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INVESTIGATIVE REPORT

# Standardized, Defined Serum-free Culture of a Human Skin Equivalent on Fibroblast-populated Collagen Scaffold

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**The defined, serum-free media used in the cultivation of skin equivalents are liable to inter-laboratory variations, require the preparation of multiple additives, and are potentially difficult to replicate. In this study, we assessed the use of standardized, serum-free and bovine pituitary extract-free keratinocyte culture media in the development of a human skin equivalent. After culture at the air-liquid interface for 3 weeks on a fibroblast-populated collagen lattice, an orthokeratinized, pluristratified epithelium was produced which expressed cytokeratins, cornified cell envelope precursors (involucrin, transglutaminase 1, filaggrin) and desmosomal components (desmoglein and desmocollin 1 and 3, plakophilin 1) in a differentiation-specific manner. There was also evidence of basement membrane reconstitution with collagen IV/VII, laminin 5, α6 and β4 integrin subunit expression at the epithelial-matrix junction. Overall, our findings demonstrate that readily available, defined organotypic culture media can be used to generate a reproducible skin equivalent with hallmarks of epidermal differentiation.** ***Key words: serum-free media; skin substitute; organotypic co-culture.***

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Skin equivalents reconstructed in three-dimensional organotypic cultures with a dermal substitute mimic the pluristratified epithelial architecture and differentiation characteristics of the native epidermis (1). Despite the well-established clinical and research utility of tissue-engineered skin equivalents (2, 3), there is no consistent formulation for organotypic culture media. Typically, such culture media require supplementation with numerous additives, including serum, vitamins, minerals, hormones and growth factors (4–7). The number of supplements may include up to 14–16 individual components (4, 7), making media preparation a potentially laborious process. The preparation of organotypic culture media is also subject to individual laboratory preferences, resulting in variations in media composition that may be difficult to replicate. Hence, it would be advantageous to use a readily available, standardized medium that does not require the preparation of multiple supplements and is free of undefined elements.

Recently, a defined, serum-free and bovine pituitary extract (BPE)-free culture organotypic culture medium kit has been made commercially available (CnT-02-3D, CELLnTEC, Bern, Switzerland). This system requires the addition of only three supplements at the time of use. According to the manufacturer's unpublished data, the defined medium enables the construction of a pluristratified, keratinized epithelia on a filter insert cultured for 14–28 days at the air-liquid interface. However, no thorough characterization of the resultant epithelia was performed. Although filter well inserts provide a robust model for in vitro studies, they lack an optimal milieu of matrix and fibroblasts (8, 9), and cannot be adapted to in vivo transplantation–conditions which are only satisfied by the presence of a dermal layer. As such, it has not been demonstrated whether this medium could be used successfully to reconstruct a human skin equivalent in the presence of a biological dermal substitute.

The aim of this study was to construct a human skin equivalent on a dermal fibroblast-embedded collagen scaffold using standardized, serum-free and tissue extract-free media in all stages of keratinocyte culture, and to characterize the differentiation profile, basement membrane formation and desmosomal adhesion in this equivalent.

## MATERIALS AND METHODS

### *Cell culture and media*

Defined Keratinocyte Serum Free Medium (SFM) and Epidermal Keratinocyte 3D Medium (CnT-02-3D) were purchased as medium kits from GIBCO (Grand Island, NY, USA) and CELLnTEC, respectively. Included supplements were added to the media at the time of use. The calcium concentration of the supplemented media was $<0.1$ mM (Defined Keratinocyte SFM) and 1.2 mM (CnT-02-3D). Both media are chemically defined and serum- and BPE-free.

Human primary foreskin-derived keratinocytes and dermal fibroblasts were purchased from Cascade Biologics (Portland, OR, USA). Epidermal keratinocytes were cultivated in Defined Keratinocyte SFM until approximately 70% confluence and cryopreserved after the first passage; cells from the second passage were used in experiments. Dermal fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM,

Sigma-Aldrich, St Louis, MO, USA) with 10% foetal calf serum (FCS), and cells from the second to fifth passages were used.

### *Construction of the skin equivalent*

Sterile, acid-solubilized, porcine tendon-derived collagen solution (Cellmatrix® Type I-A, 3 mg/ml, pH 3.0), 10 × concentrated Dulbecco's Modified Eagle Medium/Ham's F12 mixture (1:1), and sterile buffer (NaOH 50 mM, $NaHCO_3$ 260 mM, HEPES 200 mM) were obtained from Nitta Gelatin (Osaka, Japan). The collagen solution was mixed with 10 × DMEM/F12 medium on ice, followed by neutralization with the sterile buffer (volume ratio of collagen:DMEM/F12:buffer was 8:1:1, final pH 7.3–7.4). The reconstituted collagen solution was added to a suspension of dermal fibroblasts at a cell density of $1\times10^5$ cells/ml. A total of 3 ml of the above mixture was added into 3.0 micron, high pore density, polycarbonate cell culture inserts (Becton Dickinson, Franklin Lakes, NJ, USA) placed into 6-well deep-well plates (Becton Dickinson) and incubated at 37°C for 30 min to allow jellification. The fibroblast-populated collagen lattices were submerged in DMEM with 10% FCS before rinsing and equilibration with Defined Keratinocyte SFM one day prior to the addition of keratinocytes.

To define the area for keratinocyte seeding, a glass cloning ring (internal diameter 1 cm, Asahi Glass, Tokyo, Japan) was placed onto the gel surface in which a suspension of $1\times10^6$ keratinocytes in 700 µl of Defined Keratinocyte SFM was added. After 9 h, the ring was carefully removed. Skin equivalents were submerged for 2 days in the low-calcium Defined Keratinocyte SFM before switching to high-calcium CnT-02-3D for a further 2 days of submersion. Subsequently, the cultures were raised to the air-liquid interface by removing the media from the insert and lowering the media in the bottom wells until the meniscus was level with the gel surface. CnT-02-3D media in the lower wells were renewed every 2 days. Air-lifted cultures were harvested for frozen section at 1 and 3 weeks. Three independent experiments were performed; representative results are shown.

### *Indirect immunofluorescence*

Sections 6 µm thick were collected on poly-L-lysine-coated slides and fixed in cold acetone for 10 min. Sections were air-dried, rehydrated in phosphate-buffered serum (PBS), and then blocked with 10% goat serum for 30 min at room temperature. Primary and matching isotype control monoclonal antibodies were prepared in antibody diluent (Dako, Glostrup, Denmark); dilution ratios and/or final concentrations are listed in Table SI (available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1092). Primary antibody incubation was performed overnight at 4°C in a humidified slide chamber. Subsequently, sections were incubated with Alexa Fluor 488-conjugated goat anti-mouse antibody (Invitrogen, Carlsbad, CA, USA) for 45 min at room temperature, counterstained with 4',6-diamidino-2-phenylindole (DAPI), and mounted with an anti-fade reagent. Images were captured with an Axioplan 2 fluorescent microscope (Carl Zeiss, Oberkochen, Germany) with Axiovision imaging software.

## RESULTS

### *Histology*

After seeding human epidermal keratinocytes on the fibroblast-populated collagen gel and culturing them at the air-liquid interface, the resulting epithelium displayed histological features of the native epidermis. After one week, the epithelium was approximately 9–10 cell layers thick, and layers corresponding to the stratum basale, stratum spinosum, stratum granulosum and stratum corneum of the epidermis were recognizable (Fig. 1A). There was a transition in cell morphology from a cuboidal/columnar shape in the basal layer to a flattened shape in the uppermost layers. Three weeks after air-exposure, the epithelium exhibited a well-differentiated, epidermal architecture with an increase in stratification (Fig. 1B). The orderly architecture of the skin equivalent was maintained after 4 weeks of culture at the air-liquid interface (data not shown).

### *Expression of cytokeratins and cornified envelope precursors*

The presence and spatial distribution of early and late differentiation markers as an indication of normal epidermal differentiation were assessed by immunostaining (Fig. 2 and Fig. S1 (available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1092)). Cytokeratin (CK) 14, an intermediate filament in the stratum basale of the epidermis, was expressed in the basal and the immediate suprabasal layer. The suprabasal CK pairs 10 and 1 were also expressed; however, the expression of CK 1 was spatially delayed by 1–2 cell layers relative to CK 10. CK 2e was noted in the upper spinous and granular layers. CK16, expressed in the hyperproliferative epidermis (10) and during wound healing (11), was observed in the spinous layers of the epithelium.

With respect to cornified envelope-associated proteins, involucrin was expressed in the periphery of cells from the mid-upper spinous layers, producing a honeycomb pattern of staining (Fig. 2). The immunostaining of transglutaminase 1 decorated the granular layer of the epithelium, whilst filaggrin was also localized to the granular layer (Fig. S1).

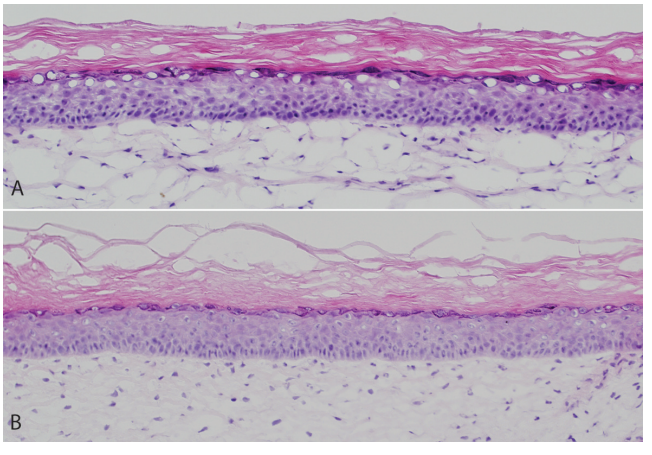


*Fig. 1.* Morphology of the reconstructed human epidermis on a dermal fibroblast-embedded collagen lattice cultured at the air-liquid interface for: (A) one; and (B) 3 weeks. (Haematoxylin and eosin (HE), original magnification × 200).

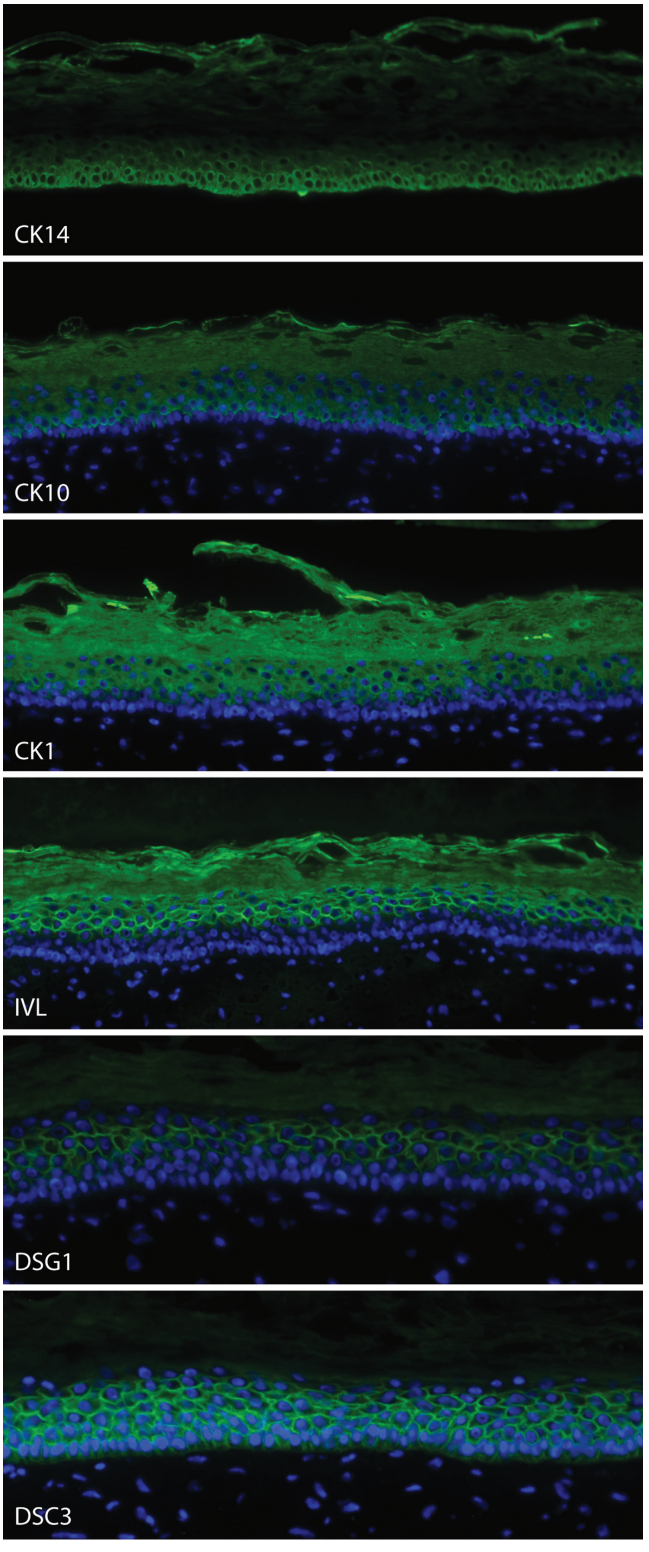


*Fig. 2.* Expression of epidermal differentiation markers and desmosome molecules in the skin equivalent. Cytokeratins (CK) 14, 10 and 1 were expressed in a differentiation-specific manner with the expression of CK 1 slightly delayed relative to CK 10. Involucrin (IVL) was expressed from the mid-spinous layer. Desmoglein (DSG) and desmocollin (DSC) isoforms specific to stratified epithelia were distributed in a differentiation-specific pattern. Immunofluorescent staining was visualized with an Alexa Fluor 488 conjugate and images were captured with an Axioplan 2 fluorescent microscope (original magnification ×200).

There was some degree of staining of the cornified layer; this phenomenon has been described previously in the immunohistochemistry of human skin equivalents (1).

No significant staining was discernible when slide sections was incubated with control isotype-matched antibodies at equivalent concentrations to the primary antibodies with images captured at equivalent exposure times (data not shown).

### *Deposition of basement membrane constituents*

The presence of basement membrane constituents at the dermoepidermal junction (DEJ) of the skin equivalent was investigated by immunostaining (Fig. 3). Antibodies directed against the α6 and β4 subunits of the α6β4 integrin decorated the DEJ. The deposition of laminin 5, a component of the anchoring filament of the lamina lucida, was also evident along the DEJ. Collagen IV and collagen VII, major components of the lamina densa and anchoring fibrils, respectively, were present along the DEJ of the skin equivalent.

### *Presence of desmosomal adhesion molecules*

As intercellular adhesion is fundamental to the structural integrity of the epidermis, we examined the presence of the desmosomal proteins which are specific to stratified epithelia. Desmoglein (DSG) and desmocollin (DSC) isoforms 1 and 3, as well as plakophilin 1, were expressed in a differentiation-specific manner in the skin equivalent (Figs 2 and S2 (available from: http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1092)). Positive staining for desmoglein 1 and desmocollin 1 were noted in the upper layers. Desmoglein 3 was detected in the lower layers, whilst there was prominent expression of desmocollin 3 in the lower to middle layers. Plakophilin 1 was noted in the immediate suprabasal layers, but was not obvious in the layers corresponding to the stratum granulosum.

## DISCUSSION

Skin equivalents constructed in organotypic cultures have been traditionally reliant on serum and/or BPE-based media. These formulations introduce undefined elements into the culture which are disadvantageous in biopharmacological testing and clinical applications (2). This has led to the development of serum-free organotypic cultures; however, there was often a period of submersion in a serum-supplemented environment with a switch to serum-free media during the submersion period (12, 13) or on air-exposure (8, 14–21). Chen et al. (7) described a complete serum-free organotypic co-culture model but keratinocytes were initially propagated as a monolayer in a serum

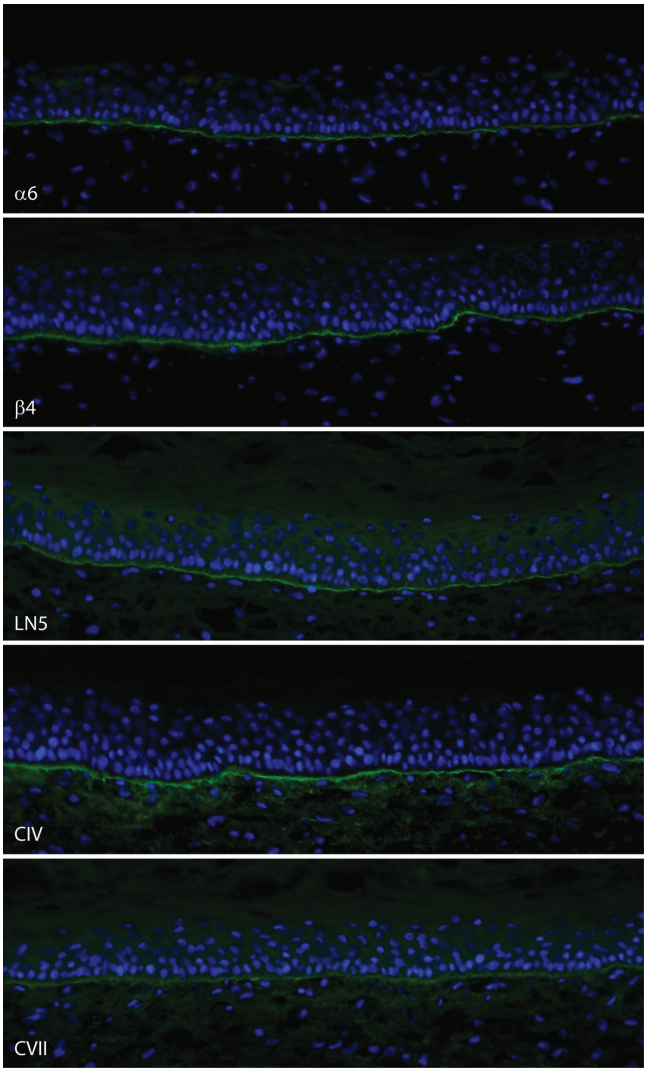


*Fig. 3.* Localization of basement membrane proteins in the skin equivalent. Immunostaining showed that constituents of the basement membrane including the subunits of the α6β4 integrin, laminin 5 (LN5), collagen IV (CIV) and collagen VII (CVII) were present along the dermoepidermal junction. Immunofluorescent staining was visualized with an Alexa Fluor 488 conjugate and images were captured with an Axioplan 2 fluorescent microscope (original magnification ×200).

or BPE-containing environment, with a potential carry-over of undefined elements into the organotypic culture. In our system, serum and BPE were absent in all stages of keratinocyte culture, including initial monolayer expansion, and in the submerged and air-exposed stages of the organotypic culture. Recently, Mujaj et al. (22) described the preparation of a defined, serum-free reconstituted epidermis on a decellularized dermis using keratinocytes that had been propagated in a defined medium supplemented with a combination of recombinant proteins. However, it was noted that only short-term epidermal reconstitution was shown (7 days of air-exposure), and that CK1 appeared to be irregularly expressed in the uppermost layers of the equivalent.

In our study, the reconstituted epithelium in defined media displayed a close histological resemblance to the native epidermis as early as one week after air-exposure, and a well-organized epidermal architecture was maintained after 4 weeks of culture at the air-liquid interface. The epithelium demonstrated an ordered process of epidermal differentiation, exemplified by the change in cell morphology from cuboidal/columnar cells in the basal layer to more flattened cells beneath the anucleate cornified layer. This was paralleled by an appropriate spatial distribution of early (C10) and late (CK2e, transglutaminase 1, filaggrin) differentiation markers, though there were notable exceptions. Normally restricted to the granular layer in the epidermis, IVL expression extended into the spinous layer. In addition, the suprabasal expression of CK1 was delayed by 1–2 cell layers relative to CK10. CK16, normally absent in the homeostatic epidermis, was present in the spinous layer of the reconstructed epidermis. These phenomena have been documented previously in organotypic skin models regardless of the presence or absence of serum. The premature expression of IVL and the presence of the CK 16/6 pair may reflect the perturbed, hyperproliferative state of the epithelium in organotypic co-culture conditions (8, 10, 15, 16, 23). The uncoupling of suprabasal CK 1/10 pair *in vitro* and their normalization after transplantation onto nude mice (16) or culture on a hyaluronic acid-based dermal scaffold (23), suggests that specific dermal requirements must be satisfied in order to achieve normal epidermal differentiation.

There was a continuous, linear distribution of basement membrane constituents in this defined, serum-free skin equivalent after 3 weeks at the air-liquid interface. Previous studies indicate that the reduction or absence of serum did not affect the expression of the basement membrane proteins (20, 21); rather, the presence of fibroblasts was crucial in the production of certain constituents (19, 20). In the absence of fibroblasts, there was a lack of laminin 5, nidogen, and types IV and VII collagen at the DEJ of serum-free organotypic cultures (20). In particular, soluble factors from human dermal fibroblasts appear to influence the production of laminin 5 and collagen VII (19). Consistent with reports by El Ghalbzouri et al. (20), the incorporation of $1 \times 10^5$ fibroblasts/ml of collagen in our system was optimal for the synthesis of laminin 5 and types IV and VII collagen at the DEJ (19).

Reports of construction of skin equivalents seldom investigate the presence and spatial expression of adherens structures, despite their critical role in epidermal integrity. Components of adherens junctions and desmosomes, including the presence of E-cadherin (8), plakoglobin (9), DSC 1 (23) and DSG1 and 3 (24) have been identified in the reconstituted epidermis of serum-supplemented cultures. As such, we are unaware of reports describing the expression of desmosomal

components in skin equivalents cultured in a defined, serum-free environment. Our findings show that plakophilin 1 as well as DSG and DSC isoforms specific to stratified epithelia were present in a differentiation-specific manner in the reconstructed epidermis, and were consistent with the pattern of distribution previously described in serum organotypic raft cultures (23, 24) and in the native epidermis (25).

Serum-containing DMEM was used in the initial culture of the fibroblast-containing dermal equivalent. In order to remove residual serum from the organotypic culture system, we submerged the dermal equivalent in defined medium and rinsed the construct prior to the seeding of keratinocytes. With these measures, the colour of the collagen lattice changed from red to that of the defined keratinocyte medium, suggesting that the serum-containing DMEM was washed out from the lattice. Despite this, we cannot completely rule out the presence of residual serum components in the collagen lattice.

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*The authors declare no conflicts of interest.*

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