Formation of keratinocyte multilayers on filters under air-lifted or submerged culture conditions in medium containing calcium, ascorbic acid, and keratinocyte growth factor

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Abstract

Three dimensional (3D) cell culture is a powerful *in vitro* technique to study the stratification and differentiation of keratinocytes. However, culture conditions, including culture media, supplements, and scaffolds (e.g., collagen gels with or without fibroblasts), can vary considerably. Here, we evaluated the roles of calcium, L-ascorbic acid phosphate magnesium salt *n*-hydrate (APM), and keratinocyte growth factor (KGF) in a chemically defined medium, EpiLife, in 3D cultures of primary human epidermal keratinocytes directly plated on polycarbonate filter inserts under air-lifted or submerged conditions. Eight culture media containing various combinations of these three supplements were examined. Calcium was necessary for the stratification and differentiation of keratinocytes based on the localization of keratins and involucrin. However, the localization patterns of keratins and integrin β4 were partially disrupted and Ki67-positive basal cells almost disappeared 3 weeks after air-lift. The addition of KGF, but not APM, prevented these changes. Further addition of APM markedly improved the tissue architecture, including basal cell morphology and the appearance of keratohyalin granules and localized involucrin in the upper suprabasal cells, even after 1 week. Although the submerged culture also formed cornified epithelium-like multilayers, involucrin was localized in the cornified layer, where nuclei were often found. Based on

these results, it is most effective to culture keratinocytes at the air–liquid interface in EpiLife medium supplemented with calcium, APM, and KGF to form well-organized and orthokeratinized multilayers as skin analogues.

Key words: keratinocytes, organotypic culture, cytokeratins, involucrin, integrin β4

Introduction

The human skin is a stratified squamous epithelium that consists of four layers, each composed of one of the following cell type: basal, spinous, granular, and cornified. Basal cells have proliferative activity and are attached to the basement membrane by hemidesmosomes, which mainly comprise integrin α6β4. There are at least 18 α and 8 β subunits in human integrins, which are transmembrane receptors, and these subunits combine to form 24 distinct integrin heterodimers (Barczyk et al. 2010; Fuchs et al. 1997; Hynes 2002; Takada et al. 2007). Basal cells differentiate and migrate toward the surface, passing through the spinous, granular, and cornified layers. Keratinocytes alter the expression of keratins (K), intermediate filament-forming proteins, during their migration to the surface. Keratins represent a very large family of proteins that form heteropolymers of type I (acidic) and type II (basic-neutral) keratin polypeptides in the

human epidermis (keratinized epithelium). Basal cells in the epidermis coexpress K5 (type II) and K14 (type I) (Moll et al. 2008; Törmä 2011). As these cells move upward and differentiate, they switch off their CK5/CK14 expression and turn on the expression of CK1 (type II) and CK10 (type I) (Kim et al. 2002; Törmä 2011). The transition from the spinous to the granular layer is accompanied by the suppression of CK1 and CK10 transcription (Zhu et al. 1999). In the uppermost epidermal layers (i.e., the upper stratum spinosum and stratum granulosum), differentiated keratinocytes express CK2 (Törmä 2011). The keratin expression profile is different in non-keratinized stratified squamous epithelia of the esophagus and oral cavity. The basal cell layer expresses CK5/CK14 and CK19 (Moll et al. 2008) and all suprabasal cells express CK4 and CK13 (Moll et al. 2008). A layer of highly crosslinked insoluble proteins, termed the cornified cell envelope (Kalinin et al. 2001), forms beneath the cell membranes of terminally differentiating epidermal keratinocytes. Involucrin is a major constituent of the cornified cell envelope and localizes in the granular cell layer and various levels of the spinous cell layer, depending on body site (Watt et al. 1987).

Three dimensional (3D) keratinocyte cultures can be roughly divided into three groups: keratinocytes plated (1) on a collagen gel with fibroblasts (Asselineau et al. 1986; O'Keefe et al. 1987), (2) on a collagen gel without fibroblasts (Pasonen-Seppanen

et al. 2001; Schurer et al. 1989) or on a de-epidermized dermis (Lamb and Ambler 2013; Ponec et al. 1988; Prunieras et al. 1983; Regnier et al. 1988; Rosdy and Clauss 1990), and (3) directly on polycarbonate filters (Poumay et al. 2004; Rosdy and Clauss 1990). In organotypic cocultures of keratinocytes and fibroblasts, keratinocytes release interleukin-1 (IL-1), stimulating dermal fibroblasts, which release keratinocyte growth factor (KGF) and granulocyte macrophage-colony stimulating factor (GM-CSF); these, in turn, stimulate keratinocyte growth and differentiation (Maas-Szabowski et al. 2000; Maas-Szabowski et al. 2001; Szabowski et al. 2000). However, Poumay et al. (2004) developed the simplest 3D culture method for keratinocytes without using KGF. Human keratinocytes form well-organized and keratinized multilayers when they are directly plated on polycarbonate filter culture inserts and cultured in EpiLife medium supplemented with 1.5 mM calcium and 50 μg/ml ascorbic acid (vitamin C) under air-lifted conditions (Poumay et al. 2004). Previous studies have also shown that keratinocytes on de-epidermized and de-vitalized skin (dermal equivalent) under air-lifted conditions in EpiLife medium containing 1.4 mM calcium are unable to form multilayers (Lamb and Ambler 2013). Extracellular calcium regulates the differentiation of cultured keratinocytes (Hennings and Holbrook 1983; Hennings et al. 1983; Hennings et al. 1980; Watt and Green 1982). Ascorbic acid treatment induces a better

organization of basal keratinocytes in organotypic cocultures of keratinocytes and fibroblasts (Marionnet et al. 2006; Wha Kim et al. 2002). However, most previous 3D culture systems include undefined components, such as serum, de-epidermized dermis, and/or unknown factors produced by fibroblasts in collagen gels (Asselineau et al. 1986; Lamb and Ambler 2013; O'Keefe et al. 1987; Pasonen-Seppanen et al. 2001; Ponec et al. 1988; Prunieras et al. 1983; Regnier et al. 1988; Schurer et al. 1989).

Here, we reevaluated the roles of calcium, ascorbic acid, and KGF in 3D keratinocyte cultures by examining the morphology and localization of proliferation and differentiation markers, such as Ki67, keratins, integrin β4, and involucrin.

Materials and Methods

Cells and reagents

Adult human epidermal keratinocytes (HEKa), EpiLife medium containing 0.06 mM calcium, and human keratinocyte growth supplement (HKGS) were purchased from Gibco (Grand Island, NY, USA). The components of EpiLife medium are published (http://www.thermofisher.com/jp/ja/home/technical-resources/media-formulation.275.html), but the concentrations of each component are proprietary. Human KGF was

purchased from PeproTech (Rocky Hill, NJ, USA). L-Ascorbic acid phosphate magnesium salt *n*-hydrate (APM), a stable derivative of ascorbic acid, was purchased from Wako (Osaka, Japan). Cell culture inserts (0.4-μm polycarbonate filter, 12 mm diameter) were purchased from Merck Millipore (Darmstadt, Germany).

Cell culture

HEKa was grown in EpiLife medium containing 0.06 mM calcium supplemented with HKGS (ELGS medium). HKGS contained 0.2% bovine pituitary extract, 5 μg/ml bovine insulin, 0.18 μg/ml hydrocortisone, 5 μg/ml bovine transferrin, and 0.2 ng/ml human epidermal growth factor. Cell suspensions of HEKa (7.5×10^5 cells/ml) in ELGS medium were seeded in cell culture inserts in 24-well plates. Each insert and each well contained 0.4 ml of cell suspension (3.0×10^5 cells) and 0.6 ml of medium. Cells were grown for one or two days until they reached 100% confluence. The growth medium (ELGS) inside and outside of the insert was replaced with the 3D medium (ELGS-3D) and cells were cultured for 16–24 h to form intercellular adhesion structures. Then, inserts (up to six) were transferred to a 60-mm (diameter) culture dish containing 3.2 ml of the 3D medium and air-lifted cultures were established by removing the 3D medium in the inserts. The ELGS-3D medium was made by adding

various combinations of 1.5 mM $CaCl_2$ (Poumay et al. 2004), 0.283 mM APM, corresponding to 50 μg/ml ascorbic acid (Poumay et al. 2004), and 10 ng/ml KGF (Maas-Szabowski et al. 2000 and 2001; Szabowski et al. 2000) to the ELGS medium. In this report, 3D media containing calcium, calcium plus APM, calcium plus KGF, and calcium plus APM and KGF are referred to as medium-C, medium-CA, medium-CK, and medium-CAK, respectively. The surfaces within the inserts were kept dry following air-lift by removing excess 3D medium in the inserts. The medium was changed every 2 days and the air–liquid interface culture was maintained for up to 3 weeks. For the submerged culture, 0.2 ml of ELGS-3D medium was added to inserts and the media in both the inserts and the 60-mm dishes were changed every 2 days for up to 2 weeks.

Immunofluorescence microscopy

Filters with cultured keratinocytes were fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at 4°C, washed in PBS and cut off from the inserts. Porcine maxillary oral mucosa containing the buccal mucogingival junction, obtained from a slaughter house, was fixed with 1% paraformaldehyde in PBS overnight at 4°C and washed in PBS. The filters with cells and pig oral mucosa were sequentially soaked in 10%, 20%, and 30% sucrose in PBS at 4°C for 1–3 h each, and

then embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Japan, Tokyo, Japan). Cryosections (4 μm) were cut and mounted onto glass slides. Some sections were stained with hematoxylin and eosin. Cryosections were washed in PBS and incubated in 0.2% Triton-X 100 in PBS for 15 min for permeabilization. Subsequently, the sections were washed in PBS, and then incubated with 1% bovine serum albumin in PBS (BSA-PBS) for 15 min to block nonspecific binding. The sections were incubated with primary antibodies diluted in BSA-PBS for 1 h. Rabbit anti-keratin (K)-1 polyclonal antibody (pAb) (1:100), mouse anti-K4 monoclonal antibody (mAb) (1:100), rabbit anti-K5 pAb (1:100), rabbit anti-K14 pAb (1:100), mouse anti-Ki67 mAb (1:200), and mouse anti-involucrin mAb (1:100) were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Mouse anti-K10 mAb (1:100) and mouse integrin β4 mAb were obtained from Abcam (Cambridge, UK). Mouse anti-K13 mAb (1:100) was obtained from Nordic-Mubio (Susteren, Netherlands). Mouse anti-K2 antibody (1:200) was obtained from Progen Biotechnik GmbH (Heidelberg, Germany). After rinsing them four times in PBS, the sections were incubated with anti-mouse immunoglobulin (Ig) conjugated with Alexa 488 and anti-rabbit Ig conjugated with Alexa 568 (Molecular Probes, Eugene, OR, USA) at a 1:400 dilution in BSA-PBS for 30 min in the dark. The sections were then washed four times in PBS and mounted in

Vectashield mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Images were obtained under an LSM710 confocal laser scanning microscope (Oberkochen, Germany).

Results

Calcium is necessary for stratification

To examine the factors that induce the stratification of HEKa cells plated on the polycarbonate filter of cell culture inserts followed by air-lift for 1 week, we prepared ELGS medium (low calcium medium) containing various combinations of calcium chloride, APM, and KGF (Supplementary Fig. 1). HEKa cells cultured in ELGS medium (control) formed small cell aggregates, but did not form a continuous cell layer (Supplementary Fig. 1a). When cells were cultured in medium containing at least 1.5 mM calcium, they formed multiple layers (Supplementary Fig. 1b, e, f, h). Stratification was not observed (Supplementary Fig. 1c, d, g) when cells were cultured in the low-calcium medium, regardless of the presence or absence of APM and KGF, similar to the control (Supplementary Fig. 1a). Therefore, calcium is a key factor for stratification.

Effects of calcium, APM, and KGF on the morphology of multilayers

The multilayers induced by the air-lifted culture were stained with hematoxylin and eosin to examine the effects of calcium, APM, and KGF on multilayer morphology. The basal cell nuclei were oriented parallel to the filter membrane in multilayers induced by medium-C (Fig. 1a-a”), -CA (Fig. 1b-b”), and -CK (Fig. 1c-c”). In contrast, the basal nuclei were cuboidal in multilayers induced by medium-CAK (Fig. 1d-d”). The cornified layer was observed within one week in all culture media (Fig. 1a-d), and the thicknesses of both cornified and living cell layers in all culture media increased as the culture aged (Fig. 1a-a”, b-b”, c-c”). Keratohyalin granules were visible just below the cornified cell layer in medium-CAK after 2 and 3 weeks (arrowheads in Fig. 1d’, d”). Flattened nuclei were occasionally observed in the cornified cell layer in medium-CK after two and three weeks (arrows in Fig. 1c’, c”). Three weeks after air-lift, multilayers in medium-C and medium-CA seemed to be on the decline, as indicated by the clear decrease in the number of basal cell nuclei (Fig. 1a”, b”). However, multilayers in medium-CK and medium-CAK still had a considerable number of basal cell nuclei (Fig. 1c”, d”). The morphology of multilayers in medium-CAK three weeks after air-lift (Fig. 1d”) was similar to that of keratinized stratified squamous epithelium *in vivo*.

Proliferation and differentiation of keratinocytes in multilayers

To examine the proliferation and differentiation of keratinocytes in multilayers, we examined the localization of Ki67 (a proliferation marker) and several differentiation markers, including integrin β4, K5, and K14 as basal cell markers, K1 and K10 as suprabasal cell markers in the keratinized epithelium, and K4 and K13 as suprabasal cell markers in the non-keratinized epithelium. In control sections, non-specific staining was often observed in the cornified layer (Supplementary Fig. 2).

Ki67 was detected in some nuclei of basal cells at 1 and 2 weeks after air-lift or immersion in all culture media (Fig. 2a-e, a'-e', red stain). The basal cells in medium-CK (Fig. 2c") and -CAK (Fig. 2d") still expressed the Ki67 protein 3 weeks after air-lift, while those in medium-C (Fig. 2a") and -CA (Fig. 2b") rarely expressed Ki67. These results suggested that KGF maintained the proliferation of basal cells for at least 3 weeks.

The localization of integrin β4 (Fig. 3, green stain) was examined in multilayers 3 weeks after air-lift. Integrin β4 was localized in the basal cell membranes of the basal cells connected with the filter and along the boundaries of the basal cells and the lower suprabasal cells when cultured in medium-CK and medium-CAK (Fig. 3c, d). Staining of integrin β4 in the basal cell membranes of the basal cells decreased when

they were cultured in medium-C (Fig. 3a). The multilayer detached from the filter and integrin β4 staining disappeared for cells cultured in medium-CA (Fig. 3b). These results suggested that simultaneous treatment with calcium and KGF (medium-CK or -CAK) maintained the localization of integrin β4 in the basal cell membranes of the basal cells.

The maxillary buccal mucogingival junction is the area where the non-keratinized stratified squamous epithelium (buccal mucosa) connects to the keratinized stratified squamous epithelium (gingiva). Arrows (Supplementary Fig. 3a, b) indicate the boundary between the non-keratinized (left part) and keratinized (right part) epithelium. At the maxillary buccal mucogingival junction, K5 and K14 were localized in the basal cell layer in the non-keratinized epithelium, but in all cell layers, including the cornified layer, in the keratinized epithelium (Supplementary Fig. 3a, b, red stain). In the buccal skin, which is keratinized, K5 and K14 were localized in the basal and whole suprabasal cell layers (Supplementary Fig. 3c, d, red stain). Thus, using antibodies against K5 and K14, the proteins were detected in the basal and whole suprabasal cells in the keratinized epithelium *in vivo*, respectively.

K4 staining in the cornified cell layer in medium-CAK (Fig. 4d', d", green stain) may be nonspecific because similar staining was detected in the control

(Supplementary Fig. 2d', d", green stain). The absence of K4 was consistent with the formation of the cornified cell layer. K5 was detected in the basal and suprabasal cells in all culture media after 1, 2, and 3 weeks, and faintly in the cornified layer (Fig. 4, red stain). When cells were cultured in medium-C or -CA for 3 weeks, K5 staining in the basal and suprabasal cells was heterogeneous (Fig. 4 a", b", red stain). Flattened nuclei were occasionally observed in the cornified cell layer (arrowheads in Fig. 4c") after three weeks of culture in medium-CK.

K10 was detected in the suprabasal cells after 1, 2, and 3 weeks, and in the cornified layer with various staining intensities (Fig. 5, green stain). The localization of K10 in suprabasal cells indicated differentiation of multilayers. We noted that K10 staining in the suprabasal cells cultured in medium-CK or -CAK for 3 weeks was consistently intense and homogeneous (Fig. 5c", d", green stain), while it was heterogeneous for those cultured in medium-C or -CA (Fig. 5a", b", green stain). These results suggested that the addition of KGF maintained the appropriate localization of K10 three weeks after air-lift. Similar to K5, K14 was detected in the basal and suprabasal cells after 1, 2, and 3 weeks, and was faintly observed in the cornified layer (Fig. 5, red stain). K14 staining in the upper suprabasal cells cultured in medium-CA for three weeks was faint (Fig. 5b", red stain). Flattened nuclei were occasionally observed

in the cornified layer (arrowheads in Fig. 5c', c") after 2 and 3 weeks when cultured in medium-CK.

K13 staining in the cornified cell layer in medium-CAK (Fig. 4d', d", green stain) may be nonspecific because similar staining was detected in control cells (Supplementary Fig. 2d', d", green stain). The absence of K13 was consistent with the formation of the cornified layer. K1 was detected in the suprabasal cells in all culture media after 1, 2, and 3 weeks, and in the cornified layer with fluctuating staining intensities (Fig. 6, red stain). The localization of K1 in the suprabasal cells indicated differentiation of multilayers. K1 staining in the suprabasal cells cultured in medium-CK or -CAK for 3 weeks was consistently intense and homogeneous (Fig. 6c", d", red stain), while it was heterogeneous for those cultured in medium-C or -CA (Fig. 6a", b", red stain). These results suggested that the addition of KGF maintained the appropriate localization of K1 3 weeks after air-lift. Flattened nuclei were occasionally observed in the cornified layer (arrowheads in Fig. 6c', c") after 2 and 3 weeks when cells were cultured in medium-CK.

Induction of stratification and differentiation by submerged culture of keratinocytes

It is appropriate to culture keratinocytes at the air–liquid interface to reconstruct a skin analogue for the keratinized epithelium because the epidermis is dry. In contrast, the oral epithelium, which is always covered with saliva, is a non-keratinized epithelium, except for the hard palate and gingiva. For this reason, we compared the air-lifted culture to the submerged culture, using medium-CAK. After 1 week of submerged culture (9 days after plating), K5 and K14 were detected in the whole multilayer (Fig. 7a, b, c, red stain). K10 (Fig. 7b, green stain) and K1 (Fig. 7d, red stain) were localized in the suprabasal cell layer, while K4 and K13 were not detected (Fig. 7a, d, green stain). Intense staining of integrin β4 was localized in the basal cell membranes, and weaker signals were detected along the boundaries of the basal and lower suprabasal cells (Fig. 7e, green stain).

After 2 weeks of submerged culture (16 days after plating), the cornified layer, which often contained nuclei (arrowheads in Fig. 7a’-f’), became thicker. K5 staining decreased in the lower cornified layer (Fig. 7a’, red stain). K4 and K13 staining in the cornified layer 2 weeks after immersion (Fig. 7a’, d’, green stain) may be nonspecific because similar staining was detected in the control (Supplementary Fig. 2e’, green stain). The absence of K4 and K13 was consistent with the formation of the cornified cell layer (Fig. 7f, f’). K10 (Fig. 7b’, green stain) was detected in the suprabasal cells

and the lower cornified layer, where K14 staining (Fig. 7b', c', red stain) decreased. Integrin β4 staining after two weeks (Fig. 7e', green stain) was similar to that observed after one week (Fig. 6e, green stain).

Localization of involucrin and K2 in multilayers

Involucrin and K2 are late-differentiation markers, unlike K1 and K10 (which are early differentiation markers), and localize in the granular cell layer and various levels of the spinous cell layer at particular body sites, e.g., the interfollicular epidermis, palms, and soles. For this reason, we examined the localization of involucrin and K2 in multilayers.

One week after air-lift, involucrin was localized in the suprabasal cells, but not in the thin uppermost cornified layer (Fig. 8a, b, c, green stain). However, involucrin was only localized in the upper suprabasal cells with medium-CAK (Fig. 8d, green stain). Two weeks after air-lift, the cornified layer was easily recognized (Fig. 8f'-i'). Involucrin was localized in the suprabasal cells using medium-C and -CA (Fig. 8f, g, green stain), and more specifically in the upper suprabasal cells using medium-CK and -CAK (Fig. 8h, i, green stain). The cornified layer induced by air-lifted conditions was negative for involucrin (Fig. 8a-d, f-i). In multilayers formed under immersion

conditions after 1 week, involucrin was localized within all suprabasal cells and the thin uppermost cornified cell layer (Fig. 8e, green stain). After 2 weeks, involucrin was localized in the uppermost suprabasal cells and the cornified layer, with only a few nuclei (Fig. 8j, green stain).

One and two weeks after air-lift, K2 was detected in the upper suprabasal cells (just below the cornified cell layer) in all culture media (Fig. 9a-d, a'-d', green stain). It was noted that K2 staining in the upper suprabasal cells was intense in medium-CA after 1 week (Fig. 9b, green stain), weak after 2 weeks (Fig. 9b', green stain), and disappeared after 3 weeks (Fig. 9b", green stain). K2 was detected in the upper suprabasal cells 3 weeks after air-lift in medium-CK and -CAK (Fig. 9c", d", green stain), but not in medium-C and -CA (Fig. 9a", b", green stain). Faint green staining for K2 was occasionally detected in the cornified cell layer (Fig. 9b', b"). Under submerged conditions for 1 and 2 weeks, K2 was detected in the upper suprabasal cells, but not in the cornified cell layer often with nuclei (Fig. 9e, e', green stain). The localization of proliferation and differentiation markers is summarized in Table 1.

Discussion

In this study, we found that calcium is a necessary factor to enable the

formation of cornified epithelium-like multilayers after 2 weeks under air-lifted culture conditions, although calcium alone showed partially disturbed localization of keratins and integrin β4 and few Ki67-positive basal cells after 3 weeks. The simultaneous addition of calcium and KGF, but not calcium and APM, prevented these changes, though flattened nuclei were occasionally observed in the cornified layer. Simultaneous treatment with calcium, KGF, and APM markedly improved the tissue architecture, including basal cell morphology and the appearance of keratohyalin granules and localized involucrin in the upper suprabasal cells, even after 1 week. Although submerged cultures in medium-CAK after 2 weeks also formed cornified epithelium-like multilayers, basal cells arranged parallel to the filter and involucrin were localized in the cornified layer, where nuclei were often found.

In contrast to a previous report (Lamb and Ambler 2013), the addition of only calcium (1.5 mM) to the EpiLife medium induced the stratification and differentiation of keratinocytes, as indicated by the localization of K1, K2, K10, and involucrin and by the absence of K4 and K13. It has been reported that K5 and K14 are localized in basal cells and, more scarcely, in lower suprabasal cells (Moll et al. 2008; Törmä 2011). In this study, K5 and K14 were detected in the basal and whole suprabasal cells of multilayers induced by calcium as well as those of the keratinized epithelium *in vivo*.

Consistent with the present results, K14 was previously detected in the spinous and granular layers (Stoler et al. 1988). Therefore, the addition of calcium alone to the present 3D keratinocyte culture could form the cornified epithelium-like multilayer, based on the localization of these differentiation markers. The alteration of the extracellular calcium concentration regulates the differentiation of cultured keratinocytes (Hennings and Holbrook 1983; Hennings et al. 1983; Hennings et al. 1980; Watt and Green 1982). In fact, the epidermis shows a calcium gradient, i.e., it exhibits low levels of calcium in the basal and lower spinous cell layers, but high levels in the granular cell layer (Forslind et al. 1984; Mauro et al. 1998; Menon et al. 1985). The present results confirmed the role of calcium in keratinocyte differentiation.

Three weeks after air-lift, the multilayers formed in media containing only calcium or calcium and APM seemed to be on the decline, as indicated by the clear decrease in the number of basal cells with Ki67 and integrin β4. Thus, calcium, as well as APM, could not preserve the characteristics of the basal cells, such as the proliferation and conservation of integrin β4 in the basal membrane, after 3 weeks. The addition of KGF, however, maintained the number of basal cells expressing Ki67, and integrin β4 was still localized in the basal membrane of the basal cells. KGF is a paracrine growth factor secreted by fibroblasts in the underlying connective tissue and

stimulates the proliferation of epithelial cells expressing KGF receptors (Miki et al. 1992; Rubin et al. 1989; Werner et al. 1994). Here, KGF could be responsible for maintaining the proliferation of basal cells after 3 weeks. In the present study, basal cells were cuboidal in shape and arranged perpendicularly to the filter in medium-CAK, while they were parallel to the filter in medium-C, -CA, and -CK. Furthermore, keratohyalin granules were visible just below the cornified cell layer in medium-CAK, but not in medium CK, after two and three weeks. These results suggest that APM may improve the tissue architecture in cooperation with KGF, probably providing a good balance between keratinocyte proliferation and differentiation. Treatment with ascorbic acid induces well-organized multilayers in 3D keratinocyte cultures on fibroblast-populated collagen gels (Wha Kim et al. 2002).

The importance of the balance between keratinocyte proliferation and differentiation to induce well-organized multilayers has been reported. Retinoic acid regulates keratin mRNA and protein expression (Törmä 2011) and promotes the retention of undifferentiated keratinocyte morphology (Darmon and Blumenberg 1993). In contrast, 1,25-dihydroxy vitamin D_3 stimulates keratinocyte differentiation (Hosomi et al. 1983). Simultaneous treatment with retinoic acids and 1,25-dihydroxy vitamin D_3 induces well-organized multilayers with a prolonged lifespan, probably by ensuring a

good balance between keratinocyte proliferation and differentiation (Gibbs et al. 1996).

Involucrin is a late-differentiation marker expressed in the upper suprabasal cells in the granular cell layer *in vivo* (Gibbs and Ponec 2000; Wha Kim et al. 2002). Supplementation with APM and KGF (medium-CAK), but not KGF alone (medium-CK), resulting in involucrin localization in the upper suprabasal cells, even after 1 week. Supplementation with APM (medium-CA) also induced early (1 week after air-lift) expression of K2, a late-differentiation marker, while K2 rapidly disappeared 2 weeks after air-lift. These results suggest that APM plays a role in enhancing the differentiation of keratinocytes either alone or in cooperation with KGF.

We compared the air-lifted culture to the submerged culture, using medium-CAK. We expected that submerged culture conditions would generate a non-keratinized stratified epithelium, as reported previously (Fig. 1c in Ponec et al., 1988). However, submerged cultures yielded a cornified layer, often with nuclei. In addition, submerged cultures switched the localization of involucrin from the upper suprabasal layer to the cornified layer with nuclei. These results suggest that submerged cultures partly disturbed the differentiation of keratinocytes to the orthokeratinized epithelium. In fact, prolonged exposure to high humidity has been shown to eliminate the calcium gradient in the epidermis and generate a decrease in epidermal

differentiation-specific proteins, including involucrin (Elias 2002). Therefore, the present results confirmed that submerged cultures are not suitable for the reconstruction of skin analogues.

In conclusion, calcium is necessary for stratification and differentiation of keratinocytes. Addition of KGF, but not APM, induced a prolonged lifespan. Further addition of APM improved the tissue architecture in cooperation with KGF.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1 Effects of calcium, APM, and KGF on the morphology of multilayers

The multilayers were formed using medium-C containing Ca (a-a”), medium-CA containing Ca and APM (b-b”), medium-CK containing Ca and KGF (c-c”), and medium-CAK containing Ca, APM and KGF (d-d”). Cryosections, prepared from multilayers one (a-d), two (a’-d’), and three (a”-d”) weeks after air-lift, were stained with hematoxylin and eosin. The basal cell nuclei were oriented parallel to the filter in multilayers induced by medium-C (a, a’, a”), -CA (b, b’, b”), and -CK (c, c’, c”). In contrast, the basal nuclei were cuboidal in multilayers induced by medium-CAK (d, d’,

d”). Three weeks after air-lift, basal cells decreased in multilayers formed in medium-C (a”) and medium-CA (b”). However, multilayers in medium-CK (c”) and medium-CAK (d”) still had a considerable number of basal cell nuclei. The morphology of multilayers in medium-CAK three weeks after air-lift (d”) was similar to that of the keratinized stratified squamous epithelium *in vivo*. Arrows (c’, c”) and arrowheads (d’, d”) indicate flattened nuclei in the cornified cell layer and keratohyalin granules, respectively. The scale bar in d” represents 20 μm and applies to all images

Fig. 2 Localization of Ki67, a proliferation marker, in the multilayers one, two, and three weeks after air-lift or immersion

The multilayers were formed by medium-C (a-a”), medium-CA (b-b”), medium-CK (c-c”), and medium-CAK (d-d”, e, e’). One (a-e), two (a’-e’), and three (a”-d”) weeks after air-lift or immersion, cells were immunostained to detect Ki67 (red), and nuclei were stained with DAPI (blue). Ki67 was detected in some nuclei of the basal cells 1 and 2 weeks after air-lift in all culture media (a-d, a’-d’, red stain). The basal cells in medium-CK (c”) and -CAK (d”) still expressed Ki67 protein 3 weeks after air-lift while those in medium-C (a”) and -CA (b”) rarely expressed it. The scale bar in e’ represents

20 μm and applies to all images

Fig. 3 Localization of integrin β4 in the multilayers three weeks after air-lift

Keratinocytes were cultured for 3 weeks at the air–liquid interface in medium-C (a), medium-CA (b), medium-CK (c), or medium-CAK (d). Three weeks after air-lift, cells were immunostained to detect integrin β4 (green stain), and nuclei were stained with DAPI (blue). Integrin β4 was localized in the basal cell membranes of the basal cells as well as along cell boundaries of the basal cells and the lower suprabasal cells when cultured in medium-CK and medium-CAK (c, d). Staining of integrin β4 in the basal cell membranes of the basal cells decreased when cultured in medium-C (a). The multilayer detached from the filter and integrin β4 staining disappeared for cells cultured in medium-CA (b). The scale bar in d represents 20 μm and applies to all images

Fig. 4 Localization of K4 and K5 in the multilayers one, two, and three weeks after air-lift

The multilayers were formed by medium-C (a-a”), medium-CA (b-b”), medium-CK

(c-c”), and medium-CAK (d-d”). One (a-e), two (a’-e’), and three (a”-e”) weeks after air-lift, cells were doubly immunostained to detect K4 (green) and K5 (red), and nuclei were stained with DAPI (blue). K4 was not detected in multilayers in all culture media (a-d, a’-d’, a”-d”, green stain). K5 was detected in the basal and suprabasal cells in all culture media (a-d, a’-d’, a”-d”, red stain). When cells were cultured in medium-C or -CA for three weeks, K5 staining in the basal and suprabasal cells was heterogeneous (a”, b”, red stain). Arrowheads (c”) indicate flattened nuclei, which were occasionally observed in the cornified layer when cultured in medium-CK. The scale bar in d” represents 20 μm and applies to all images. CL, cornified cell layer; SB, suprabasal cell layer

Fig. 5 Localization of K10 and K14 in the multilayers one, two, and three weeks after air-lift

The multilayers were formed in medium-C (a-a”), medium-CA (b-b”), medium-CK (c-c”), and medium-CAK (d-d”). One, two, and three weeks after air-lift, cells were doubly immunostained to detect K10 (green) and K14 (red), and nuclei were stained with DAPI (blue). K10 (a-d, a’-d’, a”-d”, green stain) was detected in the suprabasal

cells, and in the cornified layer with various staining intensities. K10 staining in the suprabasal cells cultured in medium-CK or -CAK for three weeks was consistently intense and homogeneous (c”, d”, green stain), but was heterogeneous for those cultured in medium-C or -CA (a”, b”, green stain). K14 (a-d, a’-d’, a”-d”, red stain) was detected in the basal and suprabasal cells. K14 staining in the upper suprabasal cells cultured in medium-CA for 3 weeks was faint (b”, red stain). Arrowheads (c’, c”) indicate flattened nuclei, which were occasionally observed in the cornified layer when cultured in medium-CK. The scale bar in d” represents 20 μm and applies to all images. CL, cornified cell layer; SB, suprabasal cell layer

Fig. 6 Localization of K13 and K1 in the multilayers one, two, and three weeks after air-lift

The multilayers were formed in medium-C (a-a”), medium-CA (b-b”), medium-CK (c-c”), and medium-CAK (d-d”). One, two, and three weeks after air-lift, cells were doubly immunostained to detect K13 (green) and K1 (red), and nuclei were stained with DAPI (blue). K13 (a-d, a’-d’, a”-d”, green stain) was not detected in multilayers in any culture media. K1 (a-d, a’-d’, a”-d”, red stain) was detected in the suprabasal cells, and

in the cornified layer with various staining intensities. K1 staining in the suprabasal cells cultured in medium-CK or -CAK for three weeks was consistently intense and homogeneous (c", d", red stain), and was heterogeneous for those cultured in medium-C or -CA (a", b", red stain). Arrowheads (c', c") indicate flattened nuclei, which were occasionally observed in the cornified layer when cultured in medium-CK. The scale bar in d" represents 20 μm and applies to all images. CL, cornified cell layer; SB, suprabasal cell layer

Fig. 7 Differentiation in the multilayers formed by submerged culture

Keratinocytes grown under submerged conditions for 1 or 2 weeks in medium-CAK were immunostained to detect K4 and K5 (a, a'), K10 and K14 (b, c, b', c'), K13 and K1 (c, c'), and integrin β4 (d, d'), and stained with DAPI (blue). Some were stained with hematoxylin and eosin (f, f'). After 1 week of submerged culture, K5 and K14 were detected in the whole multilayer (a, b, c, red stain). K10 (b, green stain) and K1 (d, red stain) were localized in the suprabasal cell layer while K4 and K13 were not detected (a, d, green stain). Intense staining of integrin β4 was localized in the basal cell membranes of the basal cells (e, green stain). After 2 weeks of submerged culture, K5

staining decreased in the lower cornified layer (a', red stain). K4 and K13 were not detected (a', d', green stain). K10 (b', green stain) was detected in the suprabasal cells and the lower cornified layer, where K14 staining (b', c', red stain) decreased. Integrin β4 staining after 2 weeks (e', green stain) was similar to that observed after 1 week (6e, green stain). Arrowheads (a'-f') indicate the nucleus often observed in the cornified layer. The scale bar in e' represents 20 μm and applies to images a-e and a'-e'. The scale bar in f' represents 20 μm and applies to images f and f'. CL, cornified cell layer; SB, suprabasal cell layer

Fig. 8 Localization of involucrin in the multilayer formed in air-lifted and submerged cultures

Keratinocytes grown under air-lifted (a-d, a'-d', f-i, f'-i') or submerged conditions (e, e', j, j') for 1 or 2 weeks in medium-C (a, a', f, f'), medium-CA (b, b', g, g'), medium-CK (c, c', h, h'), and medium-CAK (d, d', e, e', i, i', j, j'). Cells were immunostained to detect involucrin (green) and nuclei were stained with DAPI (blue in a-j, white in a'-j'). One week after air-lift, involucrin was localized in the suprabasal cells, but not in the thin uppermost cornified layer (a, b, c, green stain). However, involucrin was only

localized in the upper suprabasal cells with medium-CAK (d, green stain). Involucrin was localized in the suprabasal cells with medium-C and -CA (f, g, green stain), and more specifically in the upper suprabasal cells with medium-CK and -CAK (h, i, green stain). The cornified layer induced by air-lifted condition was negative for involucrin (a-d, f-i). In multilayers formed under immersion conditions after 1 week, involucrin was localized within the all suprabasal cells containing the thin uppermost cornified layer (e, green stain). After 2 weeks, involucrin was localized in the uppermost suprabasal cells and the cornified layer with only a few nuclei (j, green stain). Arrows (j, j') indicate the nuclei, which were often detected in the cornified layer. Arrowheads indicate the surface of multilayers. The scale bar in j' represents 20 μm and applies to all images

Fig. 9 Localization of K2 in the multilayer formed in air-lifted and submerged cultures

Keratinocytes grown under air-lifted (a-d, a'-d', a"-d") or submerged conditions (e, e') for one, two, and three weeks in medium-C (a-a"), medium-CA (b-b"), medium-CK (c-c"), and medium-CAK (d-d", e, e'). Cells were immunostained to detect K2 (green stain) and nuclei were stained with DAPI (blue). One and two weeks after air-lift, K2

was detected in the upper suprabasal cells (just below the cornified cell layer) in all culture media (a-d, a'-d', green stain). K2 was detected in the upper suprabasal cells three weeks after air-lift in medium-CK and -CAK (c", d", green stain), but not in medium-C and -CA (a", b", green stain). Faint green staining for K2 was occasionally detected in the cornified cell layer (b', b"). Under submerged conditions for one and two weeks, K2 was detected in the upper suprabasal cells, but not in the cornified cell layer, often with nuclei (e, e', green stain). Arrowheads indicate the surface of multilayers. The scale bar in e' represents 20 μm and applies to all images. CL, cornified cell layer; SB, suprabasal cell layer

Supplementary Fig. 1 Induction of stratification in HEKa cells in media containing calcium

One week after air-lift, keratinocytes seeded on polycarbonate filter cell culture inserts were fixed and differential interface contrast (DIC) images were obtained. ELGS medium (a, control) was a basal medium and it was supplemented with calcium (Ca) (b), L-ascorbic acid phosphate magnesium salt *n*-hydrate (APM) (c), keratinocyte growth factor (KGF) (d), Ca plus APM (e), Ca plus KGF (f), APM plus KGF (g), and Ca plus APM and KGF (h). Regardless of the addition of APM and/or KGF, stratification was

observed when keratinocytes were cultured in media containing at least calcium. The scale bar in h represents 20 μm and applies to all images

Supplementary Fig. 2 Control of immunofluorescent staining

The multilayers were formed in medium-C (a-a”), medium-CA (b-b”), medium-CK (c-c”), and medium-CAK (d-d”) under air-lifted conditions or formed in medium-CAK under submerged conditions (e, e’). One (a-e), two (a’-e’), and three (a”-d”) weeks after air-lift or immersion, sections of these multilayers were incubated with BSA-PBS, instead of primary antibodies, and then incubated with a mixture of anti-mouse Ig conjugated with Alexa 488 and anti-rabbit Ig conjugated with Alexa 568. Nuclei were stained with DAPI (blue). In control sections, non-specific staining was often observed in the cornified layer (especially in d’, d”, e’). The scale bar in e’ represents 20 μm and applies to all images

Supplementary Fig. 3 Localization of K5 and K14 in the non-keratinized and keratinized stratified squamous epithelium *in vivo*

The porcine maxillary buccal mucogingival junction (a, b) and buccal skin (c, d) were immunostained to detect K5 (a, c) or K14 (b, d), and nuclei were stained with DAPI

(blue). In the maxillary buccal mucogingival junction (a, b), K5 (a, red stain) and K14 (b, red stain) were localized in the basal cell layer in the non-keratinized epithelium, but in all cell layers, including the cornified layer, in the keratinized epithelium. In the buccal skin (c, d), which is keratinized, K5 (c, red stain) and K14 (d, red stain) were localized in the basal and whole suprabasal cell layers. Arrows indicate the boundary between the non-keratinized epithelium (left part) and keratinized epithelium (right part). The scale bar in b represents 20 μm and applies to images a and b. The scale bar in d represents 20 μm and applies to images c and d

Table 1 Summary of the localization of proliferation and differentiation markers.

Culture	wk	K5	K14	K1	K10	K2	Inv	int β4	Ki67[d]
air-lifted									
med-C	1	B,S	B,S	S	S,C	S(u)	S		2
	2	B,S	B,S	S,C	S,C	S(u)	S		2
	3	B[a],S[a]	B,S	S[a]	S[a],C	none		B[c]	1
med-CA	1	B,S	B,S	S	S	S(u)	S		2
	2	B,S	B,S	S,C	S,C	S(u)	S		2
	3	B[a],S[a]	B,S[a]	S[a],C	S[a],C	none		none	0
med-CK	1	B,S	B,S	S	S	S(u)	S		2
	2	B,S	B,S	S	S,C	S(u)	S(u)		2
	3	B,S	B,S	S	S,C	S(u)		B	2
med-CAK	1	B,S	B,S	S	S	S(u)	S(u)		3
	2	B,S	B,S	S	S,C	S(u)	S(u)		3
	3	B,S	B,S	S,C	S,C	S(u)		B	2
submerged									
med-CAK	1	B,S,C	B,S,C	S	S	S(u)	S,C	B	
	2	B,S,C	B,S[b],C	S,C	S,C	S(u)	S(u),C	B	

Human primary keratinocytes were cultured under air-lifted or submerged condition for one, two, or three weeks (wk) in medium (med)-C containing calcium, med-CA containing calcium and APM, med-CK containing calcium and KGF, or med-CAK containing calcium, APM, and KGF. Keratin (K) 4 and K13 were not detected in multilayers. Inv, involucrin; int, integrin; B, basal cell layer; S, suprabasal cell layer; C, cornified cell layer; [a], heterogeneous staining; [b], very weak staining; [c], disruption of the staining in the basal cell membranes; u, upper half of the suprabasal cell layer; none, no staining; [d], the number of basal cell nucleus with Ki67 staining (0, none; 1, a few; 2, moderate; 3, many)

Fig.1

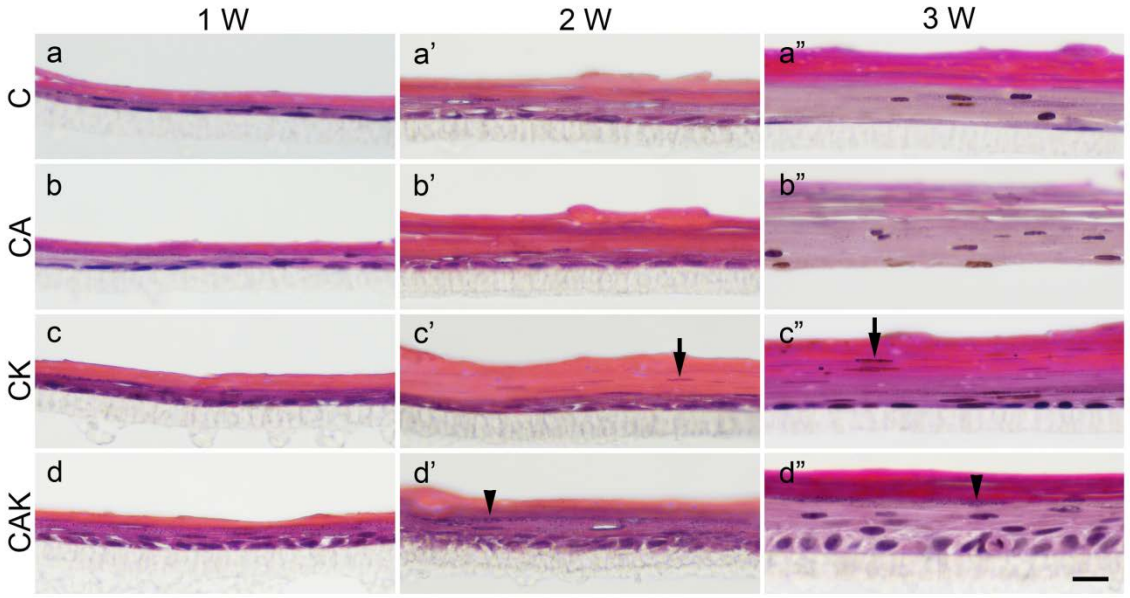
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Fig.2

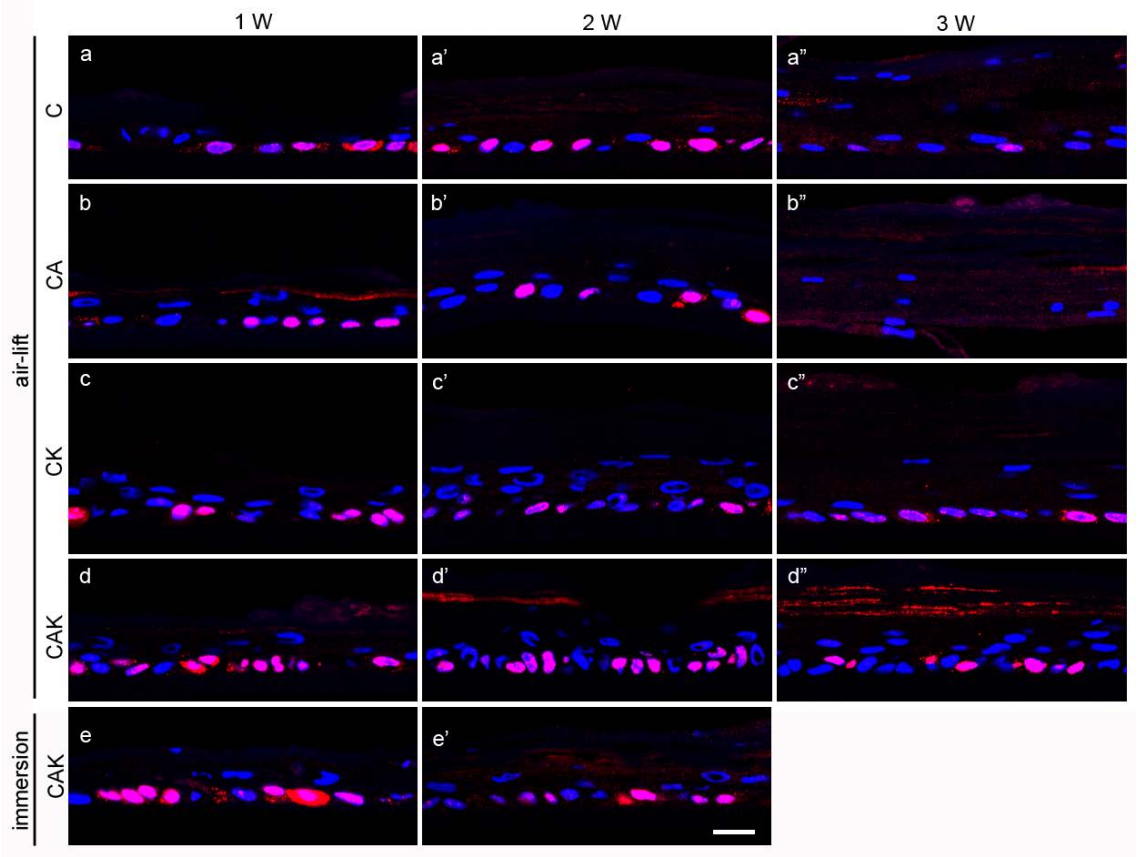
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Fig.3

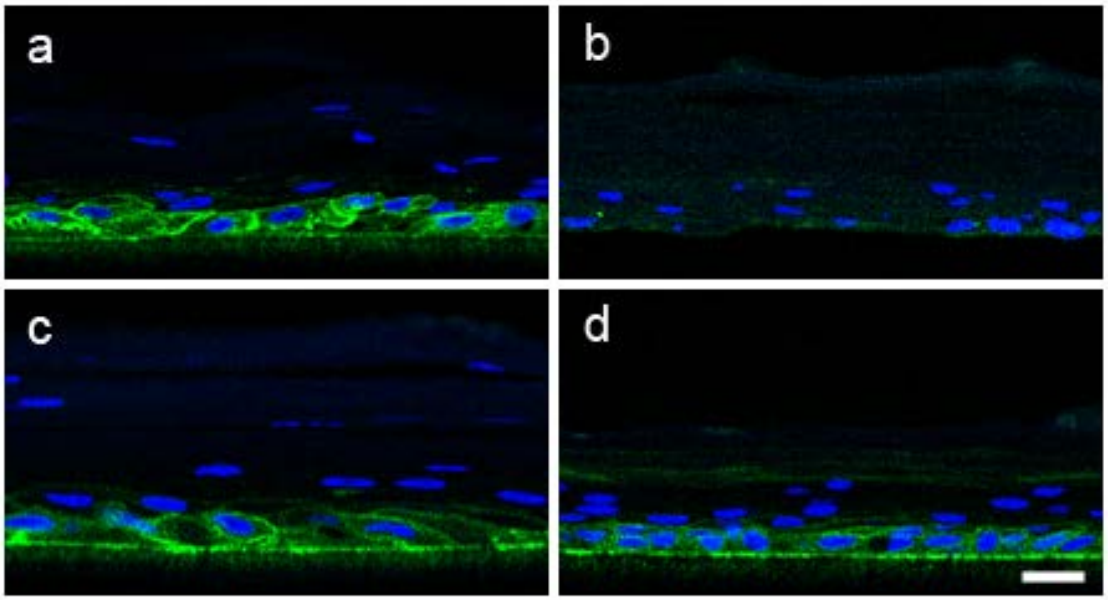
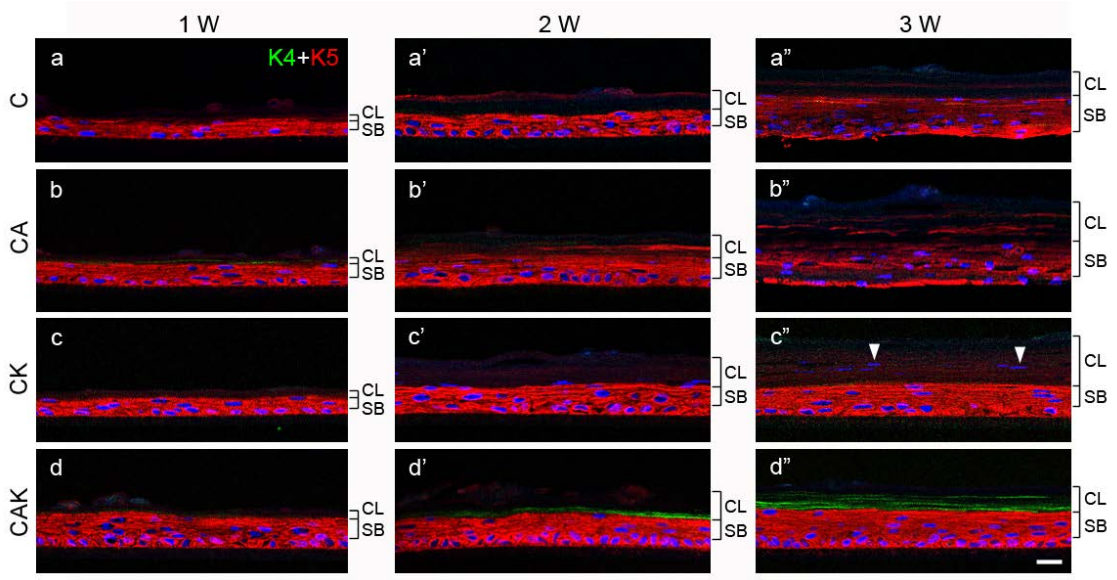


Fig.4



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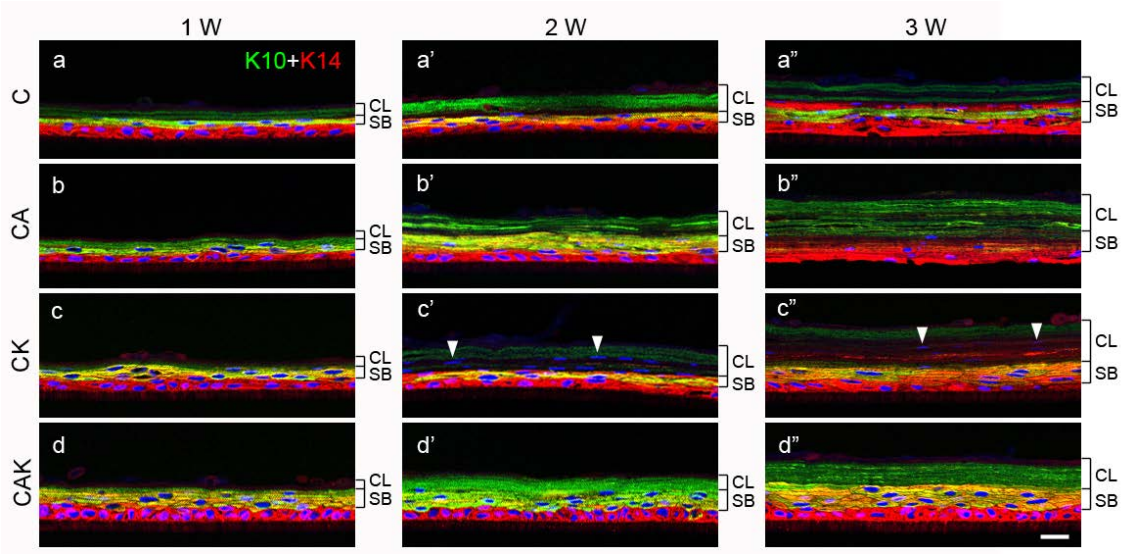
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Fig.6

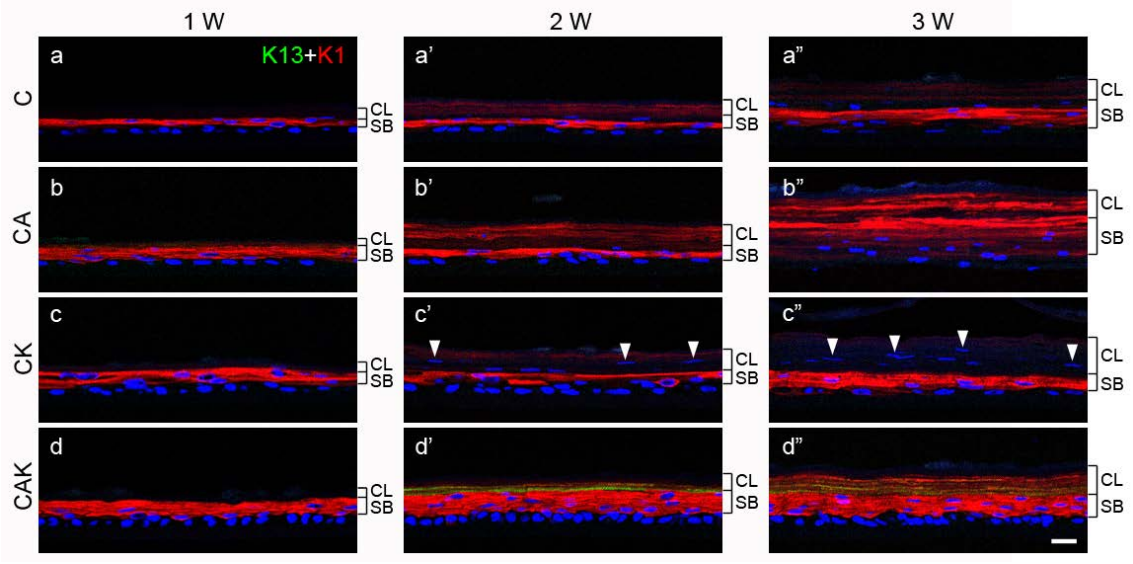
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Fig.7

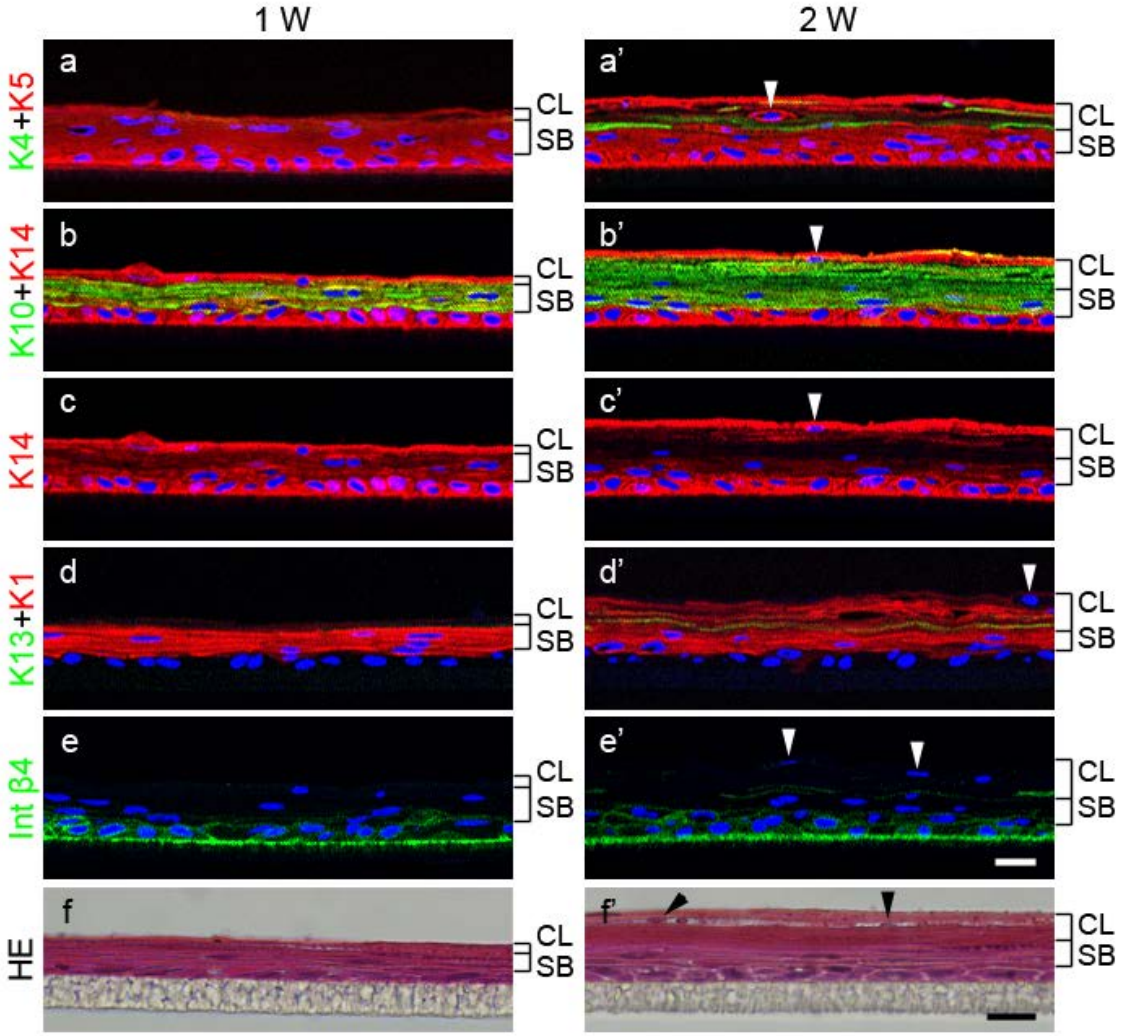

Fig.8

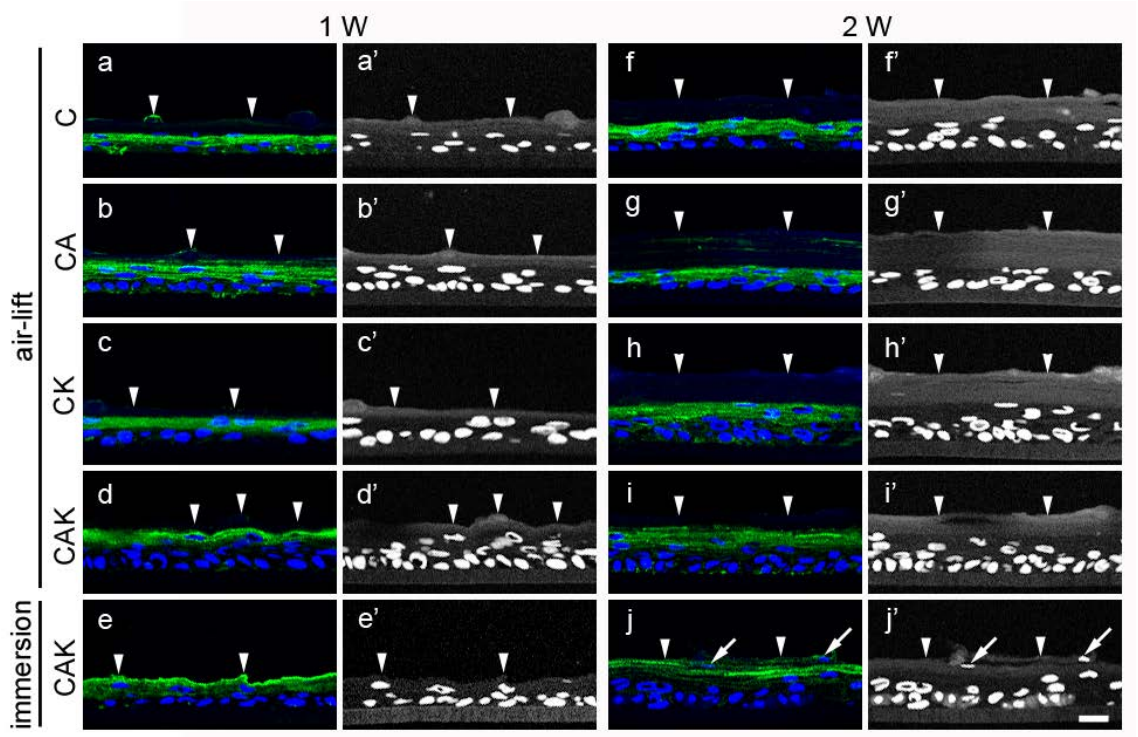
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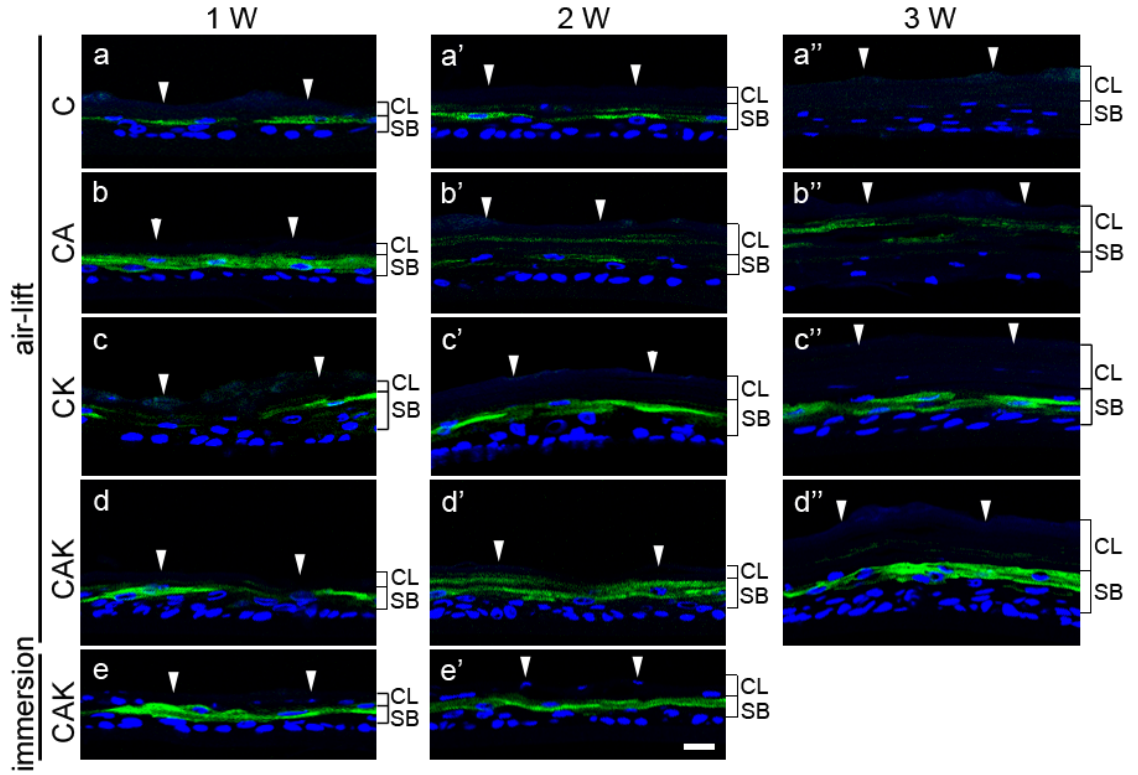
Fig.9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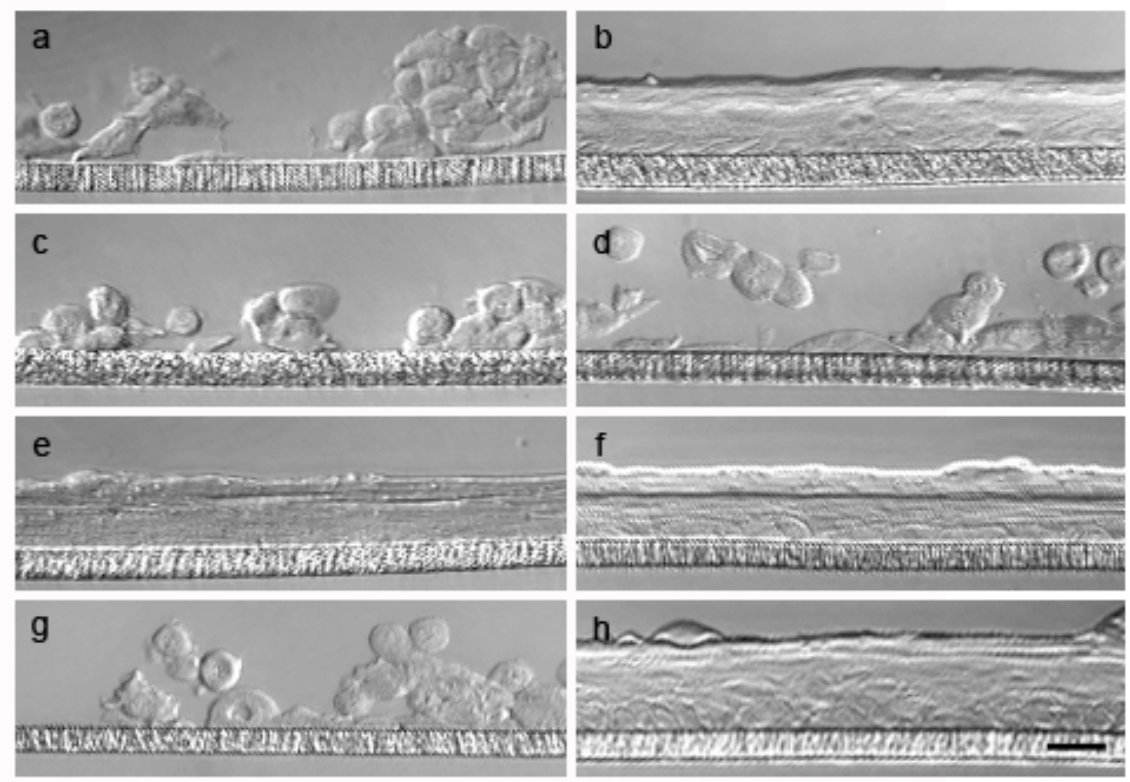
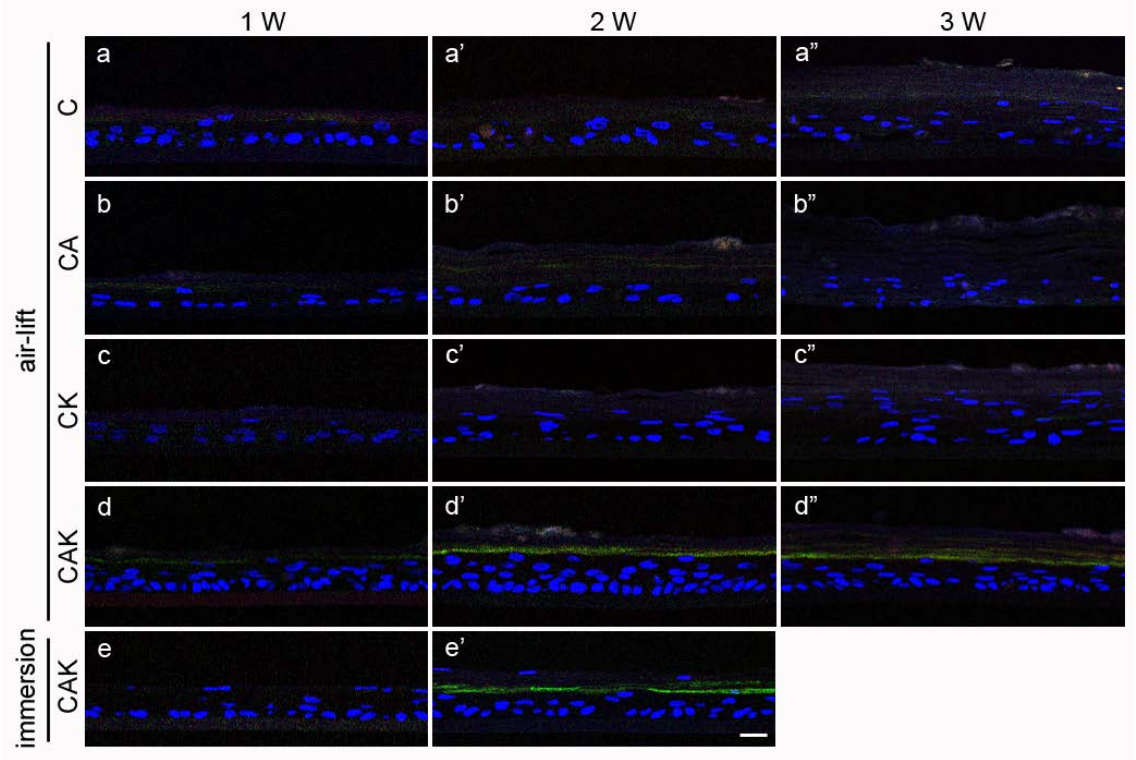
Supp Fig.1



Supp Fig.2



Supp Fig.3