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Epidermal morphogenesis in an *in-vitro* model using a fibroblasts-embedded collagen scaffold

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Summary

A novel culture system included a self-designed bi-layer 3-D collagen scaffold with different pore size on both sides and specific culture media for different culture stages. This skin equivalent culture model provides a new investigating system to study the role of extracellular matrix and growth factors including epidermal growth factor (EGF), keratinocyte growth factor (KGF), transforming growth factor beta 1 (TGF-β1), in the cell–cell and cell–matrix interactions. Keratinocytes were seeded onto the dermal equivalent and incubated under submerged condition for 5 days then proceeding to air–liquid interface cultured either with or without EGF addition. In this study, EGF has a positive effect on the keratinocyte migration and proliferation in the submerged stage. However, when 10 ng per ml of EGF was continual added in the air-lifted stage, a less organized and thin differentiated keratinocyte layers were found. Continual 10 ng per ml of EGF addition in the air-lifted stage resulted in uneven cell–matrix interface, and disorganization of the suprabasal layers. On the contrary, in the air-lifted stage without excess EGF, the epithelium cells will stratify, differentiate, and form an epidermis completed with basal, spinous, granular, and cornified layers. The results showed that time scale modulation of EGF on keratinocyte cell behavior depend on the expression of paracrine or autocrine growth factors (e.g. KGF and TGF-β1).

Introduction

In tissue engineering, the first aim of cell biologists was to find how to stimulate cell growth using sophisticated culture conditions. The success of Rheinwald and Green with long-term sub-cultivation of keratinocytes was a brilliant example of the importance of this approach [1]. When the technology achieved to make long-term sub-cultivation of normal cells possible, biologists started to emphasize their efforts on the modulation of cell growth and differentiation by different culture media [2]. Later, cells cultured in monolayer and in 3-D conditions were investigated to elucidate how cells can undergo de-differentiation and re-differentiation in a monolayer culture or 3-D culture, respectively. All these differences can be attributed to cell–cell and cell–matrix interactions, which are manifested by the synthesis and secretion of different cytokines and specific growth factors.

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Simultaneous cultivating epidermal cells on dermal equivalents provide a good model for the study of dermal–epidermal interactions [3, 4]. Because of the time required to isolate autologous fibroblasts, to insert and grow the cells into a 3-D scaffold, an alternate means to provide appropriate cytokines, growth factors, and chemokines is being investigated. When epidermal cells seeded on dermal equivalent, it showed that fibroblasts were able to stimulate keratinocytes growth, either by reorganization of collagen matrix or the production of specific growth factors [5, 6]. This reflects that growth factors arising from fibroblasts play an important role on keratinocytes migration, proliferation, and differentiation. In 2000, Gibbs et al. reported that EGF had a positive effect on the keratinocyte migration and proliferation [7]. However, Yamasaki et al. reported that high concentrations of EGF enhanced TGF-β1 mRNA expression instead. As a potent inhibitor of keratinocyte proliferation, TGF-β1 contributes to epidermal growth factor-mediated keratinocyte growth inhibition [8]. Moreover, KGF producing by fibroblasts is a paracrine mediator of epithelial cell growth [6]. In this study, we try to prepare skin equivalents *in vitro* by seeding human keratinocytes on the dermal equivalent and raised them up to the air–liquid interface to examine the effects of these growth factors on the morphogenesis of the epidermis.

A novel skin equivalent culture system will be developed in the study to mimic the *in vivo* cell–cell interaction and eventually to form a tissue-engineered skin or skin equivalent. This culture system included a self-designed bi-layer 3-D collagen scaffold with different pore size on both sides and specific culture media added at different culture periods. The lower layer is a loose layer with larger pore size for fibroblasts growth. The upper layer is a dense layer with smaller pore size and provides smoother surface for keratinocytes retention. In the submerge stage, EGF will be added into the system through the culture medium to stimulate keratinocyte migration and proliferation. In the air-lifted stage, the continual EGF addition would derail keratinocyte toward undesired differentiation pathway, which played a negative role to skin equivalent cultivation. Cell morphology, cell population, and the amount of extracellular matrix formation will be studied in the different culture stage. We suggest that the culture model of time-dependant EGF addition have a great potential in the physiological study and the cutaneous tissue engineering in the near future.

Methods

Type I collagen isolation

Porcine type I collagen was isolated as previously described [8]. Briefly, the muscle, fat and all surrounding connective tissue were removed from the harvested porcine Achilles tendon. The harvested tendon was minced, defatted (with acetone), and then cleaned with deionized water. The minced tendon was then treated with 10% sodium chloride at 4 °C for 24 h, further swollen by soaking in citrate buffer (pH 4.5) for 24 h. The treated tendon was digested with pepsin (Sigma, St. Louis, MO, USA, 1:10,000 U.T.I. from porcine stomach mucosa; 20:1 (w/w) in 0.5 M hydrochloric acid, pH = 2) at 4 °C for 24 h with constant magnetic stirring. The mixture was then centrifuged at 22,000 × *g* for 1 h at −4 °C. The precipitate can be re-digested with the pepsin until all the collagen extracted. The collagen was precipitated by adding 5% NaCl (w/v) aqua into the supernatant and followed by centrifugation at 22,000 × *g* for 1 h. The extracted collagen will be lyophilized −70 °C and stored at −20 °C for further application.

Analysis of collagen fractions by SDS-PAGE

One microgram of extracted collagen sample was dissolved in 200μl acetic acid (0.01 N) and was reduced with 10 μl 2% β-mercaptoethanol at 100 °C for 3 min. Standard type I collagen (purchased from Sigma, St. Louis, MO, USA) was treated with the same procedure and used for comparison with the extracted collagen. The electrophoresis separation of the collagen types was performed on 4–12% polyacrylamide containing 10% SDS, 40% glycerol and 0.02% bromophenol blue as described by Laemmli et al. [9]. After running at 85 V, fractionated proteins were stained with Coomassie Blue R-250 in methanol/acetic acid/water 5:2:5 (v/v/v) and destained in 15% methanol/7.5% acetic acid.

Preparation of bi-layer collagen sponge

Extracted collagen (25μg) was dissolved in 5 ml acetic acid (0.01 N) at the temperature of 4 °C. The solution (1 ml) was poured into a 9-cm Petri dish and quickly frozen at −196 °C. The collagen scaffold formed as the dense upper layer. Then, 2 ml more collagen solution was added to the top and frozen at −20 °C for 2 h followed by freeze-drying for 48 h. Through this process, a bi-layer collagen scaffold with different pore size on both sides was prepared. The bi-layer collagen scaffold was immersed in 0.01 N acetic acid solution containing 0.1% (w/w) glutaraldehyde at 4 °C for 24 h to improve the scaffold stability by intermolecular cross-linking. The cross-linked scaffold will be soaked in the solution of 0.1 M Glycine with ultrasonication to block residual aldehyde group. Then, the scaffold was rapidly frozen at −196 °C and freeze-dried for 48 h. The finally product was preserved at −20 °C for later experiments.

Sterilization

The scaffold was exposed to γ-ray irradiation for sterilization before used. Co-60 was the irradiation source with 900,000 Ci of emission energy. The scaffold was exposed to the irradiation until a total dose of 8 kGy.

Cell isolation and expansion

Primary culture of dermal fibroblasts and keratinocytes were isolated from human foreskin. The foreskin was cut into pieces with the dimension of 1.0 × 1.0 cm [2]. The small pieces of foreskin were soaked into keratinocyte serum-free medium (K-SFM Gibco, Grand Island, NY, USA) supplemented with 36 U per ml Thermolysin and 1% penicillin/streptomycin at 4 °C temperature overnight. Dermal and epidermal layer of the soaked foreskin was separated out by plastic surgery forceps. Dermis was then soaked in a 0.2% collagenase solution for dermal fibroblasts isolation. Epidermis was treated with trypsin (2.5 mg per ml in PBS) (Sigma) with intermittently shaking at 37 °C for 10 min. After centrifuged at 500 × *g* for 5 min, cell pellet was washed in PBS and re-suspended in keratinocyte serum free medium with supplements.

Dermal fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units per ml penicillin/streptomycin and 2.5μg per ml fungizone. Incubation was performed at 37 °C in 5% CO_2. The keratinocytes were cultured in keratinocyte serum free medium (K-SFM Gibco) containing with 10 ng per ml recombinant human EGF, 5 μg per ml insulin, 0.58 mg per ml L-glutamine, 5 μg per ml transferring, 100 IU per ml penicillin, 100 μg per ml streptomycin, and 2.5 μg per ml fungizone as keratinocyte growth medium (KGM). The keratinocytes were cultured on Petri dish with a density of 3×10^3 cells per cm [2] at 37 °C in 5% CO_2 for expansion.

Skin equivalent culture system

The skin equivalent culture system could be divided into two parts (dermal equivalent culture and epidermal equivalent culture with organotypic model) and contain three stages that schemed as Figure 1 and briefly described as follows.

In the first culture stage, dermal fibroblasts were seeded in collagen scaffold (5×10^3 cells per cm^2) by spinner flask and cultured in DMEM medium for 2 days to form a dermal equivalent for later organotypic culture. In the second culture stage, second-passage keratinocytes were seeded on the surface of the dermal equivalent with a cell density of 1×10^4 cells per cm^2 to form the 'bi-layer membrane for skin equivalent cultivation' (BMSE) and kept in steady state for 40 min to allow cell adhesion. Keratinocyte growth medium (KGM, with 10 ng per ml EGF) was then carefully added to the Petri dish until the BMSE submerged in the medium and cultured for 5 days to construct the basement membrane. In the third stage, the BMSE was lifted to air–liquid interface and cultured for 1–2 weeks. To elucidate the role of EGF in epithelialization, the keratinocyte growth medium in the third stage would be used with 10 ng per ml EGF addition or without EGF addition.

MTT assay

To elucidate the cell viability during three culture stage, cells were isolated as described above from the 2nd day in the first stage, the 5th day in the second stage, the 1st and 2nd week in the third

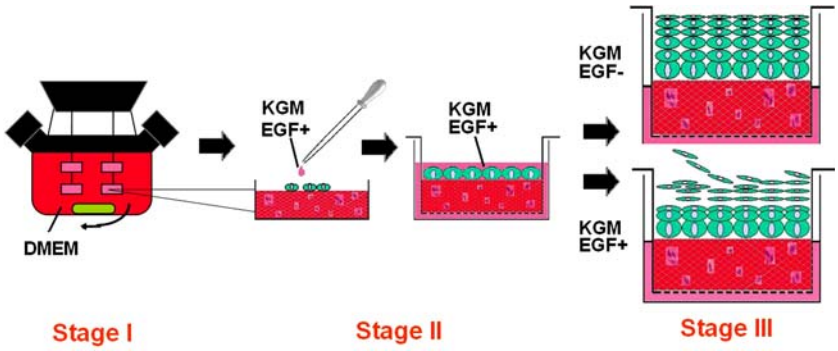

Figure 1. The flowchart of culture model scheme. Stage I: Seeding fibroblasts into the scaffold via spinner flask and then cultured for 2 days to form a dermal equivalent. Stage II: Keratinocytes were seeded on the surface of the dermal equivalent with a cell density of 1×10^4 cells per cm^2 to form the 'bi-layer membrane for skin equivalent cultivation' (BMSE) and kept in steady state for 40 min to allow cell adhesion. Keratinocyte Growth Medium (KGM, with 10 ng per ml EGF) was then carefully added to the Petri dish until the BMSE became submerged in the medium and cultured for 5 days to construct the basement membrane. Stage III: The BMSE was lifted to air–liquid interface cultural condition for 1–2 weeks. To study the role of EGF on epithelialization, the keratinocyte growth medium would be used either with 10 ng per ml EGF or without EGF in the third culture stage to reconstruct the skin equivalent.

stage, respectively. Proceeding to incubate the cells with 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Sigma) during the last 3 h at 37 °C of the incubation period. The dark blue crystals were lysed with Acid SDS (100 µl 0.01 N HCl in 10% SDS) and the absorbance at 570 nm was measured.

Histological study and scanning electron microscopy (SEM)

For light microscopic examination, the cultured skin equivalent were carefully taken out from the Petri dish and immediately fixed in 2% (v/v) glutaraldehyde solution (0.1 M, pH 7.4) for 24 h at 4 °C. Following dehydrated in graded alcohols, the specimens were embedded in paraffin, sectioned, stained with hematoxylin and eosin (HE), and then observed under optical microscope. The structure and morphology of porous collagen scaffolds and cell growth morphology on scaffolds were examined by using scanning electron microscopy (SEM).The sample was dried by critical point dehydration and then coated with gold for scanning electron microscope (JXA-840; Jeol, Tokyo, Japan) examination.

Immunohistochemistry

To detect the presence of P63, keratin 10, and involucrin the skin equivalents were fixed in 4% paraformaldehyde in PBS for 4 h at 4 °C, followed by treatment with 0.1 M ice-cold glycine for 1 h and overnight incubation in 0.6 M sucrose solution at 4 °C. Tissues were embedded in OCT and placed in dry ice. For immunohistochemistry, cryostat sections (8 µm) were washed with PBS. The slides were incubated with blocking solution (10% horse serum in PBS) for 1 h at room temperature. Sections were then incubated with 50 µl of mouse monoclonal antibodies, anti-p63 (1:200) and anti-K10 (1:400 dilution in blocking solution; 30 min at room temperature; Chemicon International, Temecula, CA), anti-involucrine antibodies (1:400 dilution in blocking solution; 1 h at 37 °C; Sigma). Slides were then washed five times with PBS and incubated with 50 µl horse antimouse biotinylated antibody (1:200 dilution in blocking solution) for 30 min at room temperature. The slides were then incubated with avidin-FITC for 30 min and washed five times with PBS then sealed with coverslip for immunofluorescence examination. Images were captured using a CCD camera attached to the microscope and image analysis was performed using Quips Smart Capture FISH Imaging software (Vysis).

Real-time PCR analysis

Fibroblasts were first seeded into collagen scaffold. Next, keratinocytes were plated onto the top of collagen scaffold and cultured in KGM contained

in 10 ng per ml EGF for 5 days. The organotypic cultures were raised to the air–liquid interface then cultured in KGM for 1–2 weeks in the absence or presence of 10 ng per ml EGF.

Skin equivalents were washed with cold PBS and newly formed epithelia were mechanically detached from fibroblast-containing collagen matrix. Total RNA was extracted by using RNeasy Mini Kit (Qiagen, Santa Clarita, CA, USA) following the manufacturer's instructions. Concentration and purity of RNA were determined at the optical density of 260 and 280 nm, respectively. The 260/280 ratios were kept in the 1.8–2.0. cDNA was synthesized by reverse-transcription of 2 μg total RNA with Omniscript RT kit (Qiagen), using oligo(dT) primer and random hexamers (Promega, Madison, WI, USA). Real-time PCR was performed on the ABI Prism 7000 sequence detection system (Applied Biosystems, Inc., Foster City, CA, USA). The KGF primers, 5′-CTG GCC TTG TCA CGA CCT GTT TCT-3′(forward) and 5′-CCC TTT CAC TTT GCC TCG TTT GTC-3′(reverse); TGF-β1 primers, 5′-CGA GCC TGA GGC CGA CTA C-3′(forward) and 5′-AGA TTT CGT TGT GGG TTT CCA-3′(reverse); GAPDH primers, 5′-CCA GGA AAT GAG CTT GAG AAA GT-3′(forward) and 5′-CCC ACT CCT CCA CCT TTG AC-3′(reverse). Quantitative real-time PCR was normalized to the copies of GAPDH mRNA from the same sample. Data were analyzed by Sequence Detector software (Applied Biosystems, Inc.). All PCR assays were performed in triplicate.

Results

The isolated type I collagen from porcine Achilles tendon was identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The α band (95 kDa) and β band (190 kDa) of isolated collagen were identical to the SDS-PAGE patterns of commercialized type I collagen (SD) (Figure 2). It means that the isolated collagen is essentially free from other proteins.

The longitudinal cross section of bi-layer type I collagen scaffold was shown in Figure 3a. The pore size in the scaffold was uniformly distributed and incrementally changed from lower part to upper part in the whole observed area (Figure 3a). It shows that the freeze-dried porous collagen

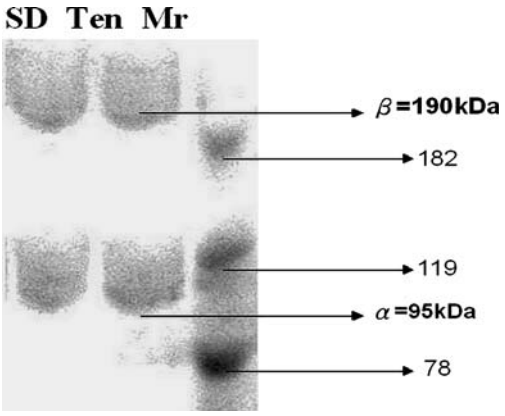

Figure 2. SDS-PAGE pattern of isolated type I collagen. The α band (95 kDa) and β band (190 kDa) of isolated collagen were identical to the SDS-PAGE patterns of commercialized type I collagen. SD: commercialized type I collagen as standard; Ten: type I collagen isolated from the porcine Achilles' tendon; Mr: reference marker.

scaffolds possess controlled small pores on the surface, middle-sized pores in upper layer, and thoroughfare pores in lower layer. The surface pore size of the upper layer was in the range of 1–5 μm (Figure 3b and c) and inner pores of upper layer (10–30 μm, Figure 3b), and inner pores of lower layer (100–200 μm, in Figure 3a, lower part). The micropores on the surface could act as a channel for the growth factors or cytokines transportation, which were secreted by the fibroblasts stayed in the scaffold. The fibroblasts in the scaffold and keratinocytes on the surface could communicate or cross talk with each other.

The flowchart of culture model scheme was shown in Figure 1. First, the dermal fibroblasts seeded in the collagen scaffold proliferated well and further developed into a dermal equivalent. In the second culture stage, the keratinocytes were seeded on the surface of the dermal equivalent. After seeded, the keratinocytes should well attach on the top surface and exhibit characteristic morphology (Figure 4a). After 5 days' submerged culture, the keratinocytes proliferated and confluenced to form the keratinocyte sheet on the dermal equivalent (Figure 4b). The dermal fibroblasts in the collagen scaffold migrated to the pores for spreading and proliferation (Figure 4c). The morphology of the reconstructed epidermis revealed significant differences in presence or absence of EGF in the air-lifted stage. After 2 weeks of air exposure without EGF addition, a well-differenti-

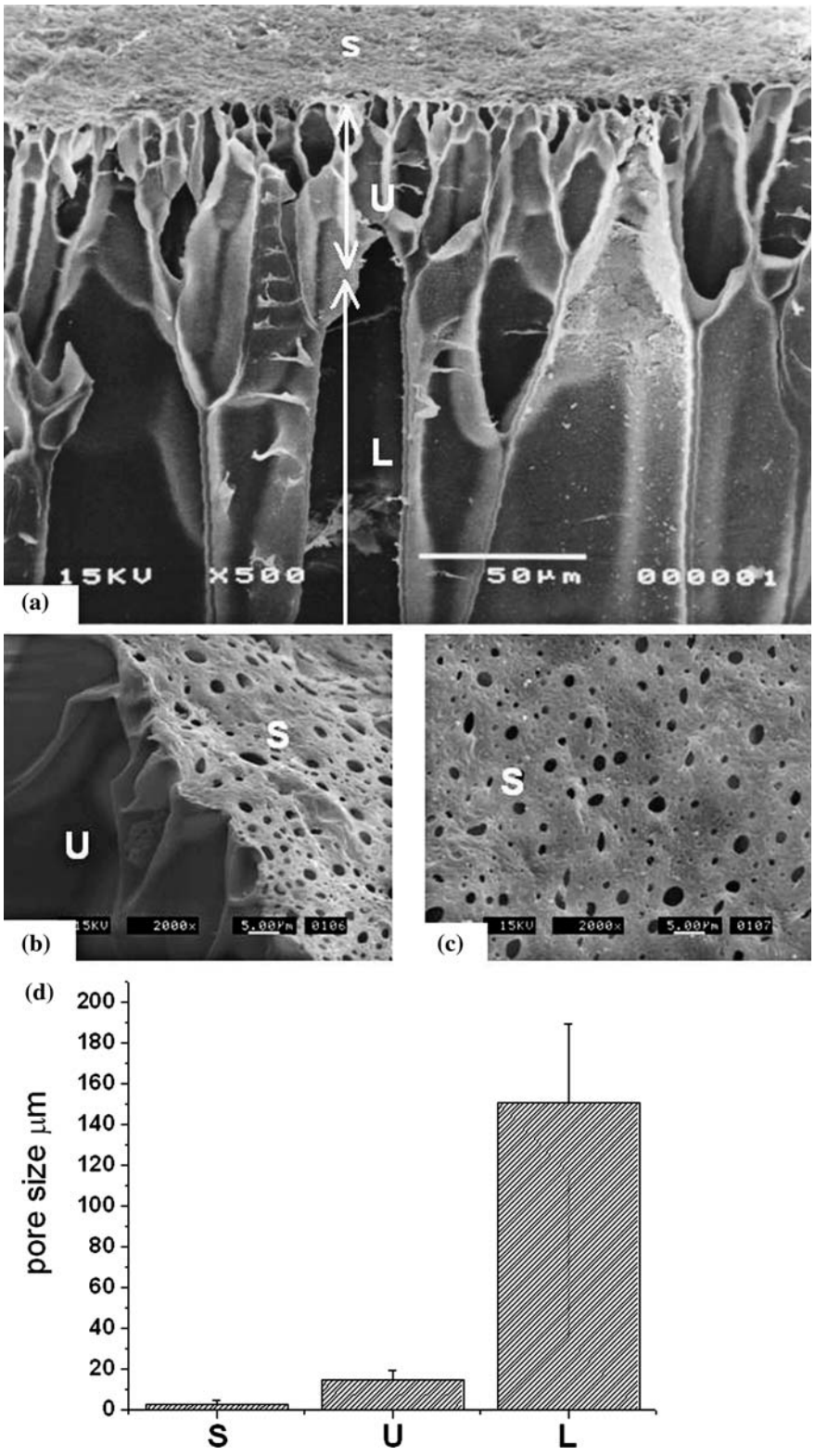

Figure 3. The picture of the (a) longitudinal cross section of bi-layer collagen scaffold from lower layer to upper layer, (b) the upper layer of the bi-layer collagen scaffold, and (c) the surface of the scaffold. (d) The distribution of pore size and mean pore diameter on each layer analyzed by SEM and computer-added imaging system, S: surface of upper layer; U: upper layer; L: lower layer. $n = 12$.

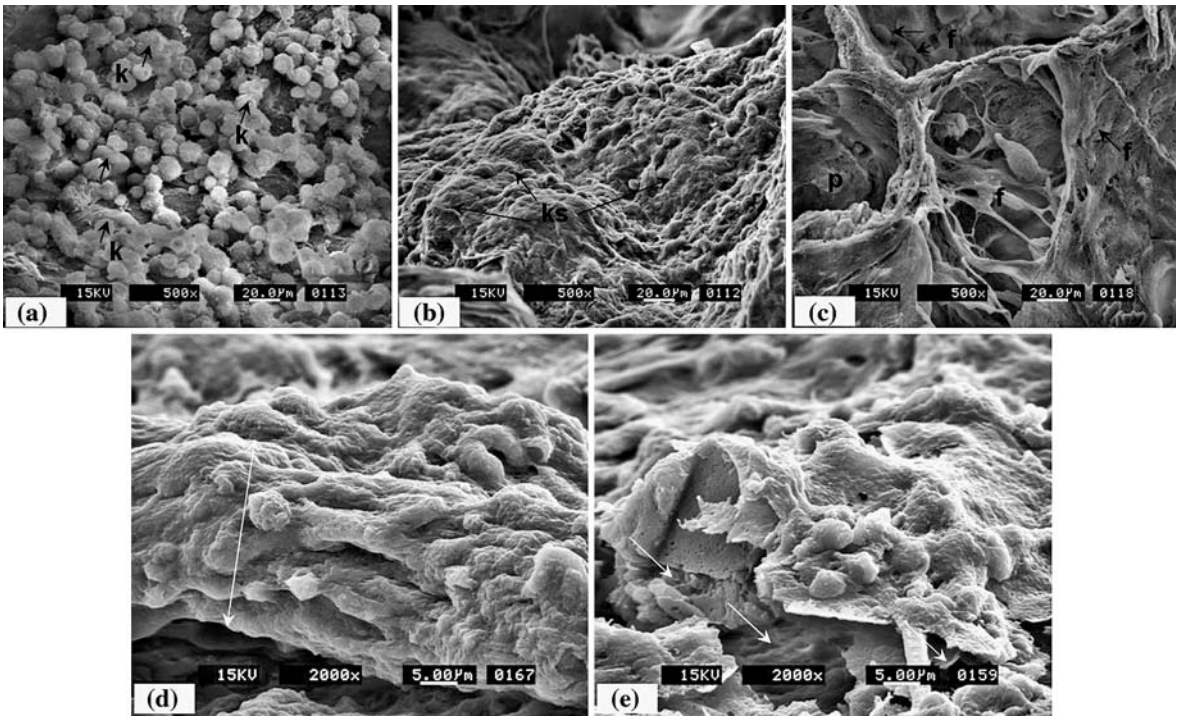

Figure 4. SEM photographs at the second culture stage and the third culture stage. (a) After seeded for 2 h, keratinocytes well attached on the top surface with round shape; (b) At the day 5 in the submerge culture stage, keratinocytes proliferated and confluenced to a cell sheet and covered on the dermal equivalent layer; (c) Dermal fibroblasts keep proliferating and migrating into the interconnecting pores. (d) After cultured for 2 weeks in the third culture stage without EGF addition, keratinocytes differentiated, stratified and organized into epithelium-like structure (arrow); (e) After cultured for 2 weeks in the third culture stage with EGF addition, keratinocytes showed uneven cell–matrix interface and disorganization of suprabasal layers (arrows). k: keratinocyte, ks: keratinocyte sheet, f: fibroblast, p: pore of scarfold.

ated and organized epithelium could be observed. The epithelium exhibited the following features: small, mostly cuboidal cells with the formation of stratum spinosum, stratum granulosum, and stratum corneum-like layers (Figure 4d). However, continuous presence of 10 ng per ml of EGF in the air-lifted stage resulted in uneven cell–matrix interface, disorganization of suprabasal layers (Figure 4e).

When the culture medium did not contain EGF in the third culture stage, the keratinocyte sheet on the dermal equivalent developed into continuous epidermis-like structure, which contained basement membrane, stratum spinosum, stratum granulosum, and stratum corneum (Figure 5a). However, the keratinocyte sheet formed only a thin atrophic epithelium if 10 ng per ml of EGF was constantly added into the culture medium as seen in Figure 5b. The thickness of epidermis decrease about 28% in the medium of containing 10 ng per ml of EGF compare to EGF-free medium in the third culture stage (Figure 5c).

In this study, we checked the marker p63 to identify the keratinocyte stem cell and checked terminal differentiation markers, such as involucrin and keratin 10 (K10), to characterize the stratified keratinocyte or reconstructed epidermis (Figure 6). Keratin 10 stain all showed positive in the EGF-added group (Figure 6a) and in the without EGF-added group (Figure 6b), but the thickness in EGF-added group was thinner than the one of without EGF-added group. Involucrin-positive stain had the same results that all showed positive for the two groups, but the without EGF-added group was thicker than the EGF-added group (Figure 6c and Figure 6d). From the Figure 6e and f, keratinocytes all showed p63-positive no matter in EGF-added group or without EGF-added group. We could tell from the p63-positive results that keratinocyte on the layer remained its

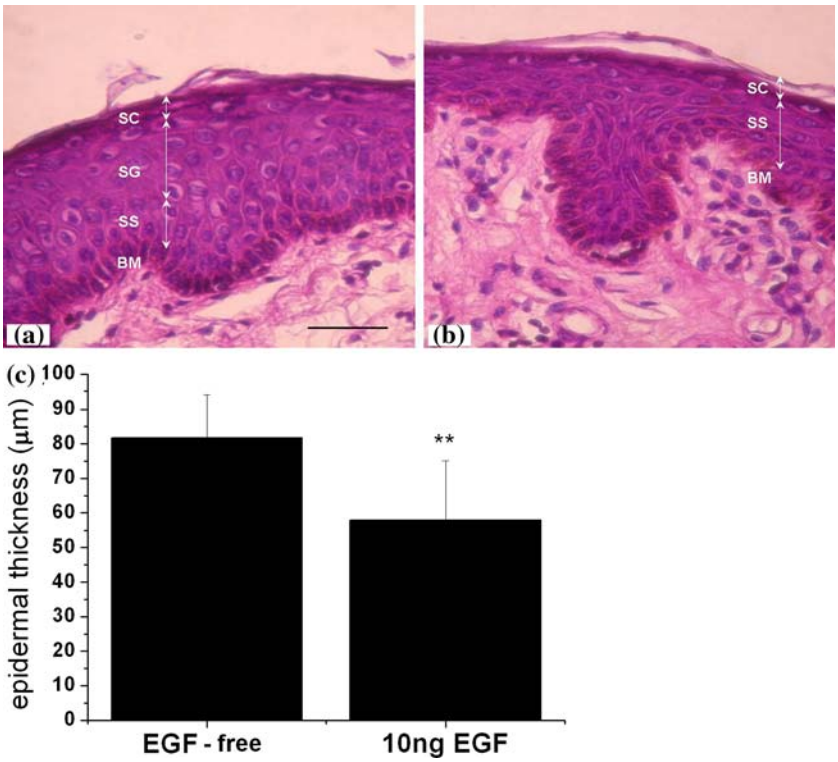

Figure 5. Histological examination of the differentiated keratinocyte sheet cultured for 2 weeks at the third culture stage. (a) Without EGF addition. The keratinocyte layers thicken in continuous epidermis-like structure, which contained basement membrane, stratum spinosum, stratum granulosum, and stratum corneum. Bar = 25 μm. (b) Added 10 ng per ml EGF in the culture medium. The keratinocyte sheet formed only a thin atrophic epithelium if 10 ng per ml EGF was constantly added into the culture medium. (c) The thickness of epidermal equivalent without and with EGF addition. To determine the epidermal thickness, five positions were randomizedly selected from each slice. A total of 50 digitized images were analyzed. Epidermal thickness with EGF addition is about 28% lower than that of without EGF addition. $^{**}p < 0.01$. BM: basement layer, SS: stratum spinosum layers, SG: stratum granulosum layers, and SC: stratum corneum layers.

phenotype of stem cells that would further develop into stratified epidermis-like structure. If the keratinocytes on the layer lost the phenotype, it is not possible to differentiate into different epidermal cells and developed into suprabasal layers. Figure 6g summarized the fluorescent intensity of K10, involucrin, and p63 in the groups of with EGF addition and without EGF addition. The fluorescent intensity of the three stains on the no EGF-added group is higher than those of the EGF-added group.

In this culture system, proliferating fibroblasts should express necessary growth factors by the way of paracrine to promote keratinocyte proliferation, differentiation, and organized into an epidermis-like structure. Fibroblast activity in the culture system was evaluated by MTT (Figure 7) and correlative mRNA expression (Figure 8a). In Figures 7 and 8, the abbreviation of FI-2d is dermal fibroblast cultured at the 1st stage for 2 days, FII-5d is dermal fibroblast cultured at the 2nd stage for 5 days, FIII-1w is dermal fibroblast cultured at the 3rd stage for 1 week, FIII-2w is dermal fibroblast cultured at the 3rd stage for 2 weeks, KII-5d is keratinocyte cultured at the 2nd stage for 5 days, KIII-1w is keratinocyte cultured at the 3rd stage for 1 week, and KIII-2w is keratinocyte cultured at the 3rd stage for 2 weeks.

As shown in Figure 7a, b, and c, the activity of dermal fibroblast was gradually increased from the first culture stage to the third culture stage. In Figure 7c, the activity of the dermal fibroblast cultured in EGF-containing medium for 1 week was higher than that of in EGF-free medium but no significant difference between the two groups cultured for 2 weeks. In Figure 7d, the viability of

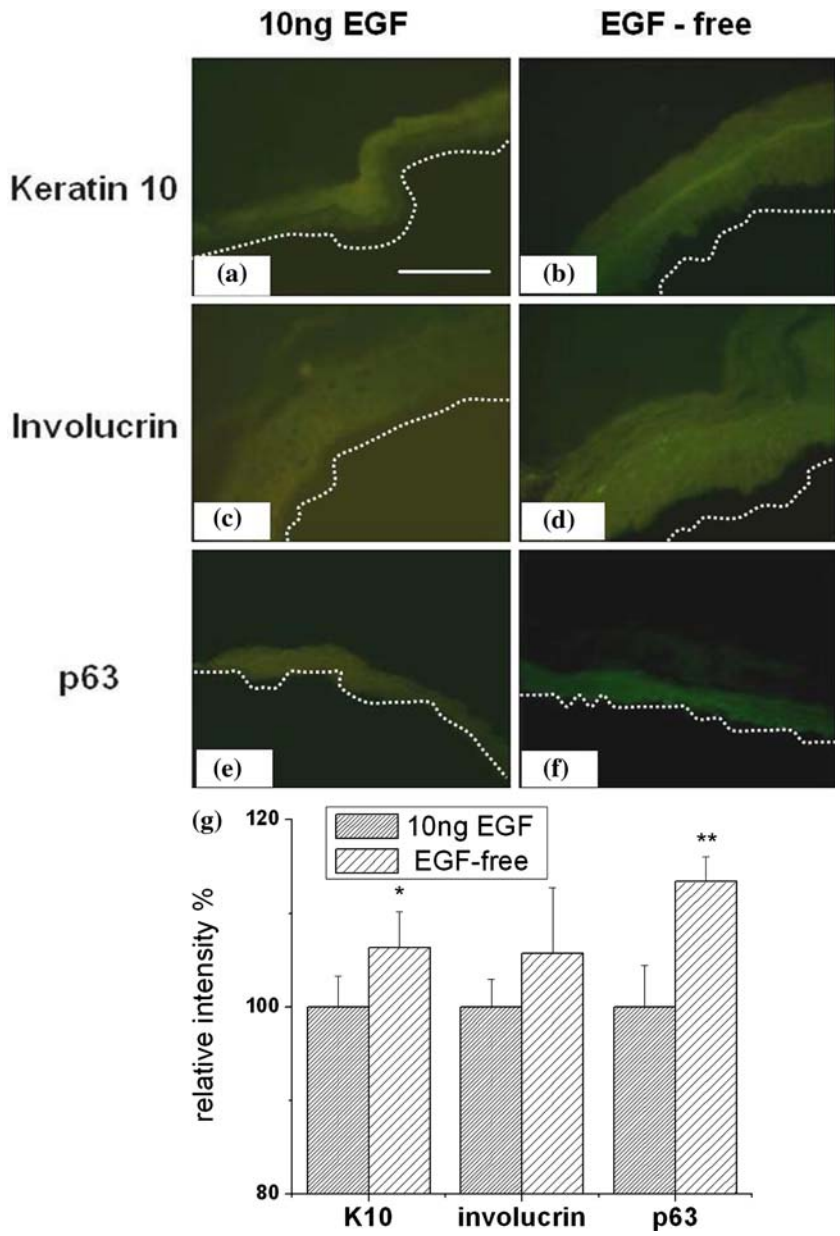

Figure 6. Immunohistochemical stains of reconstructed epidermis with EGF addition and without EGF addition at the 2nd week of the third culture stage. The Keratin 10 on the reconstructed epidermis (a) with EGF addition and (b) without EGF addition; The involucrin stain on the reconstructed epidermis (c) with EGF addition and (d) without EGF addition; The p63 stain on the reconstructed epidermis (e) with EGF addition and (f) without EGF addition; (g) The fluorescence intensity for each stain was determined by Quips Smart Capture FISH Imaging Software (Vysis). p63: keratinocyte stem cell marker, involucrin and keratin 10: terminal differentiation markers. Results are mean ± SD of 10 samples. $*p < 0.05$, $**p < 0.01$; bar = 50 μm.

keratinocyte cultured in EGF-containing medium for 1 week and 2 weeks was lower than those in EGF-free medium in the third culture stage.

In the organotypic culture system, we can expect that the dermal fibroblast should express several growth factors such as KGF and TGF-β1 to affect keratinocyte proliferation and differentiation. As shown in Figure 8a, TGF-β1 mRNA expression of dermal fibroblast in EGF-containing medium was 4-fold and 5-fold stronger than that of in EGF-free medium at the 1st week and the 2nd week, respectively, in the third culture stage.

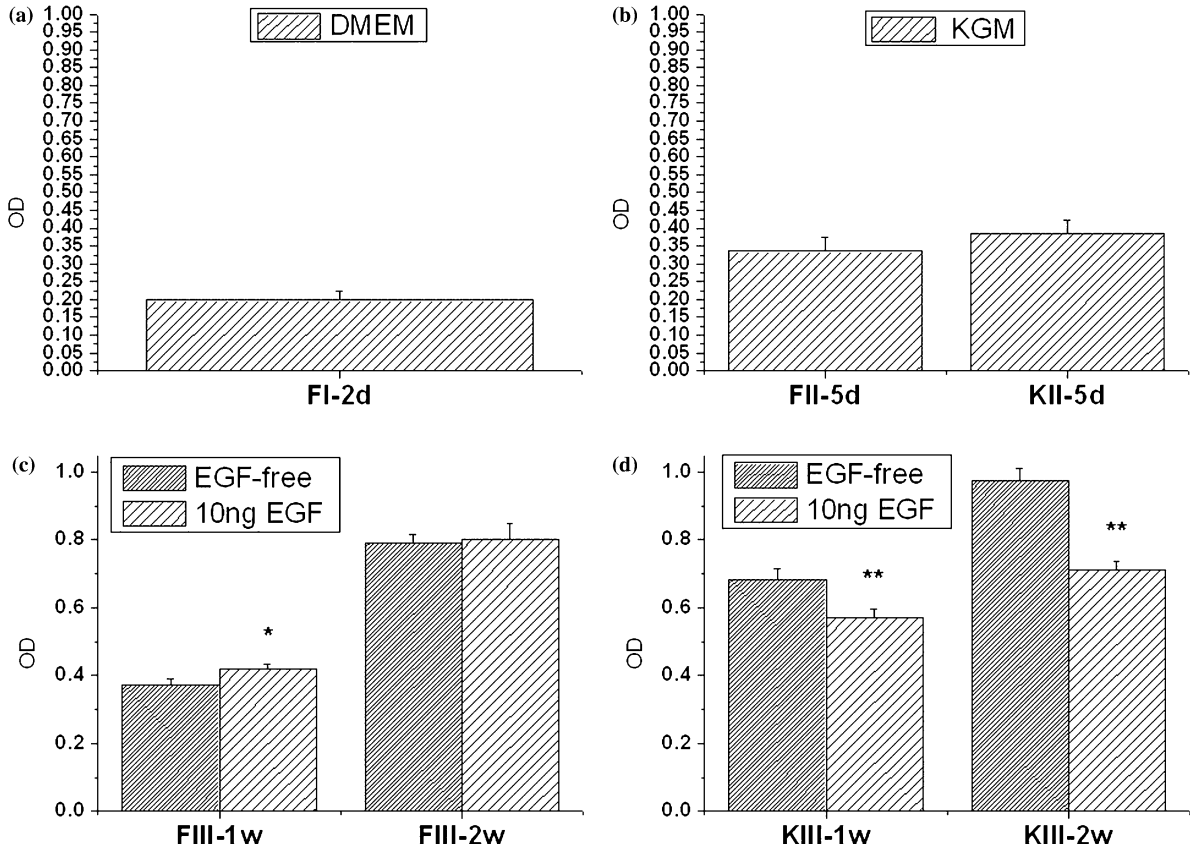

Figure 7. (a) The fibroblast viability at the 2nd day in the first stage. (b) The cell viability at the fifth day in the second stage. (c) The viability of dermal fibroblast at the 1st week and 2nd week in the third stage. (d) The viability of keratinocyte at the 1st week and 2nd week in the third stage. Results are mean ± SD of 10 samples in three different experiments. $*p < 0.05$, $**p < 0.01$.

KGF mRNA expression of dermal fibroblast in the EGF-containing medium was 2-fold stronger that that of in EGF-free medium at the 1st and the 2nd week in the third culture stage. In Figure 8b, the mRNA expression of TGF-β1 on keratinocyte in EGF-containing medium was 14-fold stronger than that of in EGF-free medium at the 1st and 2nd weeks in the third culture stage. The results strongly showed that the EGF addition in the medium highly influenced the TGF-β1 expression of keratinocyte in the third culture stage.

Discussion

The natural polymer of telopeptide-poor collagen has the advantage of more biocompatible, biodegradable, less antigenic and less toxic than synthetic polymers. The animal derived collagen needs to be modified to ensure its long-term stability in the human body. Porous type I collagen matrix not only can provide guidance for epithelial cells and fibroblast but also allow optimal migration and cell orientation. In this study, we successfully fabricated bi-layer 3-D collagen scaffold with upper dense layer and lower loose layer. The micropores existed in the dense layer could act as the channel for cytokines transportation which improved the communication and interaction between keratinocytes and dermal fibroblasts. As the previous studies, dermal fibroblasts could secret cytokines to promote keratinocytes proliferation and differentiation [10, 11]. In the study, the keratinocytes attached well and progressively proliferated on the dermal equivalent in the second stage (Figure 4a and b), which was due to the better communication between these two cells. The porous collagen scaffold provided a space for dermal fibroblasts proliferation and migration (Figure 4c).

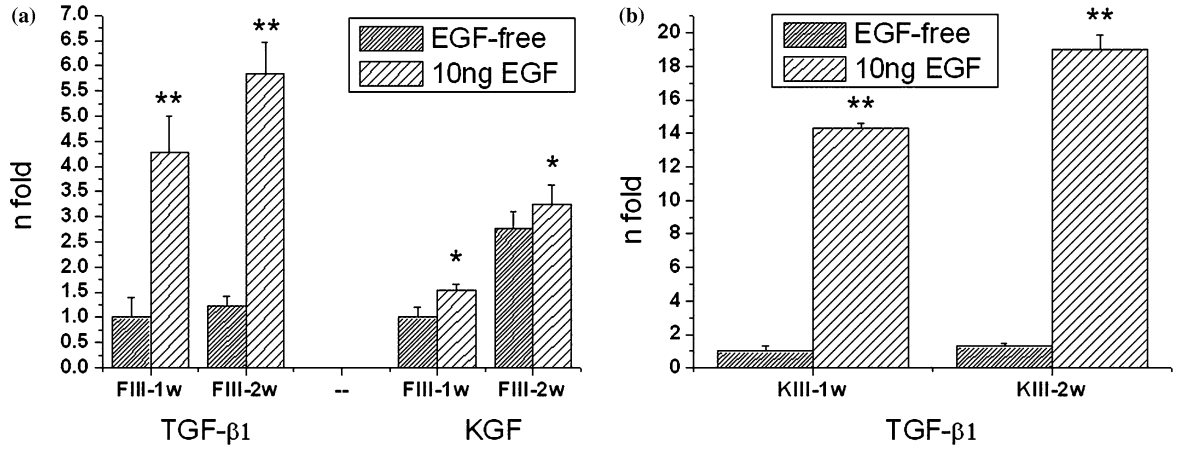

Figure 8. Effect of EGF on mRNA expression of fibroblasts and keratinocyte. (a) TGF-β1 and KGF mRNA expression of dermal fibroblasts (with EGF addition and without EGF addition) at the 1st week and 2nd week in the third culture. (b) TGF-β1 mRNA expression of epidermal keratinocytes (with EGF and without EGF addition) at the 1st week and 2nd week in the third culture stage. Expression levels were determined by real time RT-PCR. mRNA values are calculated based on GAPDH expression. Results are mean ± SD of 10 samples in three different experiments. $*p < 0.05$, $**p < 0.01$.

In previous study, EGF plays both positive and negative effects on keratinocytes migration, proliferation and differentiation [12, 13]. In the second culture stage, EGF was added into the culture medium to help the keratinocytes migration, which enlarged the cell colonies and promoted uniform distribution on the epidermal layer. EGF also induced dermal fibroblasts to secrete KGF to help keratinocytes proliferation [14, 15]. However, it also played a negative role on the keratinocytes differentiation, which could damage the epithelialization processes in the third culture stage (Figure 4e). The immunohistochemical stain of involucrin (Figure 6c and d) and K10 (Figure 6a and b) on keratinocyte all showed positive that were used to identify the differentiated layers, but EGF addition resulted in thin differential suprabasal layers. The results showed that fibroblasts cultured in EGF-containing medium would upregulate TGF-β1 expression, which modulate the onset of keratinocytes differentiation and simultaneously inhibited keratinocyte proliferation (Figures 8a and 7c). In the third culture stage, the increasing of keratinocytes proliferation in the EGF-free medium is due to less TGF-β1 expression. Furthermore, KGF expression of dermal fibroblast increaseing from 1 to 2 weeks also contributed to the increasing of keratinocyte proliferation in the third stage (Figure 8a). As a potent mitogen *in vitro* for human keratinocytes, KGF was suggested to promote keratinocytes proliferation in the early air-lifted culture stage, and to inhibit the terminal differentiation and apoptosis of cultured human keratinocytes [16, 17]. It is also found that high dose EGF would induce over expression of TGF-β1, which could act in a negative feedback autocrine manner for keratinocytes growth [12]. This study demonstrates that over express of TGF-β1 results in the suppression of high-dose EGF-induced keratinocytes growth.

In this study, higher fibroblasts activity (Figure 7c) could induce more KGF expression to support keratinocyte differentiation in EGF-free medium. The highly expression of TGF-β1 would inhibit the kerationcytes proliferation in the air-lifted stage. Therefore, EGF addition in the air-lifted stage was needless which caused to the thin atrophic epithelium (Figures 4e, 5b, 6a, and c). We believed that the keratinocyte cultured on the dermal equivalent with EGF-free medium should have a better differentiation and epithelialization (Figures 4d, 5a, 6b, d, and f). In the new cultured system, keratinocyte was proven by p63-positive to keep stem cell phenotype.

From our study, we suggest that EGF is needed during the submerged stage to increase keratinocyte proliferation to form epithelial sheet. However, the continuous presence of EGF during the subsequent air-lifted stage would inhibit keratinocytes proliferation. We believed that the KGF expressed by fibroblasts was enough to maintain

the proliferation and differentiation of keratinocytes in the air-lifted stage. After several days of air exposure without EGF addition, some characteristic features of the epithelium could be observed; i.e., small, mostly round basal cells with stratum corneum-like layers formation. The newly formed epithelium was well differentiated and multi-stratified. Moreover, several studies have shown that the presence of a dermal layer is essential for the regulation of growth and differentiation of the cultured keratinocytes [18, 19]. Further studies conducted in our group would focus on the importance of fibroblast-derived soluble factors that regulate the proliferation and differentiation of keratinocytes.

Conclusions

From this study, we suggest that EGF is needed during the submerged stage to increase keratinocyte proliferation and to from a epithelial sheet. However, continuous presence of EGF in the air-lifted stage is not necessary due to KGF expressed from dermal fibroblast is enough for keratinocyte proliferation and differentiation. The excessive EGF stayed in the culture medium may result in uneven cell–matrix interface and disorganization of suprabasal layers. Besides, the excessive EGF induces keratinocyte and dermal fibroblast to overexpress TGF-β1 at the third stage, which could act in a negative feedback autocrine and/or paracrine manner for keratinocytes proliferation. In brief, this new-designed skin equivalent culture system can be used in skin equivalent tissue engineering.

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