Superficial Dermal Fibroblasts Enhance Basement Membrane and Epidermal Barrier Formation in Tissue-Engineered Skin: Implications for Treatment of Skin Basement Membrane Disorders

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Basement membrane is a highly specialized structure that binds the dermis and the epidermis of the skin, and is mainly composed of laminins, nidogen, collagen types IV and VII, and the proteoglycans, collagen type XVIII and perlecan, all of which play critical roles in the function and resilience of skin. Both dermal fibroblasts and epidermal keratinocytes contribute to the development of the basement membrane, and in turn the basement membrane and underlying dermis influence the development and function of the epidermal barrier. Disruption of the basement membrane results in skin fragility, extensive painful blistering, and severe recurring wounds as seen in skin basement membrane disorders such as epidermolysis bullosa, a family of life-threatening congenital skin disorders. Currently, there are no successful strategies for treatment of these disorders; we propose the use of tissue-engineered skin as a promising approach for effective wound coverage and to enhance healing. Fibroblasts and keratinocytes isolated from superficial and deep dermis and epidermis, respectively, of tissue from abdominoplasty patients were independently cocultured on collagen–glycosaminoglycan matrices, and the resulting tissue-engineered skin was assessed for functional differences based on the underlying specific dermal fibroblast subpopulation. Tissue-engineered skin with superficial fibroblasts and keratinocytes formed a continuous epidermis with increased epidermal barrier function and expressed higher levels of epidermal proteins, keratin-5, and E-cadherin, compared to that with deep fibroblasts and keratinocytes, which had an intermittent epidermis. Further, tissue-engineered skin with superficial fibroblasts and keratinocytes formed better basement membrane, and produced more laminin-5, nidogen, collagen type VII, compared to that with deep fibroblasts and keratinocytes. Overall, our results demonstrate that tissue-engineered skin with superficial fibroblasts and keratinocytes forms significantly better basement membrane with higher expression of dermo–epidermal adhesive and anchoring proteins, and superior epidermis with enhanced barrier function compared to that with deep fibroblasts and keratinocytes, or with superficial fibroblasts, deep fibroblasts, and keratinocytes. The specific use of superficial fibroblasts in tissue-engineered skin may thus be more beneficial to promote adhesion of newly formed skin and wound healing, and is therefore promising for the treatment of patients with basement membrane disorders and other skin blistering diseases.

Introduction

THE BASEMENT MEMBRANE is a highly dynamic and specialized adhesive structure at the dermal–epithelial junction that binds the epidermis to the underlying dermis and provides resilience to the skin. It functions as an exchange zone between epidermal keratinocytes and dermal fibroblasts, and serves as a reservoir of growth factors and cytokines. The basement membrane contains laminin, nidogen, collagen types IV and VII, and the proteoglycans, perlecan and colla-

gen XVIII,^{1,2} in addition to the extracellular matrix proteins—collagen type I and III, tenascin, and fibrillin-1. Both keratinocytes and fibroblasts contribute to the formation of the basement membrane.^{3–5} The basement membrane and the underlying dermis play critical roles in the maturation and function of skin by regulating keratinocyte growth and terminal differentiation. The barrier property of the epidermis is due to the presence of tight junctions and lipids, such as ceramides, cholesterol esters, and free fatty acids, which accumulate during epidermal differentiation. Regulation of

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epidermal differentiation may either be through fibroblast-derived diffusible factors that act on keratinocytes in a paracrine manner or interactions of keratinocytes with basement membrane proteins.^{6,7}

Dermal fibroblasts are heterogeneous; they consist of superficial, deep and hair follicle fibroblasts, each of which exhibit distinct characteristics when cultured independently.⁸ Superficial and deep dermal fibroblasts differ in physical and biochemical characteristics such as production of collagenase, procollagen type I and III, TGF- β 1, and proteoglycans such as decorin, versican, and fibromodulin.^{9,10} Further, recently, our research showed that superficial fibroblasts are antifibrotic, while deep fibroblasts are profibrotic and both differentially remodel collagen-glycosaminoglycan (C-GAG) matrices.^{9,10} In addition, compared to superficial wounds, deep dermal wounds were found to express lower amounts of the antifibrotic molecules—decorin, fibromodulin, TGF- β 3, and Thy-1, but higher TGF- β type II receptors.¹¹ Furthermore, studies in 2D culture showed that site-matched superficial and deep dermal fibroblasts differ in the production of certain cytokines and in their interactions with keratinocytes; superficial fibroblasts release higher levels of GM-CSF, KGF, and IL-6, and form a continuous basement membrane compared to deep fibroblasts.⁸ Epidermal growth and differentiation is influenced by dermal fibroblasts; however, the specific effects that superficial and deep dermal fibroblasts have on keratinocytes in a 3D microenvironment are not understood.

In the basement membrane, collagen type IV, laminin-5, nidogen, and perlecan form a network that functions as a barrier between the epidermis and dermis. Collagen type VII, another essential component of the basement membrane, forms fibrils that anchor the basement membrane to the underlying dermis. Disruption of the basement membrane results in skin fragility, and blistering of the skin and mucous membranes, which is a serious problem in children with epidermolysis bullosa (EB), a family of severe life-threatening congenital skin basement membrane disorders.¹² Some forms of EB such as the junctional form are lethal at the neonatal stage, while the dystrophic form results in years of severely painful blistering and repeated wounding in children. These wounds typically heal with fibrosis and scarring; the newly formed epidermis poorly adheres to the underlying dermis, and therefore the skin blisters and disadheres at the slightest trauma. This lack of adherence is due to defects in basement membrane proteins such as laminin-5 and collagen type VII¹³; mutations in genes encoding laminin-5 lead to severe blistering as seen in Herlitz' junctional EB.¹⁴ Mutations or deficiencies of collagen type VII as in bullous systemic lupus erythematosus or dystrophic forms of EB¹⁵ result in a paucity of collagen type VII anchoring collagen fibrils¹⁶ leading to skin fragility and extensive blistering. Patients with skin basement membrane disorders constantly suffer from severe blisters and recurring wounds throughout the body and associated infections. In addition to experiencing excruciating pain, they are prone to fatal sepsis and therefore need on-going wound care, hospitalization, and healthcare support.

Skin basement disorders such as EB are devastating and currently there are no clinically proven therapies for effective management and treatment of the disease. Existing treatment options are mainly palliative and include surgical debridement, analgesia, and bandaging most of the body surface to protect from friction and prevent infection. Studies in animal

models of EB have attempted delivery of wild-type collagen VII to wound sites by direct injection of either the recombinant protein or cells that have minigenes or are gene modified using retroviral, lentiviral, transposon, ϕ C31-based integrase vectors, but have had limited success.^{17–19} Treatment of collagen type VII knockout mouse models of EB with bone marrow-derived cells have shown improvements in some symptoms of the disease.^{20,21} However, for clinical realization of gene therapy and stem cell-based approaches, technical and safety issues such as oncogenic potential of the vectors or cells need to be addressed.²² Fibroblast therapy has been proposed as an alternative to circumvent these problems; recently, use of fibroblasts in a collagen VII-hypomorphic mouse model for recessive dystrophic EB showed that small increases in collagen VII significantly stabilized the skin against shearing forces and reduced the disease phenotype, suggesting that full restoration of collagen VII may not be critical for improvement in function and quality of life of EB patients.²³

There is an urgent need to develop new therapeutic strategies for effective wound management and cure of EB and other skin basement membrane disorders. We propose the use of tissue-engineered skin as a promising approach, since it not only would provide effective coverage at wound sites, but also promote healing and adherence of newly formed skin due to the presence of exogenous fibroblasts and keratinocytes. Moreover, the use of tissue-engineered skin to treat burn patients has yielded favorable clinical outcomes for wound coverage and pliability.²⁴ Currently available tissue-engineered skin consists of a heterogeneous population of dermal fibroblasts with keratinocytes; however, recent studies have shown differences in fibrotic characteristics and cytokine expression in the different subpopulations of dermal fibroblasts.^{8,10,25} The aim of this study therefore was to assess differences in basement membrane, epidermis formation, and barrier function of tissue-engineered skin containing superficial and deep dermal fibroblasts independently cocultured with keratinocytes.

Materials and Methods

Preparation of C-GAG matrices

Acellular C-GAG matrices were prepared by freeze-drying a coprecipitate of type I collagen and chondroitin-6-sulfate as reported previously.¹⁰ Briefly, collagen powder (0.5 wt%; Devro Pty. Ltd.) was coprecipitated with chondroitin-6-sulfate (0.05 wt%; Sigma) in 0.5 M acetic acid, degassed under vacuum (2 h, room temperature), cast into sheets, frozen to -40°C , and freeze-dried (FreeZone⁶Plus; Labconco) to produce highly porous matrices. The matrices obtained were cut into 30-mm discs and crosslinked by dehydrothermal treatment under vacuum (140°C , 48 h) in a drying oven (APT.Line VD; Binder GmbH). Thereafter, the matrix discs were rinsed with phosphate-buffered saline (PBS; twice, 15 min each) and subsequently with a cell culture medium (the Dulbecco's modified Eagle's medium [DMEM], 10% fetal bovine serum [FBS], 1% antibiotic-antimycotic; twice, 15 min each).

Preparation of tissue-engineered skin

Tissue-engineered skin was prepared by culturing superficial or deep dermal fibroblasts and keratinocytes on

dehydrothermal-treated C-GAG matrices. The cells were obtained from lower abdominal tissue of patients ($n=3$) who underwent elective abdominoplasty surgery following informed consent. The protocols for human tissue sampling used in this study were approved by the University of Alberta Hospital's Health Research Ethics Board. Superficial and deep dermal fibroblasts and keratinocytes were isolated as reported previously¹⁰; briefly, tissue samples were horizontally sectioned into five layers (referred to as layers 1–5) using a dermatome (Padgett Instruments) set approximately at 0.5 mm. The superficial dermal layer (layer 1) was treated overnight with 25 U/mL dispase (Gibco) at 4°C to remove the epidermis, which was digested with trypsin to isolate keratinocytes. On the other hand, the superficial dermal layer and the deep dermal layer (layer 5) were separately treated with 455.3 U/mL collagenase (Gibco) for 18 h at 37°C, 60 rpm to isolate the superficial (SF) and deep (DF) dermal fibroblasts, respectively. The keratinocyte (K) and fibroblast cell suspensions were passed through 100- μ m cell strainers and centrifuged at 800 rpm for 10 min. The epidermal and dermal cell pellets were then resuspended in the respective cell culture media and serially expanded in tissue culture flasks till the desired number of K, SF, and DF was obtained. Passage 4 SF or DF were seeded onto crosslinked C-GAG discs at a density of 0.5×10^6 cells/cm² and cultured (at 37°C, 5% CO₂). Two days later, K were seeded on top of the fibroblast-populated matrices at a density of 1.0×10^6 cells/cm² and the medium was replaced with a coculture medium containing serum (DMEM-HG and nutrient mixture F-12 Ham [3:1], 2 nM triiodothyronine, 5% FBS, 0.5% insulin-transferrin-selenium-G supplement, 1 nM cholera toxin, 10 ng/mL EGF, 0.4 μ g/mL hydrocortisone, 5 μ g/mL transferrin, 1% antibiotic-antimycotic²⁶). Matrices with fibroblasts alone were used as controls, wherein the number of cells was adjusted so as to make the number of cells on the matrices comparable to those with K. The submerged culture was continued for five additional days and then lifted to the air-liquid interface on a steel platform to enable epidermal stratification. The tissue-engineered skin and the respective controls were used in assays at different time points as described below.

Assessment of cell viability

Five-millimeter punch biopsies were collected at day 7, 14, and 21 of culture and placed in separate wells in a 24-well microtitre plate; C-GAG matrices without cells were used as controls. A standard 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to assess viability of cells in the matrices. Briefly, the punch biopsies were incubated with 250 μ L of tissue culture medium containing MTT substrate (5 mg/mL) for 4 h at 37°C, and the obtained MTT-formazan product was dissolved in 200 μ L of DMSO and quantified at 570 nm using a microplate reader (THERMOMax; Molecular Devices). The values are shown as mean optical density $\pm$ standard error.

Immunohistochemical analysis

For immunohistochemical staining of cytokeratin and laminin-5, 5-mm punch biopsies were collected at days 7, 14, and 21 of culture; keratinocyte-free C-GAG matrices were included as one of the controls. Briefly, the samples were

fixed with 4% paraformaldehyde (12 h) and 70% ethanol (12 h), paraffin embedded, sectioned at 5 μ m, and mounted on microscope slides. The sections were then deparaffinized with xylene and rehydrated in descending series of ethanol, and boiled in a citrate buffer (10 mM, pH 6.0) for 20 min for epitope retrieval. Subsequently, the sections were blocked with 10% BSA in PBS for 60 min to avoid nonspecific protein binding, and incubated overnight at 4°C with primary mouse anti-cytokeratin or laminin-5 antibodies (1:50 dilution; Dako), and then washed with PBS (3 $\times$, 5 min each); nonimmune human IgG (1:50 dilution) was used as negative control. Endogenous peroxide activity was quenched with 0.3% H₂O₂ (15 min) and the sections were incubated with a fluorescent secondary goat anti-mouse Alexa Fluor 488 antibody (1:500 dilution, 60 min, 25°C; Invitrogen). The stained sections were then mounted with ProLong[®] Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen), and viewed by fluorescent microscopy at 100 $\times$ and 200 $\times$ magnifications and photographed. The thickness of epidermis formed and relative immunofluorescence assessment were done using NIH ImageJ.

Gene expression studies

For gene expression analysis, 5-mm punch biopsies were collected at day 7, 14, and 21 of culture, and snap-frozen in liquid nitrogen; C-GAG matrices with fibroblasts, but no K were used as controls. The frozen samples were homogenized (2000 rpm, 2 min, Mikro-Dismembrator S; B. Braun Biotech International) and treated with Trizol reagent (Invitrogen); the resultant supernatant was stored at -80°C . Total RNA was then extracted from all the supernatants using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions, and quantified using Nano Drop 1000 spectrophotometer (Thermo Fisher Scientific). RNA extracts (0.5 μ g) from the samples were used for first-strand cDNA synthesis using M-MLV Reverse Transcriptase (Invitrogen) by incubation at three different conditions (25°C for 10 min followed by 37°C for 50 min and 70°C for 15 min). The resulting cDNA was used as a template for quantitative real-time polymerase chain reaction (qRT-PCR) amplification of genes of interest; the gene-specific primers were designed using Primer Express 3.0 (Table 1). qRT-PCR was done using Power Sybr Green[™] PCR master mix (ABI) in a total reaction volume of 25 μ L containing 5 μ L of a 1:10 dilution of cDNA product from the first-strand reaction and 1 μ M gene-specific forward and reverse primers on a StepOne Plus qRT-PCR system (ABI). The amplification conditions included denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing and primer extension at 60°C for 30 s. Amplification of genes was measured in terms of cycle threshold (C_T) values; the obtained C_T values were normalized with the C_T value of the house-keeping gene, hypoxanthineguanine phosphoribosyl transferase (HPRT). Gene expression levels are shown as mean fold change $\pm$ standard error. Quantitation was performed in the linear range of amplification as per standard procedure.

Assessment of barrier function

Epidermal barrier function was assessed by measuring surface electrical capacitance (SEC) of the C-GAG matrices on day 7, 14, and 21 of culture, using a Nova Dermal Phase Meter

TABLE 1. GENE-SPECIFIC PRIMERS USED IN THIS STUDY

Gene	Forward primer	Reverse primer
Keratin-5	GCTGAGAGCCGAGATTGACAA	TCCGCAATGGCGTTCTG
E-cadherin	CAGTGAACAACGATGGCATTTT	ACTGCTGCTTGGCCTCAAA
Nidogen	AAGGTGAAAGGAAGGATCTTTGTG	ACGTAAGAGTGGAGGTCAGTGTCT
Collagen type IV	TGCCAGGCGCAATGATAAA	GGCTGACGGGCATCATG
Collagen type VII	CTGAGGAGCTGAAGCGAGTTG	CCTCAAGATGCTGAAGTCATTGA
Laminin-5	GCTCCTGAGGACTGAAGTGAAAAC	AGACTCCCGTGGCATTGC
Perlecan	ATTCCCGGAGACCAGGTTGT	CTTCCGAGCCCACATCCA
Collagen type XVIII	GGGCCAGATGCCAACAGT	GCAGTGAGAAGTCACGGAAGAA
Hypoxanthine guanine phosphor-ribosyl transferase	GACCAGTCAACAGGGGACA	ACACTTCGTGGGGTCCTTTT

Primers required for specific amplification of genes of interest were designed using Primer Express 3.0, and were used in quantitative real-time polymerase chain reaction.

(DPM 9003; NOVA Technology Corporation). The measurements were taken at four different sites on each of the matrices, and expressed as mean DPM units $\pm$ standard error.

Statistical analysis

All experiments were conducted in triplicate and data are expressed as mean $\pm$ standard error. Statistical analysis was done using ANOVA with significance set at $p < 0.05$, $p < 0.01$, and $p < 0.001$ using Microsoft Excel 8.0.

Results

Viability of cells on C-GAG matrices

SF, DF, and K were isolated from lower abdominal tissue of three abdominoplasty patients and serially expanded. Passage 4SF and DF were then independently cultured or cocultured with K on crosslinked C-GAG matrices, and assayed at different time points for cell viability. C-GAG matrices with SF or DF alone were used as controls for fibroblasts, while matrices without any cells were used as cell-free controls. Cell viability was observed to be significantly higher for matrices with SF and K, DF and K, and SF, DF, and K compared to those with SF and DF alone at day 14 and 21 ($p < 0.001$). Cell viability was not significantly different for SF and K, DF and K, and SF, DF, and K (Fig. 1).

Epidermis formation in tissue-engineered skin

Tissue-engineered skin containing SF and K, DF and K, and SF, DF, and K were assessed at different time points for formation of epidermis by immunofluorescence staining for cytokeratin, a marker for keratinocytes. At all time points, epidermis for tissue-engineered skin with SF and K was observed to be continuous, better defined, and thinner compared to that with DF and K, which was intermittently broken (Fig. 2A–C; day 7 data not shown). To further understand differences in epidermis formation, gene expression of the specific epithelial proteins, E-cadherin and keratin-5, was examined at day 7, 14, and 21, by qRT-PCR. Tissue-engineered skin with SF and K expressed more E-cadherin than that with DF and K, which was significant ($p < 0.01$) on day 14 and 21 (Fig. 3A). Tissue-engineered skin with SF, DF, and K had similar expression of E-cadherin compared to that with SF and K. Matrices with fibroblasts alone did not express E-cadherin. The trend was similar for keratin-5, tissue-engineered skin with SF and K expressed more keratin-5 than that with DF and K, which was significant ($p < 0.01$) on day 14 and 21 (Fig. 3B). Tissue-engineered skin with SF, DF, and K had slightly higher expression of keratin-5 compared to that with SF and K, but this was not significant. Matrices with fibroblasts alone did not express keratin-5.

FIG. 1. Viability of SF and DF cultured with or without K on collagen–glycosaminoglycan (C-GAG) matrices. SF and DF were cultured for 21 days with or without K on crosslinked C-GAG matrices, and their viability was assessed by MTT assay on day 7, 14, and 21. Each bar represents mean $\pm$ standard error cell viability ($n = 3$ abdominoplasty patients). Significant differences in cell viability for matrices with SF and K, DF and K, and SF, DF, and K compared to those with SF and DF alone at day 14 and 21 ($p < 0.001$). MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; SF, superficial dermal fibroblasts; DF, deep dermal fibroblasts; K, keratinocyte.

FIG. 2. Immunofluorescent staining of the epidermis formed in tissue-engineered skin. 4',6-diamidino-2-phenylindole (DAPI) and cytokeratin staining were done, and then the images were merged. **(A)** Week 2 staining for tissue-engineered skin with SF and K, DF and K, or SF, DF, and K at 100 $\times$ magnification. Scale bar = 100 μ m. Blue indicates DAPI staining of nuclei on matrices, while green indicates the presence of cytokeratin. **(B)** Week 3 staining for tissue-engineered skin with SF and K, DF and K, or SF, DF, and K at 100 $\times$ magnification. Scale bar = 100 μ m. Blue indicates DAPI staining of nuclei on matrices, while green indicates the presence of cytokeratin. **(C)** Epidermal thickness of tissue-engineered skin with SF and K, DF and K, and SF, DF, and K at day 7, 14, and 21. Significant differences between tissue-engineered skin with SF and K, and DF and K at all times ($p < 0.001$). Color images available online at www.liebertpub.com/tea

FIG. 3. Assessment of relative gene expression of epidermal proteins in tissue-engineered skin by quantitative real-time polymerase chain reaction (qRT-PCR). mRNA was isolated on day 7, 14, and 21, and reverse transcribed using specific primers (Table 1). cDNA was subsequently amplified and relative gene expression was analyzed by qRT-PCR. Each bar represents mean $\pm$ standard error relative gene expression ($n=3$ abdominoplasty patients). **(A)** Relative gene expression for E-cadherin (ECAD). Significant differences between tissue-engineered skin with SF and K, DF and K, or SF, DF, and K on day 14 and 21 ($p<0.01$). **(B)** Relative gene expression for Keratin-5 (K5). Significant differences between tissue-engineered skin with SF and K, DF and K, or SF, DF, and K on day 14 and 21 ($p<0.01$).

Basement membrane formation in tissue-engineered skin

Tissue-engineered skin containing SF and K, DF and K, and SF, DF, and K were assessed at different time points for formation of basement membrane by immunofluorescence staining for laminin-5, a critical basement membrane protein. Tissue-engineered skin with SF and K had more laminin-5 compared to that with DF and K at all time points (Fig. 4A–C; day 7 data not shown). Tissue-engineered skin with SF, DF, and K had similar levels of laminin-5 compared to that with SF and K. Matrices with fibroblasts alone did not have laminin-5. To further investigate differences in basement membrane formation, gene expression of four different basement membrane proteins, laminin-5, nidogen, collagen type VII, and IV, was examined at day 7, 14, and 21, by qRT-PCR. Tissue-engineered skin with SF and K expressed more laminin-5 than that with DF and K, which was significant ($p<0.01$, $p<0.05$) at all time points (Fig. 5A). Matrices with fibroblasts alone did not express laminin-5. Tissue-engineered skin with SF and K expressed higher nidogen than that with DF and K, which was significant ($p<0.01$) at all time points (Fig. 5B). Matrices with fibroblasts alone expressed low levels of nidogen, which was significantly lower than that expressed by SF or DF cultured with K ($p<0.001$). Tissue-engineered skin with SF, DF, and K had similar expression of nidogen compared to that with SF and K. For collagen type VII, tissue-engineered skin with SF and K had higher expression compared to that with DF and K, which was significant ($p<0.01$) at all time points (Fig. 5C). Tissue-engineered skin with SF, DF, and K had lower expression of collagen type VII compared to that with SF and K, which was significant at day 7 and 14 ($p<0.05$). Matrices with fibroblasts alone expressed significantly lower collagen type VII

than those with SF and DF cultured with K ($p<0.001$). Tissue-engineered skin with SF and K had lower expression of collagen type IV than that with DF and K, which was significant on day 14 ($p<0.01$) and 21 ($p<0.001$; Fig. 5D). Matrices with fibroblasts alone express very little collagen type IV. Tissue-engineered skin with SF, DF, and K had similar expression of collagen type IV compared to that with SF and K, with no significant differences.

Proteoglycan expression in basement membrane of tissue-engineered skin

To investigate the possible differences in proteoglycan expression in the basement membrane of tissue-engineered skin with fibroblasts and K, gene expression of the proteoglycans, perlecan, and collagen type XVIII was examined. Tissue-engineered skin with SF and K expressed less perlecan than that with DF and K, which was significant ($p<0.01$) at all time points (Fig. 6A). Matrices with fibroblasts alone did not express perlecan. Tissue-engineered skin with SF, DF, and K expressed slightly higher perlecan than that with SF and K, which was significant only at day 7 ($p<0.05$). For collagen type XVIII, tissue-engineered skin with SF and K had lower expression compared to that with DF and K, which was significant ($p<0.001$) at all time points (Fig. 6B). Tissue-engineered skin with SF, DF, and K had higher expression of collagen type XVIII compared to that with SF and K, which was significant on day 7 and 14 ($p<0.001$). Matrices with fibroblasts alone did not express collagen type XVIII.

Epidermal barrier function in tissue-engineered skin

The barrier function of the epidermis is due to the semi-permeable stratum corneum layer in the upper epidermis. In

FIG. 4. Immunofluorescent staining of the basement membrane formed in tissue-engineered skin. DAPI and laminin-5 staining were done, and then the images were merged. **(A)** Week 2 staining for tissue-engineered skin with SF and K, DF and K, or SF, DF, and K at 100 $\times$ magnification. Scale bar=100 μ m. Blue indicates DAPI staining of nuclei on matrices, while green indicates the presence of laminin-5. **(B)** Week 3 staining for tissue-engineered skin with SF and K, DF and K, or SF, DF, and K at 100 $\times$ magnification. Scale bar=100 μ m. Blue indicates DAPI staining of nuclei on matrices, while green indicates the presence of laminin-5. **(C)** Laminin-5 immunofluorescence of tissue-engineered skin with SF and K, and SF, DF, and K measured relative to that of tissue-engineered skin with DF and K. Significant differences between tissue-engineered skin with SF and K and that with DF and K at all times ($***p < 0.001$). Color images available online at www.liebertpub.com/tea

FIG. 5. Assessment of relative gene expression of basement membrane proteins in tissue-engineered skin by qRT-PCR. mRNA was isolated on day 7, 14, and 21, and reverse transcribed using specific primers (Table 1). cDNA was subsequently amplified and relative gene expression was analyzed by qRT-PCR. Each bar represents mean $\pm$ standard error relative gene expression ($n=3$ abdominoplasty patients). **(A)** Relative gene expression for laminin-5 (LAM5). Significant differences between tissue-engineered skin with SF and K, DF and K on day 7, 14, ($**p<0.01$), and 21 ($*p<0.05$). **(B)** Relative gene expression for Nidogen (NIDO). Significant differences between tissue-engineered skin with SF and K and DF and K on day 7, 14, and 21 ($**p<0.01$). Significant differences between tissue-engineered skin with SF or DF and K and matrices with fibroblasts alone at all time points ($***p<0.001$). **(C)** Relative gene expression for collagen type VII (COL7). Significant differences between tissue-engineered skin with SF and K and DF and K on day 7, 14, and 21 ($**p<0.01$). Significant differences between tissue-engineered skin with SF and K and SF, DF, and K on day 14 and 21 ($*p<0.05$). Significant differences between tissue-engineered skin with SF or DF and K and matrices with fibroblasts alone at all time points ($***p<0.001$). **(D)** Relative gene expression for collagen type IV (COL4). Significant differences between tissue-engineered skin with SF and K and DF and K on day 14 ($**p<0.01$) and 21 ($***p<0.001$). Significant differences between tissue-engineered skin with SF or DF and K and matrices with fibroblasts alone at all time points ($***p<0.001$).

FIG. 6. Assessment of relative gene expression of basement membrane proteoglycans in tissue-engineered skin by qRT-PCR. mRNA was isolated on day 7, 14, and 21, and reverse transcribed using specific primers (Table 1). cDNA was subsequently amplified and relative gene expression was analyzed by qRT-PCR. Each bar represents mean $\pm$ standard error relative gene expression ($n=3$ abdominoplasty patients). **(A)** Relative gene expression for perlecan (PERL). Significant differences between tissue-engineered skin with SF and K and DF and K at all time points (** $p < 0.01$). Significant differences between tissue-engineered skin with SF and K and that with SF, DF, and K at day 7 (* $p < 0.05$). **(B)** Relative gene expression for collagen type XVIII (COL18). Significant differences between tissue-engineered skin with SF and K and DF and K at all time points (*** $p < 0.001$). Significant differences between tissue-engineered skin with SF and K and SF, DF, and K at day 7 and 14 (*** $p < 0.001$).

human skin, a water gradient exists across the stratum corneum; hydration is lowest on the surface of the skin and highest within the distal layers. SEC can be used to directly measure surface hydration of skin, which then can be correlated to the barrier function of the epidermis.²⁷ SEC was monitored for tissue-engineered skin with fibroblasts and K

at different time points; as the skin matured at the air-liquid interface, its surface was observed to dry out due to keratinization of the epidermis and formation of an epidermal barrier layer, and therefore the SEC decreased with time. For tissue-engineered skin with SF and K, SEC values were lower than that with DF and K, which was significant ($p < 0.01$) on

FIG. 7. Surface electrical capacitance (SEC) measurements of tissue-engineered skin. Using the Nova dermal phase meter, SEC measurements of tissue-engineered skin with SF and K, DF and K, or SF, DF, and K were taken on week 1, 2, and 3. Each data point refers to mean SEC $\pm$ standard error of five independent measurements done in triplicate for $n=3$ abdominoplasty patients. Continuous line indicates SEC measurements obtained for normal human skin. Significant differences between tissue-engineered skin with SF and K, DF and K on week 2 and 3 (** $p < 0.01$).

day 14 and 21 (Fig. 7). SEC values for tissue-engineered skin with SF, DF, and K were lower than that with DF and K, but higher than that with SF and K.

Discussion

In this study, SF or DF were independently cocultured with K on C-GAG matrices, which provided a true 3D microenvironment for cellular crosstalk, and the resulting tissue-engineered skin was assessed for differences in the basement membrane, epidermis formation, and barrier function. To the best of our knowledge, this is the first study to investigate the use of a selective subpopulation of dermal fibroblasts to make tissue-engineered skin, and examine its functional characteristics. SF, DF, and K were obtained from the same patients to control for cell source variability. The C-GAG matrices were conducive for the growth of cells as was evident from cell viability and DAPI staining. Matrices with K had higher cell viability compared to those with fibroblasts alone, possibly due to the differences in rates of proliferation of K or due to specific proteins produced by K that support survival and proliferation of fibroblasts. The epidermis formed in the tissue-engineered skin with SF and K was significantly thinner and had better overall integrity compared to that with DF and K, or SF, DF, and K. These differences in epidermal characteristics are likely due to differential interaction of SF and DF with K; our previous studies have found that DF behave similar to hypertrophic scar fibroblasts and contribute to skin fibrosis.⁹ The observed increased epidermal thickness in tissue-engineered skin with DF and K is consistent with our previous finding that human hypertrophic scars have a thicker epithelium compared to normal skin.²⁸ An extended phase of re-epithelialization due to prolonged activation of K even after completion of epithelialization is believed to cause a thicker epidermis in hypertrophic scars^{29,30}; our current results suggest that DF may have a role in this activation. Interestingly, differences in expression of epidermal proteins were also observed with the different types of tissue-engineered skin especially that with SF and K had higher levels of keratin-5 and E-cadherin that likely contributed to better epidermis formation.

The basement membrane orchestrates growth factor-mediated extracellular communication, cellular adhesion, migration, and differentiation,³¹ and is therefore critical. Tissue-engineered skin with SF and K, or DF and K showed distinct expression profiles for the different basement membrane components. Interestingly, tissue-engineered skin with SF and K produced more laminin-5 compared to that with

DF and K as was evident from gene and protein expression levels. Laminin-5 mediates adhesion of basal keratinocytes,^{32,33} and is indispensable for keratinocyte migration in wounded monolayers and epidermal wound healing.^{34–36} Tissue-engineered skin with SF and K also expressed significantly more nidogen and collagen type VII compared to that with DF and K, or SF, DF, and K. Extremely low levels of nidogen expression observed for SF and DF cultured alone further confirmed previous findings^{37,38} that the fibroblast–keratinocyte interaction is essential for optimal nidogen expression. Nidogen interacts with other basement membrane components, perlecan, laminin-5, and collagen type IV, and stabilizes networks formed by laminin and collagen type IV.^{39–41} Deficiency of nidogen results in accelerated epidermal maturation and affects the deposition of laminin-5, laminin-10, perlecan, and collagen type IV in the basement membrane,³⁸ and is therefore a key fibroblast-derived factor.⁴² On the other hand, collagen type VII is secreted by both fibroblasts and keratinocytes, and it self-assembles into anchoring fibrils that extend from the lamina densa of the epidermal basement membrane into the dermis and secure the epidermal–dermal junction. Paucity of collagen type VII anchoring fibrils results in separation of the epidermis from the dermis and thus leads to the pathogenesis of EB.^{43,44} Further, tissue-engineered skin with SF and K expressed less collagen type IV compared to that with DF and K, or SF, DF, and K. Collagen type IV is an essential collagenous glycoprotein in the basement membrane.⁴⁵ Extremely low levels of collagen type IV and VII expression observed for SF and DF cultured alone further confirmed previous findings³⁷ that the fibroblast–keratinocyte interaction is necessary for their optimal expression.

Interestingly, differences in the expression profile of the basement membrane proteoglycans, perlecan, and collagen XVIII, were observed; tissue-engineered skin with SF and K expressed less perlecan than that with DF and K, but had similar levels as that with SF, DF, and K. Perlecan is a large heparan sulfate proteoglycan that plays an important role in binding several growth factors and their receptors, cell migration, proliferation, and differentiation.^{46,47} It is critical for keratinocyte survival and epidermis formation, and its deficiency results in thinner epidermis.⁴⁸ Tissue-engineered skin with SF and K expressed less collagen type XVIII compared to that with DF and K, or SF, DF, and K. Collagen type XVIII, another heparan sulfate proteoglycan, is critical for the maintenance of basement membrane structural integrity; low levels have been observed to result in a thicker basement membrane⁴⁹ and enhanced wound healing due to increased vascularization of the wound bed and reduced wound area.⁵⁰

TABLE 2. COMPARISON OF THE PROPERTIES OF THE DIFFERENT TISSUE-ENGINEERED SKIN

	<i>Epidermis formation</i>	<i>Basement membrane</i>	<i>Basement membrane proteoglycans</i>	<i>Epidermal barrier</i>
SF + K	Thinner, but more continuous than that for DF + K	Better than that for SF + DF + K or DF + K	Less compared to DF + K, but similar to SF + DF + K	Best barrier formed compared to SF + DF + K, or DF + K
DF + K	Thicker and discontinuous compared to that with SF + K	Poor compared to SF + K, and SF + DF + K	More compared to SF + K, and SF + DF + K	Poor barrier formed compared to SF + K, or SF + DF + K
SF + DF + K	Similar in thickness to that of SF + K	Better than that for DF + K	Less compared to DF + K, but similar to SF + K	Better barrier formed compared to DF + K

For the development of a structured and organized epidermis, continuous interaction of the epidermal cells with the underlying dermal fibroblasts is essential.⁶ It is possible that depending on the composition of the underlying dermal fibroblasts (superficial vs. deep) there would be differences in the microenvironment the keratinocytes are continuously exposed to, which in turn would affect epidermal morphogenesis and regeneration. To evaluate functional differences in keratinocyte growth and differentiation when the underlying dermal fibroblasts are different, we monitored SEC of the tissue-engineered skin as a measure of epidermal barrier function. Tissue-engineered skin with SF and K had better epidermal barrier function than that with DF and K, and was comparable to that with SF, DF, and K. Further, the expression of E-cadherin, which is essential for tight junction formation in the epidermis,^{51–53} was upregulated in tissue-engineered skin with SF and K compared to that with DF and K; this likely contributed to the better epidermal barrier observed in the case of the former. Moreover, increased keratin-5 expression observed for tissue-engineered skin with SF and K compared to that with DF and K suggests better epidermal stratification and therefore enhanced epidermal barrier function, since keratin-5 is essential for the intermediate filament network formation required for epidermal stratification.⁵⁴

Taken together, our results demonstrate that tissue-engineered skin with SF and K forms a better basement membrane with higher expression of dermo-epidermal adhesive and anchoring proteins, and superior epidermis with enhanced barrier function compared to that with DF and K, or SF, DF, and K (Table 2). The selective use of SF in tissue-engineered skin may therefore be more beneficial for the treatment of patients with basement membrane disorders such as EB and other skin blistering diseases. In such patients, this novel tissue-engineered skin may be used as wound dressings and as important adjuncts to surgical treatment. In the past, there have been multiple reports of the use of other kinds of tissue-engineered skin for this purpose.^{55–57} Further work in animal models is necessary for effective translation of this treatment modality to the clinic.

Acknowledgments

We thank Ms. Arlene Oatway for access to the Advanced Microscopy Facility at the University of Alberta. This work was supported by the Canadian Institutes of Health Research, Alberta Heritage Foundation of Medical Research, and the Firefighter's Burn Trust Fund of the University of Alberta.

Disclosure Statement

No competing financial interests exist for any of the authors.

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Received: March 11, 2013

Accepted: September 3, 2013

Online Publication Date: October 16, 2013