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Human single-donor composite skin substitutes based on collagen and polycaprolactone copolymer

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ABSTRACT

The development and characterization of an enhanced composite skin substitute based on collagen and poly(ε-caprolactone) are reported. Considering the features of excellent biocompatibility, easy-manipulated property and exempt from cross-linking related toxicity observed in the 1:20 biocomposites, skin substitutes were developed by seeding human single-donor keratinocytes and fibroblasts alone on both sides of the 1:20 biocomposite to allow for separation of two cell types and preserving cell signals transmission via micro-pores with a porosity of 28.8 ± 16.1 μm. The bi-layered skin substitute exhibited both differentiated epidermis and fibrous dermis in vitro. Less Keratinocyte Growth Factor production was measured in the co-cultured skin model compared to fibroblast alone condition indicating a favorable microenvironment for epidermal homeostasis. Moreover, fast wound closure, epidermal differentiation, and abundant dermal collagen deposition were observed in composite skin in vivo. In summary, the beneficial characteristics of the new skin substitutes exploited the potential for pharmaceutical screening and clinical application.

The design of tissue engineered skin equivalent described in the present work was based on a three-dimensional and organotypic culture by seeding single-donor human keratinocytes and fibroblasts on either side of a collagen-containing matrix. It is envisaged that the tissue-like character of this construction would demonstrate correct epithelial differentiation, morphology, and proliferation rates similar to that found in skin [1]. The biomaterials used for supporting skin cell growth were biocomposites comprising collagen and the synthetic α-polyester, poly(ε-caprolactone) [PCL]. Biocomposites of synthetic/natural polymers benefit from the wide range of physicochemical properties and processing techniques applicable to synthetic polymers and the cell adhesion properties of natural polymers. Among them, collagen is currently one of the most popular materials for scaffold production in soft tissue repair and reconstruction; however, the advantages of biocompatibility and cell adhesion are offset by possible toxicity caused by residual catalysts, initiators, and un-reacted or partially reacted chemical cross-linking agents that are generally employed to improve stability and mechanical properties [2]. To avoid the toxicity problems associated with chemical cross-linking of collagen whilst retaining the advantages associated with use of synthetic polymers, biocomposite films comprising collagen and PCL were produced previously by impregnation of lyophilized collagen with PCL solution followed by solvent evaporation. Changes in film morphology, from virtually pore-free coatings to open porous format, were achieved by variation of the collagen:PCL w/w ratio. A prior co-cultured skin model was established based on human keratinocytes and mouse 3T3 fibroblasts in a designed co-culture system [3].

The aim of the work was to develop enhanced skin substitutes based on a porous collagen:PCL biocomposite scaffold by means of a designed co-culture system, which mimics the anatomical structure of normal skin and provides a spatial separation of single-donor human foreskin keratinocytes and fibroblasts whilst allowing for the transduction of cell signaling with each other. Biocompatibility of the co-cultured skin substitute was further proven in vitro and in vivo.

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Materials and methods

Materials. Poly(ε-caprolactone) (PCL, MW 50,000; CAPA® 6500) was obtained from Solvay Interox, Warrington, UK. Type I acid-soluble collagen from calf skin (Cat. No. C-3511) was obtained from Sigma Chemicals Company, UK. Fibroblast culture medium contains DMEM (400 ml), 10% Fetal Calf Serum (FCS) and 5 ml penicillin/streptomycin (100U/ml and 100 mg/ml). The EpiLife® keratinocyte medium (Cat. No. M-EPI-012–5; Cat No.M-EPIcf-500 with addition of 0.06 mM calcium chloride) was obtained from Cascade Biologics®, Inc., USA.

Preparation of collagen:PCL biocomposites. Collagen solution (0.25% w/v) was prepared by dissolving type I collagen in 1% acetic acid. The dissolution of collagen was facilitated by stirring with a magnetic stirrer at room temperature. Aliquots (0.25 ml) of the collagen solution in glass vial were frozen at −20 °C for approximately 50 min and then were transferred to a freezer at −72 °C for 35 min. The frozen samples were placed in a freeze dryer (Edwards Modulyo®) at −44 °C under 42 mbar vacuum for 24 h. Aliquots (0.5 ml) of 1%, 2.5% w/v PCL/dichloromethane solution were then added to the collagen mats to prepare 1:8 and 1:20 w/w collagen:PCL biocomposites. The vials were kept closed for 30 min before removing the lids to allow solvent evaporation overnight.

Porosity measurement. The porosity of 1:8 and 1:20 (w/w) collagen:PCL biocomposites ($n = 3$) were investigated using the Automated Capillary Flow Porometer (PMI®, CFP-1200-A, Porous Materials, Inc.). In the analysis, wet biocomposite film was placed in the sample chamber before the gas was allowed to flow into the chamber. When the pressure reached a point that overcame the capillary action of the fluid within the largest pore, the bubble point, was then measured.

Primary culture of human epidermal keratinocyte and dermal fibroblasts. Single-donor primary human epidermal keratinocytes (PHEKs) and dermal fibroblasts (PHDFs) were isolated from human child foreskin samples obtained in the surgery of circumcision. Approval was obtained from the Institutional Review Board in the Tri-Service General Hospital, R.O.C. In brief, the foreskin sample was firstly transferred to 10 ml of 0.2% Dispase II solution kept at room temperature overnight and then epidermis was separated from dermis and incubated in 0.025% Trypsin–EDTA solution for 10 min. The cell pellets were then obtained by centrifugation at 1300RPM for 5 min and seeded in a fibronectin/collagen coated flask. For the dermis sample, it was firstly rinsed in 0.05% collagenase solution and incubated for 24 h. The cell pellets were then obtained by centrifugation at 1300RPM for 5 min and resuspended in fibroblast culture medium.

Preparation of co-cultured skin substitutes in vitro. To mimic the skin structure, co-cultured skin substitute was prepared by seeding single-donor PHEKs and PHDFs on either side of the 1:20 w/w collagen:PCL biocomposite in a co-culture system [3]. In brief, PHEKs (1.7×10^5cells/cm^2; P4) were firstly seeded on one side of the biocomposite for 6 days to achieve 90% confluence. Biocomposite film were then turned upside down and transferred to the co-culture system. PHDFs (2×10^4cells/cm^2; P4) were thereby seeded on the other side of biocomposite film for another 3 days to achieve 100% confluence. Finally, biocomposite films were turned upside down again, kept in submerge culture for another 6 days, and then lifted onto the air-fluid interface for another 10 days to enable keratinocyte differentiation.

Interaction of PHEKs and PHDFs in the co-culture system. It was shown that fibroblasts are stimulated to produce keratinocyte growth factor (KGF) by co-culture with keratinocytes and IL-1β, respectively, in an oral mucosal system [4]. To investigate the cell interactions between keratinocytes and fibroblasts in the co-cultured skin model, KGF production by fibroblasts was investigated under conditions of co-culture with keratinocytes or stimulation of IL-1β. In brief, PHDFs were firstly cultured in DMEM/10% FCS culture medium for 3 days, then serum-starved for 3 days in DMEM/0.5% FCS culture medium, and finally PHDFs (2×10^4cells/cm^2; P4) were seeded in different groups arranged as A–E (A: PHDF growth on TCP; B: PHDF growth on TCP with addition of IL-1β; C: PHDF growth on biocomposite; D: PHDF growth on biocomposite with addition of IL-1β; E: PHDFs co-cultured with PHEKs on either side of biocomposite) in DMEM/0.5% FCS medium. Cytokine stimulation of the fibroblast cultures was performed by addition of 10 ng/ml recombinant human IL-1β (R&D System, Oxford, UK). The culture medium was collected after 24, 48 h and stored at −80 °C. KGF proteins in the supernatants were then determined by ELISA. Absorbance was read at 450 nm by an automatic plate reader.

In vivo animal study. To investigate the biocompatibility of co-cultured skin model in vivo, three experiment groups were designed: co-cultured skin model, blank biocomposite (no cells), and retained open wound groups. All animals were acquired, housed and studied under a protocol approved by the Institutional Animal Care and Use Committee of National Defense Medical Center, R.O.C. Skin substitutes were grafted orthotopically to full-thickness skin wounds (1.2 cm in diameter) surgically created to the depth of the panniculus carnosus on the flank of athymic mice under aseptic condition. Grafts were attached with 4 sutures at the wound margin, covered with gel-type medium (DMEM/gelatin/agarose/10% FCS) and Tagaderm® film, and finally fixed with Omnifix® tapes (Hartmann Inc., Spain). All dressings and sutures were removed at one week time, and the wounds were left open to the air. The data of clinical evaluation of graft taking and frozen sections were collected at weekly intervals afterwards.

Assessment. Clinical evaluation of graft taking The wound healing process was recorded by photography periodically after a certain period of time. Furthermore, the wound size and clinical appearance were evaluated by the main investigator.

Scanning electron microscopy (SEM). The samples of skin substitute were fixed with 2.5% glutaraldehyde and then washed in 0.33 M sucrose buffer in 4 °C. After soaking in 1% osmium tetroxide, the sample was washed in 0.33 M Sucrose buffer followed by distilled water, and then were dehydrated orderly in gradient ethanol/Isoamylacetate at 4 °C. After critical point drying, samples were attached to aluminum stubs and sputter coated with gold prior to examination using HITACHI® S-3000N Scanning Electron Microscope.

Immunohistochemistry assay. PHEKs and PHDFs were labeled with monoclonal mouse anti-human involucrin antibody 1:200 (Cat. No. I8447-25; United States Biological, Inc.) and monoclonal mouse anti-human α tubulin antibody 1:200 (Cat. No. sc-5286; Santa Cruz Biotechnology, Inc.), respectively. The secondary antibody for involucrin was rhodamine-conjugated goat anti-mouse IgG 1:100 (Cat. No. I1903-08C, United States Biological, Inc.), and for α Tubulin was fluorescein (FITC)-conjugated goat anti-mouse IgG 1:100 (Lot. No. 62686; Jackson ImmunoResearch Laboratories, Inc., USA).

Gomori's trichrome staining. The frozen section of the specimen was prepared for Gomori's trichrome staining. The method was selected to stain collagen component (green) in terms of the extracellular matrix deposition in skin substitutes.

Statistical analysis. The data are analysed using the Paired *t*-test and the ANOVA two-factor with replication test. The data are shown as "mean ± SE (SE: standard error)". The result is statistically significant when the *P* value is less than 0.05 ($P < 0.05$).

Results

Porosity of biocomposites

Higher mean bubble point pore size in diameter, in terms of the maximum pore size, was measured as 47.1 ± 11.5 μm (ranging

from 22.2 to 77.6 μm) in the 1:8 group compared to that of 28.8 ± 16.1 μm (ranging from 5.6 to 75.4 μm) in the 1:20 group ($P = 0.391$). Furthermore, both groups exhibited a similar pore size of 9–10 μm in diameter at the maximum pore size distribution ($P = 0.739$).

Co-cultures skin substitutes in vitro

A bi-layered skin substitute consisting of a spatially separated epidermal layer with confluent keratinocyte sheet and an underneath fibrous dermal layer populated with fibroblasts was shown in SEM and immunohistochemistry assay (Fig. 1).

Interaction of PHEKs and PHDFs in vitro

The stimulation of KGF release from fibroblasts by either IL-1β or co-culture with keratinocytes was compared and the results revealed that the KGF release increased by addition of IL-1β in both TCP and biocomposite conditions up to 48 h ($P < 0.05$); however, less KGF production was measured in the group of co-cultured skin model compared to that of fibroblast alone on biocomposite at 24 and 48 h ($P < 0.05$; Fig. 2).

In vivo animal study

Rapid wound closure was observed in the group of co-cultured skin model at day 7 (Fig. 3A) even though the wound size decreased apparently in all three groups (Fig. 3B, D, and F). The blank biocomposite seemed to attach the wound at day 7 (Fig. 3C); however, it sloughed afterwards and exhibited incomplete wound closure (Fig. 3D) similar to the retained open wound group (Fig. 3E and F) in 14–19 days. Moreover, complete wound closure with differentiated epidermis and abundant dermal parallel-arranged fibrous collagen deposition was shown in co-cultured skin model (Fig. 4A). In contrast, the retained open wound group showed much loose collagen deposition (Fig. 4B) and moderate deposition of collagen was observed in the blank biocomposite group (Fig. 4C).

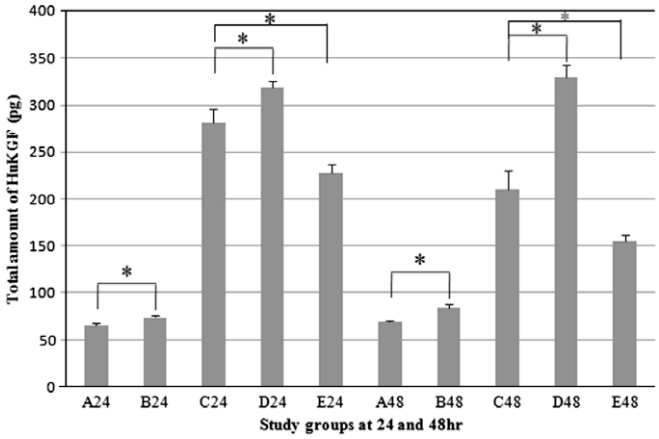

Fig. 2. KGF ELISA. The KGF release from PHDFs by stimulation of either IL-1β (10 ng/ml) or co-culture with PHEKs grown in tissue culture plastics (TCP) or 1:20 w/w collagen:PCL biocomposite was measured by ELISA at 24 and 48 h, respectively (A: PHDF growth on TCP; B: PHDF growth on TCP with addition of IL-1β; C: PHDF growth on biocomposite; D: PHDF growth on biocomposite with addition of IL-1β; E: Composite skin substitute). ($n = 5$; values are means ± SE; Paired t-test: $P < 0.05$; ANOVA: $P < 0.05$; Asterisk indicates statistically significant difference).

Discussion

An appropriate pore size of scaffold for implantation of engineered skin substitute is critical to allow for the optimal growth of native fibroblasts and endothelial cells from the wound bed. It is well known that the porous type scaffold consisting of cell adhesion molecule provides well bio-structural and mechanical supports. The infiltration and induction by host tissue will occur after engineered-tissue implantation via the release of incorporated chemotactic agents that attract the desired cells into the porous scaffold. Fibrovascular tissue thereby infiltrates the scaffold pores and cells reorganize into functional tissue as the polymer scaffold degrades [5]. In consideration of the optimal pore size of

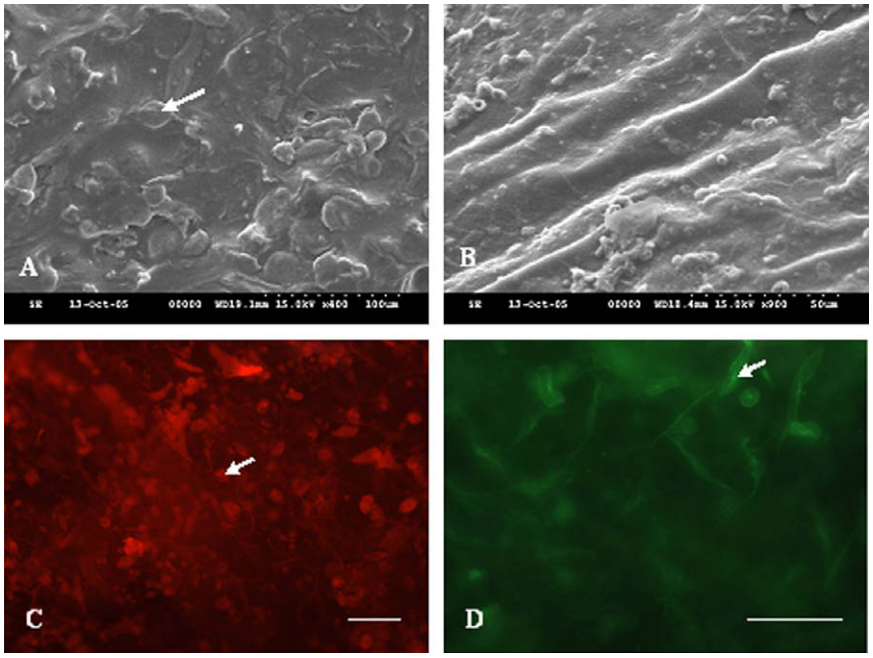

Fig. 1. Co-cultured skin model in vitro on day 35 (scale bar: 100 μm). SEM revealed a confluent PHEK (arrow) layer (A) and a dermal layer with linear fibrous structure (B). Immunostaining showed positive red fluorescence of PHEKs (arrow) (C) and positive green fluorescence of PHDFs (arrow) (D). PHEKs were labeled with mouse anti-human involucrin and rhodamine-conjugated goat anti-mouse antibodies; PHDFs were labeled with mouse anti-human α tubulin and fluorescein (FITC)-conjugated goat anti-mouse antibodies.

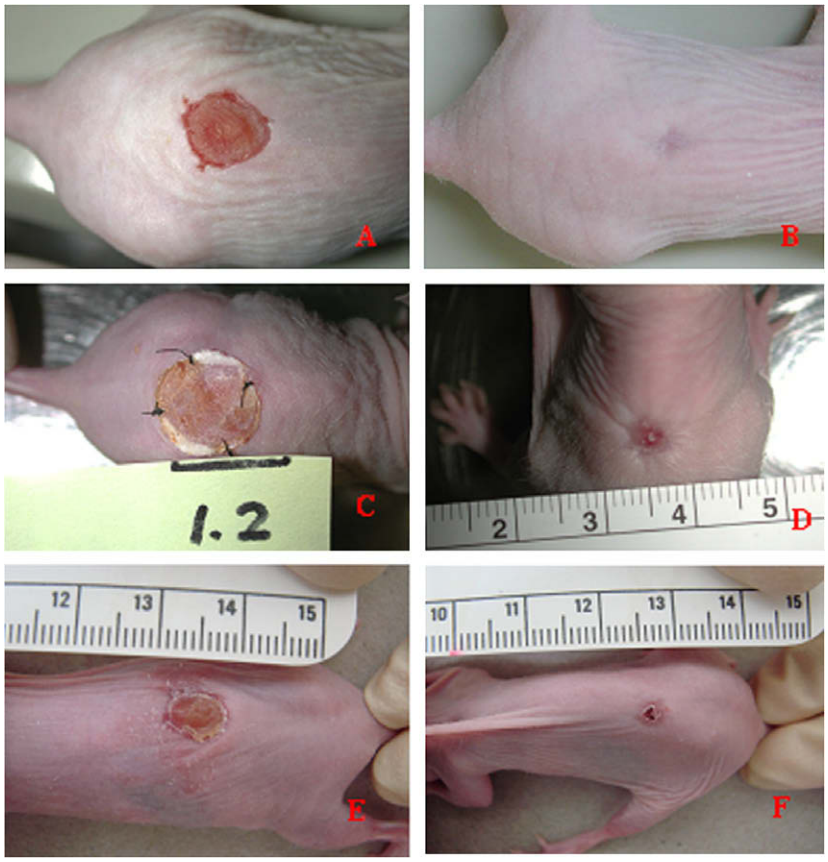

Fig. 3. Assessment of wound healing in vivo. The wound healing in groups of co-cultured skin model, blank biocomposite and retained open wound, was compared clinically. Complete wound closure was observed at day 7 (A) and normal skin appearance was shown at day 28 (B). The blank biocomposite was grafted and followed up at day 7 (C), and incomplete wound closure was observed at day 19 (D). The retained open wound group appeared dry eschar formation on the wound surface at day 7 (E) and unhealed wound at day 14 (F).

scaffold to be controlled at a diameter of 20 to 50 μm [6], the maximum pore size of both 1:8 and 1:20 collagen:PCL biocomposites appeared to fit this criteria, whilst the smaller maximum pore size distribution may prevent the direct contact between keratinocytes and fibrobalsts, and allow for cell interaction via signaling through existing pores.

Epidermal–dermal structure is the principal characteristic of normal skin tissue. Epidermal morphogenesis is modulated by cell–cell interactions via diffusible factors including cytokines and growth factors, as well as by cell–matrix interactions via cellular adhesion molecules [7]. On the other hand, the fact that fibroblast proliferation is increased in an in vitro co-culture model, compared with a fibroblast monolayer alone, implicates that the keratinocytes regulate fibroblast growth and proliferation [8]. Therefore, the design of an engineered skin substitute by co-culture of keratinocytes and fibroblasts is more realistic in mimicking the normal skin structure. In the investigation of cell interactions in the co-culture system of this work, it showed that the production of KGF from fibroblasts growing alone on either TCP or biocomposite was stimulated by cytokine, IL-1β; however, the production of KGF decreased while in co-culture condition compared to that of fibroblast growth alone on biocomposites. It indicates that the epidermal homeostasis may become more stabilized in the co-culture microenvironment following by decreased KGF production; furthermore, keratinocytes and fibroblasts are not only anatomically separated on either side of the skin substitute but also sustain interactions with each other by cell signaling.

The basic principle of organotypic co-cultures is to culture mesenchymal and epithelial cells spatially separated by seeding epithelial cells on the top surface of a matrix consisting of type I collagen, which is populated with fibroblasts [9] such as Apligraf® prepared from co-culture of allogeneic human neonatal keratinocytes and fibroblasts on type I bovine collagen gel [10] and OrCel® prepared from co-culture of allogeneic human keratinocytes and fibroblasts on type I bovine collagen sponge. Human fibroblasts are known to proliferate and secrete dermal collagen, fibronectin, glycosaminoglycans and other proteins, in effect, to enrich the dermal matrix by themselves [11]. However, while most of these update co-cultured skin constructs were developed using dermal components in gel formulations, they are prone to shrink somehow by the natural force generated by fibroblasts. A stronger biodegradable scaffold may be needed to resist the contraction force generated from fibroblasts to prevent possible scar formation in clinical application [12]. The collagen:PCL biocomposites used for developing engineered skin substitute manifests the beneficial characteristics of good cell adhesion as well as the resistance to early shrinkage of material after seeding with fibroblasts.

In comparison with the grafts of native skin, current models of engineered skin substitutes still have anatomical and physiologic deficiencies [13]. To develop an enhanced skin substitute, a brand new co-cultured skin substitute was prepared and studied in this work. Complete wound healing was observed in co-cultured skin model group at day 7; however, spontaneous regeneration of skin by contraction of the wound bed and epithelialization was noted in all groups in a longer time period due to the skin characteristics of

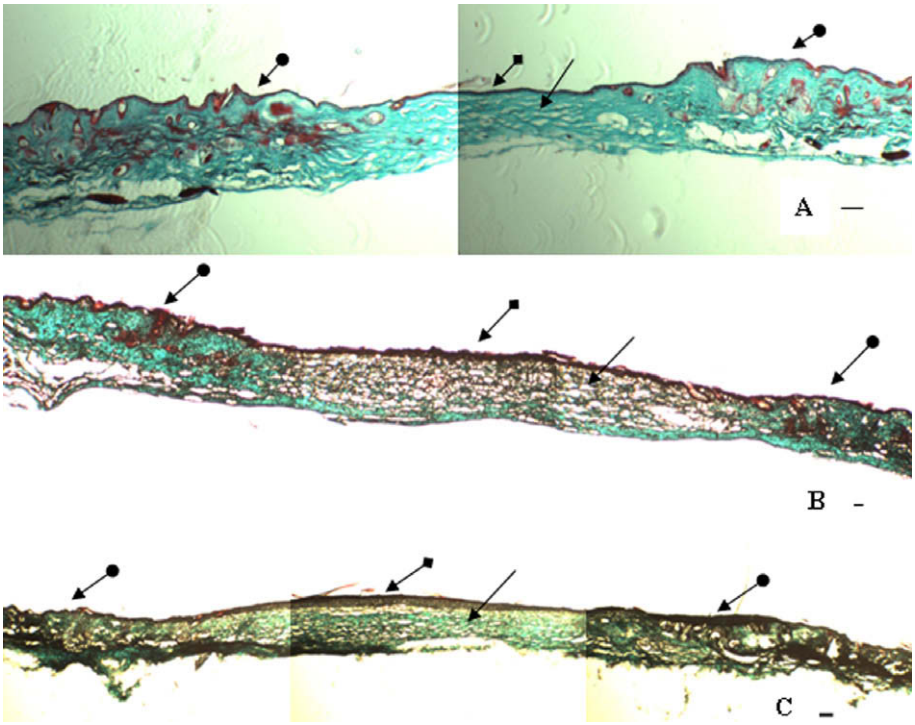

Fig. 4. Collagen deposition in the wound bed by Gomori's trichrome staining. The histology of co-cultured skin model after grafting for 225 days showed differentiated epidermis (♦→) and abundant fibrous collagen deposition (→) in the wound bed surrounded by normal mice skin structures (●→) (A: 40×; scale bar: 100 μm); The histology of retained open wound at day 34 showed complete wound closure with differentiated epidermis (♦→), however, less collagen deposition (→) was observed (B: 40×; scale bar: 100 μm); The histology of blank biocomposite group at day 34 revealed complete wound closure with differentiated epidermis (♦→) and moderate collagen deposition (→) (C: 100×; scale bar: 100 μm).

mice. Moreover, similar epidermal differentiation was observed in all three groups on day 34; however, the more dermal collagen deposition was detected in co-cultured skin substitute. The evidence indicates that the engineered skin substitute provides rapid wound closure in a mice model as well as the potential for promoting fibrotic tissue remodeling with early maturation of scar tissue and thereby less hypertrophic scar formation in wound healing process. On the other hand, no hair follicles were observed in the wound area for all three groups on day 34; however, some were found in the marginal area of co-cultured skin substitute at day 225 showing the potential for promoting follicle regeneration.

Conclusion

A bi-layered skin substitute was developed by seeding single-donor PHEKs and PHDFs on either side of 1:20 collagen:PCL biocomposite representing the beneficial characteristics of feasible porosity for cell growth, in vitro skin model for testing cell interactions and good biocompatibility in vivo. It exploited the potential for pharmaceutical screening and clinical application.

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