



A new dynamic culture device suitable for rat skin culture

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Abstract

Cultured skin has been used extensively for testing therapeutic drugs because it replicates the physical and biochemical properties of whole skin. However, traditional static culture cannot fully maintain cell viability and skin morphology because of the limitations involved with nutrient transmission. Here, we develop a new dynamic perfusion platform for skin culture and compare it with a static culture device. Rat skins were cultured in either static or dynamic condition for 0, 3, 6, 9 and 12 days. H&E, periodic acid–Schiff (PAS) and picosirius red (PSR) staining were used for skin morphology detection, immunostaining against cytokeratin 10 (CK10) for differentiation detection, immunostaining against proliferating cell nuclear antigen (PCNA) for cell proliferation detection and TUNEL staining for apoptosis detection. After culturing for 12 days, the epidermis, basement membrane, hair follicles and connective tissue were disrupted in the static group, whereas these features were preserved in the dynamic group. Moreover, compared to the static group, proliferation in the epidermis and hair follicles was significantly improved and apoptosis in dermis was significantly decreased in the dynamic group. These findings suggest that our device is effective for extending the culture period of rat skin to maintain its characteristics and viability *in vitro*.

Keywords Skin culture · Dynamic perfusion platform · Histological analysis · Proliferation · Apoptosis

Introduction

The skin organ model provides a useful platform for new drug development, toxicology experiments and physiological function research (Yanjia and Jianli 2009). At present, there are two methods of *in vitro* skin organ culture: organ culture and organotypic culture. However, none of the existing organotypic skin contains an immune system, blood vessels, or skin appendices or other functional and pathophysiologically relevant elements (Metcalf and Ferguson 2007). Further bioengineering is necessary for the implementation of adipose tissue, hair follicles and a functional vascular network (Black et al. 1999; Festa et al.

2011; Montan et al. 2010; Lindner et al. 2011). Consequently, until this technology is fully developed, native skin culture remains an attractive option.

In addition to structural characteristics of normal skin, the newborn rat skin contains only rudimentary follicles, less hair and is in the first phases of hair follicle development, which really represents follicular neogenesis (Wilson et al. 1994). It has been proved to be a useful model for the study of hair follicle elongation by means of skin organ culture (Kamiya et al. 1998) but how the skin viability and structure can be maintained in the *ex vivo* skin model remains unclear. Traditional static culture systems, including multiwell plates and Petri dishes, have frequently been used in cell culture laboratories because of advantages such as processing simplicity, low cost, disposability and easy sterilization (Ratcliffe and Niklason 2002). However, these systems suffer from serious limitations in nutrient transmission because of the lack of shear stress, which reduces the skin explants lifetime *in vitro*. Thus, experimental time has been limited in previous assays reporting the organ culture of full-thickness foetal or mature mouse skin *in vitro* under traditional static culture conditions (Hanley et al. 1997; Kamiya et al. 1998; Li et al. 1992; Paus 1991; Paus et al. 1994). To overcome this drawback, multiple dynamic bioreactors, including stirred spinner flasks (Vunjak-Novakovic et al. 1996; Neves et al.

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2005), rotating-wall vessels (Diederichs et al. 2009; Nishi et al. 2013), hollow-fibre bioreactors (Schmelzer and Gerlach 2016) and perfusion systems (Jaasma et al. 2008) have been designed. Dynamic bioreactors ensure a proper nutrient supply/waste removal, support cell growing or tissue maturation and induce controlled biomechanical stimuli, mainly shear stress, to the cells. In particular, flow perfusion bioreactors mimic the physiological elements of the *in vivo* environment and effectively increase the diffusion of nutrients and are now widely used in laboratories (Piola et al. 2013). However, current perfusion systems have their own limitations. The devices are too small to facilitate disassembly and cleaning and the separate tissue culture compartments (Atac et al. 2013; Piola et al. 2013) decrease the area of the air–water interface, reducing the dissolution rate of oxygen into the liquid. Again, it is difficult to provide a flat culture chamber to maintain the air–liquid interface. Moreover, most of these systems are used to develop skin equivalents but are not employed for long-term skin culture. Therefore, to prolong the survival time of *ex vivo* skin and construct a stable skin model for investigating the physiology and pathology of full-thickness skin and its appendages, a new perfusion device is needed that facilitates assembly, preserves sterility, provides sufficient oxygen and nutrient supply and can expose the cultured skin to mechanical stimuli.

In the present study, we develop a simple closed perfusion system with relatively large dimensions to overcome the limitations of static culture and function as a long-term dynamic device to support *ex vivo* skin culture. Furthermore, we compare perfusion culture to traditional *in vitro* assays in relation to the maintenance of rat skin to prolong the survival time of *ex vivo* skin.

Materials and methods

Animals

One-day-old SD rats ($n = 4$) were purchased from the Experimental Animal Centre, Institute of Surgery Research, Daping Hospital, Army Medical University (Chongqing, China). All animal experiments were approved by the Ethic Committee of Daping Hospital and conducted according to guidelines of the Experimental Animal Care and Use Committee at Army Medical University.

Design and assembly of bioreactor

The QSPFFD has already been granted by the US Patent; it consists of a glass box, a reservoir and a peristaltic pump connected with two silicone tubes (Fig. 1a, b). Inside the glass box, one tray (Fig. 1c (mark 4)) was placed; above this tray was a modified 24-well plate (converted from a commercially

available polystyrene culture plate) that was used to culture the rat skin (Fig. 1c (mark 2)). The medium was pumped from the reservoir into a static pressure tank (Fig. 1c (mark 1)), then to the culture area and finally over an overflow dam into a drainage trench (Fig. 1c (mark 3), d). The tray was mounted by a support bracket in the box and the height of the overflow dam and culture well plate controlled the liquid height in the tray to achieve a gas–liquid interface. The sub-millimetre microspores at the bottom of each well of the culture plate ensured medium permeability for nutrient delivery (Fig. 1c, d (mark 2)). Cultures were then fed by periodic perfusion through the peristaltic pump, which was controlled digitally every 6 h to run for 1 h. The perfusion rate of 80 ml/min was determined on the basis of CFD simulation analysis. The entire perfusion system was placed in an incubator at 37 °C and 5% CO₂ in air; 0.2- μ m pore size air filters were installed on the glass box and the open area of the reservoir (Fig. 1b (mark 5)).

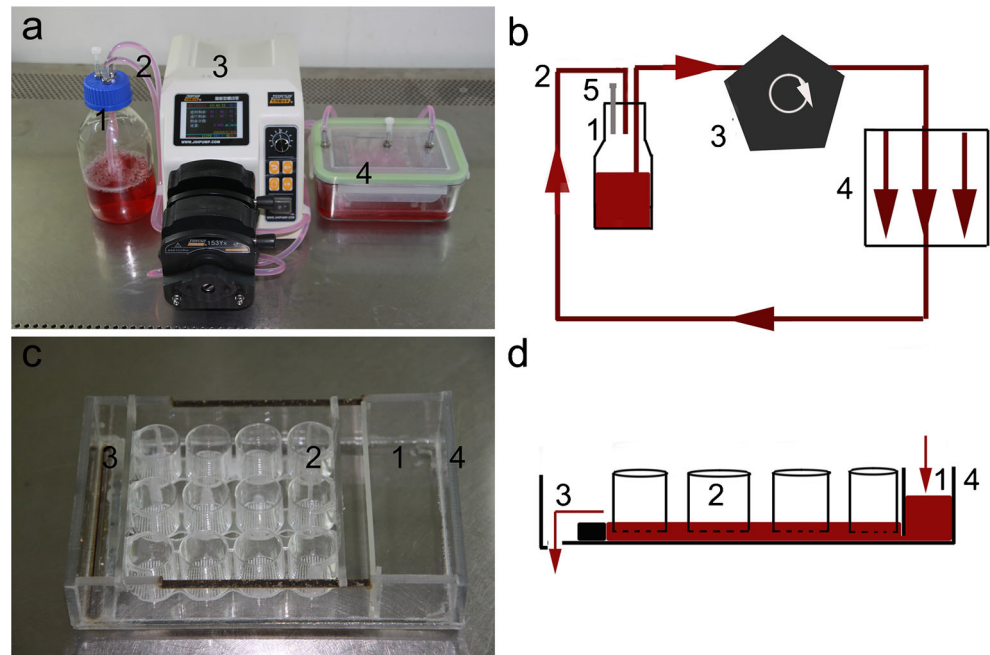
Tissue sources and culture experiments

Approximately 2.5×2 cm² dorsal skin flaps of rats were collected with scissors. Skins were sterilized in 75% ethanol for 30 s and subsequently punched to form 4-mm biopsies with a punch biopsy device (LAOA, Taiwan, China). The skin biopsies were then washed several times with sterilizing solution (PBS containing 100 units/ml penicillin and 100 μ g/ml streptomycin). The resultant skin samples were positioned in the modified 24-well tissue culture plate with the epidermis facing up and transferred to the perfusion and static systems separately. The skin biopsies were supplemented for 12 days in high-glucose (4.5 g/l) Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) with 10% foetal bovine serum (FBS, Gibco, San Diego, CA, USA) and cultured at 37 °C in 5% CO₂–95% humidified air. The total volume of 500-ml medium was not changed during the 12 days of culture for both dynamic and static cultures at the air–liquid interface. The peristaltic pump (JIHPUMP, Chongqing, China) provided a pulsatile flow of medium through a 5-mm-diameter silicone tube with a pumping rate of 80 ml/min. One skin sample was fixed immediately in 4% paraformaldehyde and embedded in paraffin and the rest were mounted in the same way on days 3, 6, 9 and 12 in the case of the time-course experiments for each of the three samples.

H&E staining

Skin samples from individual rat ($n = 4$) were fixed in 4% paraformaldehyde overnight, then dehydrated, embedded in paraffin and cut into 5- μ m-thick sections using a microtome (Leica, Wetzlar, Germany). Five sections for each sample were randomly selected and stained with standard protocols of H&E. Images for all stained sections were taken using an

Fig. 1 Quasi-static planar flow field device (QSPFFD) (Patent No. US 9902929B2) and device schematic. **a** QSPFFD and related **b** schematic diagram, indicating (1) reservoir, (2) silicone tubes, (3) peristaltic pump, (4) glass box and (5) air filter. **c** Components inside the glass box and related **d** schematic diagram: (1) static pressure tank, (2) modified 24-well tissue culture plate, (3) drainage trench and (4) tray. Red arrows show the direction of medium flow



IX71 microscope (Olympus, Tokyo, Japan). Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) was used to quantify the number of total cells, hair follicles and epidermal thickness in five independent photographs or at five random points in the skin tissue images from the same skin section ($\times 400$ magnification).

PAS staining

The paraffin-embedded tissue sections were deparaffinized, rehydrated through an ethanol series, placed in periodate solution (Solarbio, Beijing, China) for 5 min, treated with Schiff's reagent (Solarbio, Beijing, China) at room temperature for 15 min for colour development, bleached in sulphurous acid (Solarbio, Beijing, China) to remove any non-specific stain and rinsed with distilled water for 1 min.

PSR staining

Sections for picosirius red (PSR) stain were dewaxed in xylenes and rehydrated through graded alcohols, treated with Wiegert's haematoxylin (Solarbio, Beijing, China) for 8 min, washed in tap water and incubated in picosirius red solution (Solarbio, Beijing, China) for 45 min, dehydrated through graded alcohol solutions and xylenes and mounted. Sections were then observed under a light microscopy; the collagen fibres appeared as red on a pale yellow background.

Immunostaining

Skin tissue samples taken from four different rats were simultaneously harvested for immunostaining. For each sample,

five tissue sections with 5- μm thickness were randomly selected from the middle part. The sections were deparaffinized, rehydrated in distilled water, then immersed in citrate buffer solution (Beyotime, Jiangsu, China) at pH 6.0 for 10 min at 95 °C and allowed to cool down for 20 min at room temperature to reactivate masked epitopes. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min; samples were blocked with 5% BSA for 30 min at room temperature. Sections were incubated with rabbit anti-PCNA (1:800; Santa Cruz Biotechnology, CA, USA) and rabbit anti-CK10 (1:500; Abcam, USA) at 4 °C overnight. After washing in PBS, the sections were incubated with horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG (1:50; Beyotime, Jiangsu, China) for 1 h at 37 °C. DAB (Beyotime, Jiangsu, China) dye was used for colour development, followed by haematoxylin counterstaining, dehydration and application of a cover slip. The results of CK10 were analysed qualitatively; the micrographs of PCNA expression were observed by fluorescence microscopy at $\times 400$ magnification. The percentages of proliferating cells were calculated by counting the numbers of positive nuclei and total nuclei in five individual fields per section using Image-Pro Plus 6.0.

TUNEL staining

TUNEL staining of paraffin-embedded cultured skin tissue taken from four different rats was detected using an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. For each sample, five tissue sections (5- μm thickness) were randomly selected. The sections were dewaxed and rehydrated, treated for 30 min at 37 °C with 20 $\mu\text{g}/\text{ml}$ of proteinase K in PBS, then labelled by

incubation with TUNEL reaction mixture for 60 min at 37 °C in a dark environment. Finally, the sections were further counterstained with DAPI (Beyotime) and mounted on glass slides with antifade mounting medium (Beyotime). The percentage of apoptotic cells was calculated as described above to quantify the fraction of PCNA-positive cells.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software. Descriptive data presented here are the average of four independent experiments and expressed as mean \pm standard deviation (SD). Two-way ANOVA followed by Bonferroni's multiple comparison test was used. p value < 0.05 was considered statistically significant.

Results

Skin morphology evaluation

To explore rat skin structure grown under dynamic culture conditions versus static culture conditions, we first analysed the morphological changes using histochemical staining. In the dynamic culture group after 12 days of culture compared with both day 0 and the static, the skin and hair follicles were still maintained, the overall structure of skin displayed no damage and the connective tissue was also complete (Figs. 2a'–e', 3c, h). In addition, there were more viable cells in which nuclei were located closer to each other in the skin (Fig. 2e'). We observed epidermal disruption, dermal reorganization and a decrease in cell number at day 9 in the static cultures (Fig. 2c). Moreover, our data indicated that the epidermal thickness of rat skin at 12 days in the dynamic culture ($38.23 \pm 8.41 \mu\text{m}$) was significantly higher ($p < 0.001$) than in static culture ($14.28 \pm 5.29 \mu\text{m}$) although lower compared with day 0 ($48.05 \pm 6.88 \mu\text{m}$, $p < 0.01$) (Fig. 2f). The difference in total cell number between the dynamic and static groups was highly significant (862.65 ± 80.85 vs. 171.7 ± 48.21 ; $p < 0.001$) and the cell number in the skin grown in dynamic culture was slightly lower than the time 0 value (929.5 ± 117.59) (Fig. 2g). Also, a significant drop of the number of hair follicles in the rat skin with static culture was seen at day 12 compared with dynamic culture (2 ± 1.34 vs. 6.9 ± 1.21 , $p < 0.001$); it is noteworthy that no significant difference was found in the follicle number at 0 and 12 days under dynamic culture (Fig. 2h).

Basement membranes in the skin are particularly important in organization structure, stability and differentiation during development. Therefore, we evaluated the integrity of the basement membrane of the epidermis at 12 days by periodic acid–Schiff (PAS) staining (Akarca et al. 2012) and found that it was as intact as on day 0 under dynamic culture (Fig. 3a–d).

By contrast, the static culture group displayed a loss of epithelial structural integrity and the continuous lining of the basement membrane was destroyed, as shown in Fig. 3(e, f). The expression of collagen fibres by PSR staining was analysed qualitatively only on the 12th day of follow-up. Our results indicated that at 12 days, the density of collagen fibres was clearly increased in the dynamic culture and seemed better organized and distributed compared to static cultures (Fig. 3g–i).

We finally examined the expression of CK10 in epidermis as determined by immunohistochemical staining. Here, CK10 staining showed a disintegrated and weak staining in the static culture compared to day 0 and dynamic culture (Fig. 4), whereas those in dynamic culture increased in their staining intensity after 12 days of in vitro culture.

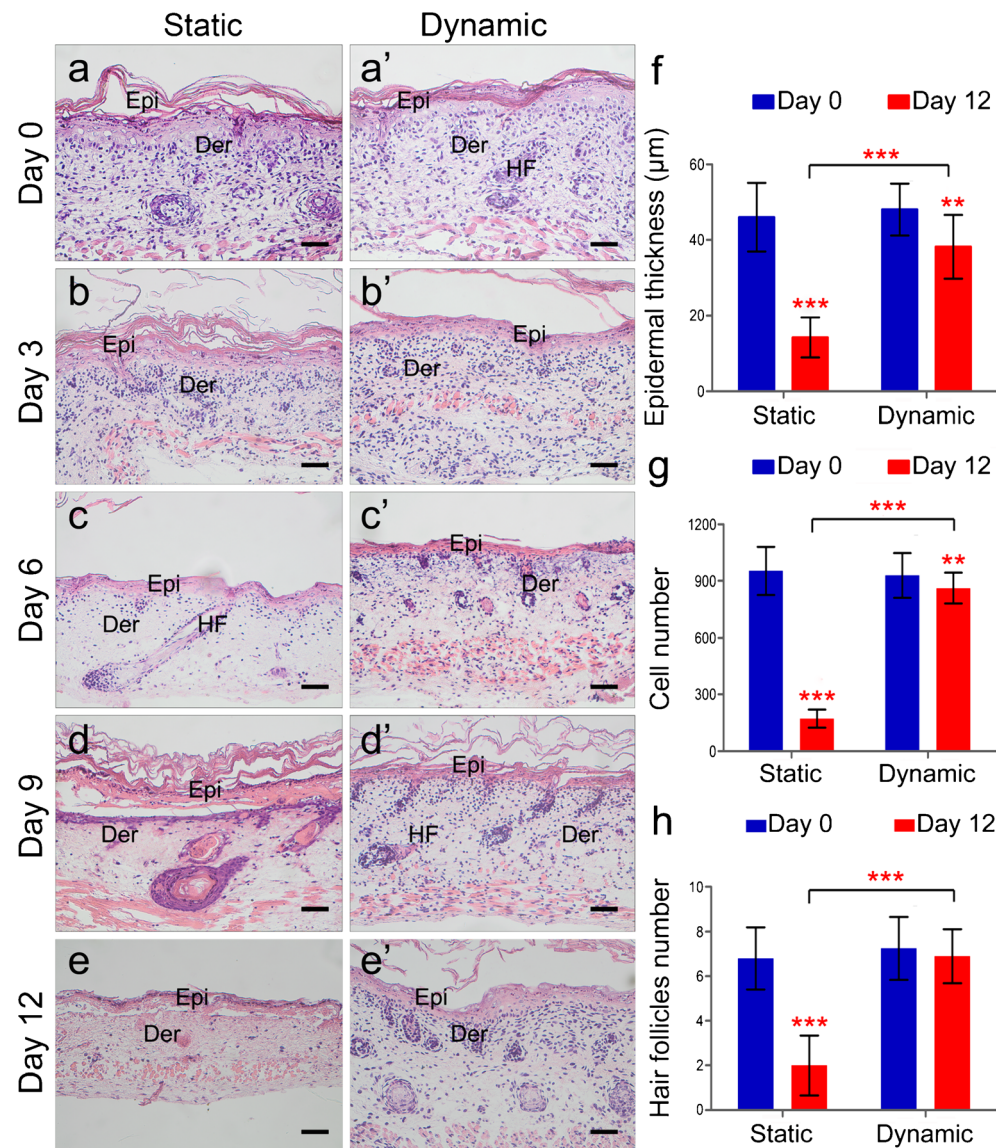
Cell proliferation during ex vivo skin culture

Proliferating cell nuclear antigen (PCNA) is a proliferation marker. Immunostaining against PCNA was used to detect cell proliferation in the two groups. As shown in Fig. 5, PCNA-positive cells were yellow-brown and positively stained cells were primarily found in the basal cell layer of the epidermis and the hair follicles (Fig. 5a–c, a'–e'). At 12 days, PCNA-positive cells were clearly observed in the dynamic culture group, whereas the PCNA expression level peaked in the static group at 6 days; thereafter, cell proliferative activity largely declined by day 9 (Fig. 5c–e'). The proliferating cells were quantified by counting the number of PCNA-positive cells and expressing that number as the percentage of PCNA-positive cells/total number of cells. The number of proliferative cells in the two groups declined sharply within 3 days; after 3 days of tissue culture, a relatively stable percentage of proliferating cells was detected in the dynamic group (from 4.03 ± 0.37 to $4.82 \pm 0.25\%$) (Fig. 5f). By contrast, the cell proliferation rates in the static group peaked at day 6 ($9.51 \pm 0.78\%$), then decreased gradually by day 12 ($0.51 \pm 0.26\%$) (Fig. 5f). A significant ($p < 0.05$) decrease in the static group was found compared to the dynamic group. In this study, PCNA-positive cells were mainly localized in the hair follicle. We next compared the difference in the amount of proliferating cells between the groups; our results indicated that the percentage of proliferating cells in the dynamic group ($14.35 \pm 5.06\%$) was significantly ($p < 0.05$) higher than in the static group ($0.88 \pm 1.20\%$) at days 12 (Fig. 5g).

Cells apoptosis during ex vivo skin culture

The number of apoptotic cells was determined using the TUNEL assay, as depicted in Fig. 6. In both groups, we observed an increase in apoptotic levels after 3 days. It was clear that TUNEL-positive cells within the dermis were more abundant in the static culture compared to the dynamic group

Fig. 2 Histological comparison of H&E stain and quantitative measurement for in vitro rat skin. H&E-stained samples of skin were observed on days 0, 3, 6, 9 and 12 after culture (a–e, a'–e'). Epi epidermis, Der dermis, HF hair follicle. The epidermal thickness (f), the cell number (g) and the number of hair follicles in the skin (h) were analysed as indicated in the bar graph. $**p < 0.01$, $***p < 0.001$ (two-way ANOVA followed by Bonferroni's multiple comparison test). All data are reported as the mean \pm SD, $n = 4$. Scale bar 50 μm



(Fig. 6c–e, c'–e'), and progressive cell death occurred over time, as expected. Specifically, the percentage of TUNEL-positive cells increased progressively at days 3, 6 and 9 and reached a peak at day 12 and there was a significant difference in the dynamic culture compared to the static culture ($p < 0.001$) (Fig. 6f). A similar pattern was observed in both epidermis and hair follicles; the percentage of TUNEL-positive epidermal cells strongly increased by day 12 and the apoptosis rate ($65.70 \pm 11.27\%$) in the dynamic group was significantly lower ($p < 0.001$) than that in the static group ($84.71 \pm 8.73\%$) (Fig. 6f, g). Furthermore, the percentage of apoptotic cells detected in the hair follicles in the dynamic group increased by only 22% ($22.39 \pm 3.46\%$) by day 12, nearly equivalent to day 0 ($18.75 \pm 2.19\%$), compared to more than 70% ($76.03 \pm 4.21\%$) in the static culture, with a significant difference between the groups ($p < 0.001$) (Fig. 6h).

Discussion

We developed a novel closed culture system, referred to as the quasi-static planar flow field device (QSPFFD), which was used to culture tissue-engineered skin. We further confirmed that the device can be applied to the cultures of skin, which is beneficial for rat skin viability.

As an ex vivo skin organ culture system, the QSPFFD has several important advantages. First, a fully closed culture system reduces the risk of biological contamination and the simple structure makes the process of assembly and disassembly convenient, enhancing the efficacy of sterilization. Second, the liquid flow in the system can enhance mass transport and produce a shear force that ensures nutrient supply and waste removal. Kamiya et al. cultured mouse skin using 24-well plates to characterize the mechanisms of hair follicle elongation including the response to minoxidil (Kamiya et al. 1998).

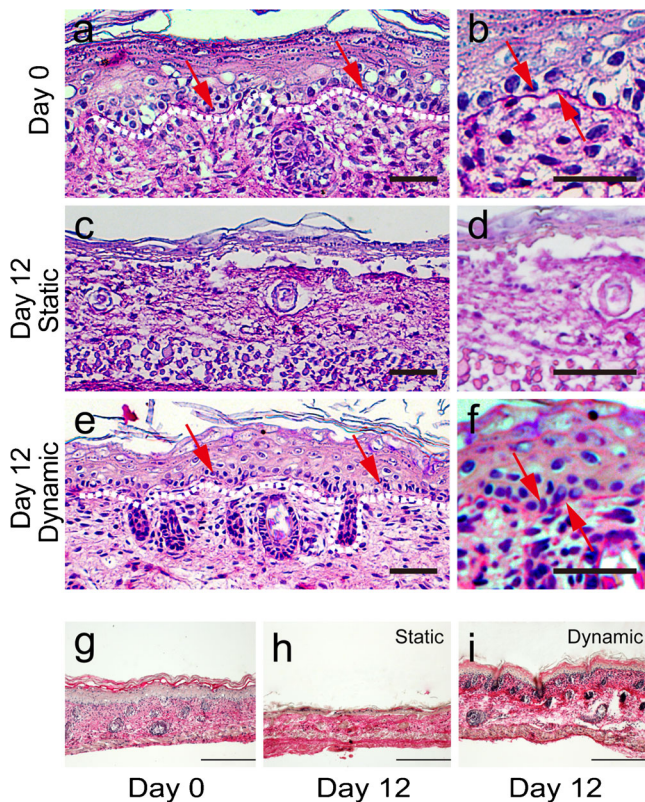


Fig. 3 Periodic acid-Schiff (PAS) and picosirius red (PSR) staining of rat skin. PAS staining of the static and dynamic groups for day 0 and day 12 samples (a–f). Red arrows point to the intact basement membranes. PSR staining of the static and dynamic groups for day 0 and day 12 sample (g–i). Scale bar 50 μm (a–f) and 200 μm (g–i)

However, their experiment was conducted for only 3 days, which is not suitable for discovering changes in the entire metabolic cycle of hair follicles. The thickness of the sample cultured in such static systems drastically hinders the mass transfer because it is more adequate to supply a 10- μm tissue thickness for passive diffusion in the static culture systems. Instead, provided that the tissues are thicker than 100–200 μm , the supply of oxygen and soluble nutrients could become extremely limited (Ratcliffe and Niklason 2002). Similar observations were also reported with cellular spheroids, whereby under static conditions, they generally construct a necrotic centre, surrounded by a rim of viable cells, when the size is larger than 1 mm in diameter (Sutherland et al. 1986). Compared to traditional *in vitro* assays, external mass transfer limitations can be reduced by our perfusion device, which produces low levels of shear stress to overcome diffusion limitations. Earlier studies of the QSPFFD through computational fluid dynamic (CFD) simulation analysis revealed that the velocity was 80 ml/min and the shear stress was 8 mPa; in this case, fibroblasts were significantly more viable than static controls. Third, the air–liquid interface was easily maintained in the QSPFFD by controlling the height of the overflow dam and culture well plate (Fig. 1d). It has long been known that

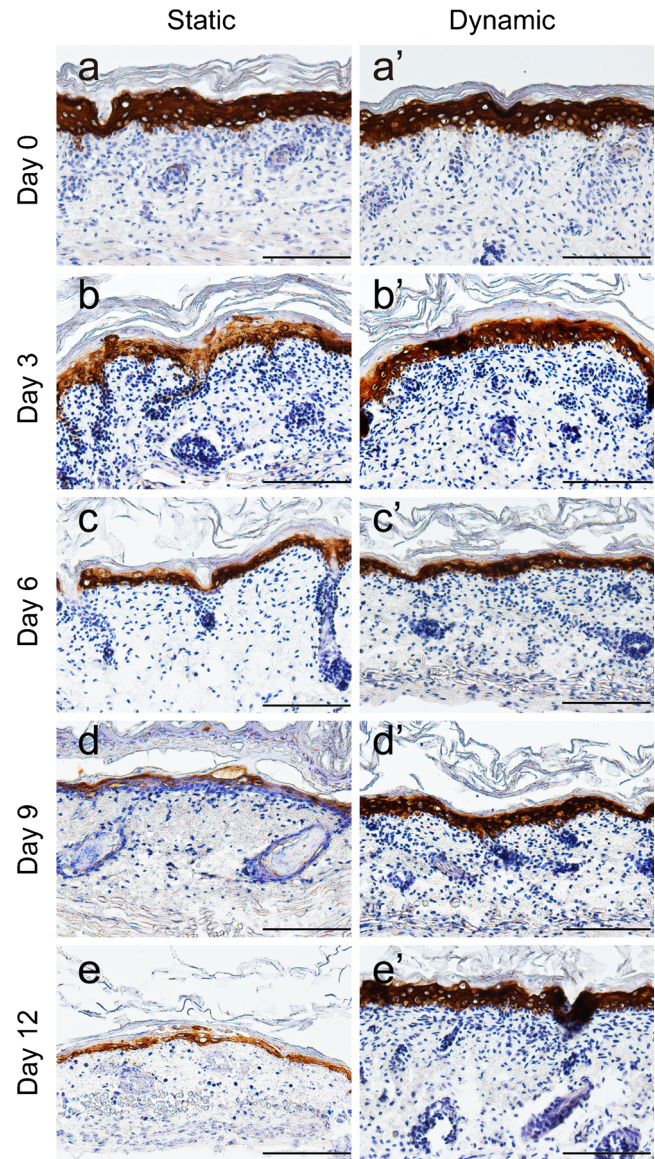
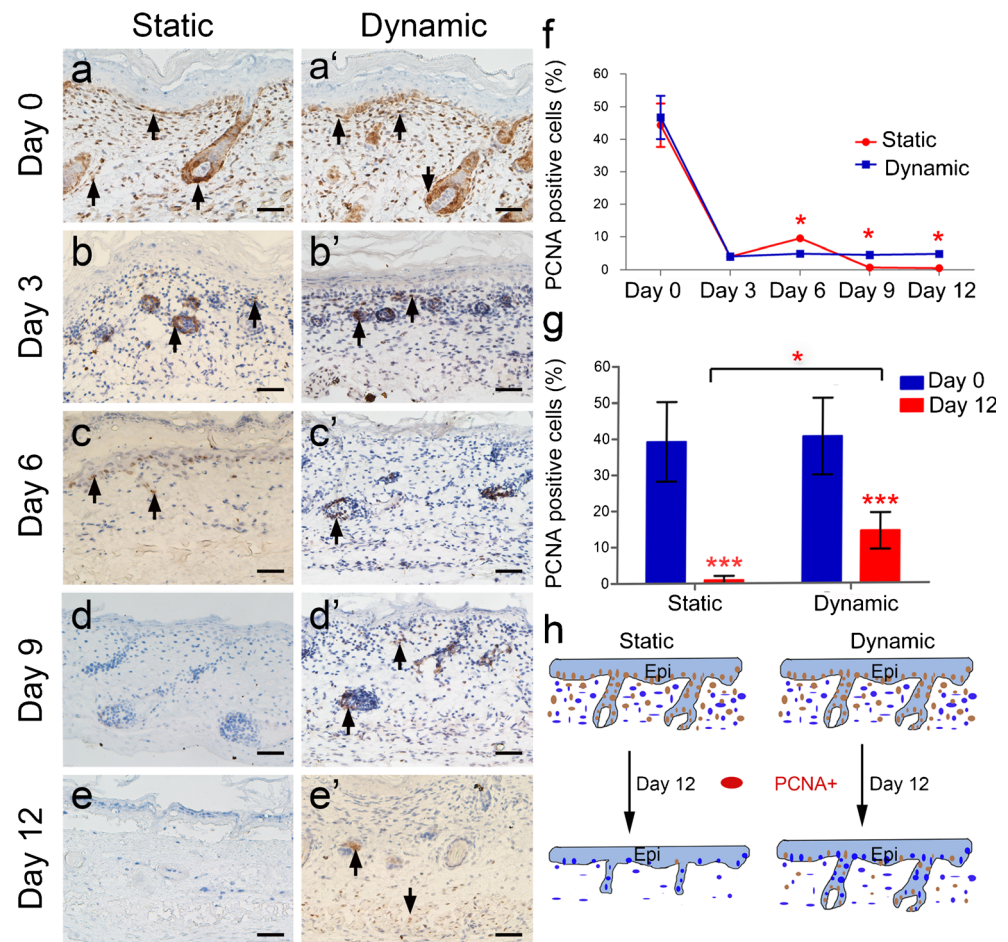


Fig. 4 Immunostaining against CK10 in cultured skin. Immunohistochemical detection of CK10 was applied for evaluating epidermal differentiation in the *ex vivo* skin (a–e, a'–e'). Scale bar 100 μm

engineered skin substitutes must be incubated at the air–liquid interface to enhance organization and differentiation of air-exposed stratified epithelium that is capable of providing the barrier function that normal skin achieves (Parenteau et al. 1992; Sun et al. 2006). Additionally, research has shown that when skin biopsies are submerged in the flow, the shear stress leads to premature degradation of the epidermis (Wagner et al. 2013). Atac et al. successfully developed a microfluidic chip system for native skin in which culture is incubated at the air–liquid interface and prolonged the maintenance of the native state of the tissue (Atac et al. 2013). Thus, a flat culture chamber is necessary for rat skin culture. Fourth, a uniform flow field is necessary to ensure a uniform supply of nutrients. The QSPFFD employed a

Fig. 5 Immunostaining against PCNA in cultured skin. PCNA assay for proliferation (a–e, a'–e') was used to evaluate the viability of the tissues. Black arrows point to the PCNA-positive cells. (f) The stages of the percentage of PCNA-positive cells in the dynamic group compared to the static group over a duration of 12 days. (g) Bar graph represents the percentage of PCNA-positive cells for hair follicles over the same period of time. (h) Schematic of the distribution of PCNA-positive cells. All data are reported as the mean \pm SD, $n = 4$. * $p < 0.05$, *** $p < 0.001$ (two-way ANOVA followed by Bonferroni's multiple comparison test). Scale bar 50 μ m



hydrostatic tank design to convert the fluid dynamic pressure of imports into static pressure; the values were between 31.0 and 30.6 Pa in the culture area from upstream to downstream, which indicates that the distribution of static pressure is uniform. In conclusion, these features indicate that the device is suitable for extended maintenance of full thickness skin.

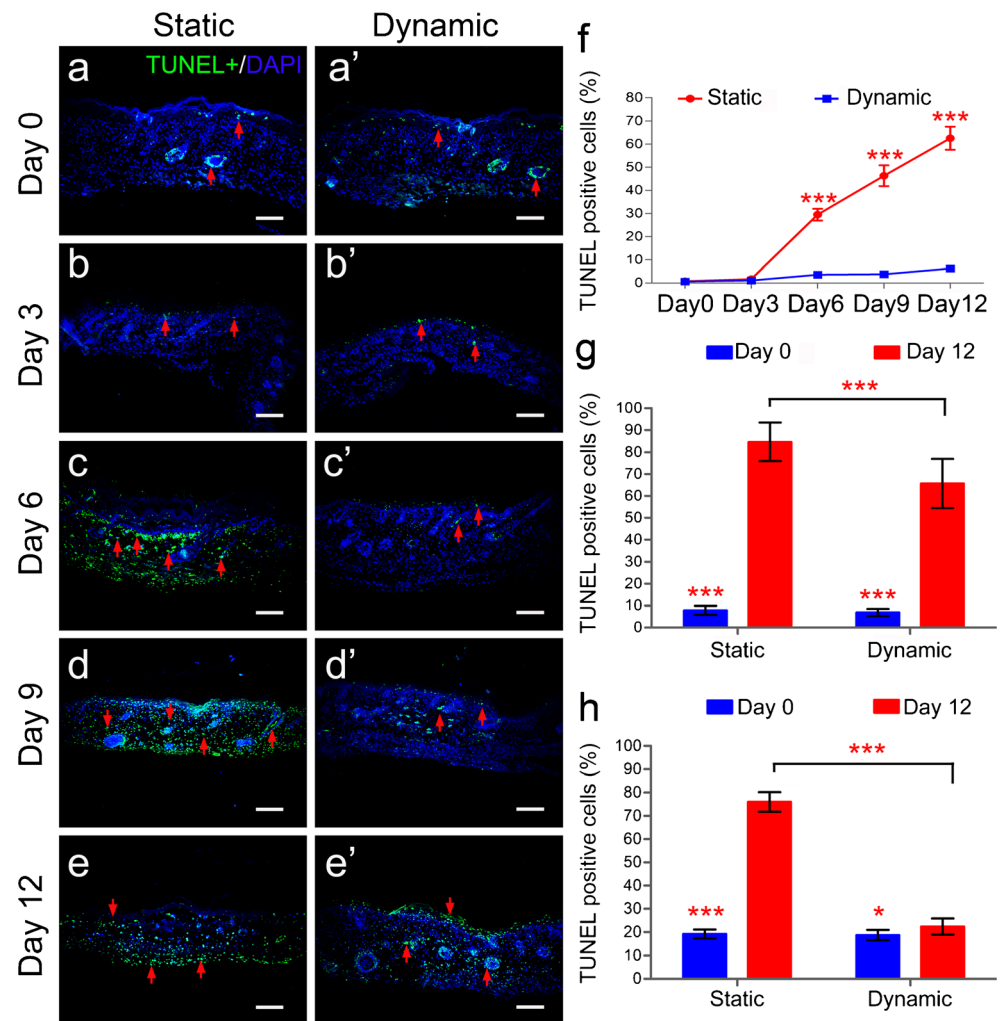
In this study, we evaluated rat skin maintenance in dynamic culture compared to static culture conditions. We showed that the epidermis and dermis were intact and normal morphology of the skin was maintained after 12 days in dynamic culture. H&E staining indicated that viable cells were retained in the skin tissue. PAS and PSR staining further indicated the preservation of epidermal and dermal structural integrity after in vitro culture (Figs. 2a'–e', 3c, h). CK10 was used as the marker of epidermal differentiation and the expression levels in the dynamic group showed that the ex vivo skin still possessed toughness and rigidity (Fig. 4a'–e').

In addition, the residing cells in the ex vivo skin stained positive for proliferation (Fig. 5e'), indicating that the tissue was viable and that the cells maintained their ability to proliferate. The experimental results revealed a distinct difference

between the static and perfusion cultures and confirmed that the dynamic device generally preserved the structure of the skin (Figs. 2, 3 and 4), maintained cell proliferation (Fig. 5), delayed cell apoptosis (Fig. 6) and prevented tissue disintegration. We were able to prolong the culture period of in vitro skin in the QSPFFD.

The ability to grow and preserve any tissue is critically dependent on a supply of nutrients (Sun et al. 2006). Our results suggest that the increase in the supply of nutrients caused by mechanical forces in the dynamic device is important for biological behaviour of cells and extracellular matrix components. Mechanical stress not only induces fibroblast proliferation but also promotes collagen production (Ladd et al. 2009). In the perfusion group, the cells within the skin tissue maintained their ability to proliferate in the epidermal and dermal regions at day 12 (Fig. 5e'), and bright red collagen fibres increased and were similarly distributed to the day 0 tissues as demonstrated by PSR staining (Fig. 3g, h), indicating that a mechanical force generated by the dynamic culture is transmitted throughout the dermis and transduced to the fibroblasts via the components of the extracellular matrix (Ladd et al. 2009). This study provided evidence that shear stress plays a critical role in prolonging the longevity of the rat skin.

Fig. 6 TUNEL staining in cultured skin. TUNEL (green)/DAPI (blue) staining of representative sections (a–e, a'–e') for apoptotic cells at different time points of skin cultures. Red arrows point to the apoptotic cells. (f) Percentage of TUNEL-positive cells for different durations. The bar graph represents the percentage of TUNEL-positive cells in the epidermis (g) and hair follicles (h). All data are reported as the mean \pm SD, $n = 4$. * $p < 0.05$, *** $p < 0.001$ (two-way ANOVA followed by Bonferroni's multiple comparison test). Scale bar 100 μ m



It is essential to maintain the structural integrity of the tissue for developing research on normal and diseased skin under similar conditions to those in the body. In this study, the tissue structures remained intact with minimal disruption after in vitro culture. Additionally, our results demonstrated that the ex vivo rat skin organ culture was viable for at least 12 days. Although the cell number and epidermal thickness of rat skin decreased, which may be due to the long culture time, this effect may be avoided by optimizing the duration of perfusion. The perfusion of medium affects local secreted factors and shear stress acting on the cells. The perfusion system should be designed according to cell viability requirements (Korin et al. 2009). In this study, the protocols that we applied were a short perfusion time followed by a long period of static incubation. In this research, the rat skin retained important elements of in vivo skin, such as hair follicles and an intact stratum corneum, which provide the pathway of skin permeation (El Maghraby et al. 2008). Thus, the ex vivo skin can be used as a model for transdermal delivery in topical penetration studies (Sanchez et al. 2010).

In summary, we successfully developed and tested a new perfusion device in which mechanical force is generated to

control the native state of in vitro rat skin. Despite only some preliminary data, it has been proved that we are on the right track for prolonging free skin survival in vitro and the research will provide the basis for identifying culture parameters required for the human skin. In the future, the pragmatic device even allows studying and manipulating the complex co-culture of various tissues of human origin. The challenges may be significant.

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Compliance with ethical standards

All studies were approved by the Ethic Committee of Daping Hospital and conducted according to guidelines of the Experimental Animal Care and Use Committee at Army Medical University.

Conflict of interest The authors declare that they have no conflict of interest.

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