Ex Vivo Prefabricated Rat Skin Flap Using Cell Sheets and an Arteriovenous Vascular Bundle

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Background: Recently, research on tissue-engineered skin substitutes have been active in plastic surgery, and significant development has been made in this area over the past several decades. However, a regenerative skin flap has not been developed that could provide immediate blood flow after transplantation. Here, we make a regenerative skin flap ex vivo that is potentially suitable for microsurgical transplantation in future clinical applications.
Methods: In rats, for preparing a stable vascular carrier, a femoral vascular pedicle was sandwiched between collagen sponges and inserted into a porous chamber in the abdomen. The vascular bed was harvested 3 weeks later, and extracorporeal perfusion was performed. A green fluorescent protein positive epidermal cell sheet was placed on the vascular bed. After perfusion culture, the whole construct was harvested and fixed for morphological analyses.
Results: After approximately 10 days perfusion, the epidermal cell sheet cornified sufficiently. The desquamated corneum was positive for filaggrin. The basement membrane protein laminin 332 and type 4 collagen were deposited on the interface area between the vascular bed and the epidermal cell sheet. Moreover, an electron microscopic image showed anchoring junctions and keratohyalin granules. These results show that we were able to produce native-like skin.
Conclusions: We have succeeded in creating regenerative skin flap ex vivo that is similar to native skin, and this technique could be applied to create various tissues in the future. *(Plast Reconstr Surg Glob Open 2015;3:e424; doi: 10.1097/GOX.0000000000000400; Published online 17 June 2015.)*

We have developed an innovative tissue engineering approach based on "cell sheet technology."[1–3] Thermoresponsive culture dishes enable us to control cell adhesion and detachment by changing the temperature. This allows for a noninvasive harvest of culture cells as an intact cell sheet, including the deposited extracellular matrix (ECM). Single-layer cell sheets were used in various types of tissue regeneration. Cell sheets have also been stacked to fabricate 3-dimensional tissues. To overcome thickness limitation, multistep transplantation has been used to create thick myocardium with microvascular network.[4] In vitro, we repeatedly overlaid triple-layer cardiac cell sheets on resected muscle with a femoral artery and vein as a vascular carrier and perfused the construct by a bioreactor, resulting in 12-layer tissue.[5]

Surgical closure with an auto-skin graft is the gold standard for treatment of burn patients. However, donor sites from the patient or allo-skin are limited; therefore, an alternative treatment is required. On the other hand, Green and coworkers[6] developed autologous cultured epidermis, and it has been applied to major burns[7] and large congenital pigmented nevus.[8] Cultured epidermis is inferior to skin graft because of its poor appearance, rough texture, and lack of durability. To ensure an efficient result for

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cultured epidermis transfer, it requires reconstruction of the dermal layer.

Bell et al[9] reported a 3-dimensional culture system using collagen gel and fibroblasts. They have also cultured epidermal cells on collagen-based constructs containing fibroblasts, presenting them as a thick skin substitute.[10] These skin substitutes require well-vascularized, smooth, flat recipient sites for temporary coverage. On the other hand, there has never been a report of a "regenerative skin flap" connected with microsurgical anastomosis of an artery and vein that could be reperfused immediately after transplantation.

Therefore, we propose the ex vivo prefabricated skin flap including a surgically connectable blood vessel. We isolated rat femoral vessels and fascia and sandwiched the bundle between artificial dermis, then introduced them into the chamber. Three weeks after implantation, the content of the chamber was harvested, and its blood vessel was connected to the perfusion tube in a bioreactor. Then, the construct was perfused with culture media and simultaneously overlaid with an epidermal cell sheet, which we have named "regenerative skin flap." The final products were analyzed morphologically.

Supplemental digital content is available for this article. Clickable URL citations appear in the text.

MATERIALS AND METHODS

Sprague-Dawley (SD) male rats weighing 200–300 g and GFP-positive SD neonatal rats were used. All animal experiments were conducted according to the "Guidelines of Tokyo Women's Medical University on Animal Use" (approval number 14–45). The animals were anesthetized by general anesthesia with isoflurane.

Experimental Design

A preliminary experiment was performed using the rat inguinal fat pad with femoral vessels as a vascular carrier for in vitro cell sheet transplantation and the short extracorporeal perfusion culture to confirm the effects of epithelial cell sheet polarity and optimize the perfusion conditions (n = 6). Subsequently, we fabricated the vascular bed with femoral vessels and artificial dermis in the body (n = 15), then a keratinocyte cell sheet was transferred on the isolated prefabricated bed, and the construct was media perfused in vitro to engineer a regenerative skin flap.

Keratinocyte Cell Sheet Fabrication

Epidermal cells were isolated from the back skin of 1-day-old SD GFP-positive neonates. Harvested tissue specimens were incubated in Dulbecco's modified Eagle medium (Sigma, St. Louis, Mo.) containing 500-U/mL dispase I (Godo Shusei, Tokyo, Japan) overnight at 4°C. All epithelial layers were harvested using forceps and treated with 0.5% trypsin–0.1% ethylenediamine tetraacetic acid (Sigma) for 60 minutes at 37°C to create single-cell suspensions. Disaggregated cells were filtered through a 40-μm cell strainer (BD Biosciences, Franklin Lakes, N.J.). Suspended cells were seeded on temperature-responsive cell culture inserts (CellSeed, Tokyo, Japan) and cultured using keratinocyte culture medium (KCM) for 7–8 days at 37°C. After cultivation, epithelial cell sheets were harvested from the culture inserts by reducing the temperature to 20°C.[11]

Bioreactor Setup and Tissue Perfusion Culture

The procedure is largely the same as described previously.[5] Briefly, the engineered constructs are perfused in a custom-made bioreactor containing perfusion pumps; sensors for temperature, pH, and pressure; and a process controller (ABLE, Tokyo, Japan). The construct is placed in the tissue culture chamber (Tokai Hit, Shizuoka, Japan) to become a vascular bed, and the femoral vessels are connected to 2 micro-polyurethane tubes (inner diameter, 0.3 and 0.6 mm). The constructs are perfused with KCM at 50 μL/min by the delivery pump through the inlet tube connected to the femoral artery. The outlet

waste medium collected from femoral vein is measured by an electrical balance. A portion of medium effused from the tissue is removed by another pump.

Polarity Analysis

A skin incision was made in the inguinal region, and the inguinal fat pad containing superficial inferior epigastric artery (SIEA) and vein was isolated. The femoral vascular bundle was ligated on the peripheral side. The small branches toward the muscle and the internal iliac vessels were ligated. The fat pad was harvested under heparinization (400 IU/kg, intravenous injection). Rats were then euthanized with overdoses of pentobarbital. The fat pad was placed in a tissue culture chamber and perfused with KCM at 50 µL/min through the inlet tube connected to the femoral artery. Then 2 epidermal cell sheets derived from GFP-positive neonates were transplanted onto the flap; 1 was placed as usual (cell sheet's basal side was adjacent to the fat pad surface), and the other was reversed. Three days after perfusion culture, fluorescent observation was performed on the 2 cell sheets randomly with 10 frame units. Image processing was carried out; green pixels were counted with Image J (NIH software). Tissue specimens were prepared for histology. After 1, 2 and 3 days, the medium collection rate (recovered from vein/delivered from artery) was assessed.

Vascular Bed Fabrication

In our study, we used Tanaka's report, which described in vivo prefabricated engineered flap.[12] The skin incision was made from the inguinal region, and the femoral-saphenous artery and its accompanying vein, nerve, and superficial fascia were isolated. The vascular bundle was ligated on the peripheral side. The branches and internal iliac vessels were ligated. The bundle was introduced into the chamber along with 2 sponges of artificial dermis, 1 on top and 1 underneath the vascular pedicle. The chamber was embedded between the abdominal fat and muscle folded at the inguinal ligament. The incision wound was closed, and 3 weeks after implantation, the contents of the chambers were harvested under heparinization (400 IU/kg, intravenous injection). Rats were then euthanized with overdoses of pentobarbital.

Extracorporeal Perfusion

Eleven of 15 vascular beds were extracorporeally perfused. The vascular bed was placed in a tissue culture chamber and perfused by a delivery pump through the inlet tube connected to the femoral artery. Then an epidermal cell sheet derived from GFP-positive neonates was transplanted onto the vascular bed, so that the cell sheet's basal side was adjacent to the vascular bed surface. The bioreactor setup was same as described in the "Polarity Analysis" section. Extracorporeal perfusion culture was performed for 3–10 days. The specimens were histologically evaluated.

Morphological Analyses

Samples were fixed in 4% paraformaldehyde (PFA) and routinely processed into 7-µm thick paraffin-embedded sections. Hematoxylin and eosin staining and Azan staining were performed. For immunohistochemical analyses, specimens were cryosectioned. For the detection of filaggrin, the specimens were treated with a 1:400 dilution of anti-filaggrin rabbit polyclonal antibody (Atlas Antibodies, Stockholm, Sweden). Specimens were treated with Vectastain Universal Elite ABC kit (Vector Laboratories, Burlingame, Calif.). Cell nuclei were then counterstained by hematoxylin. For the detection of basement membrane proteins, the specimens were treated with a 1:50 dilution of anti-laminin 5 rabbit polyclonal antibody (Abcam, Cambridge, UK) and a 1:50 dilution of anti-collagen type 4 rabbit polyclonal antibody (Abcam). For the detection of regenerative vessels, sections were incubated with a 1:10 dilution of anti-CD 31 rabbit polyclonal antibody (Life Technologies, Carlsbad, Calif.). For the detection of Ki 67, sections were treated with a 1:50 anti-Ki 67 rabbit monoclonal antibody (Abcam) overnight at 4°C. Specimens were then treated with both a 1:200 dilution of Alexa-Fluor-488-conjugated anti-rabbit antibodies (Life Technologies) and Alexa-Fluor-568-conjugated anti-rabbit antibodies (Life Technologies). Cell nuclei were then counterstained by ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Life Technologies).

Transmission Electron Microscopy

The samples were prefixed with 2% PFA and 2% glutaraldehyde overnight. After being washed, they were postfixed with 2% osmium tetroxide. Samples were dehydrated through a graded series of ethanol and embedded in Quetol-812 (Nisshin EM Co., Tokyo, Japan). The polymerized resins were ultrathin sectioned (70 nm thickness), stained with 2% uranyl acetate, and then secondary stained with Lead stain solution (Sigma-Aldrich Co., Tokyo, Japan). The grids were observed by transmission electron microscopy (JEM-1400 Plus; JEOL Ltd., Tokyo, Japan).

Statistical Analysis

All data are expressed as mean ± standard error (SE). An unpaired Student's t test was performed to compare the 2 groups. A P value of less than 0.05 was considered significant.
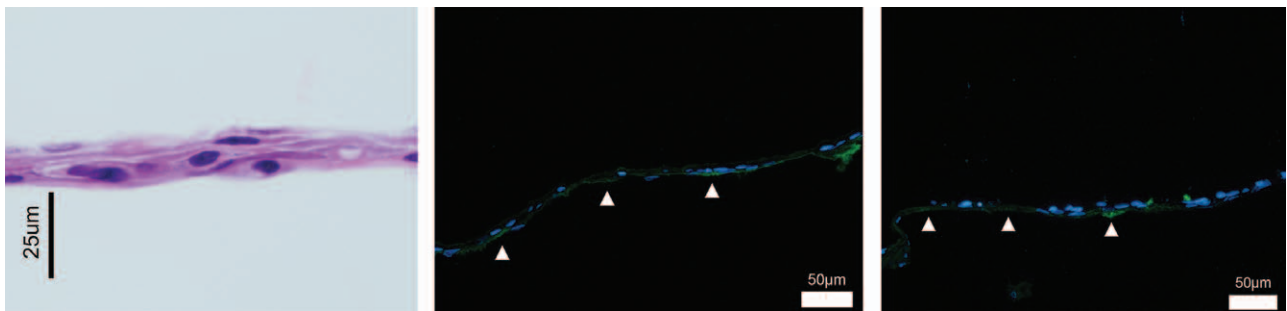
Fig. 1. Epidermal cell sheet derived from GFP-positive neonates. The cell sheets were harvested from the temperature-responsive cell culture inserts. A, The epidermal cell sheet was stained with hematoxylin and eosin. B, The epidermal cell sheet was stained with anti-laminin 332 antibody (the green color underneath the cell sheet; arrowheads). Nuclei were counterstained with DAPI (the blue color). C, The epidermal cell sheet was stained with anti-collagen type 4 antibody (the green color underneath the cell sheet; arrowheads). Nuclei were counterstained with DAPI (the blue color).
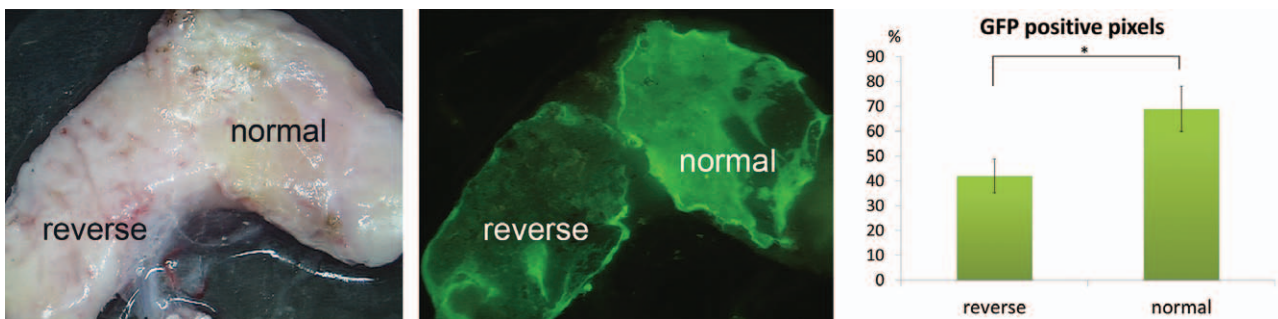

Fig. 2. Polarity analysis of epidermal cell sheets. A, Macroscopic appearance of the SIEA adiposal flap and 2 cell sheets after 3 days of perfusion culture. B, Fluorescent observation after 3 days of perfusion culture. C, GFP-positive pixels of reversed and normal transplanted groups. Asterisk indicates that the *P* value was less than 0.05. The error bars on histograms indicate standard errors of the means.

RESULTS

We also present the details concerning the number of animals and constructs. (**See Figure, Supplemental Digital Content 1**, which presents the number of animals and constructs in a flow chart, **http://links.lww.com/PRSGO/A107**.)

Keratinocyte Cell Sheet Fabrication

After 7–8 days cultivation, epithelial cell sheets were harvested from thermoresponsive culture inserts (Fig. 1A). Adhesive basement membrane proteins were identified in the basal surface of the cell sheet (laminin 332, Fig. 1B; collagen 4, Fig. 1C).

Polarity Analysis

In cell sheet polarity analysis, the SIEA adiposal flap was chosen as a vascular carrier. It is easy to harvest a flap large enough for implanting 2 cell sheets side by side. Six SIEA adiposal flaps were media perfused, where each specimen supported 2 cell sheets. (**See Figure, Supplemental Digital Content 2**, which shows macroscopic appearance of the flap immediately after transplanting 2 cell sheets (above) and its fluorescent observation (below),

http://links.lww.com/PRSGO//A108.) The culture medium was delivered from the artery of the vascular bed and was discharged from the vein. The medium collection rate fell off gradually as time passed. After 72 hours of perfusion, mean rate was 55.4% ± 4.4% SE.

The epidermal cell sheets transplanted in the normal orientation were observed to be partially keratinized after 3 days culture. On the other hand, the cell sheets transplanted upside down appeared wet (Fig. 2A). Fluorescent observation under a surgical microscope showed that the normally orientated sheets were strongly luminescent and uniform over most of the cell sheet, but the reverse sheets had weak mist-like emission intensity (Fig. 2B).

In the epidermal cell sheets, the green pixels (GFP positive) in pictures of normally transplanted specimen were 68.9% ± 9.1% SE. Those of the reverse-oriented transplant group were 41.9% ± 6.8% SE. There was a significant difference between the 2 groups ($P < 0.05$; Fig. 2C).

In the reverse-oriented transplant group, the epidermal cell sheets were rounded and detached from the bed (Fig. 3A). In contrast, engraftment of
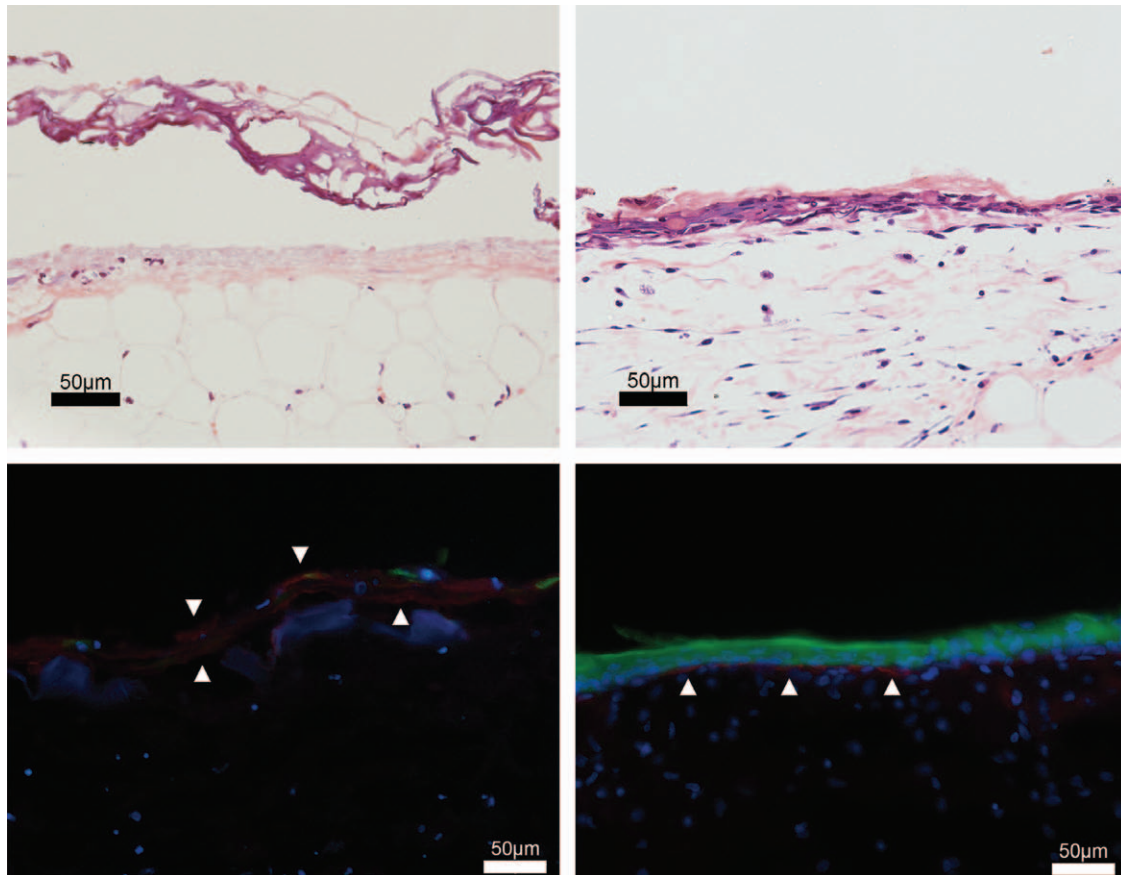
Fig. 3. Cross-sections of the epidermal cell sheets and SIEA adiposal flaps after 3 days of perfusion culture. A, Reverse-transplanted group (hematoxylin and eosin stain). B, Normally transplanted group (hematoxylin and eosin stain). C, Reverse-transplanted group. The epidermal cell sheet (green color) and laminin 332 (red color); arrowheads. Nuclei were counterstained with DAPI (blue color). D, Normally transplanted group. The epidermal cell sheet (green color) and laminin 332 (red color) arrowheads. Nuclei were counterstained with DAPI (blue color).

the epidermal cell sheets oriented normally was successful (Fig. 3B). In the former group, the cell sheet was thin and fragmented, and basal membrane protein laminin 332 was observed on both sides of the cell sheet (Fig. 3C). In the latter group, the cell sheet was uniformly thick, and laminin 332 was observed only on the basal side of the cell sheet (Fig. 3D).

Vascular Bed Fabrication

Fifteen vascular beds were fabricated. (**See Figure, Supplemental Digital Content 3**, which displays vascular bed fabrication, **http://links.lww.com/PRSGO//A109**.) The vascular bundle accompanied by fascia was sandwiched between 2 layers of artificial dermis, and the chamber was closed. Three weeks after implantation, the construct was harvested, placed in a bioreactor and extracorporeally perfused; simultaneously a keratinocyte cell sheet was transplanted onto the construct.

The implanted chambers were covered in fibrous tissue. Through the chamber pores, fibrous tissue was connected between the inside and the outside

and was carefully peeled off away from the chamber. Viable connective tissues were regenerated in 13 of 15 chambers. In 2 cases, the contents of the chambers failed to become good connective tissue and could not be perfused with medium. In these chambers, no thrombosis was observed in the arteriovenous bundles. Eleven of 13 cases were perfused in the bioreactor. Two other cases were not perfused, and immediately PFA-fixed. (**See Figure, Supplemental Digital Content. A** vascular bed was prefabricated in vivo for 3 weeks (A). The bed was injected black ink systemically (B). Cross-section of a vascular bed was stained with hematoxylin and eosin (C); it was also stained with azan (D and E). The regenerated capillaries were stained with anti-CD31 (green color, arrowheads). Nuclei were counter stained with DAPI (blue color; F), **http://links.lww.com/PRSGO/A110**.)

Extracorporeal Tissue Perfusion

Eleven of 15 vascular beds were extracorporeally perfused. (**See Figure, Supplemental Digital Content 5**,
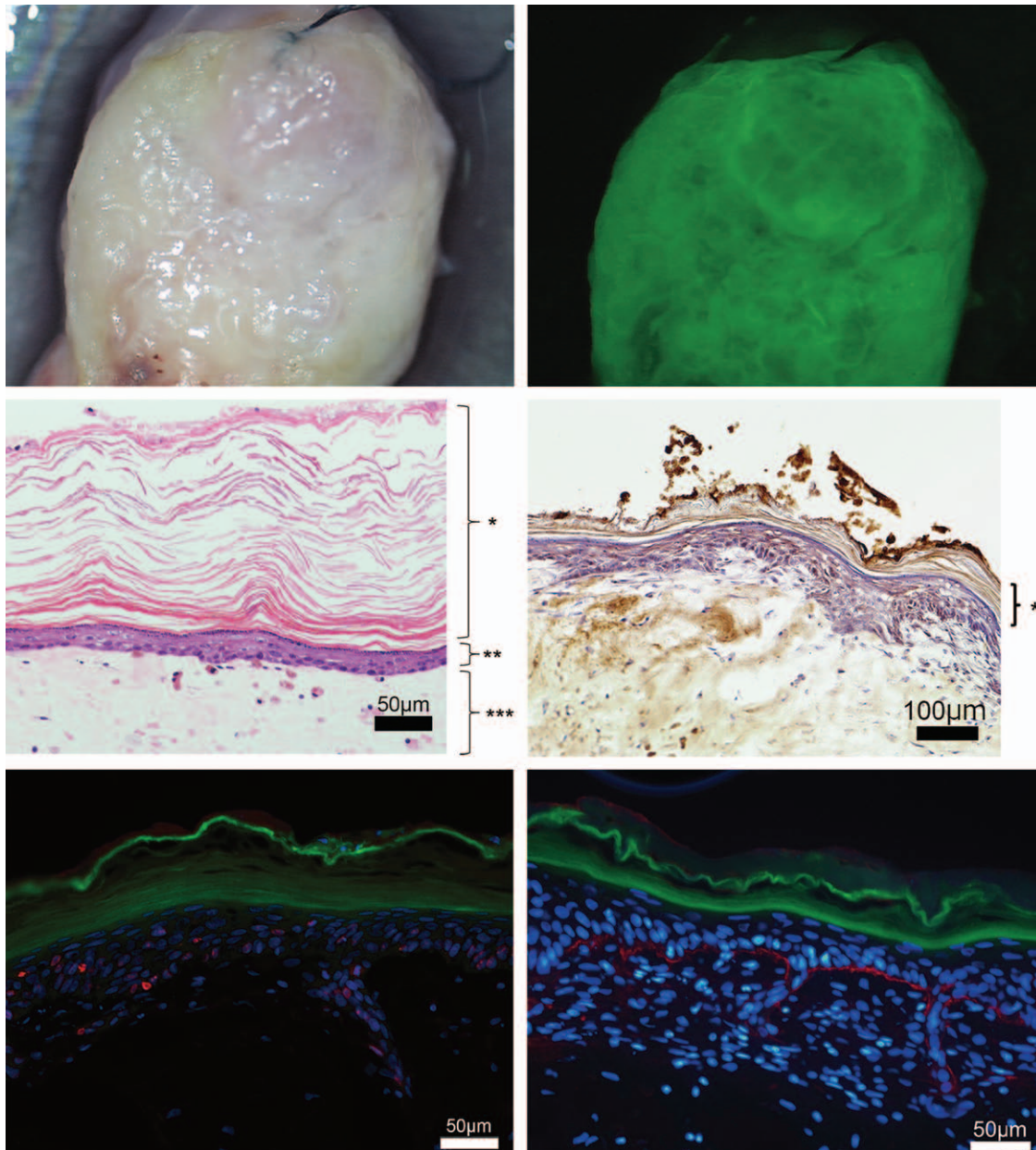
Fig. 4. Perfused skin flap model at 10 days. A, Macroscopic appearance. B, Fluorescent observation. C, Cross-section of the harvested skin flap model was stained with hematoxylin and eosin. Asterisk indicates keratinized and cast-off epidermis. Double asterisk indicates the grafted epidermal cell sheet. Triple asterisk indicates the regenerated granulation tissue. D, Cross-section of the harvested skin flap model stained with anti-filaggrin antibody. Asterisk indicates keratinized and cast-off epidermis. E, Cross-section of the harvested skin flap model was stained with anti-Ki 67 antibody (red color). F, Cross-section of the harvested skin flap model stained with anti-laminin 332 antibody (red color). Nuclei were counterstained with DAPI (blue color).

which displays (above, right) an infused culture medium (red arrow) and collected medium and blood (blue arrow), **http://links.lww.com/PRSGO/A111**) In the majority of samples, as the perfusion period became longer, leakage of medium from the tissue increased, and medium recovery from the vein was reduced.

Keratinization increased with the duration of extracorporeal perfusion. After 10 days perfusion,

the epidermal cell sheet cornified sufficiently (Figs. 4A–C). Intensity of green fluorescence was strong in the sites where epidermis keratinization was progressing (Fig. 4B). The desquamated corneum was positive for anti-filaggrin stain (Fig. 4D). In the basal cell of the epidermal cell sheet, Ki 67 was observed (Fig. 4E). Laminin 332 was deposited on the interface area of the PRS vascular bed and the
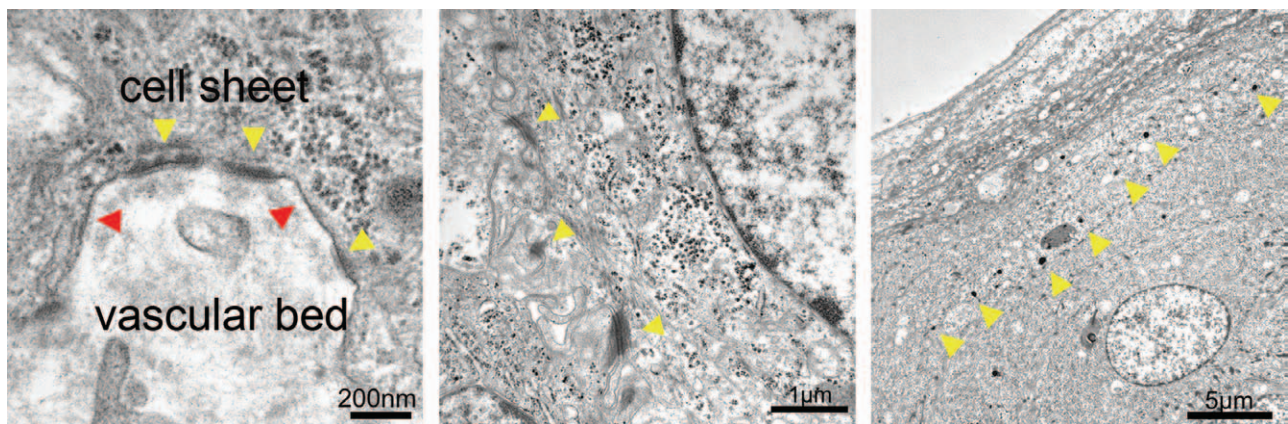
Fig. 5. Electron micrographs showing a skin flap model perfusion culture after 10 days. A, Basement membrane (red arrowheads) and hemidesmosomes (yellow arrowheads). B, Desmosomes of the prickle cell layer (yellow arrowheads). C, Keratohyalin granules in the granular layer (yellow arrowheads).

epidermal cell sheet (Fig. 4F). Observations with transmission electron microscopy showed the basement membrane and hemidesmosomes at the interface of the epidermal cell sheet and the tissue built from the collagen sponge (Fig. 5A). In the prickle cell layer, desmosomes were present (Fig. 5B). In the granular layer, keratohyalin granules were observed (Fig. 5C). These were similar to the structures observed in the native skin.

DISCUSSION

We have shown here functional skin flap-like tissues ex vivo with perfusable vascular networks, using cell sheet–based tissue engineering and a novel bioreactor system. However, it cannot be used in human clinical applications because it is necessary to bury a chamber for 3 weeks to make a vascular bed and required sacrificing the femoral vessels in our rat model. It is our final aim to fabricate a large thick flap from a small sacrifice, such as from human cutaneous veins with added scaffolds and cell sheets in a bioreactor. This study shows only the concept and core technology toward achieving our goal.

Cell Sheet Polarity

We have developed a concept of cell sheet engineering, which utilized temperature-responsive culture surfaces. Because the poly(N-isopropylacrylamide)-grafted surfaces facilitate spontaneous cell detachment, the use of proteolytic enzymes, such as dispase, trypsin, and collagenase, can be avoided. With noninvasive cell harvest, cell-to-cell junctions and ECM proteins can be preserved. Various types of ECM and membrane proteins are preserved underneath the cell sheets and play as adhesive agents when cell sheets are stacked on another cell sheet, or transferred onto other tissues and organs.[13] In particular, laminin 332 is a critical protein

not only for linking the epidermis to the dermis, but also for regenerating the basement membrane at the interface between the dermis and the epidermis.[14]

In our study, we first confirmed the adhesion of the apical and basal side of the epidermal cell sheet to the vascular carrier during tissue perfusion culture. As expected, when the cell sheet was transplanted in reverse, or upside down, engraftment of the cell sheet was poor. It was thought to be that because ECM and membrane proteins exist only underneath the cell sheet, they were unable to adhere to the vascular bed, causing the cells to slip off. When in contact with the apical–apical side, the adhesive force between the cell sheets will be weak. On the other hand, in tissue sections of transplanted cell sheets in the opposite direction, laminin 332 was observed on both sides of the cell sheet (Fig. 3C). It suggests the possibility that the polarity of the keratinocytes had changed during the 3-day culture. It is possible that there was a migration of keratinocytes, similar to that seen in wound healing.

Vascular Bed

Regenerating new vessels is difficult compared with the convenience of utilizing a preexisting vascular carrier. To date, there have been many reports on using a vascular carrier in vivo, such as fat tissue,[15] jejunum,[16] arteriovenous shunt loop,[17] and bundle.[18] Referring to these reports, we performed preliminary experiments and prepared various rat models (data not shown). An adiposal flap is not suitable as a vascular carrier for the regenerative flap because adipose tissue is fragile, and fat cells have a low tolerance for ischemia. In addition, the density of blood vessels is low resulting in the blood flow per hour per weight to be less in adipose tissue compared with muscle or fascia. On the other hand, harvesting from the intestinal tract is too invasive in human clinical applications.

So, we concluded that an arteriovenous bundle would be the most useful as a vascular carrier for clinical use. The arteriovenous bundle is simpler to construct operatively than an arteriovenous shunt loop and does not present problems with thrombus formation. To avoid compromising a major artery and accompanying vein, we considered the possibility of using a single vein because no harm could be caused from harvesting a small subcutaneous vein. It remains controversial whether sufficient angiogenesis can arise from a vein in a perfusion bioreactor.

Comparison with Existing Skin Substitute

There are currently many skin-substitute products; however, they only target partial roles in the process of wound healing. Mainly, they act as temporary biologically covering, cytokine donators, and structural proteins necessary for wound healing. After the process, the patient's skin regenerates and can, therefore, be used for serial autograft. Skin substitutes based on autologous keratinocytes and fibroblasts seem to contribute to actual skin regeneration.[19] Our skin flap substitute, on the other hand, could be transplanted to an ischemic graft bed, even onto foreign objects, if the flap vessels anastomose to the recipient's own vessels.

CONCLUSIONS

We have succeeded in making an ex vivo perfusion cultured regenerative skin flap. This tissue might be suitable for subsequent transplantation and tissue model for testing the efficacy of new drugs.

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