Research Article

Multilayered Fibroblasts Constructed by Accelerated Cellular Self-Assembly and Applications for Regenerative Medicine

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Abstract

Regenerative medicine research requires animal experiments to evaluate treatment effects. According to the 3Rs principles, alternative models have been developed and utilized to evaluate the efficacy and safety of new products. Three-dimensional (3D) cell cultures have been recognized for their relevant structures and biological functions that are akin to native tissues. They can better represent in vivo conditions than two-dimensional (2D) cell cultures. Herein, we present a fast and simple technique for the construction of 3D dermal fibroblasts (3D-DFs) without exogenous scaffolds. The 3D-DFs can be obtained within 3 days by seeding DFs at a level that exceeds their confluent density and culturing them in the presence of ascorbic acid. The 3D-DFs have a compact, multilayer structure as revealed by histology and their collagen content is drastically increased compared to the monolayer. The 3D-DF-derived extracellular matrix can serve for 3D culturing of other cells. A gap closure assay was performed with the 3D-DFs to represent a 3D-wounded dermal model. Interestingly, the multilayered structure of the 3D-DFs could be regenerated after wounding even when cultured in the absence of ascorbic acid. Moreover, skin grafting using the 3D-DFs was demonstrated in vitro using wounded in vitro human full-thickness skin models. The 3D-DFs will be potentially useful for regenerative medicine and as tissue models for in vitro studies.

1 Introduction

In vivo tissues are composed of a complex system of three-dimensional (3D) cellular multilayers of various types of cells and extracellular matrix (ECM). Animals are usually used as in vivo models for testing the toxicity and effectiveness of new products, including chemicals, drugs, consumer products and medical devices. However, animals cannot represent all human conditions since they are genetically different from humans.

Alternatives to animal experiments have been developed that include in vitro and ex vivo approaches. Since two-dimensional (2D) in vitro models composed of a monolayer of cells poorly reflect the in vivo situation, recent research has realized the significance of 3D cultures over 2D cultures due to their more relevant structure and cellular functions compared to native tissues (Griffith and Swartz, 2006; Bokhari et al., 2007; Onoe et al., 2013; Knight and Przyborski, 2015). However, methods for constructing 3D tissues are typically a complex process and still remain challenging. In general, primary cells cultured on a substrate propagate along a 2D plane since, unlike cancerous or transformed cells, they do not form a 3D multilayer once they become confluent due to contact inhibition (Takai et al., 2008). Scaffolds derived from various biomaterials can be applied for the construction of 3D tissues (Bokhari et al., 2007; Lee et al., 2009; Kim et al., 2015). Nevertheless, the presence of these scaffold materials can disturb the tissue remodeling and functions (Anderson et al., 2008; Alaribe et al., 2016), and can induce undesirable foreign body reactions following implantation (Anderson et al., 2008; Alaribe et al., 2016).
cess was achieved by seeding dermal fibroblasts (DFs) at a high density and by adding ascorbic acid to stimulate collagen production. The 3D structure was found to significantly modulate collagen production in the presence of ascorbic acid. We further show potential applications of the resulting 3D-DFs in tissue engineering (Fig. 1). The ECM could be stripped of fibroblasts by decellularization and used as a scaffold to support the 3D culture of fresh fibroblasts, instead of using synthetic materials or an animal-derived matrix. Furthermore, the multilayered structure of the 3D-DFs could be regenerated after wounding. Finally, the 3D-DFs were grafted onto a wounded in vitro commercial full-thickness human skin model. Thus, the 3D-DFs are potentially useful for in vitro studies and medical applications.

2 Materials and methods

Cell culture
Normal human DFs (ATCC, Manassas, VA, USA) at passages 9-15 were used. They were cultured in complete medium (Dulbecco’s Modified Eagle Medium containing 10% (v/v) fetal bovine serum and 2 mM L-alanyl-L-glutamine) (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere with 5% (v/v) CO₂.

Construction of 3D-DFs
The DFs were seeded onto a 6.5 mm culture Transwell (Corning, NY, USA, surface area of 0.33 cm²) at a cell density of 2 x 10⁵ cells/cm² for the construction of a single cell layer (named 1L) and cultured in a 24-well plate. The cell seeding densities used for construction of 3D-DFs with different numbers of cellular layers are shown in Table 1. The 3D-DFs were constructed at a 5L thickness by seeding DFs at a density...
of 1 x 10^6 cells/cm² (five-fold the 1L cell density) and cultured for 3 days, except where otherwise stated. After seeding, the cells were cultured in complete medium supplemented with 0.1 mM 2-phospho-L-ascorbic acid trisodium salt (SAP; Sigma-Aldrich, St. Louis, MO, USA). The volume of culture medium in the Transwell and in the well was 0.3 mL and 1.5 mL, respectively. After 24 h culture, an additional 0.5 mL of medium was added to the culture.

**Determination of collagen content**

The total collagen content of the 3D-DFs was evaluated and compared with that of 2D (monolayer) DFs. The 3D multilayer and the 2D monolayer were constructed by seeding 3.35 x 10^5 cells on the different surface areas of a 6.5 mm Transwell (0.33 cm² surface area, equivalent to 1 x 10^5 cells/cm²) and a 12-well plate (3.8 cm² surface area, equivalent to 0.9 x 10^5 cells/cm²) for the 3D multilayer and the 2D monolayer, respectively. The DFs were cultured in culture medium without SAP or with 0.1 mM SAP.

The collagen content was determined by hydroxyproline assay following previously described methods (Lee et al., 2011) with some modifications. Briefly, all samples were harvested from the culture insert by trypsinization. For the 3D-multilayer cultured in culture medium with SAP, a pipette tip was used to scratch and peel the tissue off the membrane. The collected cells were then suspended in 6 M hydrochloric acid solution at 95°C overnight (22 ±3 h). The hydrolyzed samples were dried in a vacuum concentrator and then reacted with chloramine-T and p-dimethylaminobenzaldehyde (Sigma-Aldrich). The absorbance was measured using a microplate reader at a wavelength of 555 nm (Molecular Devices, San Jose, CA, USA). Bovine type I collagen (BD Biosciences, San Jose, CA, USA) was used as a standard.

For analysis of type I collagen in the culture medium, the medium was collected on day 3 and analyzed using a human pro-collagen I alpha SimpleStep enzyme-linked immunosorbent assay kit (Cat. ab210966, Abcam, Cambridge, UK) according to the manufacturer’s protocol.

**Total cellular protein assay**

Total cellular protein content, which is a representative measure of cell number, was quantified. Cells were lysed with Nonidet P-40 (NP-40) lysis buffer (1% (v/v) NP-40, 50 mM Tris-HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaF, 0.2 mM Na3VO4, pH 8.0), collected in microtubes and disrupted using a Viva-Cell™ ultrasonic processor (SONICS, Newton, CA, USA). After centrifugation at 20,900 g, the supernatant was harvested and total protein content was determined by the Lowry method using a DC™ protein assay (Bio-Rad, Hercules, CA, USA). Briefly, 5 µL sample was added to a 96-well plate, followed by 25 µL and 200 µL of reagents A and B, respectively, and mixed well. After 15 min of incubation at room temperature, the absorbance was read at 750 nm.

**Decellularization of the 3D-DFs**

The 3D-DFs were seeded at 7L cell density and were decellularized after 3 days following a previously published method (Xing et al., 2015). Briefly, a first decellularization solution (1 M NaCl, 10 mM Tris and 5 mM EDTA) was applied to the 3D-DFs for 1 h, followed by immersion in a second decellularization solution (0.05% (w/v) sodium dodecyl sulfate (SDS), 10 mM Tris and 25 mM EDTA) for 30 min. Residual decellularized 3D-DFs were washed with phosphate buffered saline (PBS).

In the recellularization experiments, the decellularized 3D-DFs were reseeded with DFs at 5L density and cultured for 1 day in culture medium without SAP. Decellularized and the recellularized tissues were fixed with 4% paraformaldehyde (PFA) and washed three times with PBS before immunostaining.

**Gap closure assay of 3D-DFs**

After 3 days of culture, wounds were made aseptically on 3D-DFs using a 1 mm-diameter punch (Acuderm, Fort Lauderdale, FL, USA). The punched tissue was carefully removed using forceps. The wounded 3D-DFs were then cultured in fresh culture medium without SAP for 10 days, replacing the culture medium with fresh medium three times a week.

**Use of the 3D-DF as a dermal substitute on wounded skin models**

3D-DFs at 10L cell density were constructed. Rho kinase inhibitor Y-27632 dihydrochloride (Millipore, Temecula, CA, USA) was added to the 3D-DFs during the 3 days of culture to prevent tissue contraction (Gribova et al., 2016) when removing the membrane from the Transwell. The Transwell membranes containing the 3D-DFs were cut from the Transwell cup using a 6-mm punch to prepare the 3D-DF grafts. Full thickness human in vitro skin models (FTMs), consisting of dermis and epidermis (EpiSkin™, Lyon, France), were used to demonstrate dermal grafting in vitro. Full-thickness wounds were made at the center of the FTMs using an 8-mm punch. Dermis and epidermis in the cut area were removed by forceps, leaving the wounded area. The 3D-DF graft was then placed into the wounded area of the FTMs.

In another experiment, 50 µL of medium containing 1 x 10^5 DFs were seeded onto the wounded area of the FTMs, while in the control the wounded FTMs were cultured in complete medium alone without grafting.

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**Tab. 1: Cell seeding density for construction of 3D-DFs with different cellular layers used in this study**

<table>
<thead>
<tr>
<th>Number of cell layer(s) (L)</th>
<th>Cell seeding density (cells/cm²)</th>
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<tbody>
<tr>
<td>1</td>
<td>2 x 10^5</td>
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<td>2</td>
<td>4 x 10^5</td>
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<td>3</td>
<td>6 x 10^5</td>
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<td>4</td>
<td>1 x 10^6</td>
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<td>5</td>
<td>1.4 x 10^6</td>
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<td>6</td>
<td>2 x 10^6</td>
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3 Results

3.1 Determination of the optimal SAP concentration and culture period for construction of the 3D-DFs

DFs were seeded at 5L cell density to produce 3D-DFs and cultured for 3 days. The most effective concentration of SAP for obtaining the multilayered structure was evaluated across a 0-0.2 mM range. Cross-sectional images of H&E stained DFs cultured without SAP revealed a loose structure, where most of the cells did not adhere to each other (Fig. S1A). Adding SAP at a concentration of at least 0.05 mM led to the formation of a multilayered tissue. Increasing the concentration of SAP above 0.1 mM did not further improve the histological structure, so the concentration of 0.1 mM SAP was selected for further studies.

Next, a suitable culture period for construction of the 3D-DFs was investigated. The DFs were cultured in the presence of 0.1 mM SAP for 1 to 7 days (Fig. S1B). An intact multilayered structure was obtained 2 days after seeding. The tissue structure appeared even denser 3 days after seeding and became less dense 7 days after seeding. The expression of fibrillin-1, a microfibril component, and ECM glycoproteins also decreased between day 3 and day 7 (Fig. S8), suggesting disintegration of ECM components over time. The collagen level of 3D-DFs increased three-fold from day 1 to day 3 (Fig. S2). Accordingly, a condition of 0.1 mM SAP and a culture time of 3 days was selected.

Statistical analysis

The numerical data are shown as the mean ±SD derived from three independent experiments. Statistical significance of differences between means was assessed by ANOVA followed by Tukey’s post-hoc test, accepting significance at the $p < 0.05$ level.
3.2 Structure of the constructed 3D-DFs

The thickness of the 3D-DFs was adjusted by altering the seeding density (Fig. 2A). A seeding density of 2 x 10^5 cells/cm^2 yielded a monolayer of cells (1L). The thickness of the DF tissues increased proportionally to the cell seeding density. However, exceeding a 10L cell density occasionally resulted in aggregation of cells (data not shown).

To construct 3D-DFs thicker than 10L, we constructed 3D-DF by repeated cell seeding. A 10L 3D-DF was constructed first over three days, followed by reseeding 10L cell density onto the established tissue and culturing for two further days. As a result, we could double the thickness of the tissue (Fig. S3^1). However, the obtained tissue showed a less homogeneous structure and non-uniform thickness.

Expression of type I and type III collagen, the major components of dermal ECM, was visualized by immunostaining cryosections of the 3D multilayers grown in culture medium with or without SAP, as shown in Figure 2B. Both types of collagen were expressed throughout the SAP-treated sections.

3.3 Effects of SAP and the 3D cellular structure on collagen production

2D monolayer and 3D multilayer DFs, initiated by seeding the same number of DFs on different surface areas in the presence or absence of SAP, were compared in order to investigate whether multilayer structure affects collagen production. The 2D monolayer showed a 1.6-fold increase in total cellular protein content when cultured in the presence of SAP in comparison to the control, while there was no significant change in the total cellular protein content of the SAP-treated 3D multilayer compared to the control (Fig. 3A).

Treatment with SAP resulted in a 5.3- and 5.9-fold increase in the collagen level in the 2D monolayer and the 3D multilayer, respectively, (Fig. 3B). However, when the amount of collagen was normalized to the total protein amount, the increase in the level of collagen after SAP treatment in the 3D multilayer was clearly greater (ca. 1.8-fold) than in the 2D monolayer, being a 6.1- and 3.3-fold increased level in the 3D multilayer and 2D monolayer, respectively, (Fig. 3C). The amount of pro-collagen I alpha 1 in the cell culture medium of the control and SAP-treated 2D monolayers and 3D multilayers showed no statistically significant difference (Fig. S4^1).

Next, large-sized 3D-DFs were produced in a 6-well plate. An intact sheet of 3D-DFs could be peeled off the culture plate, indicating that the 3D-DF sheets are quite robust (Fig. S5^1). The 3D-DF sheets contracted after being harvested, resulting in a thicker structure.

3.4 Decellularization and recellularization of 3D-DFs

Confocal images revealed that the ECM of decellularized 3D-DFs retained its structure, as shown by type I collagen staining (Fig. 4A,B). No nucleus staining was observed, indicating the successful removal of nuclei (Fig. 4A-C). The decellularized 3D-DF was reseeded with DFs at a 5L cell density (recellularized). An intact multilayered structure of recellularized 3D-DF was obtained after 1 day of culture without addition of SAP (Fig. 4D). In contrast, a multilayered tissue could not be obtained when the DFs were seeded on a blank membrane (Fig. 4E).

3.5 Gap closure assay of the 3D-DFs

To investigate the potential use of 3D-DFs for wound healing, a gap closure assay was performed. A wound was made on 3D-DFs by puncturing them with a 1-mm punch. The inner edge of the wounded 3D-DFs contracted soon after the puncture wound was made, resulting in a larger wound than the original punched area (Fig. 5A). The wounded area appeared thick-
3.6 3D-DF grafting on wounded human in vitro skin models

Dermis grafting was demonstrated in vitro using FTMs. The dermis and the epidermis at the center of the FTMs were removed after punching (Fig. 6A and Fig. S7). The collagen gel of the FTMs contracted slightly after being punched, leaving a space on the membrane that was larger than the original punched area. The wounded areas were seeding with DFs or a 3D-DF graft was inserted onto the membrane below the wounded area (Fig. S7C).

In the control experiment (no DFs or 3D-DF added), cells were found in the wounded area (Fig. 6B, E, H, K); however, this was erased with cells as time progressed, as shown in phase contrast images (Fig. 5B), indicating that the DFs proliferate and migrate from the original 3D-DF to the wounded area. Seven days after wounding, the wounded area was completely filled with DFs and appeared no different from the unwounded area. The CLSM images revealed that the multilayered structure of 3D-DFs was renewed, as a stacking of several nuclei was evident in the wounded area. The DFs were even found in the incision of the membrane (Fig. 5C,D). Positive staining of Ki-67 was found in the wounded area (Fig. S6), indicating that cells were proliferating during the gap closure process.

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In the present study, by seeding DFs at a confluency higher than that of the monolayer and culturing them in the presence of SAP, a 3D-DF structure was formed within 3 days. The seeded DFs were stacked into a multilayered tissue and could be harvested as an intact sheet of DF tissue, which cannot be achieved when cultured in the absence of SAP.

However, after 7 days the 3D-DFs began to lose density (Fig. S1) and expression of fibrillin-1, a microfibril component, and ECM glycoproteins seemed to decrease from day 3 (Fig. S8), suggesting disintegration of ECM components over a longer culture time.

4 Discussion

In current methods for the construction of multilayered fibroblasts it takes a long time for the fibroblasts to proliferate and accumulate ECM. Since cells lose their ECM and adhesion proteins upon enzyme (e.g., trypsin) treatment during the detachment process, they cannot attach to each other. Therefore, the cells cannot be seeded at a density above the confluent density to form a 3D structure unless given an ECM or ECM components coated to the cell surface to promote cell-cell interactions (Nishiguchi et al., 2011).

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Fig. 6: Dermis graft of 3D-DFs on wounded in vitro human FTM

(A) Schematic illustration of the grafting experiment. After punching, a 3D-DF graft was placed into the membrane at the site of FTM removal (inset photo), or the site was seeded with fibroblasts. (B-J) Representative CLSM images of FTM 14 days after wounding. (K-M) Representative H&E stained cross-sectional images. The wound (B, E, H, K) without grafting or cell seeding (control), (C, F, I, L) seeded with fibroblasts and (D, G, J, M) grafted with the 3D-DF. Images show the interface between the edge of the FTM and the membrane area from which the FTM was removed (red dashed box in A). F, FTM; M, membrane, D, 3D-DFs. All scale bars are 250 µm.
The accelerated cellular self-assembly method enables construction of multilayered DF tissues by seeding cells at up to a 10L cell density (approximately 50 µm in thickness). To achieve this, we seeded a second layer onto the constructed tissue to obtain a double thickness tissue layer (Fig. S2). However, this method results in poorer tissue structure and needs to be further improved.

The presence of ascorbic acid is known to increase the collagen production in fibroblasts. We investigated the effect of the multilayer structure of DFs on SAP-induced collagen production. Distinctly higher levels of collagen were produced in the 3D multilayer compared to the 2D monolayer although the total protein concentration in the 3D multilayer remained constant (Fig. 3). It was not surprising that the total protein of the 2D monolayer increased when treated with SAP, since ascorbic acid also stimulates cell proliferation (Yamamoto et al., 1992; Phillips et al., 1994). However, the total protein content of the control and SAP-treated 3D multilayer showed no significant difference (Fig. 3A). The higher cell density of the 3D multilayer probably inhibited cell proliferation due to contact inhibition of fibroblasts (Guelstein et al., 1973; Takai et al., 2008).

It was previously found that the amount of DNA in 3D multilayers of fibroblasts constructed by a layer-by-layer technique remained stable during 4 weeks of culture, while that in fibroblast monolayers continued to increase (Chetprayoon et al., 2013). Moreover, the rate of total protein synthesis declines when the cells are in a quiescent state (Aumailley et al., 1982; Fuge et al., 1994; Chen et al., 2012). The collagen level in the SAP-treated 2D monolayer was not significantly greater than in the control 2D monolayer when normalized to the total protein level. However, the ability of SAP to stimulate collagen production was drastically increased when the DFs were in a 3D multilayer although total protein synthesis remained constant. This is consistent with a previous report that the synthesis of collagen as a proportion of the total protein level increases as the culture becomes confluent or the cell density increases (Aumailley et al., 1982). In addition, fibroblasts cultured in a 3D structure might have improved cellular functions and thus be better stimulated by SAP. Unlike a monolayer, where cells are cultured on a rigid surface, cells grown in a 3D environment are surrounded by other cells and intercellular matrices and so have increased cell-cell and cell-matrix interactions, leading to improved cellular functions that better reflect those of in vivo tissues (Petrie and Yamada, 2012; Edmondson et al., 2014; Hwae et al., 2015). Such a phenomenon was also observed in the increased elastin synthesis in fibroblasts cultured in 3D gels as compared to 2D cultures (Derrick et al., 2013).

Even though it was expected that the DFs in the 3D multilayer would not be proliferating, the 3D-DF multilayer could be regenerated after wounding, and evidence of cell proliferation was found in the wounded area (Fig. 5 and S6). In contrast, the DF monolayers do not form a multilayer, even after having been cultured for a month (Chetprayoon et al., 2013). Unlike the DF monolayer, the DFs in the 3D structure in this study proliferated and migrated into the wounded area to form a multilayer without the aid of SAP (Fig. 5B). We have to note that the 3D-DFs represent only a part of the in vivo wound healing system since they are composed of only fibroblasts. Other cell types, such as immune cells or a vascular network, may be introduced to the 3D-DF in future studies to better represent in vivo wound healing. Nevertheless, the 3D-DFs can potentially be a useful in vitro 3D model of gap closure during the wound healing process to study the efficacy of drugs or other compounds.

The constructed 3D-DF was decellularized to obtain an ECM scaffold. The resulting ECM showed a 3D meshwork of collagen that can serve as a scaffold for culturing other cells. To test the ability of the decellularized 3D-DF to support cell growth, fresh DFs were seeded onto ECM scaffolds at an over-confluent density. Multilayered DFs were obtained after 1 day of culture. A cell-derived matrix has an advantage over a synthetic matrix due to its better biocompatibility and its containing components that regulate cellular functions, including cell attachment, proliferation, migration and differentiation, as in the native tissue environment (Theocaris et al., 2016). Multilayered fibroblasts cultured by current methods require a long culture time, typically of more than several weeks, before they are ready to be decellularized to obtain the ECM (Scherzer et al., 2015; Xing et al., 2015; Franco-Barraza et al., 2017). The accelerated cellular self-assembly described in this study can produce ECM scaffolds within 3 days, which will be useful for various applications.

The use of the 3D-DF for skin grafting was demonstrated using human in vitro FTMs (Fig. 6) as an alternative to an animal experiment (Fig. 6). Wounded FTMs grafted with 3D-DF sections showed enhanced epidermalization, while seeding cells into the wounded area failed to result in wound closure, possibly due to an inadequate supporting matrix for the skin cells to migrate and regenerate. Moreover, seeding of single cells requires time for the cells to attach to the area, resulting in loss of some cells before they can attach. This is similar to in vivo situations, where cell therapy by injection of cells fails to deliver the cells to target sites unless biomaterials are used for cell transport (Suzuki et al., 2018). We demonstrate that the 3D-DF can be grafted onto the wounded dermis in vitro. The 3D-DF provides a supporting matrix for the FTM to re-epithelialize, especially type I collagen, which facilitates the migration of keratinocytes along the dermal matrix (Rousselle et al., 2019). Our results suggest that the 3D-DF may be suitable for use as a skin substitute for wound treatment. Since the 3D-DF can be constructed within a few days, it can be readily supplied for the urgent needs of skin grafts in wound treatment.

5 Conclusions

We present a simple concept for the fast construction of scaffold-free 3D tissues by cellular self-assembly. The process was accelerated by seeding DFs at an over-confluent density and culturing them with SAP as a collagen stimulant, enabling the formation of 3D-DFs within a few days. The synergistic effect of ascorbic acid and the 3D structure on stimulating collagen production was found to be key to achieving the 3D-DFs. ECM could be derived from the 3D-DFs and was suitable as a scaf-
fold for 3D cell culture. Furthermore, the 3D-DFs had the ability to regenerate into a multilayered tissue after wounding, suggesting a useful in vitro model for wound closure studies, and may be suitable as dermal grafts for wound treatments. The presented technique may be applicable to other cell types for construction of 3D tissues.

References


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**Conflict of interest**

The authors declare that they have no conflicts of interest.

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