**Abstract**

A three-dimensional gel-patch-like nerve-vascular reconstitution system using the Skeletal Muscle-Derived Multipotent Stem Cell (Sk-MSC) sheet-pellet was applied to the reconstitution of the severely damaged bladder wall as a non-skeletal muscle tissue, but has high demand for function. The Sk-MSC sheet-pellet was prepared by the mild detachment of expanded/confluent cells in culture with EDTA, then, collected in a tube and centrifuged. The sheet-pellet was pasted on the open thin-walled region of the damaged bladder wall made by myotomy (remove one-third of serosal smooth muscle layer associate with large disruptions of nerve-blood vessel networks retaining the mucosal layer). At 4 wk after transplantation, significant prevention of the reduction in the passive wall-tension, and the positive wall-contraction via electrical stimulation was observed in the transplanted group. Supporting these functional results, immunohistochemical and immunoelectron microscopic analysis revealed that the engrafted cells actively contributed to the reconstitution of blood vessels and peripheral nerves with differentiation into pericytes, endothelial cells, and Schwann cells. However, skeletal and smooth muscle formation was not observed. Thus, this method is potentially useful for the reconstitution of nerve-vascular networks in the bladder-wall to be retaining function such as passive tension and contractile function.

**Keywords:** Bladder-wall contraction; Intravesical pressure; Peripheral nerve reconstitution; Vascular reconstitution; Passive wall-tension

**Abbreviations:**
- Sk-MSC: Skeletal Muscle-Derived Multipotent Stem Cell
- Sk-34: Skeletal Muscle-Derived CD34+/45- Cells
- Sk-DN: Skeletal Muscle-Derived Double Negative (CD34+/45-) Cells
- NOR: Non-Treated Normal Control
- NT: Medium only Non-Cell Transplantation
- ST: Stem Cell Transplantation
- 10-BD: 10 minutes after bladder damage
- DMEM: Dulbecco’s Modified Eagle’s Medium
- FCS: Fetal Calf Serum
- IMDM: Iscove’s Modified Dulbecco’s Medium
- PFA: Parafomaldehyde
- PB: Phosphatase Buffer
- GFP: Green Fluorescent Protein
- SML: Smooth Muscle Layer
- SC: Schwann Cell

**Introduction**

The 3D-gel-patch nerve-vascular reconstitution method using expanded Skeletal Muscle-Derived Multipotent Stem Cell (Sk-MSC) sheet-pellets was developed recently [1]. Basically, skeletal muscle interstitium includes multipotent stem cells having synchronized (simultaneous) reconstitution capacity of muscular, vascular and peripheral nervous systems in severely damaged skeletal muscle [2-4]. Interstitial cells were divided into the CD34+/CD45- (Sk-34) and CD34+/CD45- (Sk-DN) fractions at the fresh isolation by collagenase, and both cells were different from satellite cells [5-8], side-population cells [9] and bone marrow-derived mesenchymal stem cells [2]. Therefore, Sk-MSCs sheet-pellets mainly contained these Sk-34 and Sk-DN cells. On the other hand, the characteristic of this sheet-pellets method is its handling, as the gel-like state allows it to be picked up with forceps, and placed onto the desired site correctly. This state also contributes to cell holding (prevent a cell spread or diffusion) and concentration of various paracrine factors in the desired site after transplantation. In addition, when this Sk-MSC sheet-pellets was applied for severely damaged skeletal muscle, engrafted cells actively reconstituted nerve-vascular networks associated with cellular differentiation into Schwann cells, perineurial cells with perineurium, endoneurium, vascular endothelial cells and pericytes, whereas a note that, skeletal myogenic differentiation capacity was wholly diminished after expansion culture [1]. Paracrine effects for around donor cells/tissues could be also expected, because of the active expression of neurogenic and vasculogenic factor mRNAs in the sheet-pellets [1]. These characteristics strongly suggested its utility as an accelerator and/or adjuvant in other non-skeletal muscle tissue regenerations, particularly requiring nerve-vascular reconstitution.

In this regard, we selected the bladder wall as a particular non-skeletal muscle tissue which is requiring nerve-vascular networks for their functional demand in vivo. The urinary bladder is the organ which is composed by smooth muscular and distensible tissues, and...
allowing collection and emition of urine. Therefore, autonomic muscle contraction, elasticity, and sensations are requisite important factors, and the deficit of these functions is directly linked to the quality of life. In fact, children with bladder exstrophy, spina bifida or myelomeningocele, or posterior urethral valves are seen as congenital anomalies, thus, various surgical treatments are often necessary because of the side effects of high pressure and hypertonic low compliance [10-13]. In these surgical treatments, disruption of nerve and blood vessel networks unavoidably occurred, and functional deficit, such as incomplete emptying of the bladder and/or extremely large capacity up-to 2000 ml, can result due to disrupted nervous control and reduction of passive tension of the wall. Thus, bladder tissue engineering intended to the reconstitution of nervous networks is necessary to conserve bladder function, including wall contraction and sensation, following various surgical treatments of the bladder.

Furthermore, surgically treated tissues in vivo naturally require re-vascularization, because invasive tissue constructs require massive delivery of oxygen and nutrients and removal of waste products during reconstitution. Therefore, reconstitution of vascular networks is also important in the damaged tissue recovery. In these regards, the solution as an accelerator and/or adjuvant which is effective for the reconstitution of nerve-vascular networks available in various tissues is desired.

In the present study, we applied the Sk-MSC sheet-pellets system to a reconstitution of nerve-vascular networks in the bladder wall as an initial attempt for the non-skeletal muscle tissue reconstitution. For this purpose, we modified the myotomy (detrusotomy) technique to make a severe damage for the bladder wall [14-17]. The serosal smooth muscle layer associate with nerve-vascular networks was removed excluding only mucosal thin layer for about one-third of total bladder surface area. This model showed a significant decline in passive wall-tension (evaluated by maximum bladder volume) and contractile function (evaluated by intravesical preser via electrical stimulation).

Therefore, the purpose of this study is to clarify whether the application of the Sk-MSC sheet-pellets transplantation prevents these functional declines following reconstitution of the peripheral nerve-vascular system without skeletal muscle tissue formation.

Materials and Methods

Animals

In the present study, two sets of experiments were performed. The first set was the mouse experiment. Green fluorescent protein transgenic C57BL/6 mice (GFP-Tg mice; C57BL/6 TgN [act EGFP] Osb Y01, provided by Dr. M. Okabe [18], Osaka University, Osaka, Japan; age, 4-8 wk) were used as donors, and wild-type C57BL/6N mice were used as recipients. For the histological and volume metric analysis of the bladder, mice were randomly divided into three groups: the non-treated normal control (NOR, n=60), medium only group (MDM, n=20), and gastrocnemius muscles of the right leg were removed and prepared for stem cell isolation. All experimental procedures were conducted in accordance with the Japan Physiological Society Guidelines for the Care and Use of Laboratory Animals, as approved by Tokai University School of Medicine Committee on Animal Care and Use.

Sk-MSC isolation and preparation of stem cell sheet-pellets

Sk-MSC were enzymatically extracted from the thigh and lower leg muscles (tibialis anterior, extensor digitorum longus, soleus, plantaris, gastrocnemius and quadriceps femoris) of GFP-Tg mice, as described previously [2,4-9]. Preparations of stem cell sheet-pellets were also performed following previous report [1]. First, whole non-minced muscles were treated with 0.1% collagenase type IA (Sigma-Aldrich, Tokyo, Japan) in Dulbecco’s modified Eagle’s medium (DMEM) containing 7.5% fetal calf serum (FCS) with gentle agitation for 30 min at 37°C, and were divided into fiber-bundles. Fiber-bundles were washed with Iscove’s Modified Dulbecco’s Medium (IMDM) containing 10% FCS, and were cultured in IMDM/20% FCS (100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 10 μg/ml gentamycin sulfate and 0.1 mM β-mercaptoethanol) for 4 d. Cultures were then treated with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA; Life Technologies, Tokyo, Japan) and filtered through 70-, 40- and 20-μm nylon filters in order to remove muscle fibers and other debris, followed by washing and re-culture in IMDM/20% FCS for 3 d until confluence. After 7 d of expansion culture totally, confluent cells were gently detached from culture dishes with 2 mM EDTA solution, while maintaining as much as cell-to-cell contact possible (Figure 1A). Sheet-like cell aggregations were then collected in a centrifuge tube, and stem cell sheet-pellets were obtained after centrifugation (Figure 1B). In this process, 100–120mg sheet-pellets could be obtained, and 50–60mg was applied per one bladder, and cell concentration was in the 1×10^5-1.2×10^5/ mg range.

For the rat autologous cell transplantation experiment, the right lower hindlimb soleus and gastrocnemius muscles were removed according to the method of compensatory muscle hypertrophy model

Figure 1: Preparation and transplantation of stem cell sheet-pellets (i-iii), and operation of bladder-damage model (A; i-v). Confluent cell-sheets were detached with 2 mM EDTA (i) and collected in a centrifuge tube (ii). Midline surface-incision of the bladder wall (limited outside from smooth muscle layer) (iv). Tag ends for both sides were spread in a transverse direction (v). Sheet-pellets were picked up with forceps and pasted on the open bladder wall (iii and vi). Measurement of bladder volume (B) and contractile ability (C). Protocol for B: (i) addition of stable water pressure; (ii) clamp pens at leak point; (iii) stop water pressure at leak point; (iv) completely draw out the bladder contents with syringe. Protocol for C: perform steps (i), (ii) and (iii) from B; (iv) add electrical stimulation through the bladder neck; (v) transduce intravesical pressure, amplify and record. Rec, Recorder; Amp, Amplifier; Trans, Transducer; Stim, Stimulator.
anti-sense primers, 0.2 mM dNTPs and 0.5 µl of cDNA. Taq buffer, 0.8 U of ExTaq-HS-polymerase, 0.7 µM specific sense and an Invitrogen SuperScript III system using dT30-containing primer (Hilden, Germany). First-strand cDNA synthesis was performed with lyzed and total RNA was purified using a QIAGEN RNeasy micro kit.

Expression of neurotrophic and vasculogenic factor mRNAs were analyzed in the prepared Sk-MSC-derived sheet-pellets. Factors and their primer sets are summarized in Table 1. Detailed methods for RT-PCR were as described previously [1,21]. Briefly, sheet-pellets were lysed and total RNA was purified using a QIAFAST RNaseasy micro kit (Hilden, Germany). First-strand cDNA synthesis was performed with an Invitrogen SuperScript III system using dT30-containing primer (see above), and specific PCR (35 cycles of 30 s at 94°C, 30 s at 60–65°C and 2 min at 72°C) was performed in a 15-µl volume containing Ex-Taq buffer, 0.8 U of ExTaq-HS-polymerase, 0.7 µM specific sense and antisense primers, 0.2 mM dNTPs and 0.5 µl of cDNA.

Bladder wall damage model and cell transplantation

Surgery and cell transplantation were performed under inhalation anesthesia (Isoflurane; Abbott, Osaka, Japan). Schematic drawing of the bladder-wall damage model and sheet-pellets transplantation is shown in (Figure 1D-1F). This model was made based on autologous method by myotomy [14-17]. However, note that our purpose was not obtained the bladder augmentation, but the induction of a broad range of nerve-vascular deficit in the bladder wall. A lower abdominal midline incision was made to expose the bladder, and a midline vertical incision was made on the surface of the bladder wall (Figure 1D). In this step, inflation with physiological saline into the bladder was performed to obtain appropriate wall tension, if necessary. Then, Tag ends of the outer layer were then spread out on both sides in a transverse direction for about one-third of total surface area. In this process, the serosal smooth muscle layer associate with nerve-vascular networks was wholly removed, excluding the inner thin mucosa layer (Figure 1E, and actual picture was shown in Figure 2F). Transplanted sheet-pellets were covered by fibrinogen based hemostatic agent (Beriplast, NIPRO, and Osaka, Japan). The actual feature of sheet-pellet transplantation was shown in figure 2F. The NT group was received only non-cell containing media and the covering of hemostatic agent.

Measurement of bladder volume and intravesical pressure

The protocol for the measurement of bladder volume and contractile ability is summarized in figure 1B and 1C. Both measurements were performed under sodium pentobarbital (40 mg/kg, with xylazine HCl 10 mg/kg, i.p.) anesthesia and body (rectal) temperature was maintained at 37 ± 1°C using a heating light throughout the experiment. Animals were fixed in the supine position and a lower abdominal midline incision was made to expose the bladder. For measurement of bladder volume (Figure 1B), mouse model was used and a 23 G needle associated with a water tube-line connected to a reservoir was inserted into the bladder and constant (45 cmH2O) water pressure was applied using warm physiological saline (Figure 1B). It was considered that this pressure was relatively higher, but sufficiently over an effect of bladder-to-urethral reflex [22], and could be obtained leak point easily. Leak points were identified at full bladder volume, and the penis was clamped (Figure 1B-ii) and water pressure was stopped (Figure 1B-iii). A 27 G needle with a syringe was inserted into the bladder and water was completely drawn for volume measurement (Figure 1B-iv). Actual pictures of the apparatus and serial technique of volume measurement were also shown in Supplemental Figure S1 to support the imagination. Bladder volume data were compared between the NOR (n=51), NT (n=6) and ST (n=6) groups at 4 wk after transplantation.

On the other hand, the measurement of bladder contractile activity was performed using the rat model (Figure 1C) according to the reason why the relationship between the size of stimulation-electrode and bladder neck. A full bladder volume was obtained as same as the volume measurement described above (Figure 1 C-i, C-ii and C-iii), and a pressure transducer (DT-XX; Becton Dickinson, Sparks, MD) was sat between the bladder and stopper (Figure 1C-iii). Custom-made Ag/Ag bipolar electrodes were then placed on the bladder neck (near the bladder branch of pelvic plexus) and electrical stimulation (1.5 V, 1.0-ms square pulse, 15 Hz, 5 s, induced by Digital Stimulator ME-6012; MEC, Tokyo, Japan) was repeatedly applied 3-4 times at 15-s intervals (Figure 1C-iv). The transducer was connected to an amplifier (AP-621G; Nihon Kohden, Tokyo, Japan) and data were recorded on a Linearorder (Mark VII, WR3101; Graphtec, Tokyo, Japan) (Figure 1 C-v). Changes in intravesical pressure were measured as bladder function parameters and values were expressed means ± SE/mmH2O. Actual apparatus was also present in the Supplemental figure 1B. Measurement of bladder contractile activity (intravesical pressure) was also performed in the NOR (n=10) group, the 10 min after bladder damage (10-BD, n=4) group, and in the NT (n=6) and ST (n=4) groups.

Macroscopic observation, immunostaining and immuno-electron microscopy

At 4 wk after surgery, macroscopic analysis was performed under a

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VEGF-Vascular Endothelial Growth Factor; PDGF-B-Platelet Derived Growth Factor B; EGF-Epidermal Growth Factor; HGF- Hepatocyte Growth Factor; TGFb-Transforming Growth Factor Beta 1; NGF-Nerve Growth Factor; BDNF-Brain derived neurotrophic factor; GDNF- Glial cell line derived neurotrophic factor; CNTF- Ciliary Neurotropic Factor; FGFB- Fibroblast Growth Factor; HPRT-Hypoxanthine-Guanine Phosphoribosyltransferase (used as housekeeping gene).
fluorescence dissection microscope (SZX12; Olympus, Tokyo, Japan). Recipient mice were given an overdose of pentobarbital (60 mg/kg, with xylazine HCl 10 mg/kg, i.p.), and a lower abdominal midline incision was made to expose the bladder. The cell implantation region was then observed and photographed through a GFP filter.

For immunostaining and immunoelectron microscopic analysis, recipient mice were perfused with warm 0.01 M PBS through the left ventricle, followed by fixation with 4% paraformaldehyde/0.1 M phosphate buffer (4% PFA/PB) after macroscopic observation (see above). The bladder was removed and post-fixed overnight with 4% PFA/PB at 4°C, and was then washed with a graded sucrose (0-25%)/0.01 M PBS series, embedded in optimum-compound (O.C.T compound; Tissue-Tek, Sakura Finetechnical Co., Ltd., Tokyo, Japan) and frozen at -80°C. Subsequently, several 7-μm sections were obtained. Localization of nerve fibers (axons) and Schwann cells was detected by rabbit polyclonal anti-Neurofilament 200 (N-200, dilution=1:1000; Sigma, Saint Louis, MO) and anti-p75 (rabbit polyclonal, 1:400, 4°C overnight; CST, Boston, MA). Blood vessels were detected with mouse anti-α-smooth muscle actin (aSMa Cy3-conjugated; 1:1500; room temperature for 1 h; Sigma, Saint Louis, MO) and rat anti-mouse CD31 (1:500, 4°C overnight; BD Pharmingen, San Diego, CA) monoclonal antibody. Reactions were visualized using Alexa Fluor-594-conjugated goat anti-rabbit and anti-rat antibodies (1:500; room temperature for 2 h; Molecular Probes, Eugene, OR).

For immunoelectron microscopy, sections were stained with rabbit anti-GFP polyclonal antibody (Molecular Probes), and anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (1:200; 4°C overnight; Dako, Carpinteria, CA). Reactions were visualized with 3,3-diaminobenzidine (DAB) after fixation in 1% glutaraldehyde/0.1 M PB. Sections were then fixed in 1% osmium tetroxide/0.05 M PB, and were prepared for electron microscopic analysis. Immunoelectron microscopy was performed as described previously [1-4].

**Statistical analysis**

Functional measurements were expressed as means ± SE. Differences between groups were tested by parametric Tukey-kramer post-hoc test. The level of significance accepted a priori was set at p<0.05.

**Results**

**Macroscopic observation of engrafted donor tissues and expressions of neurotrophic and vascular growth factor mRNAs in sheet-pellets**

A large number of adhesive and non-adhesive cells associate with long myotubes was observed in the Sk-MSCs culture after 7 days of expansion (3 days of fiber culture and 4 days of expansion culture, Figure 2A). After the mild treatment of EDTA, there was detached from plate and roll-up with keeping their cell-to-cell connections (Figure 2B). Detached sheet-like cell aggregations were collected in a centrifuge tube, and gel-like sheet-pellets were easily picked up with forceps (Figure 2C). Prepared Sk-MSC sheet-pellets for transplantation clearly expressed various neurotrophic factors (NGF, GDNF, CNTF and bFGF) and vascular growth factors (PDGF, EGF, HGF, TGF-β and VEGF) mRNAs (Figure 2D). Therefore, it was likely that paracrine effects for the peripheral nerve and vascular system were possibly expected. Typical feature of the damaged bladder and immediately after sheet-pellets transplantation is shown in Figure 2E and 2F. Engrafted sheet-pellets wholly covered about one-third of the bladder wall (Figure 2F). At 4 wk after transplantation, donor-derived GFP+ tissues actively engulfed onto the bladder wall (Figure 2G).

**Comparison of bladder volume and contraction ability**

At 4 wk after surgery, bladder volume (from mice experiment) and contraction ability (from rats experiment) were compared among the NOR, NT and ST groups (Figures 3A and 3B). Mean bladder volumes are 0.40 ± 0.02 ml (n=51) in the NOR, 0.73 ± 0.08 ml (n=6) in the NT, and 0.49 ± 0.11 ml (n=6) in the ST group, and NT showed a significantly higher value than that of the other two groups. There were no differences between the ST and NOR groups. This means significant reduction of passive wall tension strength in the NT group.

In the comparison of bladder contraction ability, which was evaluated by intravesical pressure via electrical stimulation of the bladder neck, there was 35% decrease from the NOR level was seen at 10 min after bladder damage operation (10-BD in Figure 3B). Therefore, this was considered the effect of damage operation for the bladder wall contractility in this model. This functional reduction was worsened over the subsequent 4 wk in the NT group, and the value reached a significant level (p<0.05, and 63% decrease from the NOR) finally. In contrast, the ST group showed 83% function for the NOR group finally. This was 2-fold higher value than NT group, and note that the ST group showed 18% recovery from the 10-BD value (from 35% to 17%) during 4 wk recovery period. Typical raw data of electrically induced change in intravesical pressure among three groups is shown in figure 3C. The changes in bladder volume in this rat experiment were similar to the mice case, as 1.53 ± 0.10 ml in NOR, 2.68 ± 1.13 ml in NT, and 1.61 ± 0.12 ml in ST group.

**Formation of blood vessels and peripheral nerve networks in damaged bladder wall**

For the functional changes above, we performed blood vessel...
(anti-CD31 and -αSMA) and nerve–related (anti-N-200 and -p75) immunostaining for the three groups (Figures 4-7). These histological data was obtained from mice experiments. On a histological overview of the bladder, the NOR group showed a thick smooth muscle layer, uniform nerve fiber networks, and large conduit blood vessels in the mucosal layer (arrows in Figures 4A-4C). In contrast, there were no conduit blood vessels and nerve networks, and very few smooth muscles in the damaged site of the NT bladder (Figures 4D-4F). On the other hand, active formation of conduit blood vessels (arrows in Figure 4G-4I) and peripheral nerve axon networks (arrows in Figures 4J-4L) were observed at the transplanted site of the ST bladder, while reconstitution of smooth muscle layer (SML) cannot be seen.

The detailed contribution of transplanted cells to blood vessel formation in the ST bladder wall is shown in figure 5A-5I. The GFP+ cells co-localized in the CD31+, as well as αSMA+ blood vessels and co-expression of CD31/GFP (arrows in Figures 5A-5F) and α-SMA/GFP (arrows in Figures 5G-5I) cells were evident. This indicates that transplanted GFP+ cells differentiated into endothelial cells and vascular smooth muscle cells and contribute to the formation of both small and large blood vessels incorporated with resident recipient cells. Similarly, the contribution of donor-derived GFP+ cells to the reconstitution of nervous networks was strongly observed (Figures 6A and 6B). The GFP+ cells were located close and along to the N-200+ nerve axons (arrows in Figure 6A). Similar distributions of GFP+ cells could be seen in B, and there were positive for anti-p75 (arrows), thus, representing the active differentiation of the donor cells into Schwann cells. These histological characteristics in mice were similarly observed in the rat autograft model (data not shown).

Detailed differentiation of transplanted donor cells was confirmed by immunoelectron microscopy (Figures 7A-7D). GFP+ reactions were representing black dots. Donor cells clearly differentiated into Schwann cells (SC in Figure 7A-7C), endothelial cells (EC in Figures 7A and 7B) and fibroblasts (Fb in Figure 7D). GFP+ Schwann cells were located near small (Figure 7A) and large (Figure 7B) blood vessels, including GFP+ endothelial cells. In addition, a number of Schwann cells were co-localized in non-myelinated nerve bundles (arrows in Figure 7C). These results correspond to the results of immunohistochemical analysis. GFP+ fibroblasts having rich endoplasmic reticulum were also observed (Figure 7D) in the damaged site.

**Discussion**

In the present study, the results clearly demonstrated that the Sk-MSC sheet-pellets transplantation prevented a significant increase in bladder volume as seen in the comparison between the NT and the ST groups. This is considered that the present damage operation clearly affected the passive tension and induced extension of the bladder wall. This laxation resulted in the increase of the maximum bladder volume in the NT group. In this regard, this model showed successful bladder autogenous as reported traditionally [14-17]. Paradoxically, it was obvious that the sheet-pellet transplantation prevented the reduction of passive wall-tension and extension of the bladder wall.
in the ST group. In addition, functional examination also showed a significantly inferior contraction capacity in the NT group, but the ST group showed almost same results as the NOR group (Figure 3B). Importantly, the NT group showed from bad to worse change in contractile function from 10 min to 4 wk. These results indicating that the sheet-pellets transplantation also contributed significant recovery of contractile function of the damaged bladder wall.

Reflecting these functional contributions, the ST group also showed active reconstitution of peripheral nerve and vascular networks in the damaged portion of the bladder wall (see Figures 4-6). Transplanted Sk-MSC sheet-pellets contributed to the induction of large blood vessels and the formation of small and intermediate blood vessels in the ST bladder wall. In this contribution, two mechanisms were supposed. One is a direct contribution and/or incorporation to the formation of blood vessels, represented as the cross-reactions for GFP and anti-CD31 (marker of endothelial cell) and/or anti-αSMA (marker for vascular smooth muscle cell) in small and intermediate blood vessels in the ST bladder wall (Figure 5). Differentiation into endothelial cell was also confirmed by immunoelectron microscopy. Second is an indirect paracrine effect of transplanted sheet-pellets on resident recipient vascular relating cells. Large blood vessels, as in the NOR bladder, also could be seen in the ST group, but disappeared in the NT bladder. These large blood vessels did not containing GFP+ cells. However, the Sk-MSC sheet-pellets preparation expressed various vascular growth factor mRNAs, thus, it was possible that this paracrine ability may be kept after transplantation, and facilitated vascular formation of resident recipient vascular cells. As a result, the supply of oxygen and nutrition to the treated portion may be favorably worked in the damaged site of the ST in contrast to the NT bladder wall.

Similarly, a composite population of numerous GFP+ cells and red axons stained with N-200 were observed in the ST bladder, and this appeared to be closely related to differentiation into Schwann cells represented by double labeling of p75/GFP, and this was also confirmed by immunoelectron microscopy. In addition, the sheet-
pellets preparation also expressed various neurotrophic and nerve growth factors, thus, in vivo paracrine effect may also be available. Therefore, it was considered that vigorous axonal re-growth in the ST bladder may be promised by the supply of extra Schwann cell source, neurotrophic and nerve growth factors. Sufficient axonal re-growth may induce favorable recovery of wall contraction, probably with various sensations.

GFP fibroblasts were also observed, and synthetic materials from the fibroblasts, such as collagens, may also contribute to keep bladder wall thickness, strengthening, and retain of passive wall-tension in the ST group. Unfortunately, present transplanted sheet-pellets did not reconstitute a smooth muscle layer in the damaged bladder wall. This may be reflected a little inferiority of whole bladder contractions in the ST group compared to the NOR. However, it is likely possible that the bridging of disrupted smooth muscle layer by the stable connective tissue networks may be advantages to keep mechanical continuity of the bladder wall. Then, actively reconstituted nervous networks may favorably contribute to remaining recipient smooth muscle contraction. A stable connective tissue networks may also contribute to be retaining passive wall-tension.

Tissue engineering and/or stem cell transplantation for the bladder has been attempted using various types of cells. Pluripotent embryonic stem cells may have great potential, but they are associated with several ethical and social issues, as well as teratoma formation in vivo due to their uncontrollable proliferation [23]. Thus, adult stem cells are a potentially attractive source for autologous therapy, including bone marrow-derived mesenchymal stem cells [24, 25], adipose tissue-derived stem cells [26], skeletal muscle derived stem cells [27], and the cells from lung, testis, umbilical cord and placenta [28]. It is likely that the main direction of these researches is headed to forming urothelium and smooth muscle by the combination use of scaffolds and stem cells, in contrast to our purpose as the reconstitution of nerve-vascular networks in the bladder wall which was observed in the present study was never reported. We also reported that the mixed population of skeletal muscle derived Sk-34 [5] and Sk-DN cell [9], which was included in the present Sk-MSCs, transplantation was also available for the reconstitution of bladder branch of pelvic plexus and concomitant conduit blood vessels [31]. Therefore, combined together, the Sk-MSCs are available for the reconstitution of nerve-vascular networks both in the outside and inside of the bladder wall.

For the formation of urothelium and smooth muscle, autologous bladder cells have shown superiority to other adult stem cells [32]. However, it is not always possible to use fresh and active bladder smooth muscle cells, because smooth muscle cells from neuropathic bladders have shown abnormal growth, less contractility and inferior adhesion capacity when compared to normal controls [32]. This urological background has been well documented by Stanasel et al. [28], and they also found that neural constituents and sufficient/robust blood supply within the engineered bladder tissue are important for rescuing damaged and/or transplanted tissue. In this regard, the present Sk-MSC sheet-pellets transplantation may be a good candidate for the bladder tissue engineering as the nerve-vascular reconstitution adjuvant.

In conclusion, the present results clearly indicated that Sk-MSC-derived sheet-pellets transplantation facilitated the significant recovery of contractile function and the retaining of passive wall-tension for the severely damaged bladder wall. These were supported by the active reconstitution of vascular and peripheral nerve networks associated with cellular differentiation into endothelial cells, vascular smooth muscle cells, Schwann cells and fibroblasts in the damaged portion.

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