Supporting Information

Trilayer Three-Dimensional Hydrogel Composite Scaffold Containing Encapsulated Adipose-Derived Stem Cells Promotes Bladder Reconstruction via SDF-1α/CXCR4 Pathway

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1. Supplementary Materials and Methods

Preparation of BAMG and Silk Mesh. BAMG was prepared as described in our previous work.[1-2] Briefly, bladders were harvested from 3-month-old pigs (Shanghai Super-B&K Laboratory Animal Co. Ltd., Shanghai, China) and transported to the laboratory in 4 °C PBS. Submucosal portion of the bladder was separated from the urothelium, muscle and serosal layers via surgical delamination, and went through a decellularization process in a stirring flask (200 rpm), including sequential incubation with distilled water for 48 h at 4 °C, 0.03 % trypsin (Gibco) for 1 h at 37 °C, and finally 0.2 % (v/v) Triton X-100 mixed with 0.1% (v/v) ammonium hydroxide for 7 days at room temperature. BAMG was washed with distilled water for 2 days at 4 °C, and lyophilized overnight for storage. The silk mesh (70-mesh, Shanghai Bolting Silk, Shanghai, China) was boiled for 30 min in 0.02 M Na_2CO_3 solution and rinsed thoroughly with distilled water to remove the glue-like sericin protein.

Cells Isolation, Culture, Identification and Labeling. ASCs from Sprague-Dawley (SD) rats (Shanghai Super-B&K Laboratory Animal Co. Ltd.) were isolated, identified and labeled according to our previous study.[3] Briefly, inguinal adipose tissues were harvested from euthanize two-week-old SD rats (Shanghai Super-B&K Laboratory Animal Co. Ltd.). The adipose tissues were washed twice in 0.25% chloromycetin, cut into small pieces and digested with 0.1% type I collagenase (Sigma-Aldrich Co. LLC., MO, USA) for 1 h at 37 °C. The digested suspension was filtrated through a 200-mm nylon filter mesh (BD Falcon) and centrifugated with 160 g for 10 min at 37 °C. The centrifugated sedimentation was resuspended in DMEM containing 10 % FBS. The isolated cells were seeded in 10-cm cell culture plates

(BD Falcon) and incubated at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was refreshed every 2 days. ASCs were passaged after attaining 80-90% confluence. ASCs were identified by flow cytometry to detect CD29, CD9, CD105 and CD45, and differentiated into adipocytes and osteoblasts as described in our previous studies.[3-4] Cell Tracker CM-DiI was used to trace ASCs in vivo. Third-passage ASCs were incubated with 2 mM CM-DiI for 5 min at 37 °C followed by a 15-min incubation at 4 °C, washed twice with PBS and resuspended in the culture medium for hydrogel encapsulation.

SEM. SEM was performed according to our previous study.[2] The samples were subjected to pre-fixation with 2% glutaraldehyde for 2 h at 4 °C, twice washes with PBS, and post-fixation with 1% osmic acid for 2 h at 4 °C. The fixed samples were washed twice with distilled water, dehydrated with an ethanol gradient, and dried to a critical point (autosamdri-815, Tousimis Research Corporation, MD, USA). After sputter-coated with gold (NeoCaster, MP-1920NCTR, JEOL, Tokyo, Japan), the samples were visualized in a SEM (NeoScope, JCM-5100, JEOL) at 20-25 kV with different magnifications.

Mechanical Properties. As described in our previous study,[2] the samples were placed between two grippers of a mechanical analyzer for biomaterials (Instron 5542, Illinois Tool Works Inc., IL, USA), whose initial interval length was 10 mm and moving speed was 25 mm/min. Strain-stress curve was recorded until complete rupture of the samples.

Blood Cells Count and Serum Chemistry. Blood samples were subjected to blood cell counts using a hematology analyzer (Genius, KT-6300, Genrui Biotech Inc., Shenzhen, China)

and serum biochemical measurements using a biochemical analyzer (7180, Hitachi High-Technologies Co., Tokyo, Japan) at 2, 4 and 12 weeks post-operation.

Conscious Cystometry. Regenerated bladder function was measured by cystometry without anesthesia and restraint at 12 weeks post-implantation as described in a previous study[5]. Briefly, bladders were extruded from anesthetized rats. One end of a PE-50 tube was inserted into the bladder dome, which was fixed by a purse-string suture, while the other end was placed on a subcutaneous tunnel from abdominal opening to upper dorsum. Conscious cystometry was performed five days after bladder catheterization to minimize the effects of inflammation and surgery, and adjust the bladder to the inserted PE-50 tube.[6] SD rats experienced no restraint or anesthesia in a metabolic cage, from which the PE-50 tube was released and connected to an infusion pump and a pressure transducer of a urodynamic system (Laborie Medical Technologies, Brossard, Canada). The infusion pump filled the bladder with sterile saline at a speed of 100 μl/min. Voiding volume and intra-vesical pressure were recorded simultaneously. When micturition pattern became regular and stable, at least 3 micturition cycles were recorded. Urodynamic parameters, including bladder capacity and compliance (divide instilled volume during the filling phase by pressure change), were calculated based on cystometrograms drawn by Origin 9.0 (Origin Lab, Northampton, MA, USA).

2. Supplementary tables

Table S1. Detailed antibodies information for immunochemistry and immunofluorescence analyses

Against	Catalog	Dilution
MPO	ab9535, Abcam, Cambridge, MA, USA	1:50
CD68	ab31630, Abcam	1:150
CK	ab1747070, Abcam	1:50
α-SMA	ab32575, Abcam	1:150
CD31	ab119339, Abcam	1:100
NeuN	ab177487, Abcam	1:150

Histological and Immunofluorescence Analyses. Bladders were sampled from euthanized rats and underwent histological evaluations as our previous study.[2] Bladder samples were fixed with 4% formaldehyde overnight at 4 °C, dehydrated with a series of graded ethanol solutions, embedded in paraffin, sectioned with a thickness of 8 μm, placed onto glass slides, deparaffinized for 30 min at 60 °C and immersed in xylene, graded ethanol and distilled water, which were then subjected to H&E, MTS, immunohistochemistry for MPO and CD68, and immunofluorescence for CK, α-SMA, CD31 and NeuN. Sections for immunohistochemistry were subsequently incubated with species-matched horseradish peroxidase-conjugated secondary antibodies (Millipore, Billerica, MA, USA) and visualized using EnVision™ Detection Systems Peroxidase/DAB (K5007, Dako, Agilent Technologies, Santa Clara, CA, USA). Sections for immunofluorescence were incubated with species-matched FITC-conjugated secondary antibodies (Millipore), and nuclei were

counterstained with DAPI. Photomicrographs were acquired by a Nikon Eclipse 80i fluorescence microscope. Photometric analyses including the numbers of MPO-positive neutrophils, CD68-positive macrophages, percentages of ADA/Gel hydrogel, collagen, and corresponding positive area of CK, α-SMA and NeuN per total filed, and CD31-postive blood vessel density and diameter were performed based on 6 randomly selected fields from 3 slides by ImageJ 1.50i software (National Institutes of Health).

Table S2. Oligonucleotide Sequences used for qRT-RCR

mRNA	Sense strand (5'-3')	Antisense strand (5'-3')
SDF-1α	ATGCCCCTGCCGATTCTTTG	TTGTTGCTTTTCAGCCTTGC
CXCR4	CGGTCATCCTTATCCTGGCT	CTCTTGAATTTGGCCCCGAG
VEGF	CGTCTACCAGCGCAGCTATTG	CTCCAGGGCTTCATCATTGC
VEGFR2	CTTCATAATAGAAGGCGTCCAG	ATAAGGCAAGCGTTCACAGC
β-actin	CACGATGGAGGGGCCGGACTCATC	TAAAGACCTCTATGCCAACACAGT

qRT-PCR. As previously described in our previous work,[2] cDNA was reversely transcribed using Hiscript Reverse Transcriptase (Vazyme Biotech Co. Ltd., Nanjing, China) form total RNA extracted from sampled bladder by TRIzol (Invitrogen Inc.). cDAN product was diluted 10 times, of which 4 μl served as templates for qRT-PCR. The abundance of targeted mRNA was evaluated in an Eco^{TM} Real-Time PCR System (ABI7900, Illumina, Inc., San Diego, CA, USA) using Power SYBR Green PCR master mix (2×) (Vazyme Biotech Co. Ltd.) according to a protocol: 1) 50 °C for 2 min; 2) 95 °C for 10 min; and 3) 40 cycles at 95 °C for 30 s and 60 °C for 30 s. Target mRNA levels were normalized to the housekeeping genes of rat β-actin as an internal control for quantification by $2^{-(\Delta\Delta Ct)}$. Each assay was performed in triplicate from three bladders.

Table S3. Detailed information of antibodies for Western blot analysis

Against	Catalog	Dilution
VEGF	ab32152, Abcam	1:1,000
VEGFR2	Sc-6251, Santa Cruz Biotechnology, Inc., Dallas, TX, USA	1:300
SDF-1α	ENT4225, Elabscience Biotechnology Co., Ltd., Wuhan, China	1:500
CXCR4	ab124824, Abcam	1:100
AKT	AF6261, Affinity Biosciences Inc., OH, USA	1:1,000
p-AKT	AF0016, Affinity Biosciences Inc.	1:1,000
ERK 1/2	AF0155, Affinity Biosciences Inc.	1:1,000
p-ERK 1/2	AF1014, Affinity Biosciences Inc.	1:1,000
β-actin	BM0627, Boster Biological Technology Co. Ltd., Wuhan, China	1:200

Western Blot Analysis. According to our previous study,[2] sampled bladders were grinded in radioimmunoprecipitation assay lysis buffer and measured total protein using a bicinchoninic acid Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Protein (30 μg) were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore), which were then blocked with 5% non-fat milk at room temperature for 2 h and incubated with antibodies against target proteins at 4 °C overnight. β-actin served as the internal control. After washes with PBS, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Boster Biological Technology Co. Ltd.) at room temperature for 2 h. Protein bands were visualized with an enhanced chemiluminescence detection kit (NCI5079, Thermo Fisher Scientific Inc.) and exposed to X-ray film (XBT-1, Eastman Kodak Company, NY, USA).

Table S4. Changes in Body Weight and Bladder Calculi of SD Rats at Different Time Points

Times (weeks)	Weight Pre-op (g)	Weight Post-op (g)	Weight Change (g)	Calculus Number (n)	Calculus Weight (μg)
2 Weeks					
BAMG-HS-ASCs	207.2 ± 8.1	285.8 ± 21.7	78.6 ± 16.9	0	0
BAMG-HS	207.1 ± 14.5	294.1 ± 15.5	87.0 ± 28.8	0	0
Cystotomy	208.3 ± 9.4	293.8 ± 10.3	85.6 ± 3.1	0	0
4 Weeks					
BAMG-HS-ASCs	208.2 ± 11.1	336.8 ± 10.0	128.6 ± 8.8	2.3 ± 1.5	5.4 ± 2.1
BAMG-HS	208.5 ± 18.6	327.6 ± 6.5	119.1 ± 25.2	2.7 ± 1.2	5.2 ± 2.0
Cystotomy	207.2 ± 11.8	336.4 ± 10.7	129.2 ± 17.8	0	0
12 Weeks					
BAMG-HS-ASCs	209.0 ± 13.0	447.8 ± 12.5	238.8 ± 24.6	4.7 ± 1.2	9.4 ± 2.1
BAMG-HS	207.0 ± 11.8	452.0 ± 9.3	244.9 ± 20.7	5.0 ± 1.0	10.3 ± 2.1
Cystotomy	208.2 ± 2.6	451.0 ± 8.0	242.9 ± 5.5	0	0

Pre-operational and post-operational body weights were recorded at each time-point. Data are presented as the mean ± standard deviation.

Table S5. Blood Cell Counts and Serum Chemistry at Different Post-operative Time-Points

Time (weeks)	WBC (10^9/L)	RBC (10^{12}/L)	ALT (U/L)	AST (U/L)	BUN (mmol/L)	Cr (μmol/L)	Na^+ (mmol/L)	K^+ (mmol/L)	Cl^- (mmol/L)
2 Weeks									
BAMH-HS-ASCs	**11.0 ± 0.6**	6.3 ± 1.1	56.2 ± 8.4	122.4 ± 14.3	6.1 ± 1.3	52.0 ± 2.2	140.3 ± 2.6	5.2 ± 0.3	105.4 ± 2.9
BAMH-HS	**11.1 ± 0.7**	6.2 ± 0.9	55.5 ± 9.8	122.7 ± 14.2	5.8 ± 0.8	48.8 ± 3.2	141.8 ± 0.3	5.0 ± 0.4	100.7 ± 0.2
Cystotomy	**10.3 ± 0.6**	6.4 ± 0.8	53.2 ± 9.2	123.2 ± 12.4	5.6 ± 1.3	51.0 ± 5.6	141.8 ± 0.8	5.2 ± 0.4	105.3 ± 2.7
4 Weeks									
BAMH-HS-ASCs	6.5 ± 0.8	6.3 ± 0.9	57.8 ± 8.3	121.1 ± 12.6	6.0 ± 0.8	52.4 ± 3.1	141.8 ± 1.5	5.1 ± 0.4	105.4 ± 2.4
BAMH-HS	6.7 ± 1.1	6.2 ± 0.1	58.2 ± 7.6	129.5 ± 22.9	6.8 ± 1.1	50.9 ± 4.4	140.0 ± 1.1	4.9 ± 0.5	103.6 ± 4.6
Cystotomy	6.2 ± 0.5	6.4 ± 1.1	55.1 ± 9.6	121.2 ± 10.4	5.9 ± 1.4	51.8 ± 5.1	140.1 ± 0.6	5.3 ± 0.8	105.2 ± 2.8
12 Weeks									
BAMH-HS-ASCs	6.3 ± 0.5	6.5 ± 0.9	56.1 ± 8.3	120.0 ± 13.4	5.9 ± 1.2	51.4 ± 2.5	138.4 ± 0.4	5.2 ± 0.4	105.3 ± 1.5
BAMH-HS	6.3 ± 0.4	6.2 ± 0.4	55.3 ± 8.6	118.5 ± 16.6	6.5 ± 1.3	51.6 ± 5.6	137.7 ± 0.5	5.5 ± 0.2	100.1 ± 2.1
Cystotomy	5.9 ± 0.6	6.5 ± 1.0	54.3 ± 8.3	123.6 ± 10.0	6.3 ± 1.4	50.0 ± 3.6	138.2 ± 1.1	5.3 ± 0.2	105.5 ± 2.2

Data are presented as the mean ± standard deviation. Data in bold indicate significant differences compared with other time-points ($P<0.05$).

3. Supplementary figures

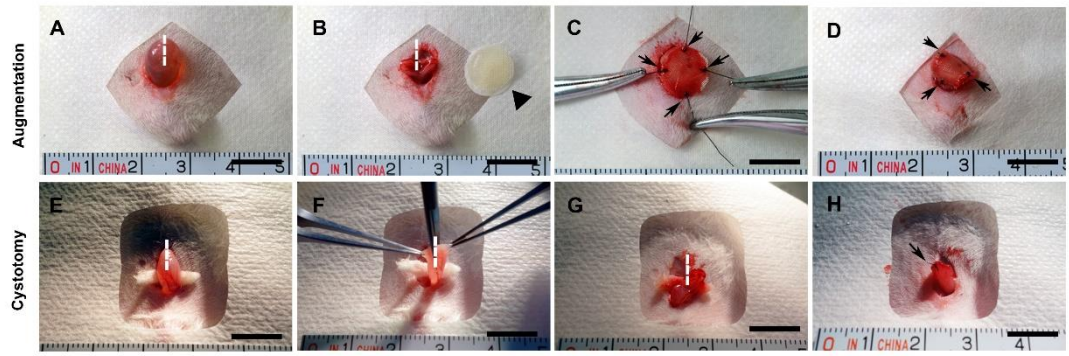

Figure S1. Representative surgical photographs of augmentation cystoplasty with ASCs-encapsulated BAMG-HS scaffold and cystotomy. Bladders were extruded from the peritoneal cavity (A and E). A 1-cm longitudinal incision was generated in the bladder apex (B and F). In the augmented groups, the circular scaffolds were anastomosed to the bladder defect with absorbable 8-0 sutures in a continuous running fashion (C). The scaffold margin was labeled with four non-absorbable 5-0 sutures (D). In addition, the incised bladders in the cystotomy group underwent immediate suture closure with absorbable 8-0 sutures and were labeled with one non-absorbable 5-0 suture (G and H). Scale bar = 1 cm. Dashed lines mark the incision area. Black triangles indicate the scaffold. Black arrows indicate the marking sutures between the native bladder and the ASCs-encapsulated BAMG-HS scaffold.

Augmentation Cystoplasty. Surgical procedures of augmentation cystoplasty were performed according to our previous study.[2] Briefly, anesthesia was induced by intraperitoneal injection of 30 mg/kg pentobarbital. The bladder of the anesthetised

rats was mobilized outside peritoneal cavity (Figure S1 A and E). The apex of the mobilized bladder was longitudinally incised at midline (approximately 1 cm) (Figure S1 B and F). In the BAMG-HS-ASCs and BAMG-HS groups, bladder defects, marked by 4 non-absorbable 5-0 polypropylene sutures, were anastomosed with scaffolds by continuous absorbable 8-0 polyglactin sutures (Ethicon, Johnson & Johnson Services, Inc., NJ, USA) (Figure S1 C and D). In the control group, bladder defects were anastomosed immediately after incision (Figure S1 G and H). After filling the augmented bladder with sterile saline via a 30-gauge hypodermic needle, regions where leaks occurred in the anastomosis sites were reinforced with interrupted absorbable 8-0 polyglactin sutures. After surgery, the rats were carefully monitored for pain or discomfort, manifested as shivering or scratching and gnawing at the abdominal incision, 3 times a day for the initial 3 days after surgery, twice a day for the post-operational 3-5 days and then once a day thereafter. Analgesic therapy typically continued for 3 days after surgery, which was managed by daily subcutaneous injections of 0.5-1.0 mg/kg meloxicam, and supplemented with 0.05-0.1 mg/kg buprenorphine every 8-12 h if necessary.[5]

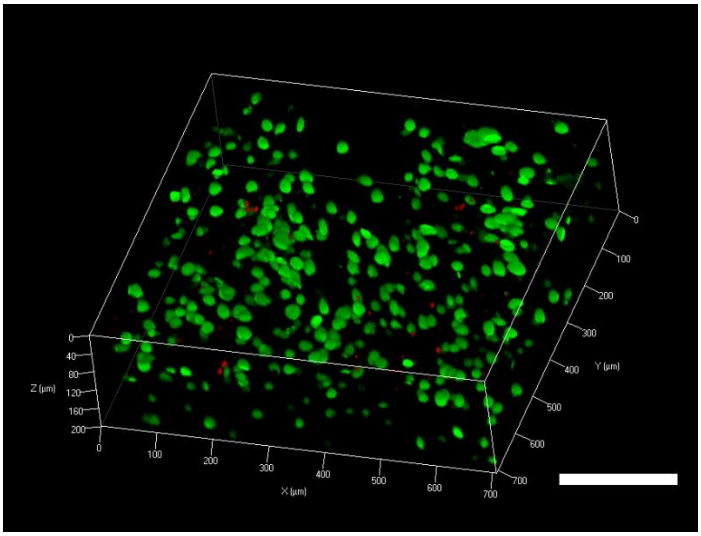

Figure S2. ASCs are well-distributed in the ADA/Gel hydrogel. A representative three-dimensional confocal photomicrograph of ASCs encapsulated in ADA/Gel hydrogel stained using Calcein-AM (viable, green) and PI (dead, red). Scale bar = 200 μm.

ASCs Distribution in ADA/Gel Hydrogel. ASCs were encapsulated in ADA/Gel hydrogel to a density of 1×10^6 cells/ml. One hundred micromillimeter of ASCs-encapsulated ADA/Gel hydrogel was slowly poured on a 48-well plate followed by incubation with 2% (w/v) $CaCl_2$ to induce cross-linking at room temperature for 10 min. The ASCs-encapsulated hydrogel was immersed in DMEM (10% FBS) and incubated in a 5% humidified CO_2 atmosphere for 24 h at 37 °C, which reacted with the live/dead Calcein-AM/ PI Double Stain Kit according to the manufacturer's protocol. The Calcein-AM and PI storage solutions were incubated at room temperature for 30 min and dissolved in Assay Buffer (1×) to reach final

working concentrations of 2 μM Calcein-AM and 4.5 μM PI. The ASCs-encapsulated hydrogel was washed with Assay Buffer (1×), incubated with 100 μl of working solution for 15 min at 37 °C, and observed under a laser scanning confocal microscope (LSM710, Carl Zeiss, Oberkochen, Germany). ZEN 2010 6.0 software (Carl Zeiss) was used to acquire the three-dimensional fluorescence photomicrographs. The viable cells labeled with Calcein-AM were detected at an excitation wavelength of 494 nm. Dead cells labeled with PI were detected at an excitation wavelength of 528 nm.

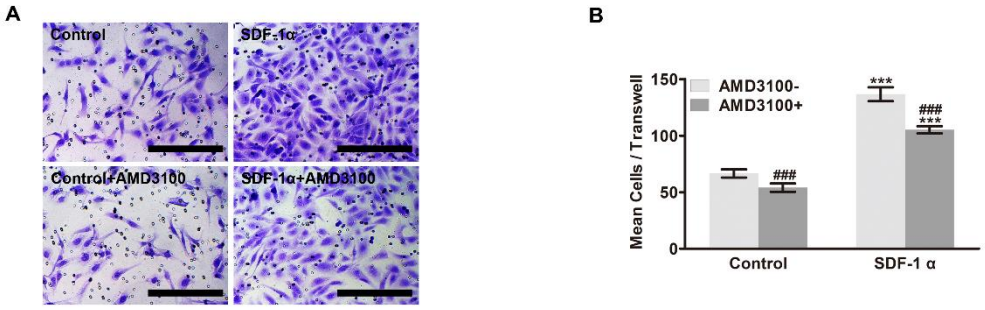

Figure S3. SDF-1α promotes ASCs migration by activating CXCR4. (A) Representative photomicrographs of ASCs migration. Scale bar = 100 μm. (B) SDF-1α promotes ASCs migration, which was inhibited by AMD3100. ***P<0.001 compared with the control group. ###P<0.001 compared with the groups treated with AMD3100.

Transwell Migration Assay. Cultured ASCs were washed twice with PBS and resuspended in fresh DMEM without FBS. ASCs suspension was counted and diluted to a concentration of 4×10^5 cells/ml for the transwell migration assay, including the SDF-1α group using cells suspension containing 20 ng/ml SDF-1α (PeproTech Inc., NJ, USA) and the control group, with or without 10 μM AMD3100 (Plerixafor, Selleck Chemicals, TX, USA). The transwell inserts were pre-loaded with 100 μl 300 μg/ml basement membrane matrix until gelatinization, and then filled with 200 μl ACSs suspension. The lower compartment of 24-well plates (BD Falcon) was filled with 700 μl DMEM (10% FBS). At 3 days after the initiation of the culture conditions, the upper compartment (8 μm pore size insert) seeded with ASCs were placed on to the well by merging the bottom of insert into the medium in the lower compartment.

The ASCs in the transwell plate were incubated at 37 °C in a 5% humidified CO_2 atmosphere. The transwell inserts were carefully removed and washed with PBS at 24 h after incubation. The ASCs on the upper side of the filter membranes that did not migrate through the pores were gently removed with a cotton swab. Migrated ASCs on the lower side of the insert filter were then quickly fixed using 5 % glutaraldehyde for 10 min at room temperature and stained with 1% crystal violet for 20 minutes. The stained specimens were visualized under the Nikon Eclipse 80i fluorescence microscope, and representative images were acquired using NIS-Elements 4.0 software. The numbers of migrated ASCs were calculated in five randomly selected fields using the ImageJ 1.50i software.

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