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Harvesting epithelial keratinocyte sheets from temperature responsive dishes preserves basement membrane proteins and improves cell survival in skin defect model.

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Abstract

Cultured epithelial autograft (CEA) therapy has been used in clinical applications since the 1980s, however, there are some issues related with this treatment that still remain unsolved. Enzymatic treatment is typically used in the collection of epithelial keratinocyte sheets, but it tends to break the adhesion and basement membrane proteins. It is thought that the loss of proteins after the enzymatic treatment is responsible for the poor survival of transplanted cell sheets. Our laboratory has developed a temperature responsive culture dish, which does not require enzymatic treatment to harvest cells. In this study, we compare morphological and survival results from rat epithelial keratinocyte cell sheets harvested by temperature reducing treatment (TT sheets) against cell sheets harvested by enzymatic (Dispase) treatment (DT sheets). TT sheets preserve keratin structure in better conditions and express higher levels of, collagen IV and laminin 5 than DT sheets. In order to evaluate the cell sheet survival after transplantation, we created an *in vivo* transplant model. Keratinocyte sheets obtained from GFP positive animals were transplanted into athymic rats. The survival rate 7 days after transplantation of TT sheet was higher than that of DT sheets. Collagen IV and Laminin 5 expression was observed in the TT sheet

transplantation group. These results indicate that the remaining basement membrane proteins are important for initial attachment and cell survival. We believe that cell sheets harvesting method using temperature responsive culture dishes provide superior cell survival and can solve one of the roadblocks in CEA´s therapy.

Keywords: cultured epithelial autograft; temperature responsive culture dish; dispase; basement membrane; transplantation; survival rate

1.Introduction

In 1975, the culture method for human epithelial cells was established (Rheinwald and Green, 1975). Cultured epithelial autograft (CEA) therapy has been used for more than 30 years since it was first reported (O'Connor *et al.*, 1981).CEA therapy has become an important treatment for severe burn patients; however, many problems remain to be solved (Atiyeh and Costagliola, 2007). In CEA therapy, one of the major complications is blistering and graft loss. This therapy is effective for reducing the burn area, however, there are large variations in graft take in the clinical literature(15%-85%) (Carsin *et al.*, 2000, Chester *et al.*, 2004, De Luca *et al.*, 1989, Donati *et al.*, 1992, Odessey, 1992, Sood *et al.*, 2010, Williamson *et al.*, 1995, Wood *et al.*, 2006). Recently, the combination of auto skin graft and CEA has been used to improve graft take (Braye *et al.*, 2000, Hayashi *et al.*, 2014). Commonly, cultured epithelial cell sheets are harvested by enzymatic treatment (dispase), which breaks down the adhesive-protein (for example integrin and keratinocyte basement membrane proteins) on the surface of the cell culture dish. Breaking down the epithelial basement membrane proteins may be the cause of the poor take. We have developed temperature responsive culture dishes, which do not need enzyme

treatment to harvest the cultured cell sheets(Okano *et al.*, 1995, Okano *et al.*, 1993, Yamato *et al.*, 2001). The temperature responsive dish is created by grafting a temperature responsive polymer, poly *N*-isoproprylacrylamide(PIPAAm) on to the surface of the culture dish by electron beam irradiation. This polymer surface reversibly changes its properties, between hydrophilicity and hydrophobicity, in response to an external stimuli. This surface is slightly hydrophobic in culture conditions at 37℃, and changes to hydrophilic below the critical solution temperature of about 32℃. The harvested cell sheets maintain the cell-cell interactions, the natural adhesive proteins and extracellular matrix (Cerqueira *et al.*, 2014, Kushida *et al.*, 2001, Kushida *et al.*, 1999, Takeuchi *et al.*, 2014, Yamato, et al., 2001). The cultured cells obtained using a temperature responsive dish are in the form of a sheet, and are ready to be used in clinical as cell sheet therapy(Nishida *et al.*, 2004, Ohki *et al.*, 2012, Sawa *et al.*, 2012).

In this study, we compared cell sheets produced on a temperature responsive dishes with cell sheets produced on standard culture dishes, requiring enzymatic treatment, to better understand their structure and the role of basement membrane proteins. In addition, we created full-thickness rat skin loss models

and transplanted both types of cultured keratinocyte sheets to evaluate the survival rate after transplantation.

2.Materials & Methods

All animal experiments were performed according to the "Guidelines of Tokyo Women's Medical University on Animal Use", and were consistent with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources (ILAR) and published by the National Research Council (2006).

2.1. Preparation of neonatal rat epithelial cell sheets

Rat epidermal cells were prepared as described elsewhere with modification (Oku *et al.*, 1994). Newborn Sprague-Dawley rats (CLEA Japan, Inc., Tokyo, Japan) and green fluorescent protein (GFP)-expressing Sprague-Dawley newborn rats (SD-TgN(Act-EGFP)CZ-004Osb; SLC, Hamamatsu, Japan) were euthanized with CO_2 gas. The skin was excised from the backs of several rats, about 2x2cm. The excised skin was chopped up and treated with dispase I (400PU/mL; GODO SHUSEI, Tokyo, Japan) diluted with PBS at 4°C overnight. Epidermis was separated from dermis using a microscope and fine cutting, and then treated with 0.25% Trypsin in phosphate buffered saline (PBS) at 37°C for 30 minutes. Cell suspension was filtered by a 40 μm cell strainer and keratinocyte cultured medium (KCM) containing 5% FCS was added to stop

further trypsin action. KCM was comprised of Dulbecco's Modified Eagle Medium and Ham's F12 medium (3:1), and contained hydrocortisone (0.4 ng/ml), triiodothyronine (2x10-9M), cholera toxin (1x10-9M), insulin (5 μg/ml), transferrin (5 μg/ml) and epidermal growth factor (10 ng/ml). The cell suspension was centrifuged at 400x g for 5 minutes. The pelleted cells were suspended in keratinocytes culture medium and seeded to a temperature responsive cell culture insert (not commercialized; CellSeed Inc., Tokyo, Japan) and a normal cell culture insert(#353090;BD falcon, Franklin Lakes, NJ) at a density of $2x10^5$ cells/cm^2, upper side of cell culture insert was filled with KCM. We didn't use 3T3 feeder cells. The culture dishes were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 7-9 days untill confluent. We cultured both dishes in the same way.

2.2 Recovery of keratinocyte sheets from dishes

Keratinocyte sheets were recovered from PIPAAm-grafted dishes by reducing the temperature and non-grafted dishes by dispase treatment as described previously (Yamato, et al., 2001).

In the temperature reduction treatment, keratinocytes were incubated at 20°C for 30minutes, the culture medium was aspirated and a Cell Shifter™ (CellSeed),

an artificial support membrane, was placed onto the cultured cells and then the Cell Shifter was slowly peeled away from the dish with tweezers. Keratinocyte sheets were harvested together with the Cell Shifter (Fig.1).

In the dispase treatment, keratinocyte sheets were detached after incubation by adding dispase I (400PU/mL) at 37°C for 10, 20, and 30minutes. Keratinocyte sheets were washed twice with culture medium and harvested using a Cell Shifter. The cultured epithelial keratinocytes which were cultured on a temperature responsive dish and harvested by reducing the temperature, called a "temperature treated cell sheet" (TT sheet). The cultured epithelial keratinocytes cultured on a normal cell culture insert and harvested by dispase treatment, called a "dispase treated cell sheet"(DT sheet). Exposure times were 10, 20 and 30 min labeled as DT10, DT20 and DT30, respectively.

2.3. Histological and immunohistochemical analyses

Cultured keratinocyte sheets were fixed with 4% paraformaldehyde, embedded in paraffin and stained with Hematoxylin–Eosin stain using conventional methods. Samples for immunohistochemistry were then embedded in optimal cutting temperature freezing compound (OCT) to make cryostat-sectioned (5μm thick) slices and positioned them on glass slides and which were then air-dried.

For immunohistochemistry, rabbit polyclonal anti-collagen IV antibodies (ab6586 ;abcam, Cambridge, England), rabbit polyclonal anti-laminin 5 (ab14509 ; abcam), mouse polyclonal anti-collagen VII (ab6312 ; abcam) and mouse monoclonal anti-pan cytokeratin (ab27988 ; abcam) were used for primary antibodies (1/200 dilutions). Fluorescently labeled secondary antibodies (Alexa Fluor 568-labeled goat anti-rabbit IgG antibodies, Alexa Fluor 568-labeled goat anti-mouse IgG antibodies (Molecular Probes, Eugene)) were used (1/1000 dilutions). The stained sections were mounted with undiluted ProLong® Gold antifade reagent containing 4',6-diamidino-2-phenylinode (DAPI)(P36935 ;Molecular Probes), and viewed by fluorescent microscopy.

2.4. Transmission electron microscopy

The samples were fixed and processed by Tokai Electron Microscopy Inc. (Nagoya, Japan) for electron microscopy analysis. The samples were fixed with 2% paraformaldehyde and 2% osmium tetroxide. After dehydration, the samples were infiltrated with propylene oxide and transferred to fresh 100% resin, and polymerized. The blocks were ultra-thin sectioned at 70nm and then sections were placed on copper grids. They were stained with 2% uranylacetate, and then rinsed, followed by secondary staining with lead stain

solution(Sigma-Aldrich Co, St. Louis, MO). The grids were observed by a transmission electron microscope (JEM-1400Plus ; JEOL Ltd., Tokyo, Japan). Digital images were taken with a CCD camera(VELETA; Olympus Soft Imaging Solutions GmbH, Münster, Germany). The thickness of cultured keratinocytes was measured by Image J software (NIH, Maryland USA). The cellular density of the cultured keratinocytes was measured by Win Roof Ver5 (Mitani Corporation Osaka, Japan).

2.5. Rat full thickness back skin defect model and cell sheet grafting

Six-week-old male F344 athymic rats (Charles River Japan, Tokyo, Japan) were anesthetized by 2% inhaled isoflurane. The back was shaved and washed with 70% ethanol. The back skin was excised, while the dartos fascia was saved, and a full thickness skin circle defect approximately 35mm in diameter was made using surgical scissors. Cultured epithelial keratinocyte sheets, collected from GFP-expressing Sprague-Dawley rats, were transferred with a cell shifter (15mm diameter circle) and grafted onto the back median of the skin defect, leaving the cell shifter (Fig 1). An Adaptic, Non-Adhering dressing (SIGMAX, Tokyo, Japan) in the shape of a donut, was placed over skin defect around the cell sheet. Injury part was covered with ointment and gauze cut to a 35mm

diameter circle and finally a cap. The cap used was a 35mm Petri dish (BD Falcon), where the top and side were drilled with holes, and stitched in a four-way pattern around the skin. During the following 7days, the dressing was not changed.

2.6. Graft survival rate analysis

One week after transplantation, the rats were anesthetized and coverture was removed. The transplanted section was observed with a stereomicroscope and fluorescence microscope in addition to recording images. The area around the transplantation was resected for histological analysis. Images of the transplanted tissue were analyzed to determine the relative size of the fluorescence area and to compare it with the size of the graft using the Image J software.

2.7. Statistical analysis

All data are expressed as mean ± standard deviation (SD). An unpaired Student's *t*-test was performed to compare each group. A *p*-value of less than 0.05 was considered significant.

3. Results

3.1. Comparison before cell sheet harvesting

Rat epithelial keratinocytes were successfully cultured with both temperature responsive culture dishes and normal cell culture dishes. It required 7-9 days to reach confluence in a sheet-like form from the primary culture. Before any treatment, the sheets were confirmed to be multilayer, with about three layers using an electron microscope (Fig. 2A, B). We recognized the lamina lucida and lamina densa, but there was very little hemidesmosome in the joint area. The thickness of the cell sheets which were cultured on a temperature responsive dish was 5.8±1.0μm and the sheets from a standard culture dish was 4.5±1.2μm (Fig. 2 C). There was no significant difference in the thickness of the keratinocyte sheets. The cellular density of the cell sheets that were cultured on a temperature responsive dish was 86.1±3.5% and the cellular density of the cell sheets from a standard culture dish was 82.6±6.8% (Fig. 2 D). There was no significant difference in the cellular density between the keratinocyte sheets. On both culture dishes, cell sheets were cultured with almost the same thickness and cellular density.

3.2. Comparison after cell sheet harvesting

We were able to harvest both types of sheets after collection treatment; however, where the TT sheets were unbroken (Fig. 3 A), the DT sheets were difficult to handle and were easily torn because they became fragile (Fig. 3, B, C, D). In the HE staining and pancytokeratin staining, many of the keratin structures were recognized in the TT sheets group; however, the number of structures decreased in the DT sheets groups (Fig. 3E, F, G, H, U, V, W, X). The TT sheets expressed collagen IV widely on the basal side (Fig. 3 I). However, in the DT10 sheets showed only sparse expression of collagen IV (Fig. 3 J). In the DT20 sheets, there was some expression of collagen IV (Fig. 3 K). In the DT30 sheets, there was slightly expression of collagen IV (Fig. 3 L). The TT sheets strongly expressed laminin5, which was widely observed on the basal side (Fig. 3 M). On the other hand, the DT10, DT20 and DT30 sheets showed only a slight expression of laminin5 (Fig. 3 N, O, P). In contrast, expression of collagen VII was neither observed in the TT sheets or the DT sheets (Fig. 3 Q, R, S, T).

3.3. TT sheets had a good survival rate (Comparison after transplantation)

Seven days after transplantation, the TT sheets displayed a round, horny surface in the middle of the transplanted area (Fig. 4 A). It was almost the

same shape as the cell shifter and this area presented GFP-expression (Fig. 4 E). On the other hand, the DT10 sheets had an island-shaped horny surface in the middle of transplanted area (Fig. 4 B); which also presented GFP-expression (Fig. 4 F). In the DT20 sheets, we also recognized a spotty horny surface (Fig. 4 C, G). The DT30 sheets had a punctuated horny surface (Fig. 4 D), with little GFP expression (Fig. 4 H). The GFP-expression area of the DT sheets transplanted groups was smaller than the TT sheet transplanted group. After transplantation, the area which survived(GFP expression area) in the TT sheet was 122.6± 31.6mm^2 (n=9), the DT10sheet was 68.1± 24.2 mm^2 (n=5), the DT20 sheet was 46.5± 14.8mm^2 (n=4), and in the DT30 sheet it was 21.5± 29.4 mm^2 (n=5). The difference between each of the values was shown to be significant (Fig. 4 I).

The survival area in the DT transplanted groups became smaller over time.

HE staining of the TT sheets showed that the epithelial structure survived in a multilayered configuration, about 8-layers thick (Fig. 5 A). However, the DT sheets had less epithelial structure and more granulation tissues (Fig. 5 B).

GFP positive keratinocytes were seen in the section of TT sheet grafted area (Sup Fig 1). In immunostaining, the cell sheet clearly showed expression of

collagen IV in the basal layer, and naturally, expression of laminin5 was also observed (Fig. 5 C, E). However, the DT sheets had no expression of collagen IV or laminin5 in the granulation tissue area (Fig. 5D, F). Deposition of collagen IV and laminin-5 was observed in the remaining areas of the epidermis. Expression of collagen VII was not present in either the TT sheets or the DT sheets (Fig. 5G, H).

In TEM analysis, the basement membrane including lamina lucida and lamina densa was observed in the survived areas. However, it showed less hemidesmosome expression than normal skin(Fig. 5I)

4. Discussion

Results of this study indicate that keratinocyte sheets which cultured on temperature responsive culture dish harvesting by temperature reducing treatment offers the good potential for initial survival of transplanted keratinocytes. Preserving basement membrane proteins are important for the initial take and an important factor in successful cell transplantation.

TEM results showed that there was no difference in the sheet thickness or cellular density between the cell sheet cultured on a temperature responsive dish and on a normal culture dish. It was same culture condition before the harvesting. Moreover, the lamina lucida and lamina densa were also identified in the both sheets. These results indicate that PIPAAm did not have a remarkable effect on cell culturing.

After the harvesting, the TT sheets retained collagen IV and laminin5. On the other hand, as the dispase exposure time increased, the DT sheets become fragile, and reduced the expression of collagen IV and laminin5. Lim et al. previously reported that dispase disrupts collagen VI, collagen VII, laminin, fibronectin, and another extracellular matrix components when they used an amniotic membrane treatment (Lim *et al.*, 2009). The present results are

consistent with those reported by Lim et al. The result of initial take after transplantation was significantly better for the TT sheets than the DT sheets. Transplantation results suggested that basement membrane proteins are directly related to sheet survival. Kim et al. showed that collagen IV and laminin are important factors, which affect the shape of the basal cells, and the existence of collagen IV and laminin5 is necessary for the adherence of epithelial keratinocytes and dermal matrix in the reconstruction of human oral mucosa (Kim *et al.*, 2001). TEM results confirmed the presence of the basement membrane structure, lamina lucida, lamina densa and hemidesmosomes. These results indicate that the basement membrane was reassembled after the sheet transplantation.

Regarding the basement membrane, we could not identify any collagen VII in the sheet stains. The basement membrane is based on basal lamina and anchoring fibrils. Briggaman et al. demonstrated that the basal lamina, whose major components are collagen IV and laminin, originates from the epidermis, whereas anchoring fibrils, whose major components are collagen VII and IV, originates from the dermis (Briggaman *et al.*, 1971). In vitro, epithelial keratinocytes make collagen IV and laminin5. Our results are congruent with

Briggaman's report. Collagen VII connects the dermis side with the epidermis through laminin5. It is known that an abnormality of this protein can cause diseases such as bullous epidermolysis. Collagen VII is essential to the stability of the epidermis and the dermis, which is directly related with their long term stability. Previous reports showed collagen VII is observed in 3weeks after transplantation (Smola *et al.*, 1998). Further studies are needed to clarify the causes of dropping off or blistering and the expression of collagen VII over a much longer period.

Moreover, our laboratory demonstrated that retaining cell-cell interaction and the extracellular matrix is also important for successful transplantation. Previous reports showed the sheet which harvested by temperature reducing treatment, the cell originated oral mucosal keratinocyte, preserved E cadherin and fibronectin (Kushida, et al., 1999, Yamato, et al., 2001). TT sheet transplantation has a higher cell survival rate than cell injection, which inhibits anoikis (Sekine *et al.*, 2011). Anoikis is defined as apoptosis induced by the loss of cell/matrix interactions, which could be a physiological mechanism, supporting the maintenance of tissue cell homeostasis. Therefore, preserving cell-cell interaction is also important for cell survival and preventing anoikis. On

the other hands, in the case of DT sheet transplantation, basement membrane proteins and cell-cell interactions are broken, then transplanted cells might disappear.

There are few reports of cultured keratinocyte transplantation using a temperature responsive culture dish, and only Cerqueira et al. reported its use to date (Cerqueira, et al., 2014). Cerqueira et al compared re-epithelialization and neovascularization among heterotypic three-dimensional stacked human TT sheets ("keratinocytes(KC) + dermal fibroblast(DF)", "KC + dermal microvascular endothelial cells(DMEC)" and "KC + DF + DMEC") engrafted into mouse full thickness wound defect model. They showed that stacked human TT sheet contributes the progress of wound healing, especially TT sheets containing KC + DMEC promotes re-epithelialization and the presence of DMEC directly influenced neovascularization. Furthermore, they mentioned that TT sheets transplantation could be good application for skin regeneration. On the other hand, our experiment compared the cell sheet survival and morphology *in vivo* between two types of cell sheet harvesting methodologies. The results clearly showed the superiority of TT sheet grafting to DT sheet grafting and strengthen the previous work.

The clinical reports have showed HE stains after CEA grafting, and these reports showed the immature nature of the outer layer of skin and pointed out its vulnerability (Hayashi, et al., 2014, Ronfard et al., 2000). However, these reports have not clearly proved the survival of CEA grafting. It is difficult to know in clinical settings whether the grafted cultured epithelium survives or whether new epithelialization by host wound healing replace the graft. This point is still controversial. Our results showed that transplanted sheets did survive at least 7 days. This indicates possibility of survival of CEA grafting and usefulness of TT sheet grafting in clinical settings.

In recent years, many reports mentioned culture methods using an air-liquid interface to promote differentiation and organization of keratinocytes (Frankart et al., 2012, Hanada et al., 2014). Our study confirmed only the construction of keratinocyte sheets, but differentiation of keratinocyte sheets was not assayed. Further study is needed to clarify the differentiation of keratinocyte sheets and we plan to use culture with an air-liquid interface.

In our transplantation model, we transplanted on to dartos fascia, superficial fascia, and transplanted only epithelial keratinocyte. It is thought that the existence of dermal fibroblast is important for basement membrane formation.

Interaction between fibroblasts and keratinocytes plays important role of basement membrane reconstruction (Smola, et al., 1998). Existence of fibroblast promotes laminin, collagen IV and collagen VII expressions (Lee and Cho, 2005). Keratinocytes and fibroblast synthesize and localize basement membrane. Keratinocytes up-regulates mRNA of collagen VII in fibroblasts, and fibroblast are needed for extracellular secretion and correct localization of collagen VII and Laminin 5 (Marionnet *et al.*, 2006). We thought that another transplantation model is needed for making sure of relationship with fibroblasts *in vivo*.

5.Conclusion

The results showed that the TT sheets have the ability to preserve collagen IV and laminin5. These proteins are important for the initial take of keratinocyte transplantation. We believe that TT sheet transplantation will be able to produce good results in clinical transplantation. Harvesting with the temperature responsive culture dish can be applied to CEA therapy around the world.

Conflict of interest

Tatsuya Shimizu is stake holders of CellSeed Inc. Tokyo Women's Medical University is receiving research funding from CellSeed Inc.

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Legends

Figure 1

Schema of cell harvesting and transplantation.

After cultured cells become confluent, harvesting treatment is performed. Cells which cultured on a temperature responsive dish are incubated at 20°C for 30min. Cells which cultured on a normal cell culture insert are treated with dispase at 37°C for 10, 20, and 30 minutes. After detaching treatment, a cell shifter is placed onto the cultured cells. Cultured cells are harvested together with the cell shifter. Cultured cells are transplanted onto the rat back skin defect with a cell shifter.

Figure 2

Transmission electron microscopy of a cultured epithelial keratinocyte sheet before any harvesting treatment. (A) A keratinocyte sheet cultured on a temperature responsive culture dish. (B) A keratinocyte sheet cultured on a normal cell culture dish. Both keratinocyte sheets are about three layers thick. (C) The thickness of the two keratinocyte sheets shows no significant differences.(D) The cellular density of the two keratinocyte sheets shows no significant differences.

Scale bar: 5μm

Figure 3

Macroscopic views of a temperature treated cell sheet (TT sheet) and a dispase treated sheet, and hematoxylin and eosin stain after the harvesting treatment. (A) A TT sheet is exfoliated into a sheet form without being torn. (B, C, D) A DT sheet becomes slightly fragile and can be easily broken by picking it up. Hematoxylin and eosin stained and anti-pancytokeratin antibodies (red). (E,U) A TT sheet contain a lot of keratin in the outer layer. (F,G,H,X,W,X) In the DT sheet, keratins gradually decrease and the sheet becomes thinner. Immunohistochemical analysis of cultured keratinocyte basement membrane proteins. Staining with anti-collagen IV antibodies (red: basement membrane protein), anti-laminin-5 antibodies (red: basement membrane protein), anti-collagen VII antibodies (red: basement membrane protein) and DAPI (blue: cell nuclei). (I) Collagen IV is expressed on the basal side like a belt. (J,K,L) As progress continues over time, expression is no longer observed. (M) Laminin-5 is also expressed on the basal side like a belt. (N, O, P) After the dispase treatment for 10 minutes, almost no expression of laminin-5 can be observed. (Q,R,S,T) Collagen VII is also not expressed.

Scale bar: A-D 2cm, E-T 20μm, U-X 50μm

Figure 4

Macroscopic view and fluorescent view of transplantation area 7 days after transplantation. (A, E) Most of the parts remainedintact in the TT sheet.GFP expression area was confirmed as a circular shape in the middle of the transplanted area. (B-D, F-H)The expression area gradually decreases over time in the DT sheets groups.(I) Analysis of GFP expression area.The GFP expression area in the TT sheetsgroup was significantly larger compared to the DT sheets groups.

Scale bar: 1cm

Figure 5

Pathological analysis of the cultured keratinocyte transplantation area with hematoxylin and eosin stain and immunofluorescence staining.(A) In the TT sheet, the multi-layered epithelial structure and keratinocytes can be seen. (B) The DT30 sheet has few epithelial structures and granulation tissues are visible. Immunofluorescence staining. Staining with anti-collagen IV antibodies (red), anti-laminin-5 antibodies (red), anti-collagen VII antibodies (red) and DAPI (blue: cell nuclei).(C) Collagen IV is shown under the sheet, in the basement membrane zone, and in the capillary wall of the ECM. (D) Collagen IV is only visible in the capillary wall of the ECM. (E) Laminin-5 is observed under the sheet, and in the basement membrane zone. (F) There is no laminin-5 expression. (G, H) Collagen VII is also not obviously expressed. (I) TEM of the TT sheet after transplantation. The lamina lucida (LL), lamina densa (LD), and hemidesmosomes (HD) are visible in the basement membrane zone. Expression of hemidesmosomes is low.

Scale bar: A-B,G-H 100μm, C-F 60μm, I 1μm

Supplemental Figure 1

GFP positive keratinocytes were seen in TT sheet grafted surface.

Scale bar: 50 μm

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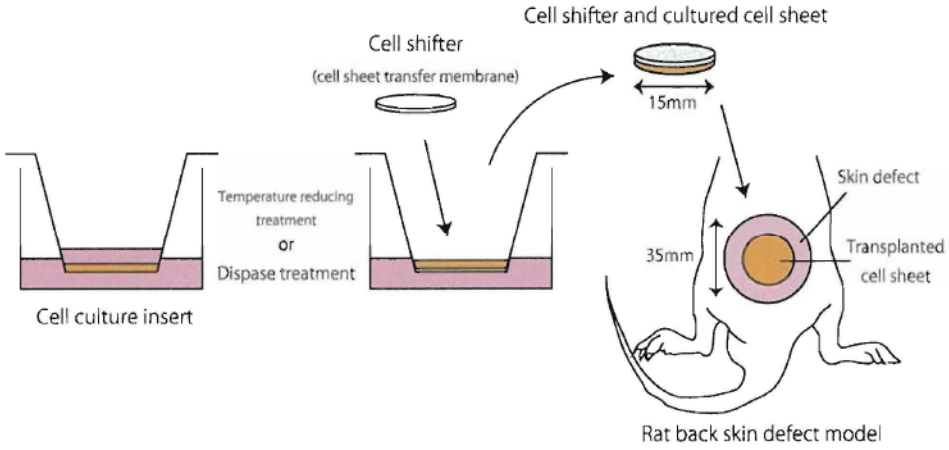

Figure 1

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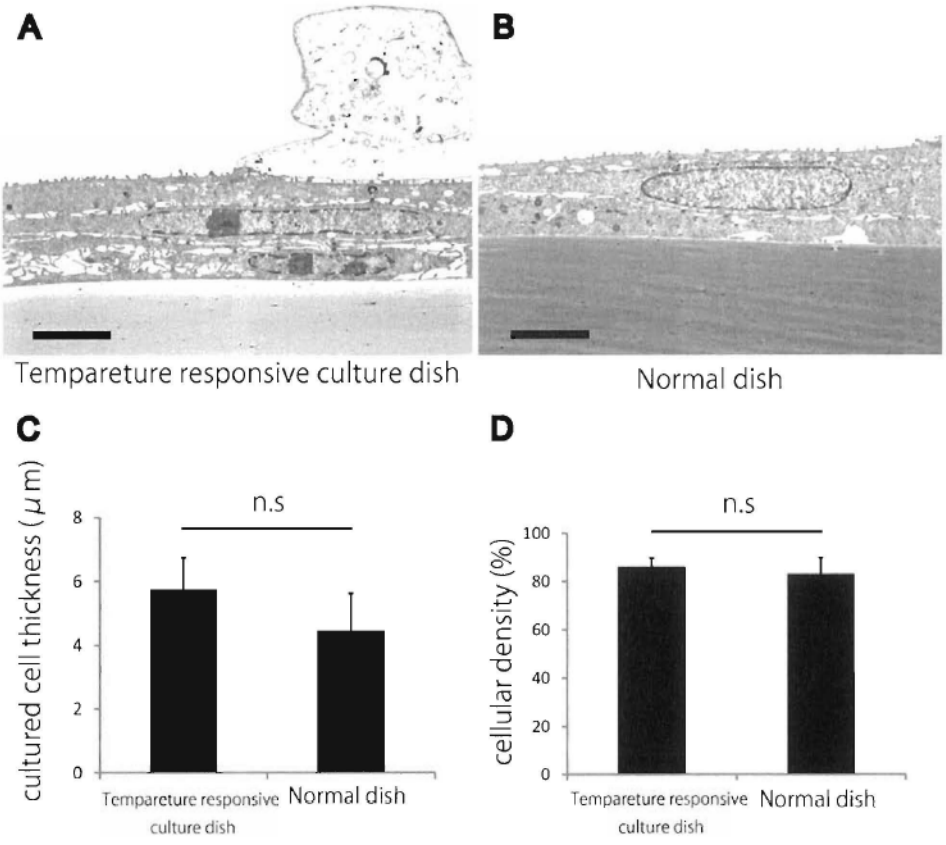

Figure 2

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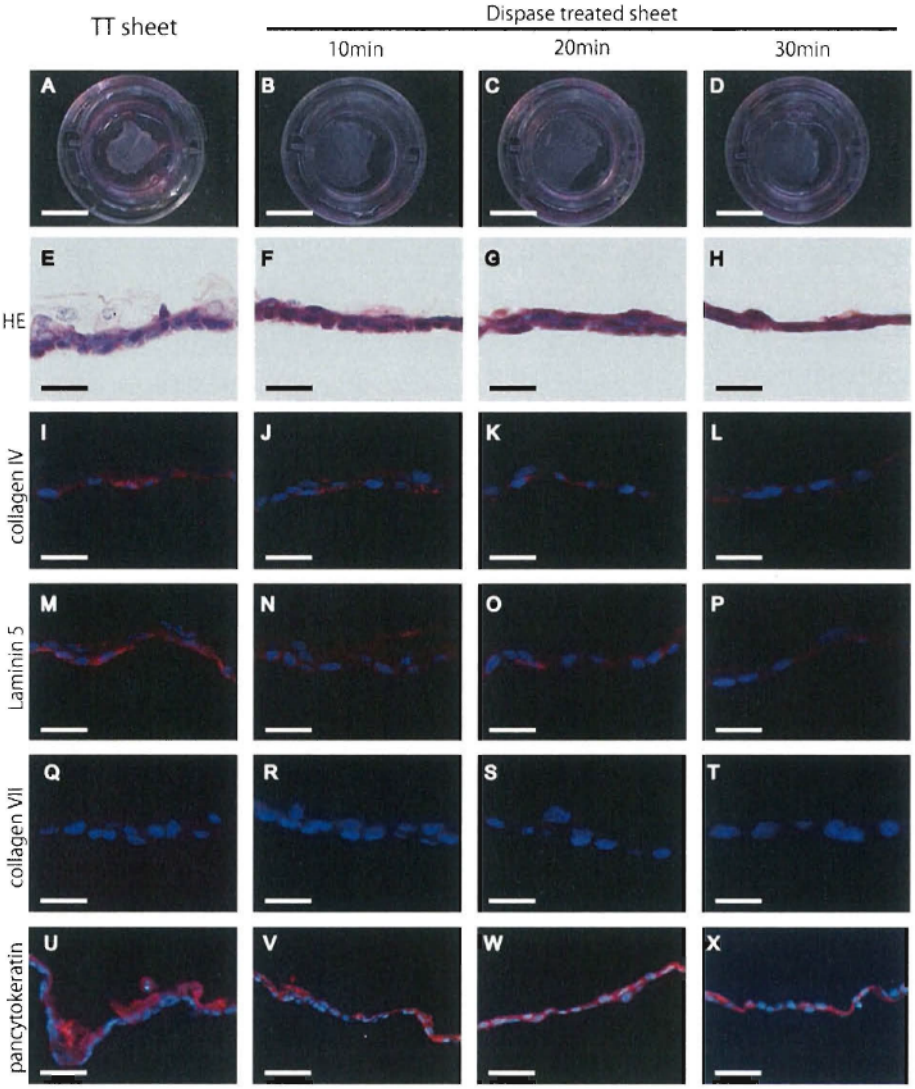

Figure 3

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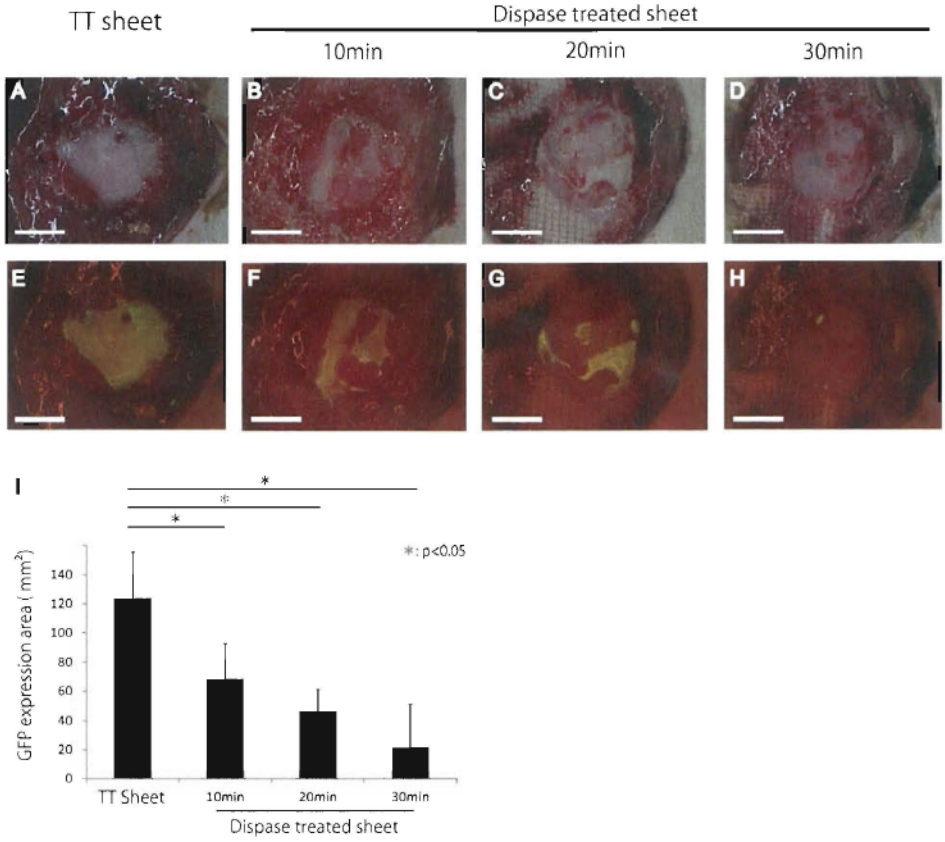

Figure 4

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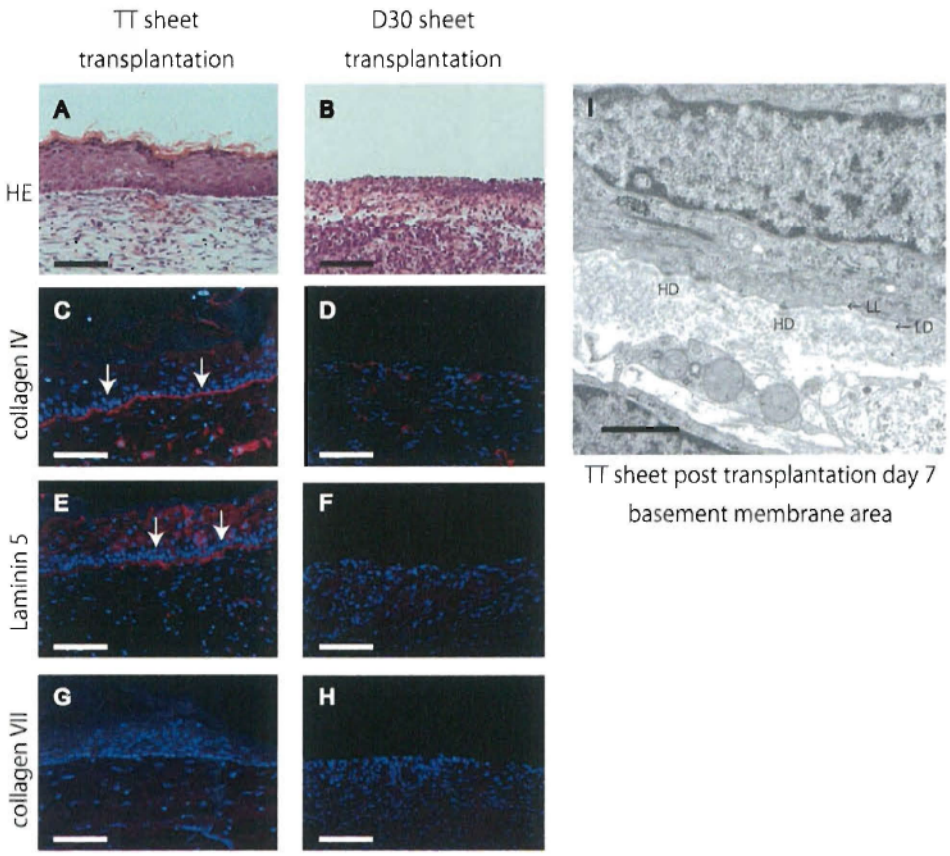

Figure 5

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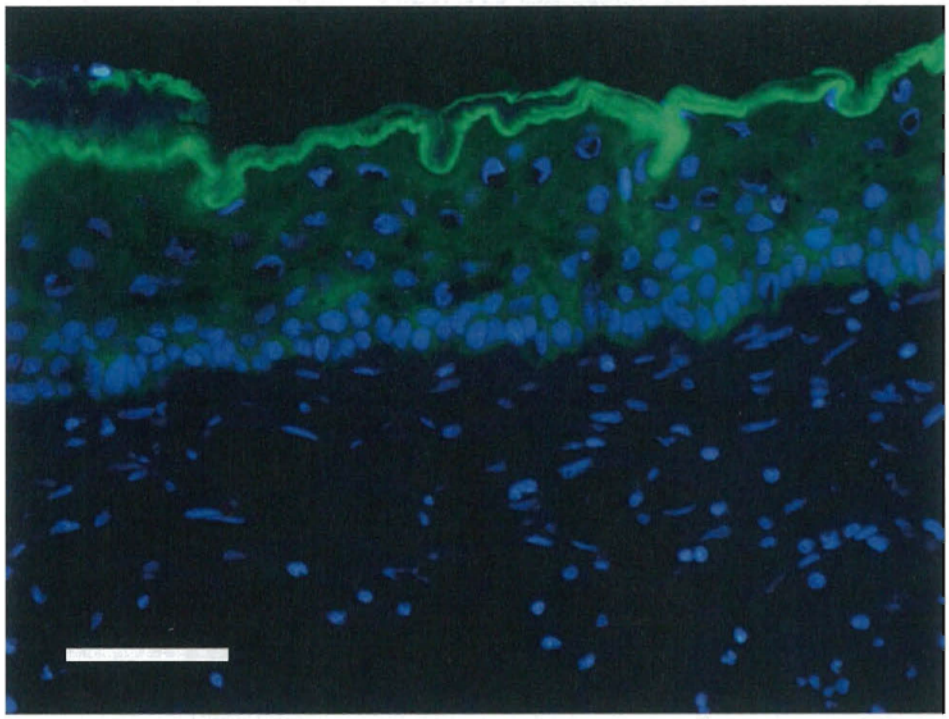

Supplemental Figure 2

69x59mm (300 x 300 DPI)