Adipose-derived stem cells from younger donors, but not aging donors, inspire the host self-healing capability through its secreta

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Abstract

Adipose-derived stem cells demonstrate promising effects in promoting cutaneous wound healing, but the mechanisms are still not well defined and contradictory views are still debatable. In the present research, we established a mouse cutaneous wound model and investigated the effects of adipose-derived stem cells in wound healing. Adipocyte, adipose-derived stem cells, and epidermal keratinocyte stem cells were isolated from younger and aged donors according to the standard protocol. The conditioned medium either from adipose-derived stem cells or from adipocytes was used to treat epidermal keratinocyte cells. The results showed that adipocytes or adipose-derived stem cells isolated from younger donors demonstrated mild advantage over those cells isolated from aging donors. Adipose-derived stem cells showed stronger stimuli than adipocytes, and the adipose-derived stem cells or adipocytes from younger donors enabled to support higher growth rate of keratinocyte stem cells. The invasion of vasculature was observed at day 10 after posttransplantation in the mice bearing the keratinocyte stem cells or combination of keratinocyte stem cells with adipose-derived stem cells; however, simply inoculating keratinocyte stem cells from aging donors did not result in vasculature formation. Adipose-derived stem cells isolated from younger donors were able to inspire the host's self-healing capabilities, and age-associated factors should be taken into consideration when designing a feasible therapeutic treatment for skin regeneration.

Keywords: Adipocyte, adipose-derived stem cells, wound healing, age-associated factor, skin

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Introduction

When skin is damaged by destructive stimuli, a dynamic process consisting of inflammation, angiogenesis, and tissue remodeling will be initiated sequentially to restore the barrier function of skin.¹ Fibrosis and scar formation are the two main forms which are commonly seen in adult skin wound healing; different cells, growth factors, and cytokines are recruited and interplay at the injury site, ending up in a closure of the skin. High concentration of growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- β), and vascular endothelial growth factor (VEGF), exerts therapeutic effects in skincare due to their important roles in wound healing.² The mutual crosstalk between these cytokines and epidermal stem cells mediates the wound healing process. The proliferation and differentiation of epidermal stem cells are influenced by many factors. Recently, adipose-derived stem cells (ADSCs) have drawn extensive attentions due to their capability of

secretion of various cytokines, such as VEGF, insulin-like growth factor (IGF), hepatocyte growth factor (HGF), and TGF- β , and all those factors play a pivotal role on restoring the damaged neighboring cells caused by destructive stimuli. Moreover, ADSCs have demonstrated diverse rejuvenation functions on skin healing. Kim and coworkers have clearly confirmed that the primary ADSCs from patient-specific skin can tremendously enhance the therapy of skin defect and rapidly restore damaged skin, meanwhile promoting wound healing.^{3,4} In a study using melanoma cells as research subject, ADSCs was approved to be able to suppress melanogenesis by manipulating the expression of tyrosinase and tyrosinase-related protein 1.⁵ In a clinical trial, lipoaspirate cells were intradermally injected and led to the increase of dermal thickness and reduction of wrinkles.^{6,7}

All of these reports approved the application of ADSCs in wound healing. However, approximately 20%–30% are ADSC in lipoaspirate cells, and the majority is adipocyte.

It is so far unknown which cell lineage in lipoaspirate cell population has the predominant roles in skin regeneration, and whether age-related physiological change influences the outcomes of application of ADSCs for wound healing. To address this issue, in this study, we systematically investigate the effect of two distinct cell lineages of ADSCs and adipocytes on promoting wound healing as well as the impact of age.

Materials and methods

Isolation of ADSCs

A written informed consent was obtained prior to harvesting the samples. The isolation of ADSCs was performed as previously described.⁸ Briefly, lipoaspirates were harvested from different donor at various age and settled in an aspiration container. The adipose tissue layer was then washed with $1 \times$ phosphate-buffered saline (PBS). After 10 min standing, the infranatant was aspirated. The washing procedure was repeated for several times until the adipose layer exhibited a yellow-gold color. Collagenase solution was mixed with the adipose fraction, followed by digestion for 1 h at 37°C with gentle swirl every 10 min. Twenty-five milliliters of the culture medium containing fetal bovine serum (FBS) was used to inactivate the collagenase by incubating at room temperature for 5 min. Stromal cell fraction was pelleted by centrifugation at $1200 \times g$ for 10 min. Ten milliliters of the culture medium was used to resuspend the pellet, and 100 μ m mesh filter was used to filter out larger tissue particles. The cells were plated into tissue culture-treated dishes and maintained in culture medium for three to four days at 37°C, 5% CO₂ without refreshment of the culture medium. For the isolation of adipocytes, the pellet of stromal cell fraction was filtered with 70 μ m nylon mesh filter and resuspended in PBS. The cell suspension was layered onto histopaque-1077 and centrifuged at $840 \times g$ for 10 min. The supernatant was discarded, and the cell band buoyant over histopaque was collected. Retrieved cell fraction was cultured overnight at 37°C, 5% CO₂ in the medium containing Dulbecco's modified eagle medium (DMEM)/F12 with 10% FBS, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin.

Isolation of epidermal keratinocyte stem cells

The epidermal keratinocyte stem cells (EKSCs) were isolated as previously reported.⁹ The procedures were briefly introduced here. Adult human scalp skin was minced and incubated in DMEM, 10% FBS, and 4 mg/mL dispase for 5 h at 37°C. Under a dissecting microscope, the bulge region of follicles which was at telogen stage was cut out and

transferred to a 15 mL tube; 0.05% trypsin-EDTA and Versene was used to digest the samples for 15–20 min at room temperature with shaking periodically. Then, 4 mL DMEM containing 10% FBS was used to stop the digestion. After centrifugation for 5 min at 800 r/min, the cell pellet was resuspended with keratinocyte medium without EGF and seeded in a culture dish containing Mitomycin C-treated 3T3-J2 feeder cells. The keratinocyte medium consisted of DMEM/F12, 10% FBS, and 1 μ g/mL hydrocortisone. After overnight cultivation, the medium was supplemented with EGF and refreshed every two days.

MTT assay

Prior to MTT (3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, the assay plates containing different cell combinations were prepared as following. Cell suspension with 75,000 cells per mL was prepared, and 100 μ L cell suspension was seeded into each well of a 96-well plate and incubated for five days. The EKSCs were inoculated into 96-well plate at day 1 in normal culture medium and at day 2 maintained in conditional medium that consisted of 50% normal medium and 50% collected culture medium with secreta of adipocyte or ADSCs and continuously cultured for extra four days. Next, 20 μ L of 5 mg/mL MTT was dispensed into each well, including one well without cells as control. After incubation for 4 h at 37°C, the media were aspirated carefully, and 150 μ L DMSO was added into each well followed by shaking on an orbital shaker for 15 min. The absorbance was measured at 570 nm on a plate reader (Biotek, USA), and the average readout was recorded from triplicate readings.

Real-time qPCR

The total RNA was isolated with Trizol according standard protocol, and the first-strand cDNA was prepared using a commercialized cDNA synthesis kit (TaKaRa, D6210A). The primer pairs for determining the mRNA level of various target genes were shown in Table 1.

Preparation of mouse wound model and transplantation of adipocyte or ADSCs

Upon approval from Animal Care and Use Committee, six-week-old immunodeficient nude mice were employed in the present research. All surgical tools were sterilized and the procedures were conducted in a biosafety cabinet. The mouse skin was decontaminated by 70% ethanol before surgery. After anesthesia, a 8-mm rounded, cutaneous wound was created with a punch biopsy device on the dorsum of the hind thighs.

Table 1 Primers of target genes

Gene type	Forward	Reverse
HGF	5'TGCTCCTCCCTTCCCTACTC3'	5'ATGCCGGGCTGAAAGAATCA3'
CK19	5'CTCAGACCTGCGTCCCTTTT3'	5'CCGTACCCCCAAAGGAAGAC3'
ADPN	5'TGGGTGAGGTGTGGAGTTCT3'	5'AGGCTCTTGCACTCAACCTC3'
GAPDH	5'GGTTGTCTCCTGCGACTTCA3'	5'TAGGGCCTCTCTTGCTCAGT3'

The wounds were divided into the following five groups: keratinocyte stem cells (KCs) (only human EKSCs were transplanted), ADSCs + KCs (both adipocyte stem cells and keratinocytes stem cells were transplanted), ACs + KCs (both adipocytes and keratinocytes stem cells were transplanted), ADSCs (only ADSCs were transplanted), and ACs (only adipocytes were transplanted). The wounds were sewn with six to eight stitches by Nylon 5-0 surgical sutures and covered with film to protect from biting or infections. For comparison among all the five groups, the wounded sites were recovered at day 2, 5, and 10, respectively, postoperation.

H&E staining

The healed wounds were excised and fixed in 10% formalin for 24 h at room temperature, followed by embedment in paraffin and section in 5 μ m. Sections were prepared through the center of the wound and stained with H&E. The sections were photographed by light microscopy. We

applied H&E stained sections to analyze the invasion of vasculature. Briefly, to calculate the average number of blood vessels from histological staining images, all images were photographed at 200 $\times$ magnification. Thirty random sections each were selected from two different regions of each implant (i.e., edge and center areas). Therefore, the total number of sections was 60 each for experimental and control groups.

Immunohistochemistry study

Sections of 4 μ m were taken from tissue array block and affixed to 3-aminopropyl triethoxysilane-coated slides and air-dried overnight at 37°C. After dewaxing and antigen retrieval, endogenous peroxidase was quenched with 3% hydrogen peroxide for 10 min. Immunohistochemistry (IHC) was performed on the two-step plus[®] poly-HRP anti-mouse/rabbit IgG detection system (ZSGB-Bio Co., Ltd). To reduce the nonspecific binding of the primary antibody, the cover slips were blocked with 3% BSA for 30 min at room

Figure 1 Evaluation of proliferative potential of primary adipocytes and adipose-derived stem cells as well as the stimulatory effects of their secreta on the growth of epidermal keratinocyte stem cells. yADCs: adipocytes isolated from younger donors; oACs: adipocytes isolated from aging donors; yADSCs or oADSCs: adipocytes-derived stem cells isolated from younger or aging donors; KCs: epidermal keratinocyte stem cells

temperature and then incubated with primary antibody in 1% BSA. After overnight incubation, the cover slips were washed thoroughly with PBST (PBS, 0.05% Tween-20). The next steps were performed according to the manual of the two-step plus[®] poly-HRP anti-mouse/rabbit IgG detection system (ZSGB-Bio Co., Ltd). The antigen-antibody complex was visualized with diaminobenzidine substrate.

Statistical analysis

All data were expressed as mean + standard deviation of three independent experiments performed in triplicate. Statistical analysis of the data was performed using

one-way analysis of variance, followed by Dunet's test to evaluate significance relative to control, $*P < 0.05$, $**P < 0.01$, $\#P < 0.05$, $\#\#P < 0.01$, $N = 3$. All statistical analyses were performed with SAS 9.1 statistical software (SAS institute, Cary, NC, USA).

Results

Evaluation of the proliferative potential of adipocyte, ADSCs, and keratinocytes by MTT assay

Adipose tissue is the primary energy resource in mammalian, and the decline in adipogenesis with advancing age is commonly observed in aging process. In order to determine

Figure 2 The mRNA level of HGF (a), CK19 (b), and ADPN (c) varied after cultivation for different time-span in adipocyte and adipose-derived stem cells, which were isolated from young and aged donors, respectively. yADCs: adipocytes isolated from younger donors; oADCs: adipocytes isolated from aging donors; yADSCs or oADSCs: adipocytes-derived stem cells isolated from younger or aging donors; KCs: epidermal keratinocyte stem cells ($*P < 0.05$, $**P < 0.01$, $\#P < 0.05$, $\#\#P < 0.01$)

whether the capability of adipocytes and ADSCs growth was influenced by age, MTT assay was conducted according to the standard protocol. Our results showed that the adipocytes or ADSCs isolated from younger donors demonstrated mild advantage over those cells from aging donors (Figure 1(a) and (b)). It was approved that the secreted from adipocyte cultures could stimulate the proliferation of epidermal KCs. In present research, we employed the conditional medium from adipocyte or ADSCs to treat the freshly extracted KCs and our data were consistent with previous reports;^{10,11} moreover, we found that the medium from ADSCs showed stronger stimuli than media from adipocytes, and the ADSCs or adipocytes from younger donors could support higher growth rate of KCs (Figure 1(c)).

qPCR analysis of target gene expression

Various target genes that were highly associated with the function of adipocyte, ADSCs, and KCs were investigated by real-time qPCR analysis. Our data showed that the relative mRNA of HGF presented an increased level in

adipocytes, ADSCs, and KCs during the continuous 10-day cultivation period regardless the aging factor (Figures 2(a) and 3(a)). On the contrary, the genes of CK19 and ADPN exhibited a declined expression (Figures 2(b), 2(c), 3(b), and 3(c)). Further analysis revealed that ADSCs overall showed higher level of HGF, CK19, and ADPN than adipocyte (Figures 2 and 3). Taking age into account, the cells derived from younger donors showed robust expression of all these three genes in comparison with the cells isolated from aged donors (Figures 2 and 3).

Moreover, in the group of KCs treated with conditional medium, the mRNA level of HGF and ADPN was significantly enhanced (Figure 3(a) and (c)) while CK19 expression was decreased (Figure 3(b)) compared with the KCs maintained in normal culture medium.

Histological study results

The wounded regions in mice models were, respectively, recovered at day 2, 5, and 10 after transplantation with different combinations of adipocyte, ADSCs with KCs.

Figure 3 The mRNA level of HGF (a), CK19 (b), and ADPN (c) varied after cultivation for different time-span in KCs in conditional media from adipocyte or adipose-derived stem cells. yADCs: adipocytes isolated from younger donors; oADCs: adipocytes isolated from aging donors; yADSCs or oADSCs: adipocytes-derived stem cells isolated from younger or aging donors; KCs: epidermal keratinocyte stem cells (* $P < 0.05$, ** $P < 0.01$, # $P < 0.05$, ## $P < 0.01$)

Figure 4 H&E staining of the wounded area at day 2, 5, and 10 posttransplantation with various cell combinations. yADCs: adipocytes isolated from younger donors; oADCs: adipocytes isolated from aging donors; yADSCs or oADSCs: adipocytes-derived stem cells isolated from younger or aging donors; KCs: epidermal keratinocyte stem cells. (A color version of this figure is available in the online journal.)

Figure 5 Expression of CD29 in the wounded regions at day 2, 5, and 10 posttransplantation with various cell combinations by immunohistochemical staining. yADCs: adipocytes isolated from younger donors; oACs: adipocytes isolated from aging donors; yADSCs or oADSCs: adipocytes-derived stem cells isolated from younger or aging donors; KCs: epidermal keratinocyte stem cells. (A color version of this figure is available in the online journal.)

Figure 6 Expression of CD90 in the wounded regions at day 2, 5, and 10 posttransplantation with various cell combinations by immunohistochemical staining. yADCs: adipocytes isolated from younger donors; oACs: adipocytes isolated from aging donors; yADSCs or oADSCs: adipocytes-derived stem cells isolated from younger or aging donors; KCs: epidermal keratinocyte stem cells.(A color version of this figure is available in the online journal.)

Figure 7 Expression of β integrin in the wounded regions at day 2, 5, and 10 posttransplantation with various cell combinations by immunohistochemical staining. yADCs: adipocytes isolated from younger donors; oADCs: adipocytes isolated from aging donors; yADSCs or oADSCs: adipocytes-derived stem cells isolated from younger or aging donors; KCs: epidermal keratinocyte stem cells. (A color version of this figure is available in the online journal.)

Figure 8 Detection of serum level of HGF, CK19 and β integrin by ELISA at day 2, 5, and 10 postgraft. yADSCs: adipocytes isolated from younger donors; oACs: adipocytes isolated from aging donors; yADSCs or oADSCs: adipocytes-derived stem cells isolated from younger or aging donors; KCs: epidermal keratinocyte stem cells (* $P < 0.05$, ** $P < 0.01$, # $P < 0.05$, ## $P < 0.01$).

The sections were stained by H&E staining. At day 5, the wounded area showed the nature of acellular dermal matrix regardless which cell subsets were inoculated (refer to the first two rows in Figure 4). The invasion of vasculature was observed at day 10 posttransplantation in the mice bearing the KCs or combination of KCs with

ADSCs (Figure 4(f), (r) and (zz)); however, simply inoculating KCs from aging donors (oKCs) did not result in vasculature formation (Figure 4(c)), and similar results were observed in the mice bearing ADCs or ADCs plus KCs. The ADSCs derived from aging donor, which was designated as oADSCs thereafter, could not facilitate the

vasculature process (Figure 4(o) and (z)). The γ ADSCs isolated from younger donors was sufficient to initiate the vasculature formation (Figure 4(r)), and the efficacy could be dramatically improved when KCs was applied simultaneously (Figure 4(z)).

Immunohistochemical result

The mouse skin tissue samples recovered from the wounded regions at different time point posttransplantation were stained by immunohistochemistry using primary antibodies against CD29, CD90, and β integrin. The data indicated that all these markers were positively expressed in all skin samples at day 2 after graft, and the expression level gradually decreased (Figures 5 to 7).

Enzyme-linked immunosorbent assay results

For determining the serum level of HGF, CK19, and β integrin after transplantation, peripheral blood samples were drawn from each mouse model and the serum was isolated through brief centrifugation. The secretion of HGF, CK19, and β integrin was investigated by enzyme-linked immunosorbent assay (ELISA). As was consistent with the result from real-time qPCR, the serum HGF level kept increasing while both CK19 and β integrin decreased during the 10-day observation period, inoculation of ADSCs plus KCs showed more remarkable effects than any other individual transplantation, dramatically increasing the serum HGF level but inhibiting CK19 and β integrin secretion (Figure 8). In consideration of aging-associated influence, the ADSCs isolated from younger donors still showed advantageous over all other cell lineages.

Discussion

Multipotent ADSCs are the most promising tools for skin regeneration, as their availability in human body is ubiquitous.¹² Numerous scientific reports have approved that ADSCs can directly differentiate into a variety of cell lineages and possess even better differentiation abilities than mesenchymal stem cells.^{13–15} A recent breakthrough in ADSCs application resides in wound healing, which needs to form complex tissues for skin regeneration. However, it is still a hypothesis that skin tissue regeneration is a direct result of ADSCs transplantation. There indeed exists another view that ADSCs are believed to inspire the host's ability to heal itself through activation of paracrine signaling pathway,¹⁶ which is contradictory to the former standpoint. Our findings in present research demonstrate to support the second view that ADSCs just acted as a stimuli, simply transplanting ADSCs was beneficial for wound healing to some extent; the healing process could be remarkably enhanced when KCs was inoculated simultaneously, indicating ADSCs accelerated the differentiation of keratinocyte stem cells. Moreover, the ADSCs from aged donors did not show any synergistic effect in facilitating KCs growth and differentiation, but young ADSCs did. All these results reveal that ADSCs play a synergistic role

on manipulating wound healing, and extremely affected by donor age.

Since the first report about ADSCs published, worldwide researchers are dedicating their efforts to identifying specific cell surface markers for characterization of ADSCs. β integrin (CD29) and CD90 are widely accepted as the consensus markers of ADSCs,^{17,18} although the profile of ADSCs surface markers varied from one research group to another group. The results of IHC showed that the expression of these markers was strongly positive right at day 2 after transplantation, and gradually went down or even disappear at day 10 postgraft, which reflected the transition of ADSCs from stem cells to differentiated cell lineages in vivo (Figures 6 and 7). It was important to note that the adipocytes in the experiment also demonstrated mild expression of these markers, which might be used as index to show the purity of the ADSCs. Although the adipocytes might be mixed with some ADSCs, the majority was still pure adipocyte and did not show cure effects as what ADSCs did. From the aspect of scientific rigorousness, it is still necessary to develop a better protocol for isolating pure adipocytes or ADSCs, like using fluorescence-activated cell sorting (FACS) or antibody-conjugated magnetic beads.

HGF is a cytokine implicated in hematopoiesis, vasculogenesis, and mammary epithelial duct formation. Based on ELISA data, either the HGF mRNA level or its serum protein level was greatly improved in the short-term observation after transplantation (Figure 8). β integrin, also named CD29, participates in the spreading, adhesion, and migration of human adipose tissue-derived stem cells,¹⁹ and interferes with the engraftment and migration of ADSCs. Our data showed that β integrin expression gradually decreased along with the time span, indicating the efficiency and migration of ADSCs decreased. Our finding supported that ADSCs can inspire the host's self-healing capabilities and would shed light on the design of novel therapy for skin regeneration.

Author contributions: NM, JB and CQ researched literature and conceived the study. WZ, HL, XZ, DL, SZ and LZ were involved in protocol development, gaining ethical approval, patient recruitment, and data analysis. NM wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

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