1 **Recovery of cryo-injured rabbit urethras by biofabricated C-shaped adipose-**
2 **derived mesenchymal cell structures**

4 **Running Title:** Recovery of cryo-injured urethra

6 Sudha Silwal Gautam MBA., MSc.,¹ Tetsuya Imamura, Ph.D.,¹
7 Mitsuru Shimamura, Ph.D.,² Tomonori Minagawa M.D., Ph.D.,¹
8 Masaki Nakazawa M.D., Ph.D.,¹ Teruyuki Ogawa M.D., Ph.D.,¹
9 and Osamu Ishizuka M.D., Ph.D.¹

11 ¹Department of Urology, Shinshu University School of Medicine, Nagano, Japan

12 ²Cyfuse Biomedical K.K., Tokyo, Japan

16 Corresponding author: Tetsuya Imamura, Ph.D., Department of Urology, Shinshu
17 University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan;
18 telephone: +81-263-37-2661; fax: +81-263-37-3082; e-mail: imatetu@shinshu-
19 u.ac.jp

22 Sudha Silwal Gautam, MBA., MSc., Department of Urology, Shinshu University
23 School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan; telephone: +81-
24 263-37-2661; fax: +81-263-37-3082; e-mail: kanchu7@gmail.com

25

26 Tetsuya Imamura, Ph.D., Department of Urology, Shinshu University School of
27 Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan; telephone: +81-263-37-2661;
28 fax: +81-263-37-3082; e-mail: imatetu@shinshu-u.ac.jp

29

30 Mitsuru Shimamura, Ph.D., Cyfuse Biomedical K.K., University of Tokyo
31 Entrepreneur Plaza, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033, JAPAN;
32 telephone: +81-3-4455-7872; e-mail: mitsuru.shimamura@cyfusebm.com

33

34 Tomonori Minagawa M.D., Ph.D., Department of Urology, Shinshu University
35 School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan; telephone: +81-
36 263-37-2661; fax: +81-263-37-3082; e-mail: minagawat@shinshu-u.ac.jp

37

38 Masaki Nakazawa M.D., Ph.D., Department of Urology, Shinshu University
39 School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan; telephone: +81-
40 263-37-2661; fax: +81-263-37-3082; e-mail: mxnakazawa@shinshu-u.ac.jp

41

42 Teruyuki Ogawa M.D., Ph.D., Department of Urology, Shinshu University School
43 of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan; telephone: +81-263-37-
44 2661; fax: +81-263-37-3082; e-mail: ogawat@shinshu-u.ac.jp

45

46 Osamu Ishizuka M.D., Ph.D., Department of Urology, Shinshu University School
47 of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan; telephone: +81-263-37-
48 2661; fax: +81-263-37-3082; e-mail: ishizuk@shinshu-u.ac.jp

49

50 **Key words:** Adipose-derived stem cell, Biofabrication, Cryo-injury, Urethra,
51 Skeletal muscle, Smooth muscle

52 **Summary Statement**

53

54 Three-dimensional bioprinter was used to biofabricate novel C-shaped
55 structures composed of adipose-derived mesenchymal cells (AMCs) and have the
56 potential to be an effective treatment for urethral recovery.

57 **Abstract**

58 Urethral tissue damage can cause stress urinary incontinence or urinary
 59 retention, and there are few long-term, effective treatments. For structural and
 60 functional recovery of urethral tissues, we designed and constructed C-shaped
 61 structures composed of adipose-derived mesenchymal cells (AMCs) by using a
 62 three-dimensional (3D)-bioprinter system, which could be transplanted in damaged
 63 urethras without obstructing the lumen. We determined if transplantation of the
 64 biofabricated structures could reconstruct the urethral tissues. AMCs were
 65 harvested from rabbits, cultured and labeled with PKH26 to form spheroids. The
 66 spheroids were assembled on a custom-designed C-shaped support by a 3D
 67 bioprinter. Urethras of rabbits were cryo-injured by spraying with liquid nitrogen
 68 for 20 seconds, incised and biofabricated structure was autologously transplanted.
 69 Control rabbits were treated similarly but without transplantation structure. Two
 70 and four weeks after surgery, the control urethras were partially constricted;
 71 however, the structure-transplanted urethras were patent. The AMCs within the
 72 structures differentiated into skeletal muscle, smooth muscle, nerve, or endothelial
 73 cells. Some cells contained growth factors and cytokines. Therefore, biofabricated
 74 C-shaped AMC structures have potential to be an effective treatment for urethral
 75 recovery.

76 **Introduction**

77 Patients with urethral tissue damage due to aging often complain about stress
78 urinary incontinence (SUI), which is one manifestation of urine storage
79 dysfunction. Also, urethral injury or post-operation stricture can induce urinary
80 retention, which is a voiding dysfunction. Long-term, safe, and effective
81 treatments for the urethral tissue damage have not been developed. Recently,
82 mesenchymal stem cell-based therapies have been attempted as treatments for SUI.
83 In our previous study, we showed that injection of autologous adipose- (Silwal
84 Gautam et al., 2014) or bone marrow-derived mesenchymal cells (Imamura et al.,
85 2011) into the cryo-injured urethra of rabbits reconstructed the functional urethra.
86 Clinical research suggests that injection of autologous adipose-derived cells into
87 the urethra of male patients with SUI has the potential to provide significant
88 benefits (Aragon et al., 2018; Yamamoto et al., 2012). Thus, autologous cells
89 including somatic mesenchymal stem cells, such as adipose- or bone marrow-
90 derived mesenchymal stem cells have the potential to repair injury-damaged
91 urethras and thus warrant further study.

92 However, there are some limitations to the cell injection methods. The cells
93 can be damaged during the harvesting and handling procedures. The most serious
94 limitation is the low survival rate of the injected cells in the recipient tissues.
95 Previously, we reported that biofabricated structures improve cell survival rates
96 during harvesting and handling and in the recipient tissues (Imamura et al., 2018;
97 Moldovan et al., 2017). In this study, we designed novel biofabricated structures
98 composed of adipose-derived mesenchymal cells (AMCs) and constructed them
99 with a three-dimensional (3D)-bioprinter. After construction, the structures were
100 inserted into the injured urethras of rabbits where they promoted healing of the
101 damaged tissues. By design, the structures were C-shaped to avoid blocking the
102 lumen of the urethra and potentially impeding the flow of urine. Here we report the
103 biofabrication methodology, the transplantation of the C-shaped structures into the
104 damaged urethras, and the reconstruction of the damaged urethral tissues through
105 cellular proliferation and differentiation. We also report evidence of paracrine
106 stimulation through the presence of growth factors and cytokines within
107 transplanted structure and adjacent tissues.

108 **Results**

109 *Characterization of AMCs within biofabricated C-shaped structures*

110 Before urethral transplantation, the PKH26-labeled AMCs within the
111 biofabricated structures (Fig. 2A) were positive for mesenchymal cell marker
112 STRO-1 antibody (Fig. 2B, C). They also expressed integrin β 1 (Fig. 2D-F) and N-
113 cadherin (Fig. 2G-I) in the structures. However, other markers, such as those for
114 skeletal muscle (myoglobin), smooth muscle (smooth muscle actin), nerve (S100),
115 and vascular endothelial cell (vWF), were not present.

116

117 *Recovery of urethral tissues after transplantation of biofabricated C-shaped* 118 *structures*

119 Two weeks after the sham operation, the cryo-injured and incised regions of
120 the sham control urethras had developed strictures (Fig. 3A). Furthermore, in the
121 sham-operated regions, there were skeletal and/or smooth muscle cells within the
122 urethral tissues, but they were sparsely distributed and disorganized (Fig. 3B). In
123 contrast, at two weeks after surgery, the urethras transplanted with a C-shaped
124 biofabricated structure were wider and straight compared to the controls (Fig. 3C).
125 The urethral tissues in the biofabricated structure-transplanted regions also had
126 skeletal and smooth muscle cells (Fig. 3D).

127 At four weeks after the sham operation, the control rabbits continued to have
128 urethral strictures that were similar to those seen at two weeks after surgery (Fig.
129 3E). While the controls had some skeletal and smooth muscle cells, they were not
130 organized into muscle layers (Fig. 3F). However, the biofabricated structure-
131 transplanted urethras, at four weeks after surgery, did not have urethral strictures
132 or other obstructions (Fig. 3G). Similar to the findings at two weeks, there were
133 numerous skeletal and smooth muscle cells, but these were now organized into
134 layered muscle-like structures (Fig. 3H).

135

136 *Survival and differentiation of AMCs within the biofabricated structures after* 137 *transplantation*

138 At both two and four weeks after transplantation, PKH26, the red fluorescent
139 dye embedded in the membranes of the AMCs before spheroid formation, was
140 present in AMCs of the transplanted structures. At four weeks after transplantation,
141 some of the PKH26-labeled cells were positive for antibodies to the skeletal

muscle marker myoglobin (Fig. 4A-C) or the smooth muscle marker SMA (Fig. 4D-F). Also, some PKH26-labeled cells were positive for antibodies to the immature skeletal muscle cell marker desmin (Fig. 4G-I) or the myoblast progenitor cell marker pax7 (Fig. 4J-L). Moreover, some of the PKH26-labeled cells migrated from the transplanted structures into the surrounding urethral regions (data not shown).

Some of PKH26-labeled AMCs were positive for the nerve cell marker S100 (Fig. 5A-C) or the afferent nerve marker CGRP (Fig. 5D-F). In addition, other PKH26-labeled AMCs were positive for the vascular endothelial cell marker vWF (Fig. 5G-I) at both two and four weeks after transplantation. The vWF positive cells with PKH26 were integrated within the blood vessel-like structures within the transplanted biofabricated structure (Fig. 5I).

AMCs growth factors and cytokines within the biofabricated structures after transplantation

At both two and four weeks after the sham operation, some cells in the control regions contained VEGF (Fig. 6A). Similarly, some cells in the transplantation regions were also positive for VEGF (Fig. 6B). In addition, the cells labeled with PKH26, indicating that they were derived from the AMCs, also contained VEGF (Fig. 6B). Another growth factor, NGF, was also detected within some cells in the both control (Fig. 6C) and transplantation (Fig. 6D) regions. The control and transplanted regions also contained the cytokines TGF- β 1 (Fig. 6E, F) and TNF- α (Fig. 6G, H). In the transplanted regions, some PKH26-labeled AMCs were also positive for NGF (Fig. 6D), TGF- β 1 (Fig. 6F), and TNF- α (Fig. 6H).

Histological changes in biofabricated structure-transplanted urethral tissues

We observed cryo-injured urethral tissues at the borders of the wound in sham-operated regions and between the biofabricated AMC structures and the recipient tissues in the transplanted regions. At two and four weeks after sham surgery in which no biofabricated structure was transplanted, the urethral wound site contained collagen fibers in the extracellular matrix along with damaged skeletal muscle and smooth muscle layers (Fig. 7A). In contrast, in urethras containing the transplanted biofabricated structures, the collagen fibers were located between the recovering skeletal and smooth muscle layers (Fig. 7B). The

175 control urethras had numerous fibroblast P4HB-positive cells, an indicator of
 176 hypoxia, within the collagen fibers (Fig. 7C) compared to the structure-
 177 transplanted urethras (Fig. 7D). The control urethras also had numerous HIF1 α -
 178 positive cells, another indicator of hypoxia (Fig. 7E), and apoptotic cells (Fig. 7G).
 179 In contrast, the structure-transplanted urethra had few HIF1 α -positive or apoptotic
 180 cells (Fig. 7F, H).
 181

182 **Discussion**

183 Biofabrication has emerged as one of the novel technologies for constructing
184 tissues and organs in tissue engineering and regenerative medicine. The process
185 holds promise for diseases and injuries that are currently difficult to treat due to
186 lack of suitable therapies. In this study, we used a 3D-bioprinter to biofabricate
187 structures from AMC-derived spheroids (Imamura et al., 2018; Moldovan et al.,
188 2017). The structures were of a suitable size, shape, and autologous cellular
189 composition to be transplanted into the cryo-injured urethras of rabbits. Thus,
190 biofabricated structures have the potential to overcome limitations of the cell-
191 injection methods. Additionally, we designed the biofabrications as C-shaped
192 structures that would prevent urinary retention after transplantation. To our
193 knowledge, the C-shaped structures are a unique and novel construct for a
194 biofabricated structure and have not been reported.

195 AMCs that were attached and proliferating on the collagen-coated dishes were
196 used to make the biofabricated structures. The cells were positive for the
197 mesenchymal cell marker STRO-1, but they did not exhibit differentiation markers
198 typical of muscle, nerve, and vascular cells that are present in the normal urethra.
199 After the C-shaped structures were formed, the AMCs expressed integrin and/or
200 cadherin, which are the cell-cell adhesion molecules. Cell-cell interactions play an
201 important role in cell differentiation, and indeed, two and four weeks after
202 transplantation, the biofabricated structures expressed markers typically found in
203 normal urethras. Thus, the AMCs within the transplanted urethral biofabricated
204 structures underwent differentiation into myoglobin-positive skeletal muscle cells,
205 SMA-positive smooth muscle cells, S100- and CGRP-positive nerve cells, and
206 vWF-positive vascular endothelial cells. These observations suggest that cell-cell
207 interactions within the biofabricated structures might be a possible mechanism to
208 enhance differentiation capacity after transplantation.

209 In addition to undergoing differentiation to form the highly specialized cells
210 and tissues typical of the normal urethra, the AMCs in the transplanted tissue
211 produced important signal molecules in the forms of growth factors and cytokines
212 that are probably essential for cellular differentiation to occur. For instance, VEGF,
213 which promotes myogenesis, muscle regeneration, and vascularization, (Frey et al.,
214 2012; Levenberg et al., 2005) was present, as was NGF, which could promote
215 reconstruction of nerve cells and/or formation of layered muscle structures

(Lavasani et al., 2006). In addition, there might be direct or indirect roles for TGF- β 1 and TNF- α that were present within the cells of the injured regions during the AMC-mediated reconstruction of the urethral muscle cells, blood vessels and nerve cells. The cytokine TGF- β is important in muscle regeneration in the absence of TNF- α , (Wynn and Vannella, 2016) while TNF- α alone promotes proliferation and fusion of myoblasts (Ji et al., 1998). However, in our study the transplanted cells contained both cytokines, suggesting the presence of a strong paracrine effect in which the two cytokines could act synergistically. Moreover, some cells that were originally surrounding the transplanted regions also produce growth factor and cytokines. Rapid conversion of the proinflammatory TNF- α -producing macrophages to an anti-inflammatory TGF- β -producing phenotype may be critical to the long-term survival of stem and progenitor cell populations in most tissues (Freytes et al., 2013; Heredia et al., 2013; Lemos et al., 2015). Furthermore, some AMCs from the transplanted biofabricated structure migrated from the transplanted regions into the tissues surrounding the transplanted areas and helped in reconstruction of the injured areas (data not shown).

Cells derived from AMCs also expressed desmin or pax7 at two and four weeks after transplantation. Desmin is a muscle-specific intermediate filament protein present in early myogenesis and is essential for the structural integrity and function of muscle (Paulin and Li, 2004). Pax7-positive cells might also play roles in regeneration and integration of muscle cells within the reconstructed muscle layers.

Urethras transplanted with the C-shaped AMC-biofabricated structures did not develop strictures that could impede the voiding of urine. Within the sham-operated control urethras, collagen fibers invaded the spaces of the damaged skeletal muscle and smooth muscle layers. There were also numerous P4HB- and HIF1 α -positive cells, indicating a significant degree of hypoxia in the damaged region. In contrast, in the transplanted urethras, the collagen fibers were located between the recovering skeletal and smooth muscle layers, and there were few P4HB- and HIF1 α -positive cells present. In addition, the structure-transplanted urethras had fewer apoptotic cells compared to the sham-operated urethras. We did not quantify the number of P4HB- and HIF1 α -positive cells or, for that matter, the number of skeletal and smooth muscle cells or other cell types in the sham-operated or the AMC-transplanted urethras. However, the absence of structural

250 recovery in the sham-operated urethras and the presence of structural recovery in
251 the transplanted urethras was verified by histological and immunohistological
252 investigations. Therefore, we hypothesize that tissue recovery mediated by the
253 AMC-transplants inhibits the formation of urethral strictures.

254 We were unable to measure urethral physiological functions in this study. The
255 most readily measured function is the leak point pressure (LPP) at which the
256 bladder pressure forces urine to leak through the urethra. However, in the sham-
257 operated controls, the presence of strictures narrowed the urethras, thus artificially
258 raising the LPP, especially in comparison to the transplanted urethras that did not
259 develop strictures. Thus, comparison of LPPs for the control and transplanted
260 animals was not valid. Nevertheless, recovery of the cryo-injured urethras by
261 transplantation of the biofabricated C-shaped AMC structures was strongly
262 supported by the gross anatomy and microanatomy observations.

263 In conclusion, we used 3-D printing to manufacture novel biofabricated C-
264 shaped structures composed of AMCs. The AMCs within the structures
265 transplanted into the cryo-injured rabbit urethras survived and promoted recovery
266 through differentiation of muscle, nerve, and vascular endothelial cells. In addition,
267 these cells contained growth factors and/or cytokines that likely enhanced
268 myogenesis, neurogenesis, and neoangiogenesis. The development of urethral
269 strictures during the recovery process was inhibited by the biofabricated AMC-
270 transplants. Therefore, transplantation of biofabricated structures derived from
271 AMCs has the potential to provide novel and effective treatments for urine storage
272 or voiding dysfunctions associated with damaged urethras.

273 **Materials and Methods**

274 *Animals*

275 Eight female New Zealand White rabbits (2.5-3.0 kg; Japan SLC, Inc.,
276 Shizuoka, Japan) at postnatal week 10 were used in this study. The rabbits were
277 treated in accord with the Guide for the Care and Use of Laboratory Animals
278 published by the US National Institutes of Health. The Animal Ethics Committee
279 of Shinshu University School of Medicine approved the protocol used in this study.
280

281 *Preparation of AMCs and spheroids for biofabrication of C-shaped structures*

282 Rabbits were anesthetized by intramuscular injection of midazolam (1.5
283 mg/kg; Astellas, Tokyo, Japan) and medetomidine hydrochloride (0.5 mg/kg;
284 Zenoaq, Orion Pharma, Orion Corporation Espoo, Finland) and further
285 anesthetized by inhalation of 3% sevoflurane (Abbot Japan Co., LTD., Tokyo,
286 Japan). Previously, we established methods of isolation and harvesting autologous
287 adipose-derived cells (Silwal Gautam et al., 2014). Briefly, intraperitoneal adipose
288 tissue (approximately 1 g) was harvested from an abdominal incision. The adipose
289 tissue was digested with 0.2% collagenase type I (Wako Pure Chemical Industries,
290 Ltd., Osaka, Japan) for 90 minutes at 37° C with moderate rotary shaking. After
291 shaking, the solution was centrifuged at 1,000 rpm for 4 minutes, and then the cells
292 were suspended in culture medium composed of 4.5 g/L glucose-Dulbecco's
293 modified Eagle's medium (Gibco, Thermo Fisher Scientific K.K., Kanagawa,
294 Japan) supplemented with 15% regular fetal bovine serum (Biowest, Nuaille,
295 France) and 0.1% penicillin-streptomycin (Gibco). After passing through a cell
296 strainer (Life Science, Corning, NY, USA), primary cultures of the suspended cells
297 were established on type I collagen-coated 10-cm culture dishes (AGC Techo
298 Glass, Shizuoka, Japan) and incubated at 37°C in humid air with 5% CO₂ for 5-7
299 days. The medium was completely changed every day to wash off unattached cells.
300 After reaching confluence, the attached and proliferating cells were sub-cultured to
301 increase the number of cells in a Collagen Type I-coated T 225 cm² Flask (AGC
302 Techno Glass).

303 To track the attached and proliferating cells in passages 2 or 3, a lipophilic red
304 fluorescent dye that binds to cell membranes, PKH26 (Sigma Aldrich, St. Louis,
305 MO, USA), was applied according to the manufacturer's protocol. The labeled
306 cells were re-suspended with Procul AD[®] that was designed for human adipose

mesenchymal stem cells (Rohto Pharmaceuticals, Osaka, Japan). To form cell spheroids, 100- μ l volumes of 4×10^4 cells/well were seeded in ultra-low attachment, U-shaped, 96-well plates (3-5 plates for each rabbit, Sumitomo Bakelite, Tokyo, Japan) and allowed to form cell aggregates at 37°C in 5% CO₂ for 5 days.

Biofabrication of C-shaped AMC structures

Biofabrication of the AMC structures was performed on a 3D-bioprinter (Regenova, Cyfuse Biomedical K.K., Tokyo, Japan) designed to create 3D-structures consisting only of cells (Imamura et al., 2018; Moldovan et al., 2017). Briefly, single spheroids, which were composed of the PKH26-labeled AMCs, were recovered from each well of the 96-well plates, and mounted on a custom designed, C-shaped 9x9 microneedle array. The formation of each biofabricated structure required 234 spheroids. The mounted spheroids, which were held in place by the microneedle array, were cultured with circulating Procul AD[®] and allowed to form spheroid-spheroid contacts at 37°C in 5% CO₂ for 7 days. On the seventh day of culture, the microneedle array was removed from the assembled biofabricated C-shaped structure (Fig. 1A).

Production of cryo-injured urethral tissues and transplantation of the biofabricated C-shaped AMC structures

The rabbits from which the adipose tissue was harvested were anesthetized as above. A urethral catheter (Fr. 8, Terumo, Yokohama, Japan) was inserted, and then the urethra was exposed through a midline lower abdominal incision. The anterior wall of the urethra was sprayed with liquid nitrogen (Cryo Pro[®], Cortex Technology, Hudsund, Denmark) for 20 s (Fig. 1B). The frozen region naturally thawed within 2-3 minutes by body temperature and/or room temperature. In this study, we define the frozen and thawed area as the cryo-injured regions. The cryo-injured regions were incised until the urethral catheter was visualized (Fig. 1C). After incision, the autologous biofabricated C-shaped AMC structure was placed around the urethral catheter (Fig. 1D). The wounded edges of the urethral tissue were sewed to the inserted C-shaped structure to secure them together and prevent the structure from falling freely into the urethral lumen or from falling out of the lumen. Finally, the abdominal incision was closed, and the urethral catheter removed. Four other rabbits served as sham-operated controls in which a urethral

catheter was inserted, and the urethra was exposed and cryo-injured. The injured region was incised until the urethral catheter was visualized, and then the wounded edges were sewn together without the insertion of a C-shaped structure. At two and four weeks after surgery, the urethras were harvested, and the rabbits were sacrificed with the injection of an overdose of pentobarbital sodium solution (Kyoritsu Seiyaku Co., Tokyo, Japan).

Histological and immunofluorescence evaluation

At two and four weeks after transplantation or sham operation, the harvested urethras were fixed in 4% paraformaldehyde for 12 hours. Then, the fixed samples were embedded in paraffin and cut in 5- μ m thick serial sections. The sections were deparaffinized with xylene, rehydrated with ethanol, and rinsed three times with phosphate-buffered saline. Each section was stained with Masson trichrome, picrosirious red, or ApopTag[®] ISOL fluorescence apoptosis detection kit (Merck Millipore, Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol.

For immunohistochemistry, some of the deparaffinized sections were immersed in 10 mM sodium citrate (pH 6.0) and microwaved at 100°C for 10 min for antigen retrieval. The sections were blocked with 1.5% non-fat milk in phosphate-buffered saline for 1 h at 4°C.

To detect differentiated cells derived from the AMCs within the biofabricated structures, each section was incubated with one of the following antibodies for 12 h at 4°C to identify the following cellular markers: myoglobin (1:200, rabbit polyclonal; Spring Bioscience, Inc., Pleasanton, CA, USA), smooth muscle actin (SMA, 1:100 mouse monoclonal; Progen Biotechnik GmbH, Heidelberg, Germany), desmin (1:200 rabbit polyclonal; Progen Biotechnik GmbH), pax 7 (1:1000, rabbit; Lifespan Biosciences, Inc., Seattle, WA, USA), S100 (1:100, mouse monoclonal; Abcam, Cambridge, United Kingdom), calcitonin gene-related peptide (CRGP, 1:800, guinea pig polyclonal; Progen Biotechnik GmbH), or vascular endothelial cell marker von Willebrand factor (vWF, 1:100 rabbit polyclonal; Abcam).

To detect the presence of growth factors and cytokines in the AMCs of the biofabricated structures, the sections were incubated for 12 h at 4°C with antibodies for each of the following markers: vascular endothelial growth factor

375 (VEGF, 1:100, rabbit polyclonal; Bioss, Woburn, MA, USA), nerve growth factor
376 (NGF, 1:500, rabbit polyclonal; Abcam), transforming growth factor- β 1 (TGF- β 1,
377 1:100, rabbit polyclonal; Abcam), and tumor necrosis factor- α (TNF- α , 1:100,
378 rabbit polyclonal; Abcam). Other sections were similarly incubated antibodies for
379 P4HB (1:50: mouse monoclonal, Novus Biologicals, Inc.) or HIF1 α (1: 50, rabbit
380 polyclonal, Proteintech Group, Inc., Rosemont, IL, USA) for 12 h at 4°C.

381 After the primary antibody reactions, the sections were incubated with the
382 respective secondary antibodies consisting of donkey anti-rabbit, -mouse,
383 or -guinea pig, each conjugated with Alexa Fluor 488 (1:250; Molecular Probes,
384 Eugene, OR, USA) for 1 h at 4°C. After rinsing, they were counterstained with 5
385 μ g/ml 4', 6-diamidino-phenylindole dihydrochloride (DAPI; Molecular Probes).

386 Biofabricated structures that were not used in a transplantation operation
387 were immunohistochemically stained as described above with antibody for
388 mesenchymal cell marker; STRO-1 (1:100, animal poly- or mono-, R&D System,
389 Inc., Minneapolis, MN, USA) and the extracellular markers anti-integrin β 1 (1:50,
390 rat monoclonal; R&D Systems, Inc.) and N-cadherin (1:100, rabbit polyclonal;
391 Bioss).

392 **Acknowledgements**

393

394 This study was supported by a Grant-in-Aid for Scientific Research (C) from Japan
395 Society for the Promotion of Science (16K11043).

396

397 **Competing interests**

398

399 No competing financial interests exist.

400

401 **Funding**

402

403 None

404

405 **Data availability**

406

407 None

References

- Aragon, I. M., Imbroda, B. H. and Lara, M. F.** (2018). Cell Therapy Clinical Trials for Stress Urinary Incontinence: Current Status and Perspectives. *Int J Med Sci* **15**, 195-204.
- Frey, S. P., Jansen, H., Raschke, M. J., Meffert, R. H. and Ochman, S.** (2012). VEGF improves skeletal muscle regeneration after acute trauma and reconstruction of the limb in a rabbit model. *Clin Orthop Relat Res* **470**, 3607-3614.
- Freytes, D. O., Kang, J. W., Marcos-Campos, I. and Vunjak-Novakovic, G.** (2013). Macrophages modulate the viability and growth of human mesenchymal stem cells. *J Cell Biochem* **114**, 220-229.
- Heredia, J. E., Mukundan, L., Chen, F. M., Mueller, A. A., Deo, R. C., Locksley, R. M., Rando, T. A. and Chawla, A.** (2013). Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration. *Cell* **153**, 376-388.
- Imamura, T., Ishizuka, O., Kinebuchi, Y., Kurizaki, Y., Nakayama, T., Ishikawa, M. and Nishizawa, O.** (2011). Implantation of autologous bone-marrow-derived cells reconstructs functional urethral sphincters in rabbits. *Tissue Eng Part A* **17**, 1069-1081.
- Imamura, T., Shimamura, M., Ogawa, T., Minagawa, T., Nagai, T., Silwal Gautam, S. and Ishizuka, O.** (2018). Biofabricated Structures Reconstruct Functional Urinary Bladders in Radiation-Injured Rat Bladders. *Tissue Eng Part A* **24**, 1574-1587.
- Ji, S. Q., Neustrom, S., Willis, G. M. and Spurlock, M. E.** (1998). Proinflammatory cytokines regulate myogenic cell proliferation and fusion but have no impact on myotube protein metabolism or stress protein expression. *J Interferon Cytokine Res* **18**, 879-888.
- Lavasani, M., Lu, A., Peng, H., Cummins, J. and Huard, J.** (2006). Nerve growth factor improves the muscle regeneration capacity of muscle stem cells in dystrophic muscle. *Hum Gene Ther* **17**, 180-192.
- Lemos, D. R., Babaeijandaghi, F., Low, M., Chang, C. K., Lee, S. T., Fiore, D., Zhang, R. H., Natarajan, A., Nedospasov, S. A. and Rossi, F. M.** (2015). Nilotinib reduces muscle fibrosis in chronic muscle injury by promoting TNF-mediated apoptosis of fibro/adipogenic progenitors. *Nat Med* **21**, 786-794.

442 **Levenberg, S., Rouwkema, J., Macdonald, M., Garfein, E. S., Kohane, D. S.,**
443 **Darland, D. C., Marini, R., van Blitterswijk, C. A., Mulligan, R. C., D'Amore,**
444 **P. A. et al.** (2005). Engineering vascularized skeletal muscle tissue. *Nat*
445 *Biotechnol* **23**, 879-884.

446 **Moldovan, N. I., Hibino, N. and Nakayama, K.** (2017). Principles of the Kenzan
447 Method for Robotic Cell Spheroid-Based Three-Dimensional Bioprinting<sup/>.
448 *Tissue Eng Part B Rev* **23**, 237-244.

449 **Paulin, D. and Li, Z.** (2004). Desmin: a major intermediate filament protein
450 essential for the structural integrity and function of muscle. *Exp Cell Res* **301**, 1-7.

451 **Silwal Gautam, S., Imamura, T., Ishizuka, O., Lei, Z., Yamagishi, T.,**
452 **Yokoyama, H., Minagawa, T., Ogawa, T., Kurizaki, Y., Kato, H. et al.** (2014).
453 Implantation of autologous adipose-derived cells reconstructs functional urethral
454 sphincters in rabbit cryoinjured urethra. *Tissue Eng Part A* **20**, 1971-1979.

455 **Wynn, T. A. and Vannella, K. M.** (2016). Macrophages in Tissue Repair,
456 Regeneration, and Fibrosis. *Immunity* **44**, 450-462.

457 **Yamamoto, T., Gotoh, M., Kato, M., Majima, T., Toriyama, K., Kamei, Y.,**
458 **Iwaguro, H., Matsukawa, Y. and Funahashi, Y.** (2012). Periurethral injection of
459 autologous adipose-derived regenerative cells for the treatment of male stress
460 urinary incontinence: Report of three initial cases. *Int J Urol* **19**, 652-659.

461

Figure Legends

FIGURE 1. Autologous C-shaped AMC structure, urethral cryo-injury, and transplantation into the injured urethra. **(A)** Biofabricated C-shaped AMC structure after removal from microneedle array. The structure was approximately 3.5 (wide)×3.5 (depth)×1.0 (high) mm, Scale bar: 1 mm. **(B)** The intact urethra was sprayed with liquid nitrogen for 20 second (arrowheads). **(C)** A small incision of approximately 5 mm was created around the cyroinjured regions (arrowheads). Asterisk, catheter in the urethral lumen. **(D)** Biofabricated C-shaped AMC structure (arrow) was implanted into the incised urethra.

FIGURE 2. Expression of mesenchymal and extracellular matrix markers of AMCs within biofabricated structures. **(A-C)** The PKH26-labeled AMCs within the biofabricated structures (A, red, arrowheads) were positive for the mesenchymal cell marker STRO-1 (B, green, arrowheads; C, merged image, arrowheads). **(D-F)** The PKH26-labeled AMCs (D, red, arrowheads) were positive for the extracellular marker integrin (E, green, arrowheads; F, merged image, arrowheads). **(G-I)** The PKH26-labeled AMCs (G, red, arrowheads) were positive for the extracellular marker cadherin (H, green, arrowheads; I, merged image, arrowheads). Blue: DAPI-labeled nuclei, Scale bar: 20 μm.

FIGURE 3. Urethral tissues transplanted with and without biofabricated structures at two and four weeks after surgery. **(A)** At two weeks after the sham operation, the gross appearance of the control urethra was narrowed and somewhat tortuous (arrowheads). **(B)** The skeletal muscle cells and/or smooth muscle cells within the control urethra were sparsely distributed and disorganized (asterisks). Scale bar: 100 μm. **(C)** At two weeks after transplantation of a AMC biofabricated structure, the gross appearance of the urethra was wider and straighter compared to the sham-operated control (arrows). **(D)** At two weeks, a urethra transplanted with a biofabricated structure had skeletal (arrows) and smooth muscle (arrowheads) cells. **(E)** At four weeks after sham surgery, the urethra had strictures that were similar to those at two weeks after surgery (arrowheads). **(F)** The walls of the sham-operated controls had some skeletal and smooth muscle cells, but they did not form typical muscle layers (asterisk). **(G)** At four weeks after surgery, the structure-transplanted

urethra had a wider lumen (arrows) that was straighter than in the controls. **(H)** At four weeks after structure transplantation, the urethra had numerous skeletal (arrows) and smooth muscle (arrowheads) cells that were organized into layered tissue structures. Scale bar: gross images (A, C, E, and G): 1 mm; Masson trichrome staining (B, D, F, and H): 100 μ m; UT, urothelium.

FIGURE 4. Differentiation of AMCs within the transplanted biofabricated structures into muscle marker-positive cells at four weeks after transplantation. **(A-C)** The PKH26-labeled cells (A, red, asterisks) were positive for the skeletal muscle marker myoglobin (B, green, arrowheads; C, merged image, asterisks). **(D-F)** The PKH26-labeled cells (D, red, asterisks) were positive for the smooth muscle marker SMA (E, green, asterisks; F, merged image, asterisks). **(G-I)** The PKH26-labeled cells (G, red, asterisks) were positive for the immature skeletal muscle cell marker desmin (H, green, asterisks; I, merged image, asterisks). **(J-L)** The PKH26-labeled cells (J, red, asterisk) were positive for the myoblast progenitor cell marker pax7, (K, green, asterisk; L, merged image, asterisks). Blue: DAPI-labeled nuclei, Scale bar: 20 μ m.

FIGURE 5. Differentiation of AMCs within transplanted biofabricated structures into nerve or vascular endothelial marker-positive cells at four weeks after transplantation. **(A-C)** The PKH26 labeled cells (A, red arrows) were positive for the nerve cell marker S100 (B, green, arrows; C, merged image, arrows). Scale bar: 15 μ m. **(D-F)** The PKH26 labeled cells (D, red arrows) were positive for the afferent nerve marker CGRP (E, green, arrows; F, merged image, arrows). Scale bar: 20 μ m. **(G-I)** The PKH26 labeled cells (G, red arrows) were positive for the vascular endothelial marker vWF (H, green, arrows; I, merged image, arrows). Blue: DAPI-labeled nuclei, Scale bar: 20 μ m.

FIGURE 6. Growth factors and cytokines within the sham-operated control and transplanted regions and in the PKH26-labeled AMCs at four weeks after surgery. The sham-operated control and transplanted regions were identified macroscopically by the presence of suture marks. **(A, B)** Within the control (A) and transplanted regions (B), some cells contained VEGF (green, arrows). In addition, the PKH26-labeled AMCs also contained VEGF (B, yellow, arrowheads).

530 (C-H) Similarly, some cells within the control (C, E, G) and transplanted regions
531 (D, F, H) contained NGF (C and D, green, arrows), TGF- β 1 (E and F, green,
532 arrows), and TNF- α (G and H, green, arrows). Some of the PKH26-labeled AMCs
533 in the transplanted regions contained NGF (D, yellow, arrowheads), TGF- β 1 (F,
534 yellow, arrowheads) and TNF- α (H, yellow, arrowheads). Blue: DAPI-labeled
535 nuclei, Scale bar: A, B: 20 μ m; C – H: 15 μ m.

536

537 **FIGURE 7.** Histological changes in sham-operated control urethral tissues and
538 biofabricated structure-transplanted tissues at four weeks after surgery. (A-B)
539 Within the control regions (A), the collagen fibers had invaded the spaces of the
540 damaged skeletal muscle or smooth muscle layers (asterisks). In contrast, in the
541 structure-transplanted regions (B), the collagen fibers were located between the
542 recovering skeletal and smooth muscle layers (arrowheads). Picrosirius red staining
543 for collagen. Scale bar: 100 μ m. (C-D) In the control urethra, some cells within the
544 collagen fibres were positive for P4HB antibody, an indicator of hypoxia (C, green,
545 arrows). However, the transplanted regions had few P4HB-positive cells (D, green
546 or yellow, arrows). Red: SMA-positive smooth muscle cells. (E-F) The control
547 regions had numerous HIF1 α -positive cells, another indicator of hypoxia (E, green,
548 arrows). In contrast, the transplanted regions had very few HIF1 α -positive cells. (F,
549 yellow, arrows). Red: SMA-positive smooth muscle cells. (G-H) The control
550 regions had numerous apoptotic cells (G, green, caspase-independent apoptotic
551 cells; red, caspase-dependent apoptotic cells; yellow, overlapping red-labeled
552 caspase-dependent and green-labeled caspase-independent apoptotic cells, arrows).
553 However, in the transplanted regions (H), there were very few apoptotic cells.
554 Unless stated otherwise, Blue: DAPI-labeled nuclei, Scale bar: 50 μ m.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7