Video Article

Creation and Transplantation of an Adipose-derived Stem Cell (ASC) Sheet in a Diabetic Wound-healing Model

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Abstract

Artificial skin has achieved considerable therapeutic results in clinical practice. However, artificial skin treatments for wounds in diabetic patients with impeded blood flow or with large wounds might be prolonged. Cell-based therapies have appeared as a new technique for the treatment of diabetic ulcers, and cell-sheet engineering has improved the efficacy of cell transplantation. A number of reports have suggested that adipose-derived stem cells (ASCs), a type of mesenchymal stromal cell (MSC), exhibit therapeutic potential due to their relative abundance in adipose tissue and their accessibility for collection when compared to MSCs from other tissues. Therefore, ASCs appear to be a good source of stem cells for therapeutic use. In this study, ASC sheets from the epididymal adipose fat of normal Lewis rats were successfully created using temperature-responsive culture dishes and normal culture medium containing ascorbic acid. The ASC sheets were transplanted into Zucker diabetic fatty (ZDF) rats, a rat model of type 2 diabetes and obesity, that exhibit diminished wound healing. A wound was created on the posterior cranial surface, ASC sheets were transplanted into the wound, and a bilayer artificial skin was used to cover the sheets. ZDF rats that received ASC sheets had better wound healing than ZDF rats without the transplantation of ASC sheets. This approach was limited because ASC sheets are sensitive to dry conditions, requiring the maintenance of a moist wound environment. Therefore, artificial skin was used to cover the ASC sheet to prevent drying. The allogenic transplantation of ASC sheets in combination with artificial skin might also be applicable to other intractable ulcers or burns, such as those observed with peripheral arterial disease and collagen disease, and might be administered to patients who are undernourished or are using steroids. Thus, this treatment might be the first step towards improving the therapeutic options for diabetic wound healing.

Video Link

The video component of this article can be found at https://www.jove.com/video/54539/

Introduction

The population of diabetic patients is increasing worldwide and reached 400 million in 2015[1]; an estimated 15 - 25% of patients with diabetes are at risk from the progression of a lower-extremity diabetic ulcer[2]. Lower-extremity diabetic ulcers are intractable and might require a prolonged therapeutic period with rehabilitation training after complete recovery. A long therapy period often results in a significant reduction in patient quality of life. Thus, new therapies that decrease or prevent aggravation must be developed for the treatment of diabetic wounds. To evaluate diabetic wound healing, we optimized a diabetic ulcer wound-healing model in rats, which mimics practical clinical conditions, and evaluated whether transplanting adipose-derived stem cell (ASC) sheets using cell-sheet engineering accelerated wound healing.

Mesenchymal stromal cells (MSCs) exhibit an excellent potential for accelerating wound healing because of their self-renewal capacity, their immunomodulatory effects, and their ability to differentiate into various cell lineages[3]. ASCs are a type of MSC derived from adipose tissue, and they exhibit several advantages over MSCs derived from other tissues, including their angiogenic potential and paracrine activity[4,5]. Adipose tissue is relatively abundant in the human body, and its accessibility allows for collection using minimally invasive procedures. Therefore, ASCs have been used experimentally for wound-healing applications[6,7].

Previous reports have shown that the direct injection of single-cell MSC suspensions into areas around wounds can accelerate wound healing[8,9]. However, despite reports of the acceleration of wound healing in diabetic ulcer models following the injection of single-cell suspensions, the survival time of transplanted cells at the wound site is not clear.

In this study, we applied cell-sheet engineering using temperature-responsive culture dishes. These dishes have the temperature-responsive polymer *N*-isopropylacrylamide covalently bound onto their surface[10]. The grafted polymer layer allows for temperature-controlled cell adhesion to or detachment from the surface of the culture dish. The surface of the dish becomes hydrophobic at 37 °C, allowing cells to adhere and

proliferate, whereas cells spontaneously detach from the surface when it becomes hydrophilic at temperatures below 32 °C. Cultured cells can be harvested as a contiguous cell sheet with intact cell-to-cell junctions and extracellular matrices (ECMs) simply by reducing the temperature; thus, proteolytic enzymes that damage the ECM, such as trypsin, are not required[11]. Therefore, cell-sheet engineering can preserve cell-to-cell connections and improve the efficacy of cell transplantation.

In addition, cell-sheet transplantation increases cell survival rates when compared to cell injection[12].In this protocol, Zucker diabetic fatty (ZDF) rats were selected as a type 2 diabetes and obesity model with delayed wound healing. ZDF rats spontaneously develop obesity at approximately 4 weeks. They then develop type 2 diabetes with obesity between 8 and 12 weeks of age, at which point they exhibit hyperglycemia associated with insulin resistance, dyslipidemia, and hypertriglyceridemia[13]. Delayed wound healing, reduced blood flow in peripheral blood vessels, and diabetic nephropathy are also observed[14,15,16]. Moreover, ZDF rats might be an appropriate model for studying the healing of intractable cutaneous ulcers, such as diabetic ulcers.

The differences between humans and rodents in wound-healing mechanisms are associated with anatomical differences in the skin. Wound healing in normal rats is based on wound contraction, whereas wound healing in humans is based on re-epithelialization and granulation tissue formation. Typically, wound splinting used in rodent models helps to minimize wound contraction and allows for the gradual formation of granulation tissue[17], although wounds in nondiabetic rats are almost completely closed by contraction. However, diabetic wound contraction in ZDF rats is impaired, and wound healing primarily occurs through re-epithelialization and granulation tissue formation; thus, this process is more similar to human wound healing[14].

Diabetic wounds with exposed bone after debridement are often encountered clinically. Previous studies have examined 12-mm diameter full-thickness skin wounds on the backs of athymic nude mice[18,19] and 10-mm diameter full-thickness skin wounds on the backs of normal mice[20]. To develop a clinical model for severe diabetic wounds, larger (15 x 10 mm^2) full-thickness skin defects with exposed bone and without the periosteum were created, as previously described[21], in rats with type 2 diabetes and obesity.

Rat ASC (rASC) sheets from the ASCs of normal Lewis rats were created through the allogenic transplantation of ASC sheets. In clinical practice, autologous transplantation is unfeasible because diabetic patients with ulcers often exhibit severe diabetic complications, such as uncontrolled high blood glucose levels and high body mass indices, and these complications cause wound-healing disorders that increase the difficulty of obtaining adipose tissue from these patients. Furthermore, ASCs from animals with diabetes exhibit altered properties and impaired function[22]. Therefore, the protocol presented here describes the allogenic transplantation of rASC sheets from normal rats and the application of artificial skin to diabetic rats.

The bilayer artificial skin used in this protocol prevents the spontaneous contraction of the wounds, promotes the synthesis of a new connective tissue matrix, and resembles the true dermis[23]. In this protocol, artificial skin is placed on an rASC sheet and fixed with nylon threads to prevent wound contraction or enlargement resulting from loose rat skin. In addition, the artificial skin provides a three-dimensional framework for the ASC sheets, maintains a moist environment for the transplanted ASC sheets and wounds, and protects the wounds from infection and external forces. Finally, a non-adhesive dressing is placed over the wound to protect it from external impact, maintain a moist wound environment, and absorb exudate.

An rASC sheet is thin, flexible, and deformable and can be adhered to moving recipient sites, such as a beating heart[24]. Cell-sheet engineering has been used for the reconstruction of various tissues and can generate curative effects[25,26]. ASC sheets that exhibit clinical therapeutic potential might accelerate the healing of many types of wounds. Moreover, the allogeneic transplantation of ASC sheets, combined with the use of artificial skin, might be applicable to the treatment of intractable ulcers or burns, such as those observed in peripheral arterial disease or collagen disease, or they may be administered to patients who are undernourished or are using steroids. This approach increases the efficiency of transplanting ASCs. The wound-healing ZDF rat model produces a severe wound condition that resembles the human wound healing process and mimics clinical conditions in a small-sized experimental animal.

Protocol

All experimental protocols presented below were approved by the Animal Welfare Committee of Tokyo Women's Medical University School of Medicine and abided by all requirements of the Guidelines for Proper Conduct of Animal Experiments.

1. Preparation of Animals, Instruments, Culture Media, and Dishes

1. Prepare complete culture medium using minimum essential medium alpha containing 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Store this for several months at 4 °C until use.
2. Use adult male ZDF rats as a type 2 diabetic obesity model. Use the fat pad of male Lewis rats to isolate adipose tissue to prepare the cell sheets.

2. Isolation and Culture of rASCs

1. **Obtain adipose tissue from Lewis rats (8 - 33 weeks old, male) under general anesthesia using isoflurane.**
    1. Prepare a rodent mechanical ventilator and 4% isoflurane. Prepare several sterile cotton tips, clean gauzes, a scalpel, a needle holder, operating scissors, a pair of forceps, and a 5-0 nylon suture. Sterilize the surgical instruments and supplies.
    2. Prepare a 100-mm Petri dish with 10 mL of sterile phosphate-buffered saline (PBS) to temporarily preserve the obtained adipose tissue. Weigh the Petri dish with PBS using a balance before starting surgery to measure the collected adipose tissue. Lay out the surgical instruments and supplies on a sterile drape.
    3. Anesthetize the rats by using isoflurane at 3 - 4% *via* a rodent mechanical ventilator and maintain the rats at 2 - 3% isoflurane during surgery. Monitor the depth of anesthesia by observing the depth and rate of respiration of each rat.

4. While wearing sterile gloves, position the rat in a supine position on a sterile drape. Shave the operative area with an electric razor and clean the abdominal section of the rat using sterile gauze soaked in 70% ethanol.
5. Create an approximately 5 cm-long skin incision with a scalpel in the lateral lower abdomen of the rat. Expose and dissect the external oblique muscle. Then, expose the epididymal adipose tissue surrounding the epididymis. Gently pull the epididymal adipose tissue out.
   NOTE: The adipose tissue can be obtained on either side of the rat's abdomen.
6. Excise only the epididymal adipose tissue, avoiding damage to the epididymis, testis, and epididymal blood vessels (**Figure S1**). Soak the excised adipose tissue in the PBS in the Petri dish to prevent dryness and weigh the tissue.
7. Close the abdominal muscle with 5-0 VICRYL and the skin with a 5-0 nylon suture. Then, return each rat that has undergone surgery to a separate cage (one rat per cage).
   NOTE: Monitor the rats until they fully recover.

2. **Isolate rASCs from 2 - 3 g of adipose tissue (excised from a Lewis rat and processed according to a previously-reported method)[27]. Briefly, enzymatically digest the excised adipose tissue with 0.1% type A collagenase at 37 °C for 1 h to obtain the stromal-vascular fraction (SVF; Figure S2).**
   1. Prepare several 100-µm cell strainers, several 50-mL tubes, several 15 mL tubes, and two scalpels on a biological clean bench. Turn on a centrifuge and warm up a water bath to 37 °C. In addition, prepare approximately 50 mL of sterile PBS in a 50 mL tube with 0.1% (final concentration) of type A collagenase.
   2. Mince the adipose tissue into small pieces using two scalpels.
   3. Move all minced adipose tissue to a 50-mL tube and add PBS to bring the total volume to 15 mL.
   4. Rest the 50-mL tube upright for 5 min. Discard the upper layer and collect the lower layer except for the debris.
   5. Add 0.1% of type A collagenase solution to the 50-mL tube with PBS containing the upper layer in order to enzymatically digest the adipose tissue.
      NOTE: Warm approximately 20 mL of PBS per 2 - 3 g of adipose tissue in a 37 °C water bath.
   6. Tilt and shake the 50 mL tube gently at 120 - 130 rpm for 1 h in a 37 °C water bath.
   7. Rest the 50 mL tube upright for 5 min after shaking.
   8. Filter the solution using a 100 µm cell strainer and pour the solution into a new 50 mL tube. Dispense the filtered solution into two 15 mL tubes.
   9. Centrifuge the solution in the 15 mL tubes for 5 min at 700 x *g*. Carefully remove the upper layer of supernatant and leave 5 mL of the lower layer of the solution. Do not aspirate the pellet.
   10. Add approximately 5 mL of complete culture medium to each 15 mL tube, up to 10 mL per 15 mL tube, and carefully re-suspend the pellet.
   11. Repeat steps 2.2.8 - 2.2.9. Then, obtain the SVF.

3. Prepare two 60 cm^2 culture dishes. Collect the SVF after two centrifugations at 700 × g for 5 min. Suspend the SVF and plate 10 mL of SVF solution on each 60-cm^2 culture dish.
4. Culture the dishes for 24 h at 37 °C in a 5% CO_2 incubator.
5. Prepare PBS and 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA), storable for several months at 4 °C until use.
6. After initial plating (24 h), wash the cells with PBS 3 times to remove unattached cells and debris. Add fresh complete medium.
7. Passage the cells that are nearly sub-confluent on day 3. Add 1 mL of 0.25% trypsin-EDTA and incubate the cells at 37 °C in a 5% CO_2 incubator for 3 - 5 min.
   NOTE: Complete the trypsinization step within 10 min to prevent cell damage.
8. Observe the cells under a light microscope after trypsinization to confirm that all the cells have thoroughly detached from the dishes. Then, add 9 mL of complete culture medium to the dishes.
9. Count the number of cells using a hemocytometer.
10. Subculture the cells at a density of 1.7 x 10^3 cells/cm^2 every 3 days until passage 3. Culture the subcultured cells at 37 °C in the humidified atmosphere of a 5% CO_2 incubator.

3. Creation of rASC Sheets

1. Prepare several 35-mm diameter temperature-responsive culture dishes. Pre-coat the dishes with FBS (or complete medium containing FBS) at 37 °C in a 5% CO_2 incubator for more than 1 h.
   NOTE: A 35 mm diameter temperature-responsive culture dish is a commercially available product.
2. **Prepare a thermo-plate and incubate at 37 °C for the following experimental procedures.**
   NOTE: Perform every procedure using temperature-responsive culture dishes on a 37 °C thermo-plate to prevent the cells from spontaneously detaching from the dish.
   1. Warm the complete culture medium used in the procedures to 37 °C prior to experimentation.
   2. Seed passage 3 rASCs derived from Lewis rats onto a 35 mm diameter temperature-responsive culture dish at a density of 1.5 x 10^5 cells/dish for 3 days using a 2 mL total volume of complete culture medium.
      NOTE: When rASCs are plated onto a new culture dish, the dish should be slowly rocked back and forth and left and right in an incubator to achieve uniform rASC seeding and a uniformly thick rASC sheet.
   3. Change 2 mL of the medium to 2 mL of complete culture medium containing 16.4 µg/mL L-ascorbic acid phosphate magnesium salt n-hydrate (AA) on day 3 after seeding to the temperature-responsive culture dishes incubated on a 37 °C thermo-plate.
      NOTE: Dissolved AA can be stored at -30 °C for months.
   4. Culture the cells for an additional 4 - 5 days and replace the medium every 2 days with fresh medium containing AA.
   5. Confirm the proliferation and generation of ASCs under a light microscope to determine whether gaps occurred between the cells in a contiguous cell sheet.

3. Transfer the cell sheets from the incubator to the benchtop for 15 - 20 min to cool them to room temperature.; observe the cells spontaneously detach as a contiguous cell sheet. Harvest the rASC sheet from the dish surface with a pair of forceps.

NOTE: Usually, rASC sheets can be handled with a pair of forceps. If necessary, a transfer membrane can be used to transfer a cell sheet from the culture dish to the wound site if the cell sheet is brittle and fragile.

4. Preparation of the Full-thickness Skin Defect Wound Model and Transplantation of rASC Sheets

1. Prepare a rodent mechanical ventilator and 4% isoflurane. Prepare several sterile cotton tips, clean gauze, 5-0 nylon suture, a scalpel, a periosteal raspatory, a needle holder, operating scissors, and a pair of forceps. Sterilize the surgical instruments and supplies. Lay out the surgical instruments and supplies on a sterile drape.
2. Prepare PBS and maintain it at room temperature, along with some artificial skin and a non-adhesive dressing. Precut the artificial skin (15 x 10 mm) and non-adhesive dressing (20 × 15 mm). Soak the inner collagen sponge layer of the artificial skin in saline before using the artificial skin.
   NOTE: The artificial skin is made of two layers: an outer silicone sheet layer and an inner collagen sponge.
3. Use ZDF rats (16 - 18 weeks old, male, 500 - 600 g) as a wound-healing model for type 2 diabetes and obesity.
4. Anesthetize the ZDF rats by inhalation of 4% isoflurane using a rodent mechanical ventilator. Induce anesthesia by using isoflurane at 4 - 5% via a rodent mechanical ventilator and maintain it at 3 - 4% during surgery. Monitor the depth of anesthesia by observing the depth and rate of respiration of each rat via a toe pinch.
5. While wearing sterile gloves, position the rat in the prone position on a sterile drape. Clean the head of the rat using sterile gauze soaked in 70% ethanol, shave the operative area with an electric razor, and clean the skin after shaving using sterile gauze soaked in 70% ethanol.
6. Create a squared full-thickness skin defect (15 x 10 mm^2) on the head of the anesthetized ZDF rats by removing the cutaneous tissue from the epidermis to the periosteum. Excise the skin and cutaneous tissue with a scalpel and remove the periosteum with a periosteal raspatory.
7. Move the rASCs on the 35 mm temperature-responsive culture dish from the incubator to a room-temperature environment immediately prior to transplantation.
8. Observe the rASCs on the 35 mm temperature-responsive culture dish spontaneously detach as a sheet from the dish surface after the formation of a wound. Harvest the rASC sheets after 7 days of culturing on temperature-responsive culture dishes. Remove the cultured medium and wash the rASC sheet with PBS three times. Soak the rASC sheet in PBS until transplantation to prevent desiccation.
9. Place the rASC sheet immediately over the skull defect using a pair of forceps. If the cell sheet is brittle and fragile, a membrane can be used as a scaffold for transferring the cell sheet from the culture dish to the wound site.
10. Cover the rASC sheet and defect with the artificial skin (15 x 10 mm^2) and close the wound with approximately 10 stiches using the 5-0 nylon suture. To protect the wound, place a non-adhesive dressing (20 x 15 mm^2) over the artificial skin, using 5-0 nylon sutures to keep the wound environment moist and to absorb exudates.
    NOTE: The non-adhesive dressings will be removed by the ZDF rats within a few days after application. Therefore, it is necessary to regularly monitor the rats after transplantation. Usually, the non-adhesive dressing is replaced every 2 days under general anesthesia.
11. Return each rat that has undergone surgery to a separate cage until they have fully recovered (one rat per cage). After an observation period, euthanize the rats using the approved method of isoflurane overdose.

Representative Results

This protocol attempted to establish a new cell-based therapy for intractable diabetic wounds. Briefly (as illustrated in **Figure 1**), allogeneic rASC sheets were created from normal rats using cell-sheet engineering and were then transplanted using a bilayer of artificial skin onto a full-thickness skin defect on a diabetic rat. Light microscope images of a good example of an rASC sheet (**Figure 2A**) and a bad example of an rASC sheet (**Figure 2B**) are shown in **Figure 2**. When ASCs are plated onto a new culture dish, the dish should be slowly rocked back and forth and left and right in an incubator to achieve uniform rASC seeding and a uniformly-thick rASC sheet (**Figure 2A**). If the rASCs cannot be uniformly attached and cultured on the surface of the culture dish, the sheet cannot be collected as a contiguous ASC sheet (**Figure 2B**). **Figure 3** shows ASC sheets that have been harvested as a contiguous cell sheet at room temperature because the ASCs were uniformly attached to the dish surface. Usually, rASC sheets can be handled with a pair of forceps. If necessary, a transfer membrane can be used to transfer a cell sheet from the culture dish to the wound site, such as if the cell sheet is brittle and fragile.
Figure 4 depicts the ZDF rats used as a diabetic wound-healing model and the transplantation of allogeneic rASC sheets combined with artificial skin. An rASC sheet is soft and flexible, adjustable in size, and capable of being extended to every corner of the wound site with a pair of forceps (**Figure 4A-F**). The rASC sheet-covered defect was also covered with artificial skin (15 x 10 mm^2) and sutured with approximately 10 stitches using a 5-0 nylon suture (**Figure 4G**). To protect the wound, a moist wound environment was maintained and exudates were absorbed, a non-adhesive dressing (20 x 15 mm^2) was placed over the artificial skin, and 5-0 nylon sutures were applied (**Figure 4I**). The non-adhesive dressing is often removed by the ZDF rats within several days of application. Therefore, the rats must be monitored after transplantation. Usually, the non-adhesive dressing is replaced every 2 days under general anesthesia. Please click here to view a larger version of this figure.

The macroscopic photographs in **Figure 5** are the representative results of the transplantation of an rASC sheet. In our previous study, the average wound area in the rASC sheet transplantation group (**Figure 5B**) was significantly smaller than in the control group (**Figure 5A**). For the controls, only artificial skin was used to cover the wound, without the transplantation of an rASC sheet. These images were taken on the 14th day after the creation of the wound (n = 6 in each group)[31].

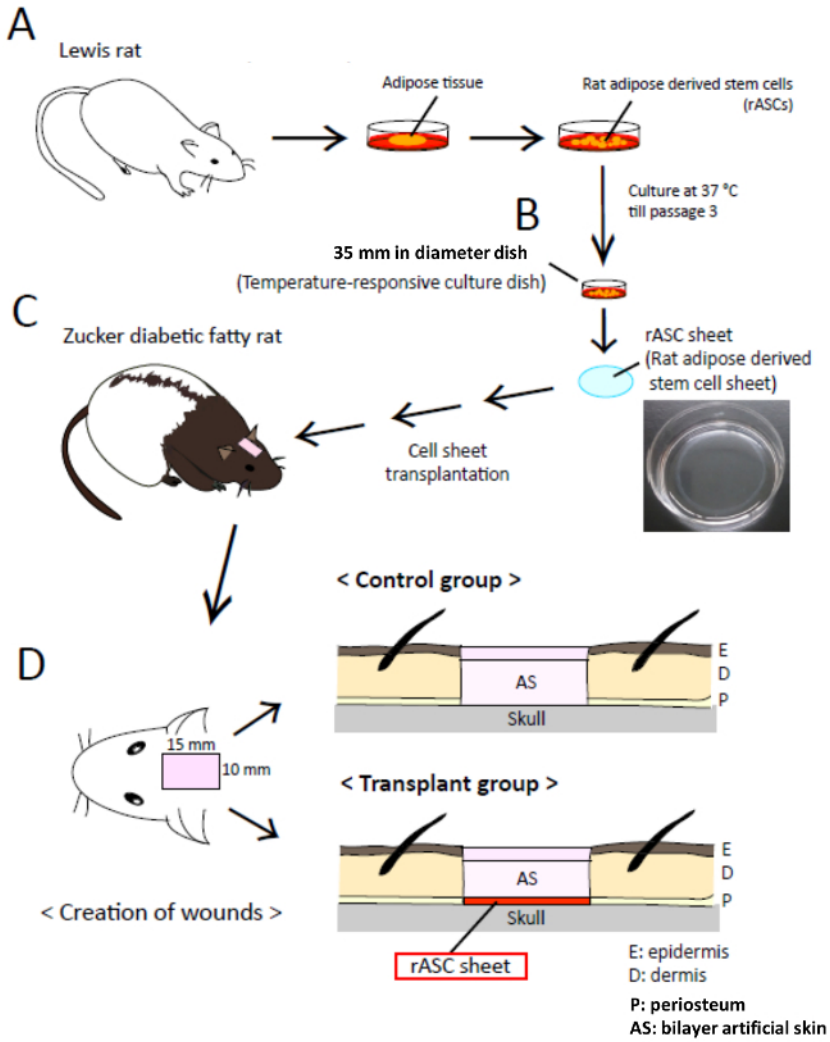

Figure 1: Schematic of the Experimental Transplantation Procedure. Schematic of the experimental transplantation procedure performed with an allogeneic rat adipose-derived stem cell (rASC) sheet and artificial skin in a rat wound-healing model of type 2 diabetes and obesity. (**A**) Rat adipose tissue was surgically excised from normal Lewis rats. rASCs were isolated and seeded onto a 60 cm^2 culture dish and cultured at 37 °C in a 5% CO_2 incubator for 7 - 8 days. (**B**) rASCs were subcultured every 2-3 days, and passage 3 rASCs were seeded onto a 35 mm diameter temperature-responsive culture dish. The cells were cultured in complete medium containing 16.4 µg/mL L-ascorbic acid phosphate magnesium salt n-hydrate (AA) at 37 °C in a 5% CO_2 incubator for 7 - 8 days. The rASCs were harvested as a contiguous rASC sheet by reducing the temperature to 20 °C. (**C**) rASC sheets transplanted onto a 15 x 10 mm^2 full-thickness skin defect with exposed bone on the heads of rats exhibiting diabetes and obesity (Zucker diabetic fatty (ZDF) rats) used as a wound-healing model. (**D**) An rASC sheet was placed on the skull directly over the defect and covered)with a 15 x 10 mm^2 sheet of bilayer artificial skin, which was sutured into place with 10 nylon (5-0) sutures. Diabetes 2015;64: 2723-2734; with permission. Diabetes (c) copyright (2015) by the American Diabetes Association. Please click here to view a larger version of this figure.

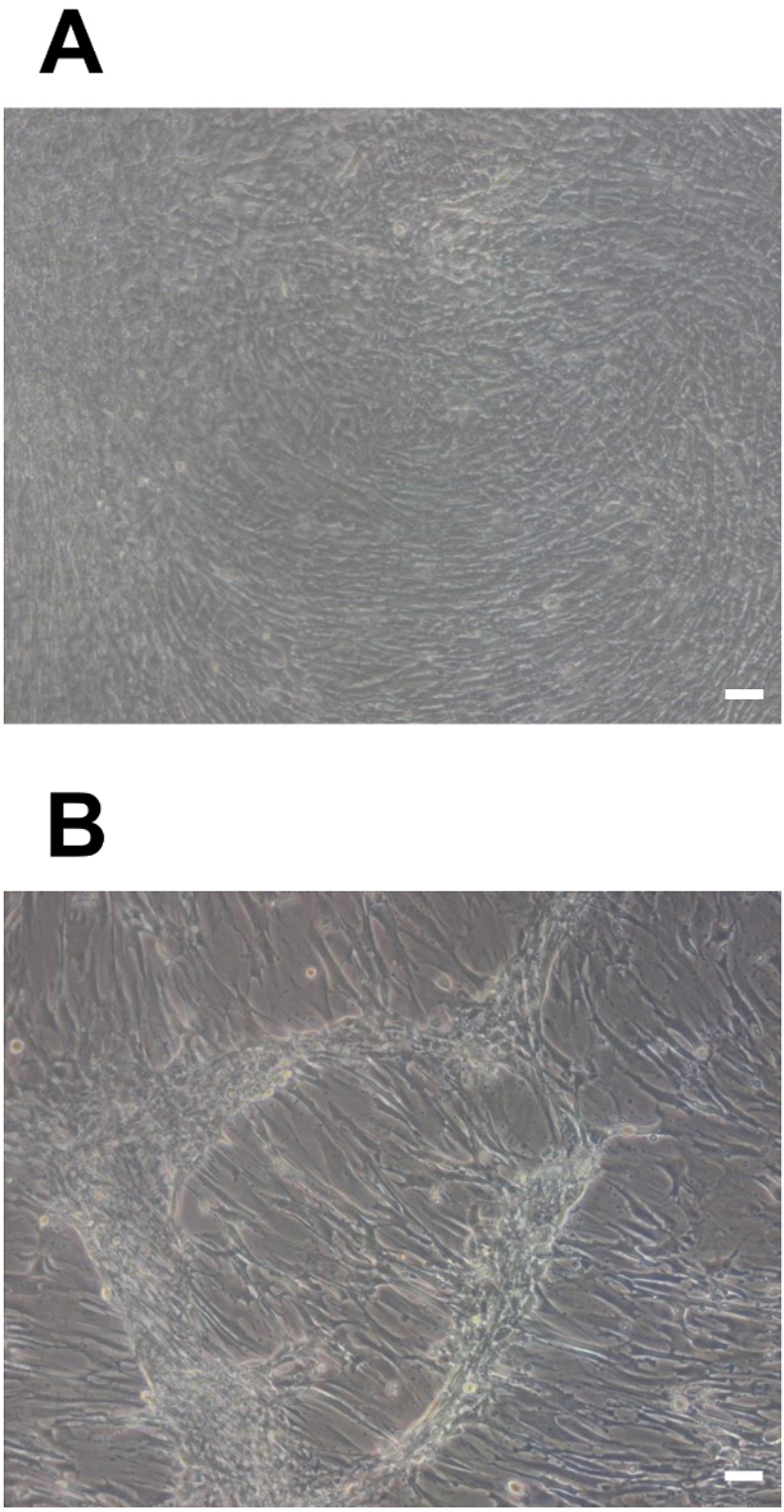

Figure 2: Light Microscope Images of ASCs. Light microscope images of ASC proliferation to the edge of culture dishes, without gaps between the ASCs. (**A**) An rASC sheet with a uniform thickness in all directions 7 days after the start of culturing (**A**). (**B**) An rASC sheet without uniform seeding. A contiguous rASC sheet cannot be obtained on day 7 after the start of culturing (**B**). Scale bars = 100 µm. Please click here to view a larger version of this figure.

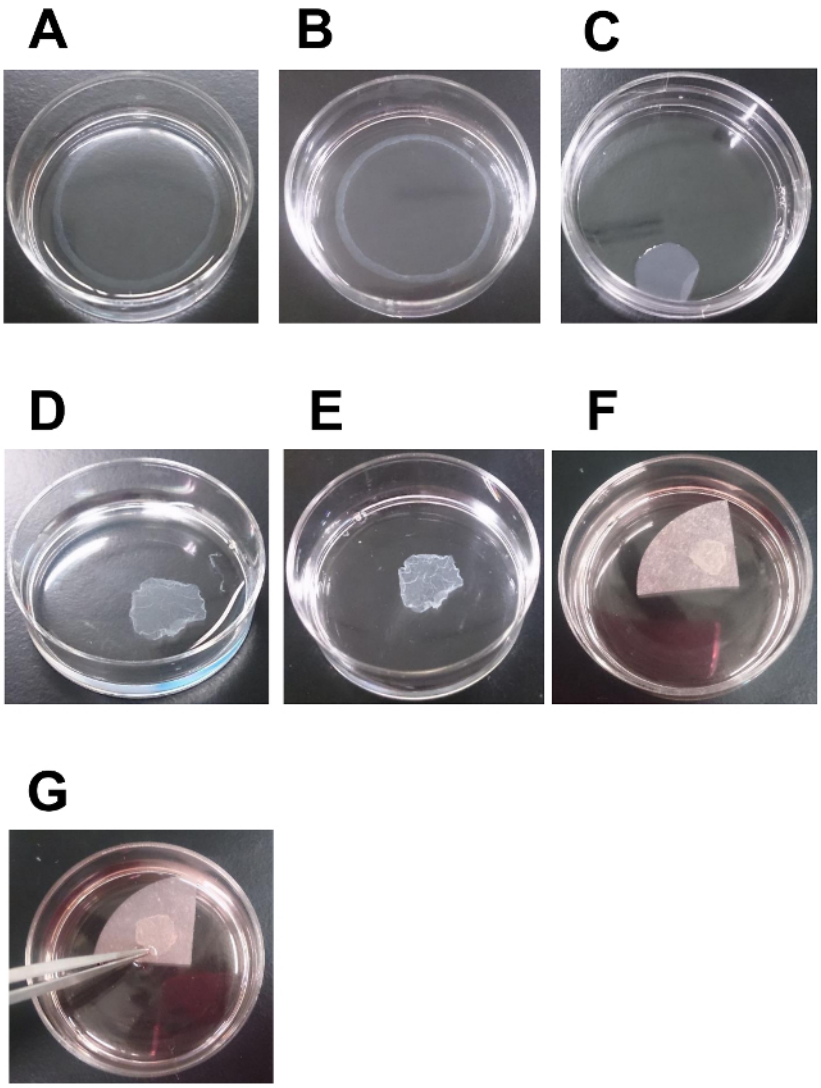

Figure 3: Time-lapse Images of rASC Sheet Statues at Room Temperature. Time-lapse images of the status of an rASC sheet at room temperature. rASCs on a 35 mm diameter temperature-responsive culture dish spontaneously and gradually detached from the dish surface at room temperature (approximately 20 °C) and were harvested as a contiguous sheet. (**A**) Approximately 5 min after moving the temperature-responsive culture dish to room temperature. (**B**) Approximately 10 min after moving the 35 mm diameter temperature-responsive culture dish to room temperature.(**C**) Approximately 20 - 30 min after moving the temperature-responsive culture dish to room temperature. This is a good-quality rASC sheet (**C**). (**D-E**) rASC sheet status approximately 20 - 30 min after moving the temperature-responsive culture dish to room temperature. This rASC sheet is of average quality (**D**). (**F-G**) rASC sheets are usually handled with a pair of forceps. If the cell sheet is brittle and fragile, a membrane can be used as a scaffold for transferring the cell sheet from the culture dish to the wound site. Please click here to view a larger version of this figure.

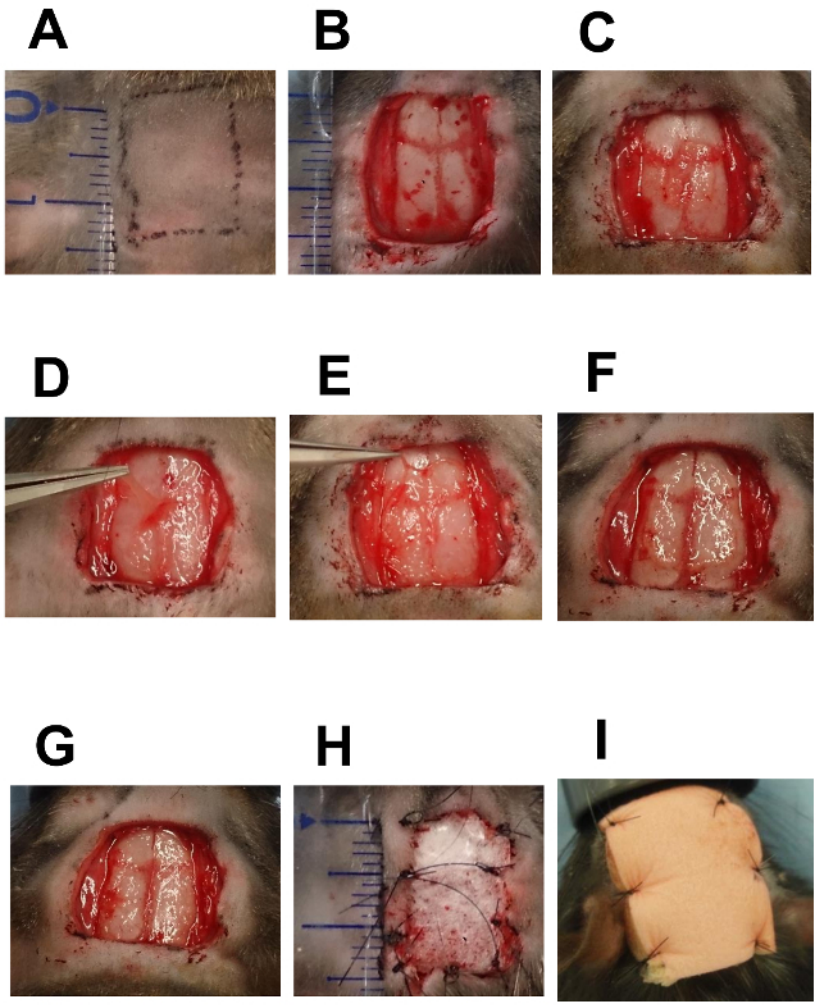

Figure 4: Time-series Images of Wound Creation and rASC Sheet Transplantation with Artificial Skin and a Non-adhesive Dressing
Time series images of wound creation and rASC sheet transplantation with artificial skin and a non-adhesive dressing. (**A**) The heads of ZDF rats were shaved with an electric razor. After shaving the body hair, check marks (15 x 10 mm^2) were drawn using an oily hydrophobic pen. (**B**) A full-thickness skin defect (15 x 10 mm^2) was created on the head of an anesthetized ZDF rat by removing cutaneous tissue from the epidermis to the periosteum. The skin and cutaneous tissue were excised with a scalpel, and the periosteum was removed with a periosteal raspatory. Using gauze moistened with sterile saline, pressure was applied to stop the bleeding after excision. (**C**) rASC sheet transplantation. An rASC sheet was placed over the defect immediately above the skull of the rat using a pair of forceps. (**D-G**) Adjusting rASC sheet extension to match wound size. The rASC sheet is flexible, adjustable, and can be extended to every corner of the wound site using a pair of forceps. For wider wounds, two or three flexible rASC sheets can be stacked. (**H**) Suturing the artificial skin covering the rASC sheet. The defect and the transplanted rASC sheet were covered with artificial skin (15 x 10 mm^2), which was sutured with 10 stitches using 5-0 nylon sutures. (**I**) Suturing of the non-adhesive dressing (20 x 15 mm^2) to the wound site covered with artificial skin. To protect the wound, non-adhesive dressing (20 x 15 mm^2) was placed over the artificial skin with 5-0 nylon sutures. Diabetes 2015;64: 2723-2734; with permission. Diabetes (c) copyright (2015) by the American Diabetes Association. Please click here to view a larger version of this figure.

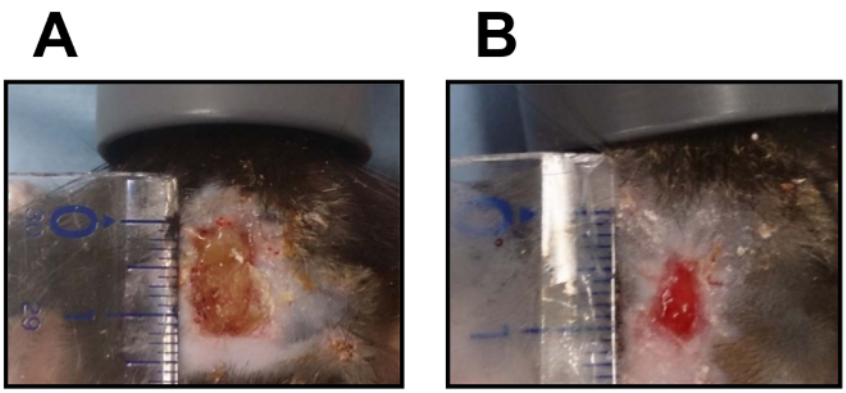

Figure 5: Macroscopic Images of Full-thickness Skin Defects. Macroscopic photographs of full-thickness skin defects without the transplantation of an rASC sheet (**A**) and with the transplantation of an rASC sheet (**B**) 14 days after creation of the wound. Please click here to view a larger version of this figure.

Discussion

The most critical steps for successfully culturing an rASC sheet are as follows: 1) The temperature must be maintained at approximately 37 °C during culturing on the temperature-responsive culture dishes. During the creation of an rASC sheet, every procedure was performed on a 37 °C thermo-plate, and every reagent was warmed to 37 °C to prevent the cells from spontaneously detaching from the dish[31]. 2) The recipient ZDF rats must be monitored to prevent the removal of the non-adhesive dressing, which is critical for the successful transplantation of rASC sheets. If the dressing is removed, a new non-adhesive dressing must be applied to prevent the transplanted ASC sheets from detaching from the wound site.

Using this procedure, rASC sheets were generally obtained within 5 - 7 days of seeding passage 3 cells on temperature-responsive culture dishes. The culture time required to generate an rASC sheet can be adjusted according to the initial cell density and the time at which the complete culture medium containing AA is applied. If an rASC sheet detaches from the dish during cell culturing, the rASC sheet should be remade, and additional dishes should be prepared in the event of cell detachment.

The limitations of this protocol are as follows: 1) Strict temperature management must be applied to maintain an approximate temperature of 37 °C during the entire process when using temperature-responsive culture dishes. 2) After obtaining an rASC sheet, special medical devices must be used to maintain moist conditions, because the rASC sheet is sensitive to dry conditions. 3) Post-operative management, including daily observation of the condition of the recipient rat, is required.

Larger wounds with exposed bone are often observed clinically. For example, traffic accident trauma, burns, infected wounds, and damaged or necrotic wounds after debridement can develop into large wounds with exposed bone. Here, a clinical model of severe wounds was developed using a large, full-thickness skin defect with exposed bone in rats with type 2 diabetes and obesity. This model has the potential to be used as a standard model for evaluating wound healing in diabetic rats.

Artificial skin is a commercially available medical device for full-thickness skin defects after debridement, and recombinant basic fibroblast growth factor (bFGF) has been widely used for wound healing to promote angiogenesis and granulation. These two treatments have been used to achieve considerable therapeutic results, even for chronic wounds, such as diabetic wounds. It has been reported that ASCs secrete angiogeneic growth factors[28], such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and bFGF, which could contribute to neovascularization[29,30] and accelerate wound healing. Our previous study confirmed that ASCs continuously secrete these growth factors[31]. Therefore, ASC sheets, combined with artificial skin, have the potential to be used as a new therapeutic option for accelerating vascularization and wound healing[31], and these sheets might be applicable to the treatment of many types of intractable ulcers or burns in human clinical settings in the future.

Disclosures

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References

1. International Diabetes Federation. IDF Diabetes Atlas, 7 ed. *Brussels, Belgium: International Diabetes Federation.* http://www.diabetesatlas.org/ (2015).
2. Boulton, A. J., Vileikyte, L., Ragnarson-Tennvall, G., & Apelqvist, J. The global burden of diabetic foot disease. *Lancet.* **366** (9498), 1719-1724 (2005).
3. Zannettino, A. C. *et al.* Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. *J Cell Physiol.* **214** (2), 413-421 (2008).
4. Kern, S., Eichler, H., Stoeve, J., Kluter, H., & Bieback, K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells.* **24** (5), 1294-1301 (2006).
5. Casteilla, L., Planat-Benard, V., Laharrague, P., & Cousin, B. Adipose-derived stromal cells: Their identity and uses in clinical trials, an update. *World J Stem Cells.* **3** (4), 25-33 (2011).
6. Zuk, P. A. *et al.* Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.* **7** (2), 211-228 (2001).
7. Zuk, P. *The ASC: Critical Participants in Paracrine-Mediated Tissue Health and Function.* (2013).
8. Nie, C. *et al.* Locally administered adipose-derived stem cells accelerate wound healing through differentiation and vasculogenesis. *Cell Transplant.* **20** (2), 205-216 (2011).
9. Shin, L., & Peterson, D. A. Human mesenchymal stem cell grafts enhance normal and impaired wound healing by recruiting existing endogenous tissue stem/progenitor cells. *Stem Cells Transl Med.* **2** (1), 33-42 (2013).
10. Okano, T., Yamada, N., Sakai, H., & Sakurai, Y. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(N-isopropylacrylamide). *J Biomed Mater Res.* **27** (10), 1243-1251 (1993).
11. Yamato, M. *et al.* Thermo-responsive culture dishes allow the intact harvest of multilayered keratinocyte sheets without dispase by reducing temperature. *Tissue Eng.* **7** (4), 473-480 (2001).
12. Sekine, H. *et al.* Cardiac cell sheet transplantation improves damaged heart function via superior cell survival in comparison with dissociated cell injection. *Tissue Engineering Part A.* **17** (23-24), 2973-2980,doi:10.1089/ten.tea.2010.0659 (2011).
13. Kuhlmann, J. *et al.* Intramyocellular lipid and insulin resistance: a longitudinal in vivo 1H-spectroscopic study in Zucker diabetic fatty rats. *Diabetes.* **52** (1), 138-144 (2003).
14. Slavkovsky, R. *et al.* Zucker diabetic fatty rat: a new model of impaired cutaneous wound repair with type II diabetes mellitus and obesity. *Wound Repair Regen.* **19** (4), 515-525 (2011).
15. Oltman, C. L. *et al.* Progression of vascular and neural dysfunction in sciatic nerves of Zucker diabetic fatty and Zucker rats. *Am J Physiol Endocrinol Metab.* **289** (1), E113-122 (2005).
16. Coppey, L. J., Gellett, J. S., Davidson, E. P., Dunlap, J. A., & Yorek, M. A. Changes in endoneurial blood flow, motor nerve conduction velocity and vascular relaxation of epineurial arterioles of the sciatic nerve in ZDF-obese diabetic rats. *Diabetes Metab Res Rev.* **18** (1), 49-56,doi:10.1002/dmrr.257 (2002).
17. Galiano, R. D., Michaels, V., Dobryansky, M., Levine, J. P., & Gurtner, G. C. Quantitative and reproducible murine model of excisional wound healing. *Wound Repair Regen.* **12** (4), 485-492 (2004).
18. Lin, Y. C. *et al.* Evaluation of a multi-layer adipose-derived stem cell sheet in a full-thickness wound healing model. *Acta Biomater.* **9** (2), 5243-5250 (2013).
19. McLaughlin, M. M., & Marra, K. G. The use of adipose-derived stem cells as sheets for wound healing. *Organogenesis.* **9** (2), 79-81,doi:10.4161/org.24946 (2013).
20. Cerqueira, M. T. *et al.* Human adipose stem cells cell sheet constructs impact epidermal morphogenesis in full-thickness excisional wounds. *Biomacromolecules.* **14** (11), 3997-4008 (2013).
21. Koga, Y. *et al.* Recovery course of full-thickness skin defects with exposed bone: an evaluation by a quantitative examination of new blood vessels. *J Surg Res.* **137** (1), 30-37 (2007).
22. Cianfarani, F. *et al.* Diabetes impairs adipose tissue-derived stem cell function and efficiency in promoting wound healing. *Wound Repair Regen.* **21** (4), 545-553 (2013).
23. Matsuda, K., Suzuki, S., Isshiki, N., & Ikada, Y. Re-freeze dried bilayer artificial skin. *Biomaterials.* **14** (13), 1030-1035 (1993).
24. Miyahara, Y. *et al.* Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. *Nat Med.* **12** (4): 459-65. (2006).
25. Iwata, T. *et al.* Cell sheet engineering and its application for periodontal regeneration. *J Tissue Eng Regen Med.* (2013).
26. Elloumi-Hannachi, I., Yamato, M., & Okano, T. Cell sheet engineering: a unique nanotechnology for scaffold-free tissue reconstruction with clinical applications in regenerative medicine. *J Intern Med.* **267** (1), 54-70 (2010).
27. Watanabe, N. *et al.* Genetically modified adipose tissue-derived stem/stromal cells, using simian immunodeficiency virus-based lentiviral vectors, in the treatment of hemophilia B. *Hum Gene Ther.* **24** (3), 283-294 (2013).
28. Kim, W. S. *et al.* Wound healing effect of adipose-derived stem cells: a critical role of secretory factors on human dermal fibroblasts. *J Dermatol Sci.* **48** (1), 15-24 (2007).
29. Nakagami, H. *et al.* Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. *Arterioscler Thromb Vasc Biol.* **25** (12), 2542-2547 (2005).
30. Asahara, T. *et al.* VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J.* **18** (14), 3964-3972 (1999).
31. Kato, Y. *et al.* 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. *Diabetes.* db141133,doi:10.2337/db14-1133 (2015).

