

# Effects of Human Adipose-derived Stem Cells on Cutaneous Wound Healing in Nude Mice

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**Background:** Despite numerous treatments available for deteriorated cutaneous wound healing such as a diabetic foot, there is still the need for more effective therapy. Adipose-derived stem cells (ASCs) are mesenchymal stem cells, which are self-renewing and multipotent. Mesenchymal stem cells have the potential for tissue repair and regeneration. **Objective:** To investigate the effects of human ASCs on the healing of cutaneous wounds in nude mice. **Methods:** 15-mm round full-thickness skin defects were generated on the back of BALB/c nude mice. The mice were divided into three groups for wound coverage: (i) human ASCs-populated collagen gel, (ii) human dermal fibroblasts-populated collagen gel, and (iii) collagen gel alone. Wound contraction was prevented with a splint method. Wound size was measured 10 days after injury. At 28 days histological analysis was performed. **Results:** Both ASCs and dermal fibroblasts accelerated wound closure, but dermal fibroblasts were more effective than ASCs. At 28 days, the dermal portion of ASCs or dermal fibroblasts wound scars were thicker than collagen gel wound scars. **Conclusion:** ASCs and dermal fibroblasts stimulate cutaneous wound healing and improve scar thickness. (*Ann Dermatol* 23(2) 150~155, 2011)

## -Keywords-

Adipose tissue, Fibroblast, Mesenchymal stem cell, Mouse, Wound healing

## INTRODUCTION

Wound healing involves a complex chain of events with interactions among different cells and tissues. The progression of this wound healing process is impaired in a number of medical conditions, including diabetes<sup>1</sup>. Although new therapeutic methods have been developed, the treatment of many chronic wounds remains unsatisfactory, and more effective treatment strategies are needed.

Bone marrow-derived mesenchymal stem cells (BMSCs) are multipotent stem cells capable of differentiating into numerous cell types, including osteoblasts, chondrocytes, and adipocytes<sup>2-4</sup>. BMSCs can accelerate wound healing<sup>5,6</sup>, but BMSC transplantation requires harvesting a large amount of bone marrow under general anesthesia, which may lead to severe complications.

Adipose-derived stem cells (ASCs) represent an alternative source of multipotent cells with similar characteristics to BMSCs<sup>7,8</sup>. ASCs are easier to isolate and relatively abundant, which may make them a potential source for wound repair and regeneration. The effects of ASCs on cutaneous wound healing have not been clearly demonstrated. Autologous ASCs improved wound healing in healing-impaired *db/db* mice<sup>9</sup>, and human ASCs accelerated wound closure in nude mice<sup>10</sup>. However, wound contraction was not prevented in both animal experiments, so it is uncertain whether ASCs enhanced the wound healing process including re-epithelialization and granulation tissue formation. In this study, we examined the benefit of human ASCs in wound healing on a nude mice wound model with a contraction-preventing splint method. Healing was measured after excisional wounds were covered with ASCs-populated collagen gels (CG), human dermal fibroblasts (DFs)-populated CG, or CG alone.

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## MATERIALS AND METHODS

All the experimental protocols were approved by the Institutional Review Board and Institutional Animal Care and Use Committee of the Seoul National University Hospital.

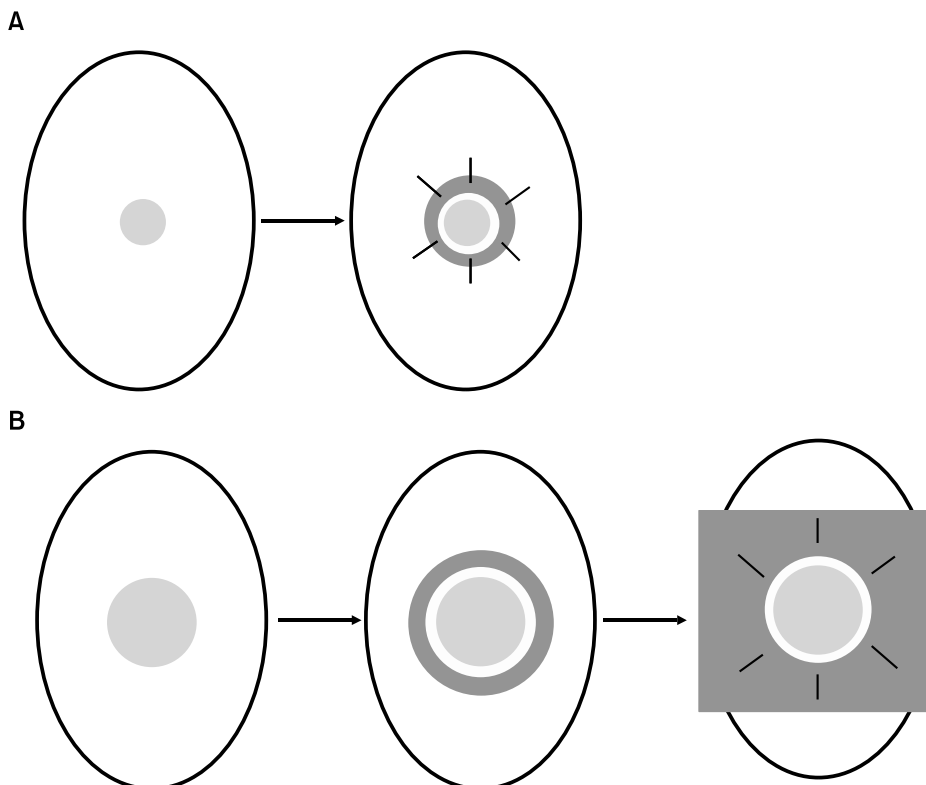
### Isolation and culture of ASCs, DFs and HaCaT cells

Human subcutaneous adipose tissues were acquired from elective liposuction from healthy female donors with informed consent. The suctioned fat was digested with 0.075% collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline under gentle agitation for 30 min at 37°C, and centrifuged at  $800 \times g$  for 10 min to obtain the stromal cell fraction. The pellets were resuspended, passed through a 100  $\mu\text{m}$  mesh filter (Millipore, Billerica, MA, USA) to remove connective tissues, and cultured at 37°C in 5%  $\text{CO}_2$  in Dulbecco's modified Eagle media (DMEM) supplemented with 10% fetal bovine serum, 100 mg/ml streptomycin, and 100 U/ml penicillin. After 3 days, culture medium was changed to MesenPRO RS™ Medium (Invitrogen, Carlsbad, CA, USA), and was replaced every 3 days. The primary cells were cultured until 80% confluence and were defined as "Passage 1". ASCs were used for the experiment at passage 4.

For harvesting human DFs, foreskin was obtained from donors under informed consent, and DFs were cultured as previously described<sup>11</sup>. DFs at passages 4~6 were used for the experiment. HaCaT cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 mg/ml streptomycin, and 100 U/ml penicillin at 37°C in 5%  $\text{CO}_2$ .

### Preparing cell-populated collagen gels

Before preparing the gels, 1 ml of 0.1% bovine serum albumin (Sigma-Aldrich) solution was added into each well of a 48-well plate and incubated at 37°C for 1 h. A collagen solution mixture was prepared by quickly mixing eight volumes of collagen type I solution (Nitta Gelatin, Tokyo, Japan) with one volume of 10-fold concentrated DMEM and one volume of sodium bicarbonate (22 mg/ml). ASCs or DFs ( $1 \times 10^6$ ) were added to 300  $\mu\text{l}$  of collagen solution mixture, and gently mixed. Collagen solution mixture with ASCs, DFs or alone was poured into a 48-well plate after bovine serum albumin solution was removed, and allowed to gel for 30 minutes in a 5%  $\text{CO}_2$  atmosphere at 37°C. After 30 minutes, culture media was poured into each well, and the CGs were detached from the surface of the well by rimming the gels with a sterile needle and gently swirling the plate.



**Fig. 1.** Schematic representation of wound model. (A) Galiano's model. 6 mm round wounds were created on the mouse dorsum. The donut-shaped silicone sheet splint was centered on the wound, fixed with an immediate-bonding adhesive and interrupted nylon sutures. (B) Modified model used in our experiment. 15 mm wounds were made on the dorsal skin of mouse. A flexible plastic splint was fixed to the skin around the wound with an adhesive film and interrupted 6-0 nylon sutures.

## Wound healing model and cell transplantation

Six to eight-week-old male BALB/c nude mice (Orient Bio, Sungnam, Korea) were used. Mice were anesthetized using an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (15 mg/kg). The wound model which was suggested by Galiano et al.<sup>12</sup> was used with some modifications. A 15 mm round full-thickness excisional wound was made on the back of mice using an Iris scissor. A donut-shaped flexible plastic splint with a 16 mm inner and 18 mm outer diameter was fixed to the skin around the wound with an adhesive film (3M Ioban™ 2, 3M, St. Paul, MN, USA) and interrupted 6-0 nylon sutures (Fig. 1). The mice were divided into three groups depending on what covered the wound: (i) ASCs-populated CG (n=4), (ii) DFs-populated CG (n=4), and (iii) CG alone (n=4). Each CG was applied on the wound, and the occlusive dressing (Tegaderm, EM, St. Paul, MN, USA) was placed to cover the wounds. Each animal was then housed in its own cage.

## Wound size measurements

Digital photographs of wounds were taken on day 10 after surgery. Wound photographs were shown on the computer monitor with an image analysis program (ImageJ, National Institutes of Health, Bethesda, MD, USA), and the border of unhealed area that was not re-epithelialized was traced manually. The size of the traced area was calculated automatically by the software.

## Histological analysis

For histological analysis of wound scars, the mice were euthanized at 28 days, and the whitish scars were excised, bisected, and fixed in 10% formalin. The samples underwent routine histological processing with hematoxylin and eosin.

## Statistical analysis

Results were expressed as means  $\pm$  SEM. A Mann-Whitney U test was used for statistical analysis, and a value of  $p < 0.05$  was considered statistically significant.

## RESULTS

### Wound size measurements

The size of unhealed wounds not re-epithelialized was measured. At 6 days after the injury, it was difficult to determine the boundary of re-epithelialization. The difference in size of the unhealed wound between groups was most obvious on day 10 (Fig. 2A). The size of the wound after ASCs treatment was significantly smaller than

CG alone ( $28.63 \pm 5.05 \text{ mm}^2$ ,  $54.63 \pm 5.69 \text{ mm}^2$ ,  $p < 0.05$ ). DFs treatment healed the wounds significantly faster than both ASCs and CG alone ( $11.09 \pm 2.71 \text{ mm}^2$ ,  $p < 0.05$ ) (Fig. 2B).

## Histological analysis

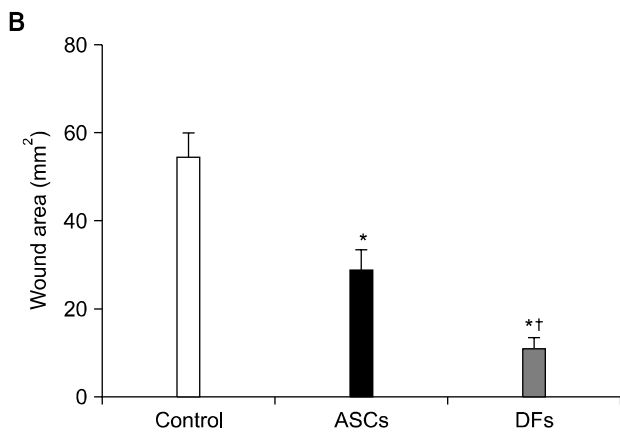
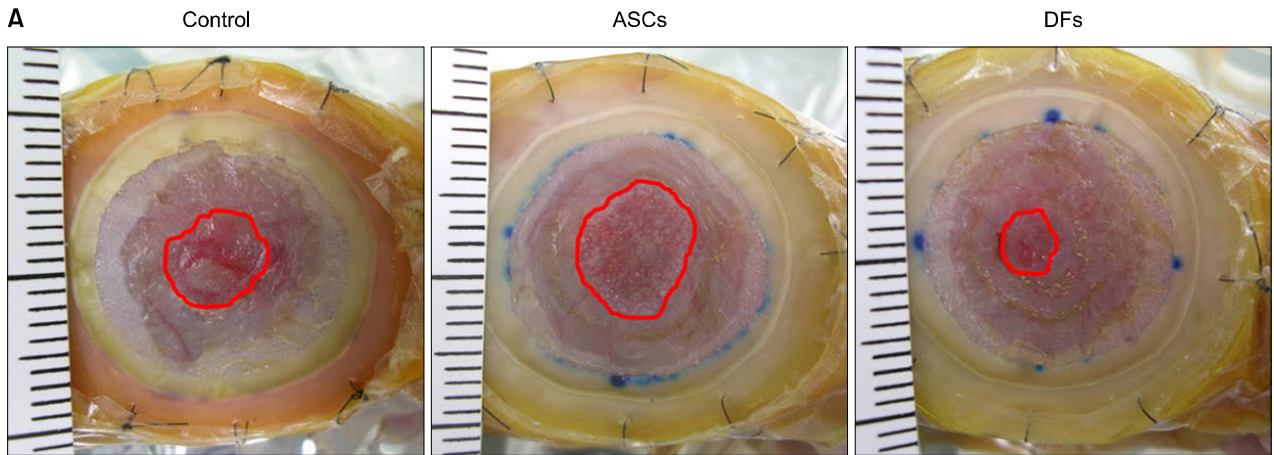
At 28 days after surgery, the wounds that became a whitish scar were excised for histological examination (Fig. 3A). The dermal portion of ASCs or DFs wound scars was thicker than CG wound scars ( $84.50 \pm 4.39 \mu\text{m}$ ,  $75.78 \pm 4.52 \mu\text{m}$ ,  $51.61 \pm 2.31 \mu\text{m}$ ,  $p < 0.05$ ) (Fig. 3B).

## DISCUSSION

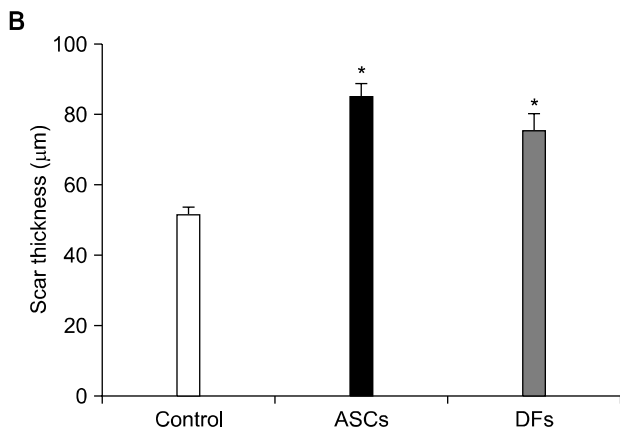
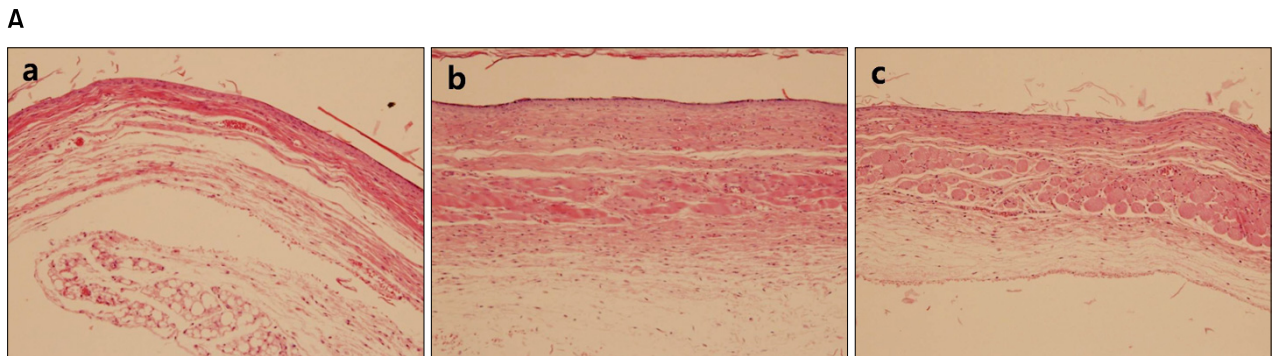
Mice are commonly used in wound healing models, but with some limitations. Because wound contraction makes the defect close rapidly, it is difficult to measure re-epithelialization and granulation tissue formation. Galiano et al.<sup>12</sup> suggested preventing contraction by fixing donut-shaped silicone splint around the wound. We used Galiano's model with some modifications. To make it easier to distinguish differences in wound closure, we made a larger wound of 15 mm diameter compared with the 6 mm wound used in Galiano's model. We were able to prevent wound contraction successfully and accurately measure wound closure.

We showed that ASCs promoted healing of full thickness wounds in nude mice, and, unexpectedly, DFs closed the wounds more rapidly than ASCs. ASCs combined with the atelocollagen matrix improved wound healing in healing-impaired *db/db* mice, but only had minor effects in normal (*db/+*) mice, perhaps because the closure assay was not sensitive enough to detect a difference in normal mice<sup>9</sup>. Preventing wound contract can improve assay sensitivity, as we showed here in healing-competent nude mice.

There have been several reports suggesting that DFs may be able to accelerate cutaneous wound healing. DFs seeding in conjunction with a collagen sponge matrix facilitated dermal and epidermal wound healing compared with collagen sponge only<sup>13</sup>. Skin substitutes with dermal components containing DFs induced the proliferation and differentiation of keratinocytes and the formation of basement membrane, accelerating re-epithelialization<sup>14-16</sup>. These reports support our result of positive effects of DFs on wound healing. However, Wu et al.<sup>5</sup> reported that DFs did not improve wound healing in Galiano's wound model. To avoid immunorejection, we used BALB/c nude mice that have defective T cell immunity. Wu et al.<sup>5</sup> used allogeneic cells, DFs derived from C57BL/6 mice on immunocompetent BALB/c mice,



**Fig. 2.** Both adipose-derived stem cells (ASCs) and dermal fibroblasts (DFs) accelerated wound closure, but DFs were more effective than ASCs. (A) BALB/c nude mice were wounded and treated with collagen gel alone (control) or ASCs or DFs in collagen gel. After 10 days, photomicrographs were taken. The red line is the margin of unhealed wound that was not re-epithelialized. (B) The size of unhealed wound area was calculated with an image analysis program. The values shown are the means  $\pm$  SEM of 4 mice per group. \* $p < 0.05$  vs. collagen gel group; † $p < 0.05$  vs. ASCs group.



**Fig. 3.** Adipose-derived stem cells (ASCs) or dermal fibroblasts (DFs) wound scars were thicker than collagen gel alone. (A) At 28 days after surgery, whitish scars of collagen gel (a), ASCs in collagen gel (b) or DFs in collagen gel (c) wounds were harvested and processed for histological examination. (B) The thickness of dermal portion of wound scars was measured with an image analysis program. The values shown are the means  $\pm$  SEM of 4 mice per group (H&E,  $\times 200$ ). \* $p < 0.05$  vs. collagen gel group.

which may elicit an immunogenic response. In a porcine wound model, allogeneic DFs induced more inflammatory reactions and scar formation than autologous ones<sup>17</sup>, indicating that transplanted DFs can be influenced by the immune response of the host, which may lead to different healing outcomes.

Wound healing depends on re-epithelialization, granulation tissue formation, and angiogenesis mediated by keratinocytes, DFs, and endothelial cells. It has been shown that ASCs affect DFs and endothelial cells in a paracrine fashion. Conditioned medium of ASCs promotes different functions of DFs including proliferation, migration, and production of extracellular matrix<sup>10</sup>. Proangiogenic factors such as vascular endothelial growth factor are secreted at high levels from ASCs, so conditioned medium of ASCs accelerates proliferation of endothelial cells *in vitro*<sup>18,19</sup>. This paracrine effect of ASCs on endothelial cells may contribute to wound healing by promoting angiogenesis *in vivo*.

The dermal portion of ASCs- or DFs-populated CG wound scars was thicker than CG wound scars 28 days after surgery. Therefore, when scar thickness is an issue, particularly problematic atrophic scars, ASCs or DFs in dermal skin substitution may be useful. Although DFs showed greater effects in wound healing than ASCs, DFs have some clinical limitations. Autologous DFs require self skin for isolation. Although allogeneic DFs are relatively easy to obtain, their immunogenicity could reduce their effects on wound healing. Autologous ASCs are easily obtained by liposuction. BMSC have low immunogenicity and immunoregulatory action by suppressing the functions of immune cells, including T cells<sup>20,21</sup>. Because ASCs have similar characteristics to BMSC, allogeneic ASCs might not be affected by the host immune response, and might be able to accelerate wound healing like autologous ones.

In conclusion, ASCs and DFs accelerated wound healing, and DFs showed a greater effect. ASCs and DFs application formed a thicker dermal portion of scars than control.

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