Original Article

Autologous and heterotopic transplantation of adipose stromal vascular fraction ameliorates stress urinary incontinence in rats with simulated childbirth trauma

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ABSTRACT

Introduction: Autologous transplantation of adipose stromal vascular fraction (SVF) is a cost-effective and technically accessible option for cell therapy. Clinical study of SVF transplantation for male stress urinary incontinence (SUI) is underway, but the effectiveness remains unknown for female SUI, majority of which is caused by childbirth trauma.
Methods: Vaginal Distension (VD) rats were generated as *in vivo* model for female SUI. To quantitate the severity of SUI, leak point pressure (LPP) was measured by placing a bladder catheter. There was a characteristic waveform of LPP with two-peaks, and we counted the second peak as an LPP value. Adipose SVF was separated from inguinal fat and delivered into external urethral sphincter (EUS) through transperineal injection. LPP was measured 7 or 14 days after SVF transplantation. Tissue damage and collagen synthesis around the EUS were visualized by Masson's trichrome and eosin staining. Antibody against α-smooth muscle actin (α-SMA) was used to stain smooth muscle or activated stromal cells. Donor SVF cells were distinguished from recipient EUS tissue by tracking with *GFP* transgene.
Results: VD procedure decreased the frequency at which the normal LPP waveform appeared and lowered the LPP value. SVF injection normalized the waveform as well as the level of LPP. VD disrupted histological structure of EUS and SVF failed to differentiate into striatal muscles. Instead, SVF increased α-SMA positive cells and collagen synthesis but the phenomena depended on VD stimulus. GFP tracking indicated that the transplanted SVF cells persisted for four weeks and synthesized α-SMA protein simultaneously.
Conclusions: Autologous transplantation of adipose SVF displayed bulking effects through collagen synthesis. However, such heterotopic activation was dependent on tissue damage.
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1. Introduction

Stromal cells are heterogeneous cell population which support organ homeostasis [1]. During tissue damage, stromal cells are rapidly activated and intensively reorganized, to achieve the optimized tissue repair [1]. Adipose stromal vascular fraction (SVF) is one of the most abundant and accessible stromal cell sources and the feasibility for cell therapy has been widely studied [2]. Clinical study of SVF transplantation for male stress urinary incontinence (SUI) is ongoing [3–6], but the feasibility remains unknown for female SUI. In Japan, SUI affects around 13% of women over 40 years old, majority of which is caused by childbirth trauma [7]. In this study, we investigated whether autologous and heterotopic SVF injection has therapeutic benefits against SUI, using simulated childbirth trauma (vaginal distension) model rats.

2. Methods

2.1. Animals

Virgin female rats of Sprague−Dawley (SD) strain at age 9−12 weeks weighing 240−320 g (CLEA Japan, Tokyo, Japan) were randomized into 6 groups for measuring leak point pressure (LPP): VD + No injection, Vaginal Distension (VD)-treated without Stromal Vascular Fraction (SVF) injection; VD + SVF, VD treated with SVF injection; VD + SVF + NPs, VD treated with SVF plus low-molecular-weight heparin/protamine nanoparticles (NPs) injection; Sham + no injection, sham-treated without SVF injection; Sham + SVF, sham-treated with SVF injection; Sham + SVF + NPs, sham-treated with SVF plus SVF + NPs injection. To track the donor cells after transplantation histologically, GFP-transgenic Lewis rats (LEW GFP; LEW-$Tg^{(CAG-EGFP)1Ys}$, a gift from Drs. Xiao-Kang Li and Hisashi Ueta) and its congenic Lewis rats (Japan SLC, Shizuoka, Japan) were used. We used LEW GFP and Lewis rats at age 10−12 weeks weighing 180−200 g bred in our animal experimentation facility. All animal experiments adhered to the *Guidelines for Animal Experimentation of Dokkyo Medical University*, with all efforts made to minimize the animal numbers and suffering.

2.2. Preparation of NPs

Low-molecular-weight heparin/protamine nanoparticles (NPs) were synthesized as described previously and used as extracellular scaffolds [8,9].

2.3. Preparation of SVF and SVF/NPs aggregates

Stromal vascular fraction (SVF) from autologous rats were prepared as previously described [10,11] with several modifications. Briefly, female SD rat's adipose tissue from the inguinal region was removed, minced, transferred to 'C tubes' (Miltenyi Biotec, Bergisch Gladbach, Germany) for enzymatic digestion. The solution contains 0.1% (weight/volume) collagenase type I (Wako Pure Chemical Industries, Osaka, Japan) and 0.2% (w/v) Dispase II (Thermo Fisher Scientific) in phosphate buffered saline (PBS). The minced tissue was kept at 37 °C for 1 h in shaking water bath (Taitec, Saitama, Japan) and mechanically stirred at every 10 min, using a MACS Dissociator (Miltenyi Biotec, installed software program "m_brain01-02"). The cell suspension was sieved through a 100 μm cell strainer (BD Falcon, Franklin Lakes, NJ, USA) and mature adipocytes and the debris were removed through the washing by PBS. Cells were subsequently centrifuged at 420 g for 5 min and resuspended in Dulbecco's Modified Eagle Medium (DMEM, Wako). The cell viability was determined with trypan blue exclusion method (Luna automated cell counter; Logos Biosystems, Anyang, South Korea). SVF (1.25×10^7 live cells per rat) were mixed with 100 μL of NPs (60 mg/mL) and incubated for 1 h at 37 °C to prepare SVF/NP aggregates.

2.4. Vaginal distension

We adopted the Vaginal Distension (VD) procedure as a tractable and reproducible rat model for human childbirth trauma [12]. Rats underwent VD under intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg) anesthesia as previously described [13,14]. A 10-Fr Foley balloon catheter (Create Medic, Yokohama, Japan) with the tip cut off was inserted into the vagina and the vagina orifice was closed with suture with 5/0 polypropylene (Prolene, Ethicon, New Brunswick, NJ, USA). The balloon was slowly distended with water to 3 mL [13]. After entire 4 h [15], the catheter was deflated. Also sham-distended rats were inserted the catheter into the vagina for 4 h but the balloon was not inflated.

2.5. Cell transplantation

Fig. 1a shows a schematic diagram of the method for the cell transplantation. After rats were anesthetized with ketamine and xylazine as described above, autologous SVF suspended in DMEM (2.5×10^6 cells; 20 μL per site) was injected in the 2, 4, 6, 8, and 10 o'clock positions around the urethral meatus using a 27-gauge needle (transperineal injection). The depth of injection (around 5 mm) was adjusted in advance so that the SVF spread around the external urethral sphincter (EUS) reproducibly.

2.6. Catheter implantation

On the day of LPP measurement, all rats underwent suprapubic bladder catheter implantation as previously described [16,17]. The rats were anesthetized as described above. A small incision was made in the bladder dome, and the polyethylene catheter (PE-50 tubing with a fire-flared tip) was inserted inside the bladder and secured with ligature through a bladder incision. The spinal cord was transected at the Th8−Th9 thoracic spinal level to prevent the spinobulbospinal voiding reflex, while maintaining urethral closure function intact [18].

2.7. Leak point pressure testing

Fig. 1b shows schematic diagram of LPP testing. The rats were placed into a sling-suit harness (Lomir Biomedical, Malone, NY, USA) and allowed to recover from the anesthesia for about 2 h, so that LPP testing was performed in an awake condition. The bladder was connected to a saline syringe via a bladder catheter. Intravesical pressure was monitored via a three-way stopcock which is connected to a pressure transducer. A data acquisition software recorded the pressure with sampling frequency at 10 Hz (Chart®, ADInstruments, Castle Hill, NSW, Australia) on a computer system equipped with an analog-to-digital converter (PowerLab®, ADInstruments, Dunedin, New Zealand). At the beginning the bladder was filled with saline until the initial leakage was observed from the urethral meatus. Hereafter, the 'voiding (urination)' could be easily distinguished from the 'leakage' because the pressure increase lasts longer during the voiding, while the leakage happened without such duration [17]. Subsequently saline was infused rapidly (3 mL/min) until a voiding was observed. The maximum pressure culminated the voiding was considered as LPP. We noted that the sham treated rats displayed 'two peaks' waveform for LPP (Fig. 1c). The second peak was larger than the first and when it was observed, it seemed that the animal was holding the voiding back (data not shown). We presume that the second peak reflects the major resistance for urinary continence and the sphincter is contributing to the peak. In fact, the level of the second peak gradually diminished over multiple testing, suggesting that the striatal muscle function declined due to overwork fatigue (data not shown). Importantly, VD treated rats frequently lost the second peak and numerical data was taken contingent upon observing such second peaks. The average of 4−12 consecutive LPPs was used as a data point of each animal. A 30-min interval was used for every three measurements to avoid the fatigue due to multiple testing.

2.8. Histology

Immediately after LPP testing, the animals were euthanized and the urethra and vagina were dissected *en bloc* at the level of the EUS for histological analysis. Tissues were immersion-fixed in 10 times-diluted neutral-buffered formalin (Original concentration is around 37% formaldehyde, Wako Pure Chemical Industries Ltd., Osaka, Japan). Subsequently, tissues embedded in paraffin, sectioned transversely (5 μm), and stained with Masson's trichrome (M-T) plus
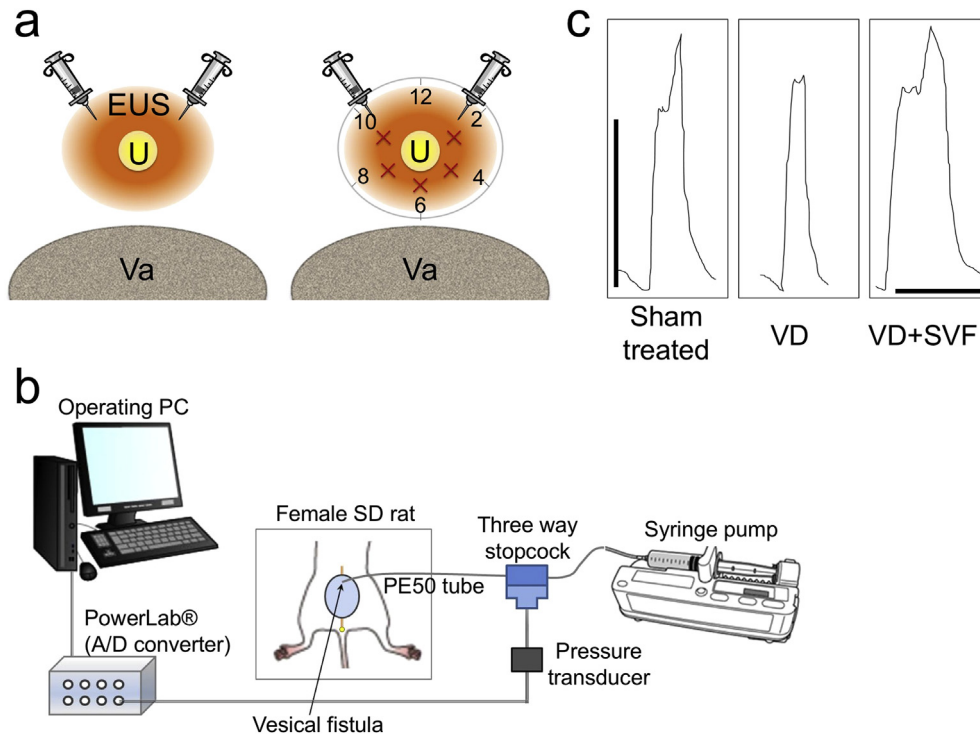
Fig. 1. Experimental methodology used in this study. (a) Schematic diagram for the cell transplantation. Transperineal injection was performed around the urethral meatus and the depth was precisely adjusted, so that the SVF cells reach around the EUS. (b) Schematic diagram of the equipment for LPP measurement. (c) Representative waveforms of the LPP. Vertical scalebar: 100 mmHg. Horizontal scalebar: 30 s. U: urethra; Va: vagina; EUS: external urethral sphincter; LPP: leak point pressure; SVF: stromal vascular fraction; VD: vaginal distension.

eosin. Immunoreactivity against anti-α-smooth muscle actin antibody (α-SMA; 1:500, rabbit polyclonal; Abcam, #ab5694, Cambridge, United Kingdom) was visualized with polymer-conjugated secondary antibody and Horse Radish Peroxidase (EnVision + System, Dako, Glostrup, Denmark). α-SMA is synthesized both by smooth muscle cells as well as activated fibroblasts. To discriminate the two possibilities, we tried to stain the serial sections with fibroblast specific protein-1 (FSP-1), but we failed to obtain the antibody with enough titer against rat FSP-1 (data not shown). Thus, we do not exclude the possibility that α-SMA positive cells are either smooth muscle cells or activated fibroblasts. For qualitative histological analysis, we used BZ-X700 microscope (Keyence, Osaka, Japan) to capture images. α-SMA-positive area and M-T-positive area were quantified as the percentage of the total tissue area in a high power field (400×) using Adobe Photoshop CS6 (Adobe Systems, San Jose, CA, USA) [19]. A threshold was selected and used to identify positive pixels on each slide. The percent area was calculated as the percentage of positive pixels relative to the total number of pixels in each view. Adjacent tissue sections were stained with hematoxylin and eosin.

In order to track the cells, the urethra and vagina samples were stored for four weeks after SVF transplantation (donor: LEW-GFP, recipient: Lews *wild type*). The tissue was fixed in the diluted neutral-buffered formalin (3.7% formaldehyde), embedded in paraffin and the serial sections were stained with anti-GFP antibody (1:250, rabbit polyclonal; Abcam, #ab6556) followed by EnVision+ visualization.

2.9. Statistical analyses

Mean values of LPP of six groups were compared using SPSS software (IBM, Armonk, NY, USA). Effects of SVF injection varied from experiment to experiment due to the smaller size of the EUS comparing to humans. We found that the LPP values do not follow normal distribution after injection (Fig. 2). Therefore, we adopted non-

parametric analyses to determine the significance of the procedure: Friedman rank test followed by Wilcoxon singed-rank test. On the other hand, we chose tissue samples for histological analysis (based on LPP recovery) and percent-area calculated data of each group almost followed normal distribution (Fig. 4). We adopted conventional Fisher's least significant difference procedure for multiple comparisons: one way analysis of variance followed by student *t* test.

3. Results

3.1. Vaginal distension decreased leak point pressure: validation of a model animal

To investigate the effect of Stromal Vascular Fraction (SVF) injection against female stress urinary incontinence, we adopted the Vaginal Distension (VD) experiment as an *in vivo* model for human childbirth trauma. In sham-treated rats, we observed characteristic 'two-peaks' waveform of Leak Point Pressure (LPP, see methods 2.7 and Fig. 1c). VD treatment decreased the frequency at which the second peak appeared (data not shown) and when we measured the average LPP, the value was significantly lower than sham-treated rats (Sham + No injection: 151.87 ± 10.09 vs. VD + No injection: 125.45 ± 7.53, p = 0.028, Fig. 2a). The decreased LPP was observed fourteen days after VD procedure (Sham + No injection: 156.19 ± 12.05 vs. VD + No injection: 125.57 ± 4.95, p = 0.005, Fig. 2b), indicating that the trauma was too severe to be recovered through spontaneous cure.

3.2. Transplantation of adipose SVF normalized the LPP decline caused by VD

Next, we evaluated the effects of SVF injection against LPP. LPP values seven days after surgical procedure are as follows: Sham + No injection, 151.87 ± 10.09; Sham + SVF, 154.35 ± 9.00;
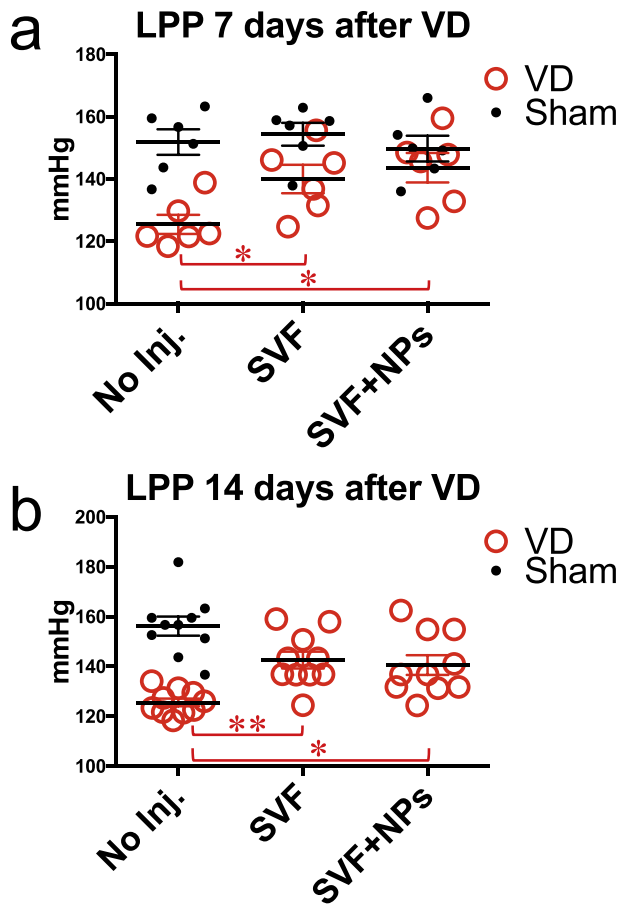
Fig. 2. VD decreased LPP values, which heterotopic SVF cells normalized. (a) The scatter-plot for LPP values of 7 days after VD treatment (n = 6). (b) The scatter-plot for LPP values of 14 days after VD treatment (n = 10). *p < 0.05, **p < 0.01: significantly different between the test group (No Inj.) vs. control group (SVF or SVF + NPs). LPP: leak point pressure; VD: vaginal distension; SVF: stromal vascular fraction; NPs: low-molecular-weight heparin/protamine nanoparticle.

Sham + SVF + NPs, 149.77 ± 10.13; VD + No injection, 125.45 ± 7.53; VD + SVF, 139.98 ± 11.17; VD + SVF + NPs, 143.62 ± 11.61 (Fig. 2a). Friedman rank test detected the significant change in LPP values among six groups (p = 0.015). Specifically, LPP was comparable between Sham + No injection and Sham + SVF

(Fig. 2a, p = 0.600 by Wilcoxon signed ranks). On the other hand, there was a significant increase from VD + No injection to VD + SVF groups (Fig. 2a, p = 0.028). Extracellular scaffolds (NPs) neither augmented nor diminished the effects of SVF *per se* (VD + SVF vs. VD + SVF + NPs, p = 0.752). Next, we prolonged the experimental schedule to see how long the transplantation effects continue. LPP values fourteen days after surgical procedure are as follows: Sham + No injection, 156.19 ± 12.05; VD + No injection, 125.57 ± 4.95; VD + SVF, 140.91 ± 11.63; VD + SVF + NPs, 138.38 ± 11.63 (Fig. 2b). Again, experimental procedures significantly affected LPP values (Fig. 2b, p < 0.001 by Friedman rank). There was a significant increase with VD + No injection and VD + SVF groups (Fig. 2b, p = 0.009 by Wilcoxon signed rank). Addition of NPs did not affect the effects of SVF (VD + SVF vs. VD + SVF + NPs, p = 0.646). Thus, SVF injection ameliorated the LPP decline for at least two weeks in experimental model rats.

3.3. SVF transplantation under tissue damage increased collagen synthesis

Next, we evaluated the influence of VD procedure and SVF injection through histological analyses. In sham-treated rats without SVF injection, α-smooth muscle actin (SMA) positive cells were surrounding the urethra (Fig. 3, see the column of Sham + No inj.). Eosin counter-staining visualized striatal muscle (sphincter) in red color adjacent to α-SMA positive cells (Fig. 3, Sham + No inj.). While VD procedure disrupted the organized structure of external urethral sphincter (EUS, arrowheads in Fig. 3, VD + No inj.) [13,15], VD *per se* hardly influenced on immunoreactivity against α-SMA (Fig. 3, VD + No inj.). In the presence of VD, SVF injection increased α-SMA positive cells around the disrupted EUS area but did not restore the striatal muscle structure (Fig. 3, VD + SVF). The percent of α-SMA positive pixels are as follows: Sham + No injection, 9.19 ± 1.34; Sham + SVF, 9.93 ± 1.03; Sham + SVF + NPs, 8.17 ± 0.84; VD + No injection, 8.62 ± 1.16; VD + SVF, 14.09 ± 1.57; VD + SVF + NPs, 13.87 ± 1.54 (Fig. 4a) On the other hand, VD plus SVF injection increased collagen fibers (visualized in blue color in Masson's Trichrome staining) around α-SMA positive cells (Fig. 3, VD + SVF). The percent of Masson' Trichrome positive pixels are as follows: Sham + No injection, 35.68 ± 7.22; Sham + SVF, 31.59 ± 5.39; Sham + SVF + NPs, 48.74 ± 6.58; VD + No injection, 34.46 ± 9.23; VD + SVF, 48.32 ± 8.33; VD + SVF + NPs, 50.57 ± 8.05 (Fig. 4b) However, such SVF function seemed context dependent. In the absence of VD, SVF injection slightly increased α-SMA positive cells
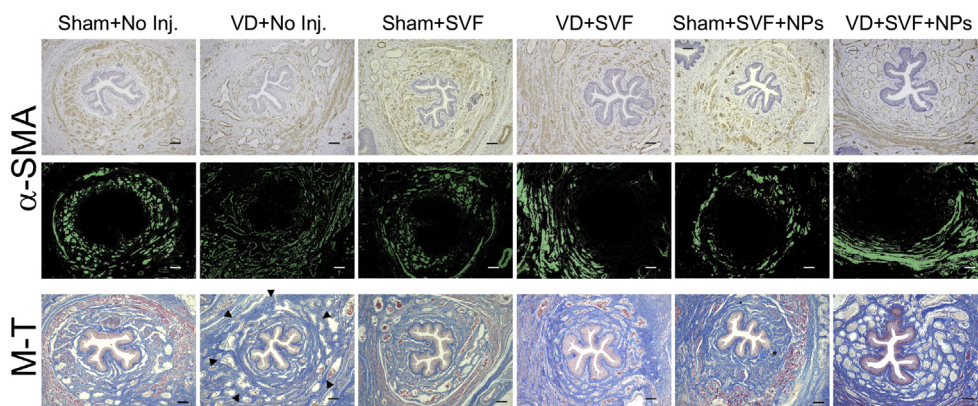
Fig. 3. VD disrupted external urethral sphincter and heterotopic SVF cells failed to restore the muscle fibers. Representative light micrographs of transverse sections of the urethras with immunohistochemistry against α-SMA and M-T plus eosin staining after VD treatment. Arrowheads indicate the approximate region where the disrupted sphincter could have existed. The images in the middle row are modified/edited by Adobe Photoshop CS6 for quantitative analysis (Green color: α-SMA immunoreactivity). Scale bar: 100 μm. α-SMA: alpha-smooth muscle actin; M-T: Masson's trichrome; VD: vaginal distension; SVF: stromal vascular fraction; NPs: low-molecular-weight heparin/protamine nanoparticle.
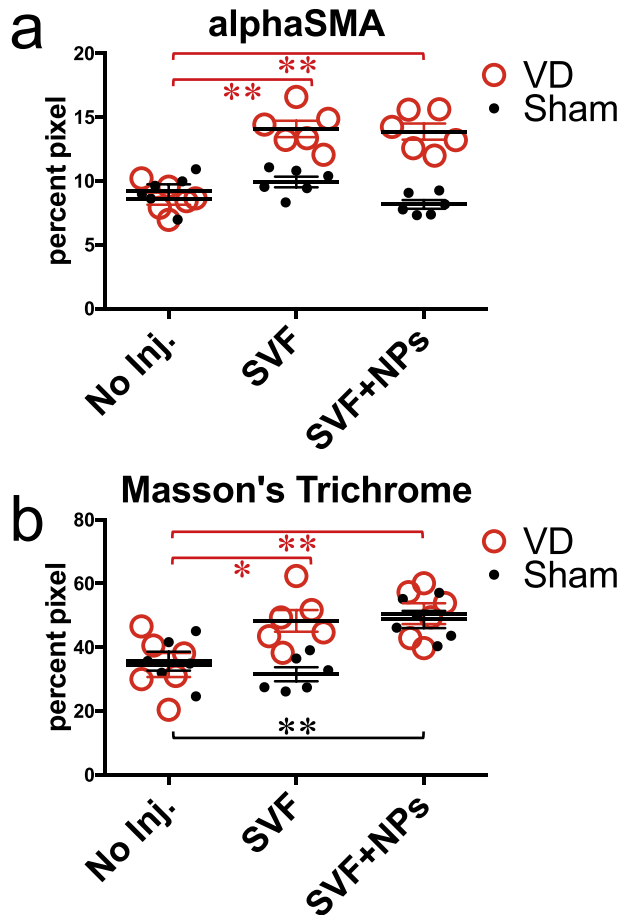
Fig. 4. Heterotopic SVF cells increased α-SMA positive cells and collagen synthesis around the damaged sphincter. (a) The percent of areas for α-SMA positive. (b) The percent of areas for collagen fibers (blue staining by M-T). The scatter plot of each percent pixels (n = 5). *p < 0.05, **p < 0.01: significantly different between the test group (No Inj.) vs. control group (SVF or SVF + NPs). SMA: smooth muscle actin; M-T: Masson's trichrome; VD: vaginal distension; SVF: stromal vascular fraction; NPs: low-molecular-weight heparin/protamine nanoparticle.

around EUS area but the augmented collagen synthesis was not observed (Figs. 3 and 4, Sham + SVF). Addition of cellular scaffolds displayed negligible effects against α-SMA positive cells but increased the collagen synthesis without VD procedure (Figs. 3 and 4, Sham + SVF + NPs).

3.4. Cellular kinetics of injected SVF cells tracked with GFP transgene

Finally, we tracked transplanted SVF cells with Green Fluorescent Protein (GFP) transgene. SVF cells from GFP transgenic inbred rats were transplanted into the congenic rats and the cellular fates thereafter were analyzed. We successfully identified transgenic GFP protein four weeks after transplantation (Fig. 5). GFP positive cells synthesized α-SMA protein simultaneously, suggesting that the increased α-SMA positive cells around disrupted EUS (Fig. 3) are transplanted SVF cells. GFP and α-SMA double positive cells were observed for at least four weeks after injection in the absence of cellular scaffolds.

4. Discussion

4.1. Transplantation of SVF for SUI: a plausible mechanism

A recent large animal study confirmed that the SVF is a safe and effective treatment for SUI [20]. SVF contains substantial
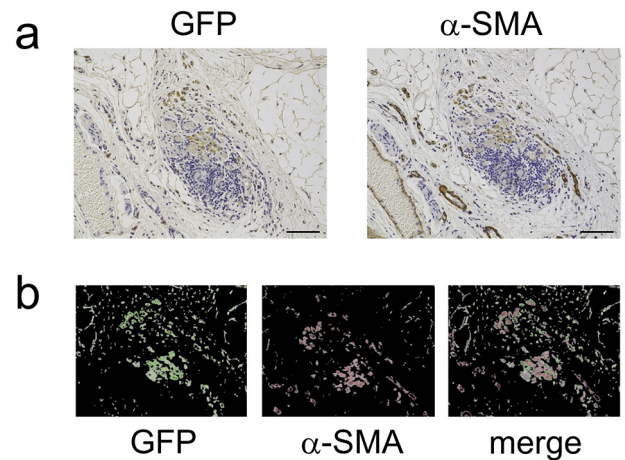
Fig. 5. Heterotopic SVF cells survived for four weeks and synthesized α-SMA. (a) Representative light micrographs of urethras four weeks after SVF transplantation (donor: LEW-GFP, recipient: *wild type*-LEW rats) (b) The micrographic images are modified/edited by Adobe Photoshop CS6. Substantial number of GFP positive cells synthesized α-SMA. Scale bar: 100 μm. GFP: green fluorescent protein; α-SMA: alpha-smooth muscle actin.

number of adipose derived stromal/stem cells (ADSCs) as well as leukocytes and endothelial cells [2]. Preceding small animal studies have reported the feasibility of cultured ADSCs for SUI, highlighting the three major mechanisms [21–23]: 1) ADSCs synthesize extracellular matrices such as collagen and elastin [21,24]. 2) Azacitidine pretreated ADSCs differentiate into myoblasts and regenerate the sphincter [21]. 3) ADSCs plus recombinant nerve growth factor regenerate pudendal nerves and the sphincter [23]. In this study, we adopted autologous SVF injection instead of *in vitro*-expanded ADSC injection. Before we used the VD as a female SUI model, we had examined the effects of SVF injection using pudendal nerve transection (PNT) model. Although we had confirmed that the LPP decreased reproducibly by PNT, the effects of SVF transplantation had not been remarkable (data not shown). Therefore, we exclude the third possibility (pudendal nerve regeneration) as a major mechanism of SVF transplantation. In addition, based on histological analysis of this study, we exclude the second possibility (regeneration of the sphincter) and support the first mechanism (synthesis of extracellular matrices).

4.2. Context dependency of transplanted SVF function

Cell therapy against SUI has long history. The safety and efficacy of muscle derived progenitor/stem cells (MDCs) transplantation has been already studied in humans [25–27]. Using rat models of SUI, Kwon et al. compared the efficacy of MDCs and fibroblasts, and concluded that the MDCs were superior to fibroblasts in terms of improvement of muscle contractility [28]. Although fibroblasts transplantation did have superior LPP recovery, authors also pointed out the obstruction due to excess bulking effects [28]. ADSCs contain fibroblast-like cells and once activated, they synthesize extracellular matrices such as collagen and elastin [21,24]. On the other hand, we observed that SVF hardly changed LPP level without VD treatment (Fig. 2). This is presumably because SVF contains less specialized cell population (containing 15–30% resident stromal cells) [2] and functionally neutral without the tissue damage (VD). Thus, stromal cells are functionally more plastic than cultured fibroblasts (already specialized for collagen synthesis), making the transplantation effects context dependent.

4.3. Clinical perspective

Although the efficacy of MDCs transplantation has been shown, it requires expensive facility such as cell processing center, which most hospitals do not afford. SVF is a promising alternative because the SVF preparation has been automated and widely used by cosmetic surgeons. In Japan, clinical trials using autologous SVF is ongoing, although the target condition is limited to male patients [3–6]. Previous study concluded that autologous 'fat injection' failed to improve SUI in female patients [29]. We presume that the material fat is inferior to adipose SVF in terms of 'durability,' because the majority of bulking volume of fat is mature adipocytes, not the stromal cells. As we have shown, SVF cells persisted to survive for at least four weeks, despite the heterotopic microenvironment. This could be explained by the fact that the stromal cells are much more plastic and adaptable than the mature adipocytes are. Therefore, the feasibility of SVF transplantation for female patients could be investigated in the clinical settings.

5. Conclusions

Autologous transplantation of adipose SVF displayed bulking effects against female SUI through collagen synthesis. However, such heterotopic activation was dependent on tissue damage.

Conflicts of interest

None.

Acknowledgments

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