Acellular Urethra Bioscaffold: Decellularization of Whole Urethras for Tissue Engineering Applications

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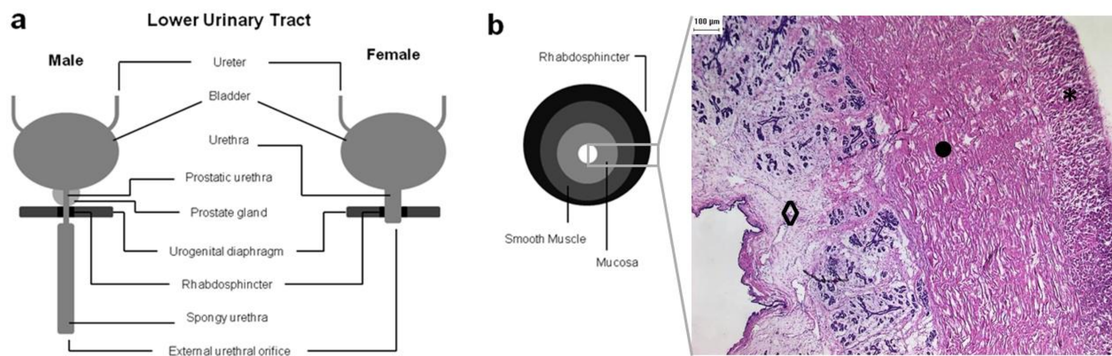
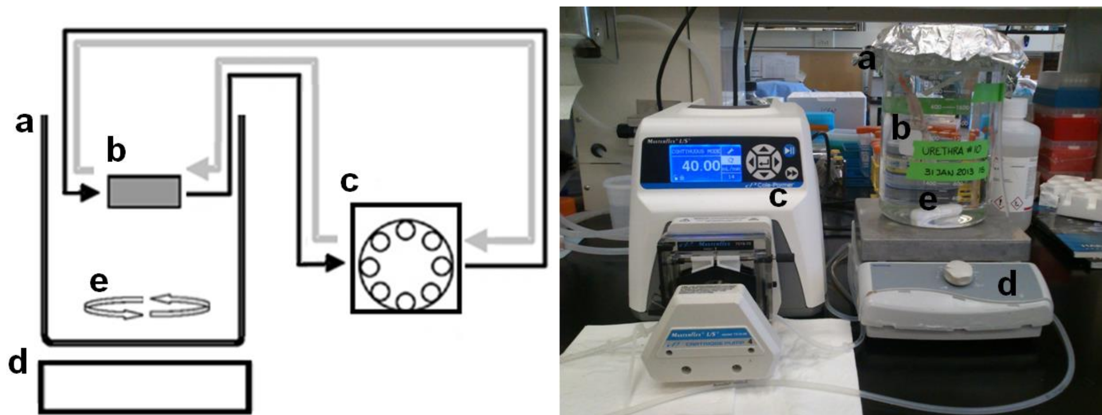
Supplementary Figures



Supplementary Figure S1. Urethra anatomy. (**a**) Schematic representation of the anatomical structure of male and female urethra (**b**) and cross-sectional organization of muscle (●, smooth and *, skeletal or RBS) and mucosal (◊) tissues in the urethra. Scale bar: 100µm. Image obtained through H&E staining.



Supplementary Figure S2. Decellularization apparatus based on mechanochemical action. Detergent solution (or distilled water) was placed inside the (**a**) vessel, where the (**b**) urethra was submerged. The solution was pumped by a (**c**) peristaltic pump and recircularized at a rate of 40 mL/min. Through a (**d**) magnetic stirrer plate the solution was (**e**) agitated at 60rpm. The process was repeated for 5 days. By the end of the first 48h, the direction of perfusion was changed (from the black arrows to the grey arrows) to allow a more efficient decellularization.

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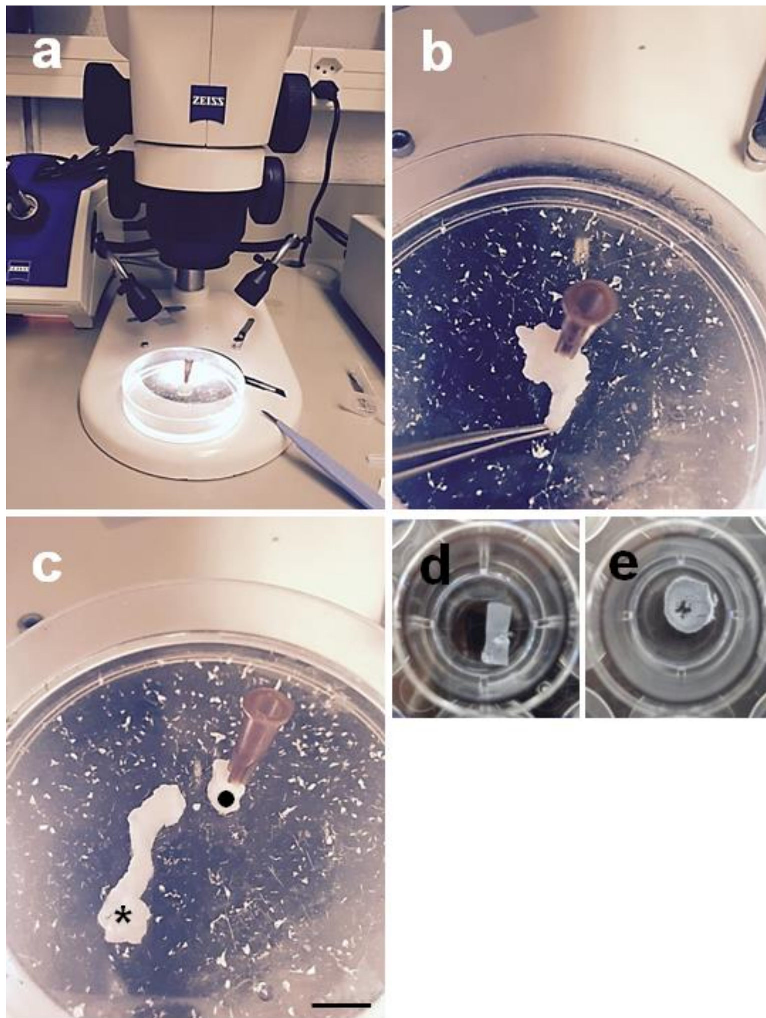


48 **Supplementary Figure S3. Microdissection of the urethra bioscaffolds to prepare**

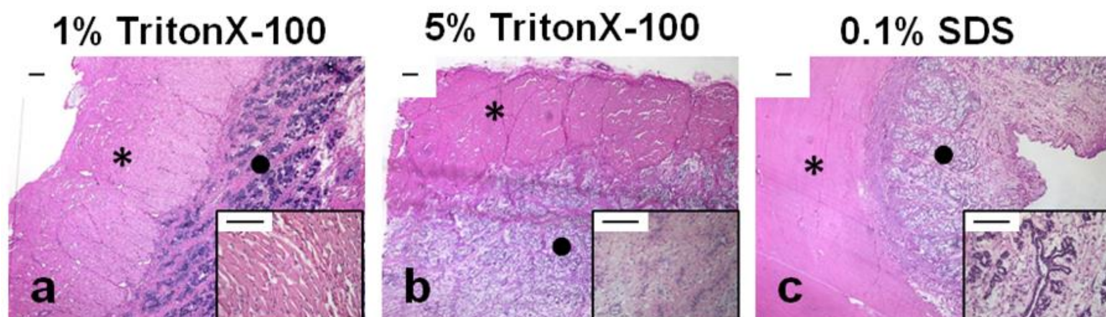
49 **sections with different muscle ECMs.** (**a**) Microdissection was performed under a stereo

50 microscope and the bioscaffold was pinned on a gelatin coated plate. (**b-c**) RBS (*) was

51 detached from the smooth muscle layer (●) and (**d**) skECM and (**e**) smECM sections,

52 respectively were produced. Scale bar: 1 cm.

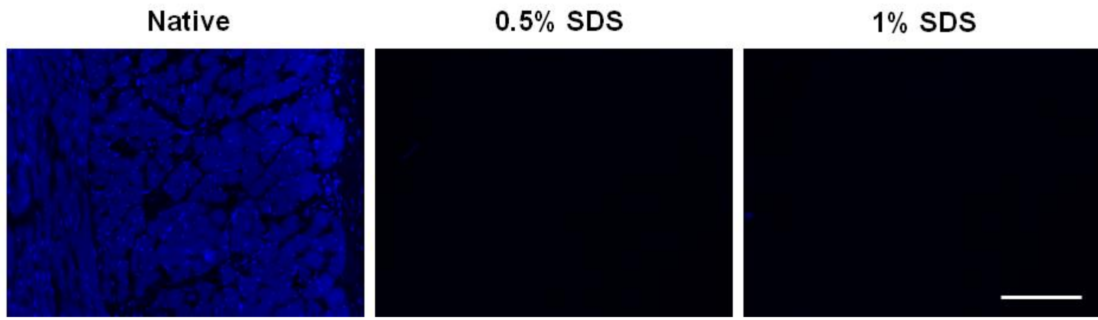
53



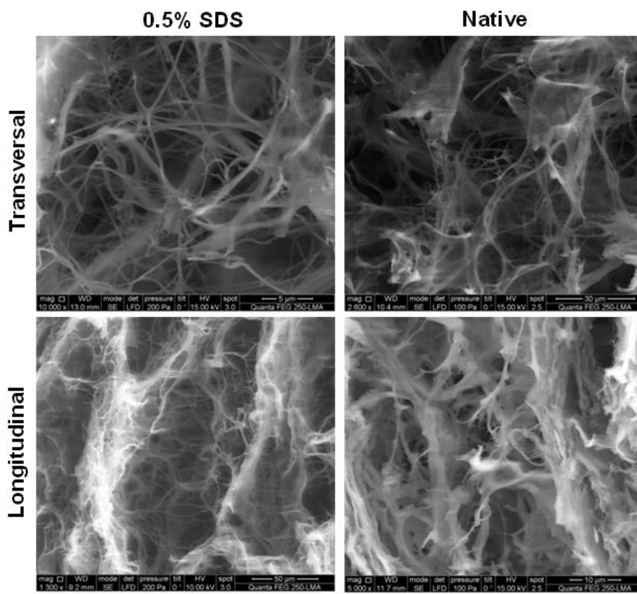
54 **Supplementary Figure S4. H&E staining of urethras exposed to decellularization.** (**a**) 1%

55 Triton X-100, (**b**) 5% Triton X-100 and (**c**) 0.1% SDS did not decellularize the urethras. Images

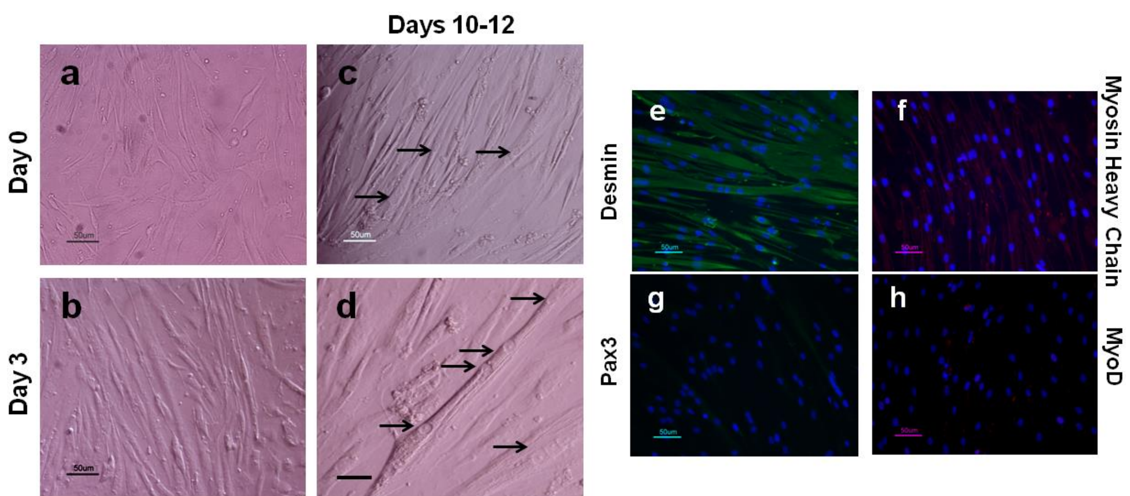
56 are from 5 µm thick sections (*, muscle and ●, mucosa). Scale bar: 100 µm.



Supplementary Figure S5. DAPI staining of decellularized urethra bioscaffolds. Images are centred on the RBS layer. Images are from 5 µm thick sections. Scale bar: 100 µm.



Supplementary Figure S6. SEM of decellularized urethra bioscaffolds. Longitudinal and transversal cuts of native urethra and 0.5% decellularized bioscaffold.

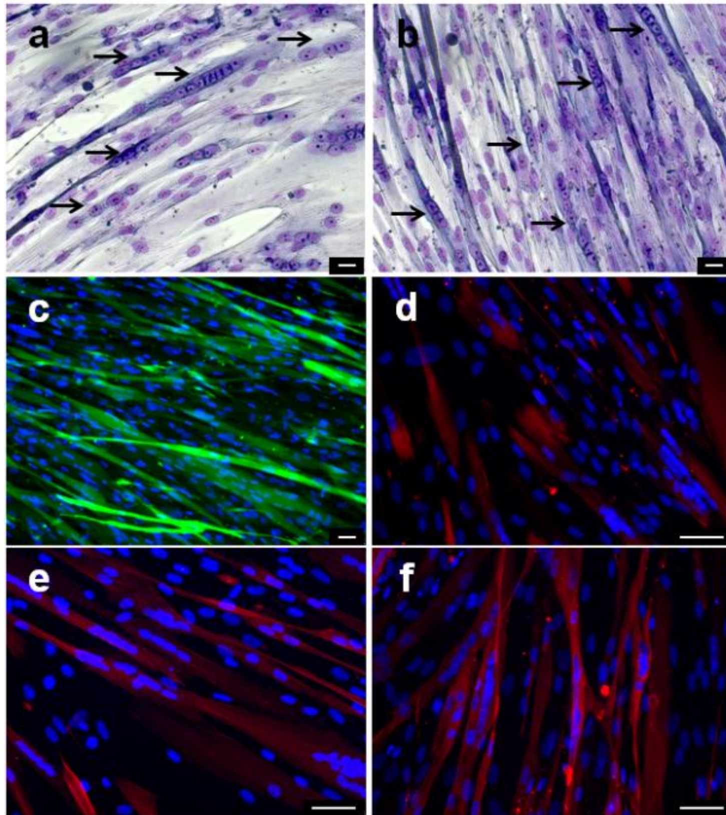


Supplementary Figure S7. MPCs (ZenBio®) fiber formation *in vitro*. Cells changed their morphology after exposure to the differentiation medium at (**a**) days 0 and (**b**) 3. Between (**c-d**)

66 days 10 and 12 cells fused and produce multinucleated fibers (black arrows). Fibers expressed

67 (**e**) desmin and (**f**) myosin heavy chain while showing no (**g**) Pax3 and (**h**) MyoD. Nuclei were

68 stained with DAPI (in blue). Scale bar: 50 µm.



69

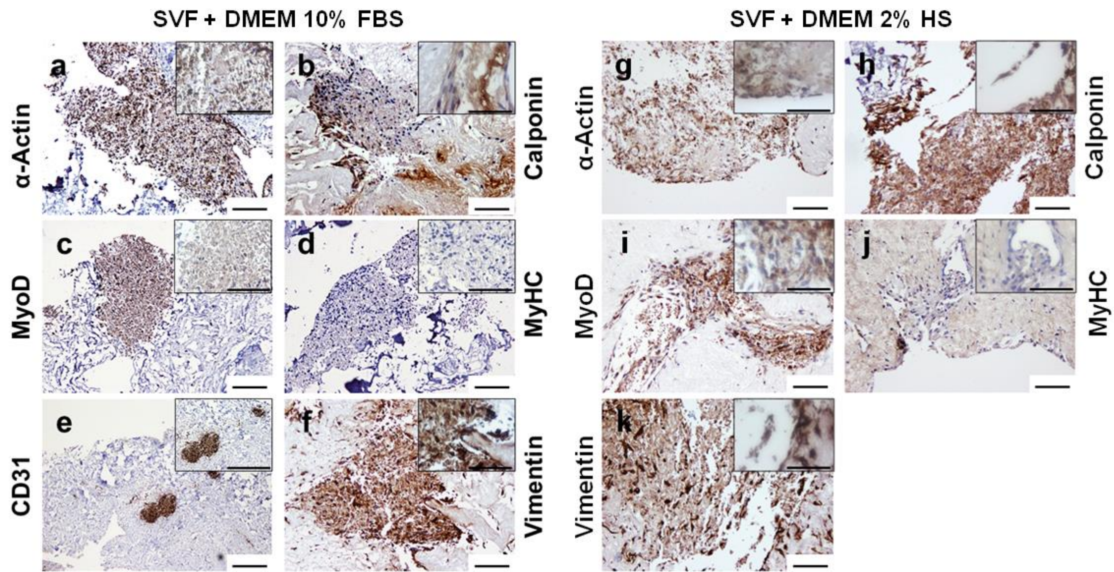
70 **Supplementary Figure S8. MPCs (isolated at USZ) *in vitro* fiber formation.** After (**a-b**) 14

71 days in culture, MPCs fused and produced multinucleated fibers (black arrows) while

72 demonstrating (**c**) viability through calcein AM staining and the expression of (**d**) desmin, (**e**) α-

73 actinin and (**f**) MyHC (all in red) through immunofluorescence staining. Nuclei were stained with

74 DAPI (in blue). Scale bar: 50 µm.



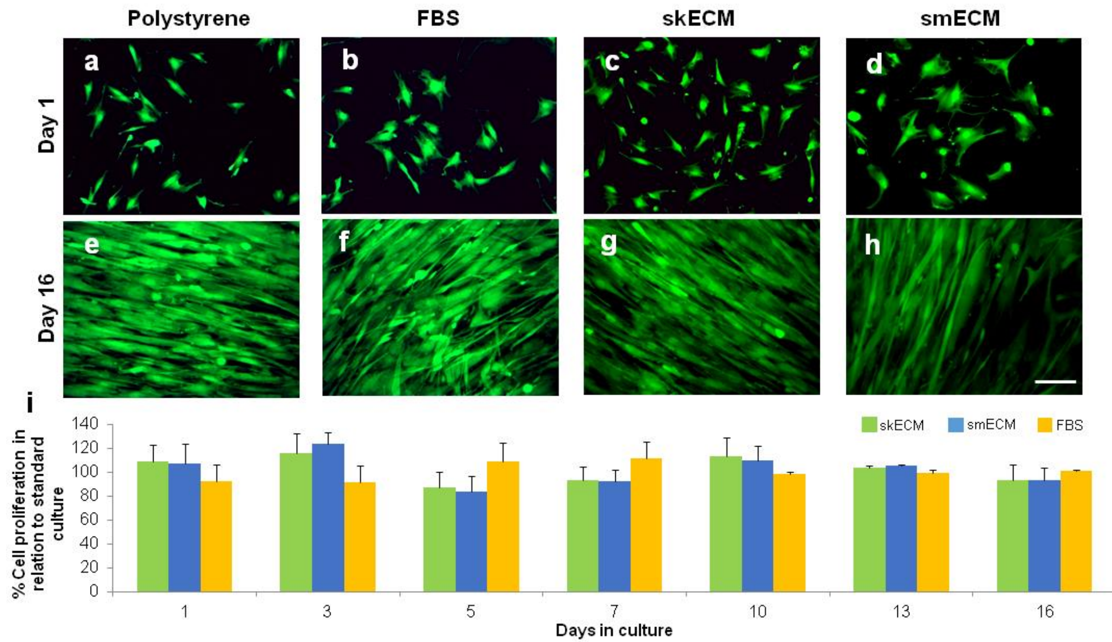
75

76 **Supplementary Figure S9. Recellularization of urethra bioscaffolds with SVF populations.**

77 Cultures were performed using (**a-f**) DMEM + 10%FBS or (**g-k**) DMEM + 2% HS. Presence of (**a**

78 and **g**) α-actin, (**b** and **h**) calponin, (**c** and **i**) MyoD, (**d** and **j**) MyHC, (**e**) CD31 and (**f** and **k**)

79 vimentin was assessed through immunohistochemistry. Scale bar: 100 µm.



80

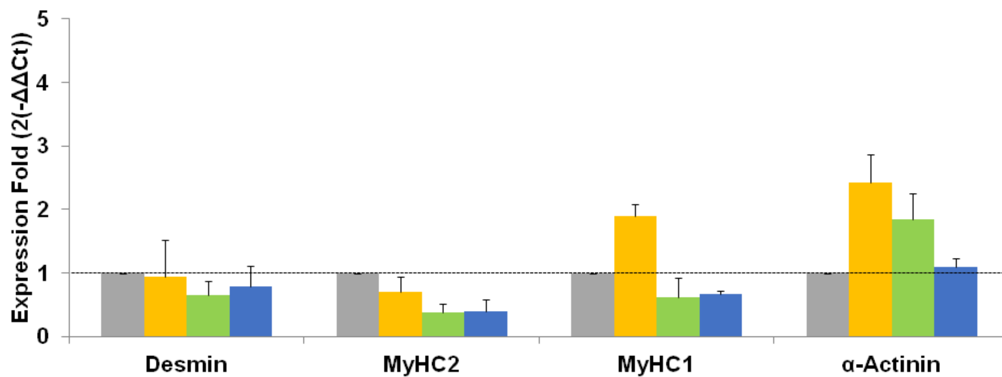
81 **Supplementary Figure S10. Cell viability and proliferation on urethra ECM coatings.** (**a-h**)

82 Cell viability was assessed using Calcein AM staining. Scale bar: 150 µm. (**i**) Cell proliferation

83 was assessed using WST-1 assay (Roche) at 450 nm in relation to MPCs grown in non-coated-

84 polystyrene: skECM (green), smECM (blue) and FBS (yellow) coatings. Results as mean ± SEM

85 (n=3), $p > 0.05$ for all time points, ANOVA.



86

87 **Supplementary Figure S11. Expression of skeletal muscle-related genes in MPCs on**
88 **urethra ECM coatings**. The expression of desmin, MyHC and α-actinin genes was quantified
89 through RT-qPCR on MPCs after 14 days of differentiation in non-coated polystyrene (grey),
90 FBS (yellow), skECM (green) and smECM (blue). Dashed line marks the expression of the gene
91 in the reference condition, non-coated polystyrene (grey). Results as mean ± SEM (n=3), $p >$
92 0.05 for all conditions, ANOVA.

93

94 **Supplementary Table S1.** Data for the estimation of the pressure (P) to which the urethra is
95 subjected with the perfusion decellularization method.

14 Gauge Internal Diameter	0.0016 m
14 Gauge Length	0.0450 m
Area	2.01×10^{-6} m^2
ρ Water	1000 Kg/m^3
ρ Triton X-100	1070 Kg/m^3
ρ SDS	1030 Kg/m^3
Flow Rate (Q)	6.67x10-7 m^3/s
Velocity (V)	0.33 m/s

96

97 **Supplementary Table S2.** Macroscopic assessment of the outcome of the decellularization
98 process using each detergent solution tested (+ for positive decellularization, organ become
99 white/transparent; – for negative decellularization, organ did not become white/transparent).

Decellularization Solution	**Results**
1% Triton X-100 + 0.1% NH_4OH	-
5% Triton X-100 + 0.5% NH_4OH	-
0.1% SDS	-
0.5% SDS	+
1% SDS	+

100

101 **Supplementary Video S1. MPCs fiber formation *in vitro*.** After 12 days of induction MPCs

102 started to contract. Scale bar: 100 µm. Time frame: 9 seconds.

103

104 ## Supplementary Methods

105 **Decellularization of Urethra Tissue – DAPI Staining**

106 Five µm thick sections of native (n=4) and decellularized (n=5, 0.5% SDS and n=2, 1% SDS)

107 urethras processed for DAPI staining. DAPI (1:100, Sigma®) was incubated for 1 h at room

108 temperature. Slides were mounted with ProLong® Gold Antifade (Invitrogen™). Images were

109 captured on a Leica Microsystems DM4000B upright microscope (Leica Microsystems Inc.,

110 Wetzlar, Germany) with Image-Pro® Express software (Media Cybernetics Inc., Rockville, MD,

111 USA).

112

113 **Isolation, Expansion and Differentiation of Cells for Recellularization and Seeding**

114 **Muscle Progenitor Cells (MPCs)**

115 MPCs were purchased (Zenbio®) or isolated from muscle tissue obtained from healthy donors

116 after informed consent at USZ in Switzerland[25]. MPCs from Zenbio® were plated at 5000

117 cells/cm^2 and expanded using SkMGM (ZenBio®) for at least two cell passages. MPCs isolated

118 at USZ were plated at 5000 cells/cm^2 and expanded for at least one cell passage using growth

119 medium DMEM/F12 (Gibco®) supplemented with 18% FBS (Gibco®), 10 ng/ml hEGF

120 (Sigma©), 1 ng/ml hbFGF (Sigma©), 10 µg/ml human insulin (Sigma©) and 0.4 µg/ml

121 dexamethasone (Sigma©)).

122 **Stromal Vascular Fractions (SVF)**

123 SVF were isolated from discarded liposuctions of healthy donors after informed consent at

124 "Clínica de Todos-Os-Santos" in Lisbon, Portugal using a standard enzymatic protocol[26]. SVF

125 populations were immediately used for recellularization.

126 **Bone Marrow Mesenchymal/Stromal Stem Cells (BM MSCs) and Fibroblasts**

127 BM MSC were isolated from bone marrow aspirates obtained from healthy donors at Instituto

128 Português de Oncologia Francisco Gentil in Lisbon, Portugal (IPOFG-L), after informed consent

129 from an approved protocol by the Ethical Review Board of IPOFG-L[27]. BM MSC and mouse

130 L929 fibroblasts (Leibniz-Institute DSMZ) were plated at 3000 cells/cm^2 and expanded using
131 DMEM + 10% FBS for at least one cell passage. All cultures were maintained at 37ºC and 5%
132 CO_2 with medium changes every 3-4 days.
133

134 **Microdissection of Urethra Bioscaffolds**

135 The RBS layer of whole decellularized urethras (n=4) was isolated from the remaining
136 bioscaffold through microdissection (Supplementary Figure S3). Transversal fragments of the
137 area containing the RBS layer were cut and pinned on gelatin-coated plates. Microdissection
138 was carried out under stereo microscope (Supplementary Figure S3a, Zeiss Stemi 2000-c, Carl
139 Zeiss©) and using a Zeiss KL1500 LCD cold light source (Carl Zeiss©). A small incision was
140 made on the most external layer of the bioscaffold (Supplementary Figure S3b) using a
141 disposable scalpel. The RBS was detached from the remaining bioscaffold using the blunt end
142 of the scalpel (Supplementary Figure S3c), creating two bioscaffolds – skECM (skeletal muscle
143 ECM, Supplementary Figure S3d) and smECM (smooth muscle ECM, Supplementary Figure
144 S3e).
145

146 **MPCs Fiber Formation**

147 Fiber formation was induced using DMEM/F12 + 10% FBS (Gibco®) (DMEM + 2% HS (Gibco®)
148 for the MPCs from Zenbio®) for 14 days at 37ºC and 5% CO_2 with medium changes every 3
149 days. Fiber formation was confirmed through immunofluorescence staining using anti-desmin
150 (1:50, Sigma©), anti-Pax3 (1:100, Santa Cruz Biotech.), anti-MyoD (1:100, BD Biosciences),
151 anti-α-actinin (1:500, Sigma©) and anti-MyHC (1:1, DSHB, University of Iowa, IO, USA).
152 Secondary antibodies FITC-conjugated goat anti-mouse (Abcam®, Cambridge, UK), FITC-
153 conjugated donkey anti-goat, Alexa-546-conjugated goat anti-mouse, Alexa-546-conjugated
154 goat anti-rabbit (all 1:500, all from Invitrogen™) and Cy3-conjugated sheep anti-mouse (1:1000,
155 Sigma©) were used. Cell nuclei were counterstained using DAPI (1:10000, Sigma®). For Pax3
156 staining all solutions were prepared using FBS instead of NGS. Images were acquired using
157 Leica DM6000 B fluorescence microscope and the LAS AF software both from Leica
158 Microsystems Inc.
159

160 **Cell Viability Analysis**

161 Cultures were washed with PBS and incubated in culture medium containing Calcein AM
162 (1:1000, Sigma©) for 30 min. Images were acquired using Leica DM6000 B fluorescence
163 microscope and the LAS AF software both from Leica Microsystems Inc.

164

165 **RT-qPCR**

166 Total RNA was isolated with SV Total RNA Isolation System Kit (Promega) according to
167 manufacturer's instructions. RNA concentration was determined through spectrophotometry at
168 260 nm using NanoDrop Lite (Thermo-Scientific™). RNA was reverse transcribed with random
169 primers using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) and
170 the T3000 Thermocycler (Biometra). Pre-designed primers for human desmin, MyHC1, MyHC2
171 and α-actinin were purchased from Life Technologies™. The analysis was normalized to the
172 endogeneous gene S18. Real Time quantitative Polymerase Chain Reaction (RT-qPCR) was
173 performed using RotorGene RG3000 and data was acquired using RotorGene software (both
174 from CR Corbett Research). RT-qPCR was performed based on TaqMan assay, TaqMan Fast
175 Advanced Master Mix (Applied Biosystems™). Detection was made with Fluorophor FAM and
176 wavelength of 495 nm. The expression fold of each gene was calculated using Supplementary
177 Equation (S1), where ΔCt is the C_{target} - $Ct_{endogenous}$ (Ct, threshold cycle). The cultures using non-
178 coated polystyrene were used as the reference condition for all calculations.

179 $$Expression\ Fold = 2^{(-(\Delta Ct_{target} - \Delta Ct_{reference}))}$$ Supplementary Equation (S1)