***In vivo* effects of human adipose-derived stem cells reseeded on acellular bovine pericardium in nude mice**

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Abstract

Tissue-engineered biologic products may be a viable option in the reconstruction of pelvic organ prolapse (POP). This study was based on the hypothesis that human adipose-derived stem cells (hASCs) are viable in acellular bovine pericardium (ABP), when reseeded by two different techniques, and thus, aid in the reconstruction. To investigate the reseeded of hASCs on ABP grafts by using non-invasive bioluminescence imaging (BLI), and to identify the effective hASCs-scaffold combinations that enabled regeneration. Thirty female athymic nude mice were randomly divided into three groups: In the VIVO group, ABPs were implanted in the subcutaneous pockets and enhanced green fluorescent protein luciferase (eGFP-Luc)-hASCs (1×10^6 cells/50 μ L) were injected on the ABP at the same time. In the VITRO group, the mice were implanted with grafts that ABP were co-cultured with eGFP-Luc-hASCs *in vitro*. The BLANK group mice were implanted with ABP only. The eGFP-Luc-hASCs reseeded on ABP were analyzed by BLI, histology, and immunohistochemistry. The eGFP-Luc-hASCs reseeded on ABP could be visualized at 12 weeks *in vivo*. Histology revealed that the VIVO group displayed the highest cell ingrowths, small vessels, and percent of collagen content per unit area. Desmin and α -smooth muscle actin were positive at the same site in the VIVO group cells. However, few smooth muscles were observed in the VITRO and BLANK groups. These results suggest that hASCs reseeded on ABP *in vivo* during surgery may further enhance the properties of ABP and may promote regeneration at the recipient site, resulting in a promising treatment option for POP.

Keywords: Adipose-derived mesenchymal stem cells, acellular bovine pericardium, bioluminescence imaging

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Introduction

Surgery is the preferred treatment option for severe cases of pelvic organ prolapse (POP), stress urinary incontinence (SUI), and fecal incontinence. The lifetime risk of undergoing a surgical procedure for pelvic floor disorders is almost 11–19% in women.^{1,2} However, failure rates are high in surgeries, and prolapse recurrences are common when treated using ungrafted methods. Although the use of synthetic mesh or biological grafts for the reinforcement of POP repair is popular, complications such as infections and vaginal erosion are frequent.³ Currently, with the aim of avoiding such complications, tissue engineering and cellular therapy have been proposed for the treatment of POP.^{4–6} Tissue engineering products are developed using scaffolds and stem cells. Biocompatible scaffolds provide a matrix that favors cell adherence and promotes integration. With the gradual degradation of scaffolds, they are eventually replaced by infiltrating cells. In addition, the stem cells integrate and revitalize the scaffolds, thereby enhancing the healing process.

One such tissue engineering product is the bovine pericardium matrix implant. It is a biologic graft composed of collagen, and is largely used in heart surgery and pediatrics. It is a reliable substrate because of the effective mechanical support, biocompatibility with host tissues, and minimal tissue irritation.^{7,8} Recent studies have examined the use of acellular bovine pericardium (ABP) grafts as a scaffold in pelvic floor reconstruction.⁹ Human adipose-derived stem cells (hASCs) are a suitable option for reseeded, because it is convenient to harvest them in large quantities using minimally invasive techniques, and additionally, these MSCs maybe reduce the immune response.¹⁰ Recent data also suggest that the multipotency and proliferative efficiency of hASCs are as effective as that of bone mesenchymal stem cells, and they improve collagen production and differentiation of smooth muscle cells.^{11,12} One of the advantages of tissue engineering products is the presence of hASCs on the surface of the collagen membranes, which modulate the inflammatory reaction and weaken the attack by foreign bodies.^{13,14}

Currently, there exists an unmet need for an alternative source of mesh for severe female pelvic floor dysfunction. Clinical application of reseeded grafts in burn reconstruction, bladder tissue repair, and construction of skin paved the way for gradual acceptance of stem cell reseeded grafts in other surgical disciplines.^{15,16} However, the *in vivo* effects of hASCs reseeded on the biologic graft such as ABP, over time remains unclear. In the traditional stem cells-scaffold combinations, scaffolds were seeded with cells and cultured *in vitro*, which might lead to significant cell loss during the procedure.

Considering the promising results of the pre-clinical and clinical studies, the purpose of the present study was two-fold: (1) to track the fate of hASCs on two kinds of tissue-engineered biologic products *in vivo* over time, by using non-invasive bioluminescence imaging (BLI); and (2) to investigate the more effective hASCs-scaffold combinations.

Materials and methods

Ethics statement

This study was approved by the ethics committee of the Shanghai Sixth People's Hospital (Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University). All animal experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Ethics Committee on Animal Experiments at Shanghai Jiao Tong University School of Medicine (Permit Number: SYXK [Shanghai] 2013-0050).

Isolation and cultivation of hASCs

hASCs were obtained from the harvested adipose tissue samples of a 35-year-old woman who underwent cosmetic subdermal liposuction, after a written informed consent. The adipose tissue was digested at 37°C with mild agitation for 60 min using collagenase type I (Sigma, St. Louis, MO). Digestion was terminated with an equal volume of Dulbecco's Modified Eagle Medium (DMEM) with high glucose (Gibco, NY) containing 10% fetal bovine serum (FBS) (Gibco). Next, the suspension was centrifuged at 400 g (1500 r/min) for 10 min. The cell pellet was seeded onto a six-well plate (Corning, USA) in control medium (DMEM + 10% FBS + 1% penicillin/streptomycin) (Gibco). The culture was maintained at 37°C in a humidified incubator containing 5% CO₂. The medium was replaced every three days.

Characterization of hASCs *in vitro*

hASCs were characterized by flow cytometry and multi-directional differentiation according to the minimum criteria set by The International Society for the Cellular Therapy in 2006 for defining multipotent MSCs¹⁷: First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, MSC must express CD90, CD105, or CD73, and lack expression of CD45, CD14, CD34, or CD11b, CD79alpha or CD19 and HLA-DR surface

molecules. Third, MSC must differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro*. Specific cell surface antigen expressions (i.e. CD13, CD44, CD90, CD14, CD31, CD45; Sigma) were confirmed by flow cytometry. The verification of adipogenic and osteogenic differentiation were performed by Oil Red O staining, Alizarin Red staining, and ALP staining (Sigma), respectively.

Viral transduction and luciferase assays *in vitro*

Cell were cultivated until a 70–80% confluence was achieved, and from the third passage onwards, they were transfected with lentiviral vectors (1×10^7 transduction U/mL, multiplicity of infection [MOI] = 50), containing enhanced green fluorescent protein (eGFP) gene and Luciferase (Luc) gene (SunBio Biotech, Shanghai, China). Next, they were incubated in a six-well cell culture cluster containing 10 µg/mL polybrene (Sigma) for 24 h. The transfected hASCs were selected by flow cytometry and purified, to improve the effect of labeling. The selected cells were cultured and used for seeding the scaffolds. The eGFP expression of eGFP-Luc-hASCs was observed in the twelfth passage by fluorescence microscopy, and the transfection efficiency of hASCs was detected by flow cytometry *in vitro*. GFP transfected cells were stained by monoclonal antibody such as anti-CD13 (Sigma) and anti-CD44 (Sigma) in 100 µL phosphate-buffered saline (PBS) with 3% FBS at room temperature for 30 min. All samples were analyzed on a FACS Calibur flow cytometer (BD Biosciences). Transduction efficiency of eGFP-Luc-hASCs was assessed by GFP positive rate in CD13 and CD44 double positive cells.

The Luc expression was checked by seeding eGFP-Luc-ASCs of different numbers ($5 \times 10^4/100 \mu\text{L}$, $1 \times 10^4/100 \mu\text{L}$, $1 \times 10^3/100 \mu\text{L}$, $5 \times 10^2/100 \mu\text{L}$, $1 \times 10^2/100 \mu\text{L}$, $50/100 \mu\text{L}$, $10/100 \mu\text{L}$) were seeded onto black, clear bottom 96-well plates in 100 µL medium (DMEM + 10% FBS + 1% P/S), respectively. Before imaging, 15 µL D-luciferin solution (150 µL/mL; Sciencelight, China) was added to every 100 µL culture medium (in a 96-well plate), and after 5 min, the bioluminescent signals were detected using a highly sensitive, cooled charged coupled device (CCD) camera (Xenogen IVIS, USA).

Cell viability

eGFP-Luc-hASCs were seeded on ABP *in vitro* during the eighth passage. The cell viability was evaluated using the Cell-Counting Kit (CCK)-8 assays. eGFP-Luc-hASCs (5×10^3) were cultured on ABP, and the medium was changed every three days (on days 1, 4, and 7). At each time point, 20 µL of CCK-8 solution reagent (Dojindo, Kumamoto, Japan) was added to 200 µL medium, and the CCK-8 assay was done. The absorbance of supernatants was measured at 450 nm, using a microplate reader. eGFP-Luc-hASCs on ABP were observed by fluorescence microscopy. The cells without viral transduction and ABP were placed in the same culture condition as control group.

Culture of eGFP-Luc-hASCs on ABP *in vitro*

ABP, the collagen-based material used to support cell growth, was provided by the Shanghai Cingular Biotech Corporation (Shanghai, China). The collagen was derived from normal bovine pericardial tissue. It was further processed through a number of steps, such as decellularization, cleaning, anti-viral treatment, chemical modification, etc. Finally, it was lyophilized, packaged, and sterilized by electron-beam radiation.

About 10 mm thick ABPs were incubated in 100% FBS under standard culture conditions for 24 h. In the tenth passage, the labeled hASCs were counted and resuspended in DMEM + 10% FBS + 1% P/S. They were reseeded at a density of 1×10^6 cells per ABP scaffold in a sterile six-well plate, and then cultivated in medium for three days. The combinations were observed by scanning electron microscope (SEM; Hitachi TM-1000 tabletop microscope) and fluorescence microscope.

Ectopic implantation of tissue-engineered product

Female athymic nude mice (BALB/C-nu/nu; $n = 30$), 4–6 weeks old, were obtained from The Animal Institute, School of Medicine, Shanghai Jiao Tong University (Shanghai, China), and were raised in a specific pathogen-free environment throughout the experiment. All experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Mice were randomly divided into three groups ($n = 10$) and anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg). The surgical area was cleaned with povidone-iodine, and a sagittal midline incision was made on the dorsum skin, to create a subcutaneous pocket of 1.5×1.5 cm. In the VIVO group ($n = 10$), ABPs were implanted in the subcutaneous pockets and eGFP-Luc-hASCs (1×10^6 cells/50 μ L) were injected on the ABP at the same time. In the VITRO group ($n = 10$), the mice were implanted with grafts that were co-cultured with eGFP-Luc-hASCs *in vitro*. The BLANK group ($n = 10$) mice were implanted with ABP only as controls. The incisions were closed with non-absorbable 6/0 nylon sutures without any additional dressing.

BLI *in vivo* and analysis

To detect the behavior of cells *in vivo*, BLI was performed at 1 day, 1 week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, 8 weeks, and 12 weeks, respectively after implantation of grafts in mice. Mice were intraperitoneally injected with luciferin (150 mg/kg) (Sciencelight), and were imaged by IVIS (Xenogen IVIS). Quantification of signals was evaluated using the acquisition and analysis software Living Image (Xenogen).

Histological analysis

To confirm the results of BLI, the expression of eGFP was checked for fluorescence after 4',6-diamidino-2-phenylindole (DAPI) staining. Twelve weeks after implantation, the animals were sacrificed and the tissue samples

from light producers and non-producers were harvested for histological analysis. One histological section (6 μ m) from the post-operative frozen sections of implanted tissues was used for DAPI staining. This was followed by detection of eGFP-positive cells by fluorescence microscopy.

Masson trichrome staining was performed to detect the collagen content. In brief, three tissue specimens were fixed in 10% formalin solution, embedded in paraffin, and stained with hematoxylin and eosin (H&E), and Masson trichrome stains. The number of the vascular structures and cell ingrowths were detected by bright field microscopy. Stained images were analyzed using the Leica Qwin V3 system. For quantitative image assessments, we included 10 fields per specimen, two specimens per mouse, and 10 mice per group.

Immunohistochemistry

Immunohistochemical staining was performed on paraffin sections to analyze contractile protein expression levels and detect vascular endothelial cells. The primary antibodies used in this study were mouse anti-human monoclonal antibodies (anti- α smooth muscle actin, anti-desmin, anti-FLI-1, anti-CD31; Sigma) in accordance with the manufacturer's protocol. Paraffin-embedded tissue sections were obtained using semiautomatic microtome approximately of 4 μ m thickness. The sections were cleared by passing through two changes of xylene for 10 min each and rehydrated by passing through two changes of absolute alcohol. Then rinsed thoroughly with distilled water and kept in the distilled water until antigen retrieval. Heat-mediated antigen retrieval in pH 6.0 citric acid was performed. After washing off the redundant antibodies, the paraffin sections were treated with secondary antibodies and incubated at room temperature. All the samples were observed under bright field microscopy.

Statistical analyses

SPSS software was used to analyze the data from the images. All the quantitative values were presented as mean $\pm$ SD. Regression plots were used to represent the relationship between cell number and bioluminescence. R^2 values were described to assess the quality of the regression model. One-way analysis of variance (ANOVA) was used for multiple comparisons, followed by *post hoc* comparisons with the Newman-Keuls test. A P value of < 0.05 was considered statistically significant.

Results

Culture and characterization hASCs

Spindle shaped adherent cells could be observed during culture. After expansion, the cells were collected and immunostained for flow cytometry analysis of immune phenotype. Most cells were positive for CD13, CD44, CD90, and negative for CD14, CD31, and CD45 (Figure 1a). The immune phenotypes of these cells were consistent with the previous reports.¹⁶ Adipogenic and osteogenic differentiation showed that hASCs possessed the capacity to differentiate into multiple (Figure 1b).

Figure 1 Characterization of hASCs on their immune phenotype and pluripotency. (a) The immune phenotype was analyzed by flow cytometry, while the pluripotency was verified by adipogenic (Oil red O staining) and osteogenic (ALP and Alizarin red S staining) differentiation (b). Original magnification, $10\times$. (A color version of this figure is available in the online journal)

***In vitro* detection of eGFP-Luc-hASCs on ABP**

hASCs transfected with lentivirus were spindle shaped. The purified eGFP-Luc-hASCs were expanded, and eGFP was highly expressed in the twelfth passage cells when observed by fluorescence microscopy (Figure 2a). Transduction efficiency of eGFP-Luc-hASCs measured by flow cytometry was up to 91.12% (Figure 2b). The light production of Luc was steady, and mainly depended on the cell number (Figure 2c). The number of eGFP-Luc-hASCs was linearly correlated with light production. The slopes of the linear regression plots showed standard plots of light production *versus* cell number ($R^2=0.99$) (Figure 2d). To prepare tissue-engineered products *in vitro*, the eGFP-Luc-hASCs at passage 8 were seeded on an ABP scaffold in a sterile six-well plate. Figure 3 (a-e) represents the magnification of materials, demonstrating that a great quantity of eGFP-Luc-hASCs survived and proliferated on ABP. CCK-8 assays indicated that viral transduction and ABP did not affect cell viability ($P > 0.05$) (Figure 3f).

***In vivo* non-invasive monitoring for implants by BLI**

To analyze the behavior of hASCs seeded on ABP *in vivo*, BLI was done on eGFP-Luc ASCs that had undergone 10 passages on ABP, one day post-implantation, and thereafter at the indicated times. The results showed that both the eGFP-Luc ASCs in the two groups remained viable *in vivo* up to 12 weeks. One day after transplantation, the population of cells in VIVO group was 13-times more than that in the VITRO group. The number of cells in both the groups (VIVO and VITRO) decreased in the first three

weeks and then remained stable in the following weeks until the end of the experiment (approximately 6.12% and 0.57% of the implanted cells in VIVO and VITRO groups, respectively at 12 weeks; Figure 4a,c). The BLANK group presented no change (Figure 4d). In addition, during the weekly BLI, majority of the implanted eGFP-Luc ASCs existed at the site of inoculation. However, significant indicators were absent in other organs such as lung, liver, heart, or brain (Figure 4b).

Histological examination

All the 30 implanted mice survived the entire 12 weeks. However, each group of the samples behaved differently. To confirm the bioluminescent data and identify in which histological layer the implanted cells survived, DAPI staining sections and H&E staining were used at 12 weeks after transplantation. In two samples of the reseeded ABP group, eGFP-positive ASCs were detected in subcutaneous layer (Figure 5b, d). The number of positive cells in the VIVO group was more than that in the VITRO group. On the other hand, no eGFP reactivity was detected in the BLANK group (Figure 5f). The results of H&E staining showed that both VIVO and VITRO group displayed numerous small vessels (Figure 5a2, c2), and compared with VITRO group, the vascular density was more abundant in VIVO group. Few vessels were present in the BLANK group (Figure 5e2). In addition, as the ABP was absorbed, cell ingrowths into the ABP were demonstrated in all groups (Figure 5a1, c1, and e1). By the end of the experiment it was found that cell ingrowth was highest

Figure 2 (a) Morphology and eGFP expression of hASCs at the sixth passage. Spindle-shaped hASCs expressing green fluorescence filled the field of view. Fluorescence microscopy: magnification $10\times$. (b) Transfection efficiency of the sixth passage eGFP-Luc-hASCs. Fluorescence intensity graph of unlabeled cells (black area) and labeled cells (red area). (c) *In vitro*, BLI of serial dilution of eGFP-Luc-hASCs ($0-50,000$) seeded in a 96-well culture plate and a group of wells with unlabelled cells. The color bar describes the level of light production intensity. Lowest intensity = blue, highest intensity = red. (d) Plots of light production extracted from the images *versus* the number of eGFP-Luc-hASCs. R, correlation coefficient (eGFP, enhanced green fluorescent protein; hASCs, human adipose-derived stem cells; Luc, luciferase gene). (A color version of this figure is available in the online journal)

in the VIVO group than that in the other groups. However, the cell ingrowth was limited in the non-reseeded samples (Figure 5e1).

Masson trichrome staining was performed at the material site to detect the collagen content. Compared to the BLANK group, more collagen was present in the tissue samples from the reseeded group (Figure 6a-c). According to the collagen content assay of tissue-engineered products at 12 weeks after implantation, the highest percent of collagen content per unit area was in the VIVO group ($34.21 \pm 3.22\%$) followed by the VITRO group ($28.39 \pm 1.30\%$). The collagen content in BLANK group ($22.46 \pm 1.20\%$) was lower than that in the reseeded group (Figure 6d).

Immunohistochemical staining

At the end of the monitoring period implant sections were assessed by immunohistochemistry to detect the expression of contractile proteins and neovascularization needed for pelvic floor repair. Desmin (a marker for striated muscle) expression was observed around the ABP in the VIVO group (Figure 7). Subsequent immunostaining at the same site was positive for α -smooth muscle actin (α -SMA), a marker for both myofibroblasts and smooth muscle cells, thus indicating that significantly healthy myofibroblasts or smooth muscle cells were present in the VIVO group. α -SMA was densely positive around the ABP within implanted eGFP-Luc ASCs (Figure 7, black arrows in the

VIVO group). There were also numerous muscular vessels existing in the connective tissue adjacent to ABP (Figure 7, pentagram in the VIVO group). However, few smooth muscles were observed in the VITRO and BLANK groups (Figure 7). These results were proved by the FLI-1 (a marker for vascular endothelial cells) and CD31 (a marker for endothelial cells) staining, which suggest that the use of ABP reseeded with eGFP-Luc ASCs in mice, could regenerate smooth muscle and blood vessels.

Discussion

In current study, the viability of the hASCs reseeded on ABP was prolonged *in vivo* for up to 12 weeks. The ABPs were used as a potential biological scaffold, reseeded with hASCs, which were labeled by a double reporter lentiviral vector containing eGFP and Luc genes. These reseeded ABPs were implanted into a subcutaneous pocket. In the *in vivo* BLI, the labeled eGFP-Luc-hASCs reseeded on ABP were traceable up to 12 weeks. Further, the viability of these reseeded cells for a substantial period was also demonstrated. Hence, BLI is a useful technique for measuring cellular activity *in vivo*. Most transplanted ASCs existed in the implantation sites, suggesting that the optimal location for potentially active healing is at the repair site, because the implanted cells had very little or no tendency to migrate to other organs. These findings may be attributed to: (1) the ABP scaffold that provides an optimal microenvironment

Figure 3 (a) Scanning electron microscope of ABP. (b, c) Scanning electron microscope of the compound of hASCs-ABP, co-culture *in vitro* (3 days) (scanning electron microscope: original magnification, a, b: 100 $\times$). (d, e) *In vitro* eGFP-Luc-hASCs seeded on ABP scaffold for 3 days (fluorescence microscope: original magnification, d: 5 $\times$, e: 10 $\times$). eGFP-Luc-hASCs which expressed GFP grew along ABP. (f) CCK-8 assays. Cell proliferation between hASCs and eGFP-Luc-hASCs on ABP. (ABP, acellular bovine pericardium; CCK-8, cell counting kit-8; eGFP, enhanced green fluorescent protein; hASCs, human adipose-derived stem cells; *Luc*, luciferase gene). (A color version of this figure is available in the online journal)

Figure 4 *In vivo* bioluminescent imaging (BLI). (a) BLI analysis of two groups of eGFP-Luc ASCs on ABP at different time point after transplantation in mice. (Upper group: VIVO; Lower group: VITRO.) (b) Image of exposed thoracic and celiac cavity of eGFP-Luc ASCs inoculated mice at week 12. The mice were sacrificed by cervical dislocation, and then the implants were isolated (right plate), and the thorax and celiac cavity were exposed and imaged immediately (left plate). (c) Quantification of image data extracted from the image memory was background subtracted, and plotted versus the elapse time post-implantation. The solid line showed average values from 10 mice for photon counts for each time point. (d) BLI of three groups of mice at week 2 after transplantation. The color show the levels of light produced by implanted eGFP-Luc ASCs, and the arbitrary color bar and numbers demonstrate relative light intensities from the lowest (blue) to the highest (red). (A color version of this figure is available in the online journal)

Figure 5 H&E staining and DAPI staining sections of specimens at 12 weeks in each group. Frozen sections of implanted tissues were stained with DAPI, and green area displayed the survived transplanted eGFP-positive cells (b, d). Green fluorescence protein was expressed around the nuclei stained by DAPI. The vascular density in each group was displayed in a2, c2. a1, c1, and e1 displayed cells ingrowth into the ABP. Original magnification, 10 × (a, c, e, b, d, f), 40 × (a1, a2, c1, c2, e1, e2). (SK: skin; ABP: acellular bovine pericardium; DM: deep muscle.). (A color version of this figure is available in the online journal)

Figure 6 Masson trichrome staining in each group at 12 weeks after implantation. Collagen stained blue on staining, and could be observed in all the groups. (a, b, & c) The results of Masson trichrome staining of BLANK, VIVO, and VITRO group, respectively. Original magnification, 20 × (a, b, c). (d) Collagen content assay. VIVO group differed significantly from the other groups in the collagen content of the implanted materials ($P < 0.05$). * $P < 0.05$ vs. VITRO, ** $P < 0.01$ vs. blank. Quantitative image assay for 10 fields per specimen, two specimens per mouse, and 10 mice per group. (A color version of this figure is available in the online journal)

for cells to attach and grow; and hence, cells responded to unclear signals and were driven to repopulate a decellularized bovine pericardium scaffold; (2) long-term cell viability (both reseeded cells and host cells), which demonstrated neovascularization of the whole construct, which was adequate to support cell nutrition and reduce cell loss. These processes facilitated the successful biointegration and remodeling of the decellularized scaffold.

The amount of implanted cells decreased first and remained stable until the end of the experiment, thus, suggesting that the seeded eGFP-Luc-hASCs did not proliferate rapidly *in vivo* after transplantation. The percentage of transplanted cells was lower at 12 weeks (10%) than that on day 1, post-implantation. Despite the decrease in cell volume, there were still more than 5×10^4 cells surviving per cubic centimeter since the implantation of 2×10^6 cells according to the linear regression plots. We hypothesize that this loss could be attributed to: (1) handling procedure during transplantation leading to cell loss; (2) inflammation

after transplantation leading to cells necrosis; (3) apoptosis; and (4) possible cell migration to other tissue. Furthermore, the extent of repair by stem cells was associated with the number of surviving stem cells. Therefore, it is extremely important to ensure that reseeded cells are properly implanted; future experiments must aim at resolving this crisis.

In the present study, the VIVO group (a group in which cells were reseeded on ABP while implanting in the mice) was compared with the VITRO group (a group in which the same number of cells were reseeded on ABP *in vitro* prior to implantation). This study has demonstrated that the number of implanted cells in the VIVO group was approximately 13 times higher than that in the VITRO group on one day after transplantation, the significant difference in the VITRO group scaffold could be attributed to cell loss during co-culturing, transporting, and transplanting of the cells *in vitro*. Thus, this indicates that simultaneous transplantation of hASCs and ABP would be an optimal way to reduce cell loss. In the VIVO group, transplanted cells

Figure 7 Immunohistochemistry for Desmin, α -SMA, FLI-1, and CD31 (12 weeks after transplantation). Contractile protein (black arrows) and vascular endothelial cells (pentagram) were stained brown. The results of BLANK, VIVO, and VITRO group, respectively. Original magnification, 20 $\times$. (A color version of this figure is available in the online journal)

co-cultured with ABP *in vivo* rather than *in vitro*, and were inserted into the ABP under the internal environment.

Histology confirmed the survival of implanted eGFP-Luc-hASCs at 12 weeks after transplantation. Neovascularization and muscularis regeneration were observed on reseeded ABP, while there was none on the non-seeded ABP. In the reseeded cells, host cells could infiltrate in the ABP extensively. hASCs could attach to ABP and limit its reaction *in vivo*, which indicates that using reseeded ABP with a multipotent cell line, rather than ABP alone, is favorable for supporting cell ingrowth and neovascularization. The VIVO group was more effective than other groups, probably due to the presence of adequately implanted eGFP-Luc-hASCs. Thus, eGFP-Luc-hASCs acted as the main contributor. In addition, the hASCs secreted numerous angiogenic growth factors that promoted vascularization, thus, nourishing the injured tissue.^{18,19} This may be a possible reason for the high collagen content in the VIVO group scaffolds in this study. Ample hASCs prevented the degradation of implanted ABP, and stimulated fibroblasts

to promote the synthesis of collagen, as mentioned previously by Park *et al.*¹¹ In immunohistochemistry, plenty of muscle cells and vascular endothelial cells confirmed muscularis regeneration and neovascularization in the VIVO group, and α -SMA and desmin were detected at the same site in the VIVO group. This confirms that hASCs in the VIVO group could promote muscularis regeneration and neovascularization, presumably by the quantity effect. Therefore, ABP reseeded with ASCs during the implant operation may be more efficient than ABP implants co-cultured *in vitro* and ABP implants without cells, thus, emerging as a suitable option for POP repair.

Limitations of this study were the lack of details on the molecular mechanisms of fascia repair and regeneration of ASCs. Further, future research is proposed to be conducted in the vagina model of POP, rather than a subcutaneous pocket. The *in situ* evaluation of collagen and extracellular matrix is a reasonable approximation to estimate the efficacy of reseeded ABP. In addition, all experiments performed with only one isolation also were a limitation of

this study. And the healing characteristics of tissue-engineered products at a later time point after 12 weeks require further evaluation in future studies.

Conclusion

Based on the aforementioned results, we opine that the viability of hASCs reseeded on ABP is sustained even after 12 weeks. The hASCs reseeded on ABP *in vivo* during surgery may further enhance the properties of ABP. In addition, it may speeden the regeneration at the recipient site due to the presence of excess cells than scaffolds with cells reseeded on ABP *in vitro* prior to implant surgery. Therefore, it appears to be a promising approach for pelvic floor reconstruction.

Authors' contributions: QW, MD and JF designed the study, carried out experiments and interpreted results; PX, MH and YT carried out experiments; QW and MD wrote the manuscript. QW and MD contributed equally to this study.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare that there is no conflict of interest.

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