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MicroRNA-126 from stem cell extracellular vesicles encapsulated in a tri-layer hydrogel scaffold promotes bladder angiogenesis by activating CXCR4/SDF-1α pathway

Dongdong Xiao a,b,*,1, Mengbo Yang a,*,1, Ming Zhang a,1, Liduo Rong c, Yamei Wang c, Huan Cheng c, Xiaofeng Sui c, Soren Paludan Sheikh d,*, Mujun Lu a,*

a Department of Urology and Andrology, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200001, China
b Shanghai Key Laboratory of Tissue Engineering, Shanghai Ninth People’s Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200011, China
c Key Lab of Science & Technology of Eco-textile, Ministry of Education, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, China
d The Danish Centre for Regenerative Medicine, Odense University Hospital, Odense 5000, Denmark

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A B S T R A C T

Restricted by insufficient waterproofness and stretching resistance, hydrogel alone is seldomly applied in bladder wall regeneration. A tri-layer hydrogel scaffold comprising bladder acellular matrix graft-alginate di-aldehyde-gelatin hydrogel-silk mesh (BAMG-HS) was designed to encapsulate human adipose stem cells (ASCs)-derived extracellular vesicles (EVs), aiming to facilitate bladder regeneration. The proangiogenic composite biomaterial was evaluated in a rat model of bladder augmentation, where rats were randomly allocated to BAMG-HS-EVs (n = 6 sampled at 2, 4, and 12 weeks, respectively), BAMG-HS (n = 6, 12 weeks) and sham operation control groups (n = 6, 12 weeks). The tri-layer hydrogel scaffold has excellent mechanical and biomechanical properties, promotes angiogenesis and facilitates bladder morphological regenerations of urothelium, smooth muscle and neural fibers, and functional restoration without unmitigated fibrosis or dysregulated inflammation. Human ASCs-EVs activate bladder CXCR4/SDF-1α pathway, and enhance human umbilical vein endothelial cells (HUVEC) proliferation, invasiveness and tube-like structure formation. After internalization by HUVEC, they up-regulate microRNA-126 and inhibit RGS16 to activate CXCR4/SDF-1α pathway, thus enhancing VEGF secretion via ERK1/2 phosphorylation.

1. Introduction

Augmentation cystoplasty is indicated in neurogenic or idiopathic detrusor overactivity, including meningocoele, bladder extrophy, spina bifida and iatrogenic micro-bladders. Augmentation enterocystoplasty remains the golden standard treatment for these patients. Enterocystoplasty is widely used for bladder augmentation despite its high possibility of post-operative complications such as metabolic disturbances, mucus production and bowel disturbances [1]. Moreover, it is contradictied by intrinsic bowel disease or abnormal bowel.

Tissue-engineered bladder augmentation is a promising alternative for enterocystoplasty to avoid donor tissue morbidity. It attempted to use autologous bladder cells-seeded collagen scaffolds to replace enterocystoplasty in 2006 [2]. However, it has been confronting obstacles in both clinical trials and animal experiments ever since. It was challenged by a phase II trial reporting poor improvement of bladder capacity and compliance, and severe adverse events [3]. Three other trials applying none cell-seeded small intestine submucosa reported insufficient smooth muscle regeneration [4,5] and unfavorable improvements in bladder capacity and compliance [6]. According to a recent meta-analysis based on 28 preclinical studies, bladder capacity was not enlarged after tissue-engineered bladder augmentation in large animals [7].

The loss of histological integrity and functional deficiency is largely


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attributed to insufficient angiogenesis of regenerated bladder, which restrains the foundation for multi-layered urothelium formation, correct smooth muscle arrangement and proper innervation. Scaffold optimizations combined with bioactive factors promote bladder reconstruction [8]. Bioactive molecules and growth factors were combined with naturally derived scaffolds, synthetic polymers and their hybrids [9–11]. Meanwhile, stem cell therapy is emerging as a promising approach to overcome the insufficiency of angiogenesis with their multi-potent differentiation into endothelial cells and proangiogenic paracrine mechanisms [12]. However, stem cell therapy has potential adverse effects including immune rejection, risk of malignancy and adventitious transmission of pathogens [13]. Moreover, the meta-analysis described above demonstrated that cell seeding did not influence bladder capacity after augmentation [7]. Although it did not separate stem cells from autologous cells, emphasis has been put on pioneering new bioactive factors to be combined with scaffolds for bladder augmentation and digging into detailed mechanisms.

Mesenchymal stem cells (MSCs) have been widely used in promoting bladder reconstruction. Although stem cell therapy initially aims to replace impaired bladder tissues by multiple differentiation, accumulative evidences have suggested that paracrine mechanism of MSCs plays a major role in promoting morphological and functional recovery of post-operative bladder by secreting bioactive factors [14]. Unfortunately, main effective components of MSCs and their underlying mechanisms in promoting bladder regeneration angiogenesis remain elusive. Extracellular vesicles (EVs) derived from MSCs have been recently identified as paracrine effectors of angiogenesis in regenerative medicine [15]. EVs are nanoparticles released from various types of cells that mediate intercellular communication. EVs contain and convey various bioactive molecules, such as peptides, proteins, nucleic acids and lipids of original cells. They are further transmitted to recipient cells and induce pro- or anti-angiogenic signaling cascade in a paracrine or endocrine manner [16]. Roles of EVs derived from MSCs in cellular signaling during vascular development, growth, and maturation have been extensively documented, which has opened up new perspectives in their therapeutic application in regenerative medicine and tissue engineering as drug delivery vehicles modulating vascularization [16]. Adipose-derived stem cells (ASCs) serve as the optimal resources for EVs isolation because of less ethical limitations, less invasive isolation, and more abundant resources than other stem cells [17]. Our previous study introduced a tri-layer BAMG-algininate di-aldehyde (ADA)-gelatin (Gel)/silk mesh (BAMG-HS) composite scaffold, and demonstrated its ability to encapsulate and convey ASCs in promoting bladder regeneration angiogenesis. [18] We hypothesized that human ASCs-EVs exhibit a proangiogenic effect in combination with BAMG-HS composite scaffold for bladder augmentation. We validated the feasibility of delivering human ASCs-EVs through the hydrogel scaffold in promoting bladder regeneration angiogenesis, thus facilitating morphological and functional regeneration. We further investigated the detailed association between their key proangiogenic effector microRNA-126 (miR-126) and CXCR chemokine receptor 4 (CXCR4)/stromal cell derived factor-1α (SDF-1α) pathway.

2. Experimental section

2.1. EVs isolation and characterization

Human ASCs-EVs were isolated according to a previous study [19]. Informed consent was obtained from donors in written forms. Briefly, human ASCs were cultured with exosome-free fetal bovine serum (FBS), which was ultra-centrifugated at 120,000 g overnight at 4 °C prior to use (human ASCs culture: Supporting Information). Serum-free conditioned medium of human ASCs (fourth passage) was collected and subjected to centrifugation in sequence at 300 g for 10 min at 4 °C and 16,500 g for 10 min at 4 °C to remove cells and their debris, and further ultra-centrifugated at 120,000 g for 70 min at 4 °C to pellet human ASCs-EVs (Rotor type: SW 32 ti, k-factor:204, Optima L-100 XP ultracentrifuge, Beckman Coulter Inc., CA, USA). After treatment by bicineharonic acid Protein Assay Kit (Beyotime Biotechnology, Shanghai, China) according to its manufacturer’s instructions, total protein contents of human ASCs-EVs were extrapolated against the standard curve in a spectrophotometer (Varioskan, Thermo Fisher Scientific Inc., MA, USA) at an absorbance of 562 nm. Additionally, human ASCs-EVs were characterized by transmission electron microscope (TEM), nanoparticles tracking analysis, flow cytometry and DNA quantification (Supporting Information).

2.2. Fabrication of BAMG-HS and EVs encapsulation

BAMG-HS scaffold was fabricated according to our previous study [18]. Briefly, 8% (w/v) ADA (30% oxidation degree) was synthesized from sodium alginate and covalently cross-linked with Gel with an ADA/Gel weight ratio of 30:70 under vortex mixing at 60 °C. The scheme of ADA/Gel fabrication was drawn by ChemDraw Pro (version 14.0, CambridgeSoft Corporation, CA, USA). The mixed solution was incubated with 2% (w/v) calcium chloride (CaCl2) for 10 min at room temperature for gelation with or without encapsulation of 5.0 × 10⁹ particles/ml human ASCs-EVs. BAMG was fabricated from the decellularization process of porcine bladder submucosa, and the silk mesh was a knitted mesh of silk fibroin fibers after degumming according to our previous study [20]. One hundred microliters of human ASCs-EVs-encapsulated ADA/Gel hydrogel were poured slowly between one round piece of sterilized BAMG and silk mesh (both diameter: 10 mm) and was incubated with 2% (w/v) CaCl2 as described above.

2.3. Hydrogel and scaffold characterization

Fourier Transform Infrared (FTIR) spectroscopy analyses were carried out using PerkinElmer Spectrum Two (PerkinElmer, Inc., CA, USA) equipped with an attenuated total reflectance accessory. The spectra of ADA and alginate, and all hydrogel were recorded in the 2000–600 cm⁻¹ range, and 4000–400 cm⁻¹ range at a resolution of 4 cm⁻¹, respectively. Rheological analyses were performed at 37 °C on an HAAKE MARS Rheometer (Thermo Fisher Scientific Inc.) with a parallel plate set-up and gaps set at 2 mm. Its platform was sealed with silicone oil to prevent evaporation of water. The storage and loss moduli (G’ and G″, respectively) were measured and compared against strain and frequency sweeping. The ADA/Gel hydrogel was frozen in liquid nitrogen, snapped, freeze-dried, coated with gold vapor and examined under a scanning electron microscope (SEM) (in TM-3030, Hitachi, Ltd., Tokyo, Japan). The distribution of PKH26-labelled human ASCs-EVs were observed under confocal microscopy. BAMG-HS, BAMG and silk mesh were dried to critical point, gold-sputtered and observed under a SEM (NeoScope, JCM-5100, JEOL, Ltd., Tokyo, Japan). The elastic modulus and maximal load of BAMG-HS with or without human ASCs-EVs were measured and compared by a mechanical analyzer (Instron 5542, Illinois Tool Works Inc., IL, USA) at an initial interval length of 10 mm and a moving speed of 25 mm/min, which were performed in triplicate.

2.4. Experimental animals

Welfares of experimental animals were guaranteed and supervised by the Experimental Animal Ethical Committee of Shanghai Ninth People’s Hospital (licence number: HKDL[2016]149). All experiments were performed in accordance with all national and local regulations. Body weight-matched 8-week-old Sprague-Dawley (SD) rats (Shanghai Sippr BK Laboratory Animals Ltd., Shanghai, China) were acclimated for 1 week, labelled with numbers and then randomly divided into BAMG-HS-EVs group (bladder augmentation with human ASCs-EVs-encapsulated BAMG-HS, n = 18), BAMG-HS group (bladder augmentation with BAMG-HS, n = 6) and cystotomy control group (sham operation, n = 6) by referring to random number table. The sample size was
decided according to both high impact articles and our previous published articles in the field of bladder augmentation [18,20–22]. Investigators were not aware of sample allocation to ensure blinding. The rats had free access to food and water, and were housed in a pathogen-free environment with 20–22 °C temperature, 40–70% humidity and 12/12 hr day/night cycle. Systemic and local safety, and efficacy of human ASCs-EVs-encapsulated BAMG-HS for bladder augmentation were evaluated comprehensively (Supporting Information). Body weight was monitored pre- and post-operation. Bladder calcius, liver and renal functions, and electrolytes were measured at each time-point. Bladder morphological regeneration was assessed by gradually magnified views in retrograde cystography, gross bladder appearance, gross bladder longitudinal section and bladder regenerated area, as well as hydrogel degradation, fibrosis and inflammation of myeloperoxidase (MPO)-positive neutrophils and CD68-positive macrophages. The regeneration of bladder wall was evaluated by cytokeratin (CK)-positive fied views in retrograde cystography, gross bladder appearance, gross bladder longitudinal section and bladder regenerated area, as well as hydrogel degradation, fibrosis and inflammation of myeloperoxidase (MPO)-positive neutrophils and CD68-positive macrophages. The regeneration of bladder wall was evaluated by cytokeratin (CK)-positive.

Bladder morphological regeneration was assessed by gradually magnified views in retrograde cystography, gross bladder appearance, gross bladder longitudinal section and bladder regenerated area, as well as hydrogel degradation, fibrosis and inflammation of myeloperoxidase (MPO)-positive neutrophils and CD68-positive macrophages. The regeneration of bladder wall was evaluated by cytokeratin (CK)-positive.

The 24-well plate was incubated for 30 min at 37 °C to solidify the membrane matrix. Human umbilical vein endothelial cells (HUVEC, purchased from cell bank of Chinese Academy of Science, Shanghai, China) were trypsinized, counted and seeded on the basement membrane matrix with 1.0 × 10^5 cells per well.

Firstly, HUVECs were incubated with serum-free Roswell Park Memorial Institute (RPMI) 1640 (Shanghai Basalmedia Technologies Co., Ltd., Shanghai, China) as negative control group, serum-free RPMI 1640 medium supplemented with 0.5, 1.0, 3.0 and 5.0 × 10^6 particles/ml of human ASCs-EVs, and 20 ng/ml of vascular endothelial growth factor (VEGF) as the positive control group at 37 °C in an atmosphere of humidified carbonate dioxide (CO₂). Secondly, HUVECs were incubated with human ASCs conditioned RPMI 1640 medium, and RPMI 1640 medium supplemented with 5.0 × 10^6 particles/ml human ASCs-EVs which was then added with or without 10 μM of AMD3100 (Pllexer-afor, Selleck Chemicals, TX, USA). Tube networks were visualized after 24 hr using an inverted microscope (IX51, Olympus Co., Tokyo, Japan) attached to a digital camera. Five microphotographs were taken at randomly selected fields in each group.

2.6. EVs internalization by HUVECs

Human ASCs (fourth passage) was stained using CM-Dil (Invitrogen Inc., Carlsbad, CA, USA) in advance (Supporting Information). EVs were derived from the stained human ASCs by ultra-centrifugation as described above. HUVECs were cultured in unconditioned serum-free RPMI 1640 medium supplemented with the stained human ASCs-EVs for 30 min, 3 hr and 6 hr, and the stained human ASCs conditioned medium for 6 hr at 37 °C in 5% humidified CO₂. HUVECs with internalized CM-Dil were photographed. Then CM-Dil-positive areas per total field were calculated based on three randomly selected fields in each group.

2.7. DNA cloning and transfection for ectopic expression or knockdown experiments

Human G-protein signaling 16 (RGS16) gene fragment was amplified from pGEM-RGS16 (HG14592-G, Sino Biological Inc., Beijing, China) and cloned into pBF-ad shuttle vector (Wuhan Biofavor Biotechnology Co., Ltd., Wuhan, China). The fragment was validated by sequencing and inserted into multiple cloning sites in pBF-ad shuttle plasmid. Three independent small hairpin (sh) RNAs against human RGS16 were used for knockdown experiments, which were validated and cloned into pB.1 vector (Wuhan Biofavor Biotechnology Co., Ltd). Human miR-126 agomir and antagonir (Shanghai GenePharma Co., Ltd., Shanghai, China) were used to manipulate the expression of HUVEC miR-126. Oligonucleotide sequences of primers for human RGS16 amplification, shRNAs against human RGS16, and human miR-126 agomir and antagomir were listed in Table S3. We transfected HUVEC with RGS16 cDNA construct or shRNA constructs with miR-126 agomir or antagonir using Lipofectamine 2000 (Invitrogen Inc.) according to its manufacturer’s instructions. The transfected cells were harvested after 24 to 48 hr, and their total proteins and RNAs were isolated for further analysis. Knockdown efficiency and ectopic expression were determined by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analysis.

2.8. qRT-PCR

Transcriptional levels of major angiogenic factors were compared between human ASCs and derived EVs, including CXCR4, SDF-1α, VEGF, angiopoietin-1 (Ang-1), hypoxia inducible factor-1α (HIF-1α) and platelet-derived growth factor (PDGF), as well as miR-126, microRNA-21 (miR-21) and microRNA-296 (miR-296). Transcriptional levels of miR-126, CXCR4 and SDF-1α were compared in HUVEC treated with phosphate-buffered saline (PBS), human ASCs-EVs (5.0 × 10^6 particles/ml) alone or supplemented with miR-126 antagonir and agonir. Transcriptional levels of rat miR-126a-3p, Cxcr4, Sdf-1α, VEGF and Vegf receptor 2 (Vegfr2) were compared in bladders sampled from BAMG-HS-EVs, BAMG-HS and cystotomy groups. Descriptions of primers are presented in Tables S4 and Table S5.

2.9. Western blot analysis

Protein expressions of Alix, CD63, CD81 and calnexin were compared between human ASCs and derived EVs. HUVEC was cultured in six groups for 24 hr at 37 °C in humidified CO₂, including incubation in serum-free RPMI-1640 with PBS, human ASCs conditioned medium, and human ASCs-EVs (5.0 × 10^6 particles/ml) alone or supplemented with 10 μM of AMD3100, 10 μM of LY294002 (MedChemExpress, NJ, USA), and 10 μM of PD0358059 (MedChemExpress). Protein expressions of CXCR4, SDF-1α, phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), ERK1/2, phosphorylated phosphoinositide 3-kinase/protein kinase B (AKT), AKT, VEGF and VEGFR2 were compared. HUVEC was treated with PBS or human ASCs-EVs, and were untreated or treated with miR-126 antagonir with RGS16 downregulation by plasmid, or miR-126 agonir with RGS16 upregulation by plasmid. The protein expressions of RGS16, CXCR4, SDF-1α, phosphorylated ERK1/2, ERK1/2, phosphorylated AKT, AKT, VEGF and VEGFR2 were evaluated. These proteins expressions were also compared among bladders sampled from BAMG-HS-EVs, BAMG-HS and cystotomy groups. Antibodies’ information is described in Table S6.

2.10. Statistical analysis

Data are presented in mean ± standard deviation. GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA) was used to calculate data, perform statistical analyses and draw corresponding statistical graphs. Two-tailed Student’s t-test or one-way analysis of variance with Tukey’s post hoc test was selected accordingly. A P value of <0.05 was regarded as statistical significance.
3. Results

3.1. Human ASCs-EVs isolation and proangiogenic effect in vitro

Human ASCs-EVs presented heterogeneous round particles (Fig. 1A), with a mean diameter of 210.8 ± 87.2 nm (Fig. 1B). EVs membrane bound protein markers CD63 and CD81 were 16.90 ± 0.64 %, and 69.89 ± 0.49 %, respectively; mesenchymal stem cell protein markers CD29, CD44, CD73, CD90, and CD105 were 27.10 ± 1.29 %, 17.69 ± 0.98 %, 4.20 ± 0.175 %, 63.15 ± 0.26 %, and 7.33 ± 1.69 %, respectively; and hematopoietic stem cell protein markers CD34 and CD45 were 0.74 ± 0.11 % and 0.41 ± 0.05 %, respectively (Fig. 1C). EVs internalized proteins Alix, and membrane proteins CD31 and CD81 were highly expressed in human ASCs-EVs with little expression of the endoplasmic reticulum protein calnexin, which was conversely highly expressed in human ASCs (Fig. 1D). Residual DNA was almost eliminated in human ASCs-EVs compared with human ASCs and their supernatants (Fig. 1E). Human ASCs-EVs promoted HUVEC proliferation at 7 days in vitro compared to serum-free medium control group (P < 0.001), but were still inferior to 10% FBS positive group (P < 0.01, Fig. 1F). In addition, human ASCs-EVs enhanced HUVEC tube formation in a concentration-dependent manner (Fig. 1G, H and I). There was no difference in closed networks or total network length between 5 × 10^8 particles/ml human ASCs-EVs and 20 ng/ml VEGF. Therefore, the concentration of 5 × 10^8 particles/ml was selected for following experiments.

3.2. Tri-layer hydrogel scaffold characterization.

Initially, alginate was partially oxidized by sodium periodate to obtain ADA, where the aldehyde group of ADA and the amino group of Gel via the imine bond to form a cross-linked hydrogel. Calcium ion (Ca^{2+}) was added to further improve the mechanical performance of the hydrogel through electrostatic interactions with the carboxyl groups of ADA/Gel hydrogel. The resultant ADA/Gel hydrogel was transparent, flexible, and round disc with a diameter of 1 cm in gross appearance (Fig. 2A). According to the FTIR spectra, the new peak at 1730 cm\(^{-1}\) was assigned to the characteristic peak of the aldehyde group in ADA.
Fig. 2. Fabrication process and measurements of ADA/Gel hydrogel, and morphology and mechanical properties of BAMG-HS scaffold. (A) Schematic illustration of the preparation of ADA/Gel hydrogel based on ADA, Ca$^{2+}$ and Gel, and its gross appearance. Scale bar = 1 cm. (B) FTIR spectra of ADA/Gel hydrogel and its components, among which the differences are marked by arrows and grey rectangle. Rheology test on the hydrogel, (C) storage modulus $G'$ and loss modulus $G''$ on strain sweeping (frequency = 1.0 Hz), and (D) $G'$ (filled symbols) and $G''$ (empty symbols) versus frequency for the hydrogel (strain: 1%). (E) Respective SEM photomicrographs of ADA/Gel hydrogel with different magnifications. Magnification area is denoted by black rectangle. Scale bars are label in correspondence. (F) Representative confocal fluorescent photomicrograph of PKH26-labeled human ASCs-EVs distribution in ADA/Gel hydrogel. Scale bar = 200 μm. (G) Representative images of gross appearance and SEM of BAMG-HS, BAMG and silk mesh. Scale bar = 1 cm for gross appearance, 500 μm for BAMG-HS SEM image and 200 μm for BAMG and silk mesh SEM images. Each layer of BAMG-HS is distinguished by white dash lines. (H) Comparison of elastic modulus and maximum load between BAMG-HS with or without human ASCs-EVs encapsulation (n = 3 per group; ns, no significance).
compared with alginate [24], which was not prominent due to low degree of oxidation (Fig. 2 B). Besides, the peak at 889 cm\(^{-1}\) became weaker with the break of the polysaccharide ring. The typical bands of the polysaccharide backbone around 3300, 2920, and 1022 cm\(^{-1}\) were assigned to the stretching vibrations of O\(–\)H, C\(–\)H, and C\(–\)O\(–\)C bonds respectively in all samples [25,26]. For ADA/Gel hydrogel, characteristic peaks of ADA and Gel both appeared in the spectrum. Besides, the peak of the aldehyde group in ADA at 1730 cm\(^{-1}\) was disappeared, and the typical imine bond at 1642 cm\(^{-1}\) was formed between amine groups of Gel and aldehyde groups of ADA. These results demonstrated that the

![Immunofluorescence and histomorphometric assessments of bladder wall components and angioneogenesis.](image)

Fig. 3. Immunofluorescence and histomorphometric assessments of bladder wall components and angioneogenesis. (A) Representative photomicrographs of CD31-positive blood vessels, CK-positive urothelium, \(\alpha\)-SMA-positive smooth muscles, and NeuN-positive neuron buttons in the bladder regenerative region at 2, 4, and 12 weeks in BAMG-HS-EVs group, BAMG-HS group and cystotomy group at 12 weeks. “V” denotes CD31-positive neovessels. UE, urothelium; SM, smooth muscle. NeuN-positive neuron buttons are pointed out by white arrows. Scale bar = 100 \(\mu\)m. Respective histomorphometric analyses of the density (B) and mean diameter (C) of the CD31-positive neovessels, as well as the proportion percentage of CK-positive urothelium (D), \(\alpha\)-SMA-positive smooth muscle (E), and NeuN-positive neuron buttons (F) \((n = 3\) per group at each time point, \(*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001\) and ns, no significance versus BAMG-HS-EVs group at 12 weeks).

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Fig. 4. Human ASCs-EVs encapsulated in BAMG-HS facilitate bladder morphological regeneration without unmitigated fibrosis or dysregulated inflammation. (A) Representative photographs of retrograde cystography, gross appearance in vivo, whole scan of bladder longitudinal section, bladder regenerated area, hydrogel degradation, collagen proportion, MPO-positive neutrophils and CD68-positive macrophages in BAMG-HS-EVs groups at 2, 4 and 12 weeks, and in BAMG-HS and cystotomy groups at 12 weeks. White arrows denote depressions of bladder wall. Black arrows point out nonabsorbable sutures. Black dash lines mark areas of bladder regeneration and bladder incision. Reg. area, regeneration area; UE, urothelium; SM, smooth muscle; H, ADA/Gel hydrogel. Collagen is presented in blue color in Masson trichrome stain. Representative MPO-positive neutrophils and CD68-positive macrophages are denoted by black triangles and exhibited in magnification as inserted. Scale bar = 1 mm for retrograde cystography, gross appearance, and gross bladder section; scale bar = 200 μm for regeneration area; scale bar = 100 μm for hydrogel debris, collagen proportion, neutrophils and macrophages. Comparison of histomorphometric analyses for hydrogel debris degradation (B), collagen proportion (C), neutrophils (D) and macrophages densities (n = 3 per group at each time point, *P < 0.05, **P < 0.01, ***P < 0.0001, and ns, no significance versus BAMG-HS-EVs group at 12 weeks). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
cross-linking of ADA with Gel was successful. According to the rheology tests, the strain amplitude sweep test showed the elastic response of the hydrogel (Fig. 2 C). The $G'$ value was significantly decreased above the critical strain region, suggesting that the three-dimensional cross-linking network of the hydrogel was collapsed under excessive strain. This result indicated the state transition of hydrogel between solid and fluid at this critical point. The frequency sweep of the hydrogel (Fig. 2 D) was analyzed based on the strain amplitude sweep test. The hydrogel had a higher $G'$ value, which demonstrated good mechanical properties that were easy to process in subsequent applications. In line with a previous study, ADA/Gel hydrogel presented a macroporous, honeycomb-like morphology with pore sizes in a range of 10–30 μm (Fig. 2 E), which provided adequate space for cell proliferation, and oxygen and nutrient transportations [27]. The three-dimensional confocal fluorescent photomicrograph illustrated the distribution of PKH26-labeled human ASCs-EVs in ADA/Gel hydrogel layer of BAMG-HS scaffold (Fig. 2 F).

BAMG-HS was made up of three layers with a unified diameter of 1 cm: BAMG and silk mesh with in-between ADA/Gel hydrogel (Fig. 2 G). BAMG was a dense collagen-based membrane with a rough and smooth layer, serving as a waterproof barrier with its smooth side towards the intraluminal cavity. The silk mesh presented a tight knitted structure, which enhanced the stiffness and strength of the composite scaffold to resist anastomosis sutures with its side towards the abdominal cavity. The in-between ADA/Gel hydrogel delivered human ASCs-EVs to the bladder defect to facilitate bladder regeneration. Elastic modulus (5.32 ± 0.96 MPa) and maximal load (26.46 ± 2.21 N) of BAMG-HS showed no significant alterations after encapsulating with human ASCs-EVs (elastic modulus: 4.75 ± 1.46 MPa; maximal load: 28.9 ± 0.69) (Fig. 2 H).

3.3. Bladder angiogenesis and components regeneration.

Regeneration of bladder walls was further investigated by immunofluorescence photometric analyses regarding CD31, CK, α-SMA and NeuN in BAMG-HS-EVs, BAMG-HS and cystotomy groups. CD31-positive vessels in BAMG-HS-EVs group regenerated gradually and reached a level similar to that of cystotomy group at 12 weeks post-operation both in vessel density and diameters (Fig. 3 A, B, C). The angiogenesis of BAMG-HS group was significantly impeded in comparison with that of BAMG-HS-EVs group (P < 0.01 for vessel density, and P < 0.05 for vessel diameters). The regenerated bladder cavity was lined with a thin layer of CK-positive urothelium in BAMG-HS-EVs group at 2 weeks. Instead of gradual climbing regeneration, CK-positive urothelium experienced an obvious hyperplastic stage at 4 weeks and then recovered to a normal level, while that of BAMG-HS group still experienced hyperplasia at 12 weeks (P < 0.01) (Fig. 3 A, D). Sparse α-SMA-positive smooth fibers in BAMG-HS-EVs at 2 weeks were finally developed into multi-layer and well-organized smooth muscle bundle like in cystotomy groups at 12 weeks, with no significant difference with that of BAMG-HS group (Fig. 3 A, E). Despite gradual regeneration, NeuN-positive neuron buttons in BAMG-HS-EVs group were still unable to reach full regeneration as cystotomy group (P < 0.05), but regenerated more favorably than BAMG-HS group (P < 0.05) (Fig. 3 A, F). These results collectively illustrate that human ASCs-EVs facilitate full regeneration of bladder defects except for deficient innervation.

3.4. Bladder morphological regeneration, and inflammation response.

Retrograde cystography showed the contour of regenerated bladder. Depression in the bladder contour in BAMG-HS-EVs group at 2 weeks gradually disappeared at 12 weeks with a similar contour to that of cystotomy group, while it still remained in BAMG-HS group (Fig. 4 A). As demonstrated by the gross appearance of BAMG-HS-EVs group in vivo, the scaffold functioned as a lid on the defective bladder initially, which was gradually replaced by de novo host tissue with trivial contracture and fibrosis. Surrounding omentum and intestines were usually adhered to the scaffold but was separated easily by careful blunt dissection. Gross bladder longitudinal and magnified views of the regenerated areas provided a more detailed investigation into how the scaffold-facilitated implantation was developed into a complete bladder wall, consisting of urothelium, lamina propria, smooth muscle and serosa in a time-dependent manner. During the regeneration process,
Hydrogel debris of BAMG-HS-EVs group was gradually absorbed and disappeared at 12 weeks, while that of BAMG-HS group was not completely absorbed \( (P < 0.05) \) (Fig. 4 A, B). Along with the wax of smooth muscle regeneration, collagen proportion in BAMG-HS-EVs group experienced gradual waning and finally reached a physiological level, while BAMG-HS group still presented signs of excessive fibrosis \( (P < 0.05) \) (Fig. 4 A, C). Inflammatory cells represented by neutrophils and macrophages changed with different trends. MPO-positive neutrophils in BAMG-HS-EVs group were accumulated in bladder defect site at 2 weeks \( (P < 0.0001) \), which then experienced gradual decline and finally dissipated at 12 weeks (Fig. 4 A, D). However, CD68-positive macrophages in BAMG-HS-EVs group reached their highest level at 4 weeks \( (P < 0.01) \) and then recovered to a normal level (Fig. 4 A, E). On the contrary, there were excessive MPO-positive neutrophils and CD68-positive macrophages in BAMG-HS group at 12 weeks \( (P < 0.05) \) for neutrophils and macrophages, respectively. Safety of the composite scaffold was confirmed by body weight changes and bladder calculus number and weight (Table S1), as well as complete blood cells count, serum chemistry of renal and liver functions, and serum electrolytes (Table S2). White blood cells increased in all three groups at 2 weeks with no significant differences among groups, and recovered at 4 weeks, which demonstrated that their transient elevation was attributed to surgical interferences and tissue injuries. These results display that human ASCs-EVs encapsulated in the biocompatible and degradable BAMG-HS facilitate bladder gross morphological regeneration with alleviated fibrosis and inflammation.

3.5. Bladder functional restoration.

Regular micturition patterns were observed in BAMG-HS-EVs group without signs of bladder irritation, obstruction or overactivity at 12 weeks (Fig. 5 A). Compared to cystotomy group, bladder capacity \( (P < 0.0001) \), including voiding volume \( (P < 0.0001) \) and residual volume \( (P < 0.01) \), was enlarged effectively in BAMG-HS-EVs group, with no significant difference with BAMG-HS group (Fig. 5 B). The basal pressure of BAMG-HS-EVs group remained unchanged compared to BAMG-HS and
cystotomy groups. The threshold pressure increased slightly in BAMG-HS-EVs group compared to cystotomy group ($P < 0.05$), while the peak pressure was comparable between groups (Fig. 5 C). BAMG-HS group presented higher threshold pressures ($P < 0.01$), and lower peak pressure ($P < 0.0001$) than BAMG-HS-EVs group. Bladder compliance of BAMG-HS-EVs group was recovered to a physiological level, while that of BAMG-HS group was significantly lower than that of BAMG-HS-EVs group ($P < 0.001$, Fig. 5 D). Collectively, these data

Fig. 7. Human ASCs-EVs promote angiogenesis by activating CXCR4/SDF-1α pathway to increase VEGF via ERK1/2 phosphorylation. (A) Representative photomicrographs of HUVEC invasiveness in the indicated conditions. Scalebar = 100 μm. (B) Photometric analyses of HUVEC invasiveness according to (A) ($n = 5$ per group, ***$P < 0.001$, and ****$P < 0.0001$ versus human ASCs-EVs group). (C) Representative photomicrographs of tube formation assay in indicated conditions. Scalebar = 100 μm. (D) Photometric analyses of tube formation according to (C) ($n = 5$ per group, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, and ns, not significant versus human ASCs-EVs group). (E) CXCR4 and SDF-1α expressions are enriched in human ASCs-EVs compared to human ASCs as measured by qRT-PCR ($n = 3$ per group, **$P < 0.01$, ***$P < 0.001$, and ns, not significant versus human ASCs-EVs). (F) Western blot analyses of CXCR4, SDF-1α and downstream signaling proteins in HUVEC treated in indicated conditions.
exhibit that human ASCs-EVs facilitate bladder functional restoration with unchanged compliance and augmented capacity.

3.6. Human ASCs-EVs activate bladder CXCR4/signal pathway

BAMG-HS-EVs group presented the highest transcriptional levels of Cxcr4, Sdf-1α, Vegf and Vegfr2 (Fig. 6 A), as well as their protein expression levels (Fig. 6 B). Instead of phosphorylated AKT, ERK1/2 exhibited elevated levels of phosphorylation, indicating that the proangiogenic effect was putatively owed to ERK1/2 phosphorylation. The overall results reveal that human ASCs-EVs activate CXCR4/signal pathway and downstream ERK1/2 phosphorylation and VEGF secretions.

Fig. 8. MiR-126 derived from human ASCs-EVs activates CXCR4/SDF-1α pathway by inhibiting RGS16. (A) Representative photomicrographs of HUVECs pre-incubated with CM-DiI-labelled human ASCs or derived EVs. Scale bar = 50 μm. (B) Photometric analysis of CM-DiI-positive area per total area (n = 3 per group; **P < 0.01, ***P < 0.001, and ****P < 0.0001 versus human ASCs-EVs group at 6 hr). (C) Comparison of the respective abundance of miR-126, 21 and 296 between human ASCs and derived EVs as measured by qRT-PCR (n = 3 per group, ***P < 0.001 versus hASCs-EVs). (D) Relative expressions of miR-126, CXCR4 and SDF-1α in HUVECs incubated in the respective indicated conditions (n = 3 per group, *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 versus human ASCs-EVs group). (H) The abundance of RGS16, CXCR4, SDF-1α and downstream signaling proteins in HUVECs as determined by Western blot analysis in the indicated conditions.
3.7. Human ASCs-EVs enhance angiogenesis through CXCR4/SDF-1α pathway activation

Human ASCs-EVs accelerated HUVEC invasiveness (Fig. 7 A, B) and tube formation (Fig. 7 C, D) compared to control and human ASCs groups, which were inhibited by CXCR4 antagonist AMD3100. Among the major angiogenic factors, the transcriptional levels of CXCR4 and SDF-1α were significantly elevated in human ASCs-EVs compared to human ASCs (P < 0.001 and P < 0.01, respectively), but not VEGF, Ang-1, HIF-1α and PDGFD (Fig. 7 E). Human ASCs-EVs exerted a stronger effect than human ASCs in activating HUVEC CXCR4/SDF-1α pathway, downstream VEGF and VEGFR2, and AKT and ERK1/2 phosphorylation (Fig. 7 F). Human ASCs-EVs promoted CXCR4 and SDF-1α expressions, of which CXCR4 expression was inhibited by AMD3100 (CXCR4 inhibitor), LY294002 (AKT inhibitor) and PD058509 (ERK1/2 inhibitor), although the declination of SDF-1α was not obvious. Human ASCs-EVs increased VEGF and VEGFR2 expressions, which were dramatically inhibited by AMD3100 and PD058509, but not by LY294002. Phosphorylated ERK1/2 and AKT were elevated by human ASCs-EVs, which were obviously inhibited by AMD3100 and respective antagonists. These collective data demonstrate that human ASCs-EVs enhance angiogenesis through the activation of CXCR4/SDF-1α pathway, promoting VEGF secretion mainly via phosphorylation of ERK1/2.

3.8. MiR-126 mediates the proangiogenic effect of human ASCs-EVs

Cell membrane stain CM-Dil-labelled human ASCs-EVs accumulated in HUVEC gradually from 30 min to 6 hr. More CM-Dil-labelled signals were detected in HUVEC co-culture with human ASCs-EVs than human ASCs conditioned medium at 6 hr (P < 0.0001; Fig. 8 A, B). MiR-126, rather than miR-21 or miR-296, was significantly elevated in human ASCs-EVs compared with human ASCs (P < 0.001; Fig. 8 C). The no-miR-126a-3p expression in SD rats' bladder was also increased by human ASCs-EVs (Figure. S2). Human ASCs-EVs increased miR-126 expression of HUVEC (P < 0.05), which was enhanced by its agonist (P < 0.05) and reversed by its agonist (P < 0.001; Fig. 8 D). Similar trends were observed in the mRNA levels of CXCR4 and SDF-1α. Human ASCs-EVs inhibited RG516, activated CXCR4/SDF-1α pathway, upregulated ERK1/2 and AKT phosphorylation, and promoted downstream VEGF and VEGFR2 expressions (Fig. 8 E), which were neutralized by miR-126 antagonist and then reversed by inhibition of RG516. Additionally, miR-126 agonist enhanced these effects, and was counteracted by RG516 activation. The control inhibition or activation plasmid vehicles did not influence the effects of miR-126 antagonist or agonist. Besides, bladder RG516 expression was greatly inhibited by human ASCs-EVs (Fig. S5). These collective results indicate that human ASCs-EVs up-regulate miR-126 of HUVEC, and in turn inhibit RG516 to activate CXCR4/SDF-1α pathway and elevate downstream VEGF and VEGFR2.

4. Discussion

EVs are collective heterogeneous nanosized particles with a wide range of diameters, including exosomes derived from the exocytosis of endosomal membrane compartment, and microvesicles shed from the cytoplasmic membrane. However, there is no efficient separation method to distinguish different EVs subtypes without negatively affecting their integrity or therapeutic potentials [16]. As a result, this study collected human ASCs-EVs as a whole without examining exosomes and microvesicles separately.

The proangiogenic potential of human ASCs-EVs on HUVECs is verified with an optimal concentration of 5 × 10^8 particles/mL. However, it is difficult to determine the universal optimal EVs proangiogenic concentration because of various EVs production and inconsistent EVs quantification. It is reported that the optimal concentration of angiogenic ASCs-derived exosomes is 100 μg/mL [28]. However, another study argues that 1–10 μg/mL bone marrow-derived stem cells exosomes are more favorable than 100 μg/mL for tube-structure-like formation [29].

Hydrogels are physically or chemically cross-linked polymer networks with water absorption capacity. They have been extensively applied as biomaterials in tissue engineering except for bladder reconstruction because of the contradiction between water absorbance of hydrogel and water-proof requirements for bladder reconstruction [30]. The tri-layer design of BAMG, silk mesh, and in-between ADA/Gel hydrogel solves the contradiction, thus making it possible to take advantage of hydrogel unique properties in bladder regeneration, including convenient encapsulation of large amount of cell derivatives, excellent absorbance and maintenance of liquid components, nourishing the encapsulated cell derivatives with abundant water and nutrients, and three-dimensional geometry mimicking the extracellular matrix of the bladder tissue. Alginate are anionic and linear polysaccharides derived from brown algae and contain various proportions of 1,4-linked β-D-mannuronate and 1,4-linked α-L-gulurionate residues [31]. It is peroxidized to produce ADA and mixed with Gel to overcome its limitations of low cell adhesion, poor support for cell proliferation, slow biodegradation, insufficient hydrolysis and unstable mechanical properties [32]. The biocompatibility of BAMG and silk mesh has been proved in our previous study [18,33].

The tri-layer BAMG-HS composite scaffold reinforces bladder defect initially and delivers human ASCs-EVs to promote bladder reconstruction without causing metabolic disturbance, irreversible systemic or local inflammation. The ADA/Gel hydrogel degrades in accordance with the bladder regeneration rate without obvious fibrosis or contracture, and vanishes at 12 weeks. Tissue regeneration process involves multiple cells, especially inflammatory cells, working in chorus and well-organized sequence. As the first leukocyte recruited to inflammatory sites, neutrophils migrate to injured tissues and initiated healing processes [34]. In BAMG-HS-EVs group, early transient infiltration of neutrophil is observed at 2 weeks in bladder sections, which dissipate at 12 weeks. In similar, white blood cells in blood cells counts increase in all groups without significant difference among groups and recover at 4 weeks. The first signal responsible for early neutrophils recruitment is released from damaged and necrotic cells under damage-associated molecular patterns [35]. As a consequence, the acute and transient inflammation is putatively attributed to surgical interferences and tissue injuries in the early-stage of regeneration.

In addition to the gross morphology, the full regeneration of bladder wall components, especially the regenerated blood vessels, remains the prerequisite for physiological bladder function. The strong regenerative potential of urothelium has been verified in a previous study, which reports that simple application of BAMG supports the full regeneration of urothelium in both groups of rabbits underwent 30–40% and 60–70% partial cystectomy [36]. The hyperplasia of regenerated urothelium is coupled with the accumulation of CD68-positive macrophages at 4 weeks. During bladder defect regeneration, urothelium stratification requires normalization of basal and intermediate cell proliferations [37]. As well-known phagocytes, macrophages not only help to eliminate cell debris and foreign bodies, but also play crucial roles in coordinating tissue repair. [38] Therefore, we hypothesize in our previous study that the temporary urothelial hyperplasia is attributed to incomplete urothelial maturation and putatively associated with macrophages accumulation at 4 weeks [33]. Although innervation is not fully achieved in this study, the regenerated bladder consists of complete angiogenesis, cornification and stratification of urothelium and muscle layers.

The MSCs-EVs component which is responsible for their proangiogenic effects still remains inconclusive. This study demonstrates that human ASCs-EVs promote angiogenesis by up-regulating miR-126 and activating downstream CXCR4/SDF-1α pathway. The conclusion in turn highlights the paracrine mechanism of MSCs in promoting bladder reconstruction. Consistent with our study, the promoted angiogenesis and reduced fibrosis of bladder implanted with allogenic adipose- or muscle-derived stem cells seeded polyglycolic-acid scaffolds are...
attributed to paracrine mechanisms rather than cell incorporations [39]. This study demonstrates that incubation with human ASCs-EVs promotes HUVECs tube formation by internalizing them. MiR-126 is proved to be transferred from human ASCs-EVs to promote endothelial cell migration and angiogenesis. This is because of the elevated level of miR-126, which inhibits RGS16 to trigger the activation of CXCR4/SDF-1α pathway via ERK1/2 phosphorylation to secrete VEGF. SDF-1α has been well documented to promote endothelial progenitor cells to repair blood vessels in the endothelium and angiogenesis [44]. In line with our study, it is reported that obesity impairs human ASCs-EVs’ capability to promote endothelial cell migration and in vitro tube formation by reducing miR-126 to upregulate sprouty-related, EVH1 domain-containing protein-1 (SPRED-1), and downregulating VEGF and matrix metalloprotease-2 [45]. Additionally, elevated miR-126 and VEGF are observed in human placenta-derived MSCs after treatment with nitrergic oxide-releasing polymer, and are released to reveal the proangiogenic effects and ameliorate hind limb ischemia in a murine model [46].

The proangiogenic effects of miR-126 are also revealed in cells other than MSCs. HUVEC apoptotic bodies transfer miR-126 to promote SDF-1α pathway to secrete VEGF and recruit endothelial progenitor cells [40]. The lipid bilayer structure of EVs protects encapsulated genetic cargoes from enzymatic digestion, especially the small non-coding RNA with 22 nucleotide sequences termed microRNA. They are synthetized and incorporated with RNA-induced silencing complex-loading complex (RLC) proteins Dicer, HIV-1 TAR RNA binding protein and argonaute 2 to be functional and achieve efficient gene silencing [41]. Co-fractionation of RLC proteins is observed in the multivesicular body, which is the origin of EVs [42]. The horizontal transfer of microRNA modulates the phenotype and biological behavior of recipient cells by regulating mRNA degradation or inhibiting translation by binding to its partial complementary sequences to mRNA three prime untranslated region [43]. The only coding gene of human miR-126 is located in the intron of epidermal growth factor-like domain-containing protein 7, which in turn regulates the integrity of blood vessels in the endothelium and angiogenesis [44]. In our study, we choose to evaluate the regenerated bladder function in vivo, this study chooses to evaluate the regenerated bladder function in vivo without restrain or anesthesia to reflect the micturition pattern under physiological condition. Despite the therapeutic angiogenic potential of human ASCs-EVs isolated from healthy donors presented in our study and other preclinical works, there are evidences questioning the proangiogenic capability of MSCs-EVs isolated from patients with obesity and high glucose [45]. As a result, it is required to specify donor inclusion criteria and identify potent predictive markers to reduce donor-related variability on therapeutic differences.

5. Conclusion

The biocompatible and degradable tri-layer BAMG-HS composite scaffold reinforces bladder defect with sufficient mechanical strength and delivers human ASCs-EVs to the regenerated site. MiR-126 derived from human ASCs-EVs promotes bladder regeneration angiogenesis by inhibition of RGS16 to activate CXCR4/SDF-1α pathway to secret VEGF
via ERK1/2 phosphorylation, thus improving morphological and functional restorations after bladder augmentation (Fig. 9).

Author contributions

M.L. Ju, X.F. Sui, and D.D. Xiao designed this study and allocated the experimental animals. D.D. Xiao and M.B. Yang performed all in vivo animal experiments, collected all raw data and wrote this manuscript. S. P. Sheikh revised this manuscript and all figures. M Zhang cultured the cells, prepared the EVs and all in vitro experiments. L.D. Rong prepared the BAMG-HS composite scaffold. H Cheng and Y.M. Wang measured the scaffold parameters. M.B. Yang performed qRT-PCR, Western blot analyses, flowcytometry analysis and isolated human ASCs from lipoaspirate.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The authors declare that all data supporting the findings of this study are available within the paper, its Supporting Information and upon request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2021.131624.

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