Regenerative Therapy 21 (2022) 52–61

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

Development of hydroxyapatite-coated nonwovens for efficient isolation of somatic stem cells from adipose tissues

Ryota Chijimatsu [a, b, c, *, 1], Taiga Takeda [a, d, 1], Shinsaku Tsuji [d, e], Kohei Sasaki [f], Koichi Kato [f], Rie Kojima [f], Noriko Michihata [f], Toshiya Tsubaki [b], Aya Matui [d], Miharu Watanabe [d], Sakae Tanaka [b], Taku Saito [b]

[a] The University of Tokyo, Bone and Cartilage Regenerative Medicine, Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan
[b] The University of Tokyo, Sensory and Motor System Medicine, Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan
[c] Okayama University Hospital, Center for Comprehensive Genomic Medicine, 2-5-1, Shikada-chou, Kita-ku, Okayama, 700-8558, Japan
[d] CPC Corporation, 3-18-16 Minami-Aoyama, Minato-ku, Tokyo, 107-0062, Japan
[e] Avenue Cell Clinic, 3-18-16 Minami-Aoyama, Minato-ku, Tokyo, 107-0062, Japan
[f] Japan Vilene Company, Ltd., Central Research Laboratory, 7 Kita-tone, Koga-shi, Ibaraki, 306-0213, Japan

ARTICLE INFO

Article history:
Received 16 March 2022
Received in revised form
15 May 2022
Accepted 25 May 2022

Keywords:
Adipose stem cells
Explant culture
Nonwovens
Hydroxyapatite

ABSTRACT

Adipose-derived stem cells (ASCs) are an attractive cell source for cell therapy. Despite the increasing number of clinical applications, the methodology for ASC isolation is not optimized for every individual. In this study, we developed an effective material to stabilize explant cultures from small-fragment adipose tissues.

Methods: Polypropylene/polyethylene nonwoven sheets were coated with hydroxyapatite (HA) particles. Adipose fragments were then placed on these sheets, and their ability to trap tissue was monitored during explant culture. The yield and properties of the cells were compared to those of cells isolated by conventional collagenase digestion.

Results: Hydroxyapatite-coated nonwovens immediately trapped adipose fragments when placed on the sheets. The adhesion was stable even in culture media, leading to cell migration and proliferation from the tissue along with the nonwoven fibers. A higher fiber density further enhanced cell growth. Although cells on nonwoven explants could not be fully collected with cell dissociation enzymes, the cell yield was significantly higher than that of conventional monolayer culture without impacting stem cell properties.

Conclusions: Hydroxyapatite-coated nonwovens are useful for the effective primary explant culture of connective tissues without enzymatic cell dissociation.

1. Introduction

Cell-based therapy using multipotent mesenchymal stem cells (MSCs) has been demonstrated to have therapeutic effects on multiple diseases owing to their immunomodulatory, immunosuppressive, and regenerative potential [24]. Adipose tissue-derived stem cells (ASCs), a type of MSCs, can be isolated from stromal vascular fractions (SVF) of lipoaspirate or resected adipose tissue, which are considered medical waste from plastic surgery. Many clinical trials have been conducted based on the availability and therapeutic potential of ASCs, and some are already being clinically used in several countries [3].

Currently, enzymatic digestion using collagenases is widely accepted for SVF collection from lipoaspirates. Several semi-automated devices are already available [23]; however, the procedure is complicated and requires multiple-step handling [5]. The method also has several disadvantages; enzymes such as collagenase, which are mostly extracted from bacteria, are costly and often

Abbreviations: MSC, mesenchymal stromal cell; ASC, adipose stem cell; SVF, stromal vascular fraction; HA, hydroxyapatite; SEM, scanning electronic microscopy.

* Corresponding author. Okayama University Hospital, Center for Comprehensive Genomic Medicine, 2-5-1, Shikada-chou, Kita-ku, Okayama, 700-8558, Japan
E-mail address: rchijimatsu@okayama-u.ac.jp (R. Chijimatsu).

Peer review under responsibility of the Japanese Society for Regenerative Medicine.

[1] equal contribution.

https://doi.org/10.1016/j.reth.2022.05.009

poorly defined, resulting in the use of a broad range of incubation times depending on the concentration, solvent, and purity of each product. Moreover, the digestion of extracellular matrices may cause cell damage, particularly in adhesive cells [15], which reduces the viability and activity of ASCs. This is a critical issue when the initial tissue volume is limited. In fact, adipose tissue sources are mainly derived from obese donors during cosmetic surgery; however, enough tissue cannot be harvested from healthy or skinny donors owing to the low fat content and the risk of scar formation at the harvest site.

Another method to isolate MSCs from connective tissues has recently been developed for the umbilical cord [9] [11,19], synovium [16], and adipose tissue [6,21,27]. This non-enzymatic method is a cost-effective and simple procedure. where tissues are minced into small fragments and incubated in culture vessels with growth media. After the tissue fragments bind to the surface of the culture vessels, adhesive cells migrate from the fragment surface to the vessel surface and proliferate. Several reports have revealed no difference or a slight predominance of explant culture for cell yield, acquired population, and cell potential compared with enzymatic methods [11,13,16,21,37]. However, this method is associated with the risk of unstable outcomes owing to the need for careful manipulation to maintain the adhesion of tissues on the surface of culture vessels. In particular, adipose tissues naturally float in the medium; therefore, additional improvements are required. Priya et al. cultured adipose tissue fragments in low-volume media to avoid float [21], and Ghorbani et al. incubated the fragments under semi-dry conditions for 24 h to allow their tight binding on the vessel surface [6]. Thus, preventing tissue float and ensuring tissue binding are key to stabilizing the primary explant culture.

Nonwoven fabrics are often highlighted as scaffolds for tissue engineering owing to characters, such as fiber materials, orientation, and 3-dimensional structure, which provide suitable environments for cellular attachment, growth, and differentiation [8,31,32,34–36]. MSCs are known to adhere and proliferate on fiber fabrics; however, their primary explant culture has not been reported. In this study, we demonstrated the utility of a nonwoven substrate for the primary explant culture of small adipose tissue. Further, we examined the surface nanostructure composed of hydroxyapatite (HA) and the fiber composition of nonwoven materials for the trapping of tissue fragments and subsequent cell isolation.

2. Materials and methods

2.1. Human samples

Human subcutaneous adipose tissues were obtained from plastic surgery performed at the Avenue Clinic, in accordance with a protocol approved by the ethics committee of the University of Tokyo. The age, sex, and harvest site are listed in Table S1.

2.2. Preparation of nonwovens

Hydroxyapatite-coated nonwovens were produced by blowing an airflow containing HA particles heated to 250 °C (SofSera Corporation, mean particle size $\leq$5 μm) onto polypropylene/polyethylene nonwovens with a mean fiber diameter of 26 μm and a weight of 30 g/m^2. The HA content per unit area values of the nonwovens were 1.4 and 3.2 g/m^2 (Fig. 1A). The apparent densities of the HA-coated nonwovens were adjusted to 0.039, 0.064, and 0.101 g/cm^3 by mechanical pressing. HA-coated nonwovens were cut into 23 mm squares and used as scaffolds.

Of note, a patent is pending for HA-attached nonwovens.

2.3. Explant culture on nonwovens

Nonwovens were initially hydrated with 70% ethanol, washed three times with saline, and soaked in growth media. Wet nonwovens were then placed on the tissue culture well plate, and the adipose tissue fragments, which were minced into approximately 2 mm squares, were placed at the center of the nonwovens (Fig. 1B). Medium was finally added to cover the top of the fragment. Nonwovens were cultured with Nutristem MSC (SARTORIUS, Göttingen, Germany) containing 2% fetal bovine serum (FBS, Lonza, Basel, Switzerland) and 1% antibiotic solution (Nacalai Tesque, Kyoto, Japan) (referred as growth media) at 37 °C in an incubator with humidified 5% CO_2. The medium was changed every 3 days. Tissue attachment on nonwoven was microscopically assessed with three levels as follows: 1, full contact; 0.5, partial contact; 0, detached.

2.4. Conventional enzymatic isolation

Adipose tissues were minced and incubated for 1 h with gentle rotation in DMEM containing 500 unit/mL collagenase type 1 (Worthington, Columbus, OH, USA). Undissolved tissues were removed by passing through a 70 μm cell strainer, and isolated cells were cultured with growth media. Cells from approximately 300-500 mg of tissue were seeded in a well of a 6-well culture plate.

2.5. Measurement of the cell number and outgrowth area on nonwovens

Cell outgrowth was microscopically monitored at the timing of medium change and recorded the day of first observation. To estimate the number of cells on nonwovens, mitochondrial activity was measured using a Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan) (termed CCK assay). Cultures of nonwovens and explants were incubated in 500 μL DMEM supplemented with 10 μL kit containing assay reagents for 3 h at 37 °C in an incubator with humidified 5% CO_2. The absorbance of the supernatants at 450 nm was measured using a Varioskan spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Outgrowth cells on nonwovens were detached using 0.25% trypsin/EDTA (Nacalai Tesque) or Accutase (Nacalai Tesque) and incubated for 15 min. Detached and weakly attached cells were collected during three PBS washes via thorough pipetting. The number of cells collected on day 14 was measured using an autocell counter (Logos Biosystems, Simindaero, South Korea).

For quantification of the outgrowth area from the tissue fragments, the cultures were fixed with formalin overnight and then stained with crystal violet for 30 min. After a thorough washout of the excess dye, the stained outgrowth area was measured using the ImageJ software. In brief, images were binarized with Hue values for violet color and then the sum area for positive signal was calculated after noise removal.

2.6. Flow cytometry

Cultured cells were dissociated into single cells with Accutase for 3 min for monolayer culture and 15 min for nonwoven primary culture. Cells were resuspended in 0.1% BSA-PBS and incubated for 30 min at 4 °C with the following fluorescence-conjugated antibodies: PE conjugated anti-hCD73 (BioLegend, San Diego, CA, USA, 344003), FITC conjugated anti-hCD90 (BioLegend, 328107), APC conjugated anti-hCD105 (BioLegend, 323207), PE conjugated anti-hCD34 (BioLegend, 343505), and APC conjugated anti-hCD45 (BioLegend, 368511). Isotype control antibodies were used as negative controls (BioLegend; 400132, 400121, and 400111). The cells were washed with 0.1% BSA-PBS and resuspended for analysis

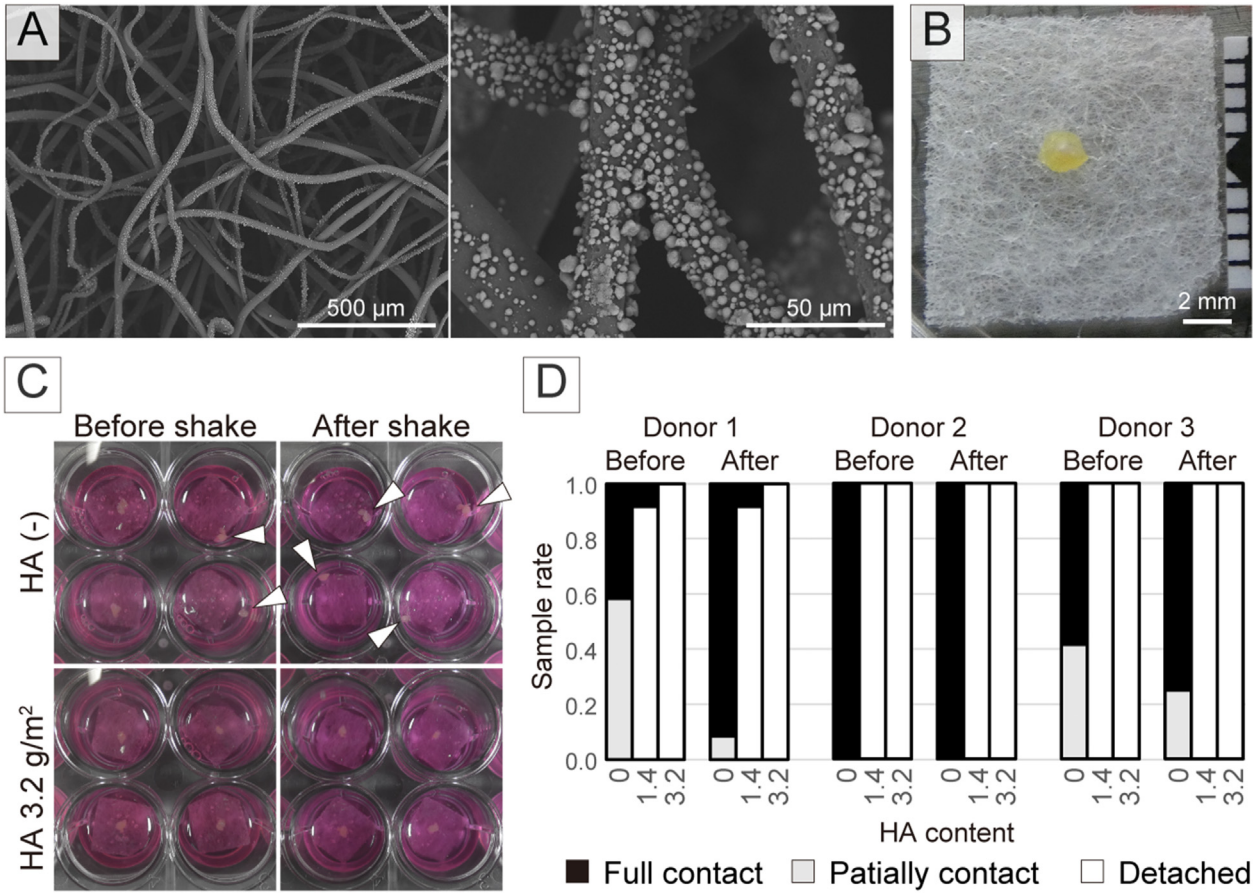

Fig. 1. Hydroxyapatite-coated nonwoven stably trap adipose tissue fragments. **(A)** SEM images of nonwovens coated with HA at 3.2 g/m^2. **(B)** Macroscopic view of the nonwovens with an adipose fragment at the center. **(C)** Macroscopic view before and after reciprocal shaking at 160 rpm for 1 min in a 24-well plate. Arrowheads indicate floating adipose tissue fragments. **(D)** Percentage bar chart of the state of tissue attachment in the shaking test. N = 12 per donors.

with CytoFLEX S (BECKMAN COULTER, Brea, CA, USA) according to the manufacturer's protocol. Data retrieved from the sorting were analyzed using CytoExpert (BECKMAN COULTER).

2.7. Differentiation

2.7.1. Induction of adipogenesis

Cells were seeded at a density of 1×10^5/cm^2 and cultured in 10%FBS-DMEM until over-confluent. Subsequently, the cells were cultured in 10% FBS-DMEM supplemented with 10 μg/mL insulin (FUJIFILM Wako Pure Chemical, Osaka, Japan), 200 μM indomethacin (FUJIFILM Wako Pure Chemical), 1 μM dexamethasone (Nacalai Tesque), and 500 μM 3-Isobutyl-1-methylxanthine (FUJIFILM Wako Pure Chemical) for seven days. Cells cultured in 10%FBS-DMEM were used as controls. To detect the formation of lipid vacuoles, the cells were fixed with 4% PFA and stained with Oil Red O solution (0.5% oil red O in 60% isopropanol) (Sigma–Aldrich, St. Louis, MO, USA). The stained dye was eluted with 100% isopropanol and the absorbance was measured at 520 nm.

2.8. Induction of osteogenesis

Cells were seeded at a density of 5×10^4/cm^2 and cultured in 10% FBS-DMEM supplemented with 10 mM β-glycerophosphate (Sigma–Aldrich), 10 nM dexamethasone (Nacalai Tesque), and 50 μg/mL ascorbic acid 2 phosphate (Sigma–Aldrich) for two or three weeks. Cells cultured in 10% FBS-DMEM were used as controls. Differentiation was confirmed by assessing the alkaline phosphatase (ALP) activity and calcium deposition. For analysis, the cells were rinsed twice with PBS, fixed with 4% paraformaldehyde (PFA), and washed with water. ALP activity was detected using the BCIP/NBT color development substrate (Promega, Madison, WI, USA). Calcium deposition was detected following incubation with 1% Alizarin Red S solution (pH 6.4) (Muto Pure Chemicals, Tokyo, Japan) for 10 min at room temperature and thorough washing with water. The colored area of the wells was measured using ImageJ software.

2.9. Induction of chondrogenesis

To obtain cell aggregated pellets, 3×10^5 cells were centrifuged in 96-deep well polypropylene plates and cultured in 10% FBS-DMEM. The next day, the medium was changed to a chondrogenic medium comprising high-glucose DMEM containing 110 μg/mL sodium pyruvate (Thermo Fisher Scientific) supplemented with 0.2 mM ascorbate-2-phosphate (Sigma–Aldrich), 40 μg/mL L-proline (FUJIFILM Wako Pure Chemical), 10 nM dexamethasone (Sigma–Aldrich), 1% ITS + Premix (Corning: 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 μg/mL selenious acid, 1.25 μg/mL bovine serum albumin, and 5.35 μg/mL linoleic acid), 10 ng/mL transforming growth factor-β3 (TGFβ3) (Peprotech, Rocky Hill, New Jersey, USA), 20 ng/mL bone morphogenic protein 2 (BMP2) (Medtronic, Dublin, Ireland), and 0.25 nM TD-198946 [2]. The pellets were maintained with 0.5 mL of chondrogenic culture medium in a humid atmosphere of 5% CO_2 at 37 °C. The medium was replaced twice per week. For assessment, the chondrogenic pellets were fixed with 4% PFA and embedded in paraffin wax. The 5-μm sections were then stained with safranin-O/fast green/hematoxylin staining dye. The positively stained area was measured using QuPATH v0.2.3.

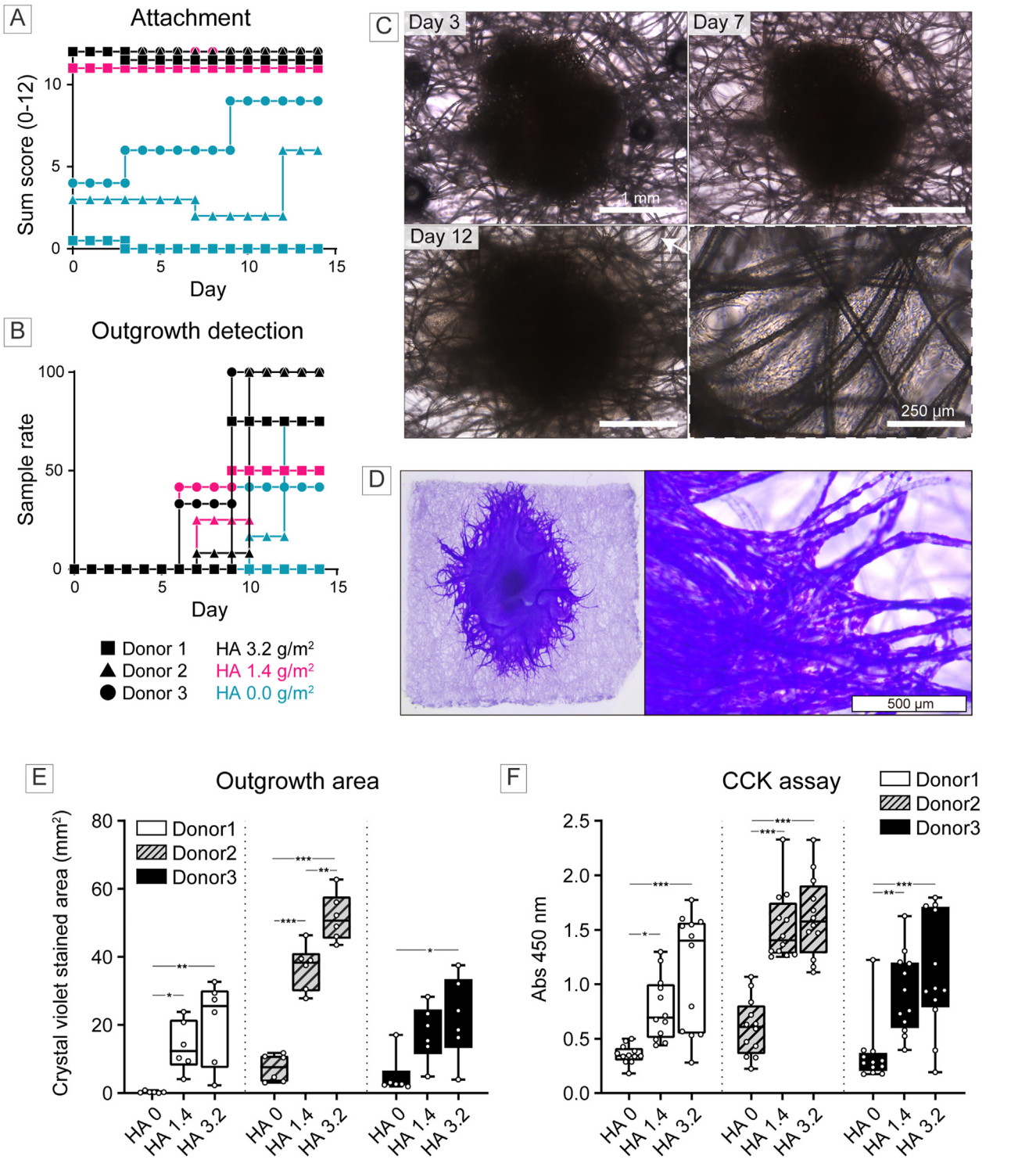

Fig. 2. Effect of hydroxyapatite content on tissue trap and cell outgrowth during primary culture. **(A)** Change in the sum score of tissue attachment over the primary culture. The score for the three-grade evaluation was used as follows: 1, full contact; 0.5, partial contact; 0, detached. N = 12 per group. **(B)** Change in the nonwoven rate at which the outgrowth was microscopically observed. **(C)** Representative microscopic views during nonwoven culture. Dense dark tissue represents a fragment of adipose tissue. **(D)** Representative image of crystal violet staining of nonwovens cultured for 14 days. **(E, F)** Effect of HA content on cell growth. Box plot showing the cell amount determined with a cell counting kit (E, N = 12 per group) and the outgrowth area stained with crystal violet (F, N = 6 per group). Significance was assessed using one-way analysis of variance with post-hoc Tukey's HSD test (*p < 0.05, **p < 0.01, ***p < 0.001).

2.10. Scanning electronic microscopy

HA-coated nonwovens were sputter-coated with an Au layer for scanning electron microscopy (SEM) analysis (TM-3030, Hitach High-Tech). Culture samples were treated with trypsin, accutase, or 500 unit/mL collagenase type 1 (Worthington) in DMEM for 15 min. Thereafter, the nonwovens were thoroughly washed with PBS and fixed with 4% PFA. Samples were dehydrated with a graded ethanol series (50/70/80/90/95/100/100%) for 30 min each, transferred to t-butyl alcohol, frozen at −28 °C, and then freeze-dried (Virtis

BenchTop K, SP Industries). The freeze-dried samples were sputter-coated with an Au layer for scanning electron microscopy (SEM) analysis.

2.11. Statistics

All data are presented as boxplots or bar plots, with each value indicated by a dot. One-way or two-way analysis of variance with post-hoc Tukey HSD test, Wilcoxon signed rank exact test, and Pearson correlation test were performed using R software. Multiple testing corrections were performed, where necessary, using the Bonferroni method. Statistical significance was indicated by a p value less than 0.05. The statistical tests used are indicated in the relevant results and figure legends.

3. Results

3.1. Surface modification of nonwovens with hydroxyapatite enables explant culture of adipose tissue

In explant culture, adipose tissues do not easily bind to culture vessels in the beginning of culture due to their floatability. Similarly, nonwovens composed of smooth fibers could not trap adipose tissue (Supplemental Video 1). However, nonwovens coated with hydroxyapatite instantly trapped adipose tissues (Supplemental Video 2), which did not detach after soaking in medium. Moreover, adhesion remained stable after vigorous shaking on a reciprocating shaker at 160 rpm for 1 min (Fig. 1C and D).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.reth.2022.05.009.

During primary culture, the hydroxyapatite coat ensured tissue binding to the nonwovens ($p = 0.0415$ among HA content in two-way ANOVA at the endpoint) (Fig. 2A). In a few cases, floating adipose tissue was found to attach and bind nonwovens without hydroxyapatite. After approximately 1 week, the outgrowth cells were observed by microscopy in HA-coated nonwovens (Fig. 2B), and their outgrowth sample rate were significantly high compared to non-coated nonwovens ($p = 0.019$ among HA content in two-way ANOVA at the endpoint) (Fig. 2C). Nevertheless the firm tissue binding, there was less outgrowth in few cultures (Fig. S1B), that may be reflected by composition of tissues like vascular and connective structure. Crystal violet staining clearly showed an outgrowth area, which was significantly higher in the hydroxyapatite coat than in the plane nonwovens (Fig. 2D and E). Moreover, the cell number, which was indicated by the measurement of mitochondrial activity, was high in HA-coated nonwovens (Fig. 2F). The content of hydroxyapatite did not significantly alter these results under the following two conditions: 1.4 g/m^2 and 3.2 g/m^2.

3.2. Higher fiber density increases outgrowth from nonwoven explant

The effect of nonwoven fiber density on cell growth was investigated. Two higher densities of fiber within nonwovens were additionally fabricated by mechanical pressing, with equal amounts of fiber material used (Fig. 3A). All three nonwovens (0.039, 0.064, and 0.101 g/cm^3) enabled explant culture without compromising the feasibility of microscopic observation (Fig. S1A). Moreover, the higher fiber density could enhance outgrowth from adipose explants in the CCK assay (Fig. 3B). There was no significance in the outgrowth area, which might reflect the three-dimensional expansion of the outgrowth within nonwovens (Fig.3C, Fig. S1B). Considering these results, nonwovens with hydroxyapatite (3.2 g/m^2) and a higher fiber density (0.064 or 0.101 g/cm^3) were used for further analysis.

3.3. Cell collection from nonwovens

When the nonwoven culture was treated with conventional trypsin solution, the shape of the attached cells changed to round

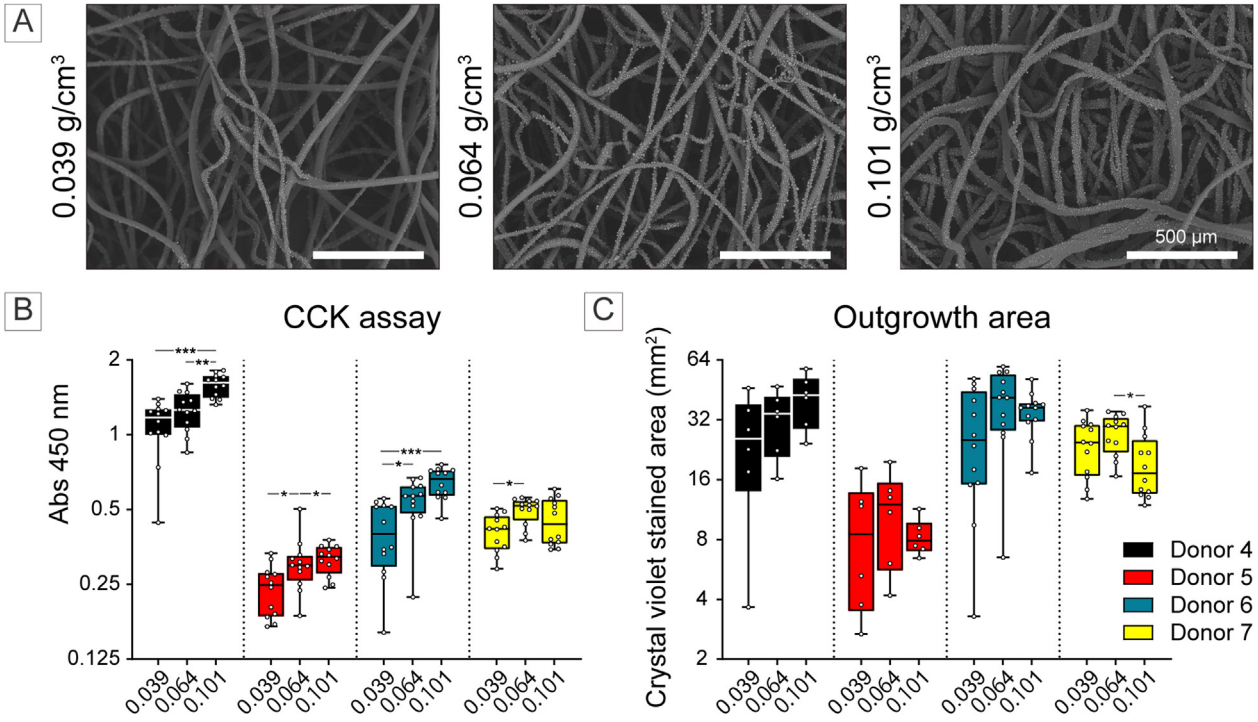

Fig. 3. Effect of fiber density on cell growth at primary culture. (A) SEM images of nonwoven fabrics with different fiber densities. (B, C) Effect of fiber density on cell growth. Box plot showing the cell amount determined with a cell counting kit (B, N = 12 per group) and outgrowth area stained with crystal violet (C, N = 6 per group). Significance was assessed using one-way analysis of variance with post-hoc Tukey's HSD test (*p < 0.05, **p < 0.01, ***p < 0.001).

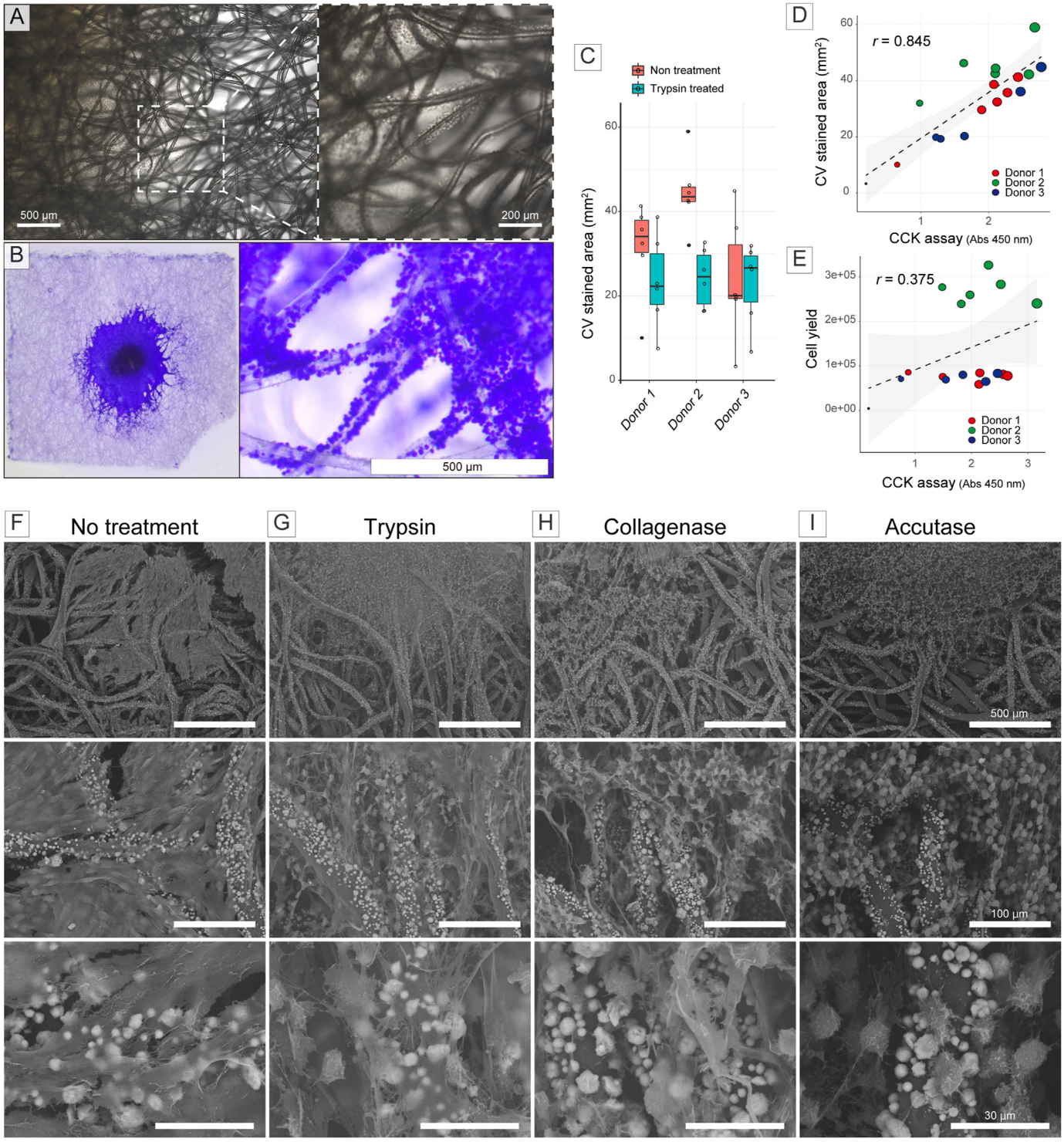

Fig. 4. Binding of outgrowth cells to nonwoven. **(A)** Microscopic views of nonwovens treated with trypsin for 5 min. **(B)** Representative images of crystal violet staining of nonwovens treated with trypsin for 15 min. **(C)** Measurement of the crystal violet-stained area of the nonwoven culture (HA, 3.2 g/m^2; and fiber density, 0.064 g/cm^3) with or without trypsin treatment. N = 6 per group. **(D)** Pearson correlation between the cell counting kit and outgrowth area in nonwoven culture (HA, 3.2 g/m2; fiber density, 0.064 g/cm3). N = 6 per group. (E) Pearson correlation between the cell counting kit and cell yield of nonwoven culture (HA, 3.2 g/m2; fiber density, 0.064 g/cm3) with trypsin treatment for 15 min. N = 6 per group. (F–I): SEM images of nonwoven cultures without enzymatic treatment (F) or with trypsin (G), collagenase (H), and accutase (I) treatment.

cells (Fig. 4A); however, cells could not be completely collected; the part of outgrowth cells remained on nonwovens with a round cell shape (Fig. 4B and C). The values from the CCK assay and outgrowth area were highly correlated (r = 0.845), however, the correlation of the collected cells with the CCK assay was weak (r = 0.375) (Fig. 4D and E).

To improve cell collection from nonwoven culture, the cell dissociation enzymes, trypsin, collagenase, and Accutase, were employed, and their effects were observed via SEM images (Fig. 4F–I). As shown in untreated nonwovens, outgrowth cells organized sheet-like connective tissues with their synthesis matrices (Fig. 4F). All enzymatic treatments caused cells to assume

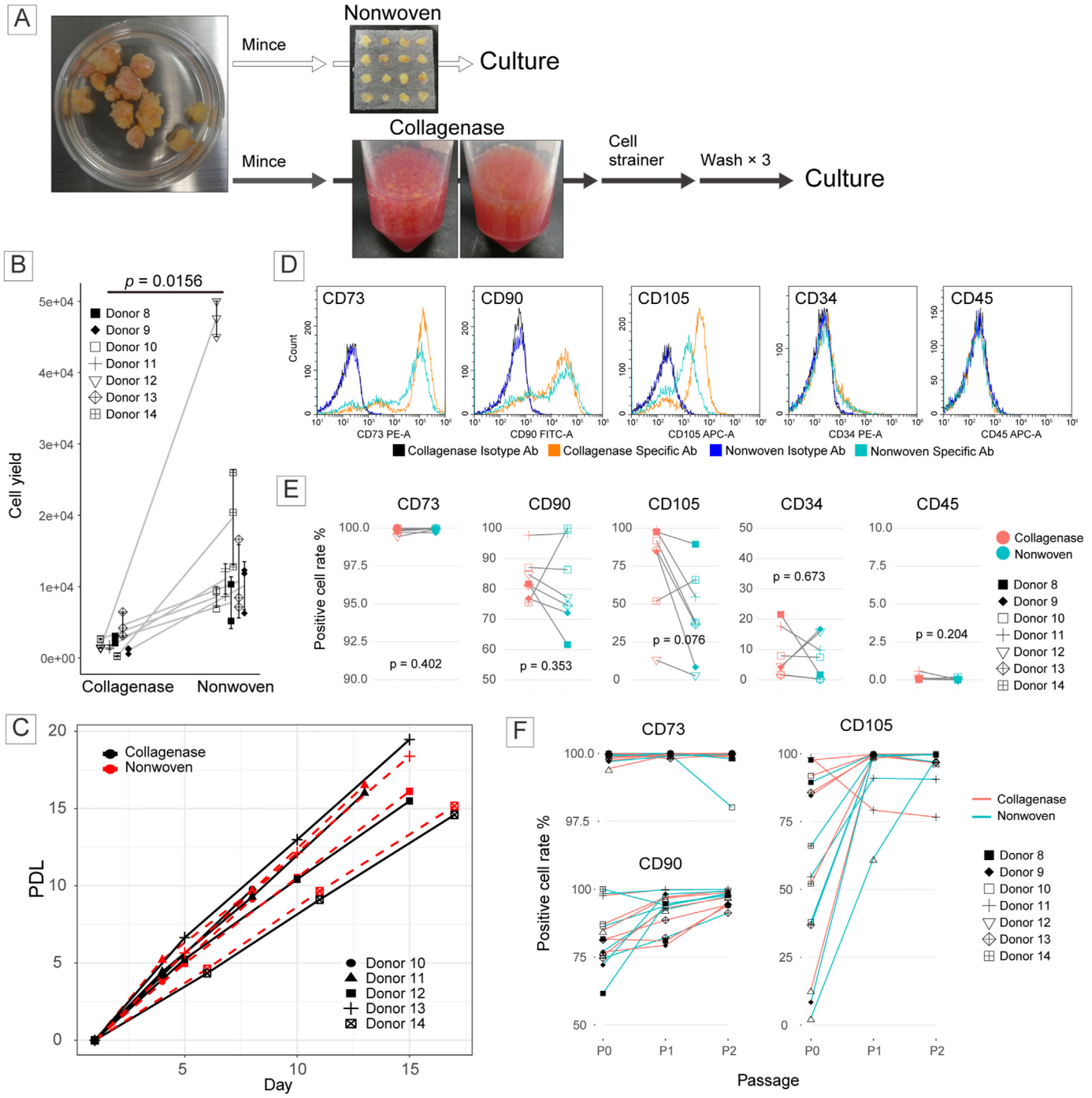

Fig. 5. Comparison of the nonwoven culture to conventional collagenase digestion in cell yield and property. (A) Schema of the comparative study. (B) Cell yield for each primary culture method from the seven donors. N = 2 or 3 replicates per group. Significance was assessed using the Wilcoxon signed-rank test. (C) Population doubling levels (PDL) after primary culture. Each dot indicates the passage day. (D, E) Flow cytometry of cells in primary culture. (D) Representative data from donor 10. Dot plot showing the positive cell rate for each surface antigen from seven donors (E). Significance was assessed using the Wilcoxon signed-rank test. (F) Change in the positive cell rate of MSC markers over passaging.

a recognizable shape and highlighted an undissected connection of cells to both the fiber surface and hydroxyapatites. Many filament structures were found to cover the material and connect cells to cells or nonwovens during trypsin treatment (Fig. 4G). Collagenase could digest extracellular matrices based on a decrease in filaments; however, cell–cell binding appeared to be insufficiently dissociated (Fig. 4H). Accutase most clearly segmented cells in a separated round shape; however, the thin filaments were not completely dissociated (Fig. 4I). Thus, the nonwoven explant culture formed the cell–matrix complex, which required stronger enzymatic treatment than the monolayer culture. Of note, adipose fragments never detached after either enzymic treatments. Based on these results, Accutase was selected for the subsequent studies.

3.4. Nonwoven culture improves cell yield at primary culture compared to conventional enzymic isolation

To evaluate the efficiency of the nonwoven culture for collecting primary cells, we compared it to conventional collagenase

methods. Seven donor samples (1603.7 ± 266.22 mg tissue) were used in this assay. Adipose tissue (50 mg) was divided into 16 pieces and placed on 23 mm square nonwovens at even intervals with two or three replicates (Fig. 5A). The remaining adipose tissue was minced and treated with collagenase digestion to serve as a conventional method for SVF collection (Fig. 5A). Dissociated cells were seeded into three wells of a 6-well culture plate (approximately 300–600 mg tissue derived cells were in a single well). At the end of culture, cells were dissociated with Accutase; detachable cells were collected from nonwovens with thorough pipetting, and almost all cells in the monolayer culture could be collected. Nevertheless, the cell yield per mg tissue was significantly higher in

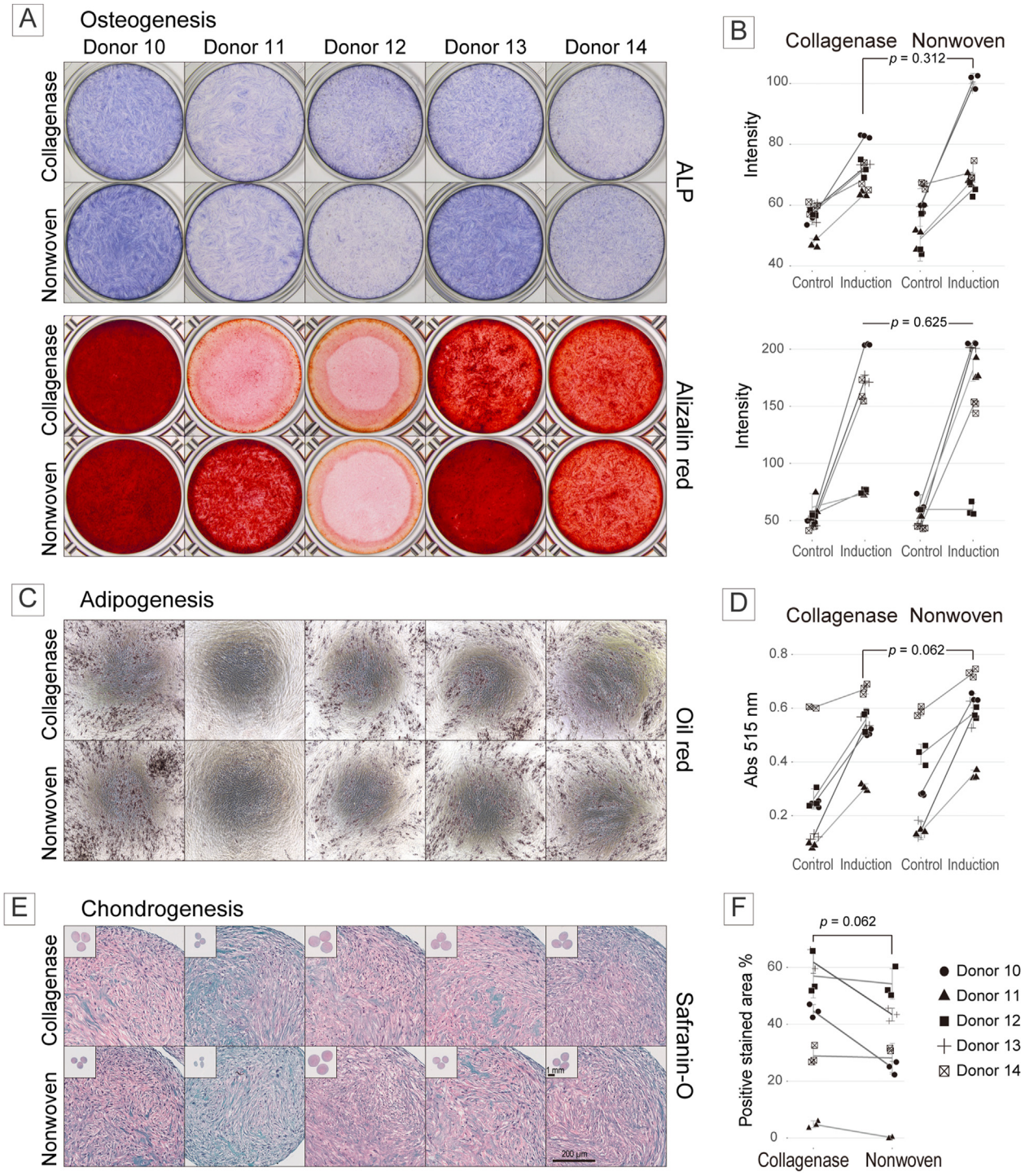

Fig. 6. Effect of the primary culture method on the differentiation potential of ASCs Osteogenic **(A, B)**, adipogenic **(C, D)**, and chondrogenic **(E, F)** induction by ASCs from five donors. Representative images from the three replicates are shown. Dot plot showing the stain intensity (B), absorbance of the eluted dye (D), and stained area rate (F). Alkaline phosphatase (ALP) activity was assessed on day 7, and calcium deposition was assessed on day 21 by Alizarin Red S staining (A). Oil droplets stained with Oil Red O (C and D) were assessed for adipogenesis. In chondrogenesis, the synthesis of proteoglycans in pellet cultures was assessed by Safranin-O staining (E, F). Significance between the primary culture methods in differentiation induction was assessed using the Wilcoxon signed-rank exact test (B, D, and F).

nonwoven cultures from all seven donors ($p = 0.0156$ between nonwovens and collagenase in Wilcoxon signed rank exact test) (Fig. 5B). However, further proliferation in monolayer culture was comparable between the primary culture methods (Fig. 5C).

Concerning the stem cell features derived from MSC studies, surface antigens for MSC markers (CD73, CD90, and CD105), endothelial markers (CD34), and hematopoietic markers (CD45) were not found to be significantly altered between the two methods even though the different time for Accutase treatment (Fig. 5D and E). Although there were some cases with low expression of CD105 in the primary culture, it converged toward a high rate during passaging (Fig. 5F). Regarding the differentiation potential for osteo-, adipo-, and chondrogenesis, there were no significant differences between primary culture methods, and their potential was dependent on the derived donor (Fig. 6A–F).

4. Discussion

In the present study, we demonstrated the utility of nonwovens for the primary explant culture of adipose tissue. Coating with hydroxyapatite particles ensured the adhesion of adipose fragments to the culture substrate by trapping tissues on nonwovens. Moreover, optimization of the fiber density further enhanced cell outgrowth, resulting in a significantly higher cell yield than the conventional SVF culture method. In the characterization of ASCs, there was no alteration between nonwoven and SVF cultures in MSC surface antigen markers and differentiation potential for osteo-, chondro-, and adipogenesis.

In tissue engineering studies, nonwovens have been investigated as scaffolds that are directly transplanted into the injured site [1,22,25,28,33]. However, to the best of our knowledge, nonwovens have never been used for explant culture of adipose tissue. In our method, explant culture could be performed using a simple procedure even with small adipose fragments without tissue floating relative to primary culture. The liberation from collagenase treatment will improve the problems related to donor-site morbidity and cost for future cell therapy. Furthermore, reduced cellular damage by enzymatic shedding is advantageous for sensitive cell types. Notably, the cell yield was obviously improved in the nonwoven explant culture. According to Hendijani et al., explant tissue releases growth factors/cytokines into the culture media, and the success of explant culture of adipose tissue may further stimulate cell growth compared with monolayer culture [12].

We showed that the formed cell–matrix complex on nonwovens was not fully dissociated via enzymatic treatment; therefore, most of the collected cells were thought to be derived from the forefront zone of outgrowth where cells highly migrated. In fact, several differences in cell size and surface antigens were observed in nonwoven culture compared with monolayer primary culture by flow cytometry. CD105, a classical MSC marker, was not enriched in the primary culture and was lower in nonwovens (six of seven donors). According to recent findings, several MSC markers are acquired during two-dimensional culture that are not expressed in vivo. A previous report also revealed a lower expression of CD105 in vivo, but its expression in vitro [14,18]. Therefore, nonwoven explant culture may be useful to provide in vivo-like MSCs. In contrast, MSC features, including proliferation, surface antigens, and differentiation, were not significantly altered in further passaged cells, similar to the findings of previous reports [10,21,37], suggesting that the cell properties are equal to those of conventional ASCs whose safety has already been defined [29].

In this study, we developed nonwovens with a hybrid fiber composed of a polypropylene core surrounded by polyethylene. Each material is generally used in a medical supply. Although hydroxyapatite was physically sunken into the polyethylene sheath without chemical bridge formation, its desorption and carry over were never seen in further culture via microscopic observation. Moreover, hydroxyapatite is a medical material used in the human body, especially in bone and teeth, and is gradually absorbed [20]. Collectively, the safety of the present nonwovens is considered to be appropriate for use in stem cell therapies.

The nonwoven components that are suitable for primary culture could still be further improved. Previous studies have revealed the effects of fiber materials [4] and orientation patterns on cell attachment and growth [7,26,30]. Several studies have focused on the surface modification of nonwoven materials to enhance biological responses, such as antibacterial [1,17] and bone regeneration [22,25]. As cells can be expanded three-dimensionally, the woven pores and thickness may be important. Moreover, fiber materials and surface structure are critical for cell attachment and migration, which should be enhanced for explant culture, especially to avoid self-organization with abundant synthesis matrices.

5. Conclusions

HA-coated nonwovens showed a high capacity for tissue trapping over the primary culture of adipose fragments without tissue floating and detachment. ASCs were migrated from tissues and three-dimensionally expanded along the nonwoven fibers. Modification of fiber density further improved the cell outgrowth. Although cells were not fully collected from nonwovens, cell yield was significantly higher than conventional collagenase dissociated primary cultures. On the other hand, stem cell properties for passaged cells in proliferation, surface antigens, and differentiation were compatible between primary culture methods.

Author contribution

Conception: Ryota Chijimatsu, Shinsaku Tsuji, and Taku Saito.

Design of the work: Ryota Chijimatsu, Shinsaku Tsuji, Sakae Tanaka, and Taku Saito.

Acquisition and analysis: Chijimatsu Ryota, Taiga Takeda, Kohei Sasaki, Tsubaki Toshiya, Aya Matsui, and Miharu Watanabe.

Interpretation of data: Ryota Chijimatsu, Taiga Takeda, Tsubaki Toshiya, Shinsaku Tsuji, and Taku Saito.

Manuscript writing and revision: Ryota Chijimatsu, Shinsaku Tsuji, and Taku Saito.

Final approval of manuscript: all authors.

Agreement to own contributions: all authors.

Declaration of competing interest

The authors declare no conflicts of interest associated with this manuscript.

Acknowledgments

We thank Reiko Honma and Junko Sugita for providing technical support. We also thank all members of the laboratory for their helpful discussions and comments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2022.05.009.

References

[1] Bai MY, Ku FY, Shyu JF, Hayashi T, Wu CC. Evaluation of polyacrylonitrile nonwoven mats and silver-gold bimetallic nanoparticle-decorated nonwoven mats for potential promotion of wound healing in vitro and in vivo and bone growth in vitro. Polymers 2021;13.

[2] Chijimatsu R, Yano F, Saito T, Kobayashi M, Hamamoto S, Kaito T, et al. Effect of the small compound TD-198946 on glycosaminoglycan synthesis and transforming growth factor beta3-associated chondrogenesis of human synovium-derived stem cells in vitro. J Tissue Eng Regen Med 2019;13(3):446–58. https://doi.org/10.1002/term.2795.

[3] Chu D-T, Nguyen Thi Phuong T, Tien NLB, Tran DK, Minh LB, Thanh VV, et al. Adipose tissue stem cells for therapy: an update on the progress of isolation, culture, storage, and clinical application. J Clin Med 2019;8:917.

[4] Edwards SL, Mitchell W, Matthews JB, Ingham E, Russell SJ. Design OF nonwoven scaffold structures for tissue engineering OF the anterior cruciate ligament. Autex Res J 2004;4.

[5] Francis MP, Sachs PC, Elmore LW, Holt SE. Isolating adipose-derived mesenchymal stem cells from lipoaspirate blood and saline fraction. Organogenesis 2010;6:11–4.

[6] Ghorbani A, Jalali SA, Varedi M. Isolation of adipose tissue mesenchymal stem cells without tissue destruction: a non-enzymatic method. Tissue Cell 2014;46:54–8.

[7] Gwiazda M, Kumar S, Świeszkowski W, Ivanovski S, Vaquette C. The effect of melt electrospun writing fiber orientation onto cellular organization and mechanical properties for application in Anterior Cruciate Ligament tissue engineering. J Mech Behav Biomed Mater 2020;104:103631.

[8] Handel M, Hammer TR, Hoefer D. Adipogenic differentiation of scaffold-bound human adipose tissue-derived stem cells (hASC) for soft tissue engineering. Biomed Mater 2012;7:054107.

[9] Hassan G, Kasem I, Soukkarieh C, Aljamali M. A simple method to isolate and expand human umbilical cord derived mesenchymal stem cells: using explant method and umbilical cord blood serum. International journal of stem cells 2017;10:184–92.

[10] He F, Chen X, Pei M. Reconstruction of an in vitro tissue-specific microenvironment to rejuvenate synovium-derived stem cells for cartilage tissue engineering. Tissue Eng 2009;15.

[11] He H, Nagamura-Inoue T, Tsunoda H, Yuzawa M, Yamamoto Y, Yorozu P, et al. Stage-specific embryonic antigen 4 in Wharton's jelly-derived mesenchymal stem cells is not a marker for proliferation and multipotency. Tissue Eng 2014;20:1314–24.

[12] Hendijani F. Explant culture: an advantageous method for isolation of mesenchymal stem cells from human tissues. Cell Prolif 2017;50.

[13] Jing W, Xiao J, Xiong Z, Yang X, Huang Y, Zhou M, et al. Explant culture: an efficient method to isolate adipose-derived stromal cells for tissue engineering. Artif Organs 2011;35:105–12.

[14] Kurth TB, Dell'accio F, Crouch V, Augello A, Sharpe PT, De Bari C. Functional mesenchymal stem cell niches in adult mouse knee joint synovium in vivo. Arthritis Rheum 2011;63:1289–300.

[15] Labinsky H, Panipinto PM, Ly KA, Khuat DK, Madarampalli B, Mahajan V, et al. Multiparameter analysis identifies heterogeneity in knee osteoarthritis synovial responses. Hoboken, NJ): Arthritis & rheumatology; 2019.

[16] Lee D-H, Joo S-D, Han S-B, Im J, Lee S-H, Sonn CH, et al. Isolation and expansion of synovial CD34−CD44+CD90+ mesenchymal stem cells: comparison of an enzymatic method and a direct explant technique. Connect Tissue Res 2011;52:226–34.

[17] Marulasiddeshwara R, Jyothi MS, Soontarapa K, Keri RS, Velmurugan R. Nonwoven fabric supported, chitosan membrane anchored with curcumin/TiO(2) complex: scaffolds for MRSA infected wound skin reconstruction. Int J Biol Macromol 2020;144:85–93.

[18] Mizuno M, Katano H, Mabuchi Y, Ogata Y, Ichinose S, Fujii S, et al. Specific markers and properties of synovial mesenchymal stem cells in the surface, stromal, and perivascular regions. Stem Cell Res Ther 2018;9:123.

[19] Mori Y, Ohshimo J, Shimazu T, He H, Takahashi A, Yamamoto Y, et al. Improved explant method to isolate umbilical cord-derived mesenchymal stem cells and their immunosuppressive properties. Tissue Eng C Methods 2015;21:367–72.

[20] Onodera J, Kondo E, Omizu N, Ueda D, Yagi T, Yasuda K. Beta-tricalcium phosphate shows superior absorption rate and osteoconductivity compared to hydroxyapatite in open-wedge high tibial osteotomy. Knee Surg Sports Traumatol Arthrosc 2014;22:2763–70.

[21] Priya N, Sarcar S, Majumdar AS, SundarRaj S. Explant culture: a simple, reproducible, efficient and economic technique for isolation of mesenchymal stromal cells from human adipose tissue and lipoaspirate. J Tissue Eng Regen Med 2014;8:706–16.

[22] Qi P, Ohba S, Hara Y, Fuke M, Ogawa T, Ohta S, et al. Fabrication of calcium phosphate-loaded carboxymethyl cellulose non-woven sheets for bone regeneration. Carbohydr Polym 2018;189:322–30.

[23] Rodriguez J, Pratta AS, Abbassi N, Fabre H, Rodriguez F, Debard C, et al. Evaluation of three devices for the isolation of the stromal vascular fraction from adipose tissue and for ASC culture: a comparative study. Stem Cell Int 2017;2017:9289213.

[24] Saeedi P, Halabian R, Imani Fooladi AA. A revealing review of mesenchymal stem cells therapy, clinical perspectives and Modification strategies. Stem Cell Invest 2019;6. 34-34.

[25] Saveleva MS, Ivanov AN, Chibrikova JA, Abalymov AA, Surmeneva MA, Surmenev RA, et al. Osteogenic capability of vaterite-coated nonwoven polycaprolactone scaffolds for in vivo bone tissue regeneration. Macromol Biosci 2021;21:e2100266.

[26] Schellenberg A, Ross R, Abagnale G, Joussen S, Schuster P, Arshi A, et al. 3D non-woven polyvinylidene fluoride scaffolds: fibre Cross section and texturizing patterns have impact on growth of mesenchymal stromal cells. PLoS One 2014;9:e94353.

[27] Sherman LS, Conde-Green A, Naaldijk Y, Lee ES, Rameshwar P. An enzyme-free method for isolation and expansion of human adipose-derived mesenchymal stem cells. JoVE; 2019, e59419.

[28] Soto-Gutierrez A, Navarro-Alvarez N, Rivas-Carrillo JD, Tanaka K, Chen Y, Misawa H, et al. Construction and transplantation of an engineered hepatic tissue using a polyaminourethane-coated nonwoven polytetrafluoroethylene fabric. Transplantation 2007;83:129–37.

[29] Toyserkani NM, Jørgensen MG, Tabatabaeifar S, Jensen CH, Sheikh SP, Sørensen JA. Concise review: a safety assessment of adipose-derived cell therapy in clinical trials: a systematic review of reported adverse events. Stem cells translational medicine 2017;6:1786–94.

[30] Trevisol TC, Langbehn RK, Battiston S, Immich APS. Nonwoven membranes for tissue engineering: an overview of cartilage, epithelium, and bone regeneration. J Biomater Sci Polym Ed 2019;30:1026–49.

[31] Tuin SA, Pourdeyhimi B, Loboa EG. Creating tissues from textiles: scalable nonwoven manufacturing techniques for fabrication of tissue engineering scaffolds. Biomed Mater 2016;11:015017.

[32] van Aalst JA, Reed CR, Han L, Andrady T, Hromadka M, Bernacki S, et al. Cellular incorporation into electrospun nanofibers: retained viability, proliferation, and function in fibroblasts. Ann Plast Surg 2008;60:577–83.

[33] Vaquette C, Sudheesh Kumar PT, Petcu EB, Ivanovski S. Combining electrospinning and cell sheet technology for the development of a multiscale tissue engineered ligament construct (TELC). J Biomed Mater Res B Appl Biomater 2018;106:399–409.

[34] Wang W, Deng D, Wang B, Zhou G, Zhang W, Cao Y, et al. Comparison of autologous, allogeneic, and cell-free scaffold approaches for engineered tendon repair in a rabbit model-A pilot study. Tissue Eng 2017;23:750–61.

[35] Wang W, Itoh S, Konno K, Kikkawa T, Ichinose S, Sakai K, et al. Effects of Schwann cell alignment along the oriented electrospun chitosan nanofibers on nerve regeneration. J Biomed Mater Res 2009;91:994–1005.

[36] Wu S, Wang Y, Streubel PN, Duan B. Living nanofiber yarn-based woven biotextiles for tendon tissue engineering using cell tri-culture and mechanical stimulation. Acta Biomater 2017;62:102–15.

[37] Yoon JH, Roh EY, Shin S, Jung NH, Song EY, Chang JY, et al. Comparison of explant-derived and enzymatic digestion-derived MSCs and the growth factors from wharton's jelly. BioMed Res Int 2013;2013:428726.