Rapid Stem Cell Extraction and Culture Device for Regenerative Therapy Using Biodegradable Nonwoven Fabrics with Strongly Oriented Fibers

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Biodegradable nonwoven fabrics that can be used to extract and culture adipose-derived stem cells (ADSCs) from adipose tissue slices without enzyme digestion are developed. The fabrics are made of fibers consisting of biodegradable poly(lactic-co-glycolic acid) (PLGA) and hydroxyapatite (HAp), which can be strongly oriented by adjusting the fiber deposition conditions. ADSCs are extracted from adipose tissue slices and cultured, and thick cell sheets are produced by placing nonwoven fabric on the adipose tissue slices or sandwiching the adipose tissue slices between two layers of fabric. Nonwoven fabrics with large anisotropy extract and culture ADSCs from adipose tissue slices more quickly than those with small anisotropy. In addition, cell sheets are produced more rapidly by cultivating cells on a culture vessel with low-cell adhesion. Finally, it confirms that ADSCs extracted and cultured from adipose tissue slices using nonwoven fabrics maintain the characteristics of mesenchymal stem cells.

1. Introduction

Between March 2016 and September 2019, we safely carried out regenerative treatments in which we transplanted autogenic cultured adipose-derived stem cells (ADSCs) into depressed lesions on the faces of five patients.[1] These regenerative treatments were implemented after our regenerative medicine provision plan (PB7150007) for the treatment was examined by the certified special committee for regenerative medicine, and the plan was accepted by the Health and Welfare Bureau of MHLW. Autogenic ADSCs cultured for 3–5 weeks in the cell processing facility (CPF) were used for the transplantations. The transplantations were performed using a method in which the autogenic ADSCs were mixed with newly extracted autologous adipose tissue and injected subcutaneously into a depressed lesion. We anticipated that the subcutaneously injected ADSCs would remain in the vicinity of the affected region and promote the regeneration of the surrounding tissues via the paracrine effect.

During the management of these five cases, we considered what should be done to promote wide adoption of this approach and identified three problems that could hinder its widespread use. First, production of ADSCs is costly. Some of the details of the production procedure cannot be described, as the method has been licensed to external companies by the intellectual property owner (University of the Ryukyus). Consequently, it is expensive to produce ADSCs for transplantation. The primary sources of expense are supplies, labor cost, quality control, and facility maintenance (Figure S1, Supporting Information). Accordingly, many research groups in addition to ours are concerned about the high costs of regenerative medicine.[2,3] To make regenerative medicine available to everyone, cell production costs must be kept low. Second, when the lesion is large or deformed, it is difficult to fill the entire affected region by injecting cells alone. In such cases, the volume necessary to fill the affected region is prepared by collecting fresh adipose tissue and mixing with cells, followed by injection.[4] However, this increases the burden on the patient because adipose tissue must be collected for both culture and transplantation. To decrease the burden on the patient, it is preferable to avoid collecting new adipose tissue, if possible. Third, it is difficult to ensure that the injected cells are delivered to the vicinity of the affected region and that they remain alive there for a long period of time. Several studies that monitored transplanted cells in vivo by various methods[5] reported that both accurate delivery and ensuring survival are challenging. These issues must be addressed because they strongly influence the outcomes of regenerative treatment. One potential solution is to transplant cells adhered to a scaffold[6] or in a self-supporting cell sheet.[7]

To solve the problems outlined above, we developed a biodegradable nonwoven fabric made from poly(lactic-co-glycolic acid) (PLGA), a biodegradable polymer, and hydroxyapatite (HAp), a calcium phosphate salt; both materials are currently used in medical devices. We found that the nonwoven fabric...
had high affinity for adipose tissue and ADSCs, and that it could be used as a scaffold for producing ADSCs quickly, in large quantities, and in a small physical volume. Use of this material should significantly decrease the cost of producing ADSCs from adipose tissue because it eliminates the need for enzyme treatment to disperse the adipose tissue, reduces passage operations, and decreases culture space (Figure S1, Supporting Information). In addition, if we use this nonwoven fabric as a filler for affected regions with large volumes or diverse shapes, it may be possible to avoid the use of adipose tissue as a filler. That is, only one adipose tissue collection would be required for therapy, decreasing the burden on patients. Furthermore, because ADSCs are firmly adhered to the scaffold at the time of transplantation, ADSCs in cell sheets on nonwoven fabric are expected to exhibit high engraftment after transplantation and remain in the vicinity of the affected region for a long period of time. Although previous studies have described seeding of adipose tissue slices on nonwoven fabric followed by extraction of ADSCs, the nonwoven fabric we developed is a new material made entirely of biodegradable polymers used in medical devices that can be transplanted directly as a sheet integrated with cultured cells.

In this study, we found that the high anisotropy of this newly developed nonwoven fabric contributed to the rapid extraction and proliferation of ADSCs. In addition, we showed that the cell density of ADSCs on the nonwoven fabric could be increased more rapidly if the ADSCs were extracted and cultured from adipose tissue slices using this nonwoven fabric on a culture vessel with low-cell adhesion. Finally, we peeled cells cultured from adipose tissue slices using this nonwoven fabric on a flat table (Figure S2, Supporting Information), and cut into circular pieces using a hollow mold of each size. The sizes of the circular pieces were 21 mm, 20 mm, 14 mm, and 32 mm, which are optimal for 24-well plates, 12-well plates, cell strippers, and 6-well plates, respectively. An example of each nonwoven fabric of 23 mm was captured using a Power Shot SX210 IS digital camera (Canon, Japan), which is shown in Figure 1a, and other sizes of nonwoven fabric A are shown in Figure S3 (Supporting Information). Figure 1a shows the nonbiodegradable polyethylene–polypropylene nonwoven fabric BMK, which was used as a control in this study. The BMK is a commercially available product (BMK-R003, 21 mm) for extracting ADSCs from adipose tissue slices. The biodegradable nonwoven fabrics were fabricated by ORTHOREBirth (Japan) and sterilized by gamma irradiation. Only 23 mm and 32 mm circles were used in this study.

2.2. Structural Observation and Analysis of Fabricated Nonwoven Fabrics

The nonwoven fabrics we developed have a structure that is predicted to improve adhesion between adipose tissue slices and fibers, as the structure is large enough to hold the seeded tissue slices firmly in the space between the fibers. Therefore, these fabrics have thicker fibers and larger voids than fabrics with subcellular-sized voids, which were created on the assumption that cells would be seeded in suspension.

Examples of 3D images of nonwoven fabrics A, B, and C obtained by confocal laser scanning microscopy observation (CLSM observation), are shown in Figure 1b. Structural observation reveals that the fiber structures differed between the fabrics. To assess the structural differences, we analyzed and compared the thickness, fiber diameter, contact point density, porosity, and anisotropy.

We observed no significant differences in thickness between A, B, and C (Figure 1c). Although B was produced by spinning for twice as long as A, the thicknesses of the two fabrics were similar. We speculated that the thickness of B may have decreased because the fiber structure was crushed by its own weight immediately after production. Fiber diameter differed significantly between A, B, and C (Figure 1d). The largest fiber diameter was C, followed by B and A in that order. Porosity did not significantly differ between A, B, and C (Figure 1e). We also evaluated contact point density because we thought it would be an important determinant of structural differences. The contact point density is the density of points where fibers come into contact with each other and intersect, and provides an index of how much the fiber structure is intertwined. The contact point density was significantly higher in B than in A (Figure 1f). We speculated that the contact point density of B may have increased because the fiber structure was crushed for the same reason that the thickness was reduced. Fiber diameter differed significantly between A, B, and C (Figure 1d). We also analyzed the anisotropy of each nonwoven fabric and found that the anisotropy of A was significantly larger than that of C (Figure 1g). The larger the anisotropy, the stronger the orientation of the fibers, whereas the smaller the anisotropy, the weaker the orientation. If the anisotropy is 1.05 or less, there is no orientation, whereas if it is 1.10 or higher, it can be considered strongly oriented. Anisotropy analysis revealed that all nonwoven fabrics used in this study had an orientation, as the smallest anisotropy was 1.17 (C). On the other hand, A and B, whose orientations were clearly visible to the naked eye, had large anisotropies of 2.54 and 2.22, and these fabrics were strongly oriented.

2.3. Comparison of cell Proliferation on Four Types of Nonwoven Fabric

We sandwiched 0.05 g adipose tissue slices between pieces of nonwoven fabric or cover glass. On the 3rd, 18th, and 36th days from the start of culture, we compared cell proliferation on...
Performing a significance test (Dunnett) for cell proliferation using A or B as the control group.

Figure 1. Fabrication, observation, and structural analysis of nonwoven fabrics. a) Circular pieces of nonwoven fabrics. b) 3D images of each nonwoven fabric. These images are superimpositions of 101 images taken at regular intervals over 300 µm in the Z-axis direction. The 300 µm step size was sufficient to capture everything from the top to the bottom of the fabric. All images taken in three locations on each fabric are shown in Figure S4b (Supporting Information). Bar: 300 µm. c) Thickness, d) fiber diameter (fiber width), e) porosity, f) contact point density, g) anisotropy of each nonwoven fabric. Significance test (Tukey–Kramer) of each pairwise comparison; only comparisons with a significant difference are marked with an asterisk.
We observed no significant difference in cell proliferation between A and the other fabrics on the 3rd day of culture, just after seeding, whereas proliferation on A was faster than on all fabrics other than B on the 18th day of culture. On the 36th day of culture, proliferation on A was significantly faster than BMK (Figure 2b). When B was used as the control, the results were almost the same. Cell proliferation on B did not significantly differ from that on other fabrics on the 3rd day of culture, was significantly faster than on cover glass and all nonwoven fabrics except for A on the 18th day, and did not significantly differ from the other fabrics on the 36th day (Figure 2b).

We suspected that the material and structure of the nonwoven fabrics might be responsible for the significant increase in the cell proliferation rate on A and B on the 18th day of culture. To explore this possibility, we focused on the structures of the fabrics. First, we examined the structures of nonwoven fabrics A, B, and C, as they all have the same fabric composition. We observed a significant difference in the anisotropies of these structures, as well as in the fiber diameters of these structures, but no differences in their thicknesses, porosities, or contact point densities between A and C or B and C. Fiber diameter significantly increased in the order A, B, C, and it would be difficult to infer a correlation between this order and the rates of cell proliferation. On the other hand, anisotropies did not significantly differ between A and B, although they were significantly different between A and C. Taking this observation into consideration, we speculated that anisotropy contributed most to the improvement in cell proliferation on the 18th day of culture on A and B. In fact, the trends in anisotropy (Figure 1g) and proliferation (Figure 2b) were similar. On the other hand, the clear difference on the 18th day of culture became smaller by the 36th day of culture. We will discuss this observation further in Section 2.5, based on the results of Section 2.4.

Based on the results described above, fabrics A and B, which had high anisotropy, were excellent materials for extracting and culturing cells from adipose tissue without enzyme treatment. Because we observed no large difference in cell proliferation between nonwoven fabrics A and B, we decided to proceed with studies using A, which has a shorter spinning time and is therefore more economical in terms of resource usage.

### 2.4. Extraction and Culture of Cells Using Low-Cell Adhesion Well Plates

A significant number of cells adhered to the surface of the cell strainer described in Section 2.3 (data not shown). Although fewer cells adhered to the cell strainer than to the nonwoven fabric, the use of a cell strainer may decrease the density of cells on the nonwoven fabrics and delay the formation of cell sheets. To resolve this problem, we investigated the use of low-cell adhesion well plates for culture with nonwoven fabrics. We anticipated that it would be easier to handle multiple fabrics using a well plate. In addition, we sought to improve the efficiencies of cell extraction and culture by preventing the adipose tissue slices from floating after seeding, and by improving adhesion between the nonwoven fabric and adipose tissue slices. To improve the efficiencies, we placed a Teflon weight...
(perforated Teflon plate), to which cells do not adhere well, on the nonwoven fabric.

First, we prepared a cell culture plate with excellent cell adhesiveness as a control and two low-cell adhesion plates as test plates. The low-cell adhesion plates were the EZ-BindShut SP 6-well plate (hereafter, EZ plate) and the ultralow attachment surface plate (hereafter, ultralow plate). We seeded 0.05 g adipose tissue slices on either one or two pieces of \( \varphi 23 \) mm circular fabric A in each well of the plates, and then cultured the tissues at 37 °C and 5% CO2 in ADSC-GM for 36 days (Figure 3a). In this experiment, when the slices were seeded on one fabric, we placed a piece of fabric over the adipose tissue slices, but when they were seeded onto two fabrics, we sandwiched the slices between the two pieces of fabric. In both covered and sandwiched seeding, the slices were seeded in the center of each piece of fabric, and then a perforated Teflon plate was placed on top as a weight. In addition, when the adipose tissue slices were sandwiched between two pieces of fabric, the orientation directions of the fibers of the upper and lower non-woven fabrics were the same.

In this experiment, when the slices were seeded on one fabric, we placed a piece of fabric over the adipose tissue slices, but when they were seeded onto two fabrics, we sandwiched the slices between the two pieces of fabric. In both covered and sandwiched seeding, the slices were seeded in the center of each piece of fabric, and then a perforated Teflon plate was placed on top as a weight. In addition, when the adipose tissue slices were sandwiched between two pieces of fabric, the orientation directions of the fibers of the upper and lower pieces were aligned to unify the seeding and culture conditions to the greatest extent possible. On the 36th day of culture, we stained nuclei and actin filaments with DAPI and Alexa Fluor 488 phalloidin and observed the cell sheet on an inverted fluorescence microscope.

Fluorescence microscopy of the cell sheets was performed on a glass-bottom dish in D-PBS. We placed the cell sheet in

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**Figure 3.** Extraction, culture, and observation of cells from adipose tissue slices using a low-cell adhesion well plate. 

- **a)** Method for seeding, extracting, and culturing adipose tissue slices on nonwoven fabrics.
- **b)** Observation of cells on nonwoven fabrics. 
- **c)** Fluorescence images of cell nuclei (DAPI staining) in cell sheets cultured in each type of 6-well plate. From left to right: high-adhesion culture plate, EZ plate (low adhesion), and ultralow plate (low adhesion). The gamma values of these images were corrected by Axiovision 4.8 to make them easier to see. The number of cell nuclei (number of cells) in (1) and (3) is displayed above each fluorescence image. Bar: 200 µm. 
- **d)** Superposition image of actin filaments and nuclei of cells in peripheral regions (1) and (3) of a cell sheet assembled on an ultralow plate. The white dotted lines indicate the edge of the cell sheet, and the double-headed arrow indicates the fiber orientation direction. Bar: 300 µm. 
- **e)** From left to right are permeation images of cells on the surface of a cell culture plate, an EZ plate, and an ultralow plate at the end of the culture period. The position where region (3) of the nonwoven fabric contacted each plate are indicated. The black lines in these figures are positioning lines (diameter and outline) for region (3) of the fabrics, drawn with a felt pen on the bottom of the well (on the outside surface of the plate). Bar: 300 µm. 
- **f)** A graph for comparing the total number of cells in regions (1) and (3) among the three cell sheets. Red column a slice of adipose tissue was covered with a piece of nonwoven fabric and cultured on each type of plate. Blue column adipose tissue slices were sandwiched between two pieces of nonwoven fabric and cultured on each type of plate.
the dish so that the seeding surface was on the bottom side and placed a cover glass from above to prevent the sheet from floating (Figure 3b). After fluorescence images (670.8 \( \mu m \times 670.8 \mu m \)) were acquired at intervals of 5 \( \mu m \) with a width of 200 \( \mu m \) or 300 \( \mu m \) in the Z-axis direction, we created a 2D image by merging all the obtained fluorescence images. Images were acquired in three areas (Figure S11, Supporting Information): the peripheral region in the direction perpendicular to the orientation direction of the fibers (1), the center (2), and the peripheral region parallel to the fiber orientation direction (3). (1) and (3) consisted of three square areas that were continuously photographed and connected around the periphery of the nonwoven fabric; the areas of the two regions were equal. Adipose tissue slices were cultivated by covering them with a piece of nonwoven fabric in various 6-well plates. Fluorescence images of cell nuclei in regions (1), (2), and (3) of the fabricated cell sheets are shown in Figure 3c. Although we observed no difference in the number of nuclei in region (2), we saw a clear difference in the total number of cells in regions (1) and (3) on the control, EZ, and ultralow plates. Regions (1) and (3) of the cell sheet fabricated on the high-adhesion cell culture plate contained few cells, but many cells were present in cell sheets fabricated on the EZ plate and ultralow plate.

As shown in Figure 3c, region (3) had an overwhelmingly higher cell density than region (1) on both types of low-cell adhesion plates. The cells in each fluorescence image were counted using MetaMorph 7.8, and the counts are shown above each fluorescence image in Figure 3c. Based on these cell numbers, we confirmed that the cell numbers of both cell sheets fabricated on a low-cell adhesion plate were larger than those fabricated on cell adhesion plates, and that the cell numbers were higher in region (3) than in region (1). This result is unsurprising due to the way in which cells adhere and extend along the fibers of the nonwoven fabric. Although the adipose tissues were different, the cells on both peripheral edges (1) and (3) adhered and extended in the direction of fiber orientation (Figure 3d), as we observed when we looked at the actin filaments and cell nuclei on the peripheral edges (1) and (3) of a cell sheet prepared under exactly the same conditions as the one fabricated on the ultralow plate in Figure 3c. Such cell adhesion and extension along the fiber orientation direction of nonwoven fabrics have been previously reported. Moreover, cells adhere and extend along the orientation direction of 3D structures other than nonwoven fabrics. We presume that cells in both peripheral edges (1) and (3) can easily migrate to the left and right from the adhesion and extension directions, as cells extend protrusions and elongate in the migration direction. That is, cells migrate from adipose tissue slices seeded in the center of the nonwoven fabric and migrate easily in parallel to the direction of fiber orientation. We believe that this is why more cells reached the parallel edge (3) than the vertical edge (1).

Moreover, when we examined transmission images of where regions (1) and (3) made contact with each plate, we found that the cells densely adhered at almost 100% confluence in both regions (1) and (3) on the control plate, but barely adhered on the two low-cell adhesion plates. Figure 3e shows an example of the what was observed in region (3) on each plate. Cell adhesion was rarely observed on the perforated Teflon plate used as a weight (data not shown). It was difficult to observe the transmitted image of region (2) on each plate, where the adipose tissue slices were pressed against the plate, due to the adhesion of an oil-like material from the adipose tissue slices.

We counted the cell nuclei in the fluorescence image shown in Figure 3c using MetaMorph 7.8 software, and graphed the total cell numbers in regions (1) and (3) of the cell sheets cultured on each plate (Figure 3f). The results from cell sheets made by sandwiching adipose tissue slices between two nonwoven fabrics are also shown in Figure 3f. The results were evaluated in exactly the same way as for cell sheets made of one piece of nonwoven fabric. As shown in Figure 3f, cell densities of cell sheets made on low-cell adhesion plates (EZ plate and ultralow plate) were >4-fold greater than those of sheets made on a cell-adhesive plate (cell culture plate) with either one or two nonwoven fabrics. Thus, the density of a cell sheet can be efficiently increased if it is made on a low-cell adhesion plate. This is largely because the number of cells escaping from the nonwoven fabric onto the plate, or crawling out of adipose tissue slices on the plate, can be reduced (Figure 3e). Moreover, more cells could be extracted and cultured when slices were sandwiched between two pieces of nonwoven fabric than when they were covered with a single piece of fabric (Figure 3f).

2.5. Effect of Fiber Orientation on Cell Migration and Proliferation

As shown in Figure 3c, when the cell sheet was fabricated using nonwoven fabric A, the cell number was larger on the peripheral edge (3) parallel to the fiber orientation than on the peripheral edge (1) perpendicular to the fiber orientation. This observation shows that cells crawling out from centrally seeded adipose tissue slices on nonwoven fabric A migrated and proliferated faster in the direction parallel to the fiber orientation than in the perpendicular direction, and that they reached the edge more rapidly. Cell migration rate and distance increases on oriented scaffolds. On the other hand, cell migration ceases or its direction is changed by contact inhibition of cell movement. When contact inhibition of cell movement is unlikely to occur, it is also advantageous for cell proliferation.

Based on this reasoning, we speculated that cell proliferation was faster on fabrics A and B on the 18th day of culture (Figure 2b) because contact inhibition of cell movement was unlikely to occur on strongly oriented fibers. Moreover, we speculated that cell proliferation on fabrics A and B did not significantly differ from proliferation on fabric C on the 36th day of culture because cell extraction and proliferation were suppressed by contact inhibition of cell movement by dense packing of cells along the fiber orientation. This can be seen in Figure 3c, which shows the cell distribution on nonwoven fabric A on the 36th day of culture.

Thus, we can conclude that with our culture method, nonwoven fabrics A and B allow rapid extraction and culturing until around the 18th day of culture. In addition, we predict that extracting and culturing cells from adipose tissue slices for a longer period of time can be effectively performed by delaying contact inhibition of cell movement on nonwoven fabrics A and B. For example, we expect that methods for seeding at multiple locations on the diameter perpendicular to the fiber orientation (see Section 2.6), and for seeding finer adipose
tissue slices evenly over the entire nonwoven fabric, would be effective because cells crawling out of adipose tissue slices tend to spread in the direction parallel to the fiber orientation, but these cells do not easily spread in the direction perpendicular to the fiber orientation.

We compared and observed cell sheets by microscopy after the same amounts of adipose tissue slices were seeded and cultured in the center of circular nonwoven fabrics with large anisotropy. In this experiment, we noticed an uneven distribution of cells on the cell sheets, and we were able to determine why such anisotropic nonwoven fabrics have a high capacity for extraction and culture of cells. We believe that the knowledge obtained in this investigation will make a major contribution to the design of nonwoven fabrics with higher cell extraction and culture capabilities.

2.6. Quality of Cells Extracted and Cultured on Nonwoven Fabric

We placed adipose tissue slices (total mass, 0.15 g) on nonwoven fabric A (ϕ 32 mm) and then placed a piece of fabric of the same size above the slices (Figure 4a). The slices were seeded on the diameter perpendicular to the orientation direction of the fibers of the fabric (Figure 4b). Also, the orientation directions of the fibers of the upper and lower pieces were aligned to make seeding and culturing conditions uniform. On day 34 of culture, we obtained a total of 6.08 \times 10^5 cells by peeling off a total of six nonwoven fabrics used to sandwich adipose tissue slices in three wells. It should be possible to further increase the number of cells collected because many cell nuclei (as revealed by DAPI staining) were still present on nonwoven fabrics after peeling and collection (data not shown). After the cells were stained for CD markers (CD29, CD34, CD44, CD45, and CD90), the percentage of cells positive for each CD marker was examined by flow cytometry (Figure 4d). CD29, CD34, CD45, and CD90 are expressed in ADSCs, but CD44 is not.[19] The level of CD34 decreased when ADSCs were cultured, eventually almost disappearing.[2,20] Single-parameter histograms of the expression level of each CD marker (Figure 4d) indicated that the cells making up the sheets were ADSCs, as 93% or more were positive for CD29, CD45, and CD90, and less than 2% were positive for CD44 and CD34. Based on these findings, we conclude that the cell sheets extracted from adipose tissue slices and cultured for 34 days on nonwoven fabric consisted almost entirely of ADSCs. We will also evaluate CD105 and CD73 as positive ADSC markers[2,19–21] in a future study.

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**Figure 4.** Estimation of CD marker of cells forming a cell sheet by flow cytometry. a) Method for seeding, cell extraction from adipose tissue slices, and cell culture on nonwoven fabric. The straight line drawn on the nonwoven fabric indicates the orientation direction of the fibers. b) Seeding positions of adipose tissue slices on a nonwoven fabric. c) Culture in a low-cell adhesion well plate. Perforated Teflon plates were placed above adipose tissue slices sandwiched between two pieces of nonwoven fabric. d) Single-parameter histograms of the expression level of each CD marker. “Normalized To Mode” on the vertical axis indicates that the highest value (i.e., the most frequent intensity) of the histograms is set to 100, to allow easy comparison. The horizontal axis is the signal intensity (area) of each dye. The gray histogram is for the isotype control, and the histograms drawn with the blue line are for each type of CD antibody-positive cell. The left end of the bar showing the percentage of each CD antibody-positive cell is set to be less than 1% of the area of the isotype control histogram.
2.7. The Numbers of Cells Extracted and Cultured Using Nonwoven Fabrics, and the Usefulness of this Culture Method

In addition to confirming the quality of ADSCs by flow cytometry, we obtained \(6.08 \times 10^7\) cells from a total of 0.45 g adipose tissue slices seeded in three wells without passage for 34 days, which is also a major achievement. Using the cell culture method that we developed, when we seeded a total of 9 g of adipose tissue slices sandwiched between two pieces of nonwoven fabric in each well of ten 6-well plates (low adhesion) and grew them for 34 days without a subculture, we obtained about \(1.20 \times 10^8\) cells. Although the cell number used in a single regenerative treatment varies greatly depending on the transplant site and transplantation method, \(1.20 \times 10^8\) cells is sufficient for regenerative treatment; the number of cells used in a single regenerative treatment is typically \(1.0 \times 10^4\)–\(1.5 \times 10^8\).[4,22] Moreover, this technique could still be further optimized, so we expect that more cells would be obtained in a shorter time if the adipose tissue slices could be made finer and more evenly seeded over the entire fabric. In addition, to get \(1.20 \times 10^8\) cells from T-75 cell culture flasks using the usual culture method, we would need liberase or collagenase treatment before seeding, two or more passages at the right timing, regular observation of the cells, and 15–20 flasks at ≥70% confluence. This would be laborious and costly using conventional culture methods (Figure S1, Supporting Information). In other words, if we take advantage of our newly developed method, the cost of regenerative treatment can be decreased because we can skip the liberase or collagenase treatment, and significantly reduce the materials and labor costs involved in the procedure (Figure S1, Supporting Information). Even when the cost of the nonwoven fabric itself is taken into consideration, the elimination of the need for liberase or collagenase treatment and subculture would greatly facilitate the production of cells in the regenerative therapy. The cells extracted and cultured using nonwoven fabric retain the properties of mesenchymal stem cells and can be generated in sufficient quantities for regenerative therapy. Therefore, we believe that this nonwoven fabric will be useful as a device for mass production of ADSCs for regenerative therapy without the need for liberase or collagenase treatment and subculture.

3. Conclusions

In this study, we succeeded in producing nonwoven fabrics A, B, and C, which have various shapes. We found that the proliferation of ADSCs extracted from adipose tissue slices and cultured using nonwoven fabrics A and B, which had high anisotropy, was significantly higher after 18 days of culture (Figure 2b). We speculated that the large anisotropy may be responsible for this increase in proliferation.

Based on the results of Figure 3c, we inferred that ADSCs on nonwoven fabrics with high anisotropy are less likely to cause contact inhibition, and we also suspect that ADSCs proliferate more rapidly on such fabrics. We suspect that contact inhibition of cell movement is primarily responsible for the reduction in cell extraction and proliferation on nonwoven fabrics A and B over longer culture periods. We predict that by seeding in this manner, the extraction and culture efficiency of ADSCs from adipose tissue slices can be improved, resulting in the production of more homogeneous ADSC sheets. In addition, we found that culturing on a low-cell adhesion plate accelerated cell proliferation and cell sheet formation.

Finally, we confirmed that a sufficient number of high-quality cells suitable for regenerative treatment could be obtained using nonwoven fabrics and the culture method we developed. This approach produces cells more easily and at a lower cost than conventional methods. Optimization of the size of adipose tissue slices and the amount of cells seeded should allow us to secure even more cells in a shorter period of time. We are also considering the use of nonwoven fabric in scaling up or stacking multiple layers of cells in a culture vessel. Moreover, cell sheets on nonwoven fabric can be made from coin size to A4 size (Figure S2 and S3, Supporting Information). We can cut the sheet into a shape that fits the affected area and easily attach it there. It is also possible to deal with large affected areas by folding or rolling the sheet. In the context of regenerative medicine, we hope that the nonwoven fabric will be a useful device for reliable delivery of stem cells throughout the affected area and keeping them active in the area for a long time.

4. Experimental Section

Cells, Reagents, and Experimental Instruments: ADSC-GM culture medium was purchased from Lonza (USA). Penicillin G potassium was purchased from Meiji Seika Pharma (Japan). Recombinant GMP-grade trypsin was purchased from Roche Diagnostics International (Switzerland). Flowmi Cell Strainers (porosity 70 µm, for 1000 µL Pipette Tips) were purchased from Merck (Germany). Paraformaldehyde (4%) in phosphate buffer solution and PBS (D-PBS, pH 7.4) were purchased from Nacalai Tesque (Japan). DAPI solution was purchased from Fujifilm Wako Chemicals (Japan). EZ-BindShut SP 6-well plates were purchased from AGC Techno Glass (Japan). Perfusion solution, CellBIND T-75 cell culture flasks, 100 mm cell culture dishes, 35 mm cell culture dishes, Costar 6-well plates (ultralow attachment surface), and Costar 6-well culture plates were purchased from Corning (USA). Petri dishes (150 mm) for suspension cell culture were purchased from Sumitomo Bakelite (Japan). Glass-bottom dishes (50 mm, φ 30 mm) were purchased from MatTek (USA). Micro cover glass (φ 22 mm round, φ 40 mm round [custom-ordered, no.1 thickness], 24 mm × 60 mm) was purchased from Matsunami Glass (Japan). Alexa Fluor 488 Phalloidin, SYTOX Blue Dead Cell Stain, and cell strainers (100 µm mesh) were purchased from Thermo Fisher Scientific (USA). PE Mouse IgG1 κ Isotype Ctrl, APC Mouse IgG1 κ Isotype Ctrl, APC Anti-Human IgG1, PE Anti-Human CD29, PE Anti-Human CD90, PE Anti-Human CD45, APC Anti-Human CD34, and PE Anti-Human CD44 were purchased from Biolegend (USA). BMK-R003 (φ 21 mm round), a nonbiodegradable polylethylene–polypropylene nonwoven fabric, used as a control for biodegradable nonwoven fabrics, was purchased from Bio Future Technologies (USA). Glass perforated plate (φ 20 mm round, 3.0 mm thickness, 1.9 g) and Teflon perforated plate (φ 30 mm round, 3.0 mm thickness, 3.8 g) were purchased from Toshin Riko (Japan). The Premixed WST-1 Cell Proliferation Assay System was purchased from Takara Bio (Japan). Milli-Q water was used for all experiments. If should be noted that nonwoven fabric A with a diameter of 23 mm (φ 23 mm), one of the biodegradable nonwoven fabrics used in this report, is already sold by ORTHOReBIRTH (Japan).

Fabrication of Biodegradable Nonwoven Fabrics: In this study, biodegradable nonwoven fabrics were used as a scaffold to extract and cultivate ADSCs from adipose tissue. The fibers that constitute the
nonwoven fabrics were composed of the biodegradable polymers PLGA and hydroxyapatite (HAp). HAp was added to increase the adhesion of cells to the fibers. For PLGA, LG85SS (inherent viscosity, 2.5–3.5 dL g⁻¹) made by Evonik Industries AG (Germany) was used. For HAp, a special-order product made by Taihei Chemical Industrial (Japan) was used. Many scaffolds containing HAp were used to culture ADSCs, and it was reported that ADSCs could be induced to differentiate into vascular endothelial-like cells,23,24 chondrocyte-like cells,8,25 osteoblast-like cells,23,26 and adipocyte-like cells.27 All raw materials and solvents were of a grade suitable for use in medical devices.

Observation of the Structure of the Nonwoven Fabrics: The 3D structure of the nonwoven fabrics made from biodegradable fibers A, B, and C on an upright confocal laser scanning microscope (CLSM) was observed. Circles of ϕ23 mm were used for these observations. 3D images of the nonwoven fabrics were obtained by confocal laser scanning microscopy imaging (CLSM imaging) (Figure 1b, and Figure S4b, Supporting Information). The CLSM used for the observation was an FV-1000 D (Olympus, Japan) with an objective lens UPLSAPO 10 × 2 NA:0.40 (Olympus). CLSM imaging of each nonwoven fabric was performed in three areas along the diameter (Figure S4a, Supporting Information). Fabrics A and B, whose fiber orientations could be clearly confirmed visually, were adjusted so that the diameter passing through the three observed areas was parallel to the fiber orientation. A total of 101 images of area 1270 × 1270 µm at intervals of 3.0 µm were observed in the Z-axis direction to ensure that the entire thickness from the top surface (in the case of an upright microscope, the surface close to the objective lens) to the bottom surface was captured.

Structural Analysis of the Nonwoven Fabrics: Thickness, fiber diameter (fiber width), contact point density, porosity, and anisotropy of each nonwoven fabric were determined by analysis of 3D images (Figure 1b, and Figure S4b, Supporting Information). These images were obtained by CLSM imaging as described above. Analyses of thickness and the fiber width of each nonwoven fabric were performed using Fluoview 4.2b (Olympus), whereas analyses of contact point density, porosity, and anisotropy were performed using Image Pro 10 (Media Cybernetics, USA).

First, the thickness of the nonwoven fabric was determined for each observation area by analyzing the 3D images that were captured at three observation areas on each nonwoven fabric. The method involved cutting out five cross-sections of the nonwoven fabric in each observation area, measuring the distance between the fiber on the top surface and the fiber on the bottom surface (i.e., the thickness) of each cross-section by Fluoview 4.2b, and averaging the thicknesses of the five cross-sections in an observed area (Figure S5, Supporting Information). Next, the fiber width of each fabric was determined by randomly selecting six fibers in each observed area, measuring the fiber widths using the line tool of Fluoview 4.2b, and averaging the fiber widths of 18 fibers in each fabric (Figure S6, Supporting Information). Furthermore, each 3D image was opened, a fiber image (Figure S7, Supporting Information) was extracted from each using Image Pro 10, and this image was used to determine the porosity (Figure S8, Supporting Information) and contact point density (Figure S9, Supporting Information). The porosity and contact point density were determined strictly in consideration of the volume of fabric in each observation area. Finally, 3D images of each fabric were converted into a 2D image with Image Pro 10, performed a Fast Fourier Transform (FFT) of these 2D images to create a spectral image using Image Pro 10, and obtained the anisotropy by approximating the ellipse (Figure S10, Supporting Information). The anisotropy is the ratio of the length of the minor axis to the length of the minor axis of the ellipse. Calculation of anisotropy was performed using a previously reported procedure.28 Naturally, the orientation angle (i.e., the angle of the minor axis of the ellipse) of the fibers of each sheet was also calculated by this method. However, the calculated orientation angle has no meaning because the standard orientation was not specified in this study.

Collections and Bacteriostatic Treatments of Adipose Tissue: Following examination approval (approval number: 810) by the University of the Ryukyus’ ethics committee for medical and health research involving human subjects, samples of human adipose tissue were collected at the University of the Ryukyus Hospital. Written consent was obtained from all human subjects from whom adipose tissue was extracted. Adipose tissues used in this study were (i) adipose tissue collected by liposuction from the inside of the right thigh of a 45-year-old male (171.6 cm, 65.6 kg, BMI 22.40) on October 15, 2018; (ii) adipose tissue collected by liposuction from the outside of the left thigh of a 43-year-old female (163.1 cm, 48.4 kg, BMI 18.19) on June 26, 2019; (iii) adipose tissue excised from a shallow part of the inguinal region of a 46-year-old male (170.0 cm, 83.8 kg, BMI 28.90) on June 17, 2020; and (iv) adipose tissue collected by liposuction from the thigh of a 56-year-old female (142.5 cm, 56.2 kg, BMI 27.90) on September 2, 2020 (Table S1, Supporting Information). After collection, each sample of adipose tissue was soaked immediately in perfusion solution containing 1% penicillin G potassium and cleaned by centrifugation (800 × g, 5 min, 4 °C). After cleaning, adipose tissue was transferred to a new 50 mL centrifuge tube containing perfusion solution with 1% penicillin G potassium and treated bacteriastically by incubation at 4 °C overnight. After the bacteriostatic treatment, we washed the sample three times by centrifugation (800 × g, 5 min, 4 °C). Bacteriostatic treatment and washing were performed to decrease the risk of contamination.

Preparation of Adipose Tissue Slices for Seeding on Nonwoven Fabrics: Adipose tissues that had been treated bacteriastically and washed were cut with sterilized scissors to make slices of 0.5–3 mm. If necessary, the tissue slices were torn with sterilized tweezers. Slices of adipose tissue weighing 0.05 g were used for seeding.

Degassing of Nonwoven Fabrics: Circles of biodegradable nonwoven fabrics (types A, B, C) were submerged, fabric made of polyethylene–polypropylene BMK-R003 (hereafter, BMK) and round cover glasses in a 100 mm cell culture dish containing 10 mL medium (ADSC-GM). A perforated glass plate was placed upon each fabric circle to prevent it from floating. Then these surfaces were degassed under reduced pressure (–0.09 MPa, 1 min) using a water flow aspirator. This surface degassing method was reported previously.29,30

Seeding of Adipose Tissue Slices on Nonwoven Fabrics and Sandwich Culture on a Cell Strainer: ϕ23 mm circles of fabrics A, B, C; ϕ21 mm circles of BMK; and ϕ22 mm round cover glasses were prepared, all of which were degassed. Two of each were placed on top of each other in a cell strainer submerged in ADSC-GM. Next, adipose tissue slices (0.05 g) prepared as described above were seeded such that they were sandwiched between two layers of nonwoven fabric or cover glass (Figure 2a). The adipose tissue slices used in this experiment were prepared from adipose tissue sample (i). Forty-five samples were seeded for each type of nonwoven fabric and cover glass. All seeded samples were placed together with a cell strainer in six 150 mm suspension-culture petri dishes containing 70 mL media and cultured at 37 °C in 5% CO2. The fiber orientation directions of the upper and lower pieces of fabric were not confirmed in this experiment.

Comparison of Cell Proliferation by Sandwich Culture: On the 3rd, 18th, and 36th day from the start of culture, the number of cells on each nonwoven fabric and cover glass was compared using preixed WST-1 cell proliferation assays (hereafter, WST-1 assays). The samples used for this comparison were three (n = 3) for each type of nonwoven fabric and cover glass. First, 8 mL medium (ADSC-GM) and 800 μL WST-1 reagent were added to each well of a new 6-well plate and mixed by pipetting. Next, samples cultivated in a 150 mm suspension-culture were transferred to a 6-well plate along with the cell strainer. At that time, one blank well was also prepared without a sample. After transferring the sample to a well plate, it was placed in an incubator (37 °C, 5% CO2) and allowed to react for 4 h. After the reaction, 100 μL reaction solution was taken from each well and transferred to a 96-well plate. Absorbance of these reaction solutions at a wavelength of 440 nm was measured.
using a Microplate reader SH-1000 lab (Corona Electric, Japan). The blank value was subtracted, the mean and standard deviation (n = 3) of samples of sandwich cultures was calculated using each type of nonwoven fabric or cover glass, and thus results of the WST-1 assay were obtained for each culture period (3rd, 18th, and 36th day from the start of culture).

**Extraction, Culture, and Staining of Cells Using Low-Adhesion Well Plates:** First, nine degassed 23 mm circles of nonwoven fabric A were prepared. Next, a cell-adhesive 6-well plate (Costar cell culture plate) and two types of low-adhesion 6-well plate (EZ-BindShut 5P and Costar polyethylene attachment surface plate) were prepared, and 4.5 mL medium (ADSC-GM) was poured into two wells of each plate. One or two pieces of degassed nonwoven fabric were placed in the two medium-containing wells. When two fabrics were placed in one well, the orientation direction of the fibers between them was matched and their peripheral portions were perfectly stacked without displacement. Finally, the 0.05 g adipose tissue slices prepared as described above were seeded in both wells of all three types of well plates. The adipose tissue slices used in this experiment were prepared from adipose tissue (iii). A total of 100 2012.4 μm × 670.8 μm square areas at intervals of 5 μm with a width of 200 μm or 300 μm in the Z-axis direction to include everything from the top to bottom surfaces of the fabric. The objective lens used for the imaging was a Plan-Apochromat 10×/0.45 M27 (Zeiss). The exposure times were as follows: reflection, 5 ms; Alexa Fluor 488 Phalloidin, 1500 ms; DAPI, 300 ms. After imaging, a single 2D image consisting of only the most focused points was created using the Extended Focus function of Axiovision 4.8 (Zeiss), the dedicated software for Observer Z1. Images were taken in three positions, (1), (2), and (3), for each nonwoven fabric (Figure S11, Supporting Information). (1) was a rectangular area of 2012.4 μm × 670.8 μm near the circumference perpendicular to the fiber orientation direction from the center of the circle; (2) was a square area of 670.8 μm × 670.8 μm in the center of the circle; and (3) was a rectangular area of 670.8 μm × 2012.4 μm near the circumference parallel to the fiber orientation direction from the center of the circle.

Areas (1) and (3) were equal in size and twice the area of (2). Areas (1) and (3) were imaged in three continuous square areas and concatenated using the Mosaic function of Axiovision 4.8 (Zeiss) (Figure 3c). The cell numbers in areas (1) and (3) were compared in each 6-well plate using a 2D fluorescence image of the cell nucleus (DAPI-stained).

On the other hand, observation of F-actin and nuclei of the periphery of (1) and (3) of each cell sheet was performed using the BZ-9000 (Keyence, Japan) equipped with the objective lens CFI Plan Apo 10x, NA 0.45 (Nikon, Japan). The cell sheet was prepared under exactly the same conditions in Figure 3c, including the culture vessel (Ultra-low plate), except that adipose tissue from a different donor (iv) was used. In areas (1) and (3), several images were taken continuously, and concatenated images were created. The image used for concatenation was based on a 3D rectangular image of 1449 μm × 1091 μm taken at intervals of 5 μm for about 300 μm in the Z-axis direction. The images were concatenated in the X direction in region (1) and in the Y direction in region (3). Each connected 3D image of regions (1) and (3) was converted into a 2D image and each area of 1091 μm × 3400 μm was cut out from the 2D image (Figure 3d). The BZ-II application (Keyence) was used for concatenation and 2D imaging of observed images. The Full Focus function of BZ-II was used for 2D imaging. As mentioned above, because the image observation method and connection method were different, the sizes of (1) and (3) differ between Figure 3d and Figure 3c. The Count Nuclei function of MetaMorph 7.8 (Molecular Devices, USA) was used to count cells in each region of the cell sheet.

In addition, the perforated plates used as weights during culture were transferred one by one to a 50 mm glass-bottom dish with the contact side facing down and observed like the cell sheets. Transparent images of cells on the surface of the three 6-well plates used in this culture were acquired using a CKX53 inverted microscope (Olympus) equipped with an objective lens UPlan FL N 4x/0.13 (PC Olympus) (Figure 3e). The observation sites on each plate were the regions where parts (1), (2), and (3) of the nonwoven fabric were in contact. In this observation, an outline of the nonwoven fabric and the positioning lines of (1), (2), and (3) were drawn at the bottom of the well (outside the plate) with a black magic marker. The positioning lines were two orthogonal diameters at the center point, drawn in the directions parallel and perpendicular to the fiber orientation direction of the fabric.

**Cell Quality Evaluation by Flow Cytometric Analysis:** First, six degassed circular nonwoven fabrics A of 32 mm were prepared. Next, 0.5–1 mL ADSC-GM was added to three wells of a Costar Ultralow-attachment surface 6-well plate, and then placed degassed pieces of nonwoven fabric A (32 mm) in the wells one by one. Three adipose tissue slices (0.05 g) were seeded on one piece of nonwoven fabric in each well, and a degassed piece of fabric of the same size was placed over it (Figure 4a). Adipose tissue slices were seeded in three locations on the diameter perpendicular to the orientation direction of the fibers of the nonwoven fabric (Figure 4b). In addition, the fiber orientations of the fabric seeded with the adipose tissue slices and the fabric placed above the slice were aligned. The adipose tissue slices used in this experiment were prepared from adipose tissue (iii). A total of 0.15 g of seeded adipose tissue slices were sandwiched between two pieces of nonwoven fabric; a Teflon perforated plate was placed on the fabrics as a weight; and extraction and culture (37 °C, 5% CO2) were initiated with a medium volume of 4.5 mL (Figure 4c). Extraction and culture were performed for 34 days, with replacement of the full volume of medium every 3–4 days. The average cell culture period for the five cases of regenerative treatment was 30.8 days;[11] it was incubated a little longer than the average period (34 days) to check the quality of the cultured cells. On the 34th day of culture, cells were detached from the fabrics by immersion in 0.1% recombinant GM-grade trypsin and shaken (60 rpm, 7 min, 37 °C) on a Wave-Si slim shaker (TAITEC, Japan). After adding medium and pipetting at room temperature, the cells were collected by centrifugation (300 × g, 5 min, 4 °C). A total of 6.08 × 10^3 collected cells were suspended in 750 μL perfusion solution. A Luna automated cell counter (Logos Biosystems, Korea) was used to measure cell numbers.
The CD markers of the cells were stained and analyzed by flow cytometry. The cells to be analyzed by flow cytometry were prepared for sensitivity adjustment and main measurement. Antibodies used for the sample preparation were PE mouse IgG1 κ isotype ctrl, APC mouse IgG1, κ isotype ctrl, APC Anti-Human CD29, PE anti-human CD90, PE anti-human CD45, APC anti-human CD34, and PE anti-human CD44. Dead cells were stained using SYTOX Blue Dead Cell Stain. Flow cytometry analyses were performed on four samples: (1) PE-stained isotype ctrl and APC-stained isotype ctrl, (2) APC-stained CD29 and PE-stained CD45, (3) APC-stained CD34 and PE-stained CD44, (4) PE-stained CD90. For each sample, 10,000 cells were analyzed except in the case of CD90; in that case, 4,850 cells were analyzed. The flow cytometer used for this evaluation was FACSVerse (Becton, Dickinson and Company, USA), and the software used for this analysis was FlowJo 10.6.2 (FlowJo, USA).

Statistical Analysis: All statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., USA). Means ± standard deviation are shown. Comparisons of nonwoven fabric thicknesses, fiber diameters, porosities, contact point densities, and anisotropies were analyzed by one-way ANOVA with the Tukey-Kramer multiple comparison test. Comparisons of cell proliferations were analyzed by one-way ANOVA with the Dunnett multiple comparison test; the control group in this analysis was A or B. p < 0.05 was considered significant. *p < 0.05; **, p < 0.01; ***p < 0.001. To ensure clear presentation of data, the figures contain only *, **, and *** to mark significance.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
The data that support the findings of this study are available in the supplementary material of this article.

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nonwoven fabric, biodegradable polymers, cell culture scaffolds, cell sheet, anisotropy

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