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Original Article

The influences of adipose-derived stem cells (ASCs) on epithelial-mesenchymal cross-talk factors in the early stage of scar formation

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Abstract: This present study was designed to explore influences of adipose-derived stem cells (ASCs) on epithelial-mesenchymal cross-talk (EMCT) factors in early stage of scar formation (EFS). Rabbit autologous ASCs were separated, and rabbit ear hypertrophic scar (HS) model was established. ASCs were injected to scar in early stage (once every five day for 3 times) after epithelization of wound surface. Then, HS appearance was observed and the HS specimens were collected for histopathological observation and EMCT factors detecting (IL-1α, TGF-β1 and KGF, and the proliferation and differentiation indexes of keratinocytes including PCAN and CK10). In the results, compared with control group, scar tissues of the experimental group were soft with flat surface and light color, HE staining showed epithelial cell number was increased with epithelial foot and dermal papilla structure, and Masson staining showed collagens were neatly arranged with lighter dyeing color, decreased density and broadened clearance. Red fluorescence was visible in dermis of experimental group, suggesting ASCs were survived after local transplantation. IL-1α and KGF were up-regulated while TGF-β1 was down-regulated in experimental group ($P<0.05$); and both CK10 and PCAN were up-regulated in epithelium ($P<0.05$). RT-PCR results showed TGF-β1 was down-regulated in experimental group, while IL-1α was up-regulated in epidermis but down-regulated in dermis ($P<0.05$). In EFS, local transplantation of ASCs can affect EMCT, inducing proliferation and differentiation of keratinocytes, improving fibroblasts' functions and promoting regeneration and healing of wound surface.

Keywords: Adipose derived stem cells (ASCs), rabbit ear scar model, epithelial- mesenchymal cross-talk, TGF-β1, IL-1α

Introduction

Hypertrophic scar (HS), whose mechanism remains unclear, is pathologically characterized by excessive generation and deposition of large amounts of connective tissue matrix such as collagen, and loss of control in proliferation of fibroblasts in epithelium and dermis of scar tissues. It is the result of excessive healing responses of wound, and can cause the damage on appearance of tissues and organs and different levels of dysfunction. HS seriously harms the people's physical and mental health, which has aroused highly attention. Currently, it is generally believed that non-surgical intervention for hypertrophic scar should be implemented in early formation of hyperplastic scar. Early treatment methods mainly include drug therapy, radiotherapy, oppression therapy, biological therapy, gene therapy, laser therapy, etc., but its curative effect is not satisfactory. MSCs have been confirmed the roles of promoting wound healing, improving the quality of wound healing and reducing the formation of hypertrophic scar, etc. in recent studies [1, 2], but its mechanism has not yet been entirely cleared. Previous studies about mechanism of scar formation mainly focus on the dermal fibroblasts.

In recent years, it has been found that epithelial-mesenchymal cross-talk plays an important role in wound healing and scar formation [3, 4]. Study found complex loop in epithelial-mesenchymal cross-talk, and the core mediators are IL-1α, TGF-β1 and KGF secreted by keratinocytes (KC) and fibroblasts [5, 6]. In vitro studies have found that MSCs can participate in the

epithelial-mesenchymal cross-talk, improve the proliferation and differentiation of KC and inhibit the apoptosis of KC, and more important, induce the KC to form stratified epithelium [7]. But it is rare reported that whether MSCs can intervene the in vivo epithelial-mesenchymal cross-talk in the early formation of HS after epithelialization of wound and induce the reconstruction of epithelium in the stage of scar proliferative stage in order to improve the quality of wound healing.

This study chose the available adipose-derived stem cells (ASCs) with extensive sources and fast proliferation as the research objects. By establishment on the model of rabbit ear scar, this study intervened the scar hyperplasia with ASCs in the early stage of scar remodeling after epithelialization of wound, and preliminarily discussed the influences of ASCs on factors related to epithelial-mesenchymal cross-talk in the wound shaping period and on proliferation and differentiation of KCs, to be expected to further explore the mechanism of MSCs in improving the quality of wound healing.

Materials and methods

Reagents and instruments

SP immunohistochemical kit was purchased from Beijing Biosynthesis Biotechnology Co., Ltd.; primary antibodies of PCAN and IL-1a were purchased from Abcam Company; TGF-β1, CK10 and KGF were purchased from Beijing Biosynthesis Biotechnology Co., Ltd.; primers of TGF-beta 1, IL-1 a primer bought in Shanghai Sangon Biotechnology Co., Ltd. Fluorescence quantitative PCR was bought from IcyclerIQ Bio-RAD Company (USA), while PCR reverse transcription instrument was bought in Thermal Company (USA).

Establishment of scar models and grouping

Nine adult New Zealand big-eared white rabbits (weighting 2-2.5 kg, aging 4 months on average) were provided by the Animal Experimental Center of the Third Military Medical University, and raised separately. Circular surgical area with diameter of 1.0 cm was designed on the ventral surface of the rabbit ears after anesthesia, four on each rabbit ear, with the interval of wound for 1.5 cm. A full-thickness skin wound was made with a scalpel along the design line; then the cartilage was fully exposed and the perichondrium was removed; and a good haemostasis was given. There were four wounds on each rabbit ear and eight on each rabbit, wrapped with sterile dressing. The dressing was changed every other day or corresponding to the conditions. Till the wound were completely epithelized. Nine New Zealand rabbits were randomly divided into the experimental group (ASCs group), the control group (PBS group), and the model group with the graphical method, and there were 3 animals (24 wounds) in each group.

Separated culture and identification of rabbit ASCs

Inguinal fat pad was incised from New Zealand big-eared white rabbits after anesthesia. The adipose tissue was rinsed repeated with PBS liquid containing double antibody, cut into slurry and placed in sterile centrifuge tubes. Then the adipose tissue was digested with 2 mg/ml of type I collagenase, and then centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded, and the sediment was added with medium. After blowing, the mixture was filtered with gauze (200 mesh), and the filtrate was implanted into culture bottle. The labeled culture bottle was placed in incubator at 37°C and 5% CO_2 for cell culture, and the cell adhesion condition was observed on the next day. When the primitive cells were fused for 80%-90%, the medium was abandoned and the cells were rinsed with sterile PBS for 3 times. 1 ml of 0.25% trypsin was added to digest in the incubator, and the digestion was observed under microscopy after 1-3 min. The cells were floated in round shape, and separated from the bottom of the culture bottle. Fresh culture medium containing 10% FBS was added, and the digestion was terminated at 1:1. The cells were transferred into the sterile centrifuge tubes, and centrifuged at 1000 rpm for 5 min. The supernatant was discarded. The cells were given passage at a ratio of 1:3, and placed in CO_2 incubator for culture, and the culture medium was changed every 2-3 days.

There are no standard labeled antibodies of rabbit ASCs surface molecule. This study identified ASCs by multi-directional differentiation. Aseptic cover glass was placed in six-well plate. And well-grown, third generation of rabbit ASCs were taken for growing on the glass slide. The

cells were observed under microscopy. When the cells were fused to 80%-90% and climbed the whole cover glass, the original medium was abandoned and the cells were rinsed by PBS buffer. The conventional medium (complete medium L-DMEM containing 10% FBS) was replaced for adipogenic induction medium, osteogenesis induction medium, and cartilage-formation induction media, for related induction culture and staining observation according to the instructions.

ASCs fluorescent label

The third generation of rabbit ASCs with active growth and in good condition were taken, washed with PBS buffer for 3 times, and digested with 0.25% trypsin for 1-3 min. And after neutralization with 10% fetal bovine serum (FBS) medium, the cells were transferred into the centrifugal tube, centrifuged at 1000 rpm for 5 min, washed by PBS and collected. Then the cells were incubated with CM-Dil in an incubator at 37°C for 30 min and observed under the fluorescence microscopy. The cells were re-suspended with PBS buffer, washed repeatedly, and centrifuged. The residual dil markers were removed, the cell suspension was prepared, and the cell density was adjusted to 5×10^6/ml to be used.

Intervention on scar formation with local transplantation of ASCs

After epithelialization of rabbit ear wound surface (21 days after modeling), the experimental group was annularly injected with 0.1 ml of the above prepared autologous ASCs suspension into the upper cartilage of rabbit HS models in the experimental group (concentration: 5.0×10^6/ml), while the control group was annularly injected with 0.1 ml of PBS buffer into the upper cartilage of rabbit HS models in the control group, once every five days, for a total of 3 times; the model group was not given any processing.

HE and Masson staining and immunohistochemical detection on scar tissues

Rabbit HS model specimens of each group were taken at 9 days after the third times of injection (40 days after modeling). And part of the tissues were fixed with 4% neutral formaldehyde, embedded with conventional paraffin, sectioned, and stained with HE and Masson staining. And the histopathological changes were observed. Immunohistochemistry was used to detect the expression changes of epithelial-mesenchymal cross-talk related factors including IL-1α, TGF-β1 and KGF, and the expression changes of epidermal layer (mainly KC) proliferation and differentiation indexes including PCAN and CK10.

RT-PCR was used to detect the expressions of IL-1α and TGF-β1 in scar tissues

Each group of scar tissues was separated into epidermis and dermis. The total RNA was extracted according to the instruction of Trizol. 1 ug total RNA was taken for reverse transcription reaction according to the kit instruction and synthesize cDNA. Then PCR amplification was carried out. Primer sequence: sequence of rabbit IL-1α primer: upstream primer: 5'TTGTTTGTTGTTGAAAGATCACCCT3', downstream primer: 5'TTTGGTAGCCATAGTCAGTAGCCCT3', the amplified fragment of about 110 bp long; sequence of rabbit TGF-β1 primer: upstream primer: 5'GGCTCACCTTCTGCCCGTCT3', downstream primer: 5'GTCTCGGTATCCCACGAAAGAAACG3', the amplified fragment of about 157 bp long; sequence of β-actin primer: upstream primer: 5'CATCCTGACGCTCAAGTACCCGA3', downstream primer: 5'CCACGCGAAGCTCGTTGTAGAA3', the amplified fragment of about 101 bp long. PCR was carried out in the following program: pre-degeneration at 95°C for 30 sec; generation at 95°C for 5 sec and annealing at 59°C for 30 s, for a total of 40 cycles; one fluorescence was taken each 0.5°C for 10 sec starting from 65°C, for a total of 81 times. It was calculated with sample Ct value as the statistical parameter, and the results were presented with mean ± standard deviation ($\bar{x} \pm s$).

Statistical analysis

Experimental results were statistically analyzed with SPSS 20.0 and presented as mean ± standard deviation ($\bar{x} \pm s$). *t*-test and homogeneity test of variances showed the experimental results were in normal distribution and the variance was homogeneous. And single-factor analysis of variance was adopted, if the differences of expression were of statistical significance, least significance difference (LSD) was further adopted for pair-wise comparison. And the distribution was not in accordance with normal distribution, non-parametric test was

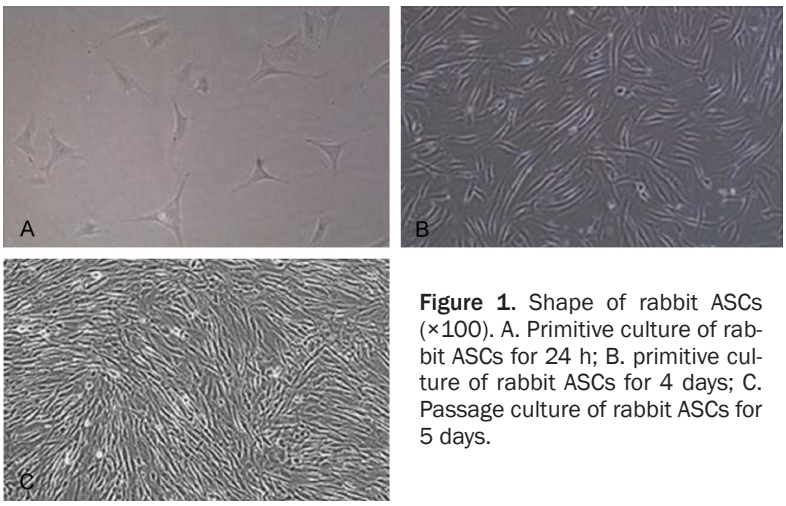

Figure 1. Shape of rabbit ASCs (×100). A. Primitive culture of rabbit ASCs for 24 h; B. primitive culture of rabbit ASCs for 4 days; C. Passage culture of rabbit ASCs for 5 days.

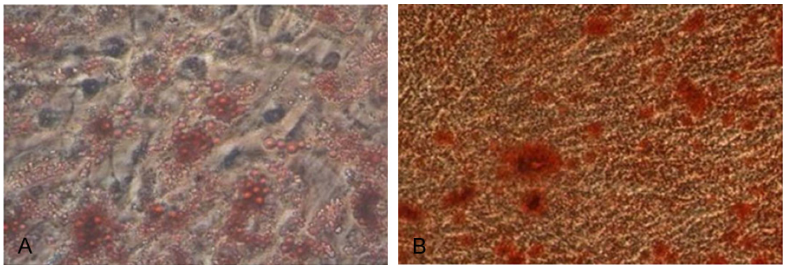

Figure 2. Multi-directional induced differentiation of rabbit ASCs. A. ASCs adipogenic induced differentiation (×200); B. ASCs osteogenesis induced differentiation (×100).

adopted for multi-samples and multiple comparisons. $P<0.05$ regards as statistically significant difference.

Results

Identification of ASCs

After 24 h of transplantation of primitives, scattered adherent cell growth was visible under inverted microscope. The cells, in short fusiform or polygon, were disorderly arranged, showing fibroblast-like growth. The cells could cover the bottom within 3-5 days. The cells were scattered irregularly, in even shape. Colony did not appear. The cells were arranged directionally, and grew fast. One generation was generated within 3-5 days. With the times of passage, the cell growth was gradually slowed down, and some cells were enlarged, with vacuole inside, and pseudopodia (**Figure 1**).

ASCs adipogenic induction: ASCs were fused into patchy or elliptic form after 2 weeks. Then the cells were gradually increased in volume, with lipid drops of different sizes in the cytoplasm. Lipid drops can account for 80%~90% of the entire volume in some cells. Oil red O staining at 21 days of induction culture showed red-stained lipid droplets (**Figure 2A**).

ASCs osteogenesis induction: Cells were in polygonal or irregular shape, distributed in clusters and grew overlapped. As the extension of time, the cell cluster was gradually increased. After 21 d of induction, the cells were stained with alizarin red S and red calcified nodules were visible (**Figure 2B**).

Gross findings and histopathological observation

Fourteen days after modeling, scar completely covered the wound surface in each rabbit ear. Compared with the control group and the model group, the scars were soft, with flat surface and light color (**Figure 3C**) in the experimental group on the 40th day. While red hard HS tissues rose above the skin were still visible in the control group and the model group.

HE staining results showed that: In the experimental group, the number of epithelial cell layer was increased, and epithelial foot and dermal papilla structure were visible in some epithelium and dermis (**Figure 4A**). While the epithelial layer was thinner in the model group and the control group as shown in HE staining, while epithelial foot and dermal papilla structure were not seen (**Figure 4B** and **4C**).

Masson staining showed that collagen was dyed blue. In the experimental group, the collagens were neatly arranged, with lighter dyeing color, decreased in density and broadened in clearance (**Figure 5A**); while in the control group and model group, blue-stained collagen fibers were disorderly arranged, in the spiral shape; a large amount of collagen was depos-

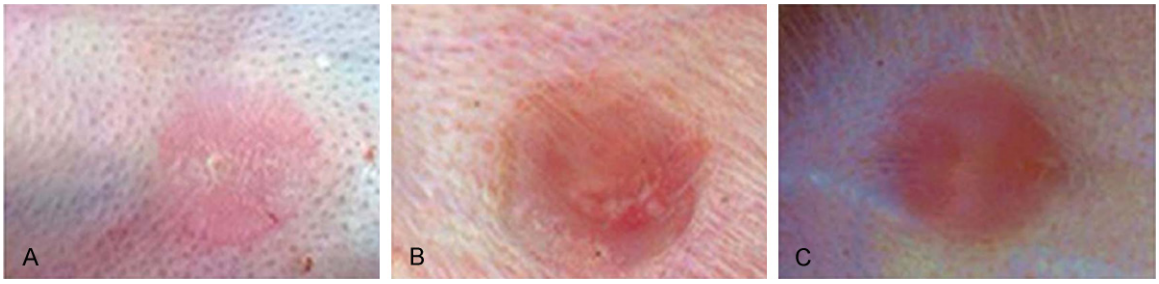

Figure 3. HS on the 40th day of modeling. A. HS of rabbit ear in the experimental group; B. HS of rabbit ear in the control group; C. HS of rabbit ear in the model group.

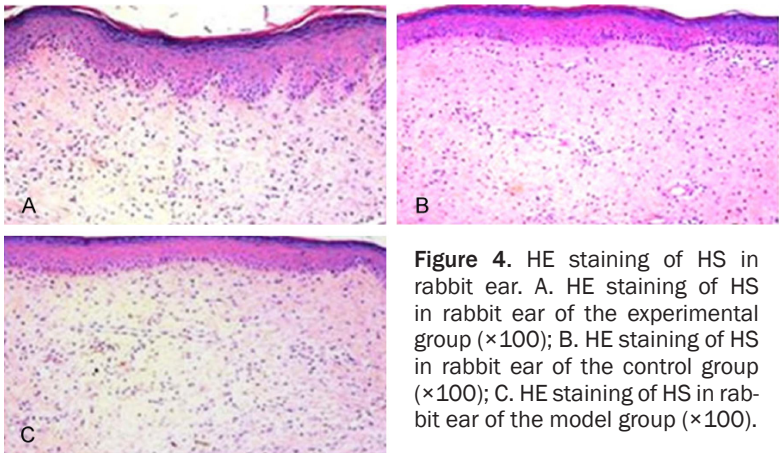

Figure 4. HE staining of HS in rabbit ear. A. HE staining of HS in rabbit ear of the experimental group (×100); B. HE staining of HS in rabbit ear of the control group (×100); C. HE staining of HS in rabbit ear of the model group (×100).

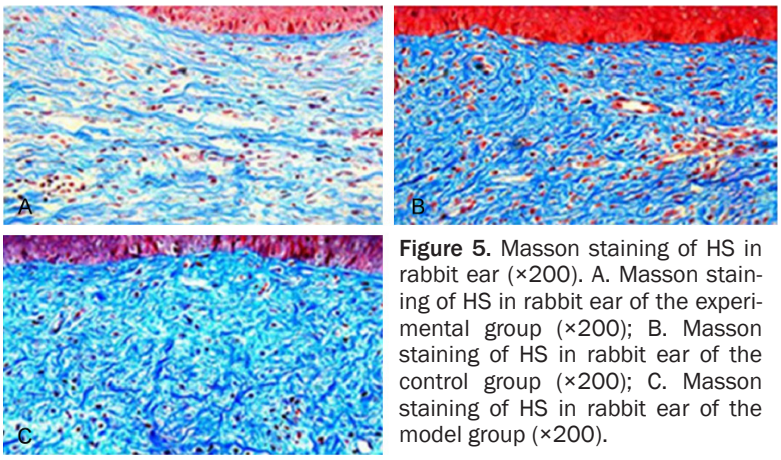

Figure 5. Masson staining of HS in rabbit ear (×200). A. Masson staining of HS in rabbit ear of the experimental group (×200); B. Masson staining of HS in rabbit ear of the control group (×200); C. Masson staining of HS in rabbit ear of the model group (×200).

ited, and the density was obviously increased compared with the experimental group (**Figure 5B** and **5C**).

Tracing on transplanted ASCs with immunofluorescence

Forty days after operation on rabbit ear HS models, immunofluorescence staining detection on the taken tissues showed: cell membrane showed red fluorescence through the dyeing of CM-Dil (**Figure 6A**); The frozen sections were observed under fluorescence microscopy and there were scattered fluorescent distribution in the experimental group, suggesting ASCs were survived in the tissues (**Figure 6C**).

Epithelial-mesenchymal cross-talk related factors expressing

In the scar tissues, IL-1α was expressed in the epithelium and dermis, TGF-β1 was expressed in the epidermis and dermis, KGF was expressed in the dermis and epithelium, PCNA was expressed in the epithelium (mainly in KC) and dermis, CK10 was expressed in the epithelium (mainly in KC) (**Figures 7-11**). Expressions of IL-1α, KGF, PCAN and CK10 were all significantly up-regulated in the scar tissues of the experimental group ($P<0.05$), while the expression of TGF-β1 was obviously down-regulated ($P<0.05$) (**Table 1**).

RT-PCR results

RT-PCR was used to express mRNA of IL-1α and TGF-β1 in the epidermis and dermis of rabbit ear scar tissues. Compared with the control group and the model group, mRNA expression of IL-1α was up-regulated in the epidermis while

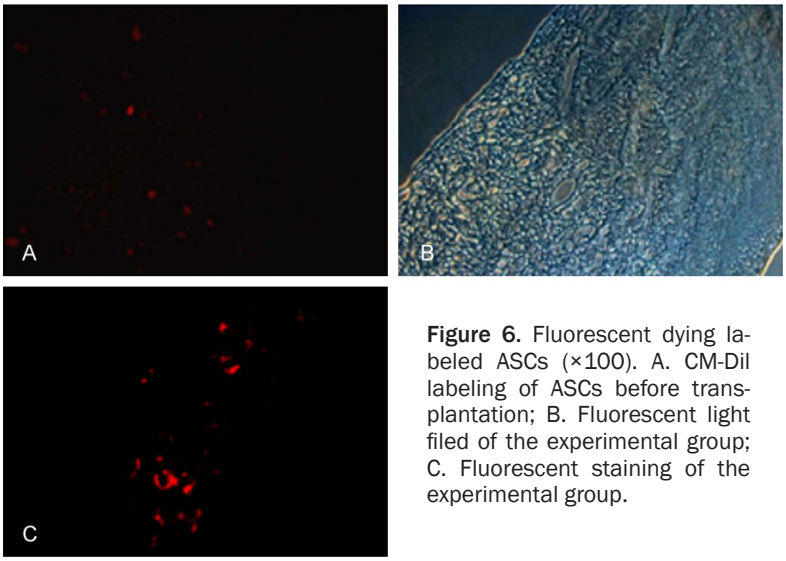

Figure 6. Fluorescent dying labeled ASCs (×100). A. CM-Dil labeling of ASCs before transplantation; B. Fluorescent light filed of the experimental group; C. Fluorescent staining of the experimental group.

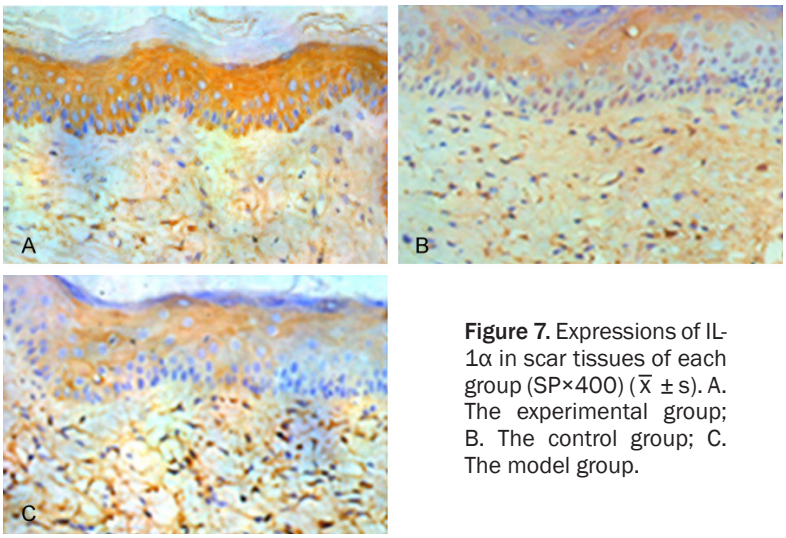

Figure 7. Expressions of IL-1α in scar tissues of each group (SP×400) ($\bar{x} \pm s$). A. The experimental group; B. The control group; C. The model group.

down-regulated in the dermis in the experimental group, with statistically significant differences ($P<0.05$); and mRNA expressions of TGF-β1 were obviously down-regulated in both epidermis and dermis, with statistically significant differences ($P<0.05$); compared with the control group and model group, there were no obvious changes in mRNA expression of IL-1α and TGF-β1 ($P>0.05$) (**Table 2**).

Discussions

Hypertrophic scar is the results of excessive repair or excessive healing after dermal damage. Its essence is dermal fibrosis disease. Hypertrophic scar is characterized by excessive generation and deposition of large amounts of connective tissue matrix and collagen, and loss of control in proliferation of fibroblasts. It can cause the damage on appearance of tissues and organs and different levels of dysfunction. And it has seriously harmed the people's physical and mental health, which has aroused highly attention. Its pathogenesis is not clear, and though the treatment methods vary in quality, their effects are not satisfactory. With the economic development and social progress, people are having higher requirement on life quality and appearance, and treatments for hypertrophic scar will undoubtedly become the focus of attention. But the research on pathogenesis, prevention and treatment of scar has been little progressed due to the animal scar models. Since 1997, when Morris [8] for the first time put forward the concept that the rabbit ears scar models were similar to HS model, this model has gradually been recognized by domestic and foreign scholars after numerous researches [9, 10]. A wound surface with diameter of 1 cm was designed on the ventral surface rabbit ear in this experiment, referenced to the previous literatures. And there were a total of 4 wounds on each ear. The skin and perichondrium were removed to establish HS models. And the results showed that average postoperative epithelization of rabbit ear wound surface was 20 ± 2 days, and the hyperplasia of scar was most obvious on postoperative 35 ± 2 days. A round, hard, red protruding plaque was obviously in the appearance. Scar hyperplasia can maintain for about 2 months, which is softened then, but there are still some hyperplasic plaques kept at the most obvious hyperplasia state. The longest duration was beyond 190 days. And further verification showed rabbit ear HS models had good similar-

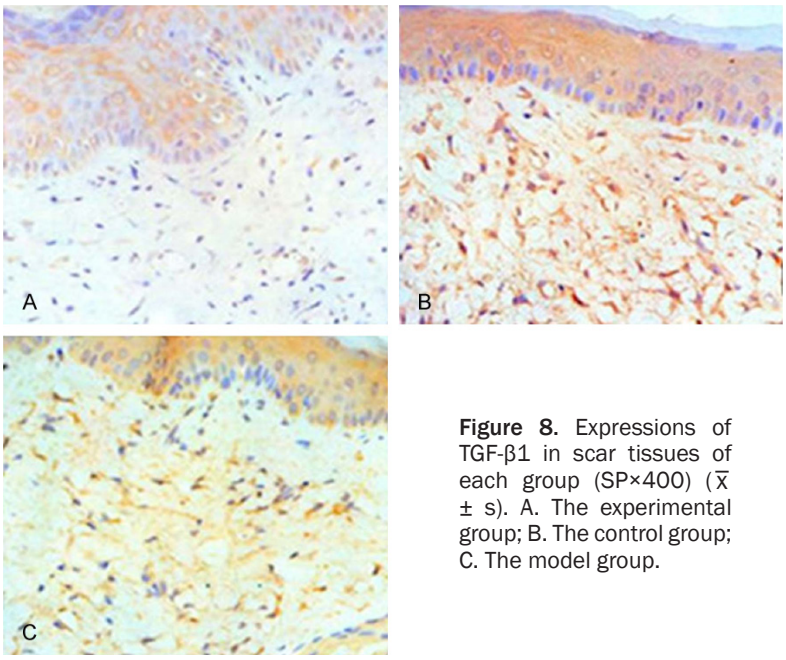

Figure 8. Expressions of TGF-β1 in scar tissues of each group (SP×400) ($\bar{x}$ ± s). A. The experimental group; B. The control group; C. The model group.

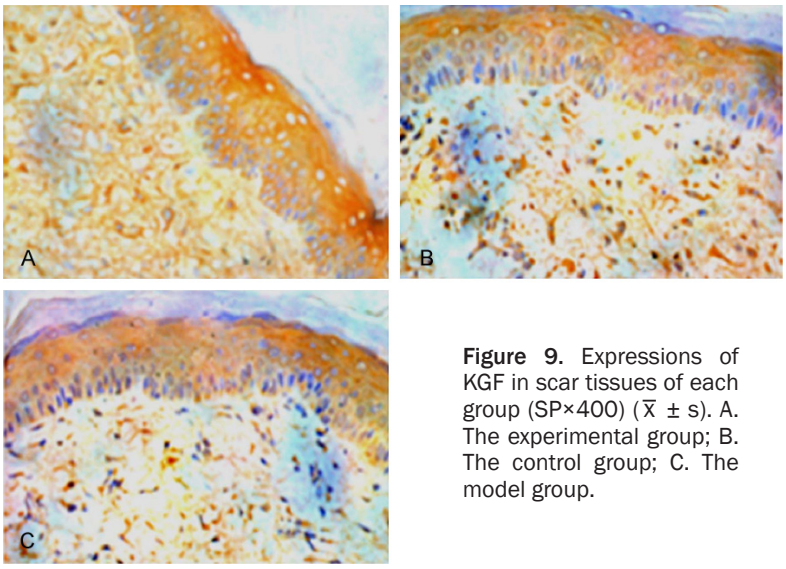

Figure 9. Expressions of KGF in scar tissues of each group (SP×400) ($\bar{x}$ ± s). A. The experimental group; B. The control group; C. The model group.

ity as human HS in the occurrence and development, which is a better animal model to study the prevention and treatment of scar at present.

The mechanism of pathological scar is not entirely clear. Previous researches about the pathogenesis of scar mainly focus on antagonism on excessive proliferation of fibroblasts and excessive generation of ECM, while relatively few on the role of KC. In recent years, people begin to realize KC, especially the information exchange between KC and fibroblasts during the healing of wound surface, namely, epithelial-mesenchymal cross-talk plays an important role in wound healing and formation of scar, which has become the research hotspot in the filed of wound healing [1-3]. Related studies have shown that epithelial-mesenchymal cross-talk plays an important role in morphogenesis of embryonic skin at development stage and in maintaining the structure integrity of adult skin. During the healing and re-epithelization of skin wound, fibroblasts can promote proliferation and migration of KCs. KCs receive the signals of fibroblasts to rebuild the functional epithelium [11], and KCs can affect the biological behavior of fibroblasts. By adjusting the phenotype of fibroblasts, the fibroblasts are more suitable to reconstruction of dermis rather than deposition of ECM. And in this way, synthesis of collagen by fibroblasts were effectively inhibited, and the occurrence of scar was reduced [12, 13]. The mentioned studies all suggested that though there are complex loops in epithelial-mesenchymal cross-talk, IL-1α, TGF-β1 and KGF secreted by KC and fibroblasts are the core mediators. IL-1α and TGF-β1 secreted by KC both can promote fibroblasts proliferation, and TGF-β1 can also promote the fibroblasts to generate collagen while IL-1α can inhibit the secretion of collagen. By secreting IL-1α, KCs promote fibroblasts to secrete factors including IL-6 and KGF, induce function changes of fibroblasts and promote wound healing. KGF secreted by fibroblasts also acts on KC to promote its proliferation and differentiation [11-14]. Hakvoort et al. used immunohistochemical method to detect the differentiation and proliferation of KC and observe the

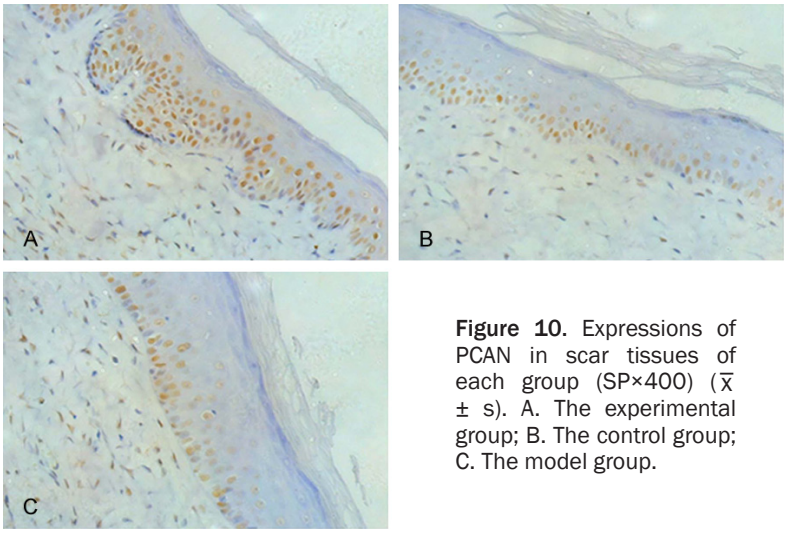

Figure 10. Expressions of PCAN in scar tissues of each group (SP×400) ($\bar{x}$ ± s). A. The experimental group; B. The control group; C. The model group.

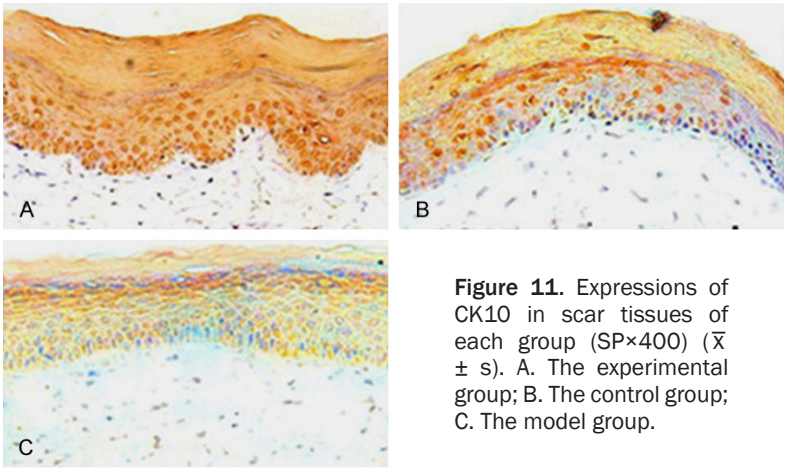

Figure 11. Expressions of CK10 in scar tissues of each group (SP×400) ($\bar{x}$ ± s). A. The experimental group; B. The control group; C. The model group.

Table 1. Expressions of epithelial-mesenchymal cross-talk related factors in the scar tissues of each group

	Experimental group	Control group	Model group
IL-1α	30.785 ± 2.830*,#	17.330 ± 2.188	18.625 ± 3.263
TGF-β1	17.304 ± 2.387*,#	38.117 ± 1.794	37.462 ± 3.396
KGF	36.582 ± 5.979*,#	23.633 ± 2.670	23.423 ± 2.814
PCAN (positive rate)	0.625 ± 0.043*,#	0.403 ± 0.074	0.395 ± 0.056
CK10	34.457 ± 2.443*,#	20.433 ± 3.437	19.497 ± 3.076

*P<0.05, compared with the model group; #P<0.05, compared with the control group.

Table 2. Changes of mRNA expressions of IL-1α and TGF-β1 in scar tissues of each group

	Experimental group	Control group	Model group
IL-1α	1.734 ± 0.081*,#	0.943 ± 0.038	1.001 ± 0.088
	0.473 ± 0.004*,#	0.984 ± 0.007	1.003 ± 0.002
TGF-β1	0.594 ± 0.0432*,#	0.919 ± 0.0422	1.003 ± 0.0423
	0.606± 0.024*,#	0.940 ± 0.067	1.000 ± 0.073

*P<0.05, compared with the model group; #P<0.05, compared with the control group.

changes of their activity in non-hypertrophic scar, hypertrophic scar and non-burnt skin of burn-wounded patients whose burn was not full-thickness at 1, 4 and 7 months after burn, and found that KC began abnormal differentiation after re-epidermization of burned skin, and in addition, this abnormal condition would continue till hyperplastic scar recovered to normal scar, suggesting KC of abnormal state after burn would induce the abnormal state of epithelial-mesenchymal cross-talk, which might be another important cause of hyperplastic scar formation [15], and effective intervention on epithelial-mesenchymal cross-talk during the wound healing may be a research direction with good prospect for prevention and treatment of HS.

The regeneration and healing of the wound have always been the goal of medical filed, and cell therapy is a major research direction of regenerative medicine. The biological effects of MSCs in promoting wound healing and inhibiting fibrosis formation have been confirmed [1, 2], and MSCs have also been found to mediate the epithelial-mesenchymal cross-talk, induce KC to highly express IL-1a, promote proliferation of KC, inhibit the apoptosis of KC, induce KC to form epithelial stratified structure, and reconstruct the epithelium [7]. CK10 is the commonly used index for differentiation of KC. Recently, Nkemcho established DED skin model, and detected the expres-

sions of CK10, CK6, PCNA, type IV collagen and vimentin in the model structure with the interaction between KC with fibroblasts and MSCs, respectively, suggesting both MSCs and fibroblasts can promote proliferation and differentiation of KC [16]. But it has not been reported whether MSCs can intervene in the in vivo epithelial-mesenchymal cross-talk in the early stage of HS formation after epidermization of wound surface, promote reconstruction of epithelium and dermis at scar hyperplastic stage, and benefits regeneration and healing. Since open wound surface might have factors (wound contamination, or infection, tissue and cell edema, etc.) influencing biologi-cal effects of transplant cells, and it needs a certain amount of time to prepare adequate autologous stem cells. Therefore, it is a cell therapy method of HS worthy of exploration, that is, intervention of scar formation with autologous stem cells in the early shaping stage of hyperplastic scar after epidermization of wound surface.

This study adopted autologous ASCs to intervene the formation of HS in rabbit ear scar models in the early scar shaping stage after epidermization of wound surface. And found that in the experimental group, the number of epithelial cell layer was increased, and the cells were in epithelial foot and dermal papilla structure; expressions of IL-1α, PCAN, CK10 and KGF in the epithelial layer were up-regulated, while expression of TGF-β1 was downregulated; these suggested that ASCs can also intervene the epithelial-mesenchymal cross-talk in vivo, promote proliferation and differentiation of KC, and induce epithelial stratified structure, which might be another important mechanism of MSCs to improve the quality of wound surface healing and is worthy of further research. And moreover, is likely to apply the cell therapy and transformation into the prevention and treatment of HS.

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Disclosure of conflict of interest

None.

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