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ORIGINAL ARTICLE

TERM

Bladder Reconstruction Using Stem Cells Seeded on Multilayered Scaffolds in a Mucosa Preserving Partial Cystectomy Model

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Recently, studies have focused more towards using biocompatible scaffolds and stem cells to augment or replace the abnormal bladder. But, due to the lack of biomaterials with appropriate thickness as a suitable scaffold for smooth muscle regeneration, several structural, mechanical, and biocompatibility problems are encountered. Therefore, we aimed to demonstrate whether human muscle-derived stem cells (h-MDSCs) seeded on multilayered polycaprolactone (PCL) nanofiber is an appropriate scaffold for bladder smooth muscle regeneration. h-MDSCs were seeded on a multilayered PCL/collagen nanofiber sheet and implanted in the bladder of a mucosa preserving partial cystectomy rat. From our findings, h-MDSCs seeded on multilayered PCL showed efficient cell seeding and proliferation. In addition, the histological and immunohistochemical analysis showed cell survival in between the multilayered nanofiber sheet, which led to smooth muscle cell regeneration with improved pro-angiogenesis in the regenerated region of the bladder. Therefore, h-MDSCs seeded nanofibers could be a promising tool in treating neurogenic bladder and related diseases. Tissue Eng Regen Med 2015;12(6):427-434

Key Words: Multilayered; Tissue engineered bladder; Mucosa preserving partial cystectomy; Human muscle-derived stem cells; Polycaprolactone

INTRODUCTION

The urinary bladder is prone to different types of diseases, injuries and anomalies. In the past, an injured bladder was treated by augmenting or replacing the abnormal section with gastrointestinal tract segment [1]. However, patient compliance to bladder treatment by using intestinal segments was poor and led to many problems including mucus secretion, stone formation, infection, and electrolyte imbalance [2,3]. Advances in regenerative medicine, material sciences, cell and stem cell biology, and tissue engineering over the past decade have enabled researchers to develop cutting-edge technology leading to the construction of different tissues [4,5]. To avoid compliance complications while improving bladder functions, numerous natural and synthetic materials have been used as bladder substitutes in clinical and experimental settings [6-10]. Atala et al. [3] achieved an effective bladder tissue by using cell implantation on a collagen/polyglycolic acid scaffold. They illustrated encouraging results in compliance, leak point pressure, cellular structure, and phenotypical characteristics in cellular transplantation on a synthetic scaffold. Similarly, Engelhardt and coworkers have demonstrated the potential of collagen-poly(lactic acid-co-ε-caprolactone) (PLCL) hybrid as a scaffold for regeneration of bladder tissue [1]. This combination of collagen and PLCL scaffolds results in good cell compatibility and mechanical strength. Over the last several years, similar types of cellular engineered constructs were implanted into the bladders of rats, dogs, pigs, and humans with promising results [11-13]. However, due to structural, mechanical, and biocompatibility problems, none of them have met the necessary expectations. Simi-

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larly, the absence of a biomaterial having proper thickness that serves as a suitable scaffold for the regeneration process complicates the treatment strategy. Therefore, we designed a multilayered polycaprolactone (PCL)/collagen nanofiber sheet and seeded human muscle-derived stem cells (h-MDSCs) on it. The integrated scaffold was implanted on a mucosa preserving partial cystectomy (MPPC) rat model. We selected PCL because it is biocompatible, flexible, and its degradation product shows little toxicity. Moreover, PCL biocompatibility can be increased by blending it with collagen [14]. PCL was arranged in a multilayered pattern to increase the surface area of the scaffolds so that the seeded cells can proliferate easily.

In this study, we investigated h-MDSCs seeded on multilayered nanofiber's impact on smooth muscle cell regeneration and bladder performance in a MPPC rat model.

MATERIALS AND METHODS

Cell culture

Isolation and culture of h-MDSCs was approved by the Institutional Review Board at Seoul National University Bundang Hospital, Seoul, Korea. h-MDSCs were isolated from a 1 cm^3 biopsy sample obtained from the rectus muscle of four localized prostate cancer patients (age range: 65–70 years) after obtaining written informed consents. Isolation and culture of h-MDSCs was performed as previously described method [15].

Fabrications of PCL/collagen nanofiber

PCL/collagen nanofiber sheets were fabricated by electrospinning technique using a combination of PCL and collagen at a ratio of 1:1 by weight. PCL (MW 80000 Da), type I collagen (calf skin), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) were obtained from Sigma-Aldrich (MO, USA). Both PCL and collagen were dissolved in HFP at a total concentration of 5% (wt/vol). These materials were used as received without further modification. The PCL/collagen mixture solution was electrospun using a high voltage power supply at 15 kV potential between the solution and the grounded surface. The solution was delivered with a 5 mL polypropylene syringe through a 22 gauge blunt tip needle at a flow rate of 0.05 mL/min using a syringe pump. Fibers were collected onto a grounded stainless steel plate at a distance of 10 cm from the syringe tip (Fig. 1). After the fabrication process, the electrospun fibers were collected gently and vacuum dried for 48 h. The synthetic PCL bladder composites had a final thickness of 30–40 μm.

h-MDSCs seeding on the scaffold

1 cm^2 PCL nanofiber sheets were uv sterilized in the clean

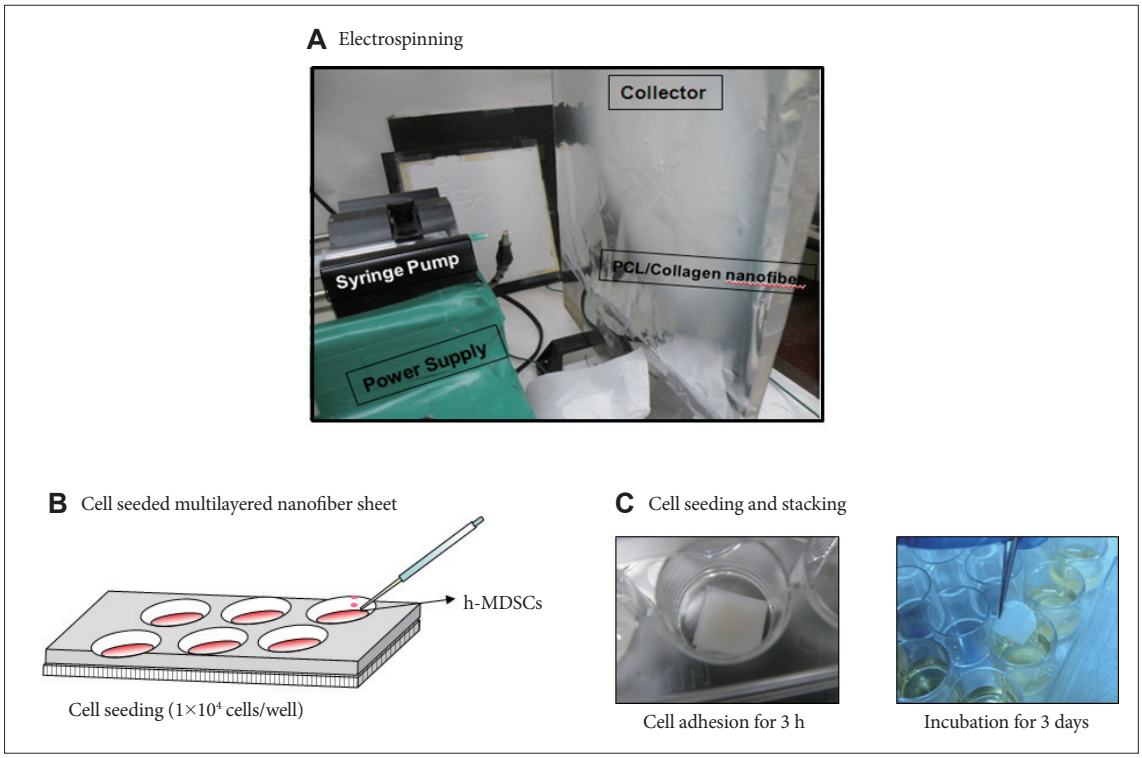

Figure 1. Diagram showing electrospinning and cell seeding. (A) Fabrication of the PCL/collagen nanofiber sheet by electrospinning. (B) Cell seeded multilayered nanofiber sheet. (C) Cell seeding and stacking. PCL: polycaprolactone, h-MDSCs: human muscle-derived stem cells.

bench. 1×10^6 h-MDSCs was seeded on the multilayered PCL/collagen nanofiber placed in a 24 well plate. The cells were attached for 3 h at 37°C, 5% CO_2 in a humidified incubator. Thereafter, cell seeded scaffold was cultured in medium containing Dulbecco's Minimal Essential Medium, 10% fetal bovine serum and 1% penicillin. Plates were incubated overnight in 37°C, 5% CO_2 humidified incubator. h-MDSCs seeded nanofibers were stacked to have a total thickness of 150−200 μm (Fig. 2).

Surface characterization of PCL nanofiber

A Scanning Electron Microscope (SEM; Model S-3000N, Hitachi, Tokyo, Japan) operated at an accelerating voltage of 25 kV was used to visualize the surface morphology of the multilayered nanofiber. For the evaluation of morphology, multilayered PCL nanofiber sheet containing h-MDSCs were fixed with 10% formaldehyde for 24 h, followed by ethanol dehydration. Samples were sputter-coated with platinum in the presence of argon gas at room temperature and visualized at ×1500 magnifications. Images of the microscope's field of view were captured.

In vivo study

10-week-old adult male Sprague-Dawley rats weighing 250−300 g were obtained from the Orient Bio Co. (Gyeonggi, Korea), and were randomly divided into four groups (N=5 per group): Normal group (N), MPPC group (MPPC), MPPC implanted with multilayered nanofiber group (NF), and MPPC implanted with multilayered nanofiber sheet seeded with h-MDSCs group (MDSC).

Mucosa preserving partial cystectomy bladder model

Rats were anesthetized with intraperitoneal ketamine (70 mg/kg body weight). Following the anesthesia, a lower abdomen midline incision was performed to visualize the urinary bladder. The bladder was partially emptied by manual abdominal pressure. Then, the bladder was held at the dome with forceps. The anterior region below the dome was scraped about 1 cm^2 area using a surgical blade to remove serosa and smooth muscle from the mucosa layer. The thin and transparent mucosa was preserved in intact condition to obtain a MPPC rat model. Finally, the omentum was sutured to the bladder to facilitate proper blood supply and the abdominal incisions were routinely closed in two layers (Fig. 3). After recovery from anesthesia, each animal was returned to its housing unit.

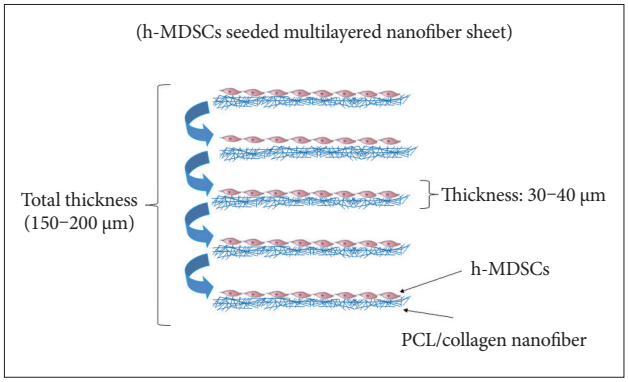

Figure 2. Schematic diagram showing the nanofiber stacking. h-MDSCs seeded on multilayered PCL/collagen nanofiber sheet that are fabricated by stacking. The cell seeded multilayered sheets are implanted in a Mucosa Preserving Partial Cystectomy rat model. PCL: polycaprolactone, h-MDSCs: human muscle-derived stem cells.

Implantation of MPPC with multilayered nanofiber (NF group)

In the MPPC bladder, a 1 cm^2 PCL/collagen nanofiber sheet was placed over the scraped region, and the nanofiber naturally attached itself to the bladder due to the fluid present in the bladder. The next step was to place another nanofiber over the first one. This process was repeated until five nanofibers were stacked one over the other and finally covered with omentum. The further steps were performed as described earlier in a MPPC rat model (Fig. 3).

PKH26 labelling of h-MDSCs

The fluorescent dye, PKH26 (PKH26 Red Fluorescent Cell Linker Kit; Sigma-Aldrich, MO, USA) was used as a cell tracker to locate the transplanted h-MDSCs. PKH26 labelling of h-MDSCs was done according to the manufacturer's protocol.

MPPC implanted with multilayered nanofiber seeded with PKH26 labelled h-MDSCs (MDSC group)

1 cm^2 PCL/collagen nanofiber sheet was placed over the scraped MPPC bladder. Then, h-MDSCs was seeded over the attached nanofiber. The process was repeated to sandwich hMDSCs between stacked nanofibers. The other procedures were performed as carried out for MPPC model (Fig. 3).

All 20 rats survived for 4 weeks after the surgical procedures without any complications during the experiments. Four weeks after the implantation, all rats were anaesthetized with intraperitoneal ketamine (70 mg/kg body weight) and their bladder were harvested for histological and immunohistochemical analysis.

Hematoxylin and eosin staining

The harvested bladder tissues were fixed in 4% paraformaldehyde for 24 h at room temperature, and then paraffin-embedded. The tissues was sectioned at 5 μm on a rotary microtome (Leica, Germany) for histological analysis. The cross sectioned tissues were mounted on slides. For the observation of collagen content, the tissues were stained with H&E (Sigma-Aldrich, MO,

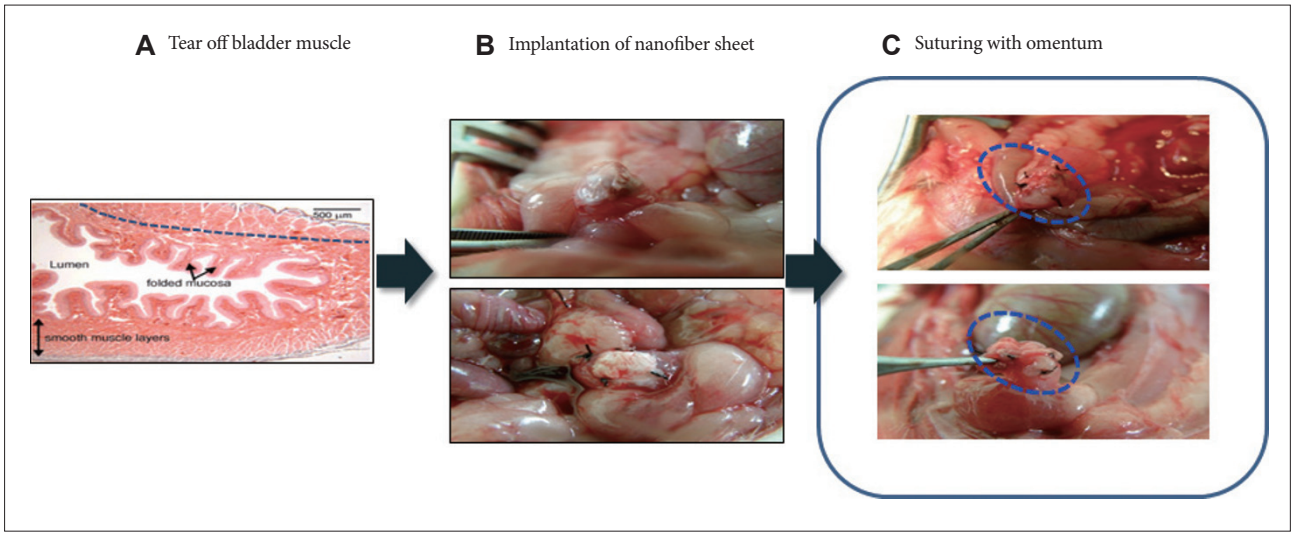

Figure 3. Macroscopic images of the implantation of the nanofiber. (A) Schematic diagram showing the tearing off the bladder muscle muscle (Adapted from http://www.histology.leeds.ac.uk/urinary/bladder.php, with permission from University of Leeds). (B and C) Macroscopic images of the implantation of multilayered nanofiber sheet on the scraped bladder and its suturing (blue circle) with omentum respectively.

USA). Histological images were obtained using a light microscope.

α-SMA, calponin, and vWF immunohistochemistry

The paraffin embedded tissue samples were sectioned in a microtome. The tissue sections were deparaffinized, rehydrated, treated with 3% hydrogen peroxide to block endogenous peroxidase, and rinsed. The slides were further incubated with antibody diluents with background reducing component (DAKO, CA, USA) at 37°C for 1 h. The sections were incubated at 4°C overnight with α-smooth muscle actin (α-SMA) (1:500; Abcam, Cambridge, UK), calponin (1:100; Abcam), and von Willebrand Factor (vWF) (1:200; Abcam) antibodies to observe the smooth muscle contents and regenerated blood vessels. For α-SMA and vWF staining, next day the samples were incubated with secondary antibodies (1:500, Alexa Fluor®488 IgG, Invitrogen, OR, USA) for 1 h at room temperature. Next, the coverslip was mounted on the slide using a mounting medium with 4,6-diamino-2-phenyl-indole (DAPI; Vector Labs Burlingame, CA, USA) to observe the cell nuclei. Digital images were obtained using an Olympus BX51 fluorescence microscope. For calponin, next day the slides were incubated with horseradish peroxidase conjugated antibodies (Gendepot, TX, USA), and then visualized with a DAB kit (DAKO). Cell nuclei were counterstained with hematoxylin. Images were captured under a brightfield microscope.

RESULTS

Cross-sectional morphology of h-MDSCs seeded PCL

SEM images were taken for the multilayered nanofiber and cell seeded multilayered nanofiber to characterize the cross-section morphology. Fig. 4A showed the stacking of the multilayered nanofibers. In Fig. 4B, h-MDSCs seeded in between the multilayered nanofibers are indicated by white arrows. From SEM observations, the multilayered nanofibers were appropriately stacked and maintained proper cell distribution.

Presence of cells in the multilayered nanofiber as shown by H&E

H&E staining of bladder after 4 weeks of implantation showed presence of cells within the multilayered scaffolds. The black arrows indicated cells localized between the multilayered PCL/collagen nanofiber and 2 asterisks (**) represented PCL nanofiber sheet. In the MDSC group, the h-MDSCs proliferated between nanofibers and there are sufficient collagen present. The cells were less in NF group compared to MDSC group. The present cells can be the seeded h-MDSCs and the migration of cells from the neighboring region of the host tissues (Fig. 5A).

Fluorescence-labeled h-MDSCs localization *in vivo*

It was observed that the N group showed an intense green color in the bladder, which indicates adequate smooth muscle in the functional bladder (Fig. 5B). In contrast, there was disrupted smooth muscle layer in the MPPC group. In the NF group, there are less cells which may have migrated from the neighboring host tissues which were negative for α-SMA. In the MDSC group, PKH-26 labeled h-MDSCs showed few merged cells (orange colored) between nanofiber, which are indicated by white arrows (Fig. 5B). Moreover, there are α-SMA positive cells at the

lower scaffolds which are the migration of smooth muscle cells from the neighboring host bladder which may be due to the chemoattractant nature of seeded h-MDSCs.

h-MDSCs promote smooth muscle regeneration

Calponin is a smooth muscle specific, actin, tropomyosin, and calmodulin binding protein thought to be involved in the

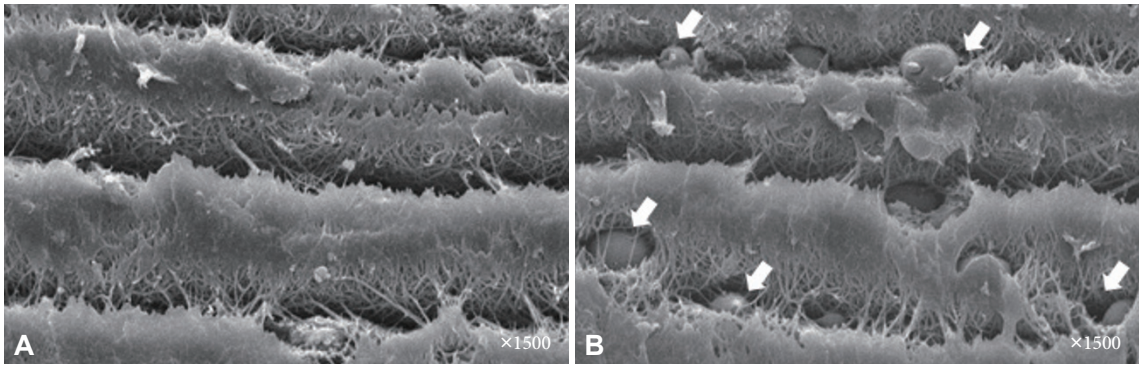

Figure 4. Cross-sectional morphology by scanning electron microscopy. (A) Multilayered PCL/collagen nanofiber sheet. (B) h-MDSCs seeded multilayered PCL/collagen nanofiber sheet. White arrows indicated the cells seeded in the nanofiber. h-MDSCs: human muscle-derived stem cells, PCL: polycaprolactone.

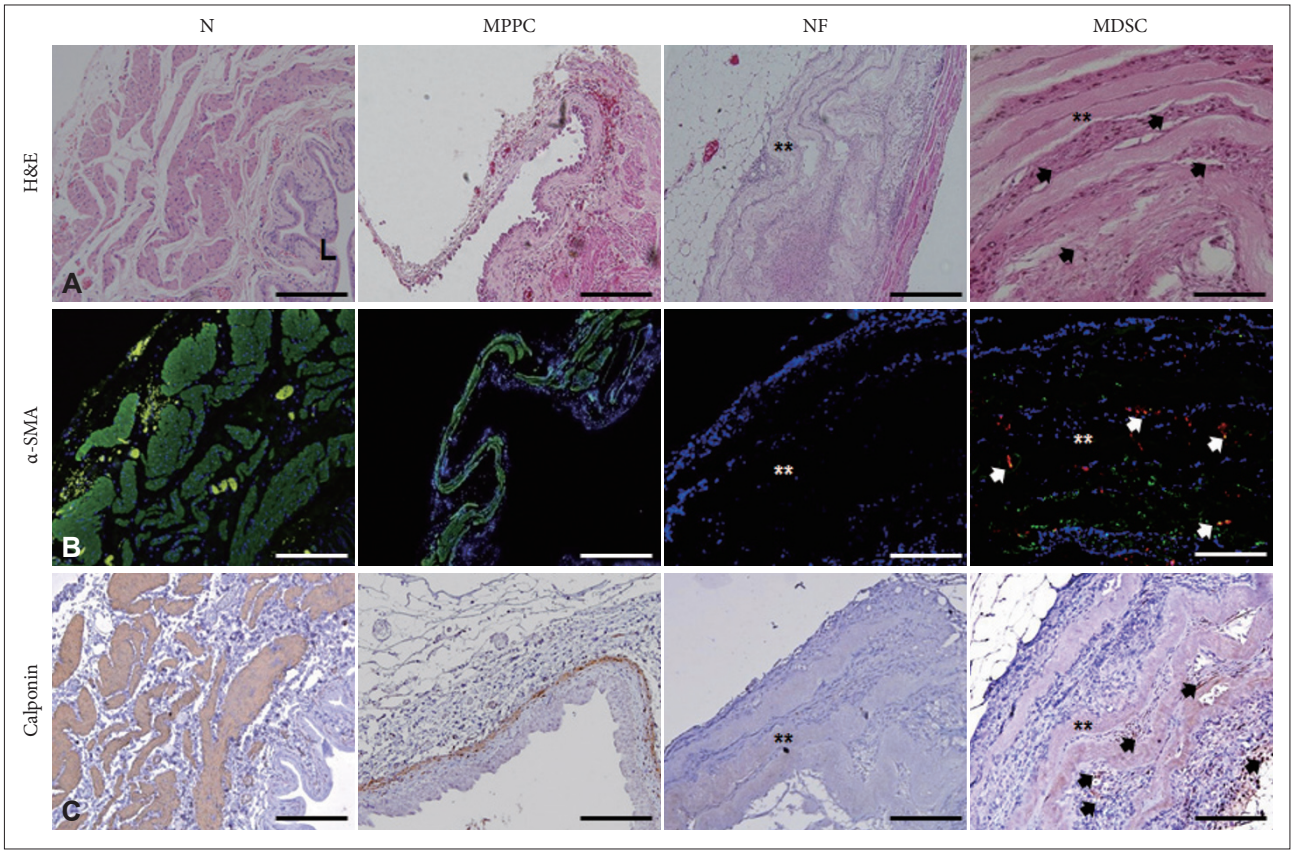

Figure 5. Hematoxylin and eosin (H&E), α-smooth muscle actin (α-SMA) staining, and calponin expression of remodelled bladder muscle 4 weeks after nanofiber implantation. (A) Morphological characteristic of the urinary bladder in H&E staining showed the enhanced muscle regeneration. Black arrows showed cells between PCL nanofiber sheet implanted in the injured bladder tissue. L: lumen of the bladder and 2 asterisks (**) represent PCL nanofiber sheet. (B) Immunohistochemical staining of the bladder tissue with α-SMA. Integrated scaffolds were stained using α-SMA (green), PKH-labeled h-MDSCs are stained red, nuclei are stained blue with DAPI, white arrow showed PKH-26 labeled h-MDSCs and 2 asterisks (**) represent PCL nanofiber sheet. (C) Immunohistochemical staining of calponin. The smooth muscle is stained brown. Black arrow showed cells between PCL nanofiber sheet implanted and 2 asterisks (**) represent PCL nanofiber sheet (N: normal group, MPPC: injury group, NF: nanofiber implanted group, MDSC: h-MDSCs seeded nanofiber implanted group). Scale bar=200 μm in all the figures. PCL: polycaprolactone, h-MDSCs: human muscle-derived stem cells, DAPI: 4,6-diamino-2-phenyl-indole, MPPC: mucosa preserving partial cystectomy, NF: nanofiber.

regulation of actomyosin as well as the regulation or modulation of contraction. Calponin expression was the highest in the N group whereas the expression decreased in injured group. There was the presence of calponin positive cells (indicated by black arrows) in between the nanofibers in the MDSC group (Fig. 5C). These calponin positive cells were responsible for smooth muscle regeneration in the injured bladder.

h-MDSCs promote vessel regeneration

The presence of vWF positive cells between the nanofibers in the MDSC group indicates that the implanted h-MDSCs survive and contributed to pro-angiogenesis in regenerated muscle region (Fig. 6).

DISCUSSION

The urinary bladder is a hollow, muscular, and distensible organ that collects urine excreted by the kidneys. Several conditions such as neurogenic bladder and bladder cancer, disturb the bladder function leading to many problems. Bladder tissue can not be easily replaced due to the difficulty of mimicking natural elasticity and urothelial permeability. Direct injection of cell suspension without biomaterials shows the difficulty in controlling the localization of transplanted cells [16,17]. There has been continuous research for the improvement of bladder tissue engineering using various types of cells and scaffolds. The major limitations of bladder regeneration are the absence of biomaterial having proper thickness that serves as a suitable scaffold for the regeneration process, the isolation and culture of urothelium cells, and finding a reliable source of healthy smooth muscle cells that can be safely harvested with minimal manipulation [18].

Researchers have demonstrated that collagen based biomatrices of small intestinal submucosa implanted in partial cystectomy rabbit model improved bladder function [19]. Yoshimoto et al. [20] have reported that PCL nanofibers have an irregular surface with varying diameters along individual fibers. Similarly, Yu et al. [21] illustrated that the bladder wall grafting in rats using salt modified and collagen coated PCL scaffolds supported smooth muscle cell growth. Recently, Del Gaudio et al. [22] evaluated electrospun scaffolds made up of poly(E-caprolactone) blended with poly(3-hydroxybutyrate) for tissue engineered urinary bladder augmentation. To date, none of them have met the necessary expectations due to structural, mechanical or biocompatibility problems. The urinary calculi formation is a common reaction to foreign material as it creates a cavity for minerals to precipitate out of urine, thus leading to stone formation [4]. In the case of thick scaffolds, seeded cells do not survive as there is no vascular growth in the inner region of the scaffold [23]. Moreover, lack of blood supply and the inflammatory processes triggered by implantable graft leads to ischemia as well as fibrosis of the graft [24,25]. Therefore, we designed a multilayered PCL/collagen nanofiber sheet with reasonable thickness and seeded h-MDSCs onto it. PCL was selected because it is biocompatible, flexible, and its degradation product show less toxicity [26]. Its biocompatibility can also be increased by blending it with collagen [14]. Collagen binds to the cell anchoring molecule, and this leads to the cytoplasmic signal transduction, which in turn leads to the proliferation of the seeded cells [27,28].

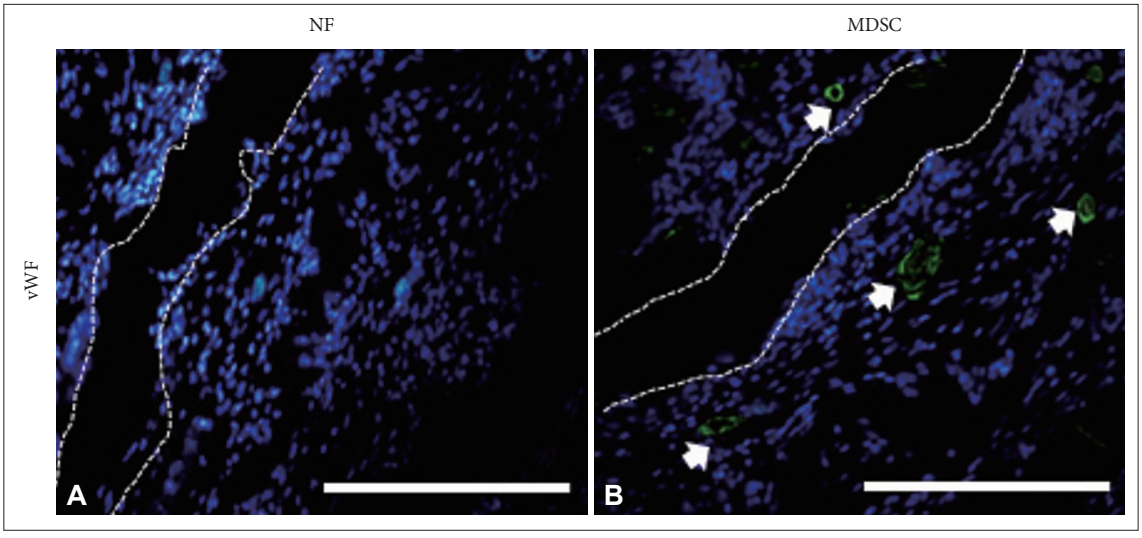

Figure 6. Immunohistochemical staining of von Willebrand Factor (vWF) at 4 weeks. (A) There are absence of vWF positive cells in the NF group. (B) There are presence of vWF positive cells, indicated by white arrow, in the MDSC group. In both the figures, the dotted line represented the PCL nanofiber. Blue is cell nucleus (DAPI). Scale bar=200 μm (NF: nanofiber implanted group and MDSC: h-MDSCs seeded nanofiber implanted group). NF: nanofiber, PCL: polycaprolactone, DAPI: 4,6-diamino-2-phenyl-indole, h-MDSCs: human muscle-derived stem cells.

PCL (30−40 μm) were stacked one over the other to get five layered PCL having a final thickness of 150−200 μm, which add advantage over the thicker scaffolds. Furthermore, the multilayered nanofiber arrangement increases the surface area of the scaffold which is suitable for cell proliferation and survival in the outer and the inner layer of the scaffold. Studies on MPPC model is poorly understood in the literature.

Stem cells hold great promise for the regenerative medicine due to their self renewal and differentiation ability [29,30]. Human stem cells do not show any immune rejection when administrated in the animal model. Our lab has previously shown the use of human ADSC in a rat for the recovery of erectile function of cavernous nerve injury without any immunological issues [31]. Recently, Lavasani et al. [32] illustrated that human muscle derived stem cells promote nerve regeneration in mice without any rejection. Therefore, we chose human stem cells. Advances in the stem cell therapy offer a therapeutic opportunity to restore smooth muscle for functional augmentation. Despite their possible use in cell therapy, practical application to muscular damage depends on the ability to control their differentiation into functional smooth muscle cells. Thus, the use of a scaffold with appropriate thickness may enhance the proliferation and differentiation of stem cell for muscle regeneration.

Here, we showed that h-MDSCs seeded on multilayered nanofiber accelerates smooth muscle repair in MPPC model by promoting the muscle regeneration as analyzed by α-SMA staining. The survival of h-MDSCs after 4 weeks within the nanofiber is affected by the interaction between the cells and their surrounding microenvironment. Moreover, the seeded h-MDSCs may stimulate the fusion of neighboring smooth muscle cells or differentiates into smooth muscle. The transplanted h-MDSCs could favor the survival and proliferation of resident bladder smooth muscle cells and vascular cells, and phenotypic conversion of the resident fibroblast and myofibroblasts to smooth muscle cells by the release of specific factors and/or cell to cell contact [33].

The co-localization of h-MDSCs and smooth muscle could be evidence of myogenic differentiation of h-MDSCs as revealed by hMDSCs tracking by PKH26 labelling. Furthermore, the attachment of h-MDSCs to PCL multilayer was also visible under an electron microscope, which indicated that PCL composite is the appropriate composite cellular carrier.

Immunohistochemical analysis showed that the calponin expression is upregulated in the MDSC group compared to the MPPC group which facilitates the regulation or modulation of contraction of actin in the smooth muscles. Similarly, neovascularization is very important for the survival of seeded cells and complete restoration of organ structure and function [34]. Baumert et al. [35] transferred the matrix into the omentum, so as to improve the vascularization of tissue engineered bladder scaffold *in vitro*. This critical vascular structure was faintly present in our model which may be due to inflammation and scarring of the remaining bladder region, and thus inhibiting proper regeneration of the bladder wall. There was minimum fibrosis and collagen deposition in the MDSC group compared to the NF group, suggesting that multilayered nanofiber sheet without h-MDSCs might cause a foreign body reaction. We observed that h-MDSCs have the potential to be the source of smooth muscle regeneration in a MPPC rat model. The mechanism of regeneration can be a fusion of the seeded h-MDSCs with the neighboring smooth muscle cell or by the transdifferentiation of the h-MDSC into smooth muscle cells.

This was a preliminary study conducted for 4 weeks. Future studies will be necessary on examining the effects of this polymer for a longer duration, confirming complete degradation of nanofiber sheet, establishing a vascular and neural supply to newly formed tissue, modulating the inflammatory response during the regeneration period. Similarly, urodynamics and contractility studies are needed to give strong evidence of bladder smooth muscle regeneration. h-MDSCs seeded multilayered PCL nanofiber promotes smooth muscle regeneration in MPPC rat model which could be promising approach in the treatment of neurogenic bladder and related bladder diseases.

In conclusion, we showed that h-MDSCs seeded multilayered PCL/collagen nanofiber sheet can lead to the regeneration of bladder smooth muscle in a MPPC rat model. Application of h-MDSCs has a promising effect on smooth muscle regeneration with minimal fibrosis during the bladder regeneration.

Acknowledgements

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

All animal experiments were approved by the Institutional Animal Care and Use Committee of The Catholic University of Korea School of Medicine, Seoul.

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