Allogeneic transplantation of an adipose-derived stem cell (ASC) sheet combined with artificial skin accelerates wound healing in a rat wound model of type 2 diabetes and obesity

Running title: Allogeneic ASC sheet therapy for diabetic ulcer

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Abstract

One of the most common complications of diabetes is diabetic foot ulcer. Diabetic ulcers do not heal easily due to diabetic neuropathy and reduced blood flow, and non-healing ulcers may progress to gangrene, which necessitates amputation of the patient’s foot. This study attempted to develop a new cell-based therapy for non-healing diabetic ulcers using a full-thickness skin defect in a rat model of type 2 diabetes and obesity.

Allogeneic adipose-derived stem cells (ASCs) were harvested from the inguinal fat of normal rats, and ASC sheets were created using cell-sheet technology and transplanted into full-thickness skin defects in Zucker diabetic fatty rats.

The results indicated that the transplantation of ASC sheets combined with artificial skin accelerated wound healing and vascularization, with significant differences observed 2 weeks after the treatment. The ASC sheets secreted large amounts of several angiogenic growth factors in vitro, and transplanted ASCs were observed in perivascular regions and incorporated into the newly constructed vessel structures in vivo. These results suggest that the ASC sheets accelerated wound healing both directly and indirectly in this diabetic wound-healing model.

In conclusion, allogeneic ASC sheets exhibit potential as a new therapeutic strategy for the treatment of diabetic ulcers.
The population of patients with diabetes mellitus is growing worldwide and reached approximately 400 million in 2013 (1). An estimated 15 to 25% of diabetic patients are at risk of developing a diabetic ulcer in their lower extremities during their life (2). Among patients with diabetic foot ulcers, 7 to 20% will subsequently require an amputation, and 85% of lower extremity amputations in diabetic patients are caused by foot ulcers (3). There is an urgent need to develop new therapies for the treatment of diabetic wounds to prevent foot ulcers from leading to amputations.

Artificial skin is one of the commercially available treatments for full-thickness skin defects after debridement. Artificial skin is typically composed of two layers, an outer silicone-sheet layer and an inner collagen-sponge layer, which act as the epidermis and dermis, respectively (4). However, difficulties are often experienced with the use of artificial skin treatments for diabetic wounds in patients with neuropathy, impaired blood flow, or relatively large wounds. In these cases, the formation of neo-dermal tissue is delayed, thus prolonging the treatment period (5).

Recombinant basic fibroblast growth factor (rbFGF) has been widely used in wound healing to promote angiogenesis and granulation. Although rbFGF successfully accelerates wound healing, it requires frequent administration given its short half-life (6,7).

Cell-based therapy has recently emerged as a new application for the treatment of ulcers (8,9). In particular, mesenchymal stem cells (MSCs) exhibit excellent potential for
increasing the rate of wound healing given their self-renewal capacity, immunomodulatory effects, and ability to differentiate into various cell lineages (10). Adipose-derived stem cells (ASCs), a type of MSCs, exhibit various advantageous properties, such as paracrine activity and angiogenic potential (11,12). ASCs have been used experimentally in wound-healing applications (13,14).

Single-cell suspensions of MSCs have been directly injected around wounds in numerous studies, and accelerated wound healing has been observed (15,16). Despite reports of improved wound healing in diabetic ulcer models following the injection of single-cell suspensions, the residence time of transplanted cells at the wound site is unclear. Yang et al. reported that the injection of single-cell suspensions resulted in the formation of island-like aggregates and visible necrosis of the injected cells (17).

Cell-sheet engineering has been developed to improve the efficacy of cell transplantation. Okano et al. reported that temperature-responsive culture dishes can be prepared by covalently grafting the temperature-responsive polymer N-isopropylacrylamide onto a culture dish surface (18). The grafted polymer layer permits temperature-controlled cell adhesion/detachment on the culture dish surface. Specifically, the surface is hydrophobic at 37°C, allowing cells to adhere and proliferate. In contrast, the surface becomes hydrophilic below 32°C, causing cells to spontaneously detach from the surface. These cells can be subsequently harvested as a contiguous cell sheet with intact cell-cell junctions and
extracellular matrix (ECM). This approach avoids the use of proteolytic enzymes, such as trypsin, that damage the ECM, and the cell sheets can be immediately transplanted to the wound site (19).

Artificial skin has been used to achieve great therapeutic results even in chronic wounds, such as diabetic wounds (20). In addition, cellular artificial skin has been developed, and a significant body of evidence supports its use in the treatment of diabetic wounds (21). As previously stated, ASCs exhibit therapeutic potential; therefore, ASC sheets with artificial skin have the potential to accelerate chronic wound healing and offer a feasible therapeutic strategy for diabetic wound treatment. The efficacy of ASC sheets with artificial skin requires further evaluation in diabetic wounds with exposed bone. In this study, the artificial skin used provided a three-dimensional framework for ASCs, maintaining transplanted ASC sheets and wounds in a moist environment, preventing wounds from spontaneous contraction, and protecting wounds from infection and external forces (22).

The aim of this study was to evaluate the efficacy of allogeneic ASC sheet transplantation with artificial skin to promote the healing and vascularization of full-thickness skin defects in a rat diabetic wound model.

**Research Design and Methods**

**Animals**
All experimental protocols were approved by the Animal Welfare Committee of Tokyo Women’s Medical University School of Medicine. Zucker diabetic fatty (ZDF) rats (ZDF-Lepr<sup>fa</sup>/CrlCrlj) were used as a type 2 diabetic obesity model. Adipose tissue was isolated from Lewis rats (LEW/CrlCrlj) and enhanced green fluorescent protein (EGFP) rats [SD-Tg (CAG-EGFP)] and used to prepare cell sheets.

**Isolation of rat adipose-derived stem cells (rASCs)**

Rat adipose-derived stem cells (rASCs) were isolated from the inguinal adipose tissue of Lewis rats (20 to 33 weeks old, male), which was processed according to a previously reported method (23). Briefly, the isolated adipose tissue was enzymatically digested with 0.1% type A collagenase (Roche Diagnostics, Mannheim, Germany) at 37°C for 1 h. The stromal-vascular fraction (SVF) was collected after centrifugation at 700 × g for 5 min. Cells in the SVF were plated on a 60-cm<sup>2</sup> Primaria tissue culture dish (BD Biosciences, Franklin Lakes, NJ, USA) and cultured in complete culture medium [α-MEM GlutaMAX (Invitrogen, Carlsbad, CA, USA) with 20% FBS (Moregate Biotech, Queensland, Australia) and 1% penicillin/streptomycin (Sigma-Aldrich, St Louis, MO, USA)] at 37°C in a 5% CO<sub>2</sub> incubator. After 24 h, debris was removed by washing with phosphate-buffered saline (PBS; Life Technologies, Grand Island, NY, USA), and fresh medium was added. The cells were passaged with 0.25% trypsin-EDTA (Life Technologies) on day 3 and transferred to a new dish.
Subcultures were plated at a density of $1.7 \times 10^3$ cells/cm$^2$ every 3 days until passage 3.

**rASC culture and characterization**

rASCs were characterized by measuring their colony-forming, adipogenic, and osteogenic abilities using previously reported methods (24). For each assay, 100 rASCs at passage 3 were plated in a 60-cm$^2$ dish and cultured in complete medium for 7 days. For the colony-forming assay, the cells were fixed with 4% paraformaldehyde (PFA) (Muto Pure Chemical, Tokyo, Japan) and stained with 0.5% crystal violet in methanol for 5 min.

For adipogenesis, the medium was switched to adipogenic medium consisting of complete medium supplemented with 0.5 µmol/L dexamethasone (DEX) (Fuji Pharma, Tokyo, Japan), 0.5 mmol/L isobutyl-1-methyl xanthine (Sigma-Aldrich), and 50 µmol/L indomethacin (Wako Pure Chemical, Osaka, Japan). After 14 days, the cells were fixed with 4% PFA for at least 1 h and stained for 2 h with fresh Oil Red-O solution (Wako). For osteogenesis, the medium was switched to calcification medium consisting of complete medium supplemented with 100 nmol/L DEX, 10 mmol/L β-glycerophosphate (βGP) (Sigma-Aldrich), and 50 µmol/L ascorbic acid (AA) (Wako). The cells were incubated for 21 days and then stained with 1% alizarin red S solution. The number of stained colonies was counted for each assay (n = 3).

**Flow cytometry assay**
One million rASCs at passage 4 were suspended in 100 µL of PBS containing 10 µg/mL of fluorescein isothiocyanate (FITC)-conjugated primary antibodies to characterize their surface marker expression (Table 1). After incubation for 30 min at 4°C, the cells were washed with PBS and then suspended in 1 mL of PBS for analysis. Cell fluorescence was evaluated using a Gallios flow cytometer (Beckman Coulter, Tokyo, Japan), and data were analyzed using the Gallios and Kaluza software (Beckman Coulter).

Creation of rASC sheets

rASCs derived from Lewis rats or EGFP rats at passage 3 were seeded on 35-mm temperature-responsive culture dishes (UpCell®) (CellSeed, Tokyo, Japan) at a density of 1.5 × 10^5 cells/dish and cultured in complete medium for 3 days. Then, the cells were cultured in complete medium with 16.4 µg/mL AA for an additional 4 to 5 days. After reducing the temperature to room temperature, the cells spontaneously detached as contiguous cell sheets and were harvested from the dishes with forceps.

Preparation of the full-thickness skin defect wound model and transplantation of rASC sheets

ZDF rats (n = 48; 16 weeks old, male, 520 to 600 g) were used as a rat wound-healing model for type 2 diabetes and obesity. rASC sheets from Lewis rats were used for
transplantation (Figure 1A-D).

The blood glucose levels of the ZDF rats were measured using a blood glucose monitor (Glutest Neo Sensor; Sanwa Kagaku Kenkyusho, Nagoya, Japan), and their body weights were monitored both before the operation and sacrifice.

ZDF rats were anesthetized by inhalation of 4% isoflurane (Pfizer Japan, Tokyo, Japan) and were ventilated with a rodent mechanical ventilator (Stoelting, Wood Dale, IL, USA). Then, 15 × 10 mm full-thickness skin defects were created on the heads of the ZDF rats by removing the cutaneous tissue from the epidermis to the periosteum (25), and the wound healing of the defects was observed at predetermined time points. ZDF rats were randomly divided into two groups, a control group and an rASC sheet transplantation group, to investigate the efficacy of cell sheets for wound healing. In the transplantation group, an rASC sheet was placed on the defect. The defects with or without an rASC sheet were covered with 15 × 10 mm of artificial skin (Pelnac®, Smith & Nephew, Tokyo, Japan). Defects were closed with 10 stitches using 5-0 silk sutures (Alfresa, Osaka, Japan; Figure 1D). To protect the wound, 20 × 15 mm non-adhesive dressing (Hydrosite plus®, known as Allevyn non-adhesive® in the United States; Smith & Nephew) was placed on top of the artificial skin with 5-0 silk sutures to maintain a moist wound environment moist and absorb exudate.

Transplantation of the rASC sheet from EGFP rats
EGFP-expressing rASCs were obtained from EGFP rats to track the fate of the transplanted cells. EGFP-expressing rASC sheets were created and transplanted into full-thickness defects in ZDF rats (16 to 18 weeks old, male; n = 2) using the same procedure described above.

**Gross wound measurement and complete wound closure time**

Gross wounds were observed, and photographs were taken obtained at 0, 3, 7, 10, and 14 days (n = 12) after the operation and every 3 to 7 days thereafter (n = 6) until complete wound closure was observed (42 days). The wound area was measured by tracing the wound margin on the photograph and calculating the pixel data using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The time to complete wound closure was assessed (n = 6).

**Tissue preparation for immunohistochemistry**

Three rats from each group were sacrificed 3, 7, 10, and 14 days after the operation. The wound areas were excised for histological analysis, and the blood vessel density was analyzed immunohistochemically. After fixation with 4% PFA for 24 h at 4°C, the trimmed specimens were decalcified with Morse’s solution [10% sodium citrate (Wako) and 22.5% formic acid (Wako)] (26) for 3 days at 4°C with gentle agitation. The Morse’s solution was
exchanged daily. After decalcification, the specimens were rinsed with PBS, embedded in paraffin, cut into 6-µm-thick sagittal sections, and stained with hematoxylin and eosin (HE). The sections of the wound area were then observed for histological analysis.

To quantify vascularization within the wound, blood vessel endothelial cells were immunohistochemically stained with an anti-CD31 antibody (rabbit polyclonal antibody; Thermo Fisher Scientific Anatomical Pathology, Fremont, CA, USA). The specimens were pretreated via heating followed by blocking with 1% BSA (Sigma-Aldrich) and then incubated with the anti-CD31 antibody at 4°C overnight. After primary antibody staining, the specimens were washed with PBS, incubated with a secondary antibody [Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) Antibody] (Life Technologies), mounted on coverslips with Prolong Gold and stained with DAPI (Invitrogen). Serial sections of the specimens were observed with a fluorescence microscope (U-RFL-T; Olympus, Tokyo, Japan), and the obtained images were analyzed with application software (DP2-BSW) (Olympus).

The blood-vessel densities of 6 animals in each group were measured 14 days after the operation by measuring the area of CD31-positive vessels in the wound area of the specimen. The center of the wound in each section was selected. Then, the vessel area in the selected field of each specimen was observed with a microscope (Eclipse E800; Nikon, Tokyo, Japan), and the area of CD31-positive vessels was quantified using ImageJ. The relative area of CD31-positive vessels (VA) was calculated using the following equation:
VA (%) = \( \frac{VA_{act}}{Af} \times 100 \),

where VA_{act} and Af are the actual area of CD31-positive vessels and the total area of the field, respectively.

EGFP-expressing rASC sheets were transplanted to trace the fate of the transplanted rASC sheets. At 3, 5, 7, and 14 days after the transplantation, the rats’ chests were opened, and their vasculature was perfused from the left ventricle with 100 mL of 2% PFA in 0.01 mol/L PBS (pH 7.4) at a pressure of 120 mmHg. The wound tissue, including the skull bone and surrounding cutaneous tissue, was then removed and soaked in 4% PFA for 24 h at 4°C. The specimens were subsequently washed with PBS and decalcified with 10% EDTA w/v (Wako) in PBS for 5 days at 4°C with gentle agitation. The EDTA solution was changed daily. After decalcification, the specimens were rinsed more than 10 times with PBS, cryo-protected in an ascending series of sucrose-PBS (15 and 30 w/v%), embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan), and snap-frozen in liquid nitrogen. Then, 14-µm-thick frozen sections were prepared with a cryostat (Leica CM 1850) (FINETEC, Tokyo, Japan) and processed for immunohistochemistry. The frozen sections were first incubated with 4% Block Ace (Dainippon Seiyaku, Osaka, Japan) and then incubated with RECA-1, a rat anti-endothelial cell antibody (Abcam, Cambridge, UK), as the primary antibody at a dilution of 1:200 in PBS containing 1% BSA (Sigma-Aldrich) at 4°C overnight. After several PBS washes, the specimens were incubated with a Cy3-conjugated secondary antibody at a 1:200
dilution (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 3 h at room temperature. Finally, the specimens were observed and photographed with a Keyence BZ-9000 fluorescence microscope (Keyence Corp., Osaka, Japan).

**ELISA of the supernatant from rASC sheets**

rASCs at passage 3 were seeded onto temperature-responsive culture dishes at a cell density of $1.5 \times 10^5$ cells/dish and cultured at 37°C for 72 h. The cells achieved sub-confluence (80 to 90%) at 72 h after seeding, and this time point was defined as day 0. Then, the medium was collected and replaced with complete medium containing 16.4 µg/mL AA on days 0, 2, and 4. The rASC sheets were transplanted on day 4 in this study. After the collected medium was centrifuged at $300 \times g$ for 3 min at 4°C, the supernatant was collected, immediately frozen, and maintained at -80°C for further experiments.

The number of cultured cells per dish was assessed on days 0, 2, and 4 ($n = 4$). The levels of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), transforming growth factor beta 1 (TGF-β1), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and keratinocyte growth factor (KGF) were determined using RRV00, MHG00, MB100B, MG100 (R&D Systems, Minneapolis, MN, USA), SE560Ra (Cloud-Clone Corp, Houston, TX, USA), and CSB-E12905r quantitative enzyme-linked immunosorbent assay ELISA kits (CUSABIO, Wuhan, China), respectively. The protein concentrations were
measured by duplicate ELISA assays, which were performed according to the manufacturer’s instructions. The amounts of the different growth factors in the supernatant were calculated to examine the amount secreted from the rASC sheets at each time point.

**Statistical analysis**

Data are expressed as the mean ± SD. Comparisons between the two groups (i.e., the transplant and control groups) were performed using Student’s \( t \)-test. In addition, \( p \)-values less than 0.05 (\( p < 0.05 \)) were considered statistically significant.

**Results**

**Characterization of rASCs**

To evaluate the characteristics of the rASCs, crystal violet, Oil red-O, and alizarin red S staining were performed to confirm rASC self-renewal, adipogenesis, and osteogenesis, respectively. rASCs exhibited colony-forming potential (Figure 2A) as well as adipogenic and osteogenic differentiation potential in vitro (Figure 2B and C), suggesting that the rASCs used in this study possessed MSC-like properties.

Flow cytometry was used to characterize cell surface markers (Figure 2D-H). rASCs strongly expressed CD29 and CD90. In contrast, low expression levels of CD11b, CD31 and CD45 were observed.
Blood glucose level and body weight of 16-week-old ZDF rats

ZDF rats in each group exhibited an average blood glucose level of greater than 250 mg/dL, and these levels were approximately 2.5-fold higher than those observed in 16-week-old non-diabetic rats.

ZDF rats exhibited an average body weight of 578.2 g in the transplantation group and 582.6 g in the control group. These body weights were approximately 1.5-fold higher than those observed in 16-week-old non-diabetic rats.

Gross wound area

Digital photographs were taken and obtained 0, 3, 7, 10, and 14 days after the operation and every 3 to 7 days thereafter until complete wound closure (Figure 3A-L). The average wound area was significantly smaller in the transplantation group than in the control group beginning on the 7th day (Figure 4A). The wounds in both groups were digitally photographed and observed until complete wound closure (n = 6). The average observed wound closure time was significantly reduced in the transplantation group compared with the control group (Figure 4B).

Histology
In low-magnification microphotographs of sagittal sections of HE-stained specimens collected 14 days after the operation (Figure 5A-D), a thin dermal layer was observed for the control group (Figure 5A). In contrast, dense connective tissue was observed in the transplantation group (Figure 5B). Higher-magnification microphotographs indicated the same results (Figure 5C and D).

**Blood vessel density**

Blood vessel density was quantified 14 days after the operation (Figure 5E-G). The blood vessel density in the wound was increased by approximately 2.5-fold in the transplantation group compared with the control group after 14 days, and the difference was statistically significant (n = 6) (Figure 5G).

**Number of rASCs per rASC sheet**

The average number of cultured cells per dish was $8.76 \times 10^5$ cells on day 4 at the time of transplantation (Figure 6A).

**Growth factor levels in the rASC culture supernatant**

VEGF, HGF, TGF-β1, IGF-1, EGF, and KGF were present in the conditioned medium from cultured rASCs at passage 3 (Figure 6B-G). Specifically, the levels of VEGF, HGF,
TGF-β1, IGF-1, EGF, and KGF per rASC sheet were 18.60, 3.27, 0.76, 2.78, 0.44, and 0.42 ng/sheet/day at day 4 (n = 4), respectively, as determined using ELISA kits for each protein. These results confirmed that the rASC sheets were secreting growth factors at the time of transplantation.

**Fate of the transplanted rASCs**

EGFP-expressing rASC sheets were transplanted into the wounds (Figure 7A and B). Fourteen days after the transplantation, the transplanted EGFP-expressing rASCs are indicated in green (Figure 7C), whereas the endothelial cells of regenerated blood vessels were stained with RECA-1 (red, Figure 7D). Overlaid microphotographs revealed that EGFP-expressing rASCs were located on the periphery of the RECA-1-positive blood vessels (Figure 7E).

**Discussion**

Although streptozotocin-induced type 1 diabetic rats without obesity are most frequently used for diabetic wound-healing studies, this study used a wound-healing model with type 2 diabetes, which is known to affect more than 90% of diabetes mellitus patients. Specifically, ZDF rats were used as a wound-healing model of type 2 diabetes and obesity in this study. These rats spontaneously develop obesity at approximately 4 weeks of age and then develop type 2 diabetes at 8 to 12 weeks, exhibiting hyperglycemia combined with
insulin resistance, dyslipidemia, and hypertriglyceridemia (27). Delayed wound healing, impeded blood flow in peripheral blood vessels, and diabetic nephropathy are also observed (28-30).

ASC sheets have been used to accelerate wound healing in previous studies. Lin et al. reported that transplantation of human ASC sheets with fibrin-coated membranes accelerated wound healing in a 12-mm diameter full-thickness skin defect on the backs of 6-week-old athymic nude mice (31,32). Cerqueira et al. reported that human ASC sheet transplantation promoted wound regeneration in 10-mm diameter full-thickness skin defects on the backs of 5-week-old normal mice (33). In contrast to previous studies, in the present study, a wider full-thickness skin defect with exposed bone (the wound area was 15 mm²) was created on the parietal region of 16-week-old ZDF rats. In addition, the periosteum, which may support the regeneration of the skin, was removed to increase the severity of the wound (25). Diabetic wounds with exposed bone are often observed clinically after debridement. To assess the clinical applicability of the method for severe diabetic foot ulcers, this study attempted to produce a wider full-thickness skin defect model with exposed bone and removed periosteum in rats with type 2 diabetes and obesity.

The differences in wound-healing mechanisms noted between humans and rodents are attributed to anatomical differences of the skin (e.g., panniculus carnosus layer). Normal rats exhibit contraction-based wound healing, whereas humans display re-epithelialization
and granulation tissue formation-based wound healing. Typically, wound splinting in rodent models helps to minimize wound contracture, which allows for the gradual formation of granulation tissue (34). Slavkovsky et al. reported that the contraction of diabetic wounds in ZDF rats is impaired, whereas non-diabetic wounds are almost entirely closed by contraction. Epithelialization also contributes to repair in this diabetic wound model in ZDF rats (28). Furthermore, the bilayer artificial skin that we used in the present study prevented wound contraction and promoted new connective tissue matrix synthesis, resembling the true dermis (35). In our study, artificial skin was placed on a recipient wound and fixed with nylon threads to reduce wound contraction and prevent wound enlargement due to the loose skin of the rat. Even if considerable contraction of the wound was observed, it appeared similar across all conditions between the control and the transplantation group except in the presence or absence of ASC sheet transplantation. Under these conditions, significant differences in wound area and vascular density were observed between the two groups. This experiment may be useful for evaluating ASC sheets.

Estrogen deficiency is associated with impaired cutaneous wound healing. Female rats exhibit a faster rate of wound healing with a higher rate of wound contraction due to their thinner skin (36). Accordingly, to reduce potential hormonal influences on wound healing, only male rats were used in this study.

ASCs exhibit several advantages compared with MSCs derived from other tissues (12).
Adipose tissue is relatively abundant in the human body. These tissues are accessible and can be collected using a minimally invasive liposuction procedure. ASCs are also abundant in adipose tissue (11). Moreover, many reports have suggested that ASCs play an important role in accelerating wound healing (37,38). Thus, ASCs are considered a good cell source for stem cell therapies.

This study investigated whether allogeneic ASC sheets together with artificial skin are effective at accelerating wound healing. Xing et al. reported that postoperative wound healing after laparotomy is impaired in obese rats, suggesting that the risk of laparotomy dehiscence may be increased by obesity (39). In clinical practice, patients suffering from diabetic ulcers often exhibit severe diabetic complications, including uncontrolled high blood glucose and a high BMI. The collection of adipose tissue from diabetic patients with acute diabetic ulcers is difficult. Furthermore, after obtaining autologous ASCs, ASC sheet preparation requires several weeks. rASCs from diabetic animals exhibit altered properties and impaired functions (40). In fact, our group attempted to create rASC sheets using rASCs from ZDF rats, but cell proliferation was slow (data not shown). Given that MSCs, including ASCs, suppress immune responses (41), rASC sheets prepared from normal rats were transplanted into defects in ZDF rats to study allogeneic transplantation. In the present study, accelerated wound healing was observed in the transplantation group, and no obvious clinical signs of immune rejection, such as erythema, other local inflammatory signs, or visible signs of necrosis as described by
Falanga et al., were observed in the transplantation group during the experimental period (42,43).

rASC sheets were successfully created from Lewis rats exhibiting a wide range of ages (8, 10, 16, 20, and 33 weeks; data not shown), and rASC sheets from 20- to 33-week-old rats were used for the transplantations. This study showed that rASC sheets from 33-week-old rats secreted growth factors and accelerated wound healing compared to the control group, suggesting that adipose tissue from middle-aged rats created functional rASC sheets. ASC sheets from patients over a wide range of ages could potentially be useful for treatment in clinical settings. In clinical practice, the age of patients undergoing liposuction operations ranges from young to elderly, and most patients are adults. This study intentionally used cells from middle-aged rats to simulate actual clinical practice.

In this study, rASC sheets contained approximately $9 \times 10^5$ rASCs at the time of transplantation. A previous study based on the topical administration of MSCs in a collagen scaffold reported that $1 \times 10^6$ MSCs were transplanted into a 6-mm-diameter wound, which was the highest dose in that experiment. The highest percentage of wound closure and angiogenesis was achieved in 1 week with this cell dose compared with lower doses, suggesting that the wound-healing effects of MSCs may be dose dependent (44). In this study, approximately the same number of rASCs was transplanted into larger defects (15 × 10 mm) as a cell sheet, and wound closure in the transplantation group was significantly accelerated 2
weeks after the operation. These results suggest that cell sheet technology might enhance the cellular activity and efficacy of cell transplantation because cell sheets are harvested with intact cell-cell junctions and undamaged ECM and can be immediately transplanted into the defect site (19). Moreover, a cell sheet is thin and exhibits sufficient flexibility to allow it to adhere well to the rough surface of a wound. In fact, cell sheet technologies have been used for the reconstruction of various tissues with curative effects (45,46).

In this study, the time to complete wound closure was significantly faster in the transplantation group, and increased blood vessel density was also confirmed in the transplantation group 14 days after transplantation compared with the control group.

The regeneration and introduction of capillaries inside the wound are important because tissue regeneration commonly requires blood flow to supply oxygen and nutrition and remove waste. Increased blood vessel density at the wound site may help promote wound healing.

Wound healing requires complex processes, including coordinated interplay between cells and growth factors; it also requires multiple growth factors to act in a synergistic manner (47). A previous study reported that the rate of VEGF secretion from surrounding cells, such as macrophages and fibroblasts, is reduced in diabetic wounds (48). Numerous physiological factors contribute to wound healing deficiency in individuals with diabetes (48).

In this study, various secreted growth factors, such as VEGF, HGF, TGF-β1, IGF-1,
EGF, and KGF, all of which play important roles in wound repair, were detected in large quantities in conditioned medium from rASC sheets. ASCs secrete angiogenic growth factors (49), such as VEGF and HGF (50), and these growth factors could contribute to neo-vascularization (51,52) and accelerate wound healing in both normal and diabetic rats (15). These findings, which are consistent with our results, suggest that the paracrine effects of transplanted rASCs might enhance neo-vascularization and wound epithelialization.

In this study, EGFP-expressing rASCs were surrounded with blood vessel endothelial cells 14 days after transplantation, suggesting that the rASCs remained at the wound site for at least 14 days and were able to differentiate into mural cells at the wound site and support the reconstitution of new blood vessels (53,54).

MSCs originate from and are natively associated with blood vessel walls, suggesting that MSCs belong to a subset of perivascular cells (55,56). Some studies have reported that ASCs have the potential to differentiate into endothelial cells (57,58). It was also previously reported that the majority of transplanted ASCs are adjacent to microvessels and differentiate into pericytes (59,60).

The results from this animal study demonstrate a promising approach for the application of ASC sheets together with artificial skin to accelerate diabetic wound healing. However, additional studies in larger animals are necessary to confirm and validate the efficacy of ASC sheets with artificial skin for clinical trials.
In conclusion, this study demonstrated that allogeneic transplantation of ASC sheets in combination with artificial skin accelerates wound healing in a rat model of type 2 diabetes and obesity. The rASC sheets displayed effective paracrine activity because they secreted angiogenic growth factors and potentially differentiated into perivascular cells to support the construction of new blood vessels. Thus, ASC sheets exhibited therapeutic value in this rat model and may be useful for accelerating the healing of diabetic ulcers in patients with type 2 diabetes and obesity.
Author Contributions

Y.K. wrote the manuscript and researched data. T.I and S.M. researched data, contributed to discussions and reviewed/edited the manuscript. M.Y., T.O., and Y.U. participated in discussions and reviewed/edited the manuscript.

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Conflict of Interest

Teruo Okano is a founder and director of the board of CellSeed, Inc. and holds technology licensing and patents from Tokyo Women’s Medical University. Teruo Okano is a stakeholder of CellSeed, Inc. Tokyo Women’s Medical University receives research funds from CellSeed, Inc.
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**Table 1.** Fluorescein isothiocyanate (FITC)-conjugated antibodies used for flow cytometry

<table>
<thead>
<tr>
<th>Target</th>
<th>Catalog No.</th>
<th>Isotype control</th>
<th>Source</th>
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<tr>
<td>CD11b</td>
<td>554982</td>
<td>Mouse IgA</td>
<td>BD Pharmingen*</td>
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<td>CD29</td>
<td>561796</td>
<td>Hamster IgM</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD31</td>
<td>MCA1334FA</td>
<td>Mouse IgG</td>
<td>AbD Serotec†</td>
</tr>
<tr>
<td>CD45</td>
<td>MCA43FA</td>
<td>Mouse IgG</td>
<td>AbD Serotec</td>
</tr>
<tr>
<td>CD90</td>
<td>MCA47FA</td>
<td>Mouse IgG</td>
<td>AbD Serotec</td>
</tr>
</tbody>
</table>

*BD Pharmingen, Franklin Lakes, NJ, USA
†AbD Serotec, Raleigh, NC, USA
Figure Legends

**Figure 1.** Schematic of the experimental procedure for transplanting an allogeneic rat adipose-derived stem cell (rASC) sheet with artificial skin into a rat wound-healing model of type 2 diabetes and obesity. (A) Rat subcutaneous adipose tissue was surgically excised from Lewis rats or enhanced green fluorescent protein (EGFP) rats. rASCs were isolated and seeded onto a 60-cm² Primaria tissue culture dish and cultured at 37°C for 7-8 days. (B) Passage 3 rASCs were seeded onto a temperature-responsive culture dish and cultured at 37°C for 7 to 8 days. rASCs were harvested as a rASC sheet with intact cell-cell junctions and extracellular matrix (ECM) by reducing the temperature to 20°C. (C) rASC sheets from Lewis rats or EGFR rats were transplanted into a 15 × 10 mm full-thickness skin defect with exposed bone created on the heads of Zucker diabetic fatty (ZDF) rats (n = 48). In the transplantation group, an rASC sheet was placed on the defect, and a 15 × 10 mm sheet of artificial skin (Pelnac®) was overlaid on the transplanted rASC sheet (n = 24). In the control group, only the artificial skin was placed on the defect (n = 24). The defect with the artificial skin was closed with 10 stitches using 5-0 silk sutures.

**Figure 2.** Confirmation of the characteristics and bipotency of rASCs obtained from normal Lewis rats. (A) One hundred cells were cultured in a 60-cm² dish for 7 days and stained with crystal violet to assess the number of cell colonies and confirm the self-renewal ability of
rASCs. rASCs were also able to differentiate into adipogenic and osteogenic lineages. (B) After being cultured in adipogenic induction medium for 2 weeks, rASCs stained positively with Oil red O. (C) After being cultured in osteogenic induction culture medium for 4 weeks, the rASCs stained positively with alizarin red S, confirming their osteogenic differentiation potential. (D-H) The expression of surface antigens on the rASCs was analyzed by flow cytometry. rASCs at passage 3 exhibited a typical mesenchymal stem cell phenotype, as they were positive for CD29 and CD90 and negative for CD11b, CD31, and CD45.

Figure 3. Macroscopic images of full-thickness skin defects. A macroscopic photograph of a typical full-thickness skin defect immediately after the creation of a 15 × 10 mm wound (A); the wound was covered with artificial skin using 10 stitches (B). Macroscopic photographs of full-thickness skin defects in the control (C, E, G, I, and K) and transplant (D, F, H, J, and L) groups at 3 (C and D), 7 (E and F), and 14 days (G and H) after creation of the wound (n = 12) and at 21 days (I and J) and 28 days (K and L) after creation of the wound (n = 6).

Figure 4. (A) Measurement and analysis of the wound area with or without rASC sheet transplantation. The empty columns represent the control group, and the red columns correspond to the transplantation group. Macroscopic photographs of the wounds were digitized, and the wound area at 0, 3, 7, and 14 days (n = 12) and 21 days (n = 6) after surgery
was quantified using ImageJ. The average wound areas at 0, 3, 7, 10, and 14 days (n = 12) and
21 days (n = 6) after the operation were 1.56, 1.42, 1.23, 1.11, 0.86, and 0.62 cm² in the control
group and 1.58, 1.30 1.16, 0.93, 0.65, and 0.25 cm² in the transplantation group, respectively.
The average wound area in the transplantation group 7 days after creation of the wound was
significantly reduced compared with the control group, indicating accelerated wound healing in
the transplantation group. (B) The times required to complete wound closure of full-thickness
skin defects are presented on a scatter diagram of the data for both the control and
transplantation groups (n = 6). The red bars on the diagram indicate the mean time to complete
wound closure for both groups. The mean times to complete wound closure were 34.2 days
(28-42 days) and 25.6 days (18-35 days) in the control and transplantation groups, respectively.
The time to complete wound closure was significantly reduced in the transplantation group
compared with the control group (p = 0.02). The asterisk (*) indicates p < 0.05.

Figure 5. Histological analysis of wounds treated with rASC sheets. Hematoxylin and eosin
(HE)-stained sagittal cross-sections of the control (A) and rASC sheet transplant (B) groups
evaluated 14 days after surgery. The green arrowheads indicate the epidermal margins. The
region between the two arrowheads corresponds to the epidermal defect. The scale bars are 500
µm. Microphotographs (C) and (D) display higher magnification images of the green square
regions of the dermal layers in (A) and (B), respectively. The scale bars are 100 µm.
Quantitative analysis of neo-vascularization of the wounds. Control (E) and transplant (F) specimens were stained with CD31, a blood vessel endothelium cell marker (green). (G) Blood vessel density in the wound area was measured at 14 days after wound creation and was calculated by dividing the area of CD31-positive vessels by the total area. The average CD31-positive area was 1.39% (SD 0.94) in the control group (E) and 3.77% (SD 1.74) in the transplantation group (F) (n = 6). The average blood vessel density in the transplantation group was increased by approximately 2.5-fold compared with the control group, and this difference was statistically significant (p < 0.001). The asterisk (*) indicates p < 0.05. The scale bar is 50 µm.

**Figure 6.** The number of rASCs per rASC sheet and the levels of growth factors secreted by an rASC sheet as measured by ELISA. rASCs achieved sub-confluency (80-90%) in a 35-mm dish 72 h after seeding, and this time point was considered as day 0. The measured time points in the remaining figures (B-E) were the same. (A) The number of rASCs per cell sheet was assessed on days 0, 2, and 4 (n = 8). The average number of rASCs per rASC sheet was $3.88 \times 10^5$, $6.59 \times 10^5$, and $8.76 \times 10^5$ cells at days 0, 2, and 4 (n = 8), respectively. The levels of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), transforming growth factor beta 1 (TGF-β1), IGF-1, epidermal growth factor (EGF), and keratinocyte growth factor (KGF) were determined in vitro with quantitative ELISA kits for these growth factors. (B)
VEGF levels were 32.58, 13.90, and 18.60 ng/sheet/day at days 0, 2, and 4, respectively (n = 4).

(C) HGF levels were 1.32, 3.73, and 3.27 ng/sheet/day at days 0, 2, and 4, respectively (n = 4).

(D) TGF-β1 levels were 0.01, 0.49, and 0.76 ng/sheet/day at days 0, 2, and 4, respectively (n = 4). (E) IGF-1 levels were 0.04, 0.77, and 2.78 ng/sheet/day at days 0, 2, and 4, respectively (n = 4). (F) EGF levels were 0.32, 0.47, and 0.44 ng/sheet/day at days 0, 2, and 4, respectively (n = 4). (G) KGF levels were 0, 0.06, and 0.42 ng/sheet/day at days 0, 2, and 4, respectively (n = 4).

**Figure 7.** Transplantation of rASC sheets prepared from rASCs isolated from EGFP rats. Bright-field (A) and dark-field (B) photographs depict the wound immediately after transplanting an EGFP-positive rASC sheet and before overlaying the artificial skin. Fluorescent microphotography (B) confirmed that the transplanted EGFP-positive rASC sheet expressed green fluorescence (green). The scale bars are 10 mm. (C) The differentiation fate of the transplanted rASC sheet, which expressed green fluorescence, was examined. (D) Frozen sections of the wounds were immunostained with rat anti-endothelial cell antibody-1 (RECA-1) (red) to visualize blood vessel endothelial cells. (E) An overlaid photograph of (C) depicting EGFP-positive rASCs (green) and (D) RECA-1-positive endothelial cells (red) revealed that the EGFP-expressing rASCs (green) were located on the periphery of the RECA-1 stained areas (red).
**FIG. 1.**

**A**

Lewis rat or GFP rat (CAG-EGFP) → Adipose tissue → Rat adipose derived stem cells (rASCs) → Cell sheet transplantation

**B**

Culture at 37 °C till passage 3

35 mm indiameter dish (Temperature-responsive culture dish)

**C**

Zucker diabetic fatty rat → rASC sheet (Rat adipose derived stem cell sheet)

**D**

15 mm → 10 mm

<Creation of wounds>

**<Control group>**

**<Transplantation group>**

E: epidermis
D: dermis
P: penostem
AS: bilayer artificial skin
FIG. 2.

Crystal Violet staining  Oil Red-O staining  Alizarin Red S staining

CD11b  CD29  CD31

CD45  CD90
A

![Graph showing wound area (mm²) vs. Post operation day. The graph compares Control and Transplantation groups with bars indicating mean and error bars. Asterisks denote statistical significance.]

B

![Graph showing Time to complete wound closure (day) for Control and Transplantation groups with data points and a line indicating median. Asterisks denote statistical significance.]

Diabetes
FIG. 5.

A Control

B Transplantation

CD31

E

F

G

Blood vessel density (%)

Control Transplantation

*