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Original Article

# Xenogeneic transplantation of human adipose-derived stem cell sheets accelerate angiogenesis and the healing of skin wounds in a Zucker Diabetic Fatty rat model of obese diabetes

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ABSTRACT

*Introduction:* Diabetic patients with foot ulcers often suffer impaired wound healing due to diabetic neuropathy and blood flow disturbances. Direct injection of human adipose-derived stem cells (hASCs) effectively accelerates wound healing, although hASCs are relatively unstable.
*Methods:* We developed an optimized protocol to engineer hASC sheets using temperature-responsive culture dishes to enhance the function and stability of transplanted cells used for regenerative medicine. Here, we evaluated the efficacy of hASC sheets for enhancing wound healing. For this purpose, we used a xenogeneic model of obese type 2 diabetes, the Zucker Diabetic Fatty rat (ZDF rat), which displays full-thickness skin defects. We isolated hASCs from five donors, created hASC sheets, and transplanted the hASC sheets along with artificial skin into full-thickness, large skin defects (15-mm diameter) of ZDF rats.
*Results:* The hASC sheets secreted angiogenic growth factors. Transplantation of the hASC sheets combined with artificial skin increased blood vessel density and dermal thickness, thus accelerating wound healing compared with that in the controls. Immunohistochemical analysis revealed significantly more frequent neovascularization in xenografted rats of the transplantation group, and the transplanted hASCs were localized to the periphery of new blood vessels.
*Conclusion:* This xenograft model may contribute to the use of human cell tissue-based products (hCTPs) and the identification of factors produced by hCTPs that accelerate wound healing.
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*Abbreviations:* hASCs, Human adipose-derived stem cells; ZDF rats, Zucker Diabetic Fatty Rats; AA, L-ascorbic acid phodpahte magnesium salt; CFA, Colony-forming assay; ITS, Insulin-Transferrin-Selenium.

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## 1. Introduction

The International Diabetes Federation reported that there were 400 million patients with diabetes worldwide in 2015 [1] and approximately 15%–25% of these patients suffer from foot ulcers [2]. Therefore, the number of patients with diabetes, including those affected by foot ulcers, will increase. Moreover, 7%–20% of patients with diabetic foot ulcers undergo amputation of their lower extremities [3]. Thus, there is an urgent need to establish effective therapies to treat diabetic foot ulcers.

Wound healing involves complex biological and molecular responses to tissue injury [4]. Under diabetic conditions, evidence indicates that multiple factors such as prolonged inflammation, decreased collagen synthesis, decreased growth factor secretion, and impaired neovascularization delay wound healing [5]. Treatment with artificial skin accelerates the synthesis of new connective tissue matrices and regenerates the dermis [6]. However, using only artificial skin to treat relatively large wounds caused by diabetic foot ulcers is difficult because of diabetic neuropathy and impaired blood flow.

Cell-based therapy using human adipose-derived stem cells (hASCs) represents a new approach to enhance wound healing [7,8]. However, cells in a single-cell suspension of hASCs migrate from the injection site to the wound area through diffusion [9,10]. To decrease the rate of migration of hASCs into the wound, we developed cell-sheet engineering using temperature-responsive culture dishes coated with the temperature-responsive polymer *N*-isopropylacrylamide. The grafted polymer layer allows cultured cells to adhere at 37 °C and spontaneously detach from the surface at <32 °C. The dish enables nonenzymatic, noninvasive harvesting of the cells as a sheet [11].

Clinical studies using cell sheets made of stem cells indicate the efficacy of this technique for treating esophageal ulcerations [12], periodontitis [13], corneal dysfunction [14], and myocardial infarction [15]. We previously generated rat adipose-derived stem cell (rASC) sheets and found that transplantation of the rASC sheet increased blood vessel density and dermal thickness, which were associated with accelerated wound healing compared with controls [16]. These results suggest that the rASC sheet combined with artificial skin offers a new approach to treat diabetic foot ulcers. We tried to create hASCs sheets under clinical settings. Moreover, the protocol of creating hASC sheets has not been reported yet.

The aim of this study was to establish the optimum protocol for creating hASC sheets and to evaluate their efficacy for accelerating wound healing. For this purpose, we employed the Zucker Diabetic Fatty Rat (ZDF rat) with full-thickness skin defects to serve as a xenogeneic model of type 2 diabetes.

## 2. Materials and methods

### 2.1. Animals

Male ZDF rats (ZDF-*Lepr*$^{fa}$/CrlCrlj) aged 16 weeks (Charles River Laboratories Japan, Kanagawa, Japan) were used to establish a wound-healing model of type 2 diabetes and obesity. The Animal Welfare Committee of Tokyo Women's Medical University approved the use of rats. We used only male rats in this study, considering that estrogen modifies wound healing [17].

### 2.2. Human subjects

This study was conducted according to the principles of the Declaration of Helsinki. The Institutional Review Board of the Tokyo Women's Medical University approved the collection of human samples and all donors provided written informed consent. Adipose tissues were present in samples of lower abdominal subcutaneous fat collected from five donors with cancer who underwent breast augmentation after mastectomy. Clinical data for each patient are presented in Table 1.

### 2.3. Isolation and culture of hASCs

Adipose tissue was digested using collagenase NB6 GMP (0.3 U/g tissue; Serva Electrophoresis, Heidelberg, Germany), at 37 °C for 1 h [18], followed by centrifugation at 700 × *g* for 5 min at 22 °C. The cells were added to a T75 flask (Falcon) and allowed to adhere to the surface. The flask contained α-MEM with GlutaMAX (Life-Technologies, Carlsbad, CA, USA), 10% FBS (Moregate Biotech, Queensland, Australia), 0.1% gentamicin (MSD, Tokyo, Japan), and 0.05% amphotericin B (Bristol-Myers Squibb, Tokyo, Japan)] at 37 °C in an atmosphere containing 5% $CO_2$ for 24 h (Passage 0). After three washes with phosphate-buffered saline (PBS) (Life Technologies, Grand Island, NY, USA), new fresh medium was added. The cells were subcultured using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA; Life Technologies) every 3 days until Passage 2 and the cryopreserved using a CELL BANKER 1 (Nippon Zenyaku Kogyo, Fukushima, Japan). The cells were stored at −80 °C for at least 1 month (Fig. 1a).

### 2.4. Proliferation assay

The numbers of cells from five donors per hASC sheet were counted using a hemocytometer at days 0, 3, 5, 7, 10, 14, and 17 after seeding on each of three temperature-responsive culture dishes (UPCell; CellSeed, Tokyo, Japan).

**Table 1**
Clinical characteristics of donors.

| Number | Donor 1 | Donor 2 | Donor 3 | Donor 4 | Donor 5 |
|---|---|---|---|---|---|
| Age (years) | 66 | 52 | 57 | 42 | 46 |
| Sex | F | F | F | F | F |
| BMI (kg/m$^2$) | 21.1 | 26.5 | 24.3 | 23.8 | 19.3 |
| Amount of adipose tissue (mg) | 2102 | 3556 | 3604 | 3416 | 3549 |
| Fasting blood glucose (mmol/L) | | 5.4 | 5.2 | 4.8 | 5.4 |
| Postprandial blood glucose (mmol/L) | 6.3 | | | | |
| $HbA_{1c}$ (%) | 5.7 | 5.8 | 5.5 | 5.5 | 5.5 |
| HbA1c (mmol/mol) | 39 | 40 | 37 | 37 | 37 |
| Creatinine (μmol/L) | 63 | 59 | 44 | 55 | 63 |
| LDL cholesterol (mmol/L) | 3.7 | 5.4 | 3.7 | 2.3 | 2.8 |
| HDL cholesterol (mmol/L) | 1.9 | 1.5 | 1.6 | 1.7 | 2 |
| Triglycerides (mmol/L) | 1.17 | 1.55 | 1.75 | 0.79 | 0.51 |

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