Engineered Bone Marrow-derived Cell Sheets Restore Structure and Function of Radiation-Injured Rat Urinary Bladders

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Abstract

Previously, we reported that implantation of isolated single bone marrow-derived cells into radiation-injured urinary bladders could restore structure and function. However, injections of isolated single cells had some limitations. Thus in this study, we produced bone marrow-derived cell sheets in temperature-responsive culture dishes that release the monolayer sheets intact. We then determined if the recovered cell sheets could restore function to irradiated urinary bladders. Twenty female 10-week-old Sprague-Dawley (SD) rats were irradiated with 2 gray once a week for 5 weeks. Bone marrow cells harvested from two male 17-week-old green fluorescence protein-transfected SD rats were placed in primary culture for 7 days. Bone marrow cell-derived outgrowths were harvested by enzymatic digestion and transferred into the atelocollagen-coated temperature-responsive culture dishes for 2 days. To harvest the secondarily cultured cells as monolayer sheets, a support membrane was put in each culture dish, and then the temperature was reduced to 20°C. Each released cell sheet was then patched onto the irradiated anterior bladder wall (n=10). As controls, cell-free sheets were similarly patched (n=10). After 4 weeks, transplanted cells were detected on the bladder walls. The cell sheet-transplanted bladders had smooth muscle layers and acetylcholinesterase-positive nerve fibers in proportions that were significantly larger than those of the control bladders. In addition, the cell sheet-transplanted bladders had reduced P4HB-positive regions of collagen synthesis and apoptosis within the smooth muscle layers. In cystometric investigations, threshold pressures, voiding interval, micturition volume, and bladder capacity in the cell sheet-transplantation group were significantly higher than those in the control group. Residual volume of the cell sheet-transplantation group was significantly lower compared to the control. There were 24 growth factor mRNAs in the cell sheet-transplanted urinary bladders that were expressed ≥ two-fold over the controls. In conclusion, cell sheet engineering has great potential to restore damaged urinary bladders.

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Introduction

Regenerative medicine of the lower urinary tract, which is composed mainly of the urinary bladder and urethra, provides great hope for patients with lower urinary tract dysfunction. Thus by applying the principles of tissue engineering and the utilization of various cells, including stem cells, we have vigorously investigated the potential for regenerative medicine to treat irreversibly damaged lower urinary tracts. Previously, we reported that implantation of allogenic bone marrow-derived cells into radiation-injured rat urinary bladders[1] or freeze-injured mouse urinary bladders[2] could reconstruct functional urinary bladders. In these studies, we established primary cultures of bone marrow cells that were harvested from femurs. From the original cells that were placed culture, bone marrow-derived cells formed monolayer outgrowths with cell-to-cell and/or cell-to-extracellular matrix (ECM) contacts. These proliferating cells were then isolated as single cells by enzymatic treatment and centrifugation. The isolated single cells, which no longer had cell-to-cell or cell-to-ECM contacts, were directly injected into injured regions of the bladder wall. The viability of the isolated single cells was slightly reduced by the enzymatic disruption and isolation.

The enzymatic treatment that produced the isolated, single bone marrow-derived cells probably also damaged cell surface adhesion molecules and receptors. Loss of these cell surface moieties could reduce adhesion of the bone marrow-derived cells injected into the damaged tissues and hinder paracellular communications that are necessary to develop smooth muscle, nerves, blood vessels, and other tissues that are needed to achieve a functional recovery. For this reason, we have now investigated a new technology that harvests the bone marrow-derived cell monolayer outgrowths without the use of enzymatic digestion. This new technology utilizes culture dishes that are covalently coated with a temperature-responsive polymer, Poly(N-isopropylacrylamide) (PIPAAm). PIPAAm exists in a hydrophobic state at 37°C, but reversibly changes to a hydrophilic state below 32°C.[3] The hydrophobic state of the surface supports cell adhesion and proliferation at 37°C. By lowering the temperature below 32°C, the cells, along with the ECM substratum, spontaneously detach from the culture dish as it becomes hydrophilic.[4-5] Thus, under low temperature condition, the cultured cells can be harvested without any enzymatic treatments or centrifugal separation.[6] The cells can be harvested as a cell sheet and retain the intact

monolayer structure with full preservation of the cell-to-cell contacts and contact with the ECM.[7-9] In addition, the cell sheets can be transplanted without suturing.

By using the temperature-responsive culture dishes, we were able to produce bone marrow-derived cell sheets that were composed of the intact bone marrow-derived cultured cells that maintained the monolayer structure, thus overcoming the problems in the transplantation of isolated single cells. In this study, we determined if the bone marrow-derived cell sheets could reconstruct functional rat urinary bladders that were damaged by irradiation.

Materials and Methods

Animals

The urinary bladders of 20 female 10-week-old Sprague-Dawley (SD) rats (Japan SLC Inc., Shizuoka, Japan) were irradiated (described below). Two male 17-week-old green fluorescence protein (GFP)-transfected Tg-SD rats (Japan SLC Inc.) were used as bone marrow cell donors. All of the rats were maintained with freely available food and water under a 12-hour alternating light-dark cycle. All animals were treated in accordance with National Institutes of Animal Health Care Guidelines and the guidelines approved by the Animal Ethics Committee of Shinshu University School of Medicine.

Production of Radiation-injured Urinary Bladders

Radiation injury to the urinary bladders was produced as we previously described.[1] Briefly, the SD rats were anesthetized with a pentobarbital sodium solution (40 mg/kg-body weight, Kyoritsu Seiyaku Co., Tokyo, Japan), and then protected with an iron shield except for a 1-cm diameter circle bordering on the pubic bone to allow radiation of the pelvic region containing the urinary bladder. The exposed area was irradiated with 2-Gy once a week for 5 weeks. Following the last radiation exposure, the rats were maintained for 2 weeks and then randomly separated into a cell sheet-transplantation group and a cell-free sheet control group (n=10 in each).

Production of Bone Marrow-derived Cell Sheets

The bone marrow-derived cells were prepared according to our previous report.[1] Briefly, the Tg-SD rats were anesthetized with a pentobarbital sodium solution, and then both femurs of each rat were harvested. After the femurs were removed, the animals were euthanized by an overdose of pentobarbital sodium solution (Kyoritsu Seiyaku Co.). Both ends of the femurs were cut and flushed with 10 ml of culture medium composed of Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY, USA) supplemented with 15% regular fetal bovine serum (Biowest, Nuaille, France) and 0.1% penicillin-streptomycin solution (Gibco). The harvested cell suspensions were centrifuged at 1000 rpm for 4 min, and then seeded onto type I collagen-coated 6-cm

culture dishes (Iwaki, Asahi Techno Glass, Chiba, Japan). The primary cell cultures were maintained at 37℃ in humid air with 5% CO_2 for 7 days. The medium was completely replaced every day and non-attached cells were discarded. This study used only the adherent and proliferating cells as bone marrow-derived cells.

Prior to secondary culture, temperature-responsive culture dishes (1.9 cm^2/well, UpCell, CellSeed Inc., Tokyo, Japan) were coated with atelocollagen (Koken Co., LTD., Tokyo, Japan) to maintain primary culture conditions. The adherent proliferating bone marrow-derived cells of the primary culture were dissociated with 0.25% trypsin (Gibco) and suspended at $1.0x10^6$ cells/ml in culture medium. The cell suspensions (1 ml) were seeded onto the atelocollagen-coated temperature-responsive culture dishes ($1.0x10^6$ cells/ml/well), and then the cells were cultured for 2 days. The culture medium was then removed, and a support membrane (CellShifter, 11-mm diameter, CellSeed Inc.) needed to harvest the cultured cells was placed on the surface of the cultured cells. Following this, the temperature of the culture dishes was reduced to 20℃ from 37℃. Five minutes later, the cells were slowly taken up with the support membrane and transplanted onto the surface of the irradiated urinary bladders as described below.

Patch Transplantation of Bone Marrow-derived Cell Sheets

Two weeks after the last radiation treatment, the recipients were anesthetized with pentobarbital sodium solution as above. The irradiated urinary bladders were exposed, and if empty, approximately 1-ml saline was infused through the urethra to distend the bladder, creating a smooth, firm surface upon which to apply the transplantation patch. For the cell sheet-transplantation group, the support membrane with cells attached was patched onto the surface of the irradiated anterior bladder wall (n=10). The patch was simply laid over the bladder surface, without any use of adhesives or sutures. The circular cell-support membrane covered approximately 90% of the anterior bladder wall surface. Five minutes later, the cells were attached on the bladder wall, and then the support membrane was slowly and gently removed. For the control group, the cell-free support membrane was similarly patched onto the irradiated bladder wall (n=10). The cell-free support membrane was produced by simply incubating it with the culture medium for 5 min before the operation. After removing the support membrane, the urinary bladders were returned to the pelvic cavity, and then

the incised region was closed. All of the rats were maintained for 4 weeks as described above.

Cystometric Investigation

Four weeks after the bone marrow-derived cell or cell-free control sheet transplantation, we performed cystometric investigations as previously described.[1] Briefly, two days prior to cystometric measurements, the urinary bladders were exposed and a polyethylene catheter (PE50, Nippon Becton Dickinson, Tokyo, Japan) was inserted. The free end of the catheter was tunneled subcutaneously and exteriorized at the back of the neck. Cystometric investigations were performed on unanesthetized, unrestricted rats placed in metabolic cages for 30 min. The catheter inserted into the urinary bladder was connected via a T-tube to a pressure transducer (P23 DC; Statham, Oxnard, CA, USA) and a syringe pump (Terumo Inc., Tokyo, Japan). Saline maintained at room temperature was instilled into the urinary bladder at a rate of 10 ml/hr. Bladder contractions were recorded continuously on a pen oscillograph (10 mm/min recording speed, Recti-Horiz-8K; NEC San-ei Instruments, Tokyo, Japan). Micturition volume, measured with a fluid collector connected to a force displacement transducer (Type 45196; NEC San-ei Instruments), was simultaneously recorded. After confirming first and second voiding, the following cystometric parameters were directly measured: basal, threshold, and micturition pressures (cmH_2O), micturition volume (ml), and voiding interval (minutes). Residual volume (ml) was calculated by subtracting the micturition volume from the saline infusion volume. Bladder capacity (ml) was calculated by adding the residual volume to the micturition volume.

After the cystometric investigations, the rats were anesthetized with a pentobarbital sodium solution (50 mg/kg-body weight, Kyoritsu Seiyaku Co.). Their urinary bladders were then removed, trimmed, processed for immunohistochemical and histological investigations, and real-time reverse transcription-polymerase chain reaction (PCR) array (described below). After the bladders were removed, the animals were euthanized by an overdose of pentobarbital sodium solution (Kyoritsu Seiyaku Co.).

Fluorescence Double-label Immunohistochemistry and Histological Investigations

The trimmed urinary bladders were fixed and embedded in paraffin. The samples were cut in 5-µm thick serial sections. For immunohistochemistry, the sections were treated according to our protocol.[1] Briefly, they were deparaffinized, and treated with 10 mM sodium citrate (pH 6.0, 100°C, 5 min) for antigen retrieval. The specimens were then coated with 1.5% normal donkey serum (Chemicon International, Inc., Temecula, CA, USA) and 1.5% non-fat milk in phosphate buffered saline (PBS) for 1 hr at 4°C.

To detect the transplanted bone marrow-derived cells, the sections were incubated for 12 hr at 4°C with GFP antibody (1:500, mouse monoclonal, Lifespan Biosciences, Inc., Seattle, WA, USA). The sections were rinsed with PBS at 4°C and then incubated with secondary antibody consisting of donkey anti-mouse IgG conjugated with Alexa fluor 488 (1:250, Molecular Probes, Eugene, OR, USA) for 1 hr at 4°C.

Following subsequent rinsing, the sections were then incubated for 12 hr at 4°C with antibodies for alpha-smooth muscle actin (SMA, 1:100, mouse monoclonal, Progen Biotechnik GmbH, Heidelberg, Germany) as a marker for smooth muscle cells. Alternatively, they were incubated with antibodies for calcitonin gene-related peptide (CGRP, 1:500, guinea pig polyclonal, Progen Biotechnik GmbH) as a marker for nerve cells. The sections were rinsed with PBS at 4°C, and then incubated with secondary antibody consisting of donkey anti-mouse or anti-guinea pig IgG conjugated with Alexa fluor 594 (1:250, Molecular Probes) for 1 hr at 4°C. Following rinsing, nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 5 µg/ml, Molecular Probes), and the sections were then coated with Fluorescent Mounting Medium (Dako Cytomation, Carpinteria, CA, USA). Other samples were similarly stained with SMA and collagen prolyl 4-hydroxylase beta (P4HB, 1:50, mouse monoclonal, Novus Biologicals, Inc.). P4HB plays an essential role in the synthesis of all collagens. The ApopTag® ISOL fluorescence apoptosis detection technique (DNase Types I & II, Merck Millipore, Merck KGaA, Darmstadt, Germany) for caspase-dependent and -independent apoptosis was applied to other serial sections. The slides were observed and photographed with a Leica DAS Microscopethe (Leica Microsystems GmbH, Wetzlar, Germany). Histochemical control of the primary antibodies conformed with our previous studies.[1,2]

Other deparaffinized sections were prepared for histological analysis. They were

stained with masson trichrome, enzyme-labeled acetylcholinesterase antibody (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan), or picrosirius red. These stained samples were observed and photographed with a common optical microscope. Sections stained with masson trichrome and acetylcholinesterase antibody were used respectively to semi-quantitatively evaluate the proportion of smooth muscle layer or nerve fiber areas in each slide. The evaluations were conducted with image analysis software (Image-Pro® Plus, Media Cybernetics Inc., Bethesda, MD, USA). Picrosirius red stain was used to observe type I and III collagen fibers.

Real-time reverse transcription polymerase chain reaction (RT-PCR) array

The mRNA expression levels of 84 growth factors in the cell sheet-transplanted and control urinary bladders were estimated by real-time RT-PCR arrays. Total RNA was extracted from trimmed urinary bladders with the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). Single strand complementary DNA (cDNA) was synthesized from 1.0 μg of the total RNA with the RT^2 First Strand Kit (Qiagen Inc.). The synthesized cDNA was added into plates of the RT^2 Profiler™ PCR Array Rat Growth Factors (Qiagen Inc.) pre-loaded with 84 growth factor primers and the following five internal standard genes: actin, beta (*Actb*), beta-2 microglobulin (*B2m*), hypoxanthine guanine phosphoribosyly transferase 1 (*Hprt1*), lactate dehydrogenase A (*Ldha*), and ribosomal protein, large, P1 (*Rplp1*). Real-time RT-PCR was performed at 50ºC for 2 min followed by 95ºC for 10 min. These were then followed by 40 cycles at 95ºC for 15 sec and 60ºC for 1 min. The mRNA expression levels of the 84 growth factors were calculated by the delta-delta method as the ratio to threshold cycle (Ct) value of the averaged internal standard genes. Ct values over 35 were considered as undetectable. The expression change for each gene in the cell sheet-transplanted urinary bladders was compared to the change in the control urinary bladders. Gene expression was considered to be upregulated if the real-time RT-PCR array indicated a > 1.0-fold increase.

Statistical Analysis

Results were expressed as means ± standard deviation. Statistical differences were determined using the Excel® Statistics program (Esumi Co., Ltd. Tokyo, Japan).

Comparisons were made by t-test. P-values less than 0.05 were considered statistically significant.

Results

Cell Sheet Engineering of Bone Marrow-derived Cells

After primary culture of the bone marrow cells on collagen-coated culture dishes for 7 days, the adhered proliferating cells exhibited a relatively uniform spindle-shaped morphology. After retrieval by enzymatic digestion and centrifugation, the bone marrow-derived cells were secondarily cultured on the temperature-responsive culture dishes for 2 days. These cells maintained the uniform spindle-shaped morphology and nearly achieved confluence (Fig. 1A). Like the cells in primary culture,[1,2] the cultured cells just prior to transplantation were positive for STRO-1 (Fig. 1A inset), a mesenchymal cell marker. When incubated at 20°C for 5 min, the monolayer of bone marrow-derived cells was released from the hydrophilic surface of temperature-responsive culture dishes and harvested on the support membrane. Cell sheets that fully preserved the monolayer structure of the cultured bone marrow-derived cells were patch transplanted onto the surface of the irradiated anterior bladder wall (Fig. 1B).

Four weeks after transplantation of the cell-free control sheet, the bladder walls appeared pale due to the absence of blood vessels (Fig. 1C). In contrast, at the same time, bladder walls receiving the cell sheet transplants had distinct, thick, blood vessels (Fig. 1D). In immunohistochemistry investigations, the transplanted cells of the sheet on the anterior surface of the bladder walls were detected by GFP-antibody staining (Figs. 1E and F). There were no identifiable smooth muscle or nerve cells that were clearly differentiated from the transplanted cells, though the endogenous smooth muscle (Fig. 1E) and CGRP-labeled nerve cells (Fig. 1F) were readily shown.

Histoarchitecture of the Reconstructed Urinary Bladders

Staining by masson trichrome showed that the control bladders receiving the cell-free sheets had a few atypical smooth muscle layers (Fig. 2A). In contrast, the cell sheet-transplanted urinary bladders had thick and typical smooth muscle layers that were composed of numerous smooth muscle cells (Fig. 2B). The proportion of smooth muscle layer areas in the cell sheet-transplanted urinary bladders, 0.28±0.02, was significantly higher than that in the cell-free sheet control urinary bladders, 0.18±0.01

($P<0.01$, Fig. 2C). Within the same regions of the serial sections, the cell-free sheet control urinary bladders had few nerve fibers that were positively stained for acetylcholinesterase (Fig. 2D). In contrast, the cell-sheet transplanted urinary bladders had more acetylcholinesterase-positive nerve fibers (Fig. 2E). The proportion of the acetylcholinesterase antibody-detected nerve fibers in the cell sheet-transplanted urinary bladders, 0.04±0.01, was significantly higher than that in the control urinary, 0.008 ±0.004 ($P<0.01$, Fig. 2F).

In the cell-free sheet control urinary bladders, the collagen fibers were largely absent except where they accumulated together in clusters (Fig. 3A). These bladders exhibited P4HB-positive regions within the remaining smooth muscle cells (Fig. 3A inset). In contrast, collagen fibers of the cell sheet-transplanted urinary bladders were distributed among the reconstructed smooth muscle layers (Fig. 3B). Within the smooth muscle layers of these bladders, the P4HB-positive regions were reduced compared to the controls (Fig. 3B inset). In addition, the cell-free sheet control urinary bladders had apoptotic cells that were induced by caspase-dependent and/or –independent pathways (Fig. 3C). While the cell sheet-transplanted urinary bladders also had apoptotic cells (Fig. 3D), the number, 4.46±0.49 cells per field was significantly lower compared to the control bladders, 13.74±1.77 cells per field ($P<0.01$, Fig. 3E).

Bladder Functions After Transplantation of Bone Marrow-derived Cell Sheets

In cystometric investigations, cell-free sheet control rats had frequent and irregular periods of micturition at intervals of less than 5 min and micturition volumes of less than 1 ml (Fig. 4A). In contrast, the cell sheet-transplanted rats voided at regular intervals of about 5 min with micturition volumes of about 1 ml (Fig. 4B), which is typical for rats with uninjured bladders.[1]

For cell-transplanted rats, the basal pressure, defined as the bladder pressure during storage of urine, was 7.29±1.13 cmH_2O (Fig. 4C), and the micturition pressure, defined as the maximum bladder pressure during urination, was 34.91±3.04 cmH_2O (Fig. 4D). These values were similar to the cell-free sheet controls, 7.12±1.25 cmH_2O (Fig. 4C) and 33.28±4.39 cmH_2O (Fig. 4D) respectively. In the cell sheet-transplanted rats, the threshold pressure, defined as the bladder pressure just prior to micturition, was 17.59±2.56 cmH_2O, which was significantly higher than that of the control rats,

10.93±0.69 cmH_2O ($P<0.05$, Fig. 4E). The voiding interval of the cell sheet-transplanted rats, 6.83±0.75 min, was significantly longer than that of the control rats, 4.30±0.61 min ($P<0.05$, Fig. 4F). In addition, the micturition volume, 1.25±0.12 ml, and bladder capacity, 1.27±0.12 ml, in the cell sheet-transplanted rats were significantly higher than for control rats, 0.73±0.13 ml (Fig. 4G) and 0.78±0.12 ml (Fig. 4H) respectively ($P<0.05$ for each). Finally, the residual volume of the cell sheet-transplanted rats, 0.01±0.01 ml, was significantly less than in the cell-free sheet control rats, 0.05±0.03 ml ($P<0.05$, Fig. 4I).

Increased Growth Factor mRNA Expressions in Bone Marrow-derived Cell Sheet-transplanted Urinary Bladders

Of the 84 growth factor mRNAs detected by the PCR array 4 weeks after transplantation, 24 in the cell sheet-transplanted urinary bladders showed at least a two-fold increase over the control urinary bladders. The overexpressed growth factor mRNAs were interleukin 3 (*Il3*), fibroblast growth factor 8 (*Fgf8*), teratocarcinoma-derived growth factor (*Tdgf1*), fibroblast growth factor 3 (*Fgf3*), fibroblast growth factor 17 (*Fgf17*), fibroblast growth factor 5 (*Fgf5*), artemin (*Artn*), nodal homolog (*Nodal*), fibroblast growth factor 4 (*Fgf4*), interleukin 2 (*Il2*), fibroblast growth factor 14 (*Fgf14*), interleukin 4 (*Il4*), fibroblast growth factor 1 (*Fgf1*), myostatin (*Mstn*), anti-mullerian hormone (*Amh*), brain-derived neurotropic factor (*Bdnf*), epiregulin (*Ereg*), growth differentiation factor 10 (*Gdf10*), fibroblast growth factor 10 (*Fgf10*), bone morphogenetic protein 8a (*Bmp8a*), leptin (*Lep*), bone morphogenetic protein 1 (*Bmp1*), left right determination factor 1 (*Lefty1*), and neurotorophin 3 (*Ntf3*). These growth factor mRNAs were divided into seven groups according to their functional implications as follows (Table 1): [1] muscle tissue development-, [2] peripheral nervous system development-, [3] angiogenesis-, [4] apoptosis regulator-, [5] cell differentiation- and/or morphogenesis-, [6] immune response-, and [7] ureteric bud development-related growth factor.

This study focused on mRNAs that were overexpressed by 2-fold; however, the expressions of some growth factor mRNAs were only slightly upregulated, ranging from >1.00- to <2.00-fold. These included interleukin 6 (*Il6*, 1.75-fold), growth differentiation factor 11 (*Gdf11*, 1.73-fold), transforming growth factor alpha (*Tgfa*,

1.65-fold), growth differentiation factor 5 (*Gdf5*, 1.46-fold), placental growth factor (*Pgf*, 1.44-fold), nerve growth factor (*Ngf*, 1.39-fold), RAB GTPase binding effector protein 1 (*Rabep1*, 1.26-fold), glial cell derived neurotrophic factor (*Gdnf*, 1.24-fold), vascular endothelial growth factor B (*Vegfb*, 1.22-fold), vascular endothelial growth factor A (*Vegfa*, 1.21-fold), bone morphogenetic protein 4 (*Bmp4*, 1.18-fold), cardiotrophin-like cytokine factor 1 (*Clcf1*, 1.16-fold), and platelet-derived growth factor alpha polypeptide (*Pdgfa*, 1.08-fold).

Discussion

In the present study, we engineered the bone marrow-derived cell sheets by using temperature-responsive culture dishes, and then we patch transplanted the cell sheets onto the irradiated anterior bladder walls. The most important finding of this study is that the cell sheets promoted the functional recovery of the irradiated urinary bladders. The cell sheet-transplanted urinary bladders had thick and distinct smooth muscle layers and acetylcholinesterase-positive nerve fibers. In addition, these bladders had reduced expression of P4HB within the smooth muscle layers, possibly indicating inhibition of the fibrosis progress compared to control bladders. The cell sheet-transplanted bladders also had a lower level of apoptosis compared to the control bladders. The cystometric investigations showed that bladder functions of the cell sheet-transplantation group were improved. Our previous study showed that isolated single bone marrow-derived cells directly injected into the irradiated urinary bladders similarly supported reconstruction of functional tissues.[1]

Between cell sheet patch transplantation and direct injection of isolated single cells, there are likely to be some differences in the mechanisms of urinary bladder reconstruction. In cell sheet patch transplantation, the bone marrow-derived cells themselves did not exhibit differentiation into the components parts of the urinary bladder walls, such as smooth muscle or nerve cells. In contrast, for injured bladders injected with isolated single cells, it was the injected cells themselves that differentiated into smooth muscle or nerve cells.[1] These results suggest that the isolated bone marrow-derived cells directly transplanted into the injured regions might act as a cell source for reconstruction of tissue structures. In contrast, the cells that were used to produce the cell sheet and then patch-transplanted onto the surface of the injured regions probably did not act as a source of cells that participated in tissue reconstruction. Thus, the bone marrow-derived cell sheets probably played other roles in the reconstruction of functional urinary bladders.

In cell sheet engineering research for cardiac dysfunction and heart failure, the cell sheets composed of cardiomyocytes,[10] myoblasts,[11-13] mesenchymal stem cells,[14] adipocytes,[15] or embryonic stem cell- or induced pluripotential stem cell-derived cardiomyocytes[16-18] induce paracrine effects. The paracrine effects include angiogenesis, cardioprotection, stem cell migration, ECM production, and anti-inflammation due to

the secretion of some growth factors, such as vascular endothelial growth factor, hepatocyte growth factor, insulin-like growth factor, adiponectin, vascular cell adhesion molecule-1 in the injured hearts.[19] The paracrine effects for these growth factors might be different among different cell sources, target organs, and/or degree of injury.[19] Therefore, we focused on the upregulated growth factor mRNAs in the cell sheet-transplanted urinary bladders compared to the cell-free sheet controls.

Compared to the control urinary bladders, the cell sheet-transplanted bladders showed at least a two-fold increase in the expression of 24 growth factor mRNAs (Table 1). We divided these growth factor mRNAs into seven groups according to their functional implications. Higher expression of the muscle tissue development-related growth factors (myostatin, growth differentiation factor 10, bone morphogenetic protein 8a, and bone morphogenetic protein 1) could be responsible for the greater proportion of smooth muscle layer areas in the cell sheet-transplanted bladders. Similarly higher expression of the peripheral nervous system development-related growth factors (artemin, nodal homolog, left right determination factor 1, and neurotorophin 3) could be responsible for the presence of larger numbers of acetylcholinesterase-positive nerve fibers. Finally, higher expression of the apoptosis regulator-related growth factors (interleukin 4 and brain-derived neurotropic factor) could determine the level of apoptosis in the regenerating tissues. Ultimately, expression of these genes could induce paracrine effects, such as angiogenesis, stem cell migration, ECM production, or anti-inflammation. In turn, these growth factors might promote growth and development of a suitable biochemical microenvironment to reconstruct functional tissues. Additionally, we detected some growth factor mRNAs for which the increased expression ranged between 1.00- and 2.00-fold. While expression for these genes was only slightly more than in the control group at 4-weeks after patch transplantation, they also might support tissue reconstruction in the early stages.

The methodology for preparing isolated bone marrow-derived cells for direct injection into damaged bladder walls has clear limitations relevant to its use in clinical therapies. The harvesting of isolated bone marrow-derived cells from monolayers by enzymatic treatment and centrifugation disrupted the cell-to-cell contact and contact with the ECM.[1,2] It also slightly decreased cell viability to 80-90%.[1,2] The mechanical manipulations required for direct injection of the isolated single cells into the recipient bladders could also cause additional trauma to the already damaged tissues. These

limitations of the direct injection method have the potential to cause marginal engraftment and suboptimal outcomes. Cell sheet engineering may be a technique that can overcome these problems.

By utilization of the temperature-responsive culture dishes, the cell sheets are produced without enzymatic treatments and centrifugal separations. Thus, the cell sheets fully preserves the monolayered structures, and the cell viabilities are not reduced during the production processes.[5-7] In the transplantation processes, use of cell sheets avoids the loss of large numbers of cells.[19] In addition, the patch transplantation is only minimally invasive. Another advantage of cell sheet engineering is that it ensures uniform cell quality and curative effects.[19] Cell sheet-based cardiac cell therapies show better effects and prolonged improvement of cardiac functions compared to the direct injection transplantation methods.[20-21] To date, the cell sheet technology has been applied to various types of organ injuries in the clinical setting, including cornea,[22] heart,[23] esophagus,[24] periodontal tissue,[25] and cartilage.[26]

In this study we patch-transplanted the cell sheets onto the anterior surface of the irradiated bladder walls. In other cases where the urothelium is damaged or the submucosal layers of patients with interstitial cystitis are inflamed, the cell sheets may be intravesically transplanted.[27-28] While the single-layer cell sheet has great potential to treat damaged urinary bladders, cell sheet engineering also has the potential to create 3-dimensional grafts composed of multi-layered cell sheets,[29-30] and/or combined with other tissues.[31-32] The multi-layered cell sheet grafts could be used to treat tissue defects or applied to produce artificial organs without the use of extremely limited biodegradable scaffolds. Some studies have reported the use of multi-layered cell sheet grafts for defects of urinary bladders and the urinary ducts.[28, 33]

This study has some limitations and the potential use of cell sheets for transplantation to damaged urinary bladders requires further investigations. There are many unanswered questions which must first be addressed before this technique advances to the clinic. For instance, we did not attempt to estimate the exact number transplanted cells or the survival rate in the walls of the rat bladders, nor do we know the fate of the transplanted cells within the recipient tissues. While we know that the transcription of certain growth factors is upregulated, we do not know which donor or recipient cells are responsible for these changes. The nature and pathways of any

paracrine effects that were induced are also currently unknown. In addition, this study detected the upregulated growth factors at only one point, 4 weeks after transplantation. In future studies, we will investigate how the expression patterns of these growth factors changes during different periods of tissue reconstruction.

In conclusion, bone marrow-derived cell sheets appear to overcome many of the limitations of direct injection of isolated single cells in the recovery of radiation-injured rat bladders. At 4 weeks after the cell sheets were patch transplanted on the irradiated bladder walls, donor GFP-labeled cells were present on the transplanted regions. Smooth muscle layers and nerve fibers were reconstructed in significantly higher proportions in the cell sheet-transplanted bladders compared to the cell-free sheet controls. In addition, the cell sheet-transplanted urinary bladders had decreased levels of fibrosis and apoptosis. In cystometric investigations, micturition patterns of the cell sheet-transplantation group were similar to those of normal rats, while the control group exhibited higher urinary frequency and smaller volumes. The threshold pressure, voiding interval, micturition volume, and bladder capacity in the cell sheet-transplantation group were significantly higher than those of the control group, and the residual volume of the cell sheet group was significantly lower compared to the control group. The cell sheet-transplanted urinary bladders expressed 24 growth factor mRNAs that were over two-fold greater than in the controls. Therefore, the bone marrow-derived cell sheets have a great potential for treating damaged urinary bladders.

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Disclosure Statement

No competing financial interests exist.

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Figure legends

Figure 1. Gross and histochemical urinary bladder appearance at 4 weeks after patch application of bone marrow-derived cell sheets and control cell-free sheets. **(A)** After enzymatic harvesting of the primary culture outgrowths and replating them in secondary cultures, the bone marrow-derived cells showed the uniform spindle-shaped morphology. Bar: 100 μm. (A inset) Just prior to transplantation, the cultured cells were positive for GFP (green) and STRO-1 (red) antibodies (arrowheads: yellow: overlapping red- and green-labeled antibodies; Blue: nuclei; Bars: 20 μm). **(B)** The bone marrow-derived cells were attached to the support membrane (arrow), and the sheets were patched onto the irradiated anterior bladder wall. **(C)** At 4 weeks after patching, there was a relative absence of blood vessels in the bladder walls receiving the cell-free sheet control patch. **(D)** At 4 weeks after receiving the cell sheet patch transplant, the bladder walls had distinct, thick, blood vessels. **(E and F)** The transplanted cells were readily detected by GFP-antibody (green, arrows) on the anterior bladder walls. **(E)** Smooth muscle cells detected as SMA-positive layers (red, asterisks) were not differentiated from the transplanted cells. **(F)** Similarly, presumed nerve fibers detected as CGRP-positive bodies (red, arrowheads) were also not associated with the transplanted cells. Blue: nuclei; Scale Bars: 20 μm.

Figure 2. Reconstructed smooth muscle layers and nerve fibers. (**A)** At 4 weeks after patching with the control cell-free sheet, the urinary bladders had a few atypical smooth muscle layers. Masson trichrome stain. **(B)** In contrast, the cell sheet-transplanted urinary bladders had thick and typical smooth muscle layers that were composed of numerous smooth muscle cells. Masson trichrome stain. (**C**) The proportion of smooth muscle layer areas in the cell sheet-transplanted urinary bladders was significantly higher than that in the cell-free sheet control bladders ($P<0.01$). (**D**) Within the same regions of masson trichrome stained serial sections, the cell-free sheet control bladders had few nerve fibers that were positively stained for acethylcholinesterase. **(E)** However, the cell-sheet transplanted bladders had more acethylcholinesterase-positive nerve fibers. (**F**) The proportion of the acethylcholinesterase antibody-detected nerve fibers in the cell sheet-transplanted bladders was significantly higher than that in the control urinary bladders ($P<0.01$). Scale bars for micrographs A, B, D, and E: 100 μm.

Figure 3. Fibrosis and apoptotic cells. (**A**) At 4 weeks after patching with the cell-free sheet, collagen fibers in the control bladders were largely absent (asterisks) except where they were accumulated together in clusters. **(B)** However, collagen fibers (arrows) in the cell sheet-transplanted urinary bladders were distributed among the reconstructed smooth muscle layers. **(A inset)** The control urinary bladders exhibited P4HB-positive regions (arrowheads, green) within the remaining smooth muscle cells (red). Scale Bar: 20 μm. **(B inset)** In contrast, the cell sheet-transplanted urinary bladders had fewer P4HB-positive regions (arrowheads, green) among the smooth muscle cells (red) Scale Bar: 20 μm Both the cell-free sheet control **(C)** and the cell sheet-transplanted urinary bladders **(D)** had apoptotic cells (arrows) induced by caspase-dependent (red) and/or –independent (green) pathways. Yellow: overlapping red-labeled caspase-dependent and green-labeled caspase-independent antibodies. Bars: 20 μm. (**E**) The number of apoptotic cells in the cell sheet-transplanted group was significantly lower than that in the control group ($P<0.01$).

Figure 4. Effects of cell sheet-transplantation on urinary bladder functions. (**A**) The cell-free sheet control rats exhibited frequent and irregular micturition with intervals of less than 5 min (upper trace: arrowheads, voiding points) and micturition volumes of less than 1 ml (lower trace). (**B**) The cell sheet-transplanted rats voided at regular intervals of about 5 min (upper trace: arrowheads, voiding points) with micturition volumes of about 1 ml (lower trace). Basal **(C)** and micturition pressures **(D)** were not significantly different between the two groups. Threshold pressures **(E)**, voiding interval **(F)**, micturition volume **(G)**, and bladder capacity **(H)** in the cell sheet-transplantation group were significantly higher than those in the cell-free sheet control group. (**I**) Residual volume of the cell sheet-transplanted rats was significantly lower than that in control rats.

TABLE 1. UPREGULATED GROWTH FACTOR mRNAS IN CELL-SHEET TRANSPLANTED URINARY BLADDERS

	Fold change
Muscle tissue development-related growth factors	
Myostatin *(Mstn)*	2.76
Growth differentiation factor 10 *(Gdf10)*	2.44
Bone morphogenetic protein 8a *(Bmp8a)*	2.33
Bone morphogenetic protein 1 *(Bmp1)*	2.22
Peripheral nervous system development-related growth factors	
Artemin *(Artn)*	4.09
Nodal homolog *(Nodal)*	3.94
Left right determination factor 1 *(Lefty1)*	2.15
Neurotrophin 3 *(Ntf3)*	2.09
Angiogenesis-related growth factors	
Fibroblast growth factor 17 *(Fgf17)*	4.21
Fibroblast growth factor 1 *(Fgf1)*	2.90
Epiregulin *(Ereg)*	2.49
Apoptosis regulator-related growth factors	
Interleukin 4 *(Il4)*	3.00
Brain-derived neurotrophic factor *(Bdnf)*	2.62
Cell differentiation- and/or morphogenesis-related growth factors	
Fibroblast growth factor 8 *(Fgf8)*	6.56
Teratocarcinoma-derived growth factor *(Tdgf1)*	5.67
Fibroblast growth factor 3 *(Fgf3)*	5.63
Fibroblast growth factor 5 *(Fgf5)*	4.11
Fibroblast growth factor 4 *(Fgf4)*	3.75
Fibroblast growth factor 14 *(Fgf14)*	3.15
Fibroblast growth factor 10 *(Fgf10)*	2.44
Leptin *(Lep)*	2.27
Immunoresponse-related growth factors	
Interleukin 3 *(Il3)*	7.23
Interleukin 2 *(Il2)*	3.52
Ureteric bud development-related growth factors	
Anti-Mullerian hormone *(Amh)*	2.71

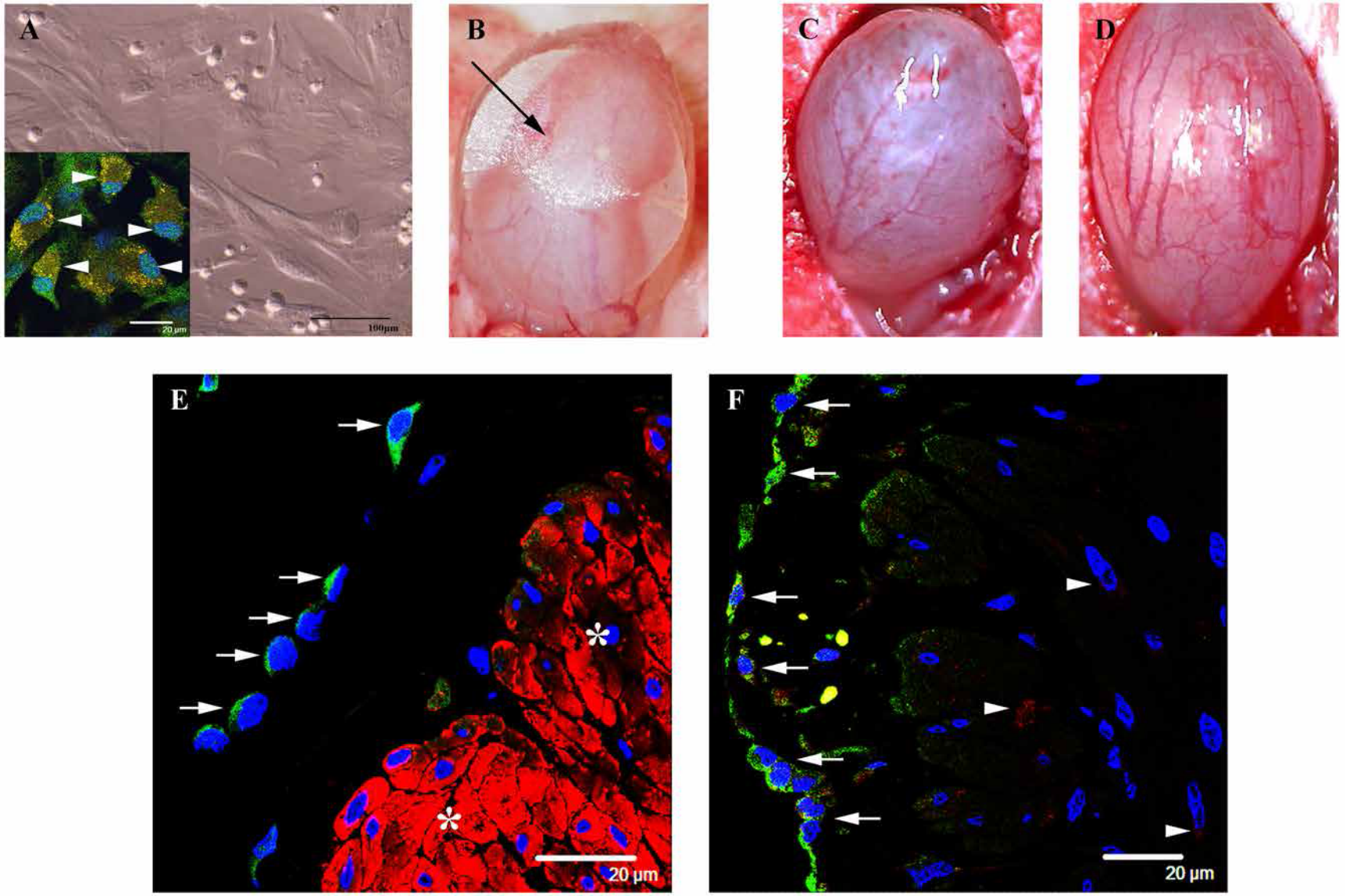

Figure 1

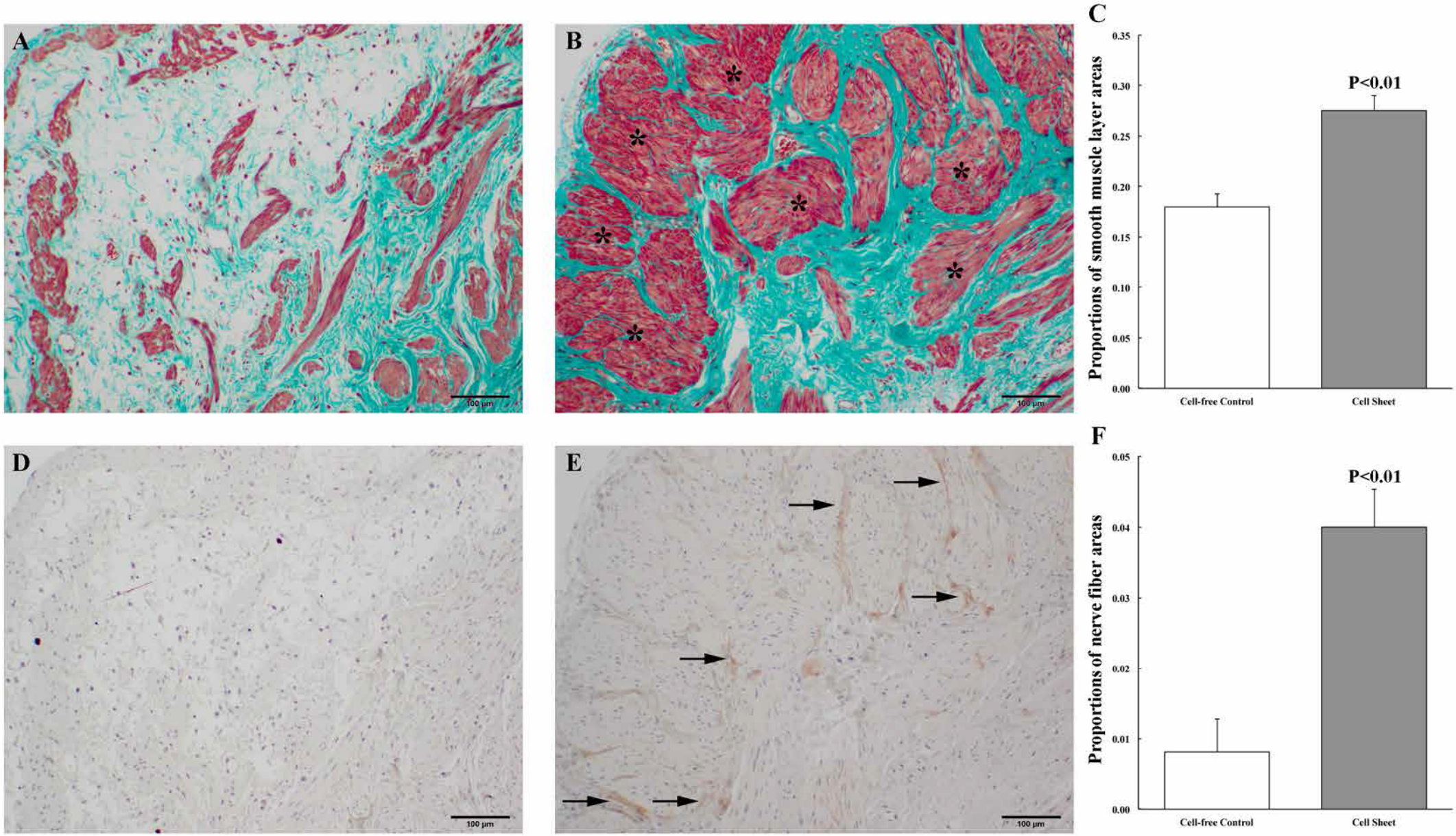
A
100 μm
B
100 μm
C
Proportions of smooth muscle layer areas
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
P<0.01
Cell-free Control
Cell Sheet
D
100 μm
E
100 μm
F
Proportions of nerve fiber areas
0.05
0.04
0.03
0.02
0.01
0.00
P<0.01
Cell-free Control
Cell Sheet

Figure 2

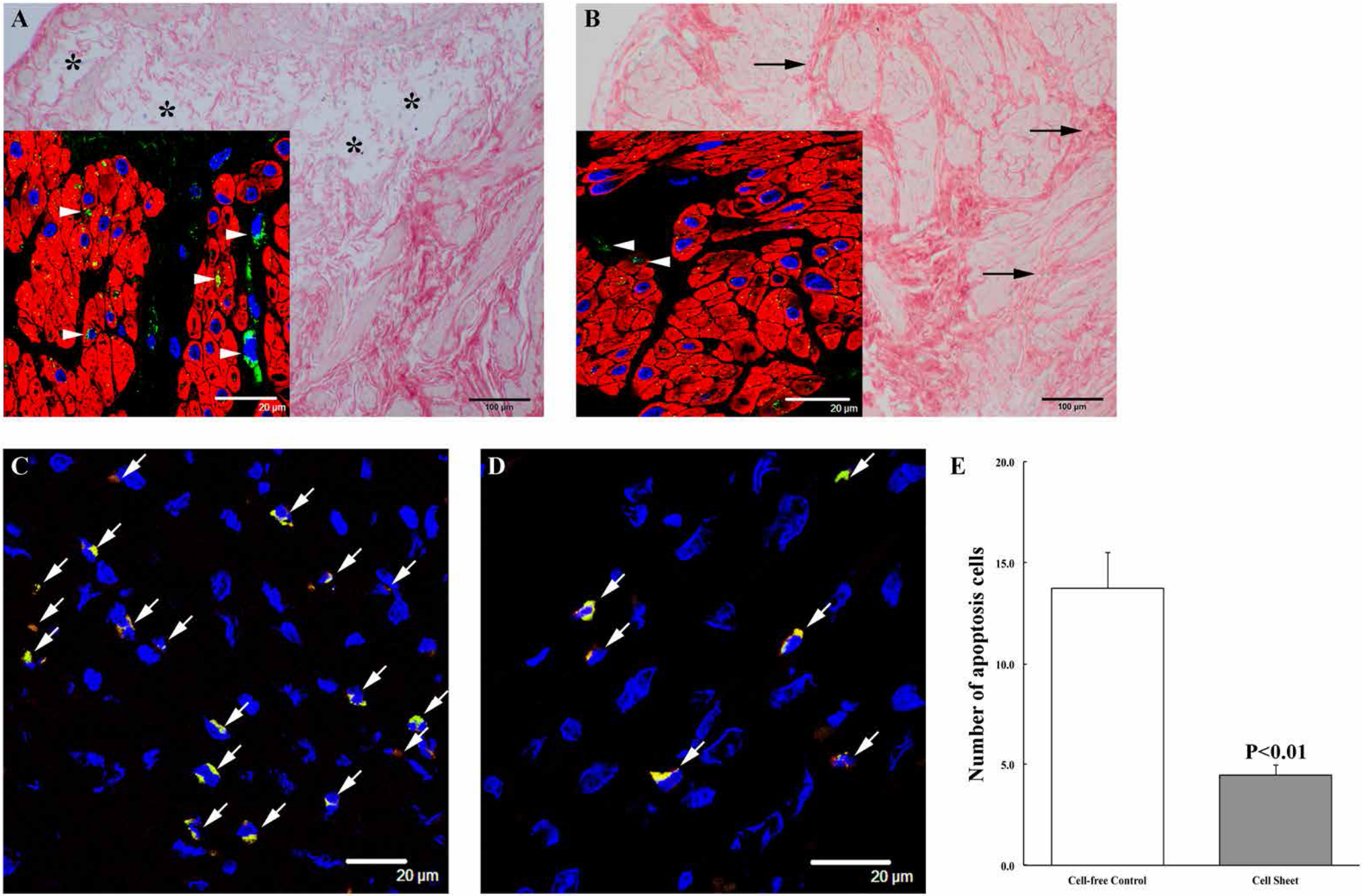
A
B
C
D
E
20 μm
100 μm
20 μm
100 μm
20 μm
20 μm
Number of apoptosis cells
20.0
15.0
10.0
5.0
0.0
P<0.01
Cell-free Control
Cell Sheet

Figure 3

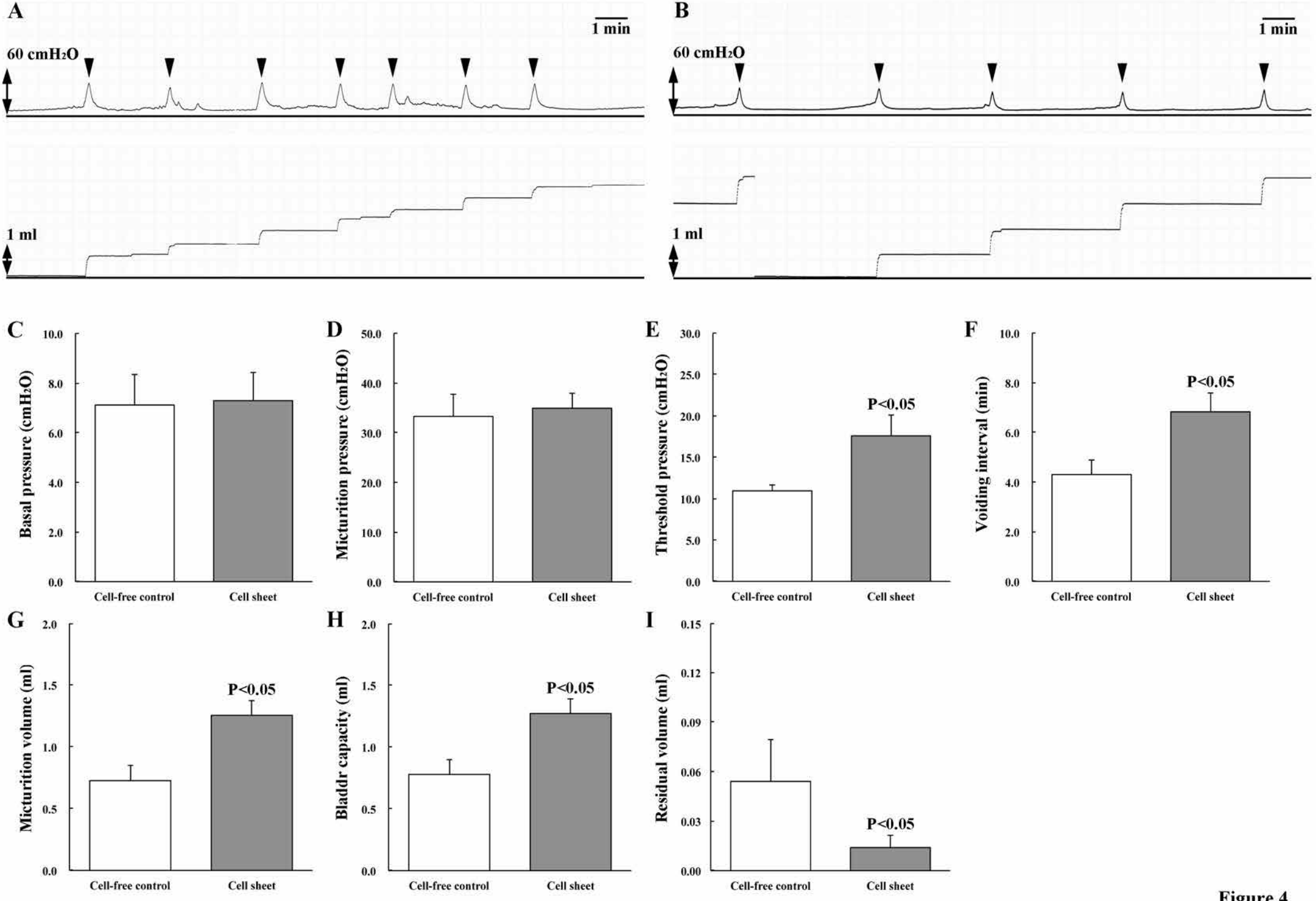
A
1 min
60 cmH2O
1 ml
B
1 min
60 cmH2O
1 ml
C
Basal pressure (cmH2O)
10.0
8.0
6.0
4.0
2.0
0.0
Cell-free control
Cell sheet
D
Micturition pressure (cmH2O)
50.0
40.0
30.0
20.0
10.0
0.0
Cell-free control
Cell sheet
E
Threshold pressure (cmH2O)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
P<0.05
Cell-free control
Cell sheet
F
Voiding interval (min)
10.0
8.0
6.0
4.0
2.0
0.0
P<0.05
Cell-free control
Cell sheet
G
Micturition volume (ml)
2.0
1.5
1.0
0.5
0.0
P<0.05
Cell-free control
Cell sheet
H
Bladdr capacity (ml)
2.0
1.5
1.0
0.5
0.0
P<0.05
Cell-free control
Cell sheet
I
Residual volume (ml)
0.15
0.12
0.09
0.06
0.03
0.00
P<0.05
Cell-free control
Cell sheet

Figure 4