C, 5% CO2. After 4 days, RNA was extracted with l mL TRIzol (Invitrogen) and treated with 20μl RNA enzyme inhibitor (RNasin), and concentration was determined by spectrophotometer. RNA was reverse-transcribed to cDNA with M-MLV (Promega); β-actin and EGFP were amplified with SYBR® Premix Ex Taq (Takarabio) (Supplementary Table 1). ΔCt was used to calculate titer in each sample. ΔCt >2 was considered significant.

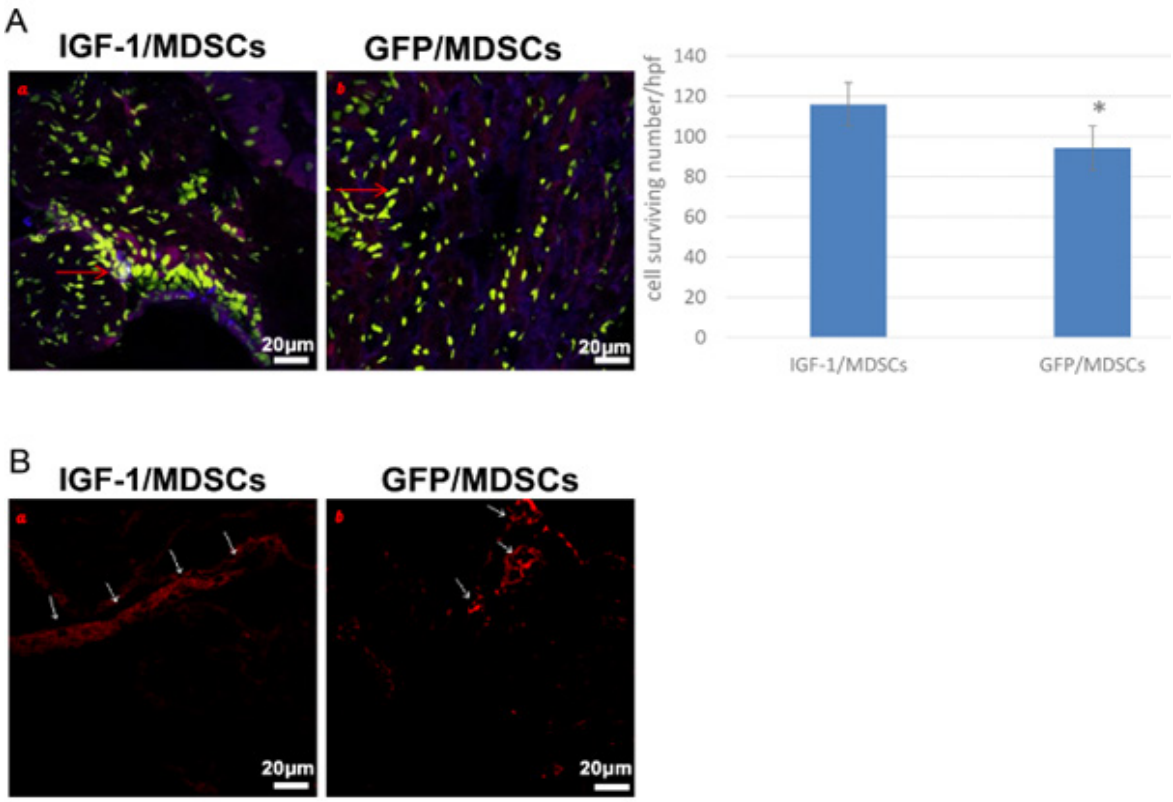

Figure 2. The effect of IGF-1 on MDSC survival and the expression of MyHC.
The isolated and transplanted MDSCs in proximal urethra were examined for the effect of IGF-1 on the MDSC survival and the expression of MyHC. **(A)** MDSC survival in IGF-1/MDSCs and GFP/MDSCs groups was shown. Red arrows denote MDSCs labelled with GFP. The error bar representing mean and 95% CI. *indicates statistical significance between two groups ($p < 0.05$). **(B)** MDSC differentiation in IGF-1/MDSCs and GFP/MDSCs groups was shown. White arrows denoted MyHC labelled with TIRTC.

Lentiviral transduction of MDSCs
P3-MDSCs were transduced with GFP-expressing lentiviral vector (GFP/MDSCs), or with GFP-IGF1-expressing bicistronic vector (IGF-1/MDSCs). Transduction efficiency was assessed by fluorescence microscopy (Olympus, Japan).

MDSCs isolation
MDSCs were obtained from the hind gastrocnemius of 3-weeks-old Wistar female rats by modified preplate technique[13]. Briefly, gastrocnemius muscles were removed, minced and dissociated with enzyme. Rapidly adhering cells (RACs) and slowly adhering cells (SACs) containing MDSCs were obtained and were further purified preplating at each passaging step.

MDSCs purity assessment
Immunostaining and flow cytometry were used to assess cell purity. CD34, CD45, Sca-1 and desmin were used to identify myogenic progenitors[14]. P3-MDSCs were washed with PBS and digested with 1% pancreatin. Digestion was stopped 10% fetal bovine serum (FBS). Cells were separated and was adjusted to 1 x 106 under microscope. Mouse anti-CD34 (rat) PE-labeled monoclonal (Santa Cruz Biotechnology) and mouse anti-CD45 (rat) PE-labeled monoclonal (Biolegend) were added respectively, and sample was assessed on flow cytometer (Becton-Dickinson, USA) after 30min incubation at 37℃. Meanwhile, cover slips were placed in the 6-well cell culture plates, where pancreatin-digested P3-MDSCs were inoculated (0.5×105/ml/well). Coverslip was treated with neutral balsam, followed by 3% hydrogen peroxide. Coverslip was blocked by goat serum for 20min, and incubated in rabbit anti-Sca-1 polyclonal (1:100, PharMingen, USA) or anti-desmin (1:100, Boster Biological Technology co.ltd, China) at 4℃ overnight. Coverslip was incubated in FITC-labeled IgG (1:64) at 37℃ in dark for 45 min. Coverslip was washed, dried, mounted, imaged at 200X magnification (Olympus CX40).

Urodynamic test
Urodynamics were examined to assess urethral sphincter function. Briefly, the rats were anesthesized to isolate the bladder. Infusion pump and pressure sensor were connected to the urodynamic equipment (MMS, Netherlands). After emptying the bladder via Grede method[15], sterile saline was infused into bladder at 5 mL/h, and voiding was observed. Once the blue urine flowed out, maximum bladder capacity (MBC) was determined. The bladder was emptied multiple times and was filled with saline to half MBC. Abdomen was gently pressed until urine was produced to determine the abdominal leak point pressure (ALPP). The bladder was emptied and re-infused three times.

H&E and Masson staining
All animals were euthanized four weeks post-injection.

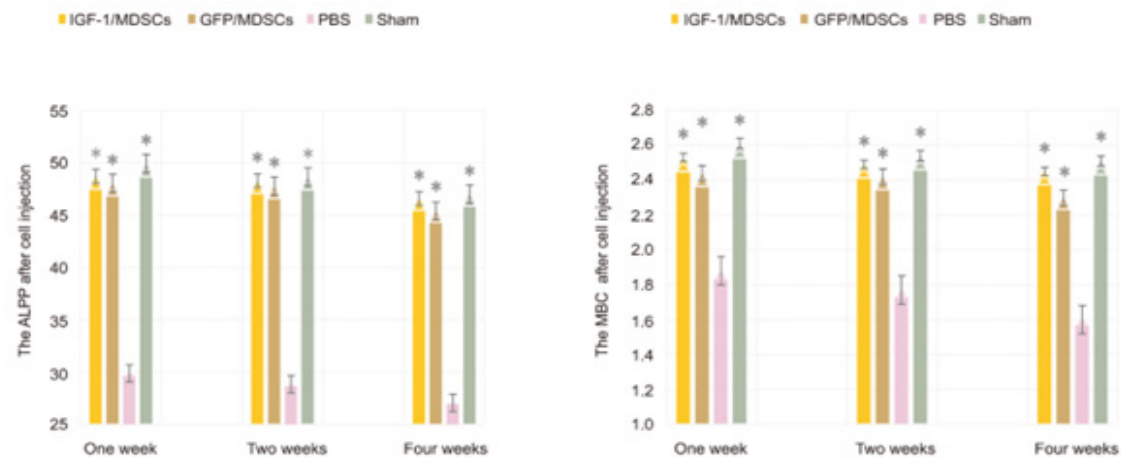

Figure 3. SUI symptoms after MDSC transplantation shown by urodynamic test.
After MDSC transplantation, ALPP and MBC were measured at different time all groups, including IGF-1/MDSCs, GFP/MDSCs, PBS, and sham. Similar results were observed in three independent experiments. *indicates significant difference as compared to the group PBS ($p < 0.05$).

To evaluate pathological changes in the urethra, proximal urethral tissues were embedded in paraffin, sectioned into 5-µm thick slices, and collected for routine H&E and Masson's trichrome staining (Sigma, USA). For each group, four specimens were imaged at 200X magnification (Olympus CX40 & Imaging Micropublisher 5.0 PTV).

Immunohistochemical analysis

Immunohistochemical analysis was performed on 8-µm thick cryo-sectioned slices and paraffin proximal urethra sections. Sections were incubated with primary antibodies at 4 °C overnight, including rabbit anti-FVI-II R Ag polyclonal antibody (Zymed, USA; 1:100), mouse anti-MyHC (rat) monoclonal antibody (Abcam, UK; 1:200) and rabbit anti-VEGF polyclonal antibody

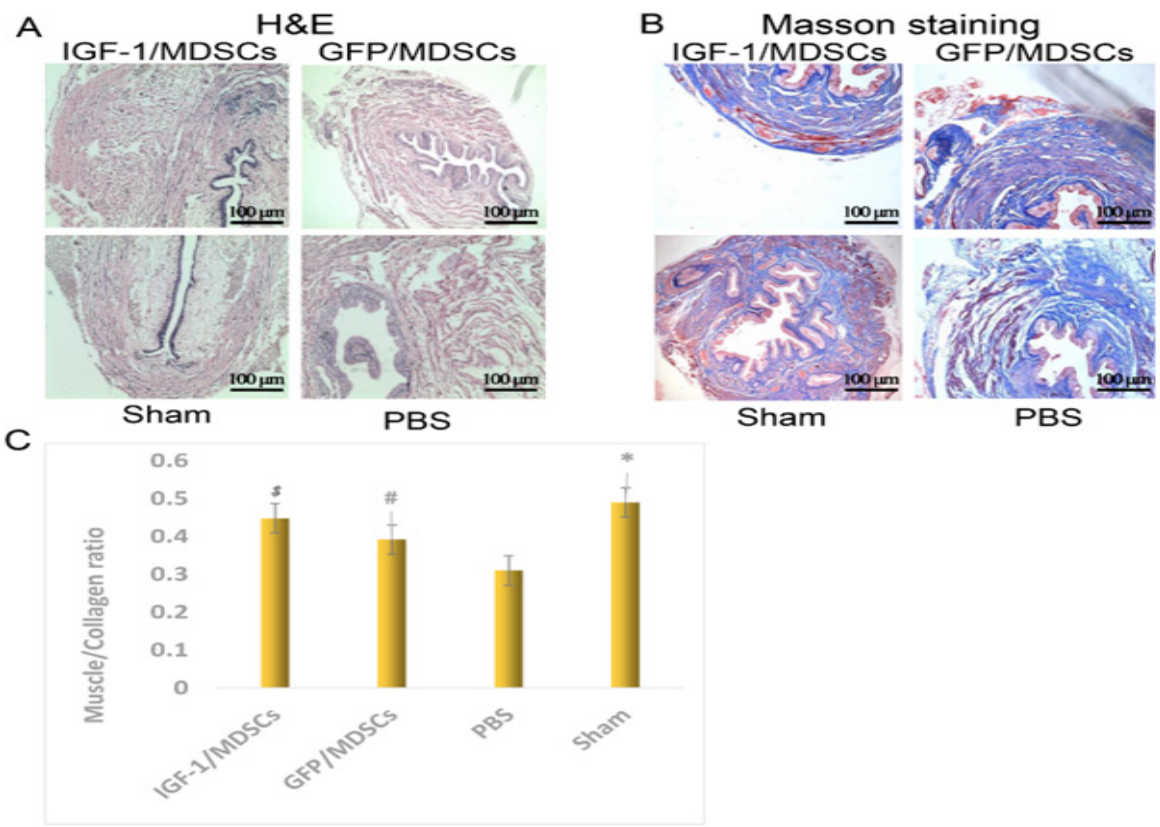

Figure 4. Pathological analysis of urethra four weeks after injection. The proximal urethral tissue samples were collected and processed for **(A)** H&E and **(B)** Masson's trichrome staining. **(C)** Muscle to collagen ratio for each of the four groups was presented in chart. $, # and *indicate significant difference in comparision to other groups ($p < 0.05$).

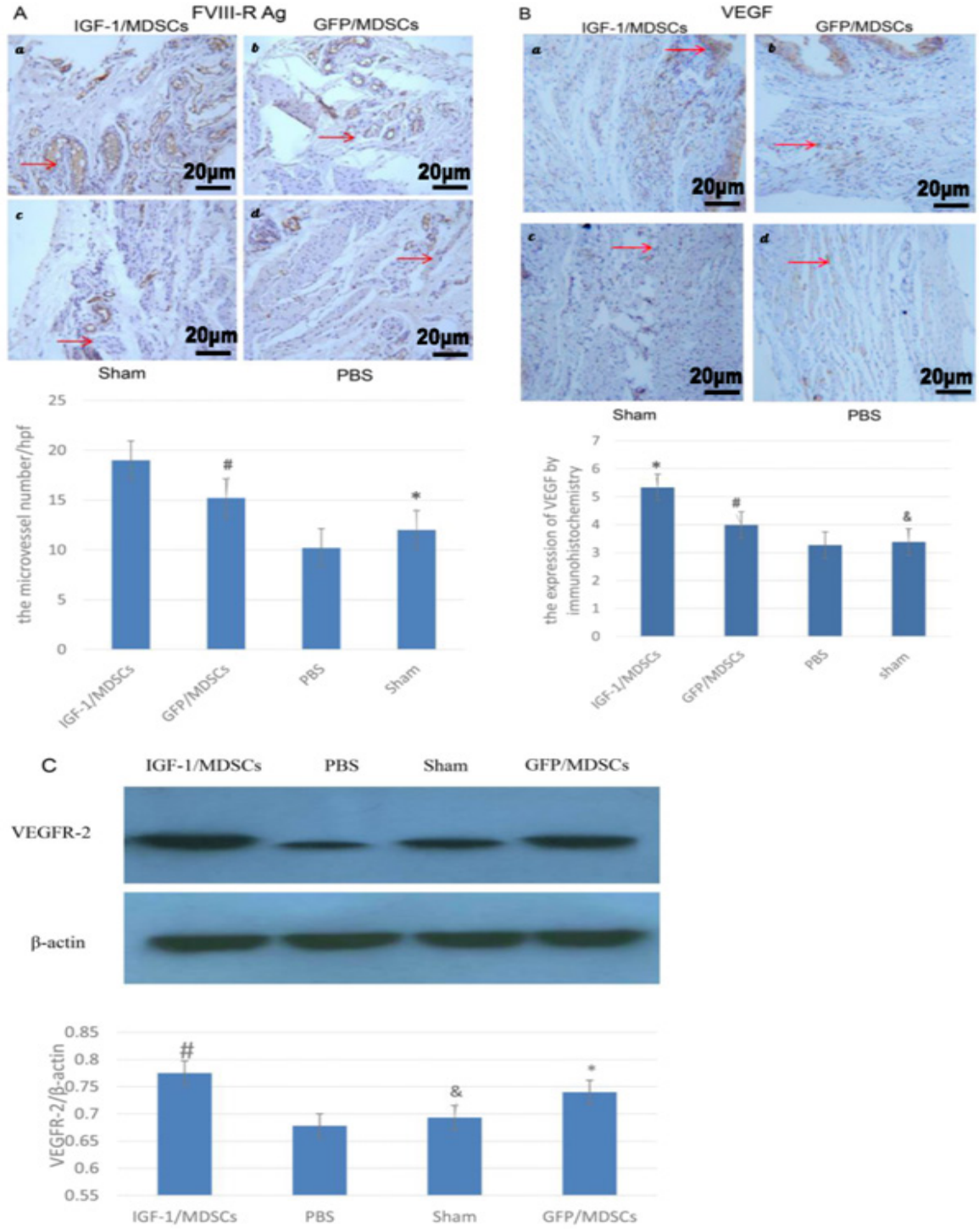

Figure 5. Microvessel density analysis of rat urethra and the detection of VEGF/ VEGFR-2. Four weeks after injection, proximal urethral tissue samples were collected from each of the four groups. Results from three independent experiments were summarized in error bar representing mean and 95% CI. #, & and *indicate significant difference as compared to other groups ($p < 0.05$).
(A) Samples were processed for microvessel density analysis via immunohistochemistry of FVIII-R Ag. The arrows indicated newborn microvessels. **(B)** The expression of VEGF was examined by immunohistochemistry. The arrows indicated VEGF positive staining. (C) Western blot analysis showed the level of VEGFR-2 in the proximal urethral tissue samples. β-actin was used as an internal control.

(Sigma, USA, 1:100). Sections were treated with corresponding secondary antibodies (1:100) for 45 min at room temperature. For each group, four specimens and five randomly selected fields were imaged under laser scanning confocal microscopy (LSCM) (M510, Zeiss, Germany), and CX40 microscope (Olympus). In the images, cells in blood vessels and the smooth muscle layer, as well as the rhabdosphincter layer in the sections, stained red while collagens stained blue. Image analysis was done as described above and quantified

using the Image-Pro Plus 6.0 image software. The software can automatically distinguish regions stained with different colors and accurately measure the areas of muscle and collagen to yield a muscle/collagen ratio.

Western blot

Total protein was extracted from urethra tissues lysates; protein concentration was determined by BCA assay. Proteins were separated by SDS-PAGE and electrotransferred to polyvinylidene fluoride (PVDF) membranes. All membranes were blocked by 3% bovine serum albumin (BSA) for 3 h at room temperature. Membranes were incubated in primary antibody (anti-VEGFR2, Abcam; 1:200) overnight at 4 °C. β-actin was used as an internal control. After incubation in secondary antibody (1:100) for 45 min at room temperature, proteins were visualized by chemiluminescence (ECL) solution.

Statistical Analysis

Results (mean ± SD) were analyzed with SPSS20.0. Normality and homogeneity of variance test of the sample were performed previous to the statistical analysis. Differences among groups and time points were analyzed by two-way ANOVA and least-significant difference (LSD) test was used as post-hoc test, whenever a significant difference was found by the ANOVA. Unpaired t-test was applied for compare means of two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Purification and lentiviral transduction of MDSCs

After purification, MDSCs expressed CD34 but not CD45 (Figure 1A a,b), eliminating the possible contamination of hematopoietic stem cells[14]. Sca-1 and desmin by immunofluorescence and immunocytochemistry respectively showed that about 60% of the cell suspension was MDSCs undergoing myogenic differentiation (Figure 1A c,d)[14]. After transduction, more than 90% of MDSCs was infected by the IGF-1-carrying lentivirus vector (2×108 TU/mL) in both GFP/MDSCs group and IGF-1/MDSCs group, as indicated by GFP fluorescence (Figure 1B). Lastly, we noticed that the expression of IGF-1 increased at higher multiplicity of infection (MOI) (Figure 1C). MOI=30 was used as the optimal condition in subsequent analyses.

IGF-1 enhances MDSC survival and differentiation

We next tested the effect of IGF-1 on the survival and differentiation of MDSCs. The isolated and transplanted MDSCs in proximal urethra were identified by GFP. MDSCs were found in the submucosa and muscular layer of middle urethra (**Figure 2A a**). Cell survival was higher in the IGF-1/MDSCs group compared to that in the GFP/MDSCs group (**Figure 2A b**; $P < 0.05$), in line with a previous study (16). Next, we assessed MyHC expression, a marker for later-stage muscle-specific protein in the IGF-1/MDSCs and GFP/MDSCs groups. As expected, IGF-1/MDSCs showed higher expression of MyHC, suggesting that IGF-1 could promote MDSC differentiation (**Figure 2B**).

Urodynamic testing

After cell injection, six rats were withdrawn from the study due to urethra congestion or infection. Abdominal leak point pressure (ALPP) and maximum bladder capacity (MBC) after cell injection were assessed at three time points (1-week, 2-week, 4-week) (Figure 3). At all time-points, ALPP and MBC in the IGF-1/MDSCs and GFP/MDSCs groups were significantly higher than those injected with PBS ($P < 0.05$), and slightly lower than sham rats ($p > 0.05$). These results suggested that MDSC transplantation could relieve SUI symptoms in rats after PNT. Furthermore, we noticed that there was an increase in ALPPs and MBC in the IGF-1/MDSCs treatment group in comparison to the GFP/MDSCs group, but the difference was not statistically significant ($p > 0.05$).

Morphological changes in urethra

H&E and Masson's trichrome staining revealed uniformly arranged tissues, including epithelium, connective tissue, striated muscle and smooth muscle in normal rat urethra (**Figures 4A and 4B**). On the other hand, urethra samples from PBS-treated SUI rats displayed denaturation, atrophy, unclear structure and sparse arrangement of striated muscles, as well as unclear collagen fibers (**Figures 4A and 4B**). After MDSC transplantation, SUI rats showed newly formed capillaries and muscle fibers with less fibrosis and clear morphology of collagen fibers, suggesting repair of damaged muscle tissues. In addition, IGF-1/MDSCs rats showed regenerated urethral morphology than GFP/MDSCs rats (**Figures 4A and 4B**). Specifically, we noticed significantly higher muscle-to-collagen ratio in IGF-1/MDSCs rats, suggesting that IGF-1 could promote the survival and differentiation of MDSCs in SUI rats (**Figure 4C**).

MDSCs pretreated with IGF-1 enhance cell neoangiogenesis

FVIII-R Ag was used as a marker to quantify microvessel density[17] and its expression was mainly detected in a triated muscle layer (**Figure 5A**). The expression of FVIII-R Ag in the IGF-1/MDSCs group and GFP/MDSCs group were significantly higher than PBS and sham rats, suggesting that MDSC transplantation could lead to the formation of more microvessels (**Figure 5A**, $P < 0.05$). Additionally, there was a significant difference in the level of FVIII-R Ag between IGF-1/MDSCs and GFP/MDSCs groups (Figure 5A, $P < 0.05$). Vascular endothelial growth factor (VEGF), which promotes the growth of new blood vessels[18], was detected in the epithelial cells and muscle cells of middle urethra (Figure 5B). The expression of VEGF in IGF-1/MDSCs rats and GFP/MDSCs rats was significantly higher than sham and PBS rats (Figure 5B, $P < 0.05$). Additionally, there was a significant difference between IGF-1/MDSCs and GFP/MDSCs groups ($P< 0.05$). Next, we examined the level of VEGFR-2, the receptor of VEGF[19]. The expression of VEGFR-2 in IGF-1/MDSCs and GFP/MDSCs groups was significantly higher than that in the PBS and the sham groups ($P < 0.05$). In addition, there was a significant difference in the level of VEGFR-2 between IGF-1/MDSCs and GFP/MDSCs groups ($P < 0.05$) (Figure 5C). Taken together, our results suggested that MDSC transplantation could trigger neoangiogenesis, which was further enhanced by IGF-1.

DISCUSSION

UI is affecting over 200 million people worldwide. As the most common type of UI, SUI was found to affect roughly one-third of the women, greatly reducing their

quality of life[20]. With the rapid development of tissue engineering techniques, stem cell therapy has shown some potential in SUI treatment[6]. MDSCs can differentiate into muscle cells without inducing agents and have been successfully used as SUI therapy[3]. However, the promising therapeutic effect of MDSCs depends heavily on the cell viability and differentiation at the transplanted site. The survival rate of those implanted cells is often limited due to negative host factors, including hypoxia, ischemia and oxidative stress. Our study showed that IGF-1 promoted viability and differentiation of MDSCs in SUI rats. Specifically, urodynamic testing showed that MDSC treatment could alleviate the symptoms in SUI rats. Additionally, IGF-1 facilitated the formation of new capillaries and muscles fibers with less fibrosis. This is the first time that IGF-1 was shown to enhance myogenic differentiation of transplanted MDSCs in the urethra after damage.

Urodynamic testing reflects the functional changes of the neural-controlled urethral sphincter and is widely used in the diagnosis of urinary incontinence. Our study showed that ALPPs and MBC were significantly higher in MDSC-treated rats and their levels would further elevate with IGF-1 treatment. The results suggested that MDSC therapy could restore urethral sphincter continence while IGF-1 further improved the efficacy of such treatment. However, previously, our group has shown that there was no significant difference in leak point pressure (LPP) after MDSC or MDSC fibrin glue (FG) mixture treatment. It is possible that fibrin glue has a short half-life time and was degraded soon after the treatment, leading to a decrease in the curative effect of the implanted cells. However, lentiviral vector transduction allowed IGF-1 to be constitutively expressed, maintaining its level in MDSCs. Subsequently, IGF-1-expressing MDSCs could efficiently alleviate the symptoms of SUI in rats by regenerating muscle fibers, thereby restoring their sphincter function.

Here we report several speculations regarding the underlying mechanisms of IGF-1 acting on urethra rhabdosphincter. An earlier study reported that IGF-1 aided in the myogenic differentiation of human urethral rhabdosphincter (RS) satellite cells via PI3-K, a signal transduction pathway that mediates metabolism, cell proliferation and survival, as well as angiogenesis[10]. Therefore, we speculated that through interaction with players of PI3-K, IGF-1 was able to upregulate protein synthesis essential to tissue regeneration, leading to restored urethra sphincter function26. Furthermore, our results suggested that IGF-1 could suppress fibrosis, which improved the efficiency of muscle regeneration after tissue damage. Lastly, IGF-1 is a potent neurotrophic factor and was reported to promote nerve regeneration. Therefore, IGF-1 might also play a role in the nerve re-domination of the urethral sphincter, improving urethral sphincter function.

Angiogenesis accelerates local blood circulation and is essential for regeneration-dependent muscle repair. Therefore, it is beneficial for the survival and growth of the implanted cells. Our results showed that the density of new capillaries, as well as the expression of VEGF/VEGFR-2 protein, was significantly higher in IGF-1-treated rats. VEGF/ VEGFR-2 pathway is a key regulator of blood vessel formation28. We speculated that IGF-1 could stimulate the production of VEGF in MDSC in urethral sphincter, promoting angiogenesis. It was previously reported that VEGF could undergo autocrine or paracrine upregulation in MDSC. Subsequently, VEGF binds to VEGFR-2 and promotes the formation of new blood vessels[21,22]. On the other hand, due to the multilineage differentiation potential of MDSCs, it is also possible that IGF-1 could stimulate MDSCs to differentiate into vessel-like structures. More experiments are needed to investigate whether angiogenesis at the implanted site correlates to the better survival and growth of MDSCs.

Lastly, our findings in the current study should be considered in the light of some limitations that need to be further explored. First, we established the SUI model by bilateral PNT, which could not represent all physiopathologic phenotypes in patients. Thus, additional studies are needed to assess the clinical application of our study. Second, our results suggested that IGF-1 facilitated the growth of transplanted cells, yet its potential adverse effect, such as tumorigenesis, remains to be addressed. Moreover, we have not examined the clinical potential of IGF-1-expressing MDSCs, as in whether it could improve the urethral sphincter function in elderly patients.

CONCUSIONS

Taken together, in this study, we expressed IGF-1 in MDSC through lentiviral transduction and we showed that IGF-1 could promote the survival, growth and differentiation of transplanted MDSCs. In addition, we showed that IGF-1-expressing MDSCs could mitigate the symptoms of SUI in rats by regenerating of muscle fibers and local blood capillaries. In summary, our study illustrated the clinical potential of IGF-1-expressing MDSCs. We hope that this study will aid in the development of stem cell therapy for patient with SUI.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

APPENDIX

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