Serum affects keratinization and tight junctions in three-dimensional cultures of the mouse keratinocyte cell line COCA through retinoic acid receptor-mediated signaling

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Abstract

Vitamin A, which is found in serum, is known to affect keratinocyte proliferation, epidermal differentiation, and keratinization. In mice, stratified epithelia in the oral cavity, esophagus, and forestomach are keratinized; however, these epithelia are not keratinized in humans. Several studies have reported that three-dimensional (3D) cultures of human keratinocytes in serum-containing medium could form keratinized epithelia. Here, we evaluated the effects of serum on morphology, the expression and localization of differentiation markers and tight junction proteins, and paracellular permeability in 3D cultures of mouse keratinocytes. We found that only 0.1% calcium-depleted serum inhibited keratinization and induced a change in the expression of differentiation marker proteins from loricrin to keratin 4; the inhibition of retinoic acid receptor-mediated signaling reversed these changes. Furthermore, the serum reduced claudin-1 protein expression and prevented its localization at occludin-positive spots on the surface of 3D cultures. On the other hand, the serum increased the protein expression of claudin-4, occludin, zonula occludens-1, and E-cadherin. These changes may contribute to the reduction of the transepithelial electrical resistance by approximately half. In conclusion, mouse keratinocytes derived from the epidermis formed non-keratinized structures in 3D cultures in response to vitamin A in serum. The results suggest that retinoic acid receptor-mediated signaling may be inhibited in the mouse epithelia in the oral cavity, esophagus, and forestomach as well as the epidermis, leading to the keratinization of these

epithelia.

Key words: retinoic acid receptor; keratinocyte; three-dimensional culture; keratinization; tight junction; claudin

Introduction

In humans, keratinized stratified squamous epithelium (SSE), which consists of the basal, spinous, granular, and cornified layers, is found in the epidermis. On the other hand, non-keratinized SSE, which consists of the basal, intermediate, and superficial layers, is found in the oral cavity, esophagus, vagina, and cornea. Keratin (K) is a family of fibrous structural proteins specific to epithelial cells and forms intermediate filaments. K10 and K1 are early differentiation markers present in spinous and granular cells in keratinized SSE (Moll et al. 2008; Torma 2011), whereas K4 and K13 are localized in all suprabasal cells in non-keratinized SSE (Moll et al. 2008). Loricrin (LOR), a late differentiation marker in the epidermis, is one of the major protein components of the cornified envelope and is localized in the granular layer of keratinized SSE, whereas involucrin is localized in non-keratinized SSE (including the buccal mucosa, esophagus, vagina, and conjunctiva) in addition to keratinized SSE (Hohl et al. 1993).

Various studies using three-dimensional (3D) cultures of keratinocytes have been

conducted. 3D culture methods vary considerably in terms of the culture medium and source of keratinocytes. To form a skin analog consisting of the epidermis and dermis, keratinocytes seeded on collagen gels containing dermal fibroblasts (Asselineau et al. 1986; O'Keefe et al. 1987) or a de-epidermized dermis (Lamb and Ambler 2013; Ponec et al. 1988; Prunieras et al. 1983; Regnier et al. 1988; Rosdy and Clauss 1990) have been cultured at the air-liquid interface. To form an epidermal analog, keratinocytes have been directly seeded on the filters of cell culture inserts and cultured at the air-liquid interface (Frankart et al. 2012; Poumay et al. 2004; Rosdy and Clauss 1990; Seo et al. 2016). Serum-free medium is commonly used for 3D keratinocyte cultures as serum contains unidentified components such as proteins, growth factors, hormones, minerals, vitamins, and lipids. Nevertheless, keratinocytes cultured on an inactivated feeder layer in serum-free medium could not form keratinized SSE in a previous study using a reconstituted skin model with de-epidermized and de-vitalized human skin (Lamb and Ambler 2013). Although the effects of serum on keratinocyte growth and differentiation were previously investigated using 2D cultures (Bertolero et al. 1986; Borowiec et al. 2013), the effects in 3D cultures have not been extensively examined thus far.

Tight junctions consist of transmembrane and cytosolic plaque proteins including claudin (CLDN), occludin, tricellulin, zonula occludens (ZO)-1, ZO-2, and ZO-3 and form a paracellular permeability barrier. Although tight junctions in the epidermis are formed in the second layer of the granular cell layers, which consist of three layers (Furuse et al. 2002;

Kubo et al. 2009; Tsuruta et al. 2002), each tight junction protein shows a distinct localization pattern from the basal layer to the granular layer (Kirschner and Brandner 2012). In non-keratinized SSE found in the cornea, esophagus, and oral cavity, CLDN1, CLDN4, and CLDN7 are widely distributed from the basal to the superficial layers, whereas occludin and ZO-1 are restricted to the superficial layer (Babkair et al. 2016; Ban et al. 2003; Nakatsukasa et al. 2010; Oshima et al. 2011, 2012; Takaoka et al. 2007; Yoshida et al. 2009).

In this study, we investigated the effects of serum on morphology, the expression and localization of differentiation markers and tight junction proteins, and paracellular permeability in 3D cultures of mouse keratinocytes.

Materials and methods

Antibodies

The primary antibodies used in this study were as follows: mouse anti-occludin (clone OC-3F10, #33-1500) antibody and rabbit anti-CLDN1 (#51-9000) and anti-ZO-1 (Mid) (#40-2200) antibodies from Zymed (San Francisco, CA, USA); rabbit anti-CLDN4 (ab53156) antibody from Abcam (Cambridge, UK); rabbit anti-E-cadherin (clone 24E10, #3195) antibody from Cell Signaling Technology (San Diego, CA, USA); mouse anti-K4 (clone 5H5, #WH0003851M1) and rabbit anti-actin (#A2066) antibodies from Sigma-Aldrich (St. Louis, MO, USA); rabbit anti-LOR (clone 19051, #905104) antibody from BioLegend (San Diego,

CA, USA).

MDCK II Tet-Off cells stably transfected with a Tet-Off regulatory plasmid were purchased from Clontech (Palo Alto, CA, USA) and grown in DMEM medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA). MDCK II cells, which are known to express CLDNs 1, 2, 3, 4, and 7, occludin, ZO-1, and E-cadherin, were used to examine antibody specifications used in this study.

Cell culture medium for COCA

CnT-Prime (CnT-PR) medium containing 70 μM $CaCl_2$ was purchased from CELLnTEC (Bern, Switzerland). For 3D cell culture (Seo et al. 2016), the 3D medium was prepared by adding 1.2 mM calcium chloride (Nacalai Tesque, Kyoto, Japan), 10 ng/ml human keratinocyte growth factor (KGF; PeproTech, Rocky Hill, NJ, USA), and 0.283 mM L-ascorbic acid phosphate magnesium salt *n*-hydrate (APM; Wako, Osaka, Japan) to CnT-PR. To remove the calcium in the serum, 500 ml of FBS (HyClone, South Logan, UT, USA) was mixed with 20 g of a chelating resin, Chelex 100 (Bio-Rad, Hercules, CA, USA) (Lichti et al. 2008). Subsequently, 0–10% (final concentration) of the Chelex-treated FBS (ch-FBS) was added to the 3D medium.

3D cell cultures of COCA

A murine epidermal keratinocyte cell line, COCA (Segrelles et al. 2011), was derived from the back skin of adult C57BL/DBA mice and grown in CnT-PR. The COCA cell line was purchased from ECACC (Salisbury, UK). For 3D cell culture, COCA cells (7.5×10^5 cells/ml) in CnT-PR (low-calcium medium) were seeded in cell culture inserts (0.4 μm polycarbonate filter, 12 mm diameter), which were purchased from Merck Millipore (Darmstadt, Germany), in 24-well plates. Each insert and well contained 0.4 ml of cell suspension (3.0×10^5 cells) and 0.6 ml of CnT-PR. The cells were grown for 1 or 2 days until they reached 100% confluence. The growth medium inside and outside of the insert was replaced with the 3D medium (high-calcium medium), and the cells were cultured for 16–24 h to form intercellular adhesion structures. Then, the inserts (up to six) were transferred to a culture dish (60 mm diameter) containing 3.2 ml of the 3D medium supplemented with 0–10% ch-FBS, and airlifted cultures were established by removing the 3D medium in the inserts. The surface within the inserts was kept dry following airlift 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. In some cases, the 3D cultures of COCA were airlifted for 1 week in 3D medium containing 1% ch-FBS and further airlifted for 1 week in the presence of 1% ch-FBS (control) or 1% ch-FBS with 0.2 μM BMS 493, an inverse agonist of pan-retinoic acid receptors (pan-RARs).

Immunofluorescence microscopy

After washing in phosphate-buffered saline (PBS), the 3D cultures of COCA were fixed with 1% paraformaldehyde in PBS for 1 h at 4°C and washed with PBS. Filters with cultured COCA cells were cut off from the inserts. Pig oral mucosa containing the boundary between lower lingual gingiva and alveolar mucosa which was obtained from slaughter facilities was fixed with 1% paraformaldehyde in PBS for 1 h at 4°C and washed with PBS. Then, they were sequentially soaked in 10%, 20%, and 30% sucrose in PBS at 4°C for 1–3 h each and embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan). Cryosections (5 μm) were cut and mounted on glass slides. Some sections were stained with hematoxylin and eosin (HE). Cells in the wells or cryosections were washed with PBS and incubated with 0.2% Triton-X 100 in PBS for 15 min for permeabilization. Subsequently, the sections were washed with PBS and incubated with 1% bovine serum albumin in PBS (BSA-PBS) for 15 min to block nonspecific binding. They were then incubated with primary antibodies diluted in BSA-PBS for 1 h in a moist chamber. The following primary antibodies were used: rabbit anti-CLDN1 (1:100), anti-ZO-1 (1:200), anti-E-cadherin (1:100), anti-CLDN4 (1:200), and anti-LOR antibodies and mouse anti-occludin (1:50) and anti-K4 (1:100) antibodies. After rinsing the sections four times with PBS, they were incubated with anti-mouse or anti-rabbit immunoglobulin conjugated with Alexa 488 or Alexa 568 (Molecular Probes, Eugene, OR, USA) at 1:400 dilution in BSA-PBS for 30 min in the dark. The sections were then washed

four times with PBS and mounted in Vectashield mounting medium containing 4′, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). The images were obtained by sequentially scanning the specimen to prevent bleed-through using a LSM710 confocal laser scanning microscope with the ZEN 2010 software (Carl Zeiss, Oberkochen, Germany). Basic system settings were: (1) objective lens: Zeiss Plan-Apochromat 63×/1.40 Oil DIC M27; fluorescence settings for DAPI: Diode 405-30 laser (405 nm) 4.0%, Ch 1, pinhole 67.2 μm (1.0 μm section), filter 415–479 nm; Alexa 488: Argon laser (488 nm) 5.0%, Ch 1, pinhole 68.5 μm (1.0 μm section), filter 492–545 nm; Alexa 568: HeNe 543 laser (543 nm) 26.0%, Ch 2, pinhole 64.9μm (1.0 μm section), filter 555–812 nm; beam splitters: MBS: MBS488/543/633, MBS_InVis: MBS -405; image size: 512 pixel (134.7μm) x 512 pixel (134.7μm), pixel size=0.26 μm; (2) objective lens: Zeiss EC Plan-Neofluar 20x/0.50 M27; fluorescence settings for DAPI: pinhole 64.9 μm (5.4 μm section); Alexa 488: pinhole 62.6 μm (5.4 μm section); Alexa 568: pinhole 57.9μm (5.4 μm section); image size: 512 pixel (424.3μm) x 512 pixel (424.3μm), pixel size=0.83 μm; other settings are the same as 63x objective lens. Using auto exposure and range indicator, master gain, digital offset (basically 0.00), and digital gain (basically 1.00–1.24) were determined. Images of HE stainings of the sections were acquired using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan) with an olympus objective lens (Ach, 60x/0.80) and an interlace scan CCD camera (Olympus DP12, 3.24 megapixel, 2048 x 1536 pixel resolution).

Gel electrophoresis and immunoblot analysis

The 3D-cultured cells were washed with ice-cold PBS and lysed with 0.2 ml of lysis buffer [62.5 mM Tris, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and 0.002% bromophenol blue] containing a protease inhibitor cocktail (Sigma-Aldrich) and a phosphatase inhibitor cocktail (Nacalai Tesque). Cell lysates (10 μl per lane) were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Precision Plus Protein Dual Color Standards (Bio-Rad, Hercules, CA, USA) were used to determine the size of the detected bands. The membranes were incubated with Blocking One (Nacalai Tesque) for 1 h followed by primary antibodies overnight at 4°C. Antibodies against CLDN1 (1:1,000), CLDN4 (1:1,000), occludin (1:1,000), ZO-1 (1:1,000), E-cadherin (1:1,000), K4 (1:1,000), LOR (1:1,000), and actin (1:2,000) were used as primary antibodies and diluted with Tris-buffered saline (TBS) [20 mM Tris (pH 7.6) and 137 mM NaCl] containing 5% Blocking One. After washing with TBS containing 0.1% Tween 20 (T-TBS), the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse Ig (1:2,000) (GE Healthcare UK Ltd., Amersham, England) for 1 h. They were then washed with T-TBS, and the bands were detected using Luminata Forte Western HRP substrate (Millipore, Billerica, MA, USA). Some membranes were reprobed after stripping the primary and secondary antibodies with

stripping buffer [62.5 mM Tris (pH 6.7), 2% SDS, and 100 mM 2-mercaptoethanol] at 50°C for 30 min.

Measurement of the transepithelial electrical resistance (TER)

After 2 weeks of airlift culture, cell culture inserts were transferred to a 24-well plate. CnT-PR medium containing 1.2 mM calcium was added to the inserts (0.4 ml) and wells (0.6 ml). The TER was measured using Millicell ERS-2 Voltohmmeter (Millipore). After TER measurement, filters with cultured COCA cells were processed for immunofluorescence microscopy, HE staining, or immunoblot analysis. TER values were calculated by subtracting the contribution of the bare filter and medium and multiplying it by the surface area of the filter. All experiments were performed twice in triplicate.

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Statistical differences between groups were determined by two-sided Welch's *t*-test. $P < 0.05$ was considered statistically significant.

Results

3D cultures of COCA formed keratinized SSE-like structures

A new 3D medium for keratinocytes (CnT-PR 3D Barrier) instead of the previous 3D medium (CnT-PR 3D) from CELLnTEC was used. In contrast to the previous 3D medium, the new medium did not induce the formation of keratinized stratified epithelium-like structures in cultures of COCA (data not shown). We attempted to form epidermal analogs from COCA by adding calcium, KGF, and APM to CnT-PR (Seo et al. 2016). As shown in Fig. 1, COCA formed epidermis-like structures in 1 week. These structures were maintained until at least 3 weeks; however, the cornified layer became loosely packed.

Serum inhibited keratinization, affected the morphology of superficial cells, and altered the expression of differentiation marker proteins

We examined effects of serum on the morphology of 3D cultures of COCA after 2 weeks (Fig. 2) considering that serum was an essential factor for the formation of SSE in a skin equivalent model in another study (Lamb and Ambler 2013). For the control, 3D cultures of COCA formed keratinized SSE (Fig. 2a). Nuclei were observed in the cornified layer when cultured with 0.01% ch-FBS (Fig. 2b, arrowheads). Keratinization was inhibited by the addition of 0.1%, 1%, and 10% ch-FBS (Fig. 2c, d, e). In the non-keratinized SSE in vivo, there were two to three layers of squamous cells on the surface of 3D cultures with 0.1% ch-FBS (Fig. 2c). Nucleated cells detached from the surface of 3D cultures were also observed (Fig. 2c). The superficial cells were hyperplastic rather than flat when cultured with 1% or 10% ch-FBS (Fig.

2d, e), and some of them were detaching from the surface (Fig. 2d, e, arrows). Several layers of flattened cells (Fig. 2d, large arrow) were observed just beneath the surface of hyperplastic cells. Large intercellular gaps were observed in the presence of 10% ch-FBS (Fig. 2e). When cultured without ch-FBS, the immunofluorescence signals of LOR (a late marker of keratinization) were localized in cells just beneath the cornified layer; however, signals of K4, a marker of non-keratinization, were not detected in nucleated cells (Fig. 3a). When 0.1% or more ch-FBS was added to the 3D cultures, signals of K4 instead of LOR were detected in all suprabasal cells in the non-keratinized SSE (Fig. 3b, c, d). Specifications of antibodies against K4 and LOR were examined by immunofluorescence localization in pig oral mucosa containing the boundary between lower lingual gingiva (keratinized) and alveolar mucosa (non-keratinized) (Supplementary Fig. 1).

Inhibition of RAR signaling restored keratinization and LOR expression when cultured in serum-containing medium

Retinoic acid (RA), which is a metabolite of vitamin A (retinol) detected in serum (De Leenheer et al. 1982; Fuchs and Green 1981), is known to affect keratinocytes (Elias et al. 1981; Groeger et al. 2016; Hatakeyama et al. 2004, 2010; Ortiz-Melo et al. 2013; Virtanen et al. 2010; Wanner et al. 1999); thus, we attempted to suppress RAR signaling using BMS 493. When cultured with 1% ch-FBS (control), the 3D cultures of COCA formed non-keratinized

SSE-like structures after 1 week (Fig. 4a), and these structures were maintained until 2 weeks (Fig. 4b). When 0.2 μM BMS 493 was added to the 3D cultures in the final week, keratinization was restored (Fig. 4c). K4 but not LOR was detected in 3D cultures without BMS 493 after 1 and 2 weeks (Fig. 5a, b). On the other hand, LOR but not K4 was detected in the presence of BMS 493 (Fig. 5c).

Serum decreased paracellular permeability and affected the expression and localization of tight junction proteins

We assessed paracellular permeability (Fig. 6), the protein expression of tight and adherens junction proteins (Fig. 7), and the immunofluorescence localization of these proteins (Fig. 8) in 3D cultures of COCA after 2 weeks. In comparison with the control, addition of 0.1%, 1%, and 10% ch-FBS resulted in a 55.8%, 66.6%, and 56.8% decrease in the TER, respectively (Fig. 6). As shown by immunoblotting, the addition of ch-FBS increased the expression of CLDN4, occludin, ZO-1, E-cadherin, and K4 but decreased that of CLDN1 (Fig. 7). The Raw data of immunoblotting are shown in Supplementary Fig. 2. In cultures without ch-FBS, occludin-positive spots were detected in cells in the first and second layers just beneath the cornified layer (Fig. 8a-d). Immunofluorescence signals of CLDN1 or CLDN4 were detected in the cornified layer (Fig. 8a’, b’). Spots positive for CLDN1 or CLDN4 were small compared with occludin-positive spots (Fig. 8a’, b’). Some occludin-positive spots were

colocalized with CLDN1 (Fig. 8a, a', a", arrows) or CLDN4 (Fig. 8b, b', b", arrows); however, almost all occludin-positive spots were colocalized with ZO-1 (Fig. 8c, c', c", arrows). ZO-1 was also localized at the cell-cell borders in the deeper layers (Fig. 8c'). E-cadherin staining at the cell-cell borders revealed that occludin-positive spots were localized in cells in the first and second layers just beneath the cornified layer (Fig. 8d, d', d"). In cultures with 1% ch-FBS, occludin-positive spots were detected in the deeper layers (Fig. 8 e-h) in addition to the surface layer (Fig. 8e-h, small arrows) of non-keratinized SSE. Occludin-positive spots in the deeper layers were colocalized with CLDN4 or ZO-1 (Fig. 8f, f', f", g, g', g", large arrows). The weak immunofluorescence signals of CLDN1 were observed in the cytoplasm (Fig. 8e'). CLDN4 and ZO-1 were also localized at the cell-cell borders in suprabasal cells (Fig. 8f', g'). Occludin-positive spots were detected at the edge of E-cadherin staining between the superficial cells (Fig. 8h, h', h", arrowheads). Controls for immunofluorescence in COCA 3D cultures were shown in Supplementary Fig. 3. Antibody specifications (antibodies against occludin, CLDN1, CLDN4, ZO-1, E-cadherin) were examined in MDCK II cells (Supplementary Fig. 4).

Discussion

In this study, we found that calcium-depleted serum (ch-FBS) inhibited keratinization in 3D cultures of a mouse keratinocyte cell line, COCA, concomitantly with a change in the

expression of differentiation marker proteins from LOR (a marker of keratinization) to K4 (a marker of non-keratinization). Furthermore, the serum reduced CLDN1 protein expression and prevented its localization at occludin-positive spots on the surface of 3D cultures. On the other hand, the serum increased the protein expression of CLDN4, occludin, ZO-1, and E-cadherin. CLDN4 was localized at the cell-cell contacts in deeper layers. These serum-induced changes may contribute to the reduction of the TER by approximately half. The inhibition of RAR-mediated signaling restored keratinization and LOR expression, suggesting that vitamin A in the serum may induce the changes in differentiation of keratinocytes.

The addition of ch-FBS induced parakeratosis, inhibited keratinization, and increased the thickness of stratified cell layers with hyperplastic cells on the surface of 3D cultures. The results suggest that ch-FBS may stimulate the growth (increased thickness of 3D cultures) and inhibit the terminal differentiation of keratinocytes (keratinization and flattening of superficial cells). Consistent with our results, BSA (one of the major components in FBS) stimulated the growth of keratinocytes in 2D cultures in serum-free and low-calcium medium in a previous study (Bertolero et al. 1986). However, whole serum containing 3.62 mM Ca^{2+} (Bertolero et al. 1984) inhibited the growth and induced the terminal differentiation of keratinocytes (flattening of cells). On the other hand, Chelex resin-treated serum, in which divalent and trivalent cations including Ca^{2+} were removed, markedly reduced the inhibitory effect on cell

growth (Bertolero et al. 1986). Calcium is known as a major and essential factor that regulates the growth and differentiation of keratinocytes (Hennings et al. 1980). In addition to calcium, components in FBS, such as platelet-derived growth factor, fetuin, and transforming growth factor β, were found to inhibit the growth and induce the terminal differentiation of keratinocytes (Bertolero et al. 1986). Taken together, some components in FBS inhibit growth and induce differentiation, whereas others induce growth and inhibit differentiation. This is consistent with the results of a previous study showing that serum facilitated stratification and heat-inactivated serum further improved cell morphology and thickness in 3D cultures (Lamb and Ambler 2013).

Keratinocyte differentiation can be regulated by various factors including Ca^{2+} (Boyce and Ham 1983), serum (Bertolero et al. 1986), 1α,25-dihydroxyvitamin D_3 (Hosomi et al. 1983; Smith et al. 1986), RA (Asselineau et al. 1989; Bernard et al. 2002; Fuchs and Green 1981; Kopan et al. 1987; Pavez Lorie et al. 2009; Virtanen et al. 2010), and confluence (Poumay and Pittelkow 1995). In previous studies, human keratinocytes cultured at the air-liquid interface in 5% (Virtanen et al. 2010) or 10% (Asselineau et al. 1985; Kopan et al. 1987) fetal calf serum (FCS), which probably contains vitamin A, formed keratinized SSE. In contrast, the use of high-calcium culture medium with 0.1% or more ch-FBS suppressed the formation of the cornified layer in this study. A study has reported that 3D human keratinocyte cultures with RA ranging from 10^{-9} M to 10^{-8} M induced the formation of orthokeratinized

SSE, whereas those with less than 10^{-9} M and more than 10^{-7} M RA induced the acceleration and suppression of keratinization, respectively (Asselineau et al. 1989). The human serum contains approximately 10^{-8} M RA (De Leenheer et al. 1982), which is suitable for orthokeratinization. If this is applied to FBS, 0.1% ch-FBS (minimum concentration for inhibiting keratinization in this study) would contain 10^{-11} M RA, which can induce hyperkeratosis. In another study, a culture medium containing 10% FCS was estimated to contain 3.6×10^{-8} M vitamin A (Fuchs and Green 1981). Based on this estimation, 0.1% ch-FBS would contain 3.6×10^{-10} M vitamin A, which can still induce hyperkeratosis. The discrepancy may be attributed to the origin of keratinocytes (humans vs. mice). The epithelia in the esophagus and oral cavity except for the gingiva, hard palate, and filiform papillae of the tongue are not keratinized in humans; however, these epithelia are keratinized in mice and rats (Barrett et al. 1998; Jones and Klein 2013). The interspecies differences in keratinization in serum-containing culture medium may depend on the sensitivity of keratinocytes to factors involved in accelerating or suppressing keratinization in the serum and/or may depend on the number and amount of factors in the serum.

Supplementation with 1% ch-FBS suppressed LOR protein expression and induced K4 protein expression concomitantly with the inhibition of keratinization in this study. In another study, keratinocytes cultured with 10% delipidated FCS, in which vitamin A was removed, induced terminal differentiation as demonstrated by the increased expression of K1

(67 kD) and reduced expression of K13 (52 kD), and the addition of 1.2×10^{-6} M retinyl acetate (acetate ester of retinol) reversed this effect (Fuchs and Green 1981). RA has been reported to increase the expression of K4 and K13 (early markers of non-keratinization) but decrease K1 and K10 (early markers of keratinization) in 3D cultures of keratinocytes (Bernard et al. 2002; Kopan et al. 1987; Pavez Lorie et al. 2009; Virtanen et al. 2010). Furthermore, treatment with 10^{-5} M RA has been reported to contribute to the hyperplastic appearance of keratinocytes and looser cell-cell-contacts (Kopan et al. 1987), as shown in cultures with 10% ch-FBS in this study. RA could increase the shedding of superficial cells and reduce desmosomes, leading to an increased intercellular space (Hatakeyama et al. 2004; Wanner et al. 1999), as demonstrated in this study (Fig. 2c, e). Based on these observations, we attempted to inhibit RAR-mediated signaling using BMS 493, an inverse agonist of pan-RARs. As expected, BMS 493 induced LOR protein expression and keratinization and inhibited K4 protein expression. The results indicated that the morphological changes (non-keratinization and hyperplastic cells) and changes in the expression of differentiation markers (from LOR to K4) were induced by ch-FBS via RAR-mediated signaling.

In this study, only 0.1% ch-FBS increased the protein expression of CLDN4, occludin, ZO-1, E-cadherin, and K4 and decreased that of CLDN1. Consistent with our results, the protein and mRNA expression levels of CLDN4 were increased, whereas those of CLDN1 were decreased when immortalized mouse keratinocytes (GE1) derived from the oral

mucosa was treated with 1 μM RA for 5 days in a previous study (Hatakeyama et al. 2010). On the other hand, the protein expression of CLDN4 was increased, whereas that of CLDN1 was not affected when the human gingival keratinocyte cell line Gie-No3B11 was treated with 15 μM RA for 1–6 h (Groeger et al. 2016). In another study, the expression of CLDN4 mRNA was reduced, whereas that of CLDN1 mRNA was not affected when the rabbit corneal epithelial cell line RCE1 (5T5) was treated with 0.1–10 μM RA for 48 h (Ortiz-Melo et al. 2013). Differences in the duration of RA treatment and/or origin of keratinocytes (species and organs) may result in different effects on CLDN expression.

In cultures with 1% ch-FBS in this study, the TER was markedly decreased, and the localization of CLDN1, CLDN4, and occludin was altered. CLDN1 staining was almost undetectable. CLDN4 was localized at the cell-cell contacts in the deeper layers. Furthermore, occludin was occasionally observed in the deeper layers as a dotted appearance in addition to the surface layer. A study found that tight junction strands were reconstituted when CLDN1 or CLDN2 was exogenously expressed in tight junction-free fibroblasts (Furuse et al. 1998). Although occludin could not form tight junction strands on its own, occludin may be incorporated into tight junction strands with CLDN1 (Furuse et al. 1998). In simple epithelia, tight junctions are formed at the most apical site of the lateral plasma membrane, and CLDN and occludin are generally concentrated there. However, some CLDN proteins have been detected along the lateral plasma membrane; for example, a study reported that CLDN7 was

localized in the lateral plasma membrane, whereas CLDN8 and occludin were localized at the apical junctional region in the epididymis (Inai et al. 2007). These findings suggest that occludin may be a suitable marker to indicate the site of functional tight junctions. Therefore, CLDN4 localized at the cell-cell contacts in the deeper layers, which was not colocalized with occludin, may not form functional tight junctions and may not contribute to an increase in the TER. In a previous study, the paracellular permeability barrier in the granular layer of the epidermis was severely damaged in CLDN1-deficient mice (Furuse et al. 2002). Taken together, the absence of CLDN1 from occludin-positive spots on the surface of 3D cultures (representing functional tight junctions) may cause a decrease in the TER when cultured with 1% ch-FBS.

Addition of 0.1% ch-FBS caused a 55.8% decrease in the TER in comparison with the control and increase of ch-FBS had no additional effect on TER (Fig. 6). As the morphological change (inhibition of keratinization) occurred by 0.1%, but not 0.01%, ch-FBS, we did not examine the effect of 0.01% ch-FBS on TER. Threshold of ch-FBS concentration to inhibit keratinization might be higher than that to decrease TER.

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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 Formation of keratinized SSE-like structures in 3D cultures of COCA. COCA cells seeded on the filters of cell culture inserts were airlifted for 1 (a), 2 (b), or 3 (c) weeks. The

cornified layer, which was stained pink by eosin and did not contain nucleoli, was observed after 1 week and became loosely packed after 3 weeks. Scale bar: 20 μm for all images

Fig. 2 Serum inhibition of keratinization in 3D cultures of COCA. COCA cells seeded on the filters of cell culture inserts were airlifted for 2 weeks in the presence of 0% (a), 0.01% (b), 0.1% (c), 1% (d), and 10% (e) ch-FBS. Nuclei were observed in the cornified layer when cultured with 0.01% ch-FBS (arrowheads in b). Non-keratinized SSE-like structures were formed with the addition of 0.1%, 1%, and 10% serum. A large number of nucleated cells were detached from the surface of 3D cultures in the presence of 0.1% ch-FBS. In addition, there were hyperplastic cells detaching from the surface (arrows in d, e). Large intercellular gaps were observed in the presence of 10% ch-FBS (e). Several layers of flattened cells (large arrow in d) were observed just beneath the surface of hyperplastic and detaching cells (arrows in d). Scale bar: 20 μm for all images

Fig. 3 Serum-altered expression of keratinocyte differentiation markers in 3D cultures of COCA. COCA cultures were airlifted for 2 weeks in the presence of 0% (a), 0.1% (b), 1% (c), and 10% (d) ch-FBS and immunostained with K4 (green) and LOR (red). LOR was localized in cells just beneath the cornified layer; however, K4 was not detected in 3D cultures without ch-FBS (a). The addition of ch-FBS induced the protein expression of K4 but suppressed that

of LOR in suprabasal cells (b, c, d). Scale bar: 20 μm for all images

Fig. 4 Restoration of keratinization by RAR inhibition in 3D cultures of COCA in the presence of 1% ch-FBS. COCA cultures were airlifted for 1 week in the presence of 1% ch-FBS (a) and further airlifted for 1 week in the presence of 1% ch-FBS (b) or 1% ch-FBS with 0.2 μM BMS 493 (c). The cornified layer was restored by inhibiting RAR-mediated signaling using BMS 493. Scale bar: 20 μm for all images

Fig. 5 Altered expression of keratinocyte differentiation markers following RAR inhibition in 3D cultures of COCA. COCA cultures were airlifted for 1 week in the presence of 1% ch-FBS (a) and further airlifted for 1 week in the presence of 1% ch-FBS (b) or 1% ch-FBS with 0.2 μM BMS 493 (c). In cultures with 1% ch-FBS after 1 week (a) and 2 weeks (b), K4 (green) was detected in all cells (a) and suprabasal cells (b), respectively; however, LOR was not detected. The addition of BMS 493 induced the protein expression of LOR (red) but suppressed that of K4 in cells just beneath the cornified layer (c). Scale bar: 20 μm for all images

Fig. 6 Serum-induced TER reduction in 3D cultures of COCA. COCA cultures were airlifted for 2 weeks in the presence of 0%, 0.1%, 1%, and 10% ch-FBS, and the TER was measured.

The addition of 0.1% ch-FBS decreased the TER by approximately half; however, a higher ch-FBS concentration had no further effect on the TER. Values are expressed as the means ± SEM. *$P < 0.05$ vs control.

Fig. 7 Serum-altered protein expression in 3D cultures of COCA. COCA cultures were airlifted for 2 weeks in the presence of 0%, 0.1%, 1%, and 10% ch-FBS and analyzed by immunoblotting. The protein expression of CLDN4, occludin (OC), ZO-1, E-cadherin (E-cad), and K4 was increased by 0.1% or more ch-FBS; however, the expression of CLDN1 was decreased

Fig. 8 Serum-altered localization of tight junction proteins in 3D cultures of COCA. COCA cultures were airlifted for 2 weeks in the presence of 0% (a-d, a'-d', a"-d") and 1% (e-h, e'-h', e"-h") ch-FBS and double-immunostained with occludin (OC; green) and CLDN1, CLDN4, ZO-1, or E-cadherin (E-cad; red). In cultures without ch-FBS, occludin-positive spots were observed in cells just beneath the cornified layer and were colocalized with CLDN1, CLDN4, and ZO-1 (small arrows in a-c, a'-c', a"-c"). Signals of CLDN1 and CLDN4 were also detected in the cornified layer (a', b'). Furthermore, ZO-1 was detected at the cell-cell borders (c'). In cultures with 1% ch-FBS, occludin-positive spots colocalized with CLDN4 or ZO-1 were detected in some suprabasal cells (large arrows in f, f', f", g, g', g") in addition to cells

at the superficial layer. The weak staining of CLDN1 was observed in the cytoplasm (e'). Occludin-positive spots were detected at the edge of E-cadherin staining (arrowheads in d, d', d", h, h', h") between the superficial cells. Scale bar: 20 μm for all images

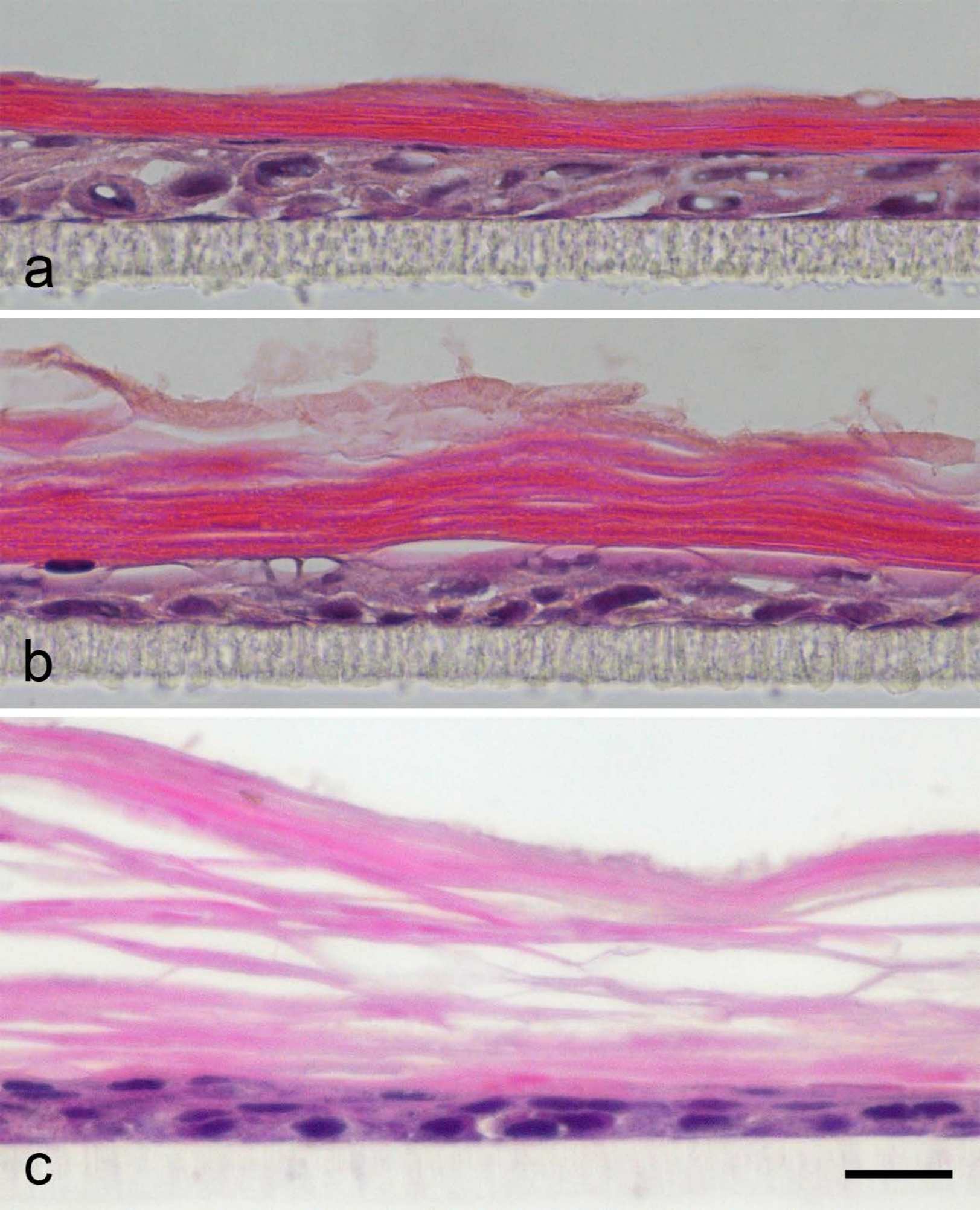
Supplementary Fig. 1 Antibody specifications examined by immunofluorescence localization in oral mucosal epithelium obtained from pig lower jaws. Cryosections were double stained with anti-K4 and anti-LOR antibodies. Arrows indicate the boundary between non-keratinized lingual alveolar epithelium (the left part) and keratinized lingual gingiva (the right part). K4 signals (green) were observed in almost all layers in non-keratinized stratified epithelium (b). A higher magnification of the lower layers of non-keratinized stratified epithelium revealed that K4 signals are faint in the basal layer (d). K4 signals were gradually decreased from the upper layers to the basal layer in the right side of the part than the arrow (b). On the contrary to K4, LOR signals (red) appeared in the upper layer corresponding to the granular layer in the right side of the part than the arrow (b', c'). Merged images are shown in a"–d". Nuclei were stained with DAPI (blue). Scale bar in b" is applied to a–a" and b–b": 50 μm. Scale bar in d" is applied to c–c" and d–d": 20 μm.

Supplementary Fig. 2 Raw data of immunoblotting shown in Fig. 7. COCA cultures were airlifted for 2 weeks in the presence of 0%, 0.1%, 1%, or 10% ch-FBS and analyzed by

immunoblotting. Cells were lysed, fractionated by SDS-PAGE, and transferred onto PVDF membranes. Immunoblotting was performed using antibodies against CLDN1, CLDN4, occludin, ZO-1, E-cadherin, and K4. Membranes were reprobed after stripping primary and secondary antibodies. Lysates obtained from COCA 3D cultures in 0% (lane 1), 0.1% (lane 2), 1% (lane 3), and 10% (lane 4) ch-FBS were used. Observed band sizes (arrowheads) are as follows: CLDNs 1 and 4, ~20 kDa; occludin, 59 kDa; ZO-1, ~225 kDa; E-cadherin, ~135 kDa; K4, 57 kDa.

Supplementary Fig. 3 Controls for immunofluorescence in COCA 3D cultures. COCA cells seeded on insert filters were airlifted for 2 weeks in the presence of 0% (a, a', a"), 0.1% (b, b', b"), 1% (c, c', c"), 10% (d, d', d") ch-FBS. COCA cells seeded on insert filters were airlifted for 1 week in the presence of 1% (e, e', e") ch-FBS and further for 1 week with 0.2 μM BMS 493 (f, f', f"). Cryosections were incubated with BSA-PBS in place of primary antibodies and then secondary antibodies (a mixture of anti-mouse and anti-rabbit Ig conjugated with either Alexa 488 or Alexa 568). Images derived from Alexa 488 (green) are shown in a–f. Images derived from Alexa 568 (red) are shown in a'–f'. Nuclei were stained with DAPI (blue). Merged images are shown in a"–f". No specific signals were observed in these controls. Scale bar: 20 μm.

Supplementary Fig. 4 Antibody specifications examined by immunofluorescence localization in MDCK II cells. Cells were double stained with mouse anti-occludin (b, c, d, e) and either rabbit anti-CLDN1 (b'), anti-CLDN4 (c'), anti-ZO-1 (d'), or anti-E-cadherin (e') antibodies. In control (a–a"), BSA-PBS was used in place of primary antibodies. Merged images are shown in a"–e". Nuclei were stained with DAPI (blue). Occludin was localized at apical junctions with CLDN1, CLDN4, and ZO-1, but E-cadherin was localized in lateral cell membrane below apical junctions. Some nuclei were heavily or weakly stained with anti-CLDN4 (c') or anti-ZO-1 antibody (d'), respectively. Scale bar: 20 μm.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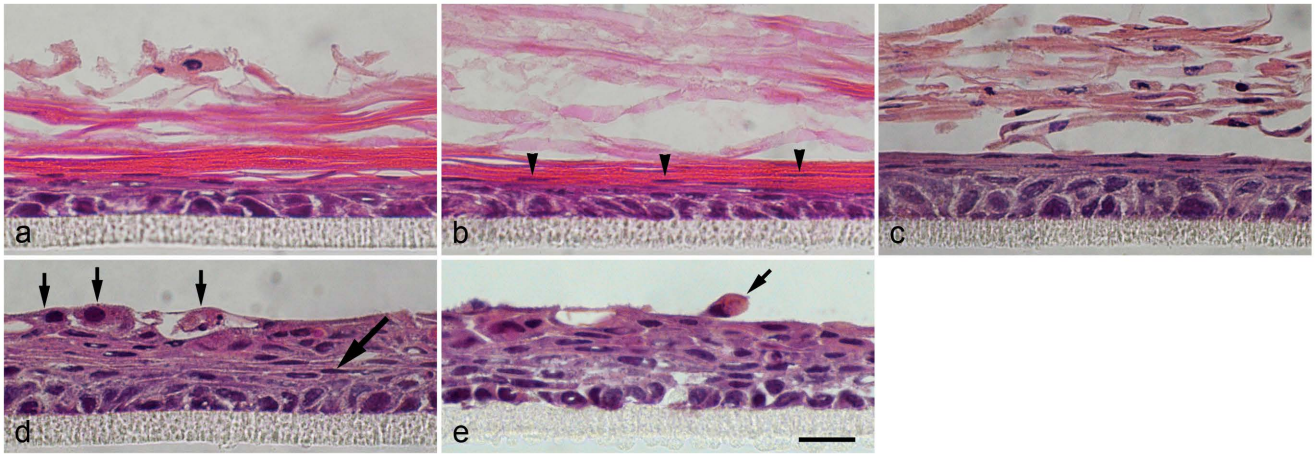
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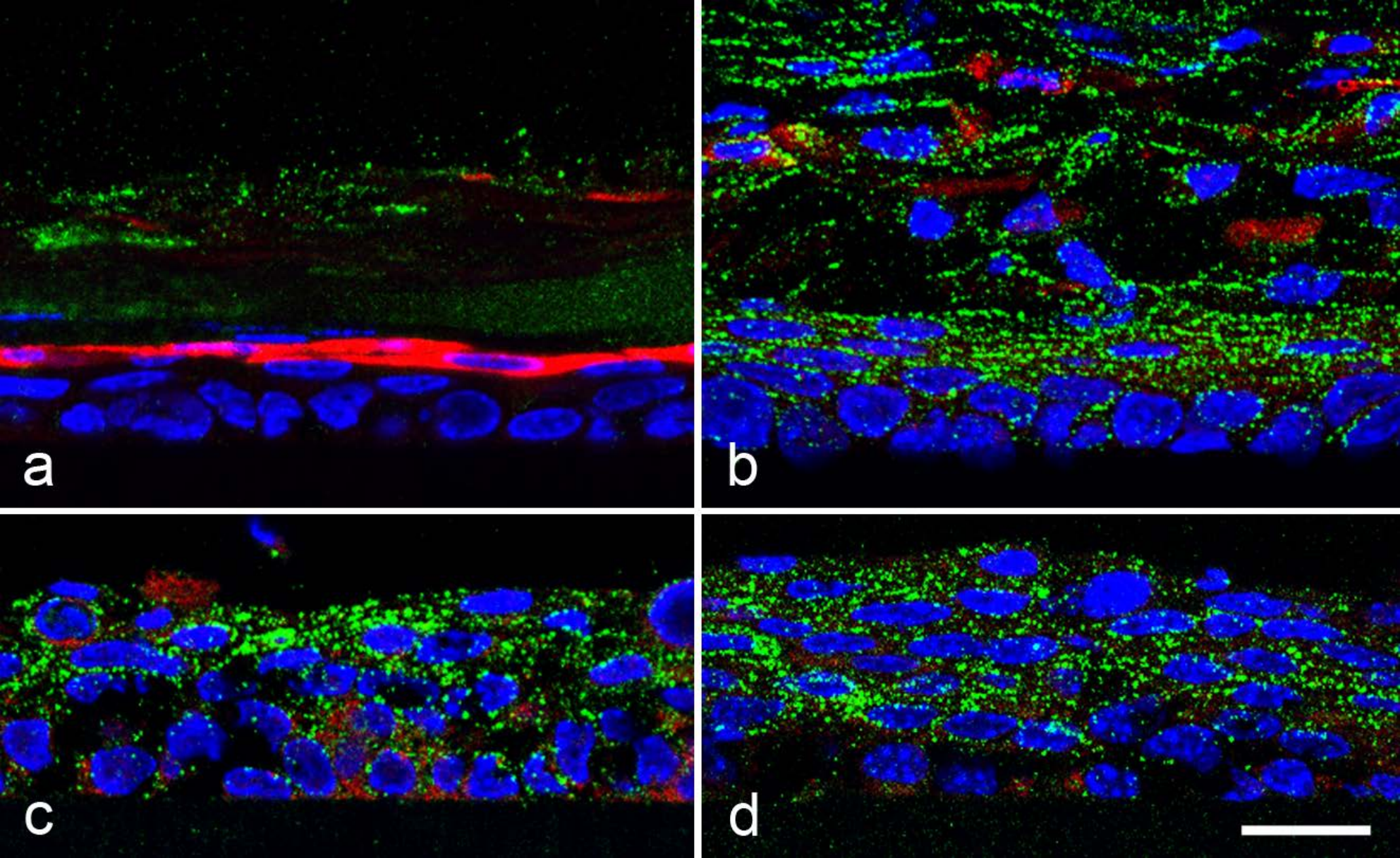
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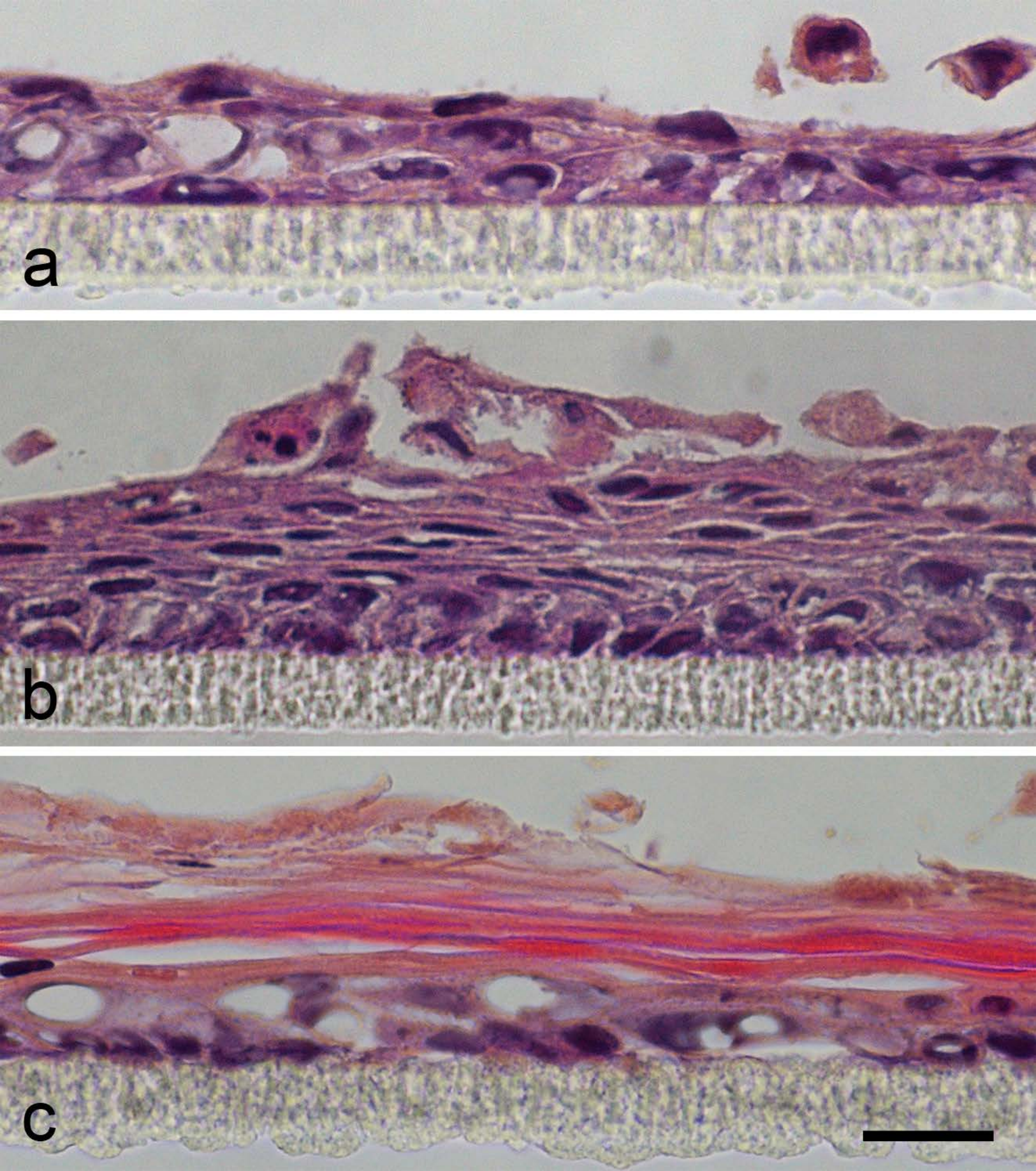
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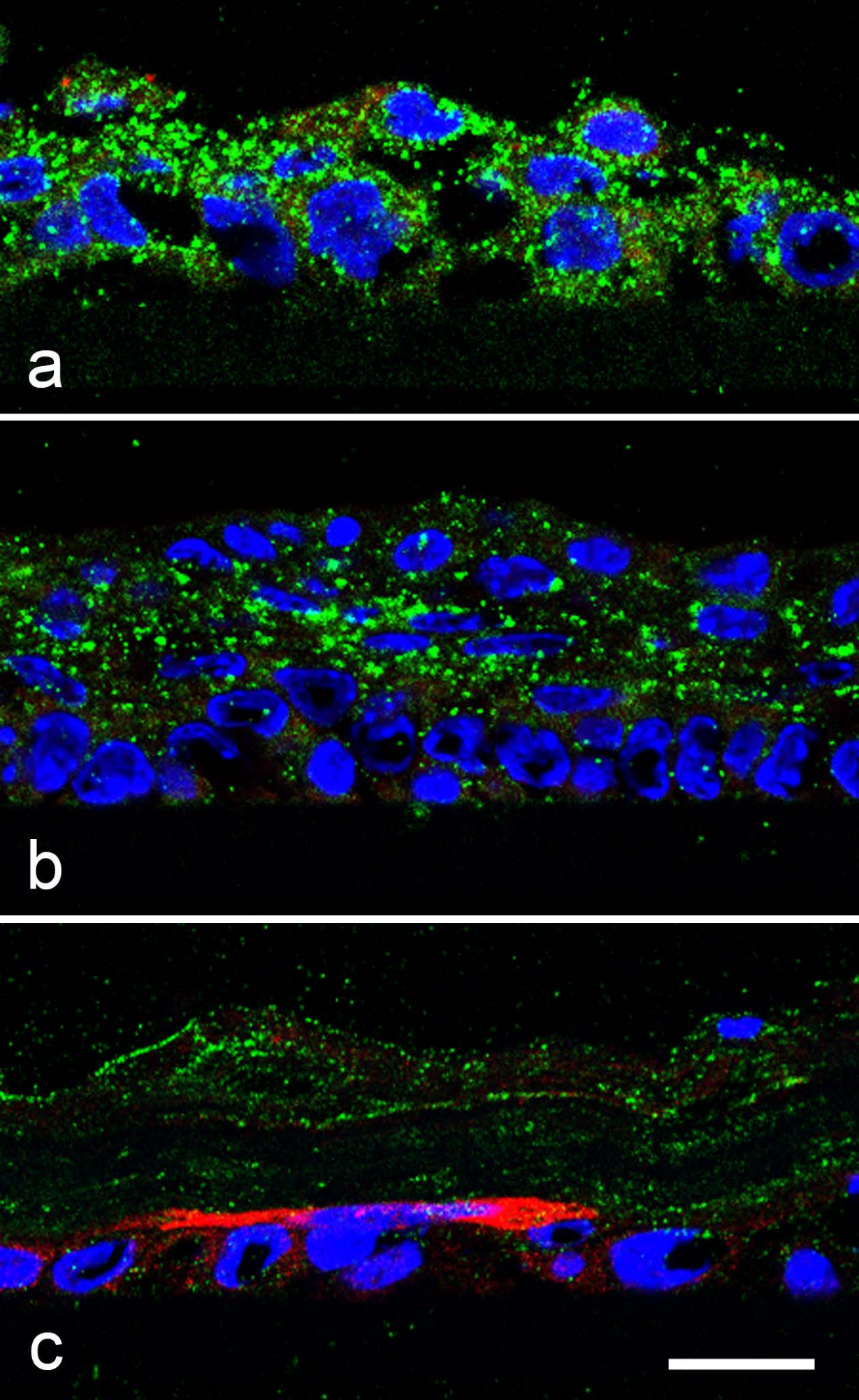
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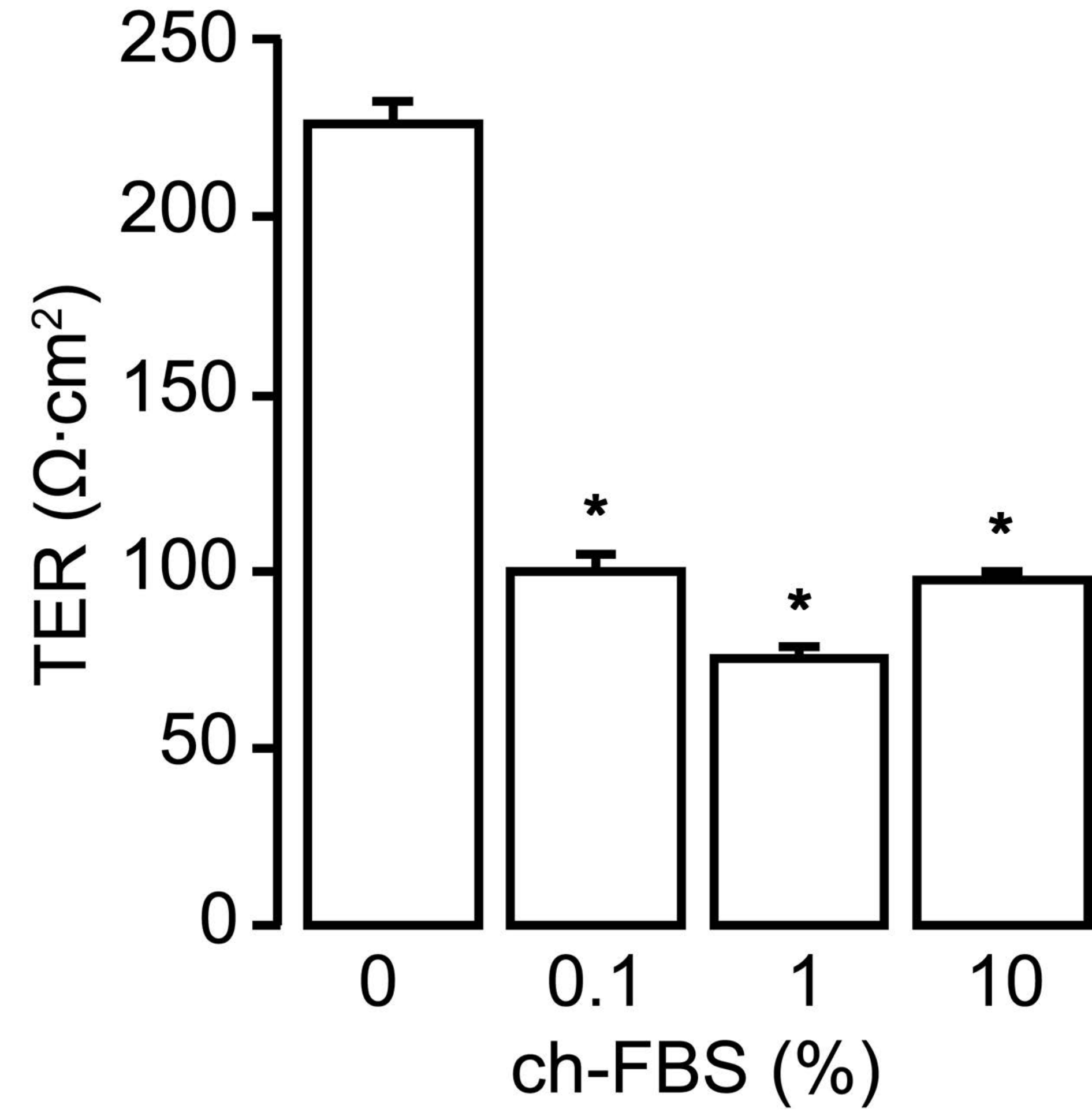
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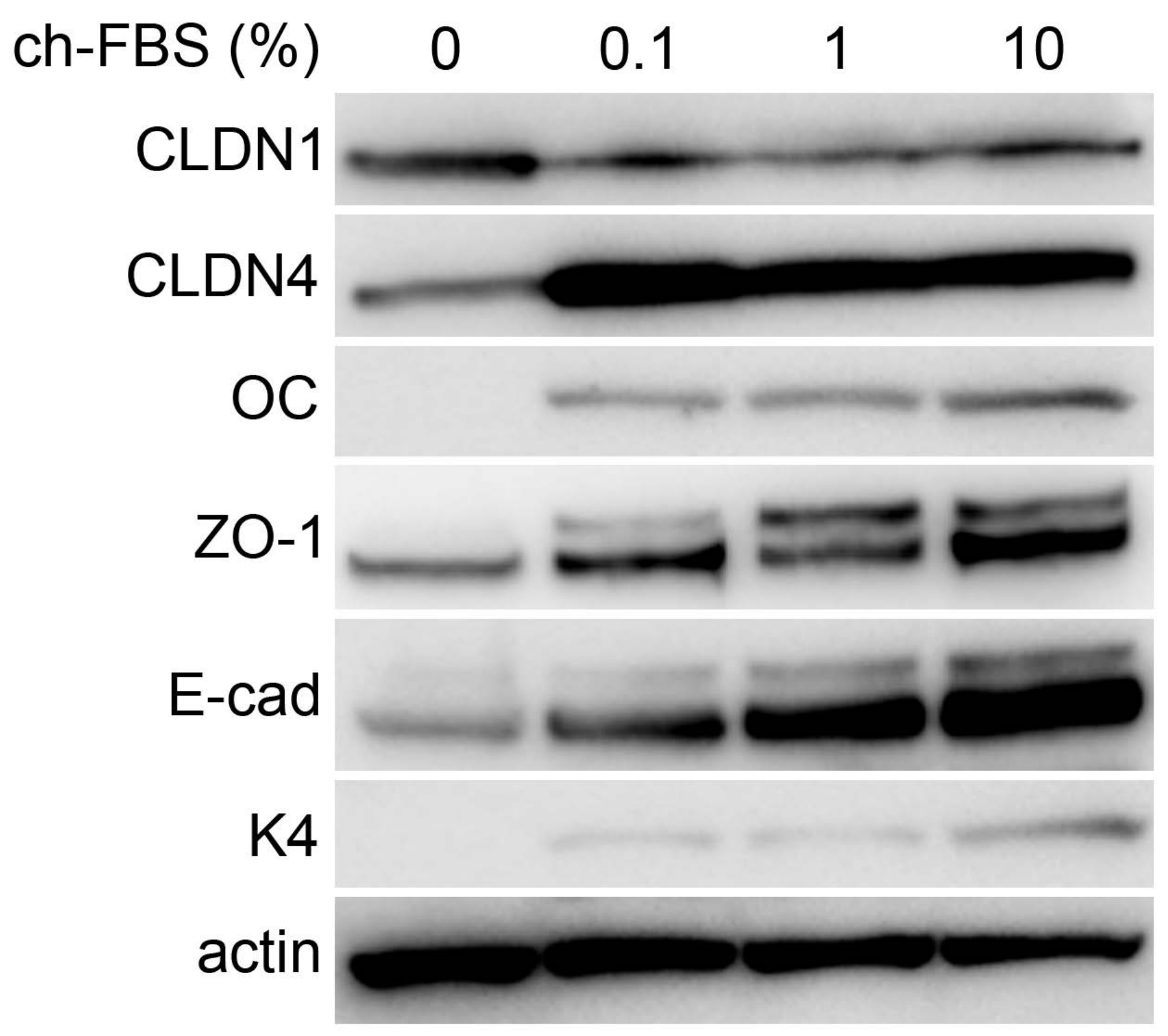
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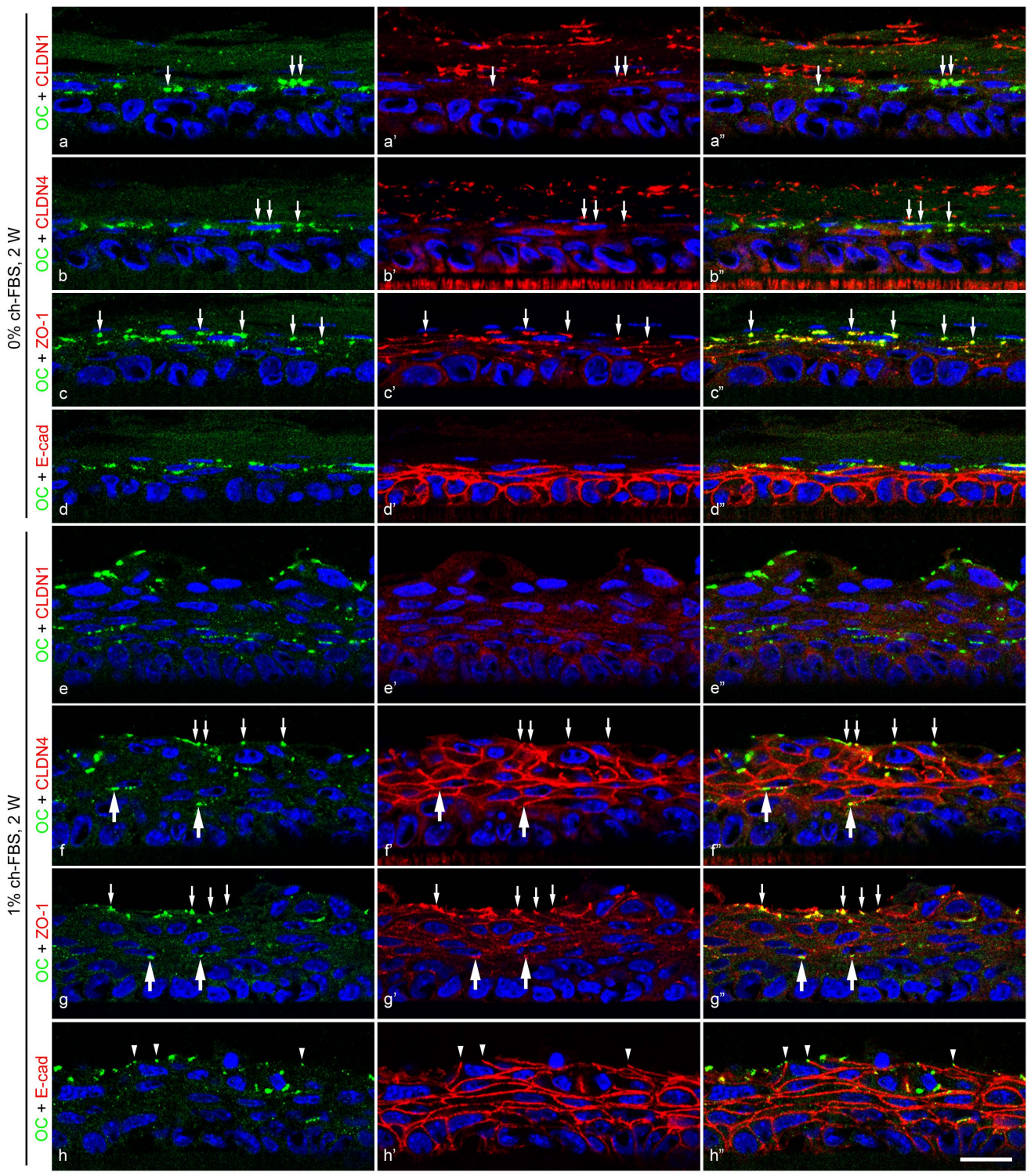
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OC + ZO-1
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