The Efficacy and Mechanism of Autologous Fat Stem Cells Combined with Microcarrier 6 Transplantation for Anal Fistula Treatment

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Research

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Abstract

Background: Adipose-derived stem cells (ASCs) function in multi-directional differentiation, proliferation, and tissue regeneration. It is not clear whether microcarrier 6 can promote the migration, differentiation, and regeneration of fat stem cells, and improve therapeutic effects for the treatment of anal fistula.

Methods: Japanese big ear rabbits were employed to establish an anal fistula model, and ASC-microcarrier 6 mixed transplantation was used as a treatment. HE staining, immunohistochemistry, and RNA sequencing were used to observe the effect of ASC-microcarrier 6 transplantation on anal fistula healing, in comparison with the ASC treatment group, rubber band operation group, and control group.

Results: HE staining indicated scattered striated muscle cells and epithelial tissues in fistula tissues of the ASC-microcarrier 6 complex group and the ASC treatment group, while a small number of lymphocytes were clustered around the microcarrier 6, and fat cell aggregation was seen in the ASC treatment group. RNA sequence analysis showed that differential genes were mainly concentrated in striated muscle cells, vascular smooth muscle, and other tissues. PI3K/AKT signaling pathway molecules were significantly enriched, granulation tissue and lymphocyte infiltration were observed in the rubber band string operation group, and a large amount of necrotic tissue was seen in the control group.

Conclusion: Microcarrier 6 is beneficial to the multi-directional differentiation of ASCs, which can provide a good environment for the survival of ASCs and promote fistula healing.

Introduction

Anal fistula is an infectious canal disease involving the rectum, anal canal, and perianal skin, and usually presents as a recurrent perianal infection. Anal fistula cannot heal spontaneously, and surgery is the main treatment for anal fistula at present [1]. However, conventional surgery is usually associated with a large wound surface and can damage the sphincter ani to varying degrees. Furthermore, surgery-related complications can lead to postoperative copracrasia, which can greatly affect the patients' life quality [2]. Developing alternative therapies to increase the healing rate of anal fistula and maximally preserve anal function has long been an urgent need [3].

Adipose-derived stem cells (ASCs) are mesenchymal stem cells that are found in adipose tissues. They possess self-regeneration and multi-differential potential and belong to the class of adult stem cells [4]. As compared to the bone marrow-derived hematopoietic stem cells, ASCs have a shorter doubling time, faster doubling speed, and display the potential of trans-germ-layer differentiation [5]. Besides, ASCs provide several advantages, such as ease of availability, abundance in the human body, and good tissue regeneration and repair effects. Under different induction conditions, ASCs can secrete a variety of growth factors through autocrine and paracrine effects. Moreover, ASCs can be induced to differentiate into many cell types, thus promoting epithelial and vascular regeneration and local blood supply reconstruction and recovery. ASCs are ideal seed cells for tissue regeneration. ASCs are widely used in tissue repair, reconstruction, and in the prevention and treatment of diseases of several systems,

including autoimmunity [6]. Clinical studies have reported the application of ASCs in the treatment of anal fistula, which has been widely considered due to several advantages, such as small wound areas, no damage to the anal sphincter, and rapid postoperative recovery [7]. However, the efficacy and safety of ASCs are not clear.

Many researchers believe that in the early stage of transplantation (before new blood vessels have been completely formed), the transplanted ASCs require a constant supply of nutrients from the extracellular matrix and interstitial fluid in the recipient tissues for their survival [8]. This explains the uncertainty regarding the success rate of ASC transplantation. Development of tissue engineering provides a new pathway to solve this problem. Microcarrier 6, manufactured by ELYON BIOTECHNOLOGIES LLC (USA), is composed of positively chargeable organic polymer composite and presents a multi-layer porous structure. Its pore size, positive charge density on its surface, and carrier particle size can be regulated by chemical synthesis. Microcarrier 6 is composed of organic compounds and displays resistance to contamination, low immunogenicity, good biocompatibility, and can be easily used in metabolic processes [9]. Our studies have shown that Microcarrier 6 complexed with cells provides a 3D multi-channel scaffold structure for the cells, so that the transplant can quickly establish a connection with recipient tissues. With an independent and organic relationship formed between Microcarrier 6 and the cells, a stable growth microenvironment can form for transplanted cells, thereby improving treatment effects [9].

According to the existing research, ASCs have been shown to play a positive role in the treatment of anal fistula, however, the efficacy requires further improvement. Microcarrier 6 can improve the survival rate and biological function of transplanted cells. ASCs and microcarrier-6 are used to repair and treat anal fistula. The therapeutic effect of ASCs combined with microcarrier 6 is observed and its mechanism is discussed.

Experimental Materials And Methods

1 Experimental materials

1.1 Experimental animals

Jinan Jinfeng Experimental Animal Co., Ltd. provided 24 4-month-old Japanese big ear rabbits, each weighing 2.5–3.5 kg. The rabbits were bred at the SPF Laboratory Animal Center of Affiliated Hospital of Jining Medical University [laboratory animal quality certificate No.: 37009700000659, license No.: SCXK (Shandong) 20180006]. All procedures of animal experiments conformed to the regulations of the Animal Ethics Committee of the Affiliated Hospital of Jining Medical University.

1.2 Reagents

RPMI 1640 medium, collagenase Ⅱ, fetal bovine serum (FBS), red blood cell lysis buffer (ACK), 1 × PBS, and trypsin were procured from Gibco (US).

1.3 Microcarrier 6 was provided by Elyon Bio-Technologies LLC (US).

2 Methods

2.1 Preparation of anal fistula rabbit model

For the preparation of the rabbit anal fistula model, referring to our previous study [10], sevoflurane was used for inhalation of general anesthesia and fixed after satisfactory anesthesia. Routine disinfection of perianal depilation was conducted, a sterile disposable cloth towel was laid, and an incision of the external anal fistula mouth from perianal to anal margin (0.5-1 cm) was performed with a self-made temperature control (shaping) electric knife. The internal anal fistula mouth is located in the anal canal 0.5–0.7 cm from the anal margin, forming a fistula between them, the temperature of the electric knife was controlled between 150–450 °C. After successful anal fistula preparation, a rubber band was implanted in the fistula. The two ends were ligated together with silk thread, and the rubber band could slide into the anal fistula. After 26 days, the rubber band was removed. Through visual inspection and probe examination of the perianal area of the rabbit anal fistula model, it was confirmed that the anal fistula model mimicked the human anal fistula model, and the model was deemed successful.
2.2 Extraction and culture of ASCs

After the rabbits received general anesthesia, the limbs were fixed, the skin of the operation area was treated, and normal disinfection was performed using iodophor, and then the sterile disposable cloth was laid. In the experimental group, a median incision about 4 cm long was made on the back of the rabbits, the subcutaneous tissue was separated, 5–20 g fat tissue was removed and placed into a sterile culture plate, and finally washed with normal saline. Excess hair and blood were removed, and cells were washed 2–3 times with PBS. The capsule, connective tissue, and blood vessels were removed under aseptic conditions. The adipose tissue was cut into 1 mm^3 pieces with small scissors, digested using 5 ml 0.05% collagenase solution in a constant temperature flask at 37 °C for 60 min, followed by the addition of RPMI medium containing 10% FBS to stop digestion. The suspension was centrifuged at 1000 rpm/min for 5 min to remove fat droplets in the upper layer and the supernatant. Cells were resuspended in PBS and passed through a 200-mesh filter. The supernatant was then centrifuged at 1000 rpm/min for 5 min and discarded. Cells were resuspended in RPMI medium containing 10% FBS, inoculated on 6-well plates, cultured in a 5% CO_2 incubator at 37 °C, and the medium was changed every 3–4 days according to cell growth. The primary cells were cultured for two weeks and then digested with 0.25% trypsin. Cell morphology was observed using an inverted microscope. Photographs were taken and cells were collected after third generation passage.

2.3 Construction of the ASC-microcarrier 6 complex

The microcarrier-6 was soaked in 75% alcohol for 24 h and washed with PBS thrice. Next, the microcarrier 6 was incubated in RPMI 1640 medium for 24 h. Subsequently, the microcarrier-6 was modified with stromal cell-derived factor-1α (SDF-1α) and vascular endothelial growth factor (VEGF); the concentration

of both was 100 ng/mL, and the incubation time was 3 h. Cells in the log-phase growth stage were harvested. Using trypan blue, the percentage of living cells was found to be above 95%, and the cell concentration was adjusted to 2×10^7/mL. The ASC suspension was mixed with the modified microcarrier-6 and cultured in a 5% CO_2 incubator at 37^0C for 24 h, until the cells reached a saturation state on the microcarrier-6 (under the microscope) [11].

2.4 Animal grouping

After successful development of the model, 14 rabbits were randomly divided into four groups: ASC-microcarrier 6 complex group (N = 4), simple ASC treatment group (N = 4), Operation group with thread hanging (N = 4), and control group (N = 2). Autologous ASCs were used for transplantation. For the ASC-microcarier-6 complex group, a mixture of 200 μl 2×10^7/ml ASC suspension and 100 μg microcarrier-6 was inoculated; the simple ASC treatment group was inoculated with 200 μl 2×10^7/ml ASC suspension; and the control group was inoculated with 200 μl 1 × PBS solution.

2.5 Surgical procedures for laboratory animals

(1) The inner mouth of the anal fistula was closed, we used an anal fistula scraper to remove necrotic tissue in the fistula and scrape the scar tissue and proliferative tissue of the inner wall of the fistula. Absorbable sutures were used to suture the inner mouth of the fistula. Physiological saline was injected from the outer mouth of the fistula to verify that the inner mouth was closed successfully. (2) ASC composite microcarrier scaffold transplantation was conducted by injecting 100 μl of tissue fluid with ASC composite microcarrier 6 scaffold into the tissue near the wound cavity of the fistula. To uniformly distribute cells, we used a multi-point injection method to inject the same amount of tissue fluid at 3, 6, 9, and 12 points around the fistula wound cavity. The non-invasive needle was replaced, and the remaining 100 μl of tissue fluid was injected through the external port into the entire fistula wound cavity. (3) The method of transplanting pure ASCs is the same as indicated in "2". (4) Operation group with thread hanging: the probe was slowly inserted from the outer opening of the fistula along the wound path of the fistula, the inner opening was penetrated and the probe end was bent, and finally pulled out from the anal opening. The thick wire connection of the tendon was tied to the probe head, and then the probe and the rubber band were pulled out. The rubber band was lifted to tighten the clamp along the base, and No. 7 silk thread was used under the vascular forceps. (5) Heavy rubber band ligation: The control group was inoculated with 200 μl of 1 × PBS. The experiment was ended by observing each feeding group daily and recording anal fistula healing.

2.6 Pathological examination and RNA sequencing

At the end of the experiment, the anal tissues (FIA-PBS, FIA-ETO, FIA ASCs, and FIA ASCs-Microcarrier 6) were fixed in 4% saline buffered formalin for pathological examination, and the same amount of tissues were frozen for RNA sequencing.

2.7 Statistical Analysis

The Student *t*-test was used to analyze the differences available in quantitative variables between the mentioned groups using the SPSS 16.0 software (SPSS, IL, USA). Herein, values of $p < 0.05$ were considered statistically significant.

Results

1.Morphological observation of ASCs

After the primary cells were cultured for 1 day, it was found that some cells were round with visible nuclei that adhered to the walls of the culture flask. On the 2nd day, the number of adherent cells increased, and some adherent cells were deformed, with an increase in cell diameter. On the 3rd day, the cells gradually elongated resembling a polygon shape. On the 7th day, the adherent ASCs presented with a uniform morphology and a long spindle shape. On the 12th day, most adherent cells were fused, presenting a colony-like distribution and fish swarm-like arrangement (Fig. 1a). Lipid droplets could be seen in certain individual cells. After two weeks of culture, the cells were uniformly distributed, and most appeared long and spindle-shaped, with nuclei in the middle. After cell passage, the cells became quickly adherent to the walls with relatively uniform morphology and few heteromorphic cells. The cells presented with a colony-like or fish swarm-like arrangement pattern (Fig. 1b).

After digestion with trypsin, ASCs became a single-cell suspension and co-incubated with microcarrier 6 for 4 h. It was then found that the ASCs adhered well to the microcarrier 6 with uniform distribution, and reached a saturation state (Fig. 1c).

2. Observation of treatment effect in anal fistula

After initiating anal fistula treatment, the healing procedure was observed on a daily basis, for a total of three weeks. All animals survived during the surgery and follow-up period (Table 1).

Table 1 Observation of treatment effect in anal fistula. Compared with the thread-drawing procedure group, the time required for healing was significantly shortened in the ASC-microcarrier 6 complex group ($t = 5.95$, $t > t_{0.01/2,4}$, $p < 0.01$); the time required for healing was also significantly shortened in the simple ASC treatment group as compared with the thread-drawing treatment group ($t = 11.88$, $t > t_{0.01/2,4}$, $p < 0.01$).

Table 1
Observation of treatment effect in anal fistula

	ASC-microcarrier 6 complex group	Simple ASC treatment group	Thread-drawing group	Control group
	(N = 4)	(N = 4)	(N = 4)	(N = 2)
Body weight (g)	3.53 ± 0.51	3.38 ± 0.54	3.30 ± 0.76	3.70 ± 0.28
Wound healing time (d)	7.00 ± 2.45	4.75 ± 0.43	15.25 ± 1.30	▯21
Healing rate (%)	100%	100%	100%	0%

There were no significant differences in the body weight between the different groups. During the observation period, all anal fistulas in the ASC-microcarrier 6 complex group, simple ASC treatment group and thread-drawing procedure group healed. When compared with the thread-drawing procedure group, the time required for healing was significantly shortened in the ASC-microcarrier 6 complex group ($t = 5.95$, $t▯t_{0.01/2,4}$, $p▯0.01$); the time required for healing was also significantly shortened in the simple ASC treatment group as compared with the thread-drawing treatment group ($t = 11.88$, $t▯t_{0.01/2,4}$, $p▯0.01$). According to a recently published clinical trial of perianal Crohn's disease, the success rate of cell therapy was 57% (95% CI 44–69%, n = 251) [12]. As compared with the control group, the ASC treatment showed a significant improvement in the healing rate of anal fistula (6–24 weeks, OR = 3.06 (95% CI 1.05–8.90); $p = 0.04$), the healing rate remained stable, and follow-up continued for a relatively long period of time (24–52 weeks, OR = 2.37 (95% CI 0.90–6.25); $p = 0.08$) [13]. In non-Crohn's perianal fistula (the second and third phases of study, n = 235), the success rate of ASC treatment in anal fistula was 71% and 57%, respectively [14]. These results agreed with our general conclusion.

The ASC-microcarrier 6 complex group exhibited a longer time to heal, when compared to the simple ASC treatment group, but the difference was statistically insignificant ($t = 1.81$, $t▯t_{0.05/2,6}$, $p▯0.05$).

3. Pathological examination

After three weeks of anal fistula treatment, the original anal fistula tissue was removed, under general anesthesia, for pathological section. In the control group, the anal fistula was not healed, abundant necrotic tissue was observed in the fistula, and considerable lymphocyte infiltration was observed with HE staining (Fig. 2d). All anal fistulas in the experimental groups healed, with the presence of fibrous granulation tissues growing at the original anal fistula site. HE staining was performed on the resected original anal fistula site. Low lymphocyte accumulation was observed around the microcarrier 6 in the ASC-microcarrier 6 complex group (Fig. 2a). This may be attributed to the fact that the ASC-microcarrier 6 complex was not yet completely absorbed by the surrounding tissues, given the short observation period, and thus immune-stimulation was produced at the local tissues. Pathological sections from the simple

ASC treatment group were found to contain many lipid vesicles (Fig. 2b), which may be due to the replacement of the fistula tract by adipose tissues differentiated from ASCs. Pathological sections from the thread-drawing procedure group showed granulation tissues and lymphocyte infiltration (Fig. 2c), which might be caused by reactive hyperplasia of tissues surrounding the fistula tract under stimulation from the thread.

4. ASC-microcarrier 6 transplantation for anal fistula mainly promotes tissue regeneration through multi-directional differentiation, and it has been found that PI3K/AKT signaling pathway molecules are significantly enriched

In this study, a whole-genome expression profiling chip was tested, and high-throughput RNA sequencing was performed to screen for differentially expressed genes. After data analysis, differentially expressed genes were observed in the ASC transplantation group and ASC-microcarrier 6 composite treatment group, compared with normal control tissues. These genes were mainly concentrated during differentiation of various tissue types, such as striated muscle, vascular smooth muscle, and epithelial cells. The differential genes are mainly concentrated in the construction and differentiation of striated muscle. Tissue regeneration for fistula repair was promoted via multi-directional differentiation. Further functional enrichment analysis of differential genes (KEGG pathway) indicated that PI3K/AKT signaling pathway molecules were significantly enriched, suggesting that the PI3K/AKP signaling pathway may play an important role in anal fistula treatment.

5. Gene expression profile difference GOterm analysis shows that ASC -microcarrier 6 promotes muscle/striated muscle differentiation and contraction

Gene expression profile differences between the anal fistula control group and ASC-microcarrier 6 treatment group were analyzed using the GOterm relationship network. The results suggested that ASC-microcarrier 6 transplantation can promote differentiation, development, and contraction of muscles in anal fistula (Fig. 3 AB).

Discussion

Anal fistula is an infectious disease occurring in the perianal region. The conventional thread-drawing procedure can ensure a satisfactory healing rate, despite the drawbacks regarding large postoperative wound surfaces and increased healing time. If the sphincter ani is severely damaged during surgery, patients may suffer from fecal incontinence, which greatly impairs quality of life [2].

ASCs are a type of mesenchymal stem cell, which are abundant in the human body, can be easily harvested, and have multi-directional differentiation, self-replication ability, and strong immune tolerance, as well as the ability to suppress CD4 + T cells [15]. Ideal applied stem cells can be the best source for stem cell transplantation and treatment [16]. ASCs can secrete a variety of growth factors, cytokines, anti-inflammatory factors, and chemokines [17]. These anti-inflammatory factors and chemokines may play an important role in defending the immune response against infectious inflammation.

Therefore, ASCs can be used as seed cells to treat anal fistula, promote wound tissue repair, and wound surface healing. It is becoming a research hotspot in injury repair and regenerative medicine. Analysis of all clinical research data employing foreign stem cell treatments for anal fistula indicate that allogeneic adipose stem cells are advantageous due to being minimally invasive, reducing anal sphincter injury, reducing pain, and decreasing hospitalization [18]. Jiang Bin *et al*. reported the use of autologous adipose stem cells to treat 23 patients with complex anal fistula. The clinical study has verified the effectiveness and safety of autologous stem cell transplantation for the treatment of anal fistula. The total cure rate was 69.57%, of which 11 cases were Crohn disease anal fistula with a cure rate of 90.91%, 12 cases of glandular anal fistula exhibited a cure rate of 50% [19]. In terms of experimental research, Ferrer *et al.* established an animal model of Crohn's disease anal fistula in dogs, and then treated them with human mesenchymal stem cells. The efficacy and safety were observed. No rejection occurred. All dogs' fistulas healed within three months. Fistula recurred in two dogs, with a cure rate of 66.67% [20]. Several scholars have also suggested introducing a novel fistula treatment in animal models. Volpe BB *et al.* attached mesenchymal stem cells to absorbable sutures and achieved significant results in the treatment of rabbit intestinal fistula models, which displayed a significant improvement over sutures alone [21]. To date, only a small sample of retrospective studies have been conducted involving the use of ASCs in the treatment of anal fistula. Research for anal fistula treatment methods is still at the exploratory stage, and no consensus has been reached concerning the origin of adipose tissues of ASCs, separation and culture workflow, or injection dose and frequency.

It has been found that the use of scaffolds can improve the survival rate and biological function of transplanted cells. A scaffold provides a reticular structure, to which the stromal cells and ASCs adhere to form a microstructure. Injection of ASCs into lesions lead to micro-environmental changes of the anal fistula, thereby producing a synergistic effect on the treatment.

Experiments show that autologous ASC transplantation can improve the prognosis of anal fistula. A comparison of pathological sections from the ASC-microcarrier 6 complex treatment and the simple ASC treatment indicated that a small number of lymphocytes infiltrated the tissues near the microcarrier 6, possibly due to the incomplete absorption of microcarrier 6 by the surrounding tissues. Thus, the amount of microcarrier 6 can be reduced depending on the number of transplanted ASCs, while local physiotherapy can be applied to the transplanted site to lower the reactivity of local tissues to the transplant.

We also performed whole-genome expression profiling microarray testing and high-throughput RNA sequencing to screen differentially expressed genes. The data indicated that the ASC-microcarrier 6 combined treatment group expressed differential genes compared to the normal controls. These genes were primarily concentrated during differentiation of various types of tissues, such as striated muscle, vascular smooth muscle, epithelial cells, and nerve cells. The differential genes are mainly concentrated in the construction and differentiation of striated muscle. Tissue regeneration for fistula repair was promoted via multi-directional differentiation. Further, KEGG pathway data indicated that PI3K/AKT signaling pathway molecules were significantly enriched, suggesting that the PI3K/AKP signaling pathway may play an important role during anal fistula healing. S Lin *et al*. found that insulin-like growth factor 1 (IGF-1) can promote endothelial cells/fat by activating the PI3K/AKT signaling pathway by observing vascular formation in stem cell co-culture systems [22]. H Zhou reports that the PI3K/AKT signaling pathway promotes paracrine functions of ASCs [23].

Conclusion

Our experiments show that the transplantation of ASCs together with microcarrier 6 is a therapeutic approach with high potential for the treatment of anal fistula: microcarrier 6 can promote ASCs differentiation and the rapid healing of anal fistula *in vivo*. However, we further need to unveil the complete mechanism of action of our approach. Furthermore, we also plan to improve the effect of ASCs transplantation for the treatment of anal fistula via optimization of microcarrier 6 concentration and degradation rate, and via modification of PI3K / Akt signaling. Last but not least, the full characterization of ASCs + microcarrier 6 safety and long-term efficacy, very important for future translation to the clinical context, will be our next step.

Abbreviations

ASCs - adipose-derived stem cells; ETO -rubber band string operation ;FBS - fetal bovine serum; FIA -anal fistula ; IGF-1 - insulin-like growth factor 1; HE - Hematoxylin & Eosin; OR - odds ratio; PBS - phosphate buffered saline; PI3K/AKT - phosphatidylinositol 3 kinase (PI3K) / protein kinase B (AKT); RNA - ribonucleic acid; SDF-1α - stromal cell-derived factor-1α; SPF - specific pathogen free; VEGF- vascular endothelial growth factor.

Declarations

Ethics approval

All procedures of animal experiments conformed to the regulations of the Animal Ethics Committee of the Affiliated Hospital of Jining Medical University.

Publication consent

Not applicable

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing Interests

All authors declare that they have no competing interests.

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Authors' contributions

Professor MC was responsible for the design and implementation of animal experiments and ASC-related experiments in this research, and was also involved in the writing of manuscripts. Professor FH guided the experimental research and conceived the manuscript. YL was mainly responsible for rabbit anal fistula model surgery. MF participated in the operation of ASCs / microcarrier 6 transplantation to rabbit anal fistula model ward and recorded. HL wrote the first draft of this article and participated in related experiments of ASCs / microcarrier. XX participated in the experimental observation and recorded the wounds, SW was responsible for the collection of experimental data. LK was the surgical assistant. YB extracted ASCs from the tissue. HT and SC extended ASCs *in vitro* and inoculated them into the scaffold. Each author participated in this research and provided valuable suggestions for this article. All authors have reviewed and approved the manuscript.

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Not applicable

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Figures

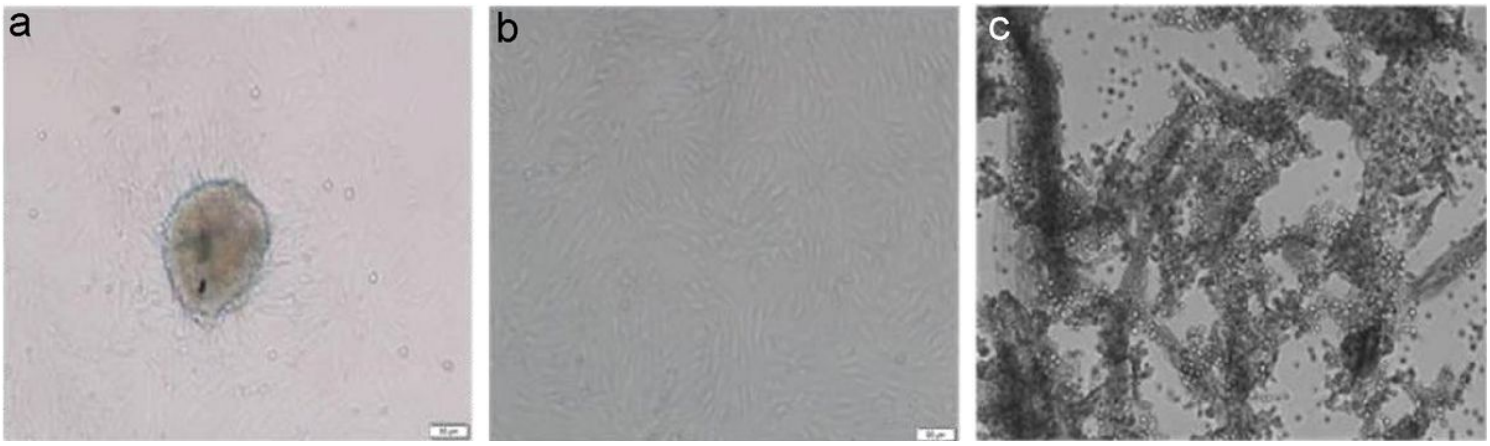

Figure 1

Morphological observation of ASCs. a) On the12th day, most adherent cells were fused, presenting a colony-like distribution and fish swarm-like arrangement. b) The cells presented with a colony-like or fish swarm-like arrangement pattern after cell passage. c) The ASCs adhered well to the Microcarrier 6 with uniform distribution and reached a saturation state.

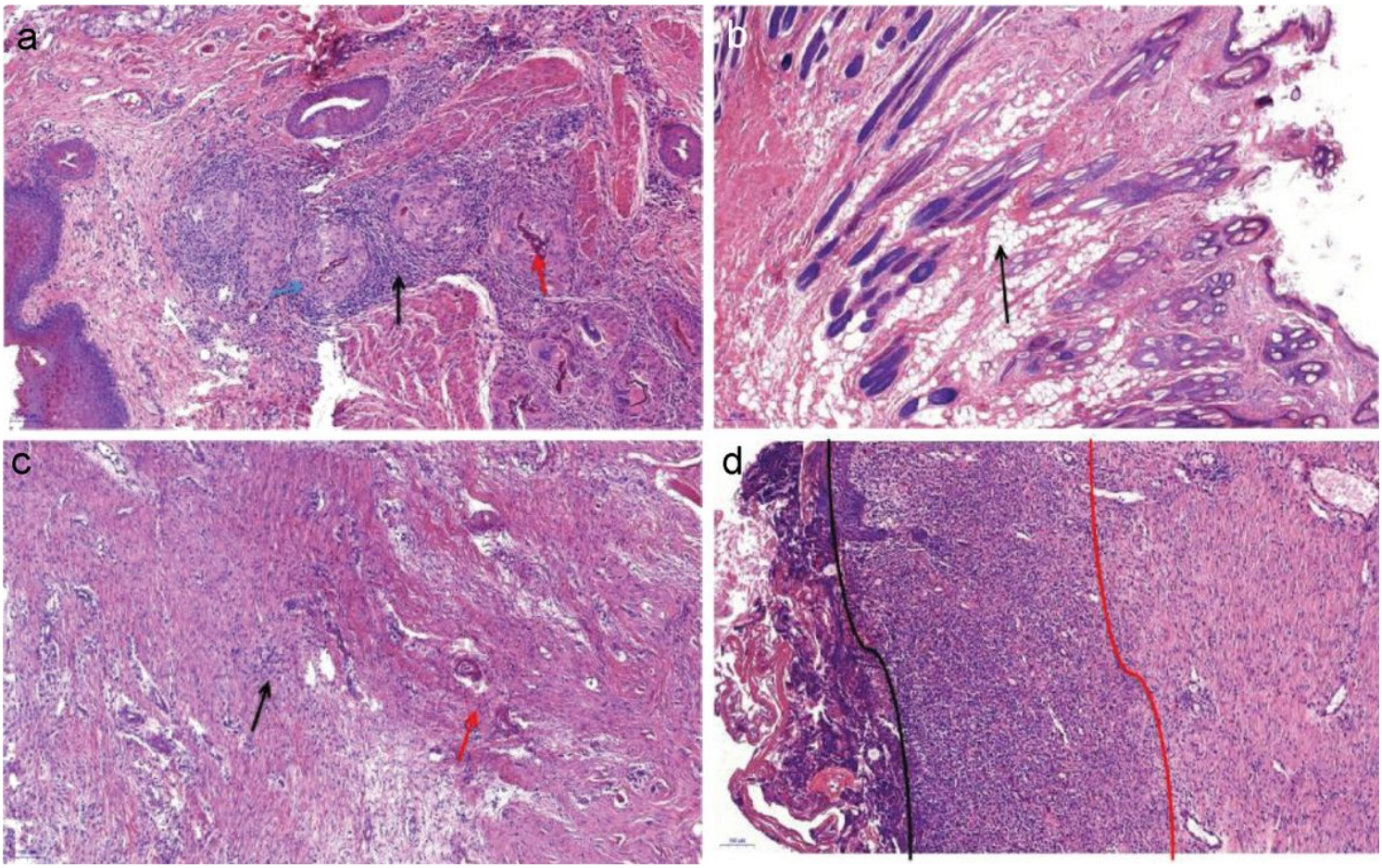

Figure 2

Pathological examination. a) Accumulation of a small number of lymphocytes (black arrow) was observed around the Microcarrier 6 in the ASC-microcarrier 6 complex group. The red arrow indicates remnant scaffold in the center of the cell and the blue arrow indicates squamous cell derived from ACSs. b) Pathological sections from the simple ASC treatment group were found to contain many lipid vesicles (black arrow). c) Pathological sections from the thread-drawing procedure group showed tissue granulation and lymphocyte infiltration (black arrow). The red arrow indicates collagen. d) HE staining revealed infiltration of many lymphocytes in the control group. The left area exhibits fibrokeratoma necrosis, the middle area depicts lymphocyte infiltration, and the right area depicts fibers and collagen. Scale Bar, 100μm.

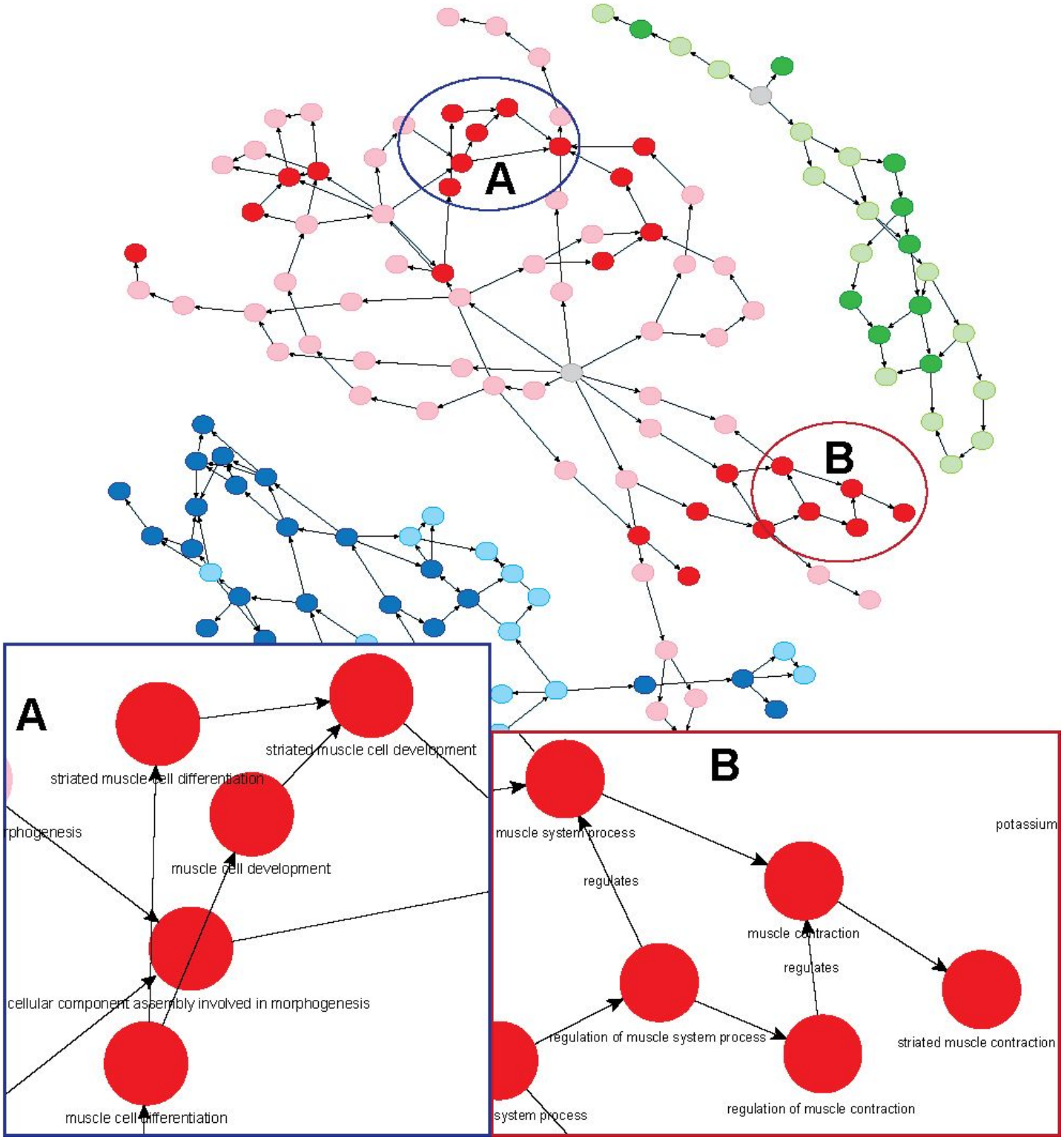

Figure 3

GOTerm relationship network A, muscle / striated muscle differentiation and development; B, muscle / striated muscle contraction Each node represents a GOterm, with different colors representing different functional classes to which it belongs, namely red for biological processes, blue for cellular components, and green for biological processes. Dark colors indicate GOterms with significant enrichment (Q-value <0.01), light colors indicate GOterms with insignificant enrichment, and gray terms indicate no enriched

GOterms. Similar to the directed acyclic graph, the relationship between GOterms can be seen. The solid arrows indicate the containment relationship, the dashed arrows indicate the regulatory relationship, the red dotted line indicates positive regulation, and the green dotted line indicates negative regulation.