Association of Caspase-14 and Filaggrin Expression with Keratinization of the Oral Mucosa and Reconstruction Culture Rat Models

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Abstract

Background and Objective: Keratinization of the oral mucosa, such as the gingiva, has been shown to be important for periodontal health. Caspase-14 is a protease that plays a role in keratinization of the epidermis. The objective of this study was to investigate whether the expression of caspase-14 is intimately linked with keratinization and to examine the effect of the main component of green tea on the improvement of keratinization in rat oral mucosal preparations.

Materials and Methods: Histological and immunohistochemical analyses and quantitative mRNA measurements of caspase-14 and its substrate filaggrin were performed using different types of rat epithelial tissue and organotypic reconstruction culture models derived from epithelial cells and fibroblasts taken from the rat oral mucosa.

Results: In the skin, palate, buccal mucosa, and esophagus, the degree of keratinization appeared to be associated with cytokeratin 10 expression. The relative protein and mRNA expression levels of caspase-14 and filaggrin were consistent with the degree of keratinization in the following order: skin > palate > buccal mucosa > esophagus. The culture models of palatal and buccal mucosa retained a stratified epithelial structure. Caspase-14 expression appeared to be greater in the palatal model than in the buccal model. Remarkably, epigallocatechin-3-gallate (EGCG) improved the localization of cytokeratins and increased caspase-14 and filaggrin expression. This expression was more intense in the palatal model than in the buccal model, indicating that both models maintain the intrinsic properties of keratinization of the mucosa from where the

cultured cells were derived.

Conclusions: These results suggest that keratinization is closely associated with caspase-14 and filaggrin expression. Our reconstruction models are promising tools for drug evaluation and show that EGCG is beneficial for improving both keratinization and the expression of the linked protease in the oral mucosa.

Introduction

Epithelial tissue protects internal tissues against exogenous physical, chemical, and microbiological damage by forming a barrier between the body and the environment and also protects against dehydration (1). In the upper layers of some types of stratified epithelia, such as the epidermis, the cells are dead, contain no nucleus, and are surrounded by a cornified envelope to form an epithelial barrier (2). Conversely, in non-keratinized epithelial tissues, including the buccal mucosa of the human oral cavity, the cells are not flattened and still contain a nucleus. Several clinical studies have indicated that in some periodontal regions, the keratinized epithelium plays a role in stabilizing the mucosa; an adequate width of keratinized epithelium is likely to be important for sustainable gingival health (3,4), and the absence of keratinized mucosa around endosseous dental implants might increase the susceptibility of the peri-implant region to plaque-induced tissue destruction (5–7).

Caspase-14 belongs to the family of aspartate-specific proteinases and is expressed in the epidermal epithelia, which is involved in keratinization and barrier formation (8–10). Previous reports have demonstrated that its transcription is upregulated by the green tea polyphenol epigallocatechin-3-gallate (EGCG) (11,12). One of the substrates of active caspase-14 is filaggrin, which plays a role in tissue hydration after its degradation into free hygroscopic amino acids (1,13). These findings underscore the importance of caspase-14 in the formation of the epidermal barrier (9,10,14). Caspase-14 is reportedly not expressed in the non-keratinized keratinocytes of the oral cavity (15); nevertheless, previous studies have paid little attention to the involvement of caspase-14 and filaggrin in the degree of keratinization in distinct regions of the oral cavity.

We hypothesized that the differential expression of caspase-14 may be intimately connected with keratinization of the oral mucosa. Generally, the pattern of keratinization in experimental animals should be considered with caution because of species differences. Nevertheless, *in vivo* and *in vitro* models derived from the same animal are useful for quantitatively comparing the relationship between the degree of keratinization and the expression of the related protease and its substrate. In the present study, instead of using gingival epithelium due to its insufficient size, we dissected distinct areas of neonatal rat oral mucosa in which different degrees of keratinization can be expected. We then compared the expression of caspase-14 and filaggrin in *in vivo* preparations of the oral mucosa with preparations of epidermal and esophageal epithelia. Next, we produced organotypic culture models using epithelial cells and fibroblasts dissociated from the regions used in the *in vivo* studies to examine whether phenotypic differences exist between these models with respect to keratinization and the distribution of caspase-14. Furthermore, we used these models to investigate the potential of EGCG for improving keratinization and the expression of caspase-14 and filaggrin. Both models proved useful for evaluating various reagents used to improve keratinization of the oral mucosa.

Materials and methods

Antibodies and reagents

The following primary antibodies were used: mouse monoclonal anti-cytokeratin 14 (CK14) (1:100; Novocastra, Newcastle, UK), anti-CK13 (Ks13.1, Progen, Heidelberg, Germany) and anti-CK10 (1:100; Dako, Glostrup, Denmark), and rabbit polyclonal anti-caspase-14 (IMG-5713, 1:100; Imgenex, San Diego, CA) and anti-filaggrin (1:50;

Bioss, Woburn, MA). EGCG was purchased from Enzo Life Sciences (San Diego, CA).

Histological and immunohistochemical examinations

Neonatal Wistar rats (7 days old) were anesthetized with isoflurane and sacrificed. Permission for the procedures used was granted by the Animal Research Committee of Fukuoka Dental College (permission number, 11009). Three animals were used for each histological and immunohistochemical experiment. The *in vivo* rat tissues or reconstructed models were fixed in 10% formalin solution, processed routinely, and embedded in paraffin. Five-micrometer-thick sections were stained with hematoxylin and eosin (H-E). For 3,3′-diaminobenzidine visualization of immunostaining, the tissue sections were incubated in 10% normal goat serum, then incubated with primary antibodies for 3 h at 4 °C, followed by incubation for 1 h with the corresponding secondary antibodies conjugated with horseradish peroxidase at room temperature. The sections were counterstained with hematoxylin. Quantification of the relative area of immunostaining was performed with the aid of ImageJ software (NIH, Bethesda, MD).

Real-time PCR measurement of mRNA levels

Rat tissues and the reconstitution models were dissected and dispersed in an RNA extraction reagent (Sepasol-RNA I SuperG; Nacalai Tesque, Kyoto, Japan) by using a homogenizer (Dispergieantrieb T10 Basic; IKA Japan K.K., Osaka, Japan). Total RNA was isolated and the mRNA was reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed on an Applied Biosystems 7500 Real-time PCR system (Grand Island, NY) using SYBR® Premix Ex Taq II (Takara Bio) and a pair

of specific primers to target rat caspase-14 (forward: 5′-CCTGGAGATGAACTTGCTGTG-3′, reverse: 5′-CTTCTTCCGGAGGGTGCTTTG-3′, GenBank accession number: NM_001191776) and profilaggrin (forward: 5′-ATGCTAGATGTGGACCACGATGAC-3′, reverse: 5′-TGTTCCCTCTTCCTCTTGGGCTT-3′, GenBank accession number: XM_003753631) under the thermal cycling conditions recommended by the manufacturer. qPCR was performed on the samples in duplicate. A standard curve was used to calculate the expression of caspase-14 and profilaggrin normalized to the endogenous control GAPDH (forward: 5′-GACATGCCGCCTGGAGAAAC-3′, reverse: 5′-AGCCCAGGATGCCCTTTAGT-3′, GenBank accession number: NM_017008), and their relative levels were compared.

Construction of rat oral mucosal models using collagen gel matrix culture

All procedures were conducted based on previous studies using skin (16–19) and oral mucosa (17,20). Oral mucosa samples were dissected from the palatal and buccal regions of 7-day-old rats (approximately 3.0 mm × 3.0 mm for the palate and 1.5 mm × 2.0 mm for the buccal mucosa) and incubated overnight at 4 °C in modified Eagle's medium (MEM) containing dispase (750 protease units/mL; Dispase II; Godo Shusei, Tokyo, Japan). Epithelial tissue was peeled and digested at 37 °C for 5 min in phosphate-buffered saline containing 0.1% trypsin solution and 0.65 mM EDTA (Gibco, Grand Island, NY). The cells were resuspended in MCDB 153 medium (Kinousei Peptide Institute, Yamagata, Japan). The mucosal lamina propria was minced into rectangular pieces and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). After fibroblasts had sprouted from the tissue,

the cells were cultured in fresh medium containing 10% FBS. The fibroblasts were cultured for 5–6 days and then subcultured 2 or 3 times.

Collagen gels were prepared on ice by mixing type I collagen (0.7 volume; Nitta Gelatin, Osaka, Japan) with a reconstitution buffer (0.1 volume; 2.2 g $NaHCO_3$ and 4.77 g HEPES in 100 mL of a 50 mM NaOH solution, pH 7.0), and 5-times concentrated DMEM (0.2 volume). Three milliliters of the gel mixed with 2.0×10^6 dermal fibroblasts was poured into a cell culture insert (2.5 cm diameter; Millicell CM; Millipore, Billerica, MA). The gel was solidified by warming at 37 °C for 30 min. Epithelial cells were overlaid on the collagen gel containing the fibroblasts. The culture insert was placed in an outer dish (10-cm diameter) containing MCDB 153 medium + DMEM (1:1) supplemented with 10% FBS and a growth factor cocktail (0.4% v/v bovine pituitary extract, 0.2 ng/mL human epidermal growth factor, 5 μg/mL transferrin, 5 μg/mL insulin, and 0.18 μg/mL hydrocortisone; Kurabo, Osaka, Japan), 250 μM ascorbic acid, and 20 μM phosphatidylethanolamine (Sigma-Aldrich, St. Louis, MO), and incubated at 37 °C in 5% CO_2. After the epithelial cells had grown to confluency, the gel surface was transferred to an air-liquid interface by removing the inner medium and reducing the amount of the outer medium to the level of the gel to avoid immersing or drying the surface of the gel (air-lift). Fresh culture medium was added every other day. The reconstruction models were used 8 days after air-lift.

Statistical analysis

Data are presented as the mean ± standard error of the mean (N, number of observations). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by a *post-hoc* test. A P value less than 0.05 was considered

statistically significant.

Results

Distinct profiles of keratinization and cytokeratin expression in rat epithelial tissues

We performed histological examination of the four types of rat stratified epithelial tissues with H-E staining (Figure 1, H-E). In the skin samples, a remarkably high eosinophilic pattern was observed in the cornified layers, and their surface was often loosened and shed, probably due to the dehydration step before paraffin embedding. The epithelial cells within the granular layers appeared to be well flattened, and dense dot-like staining of keratohyalin granules was observed underneath the cornified layer (arrowhead). Judging from the eosinophilic staining of the palatal mucosa, a cornified layer was evident, but to a lesser degree than in the skin. The granular epithelial cells were also flattened, and fewer keratohyalin granules were observed than in the skin (arrowhead). The rat buccal mucosa also exhibited keratinization (superficial layer), and its epithelial-lamina propria junction was scalloped, showing connective tissue papillae and rete ridges. It contained thicker epithelial intermediate layers compared with the other three specimens and each cell was less flattened than in the skin. A few keratohyalin granules were scattered in the upper layers (arrowheads). In contrast, a cornified layer was faint in the esophagus.

Pairs of cytokeratins are known to be region-specific; for instance, CK5 and CK14 in the basal layer, CK1 and CK10 in the suprabasal layer accompanied with orthokeratinization, and CK4 and CK13 in the suprabasal layer in non-keratinized epithelium (21). Thus, we compared cytokeratin expression by immunohistochemistry. Initially, we confirmed that no positive staining was observed in the absence of the

primary antibodies used. The anti-CK10 antibody exhibited intense immunostaining of the granular and spinous layers of the skin (Figure 1, CK10). In the palate, it elicited strong immunoreactivity in the spinous layer and moderate staining in the granular layer, whereas its immunoreactivity was almost lost in the esophagus. In the buccal mucosa, immunostaining with the anti-CK10 antibody was mottled in the intermediate area.

In marked contrast, the anti-CK13 antibody demonstrated a consistent pattern of suprabasal staining in the esophageal samples, whereas moderate staining was observed in the buccal samples (Figure 1, CK13). Specific staining was almost lost in the skin and palatal samples. The anti-CK14 antibody exhibited consistent staining in the basal and parabasal layers of the skin and in all mucosa samples (Figure 1, CK14). These results suggest that the degree of keratinization is well correlated with the expression of CK10 in different types of mucosal epithelia and the epidermis.

Expression of caspase-14 and filaggrin in rat stratified epithelial tissues

Intense caspase-14 staining was observed in the granular and spinous layers of the skin and in the spinous layer of the palate (Figure 2A). Caspase-14 staining was broader in the intermediate layer of the buccal mucosa and only slight staining was observed in the esophageal mucosa. The antibody to filaggrin recognized the granular layers, but not the cornified layers, of the skin and palatal mucosa, whereas it exhibited weak staining in the buccal mucosa and only slight staining in esophagus. Figure 2B shows the quantitative analysis of the immunostaining of caspase-14 and filaggrin from four preparations. The relative values for the immunoreactive areas of caspase-14 and filaggrin were in the following descending order: skin > palate > buccal mucosa >

esophagus. Real-time PCR analysis demonstrated that the mRNA levels of caspase-14 and profilaggrin were also in the same descending order (Figure 3).

Organotypic reconstruction models of rat oral mucosa: EGCG-induced acceleration of keratinization and caspase-14 and filaggrin expression

Next, we tested the expression of cytokeratins, caspase-14, and filaggrin during keratinization by using reconstruction models (8 days after air-lift) derived from epithelial cells and fibroblasts from the palatal and buccal mucosa of neonatal rats. The culture models retained a multilayered epithelial structure. However, the epithelial-collagen gel (including fibroblasts) junction was parallel to the surface of the models, which was different from the irregular junction observed in the *in vivo* buccal mucosa. Epithelial differentiation was not as pronounced as in the *in vivo* samples according to the degree of keratinization and the expression of CK14 and CK10 (Figure 4, EGCG(-)). Under these conditions, caspase-14 expression appeared to be greater in the palatal model than in the buccal model. Previously, EGCG was reported to upregulate caspase-14 and filaggrin expression (11,12,22). In the present reconstruction models, 100 μM EGCG significantly increased the expression of caspase-14 and filaggrin, especially in the palate (Figure 4, EGCG(+)). Furthermore, EGCG promoted keratinization, while localizing the expression of CK14 and CK10 to the basal and suprabasal layers, respectively (Figure 4). The expression of caspase-14, filaggrin, and cytokeratins and the degree of keratinization were observed to a greater extent in the palatal model compared with the buccal model (Figure 4).

Discussion

Epithelial tissue is important for protecting the underlying tissues from environmental damage, and the oral cavity is not considered an exception, while a remarkable degree of regional variation is found with respect to keratinization in various types of epithelial tissues (1,23,24). In the present study, we investigated the morphology and keratinization patterns of four types of rat stratified epithelia to identify possible differences among these tissues. In general, CK10 is known to be a specific marker for the terminal differentiation of the epidermis (21). The present study, using neonatal rat tissues, showed that CK10 expression was intense in the skin and palatal mucosa, which appeared to be orthokeratinized. Notably, the rat buccal mucosa is likely to have an intermediate characteristic of non-keratinized and keratinized epithelia. In fact, in the rat buccal mucosa, CK13, which is found predominantly in human non-keratinized epithelium (21), was present; yet the scattered expression of CK10 and the presence of keratohyalin granules were also observed. In humans, no keratinization or CK10 protein expression has been observed in the buccal mucosa (17), even though CK10 mRNA has been found in normal buccal mucosa with a much wider range of expression than CK10 protein (25). These observations denote the species differences in keratinization and cytokeratin expression in oral tissues. The present findings indicate that the distinct patterns of keratinization and cytokeratin expression in the oral cavity are likely to play an important role in site-specific protection from physical damage, although it is not yet well understood how such patterns are made.

This study demonstrated that the highest levels of caspase-14 mRNA and protein expression are in the skin, while the lowest levels are in the esophagus. Furthermore, expression to an intermediate extent was observed in the palatal and buccal tissues. Lippens et al. (15) reported that caspase-14 expression is absent in the human oral

epithelium. The present study revealed non-negligible levels of caspase-14 expression in the rat oral mucosa, being consistent with the degree of keratinization and the expression of CK10. Active caspase-14 cleaves filaggrin units into fragments (9); these degraded products are the major source of hygroscopic amino acids, which are important for maintaining hydration of the skin (13,14,26). Filaggrin protein expression was reported to be distributed in the oral epithelium of newborn rats (27). The present real-time PCR and immunostaining results clearly shows that profilaggrin transcription and filaggrin protein expression are well correlated with the expression of caspase-14. However, given the lack of human experimental data to corroborate the present results, further studies with human tissues are required to support the potential of the present findings in translational dentistry.

The organotypic reconstruction models used in this study maintained the multilayered structure of the oral mucosa, even though epithelial differentiation appeared incomplete, as judged from the expression of cytokeratins. EGCG is known to be an activator of caspase-14 (11,12) and filaggrin (22) expression. In our reconstruction models, we found that EGCG markedly increased the expression of caspase-14 and filaggrin in accordance with the promotion of keratinization and localization of cytokeratins. The finding that immunoreactivity to CK10, caspase-14, and filaggrin was more pronounced in the palatal culture than in the buccal culture suggests that the organotypic models retain the intrinsic properties of keratinization of the mucosa from where the cultured cells were derived (17). These findings support the presence of a close relationship between caspase-14 expression and keratinization of the epithelium. In addition, we found that EGCG has the potential to augment keratinization of the epithelium. Accumulating evidence suggests that EGCG has the potential to have an

impact on a variety of human diseases as an anti-infective, anti-oxidative, anti-angiogenic, and anti-tumor agent (28–30). Furthermore, EGCG could have a beneficial effect on stabilizing the oral mucosa as keratinization has been shown to have an important role in maintaining healthy gingiva (3,4). Our model is a promising tool for analyzing the effects of various reagents on keratinization of the oral mucosa. For future clinical investigation of the effects of EGCG on keratinization, further experiments using human culture models are needed to confirm the link between caspase-14 and filaggrin.

In conclusion, the rat oral mucosa contains keratinized epithelium, which is closely associated with the expression of caspase-14 and its substrate filaggrin. By using the reconstruction models to search for substances with the potential to induce keratinization, this study provides evidence that a component of green tea, EGCG, stimulates keratinization of the epithelium in the oral cavity, probably leading to the maintenance of sustainable oral health.

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Figure legends

Figure 1. Histological and immunohistochemical assessments of the skin, palate, buccal mucosa, and esophagus of rats. H-E, hematoxylin-eosin staining; B, basal layer; S, spinous layer; G, granular layer; C, cornified layer; I, intermediate layer; Su, superficial layer (1). Arrowheads denote keratohyalin granules. Anti-cytokeratin 10 (CK10), 13 (CK13), and 14 (CK14) antibodies were used.

Figure 2. Immunostaining using antibodies against caspase-14 and filaggrin in the skin, palate, buccal mucosa, and esophagus of rats. A, anti-caspase-14 and anti-filaggrin antibodies recognized stratified epithelium with a distinct pattern in these samples. B, quantitative analysis of the relative area of immunostaining for caspase-14 and filaggrin (n = 3 animals per group). *P < 0.05, **P < 0.01, ***P < 0.001; one-way ANOVA followed by *post-hoc* Fisher's PLSD test.

Figure 3. Expression of caspase-14 and profilaggrin mRNA in the skin, palate, buccal mucosa, and esophagus of rats. A, typical RT-PCR results obtained using cDNA derived from the 4 types of tissue using specific primer pairs for caspase-14, profilaggrin, and GAPDH. B, quantitative real-time PCR measurements of caspase-14 and profilaggrin mRNA in the distinct types of epithelium (n = 4 animals per group). Expression values were calculated by the relative standard curve method. **P < 0.01; one-way ANOVA followed by *post-hoc* Scheffe's test.

Figure 4. H-E staining and immunostaining in the reconstruction models using epithelial cells and fibroblasts dissociated from rat palatal and buccal mucosa. The

models were cultured in the presence (+) or absence (-) of 100 μM EGCG. A, anti-CK14, anti-CK10, anti-caspase-14, and anti-filaggrin antibodies recognized distinct patterns in the reconstruction models. B, quantitative analysis of the relative area of immunostaining for caspase-14 and filaggrin (n = 3). E(+) and E(-) denote the presence (+) or absence (-) of EGCG.*P < 0.05, **P < 0.01, ***P < 0.001; one-way ANOVA followed by *post-hoc* Fisher's PLSD test.

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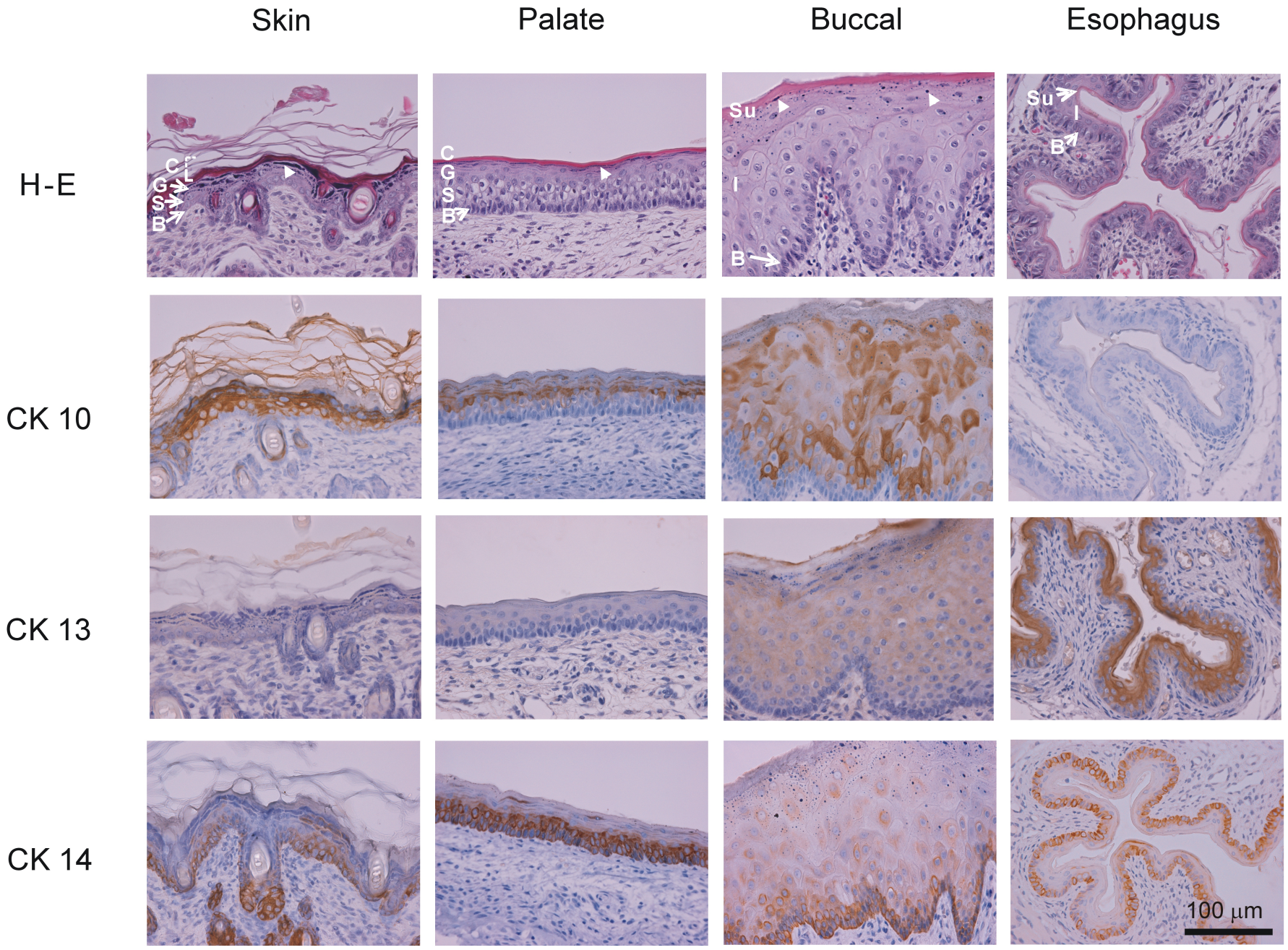

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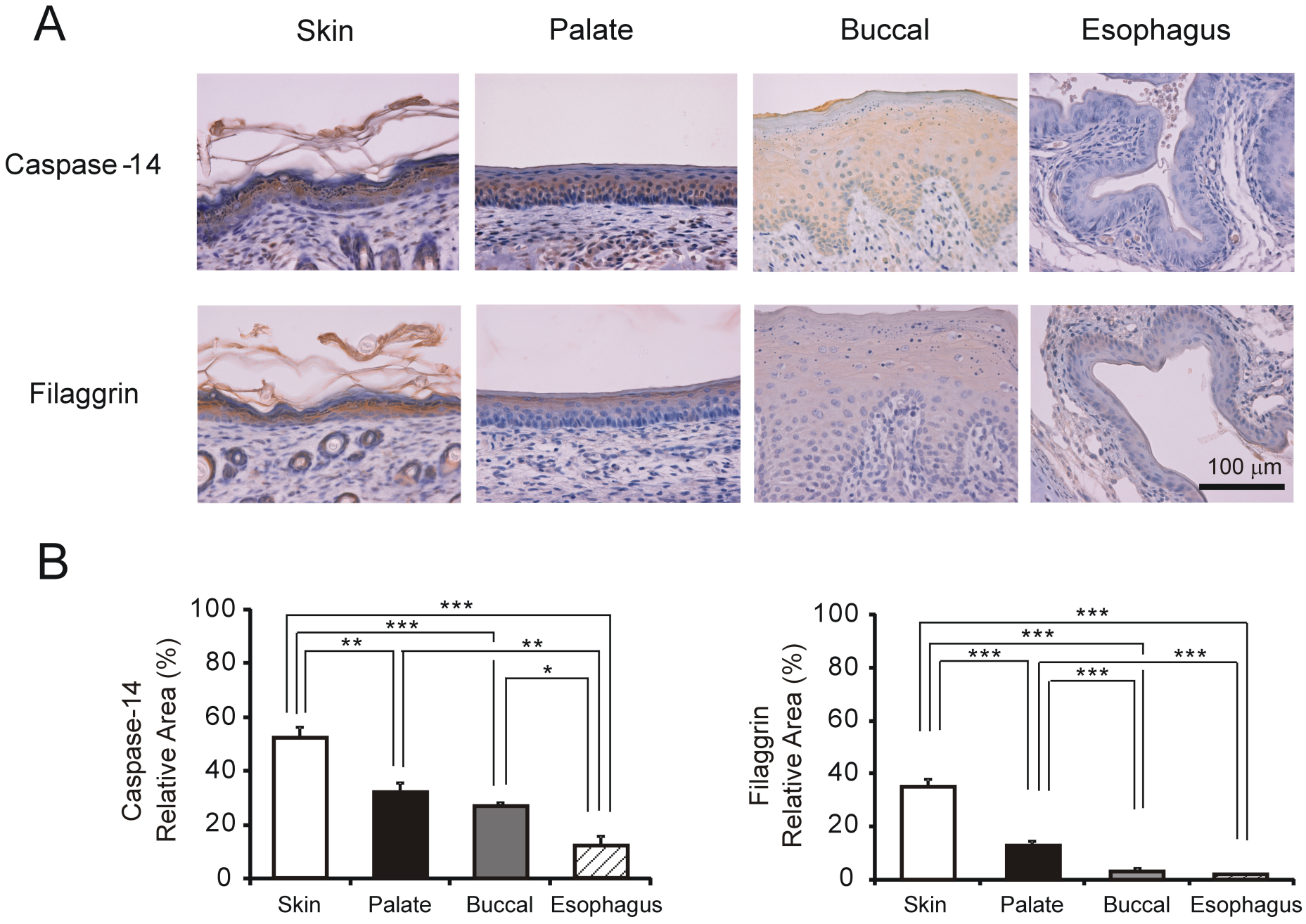

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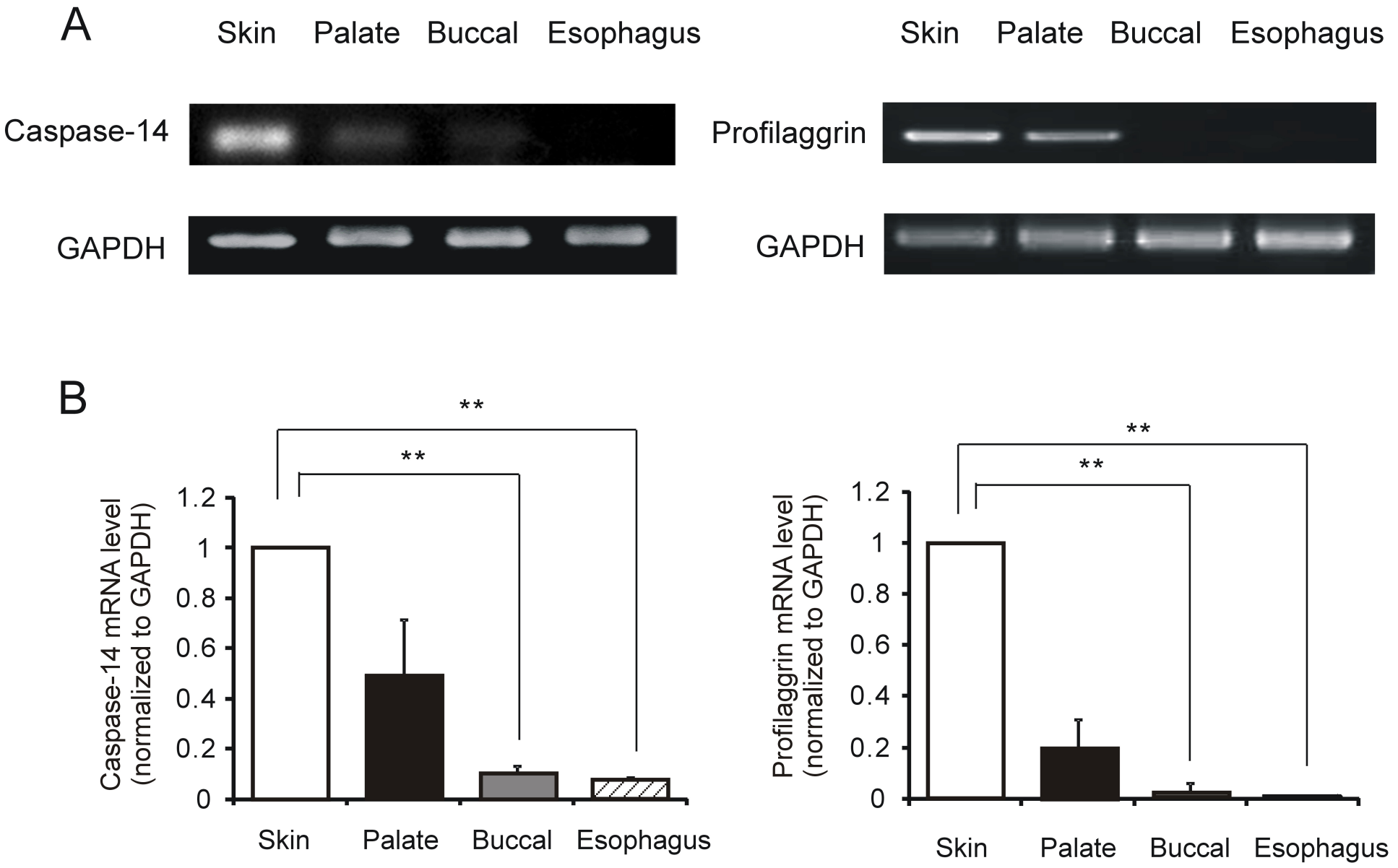

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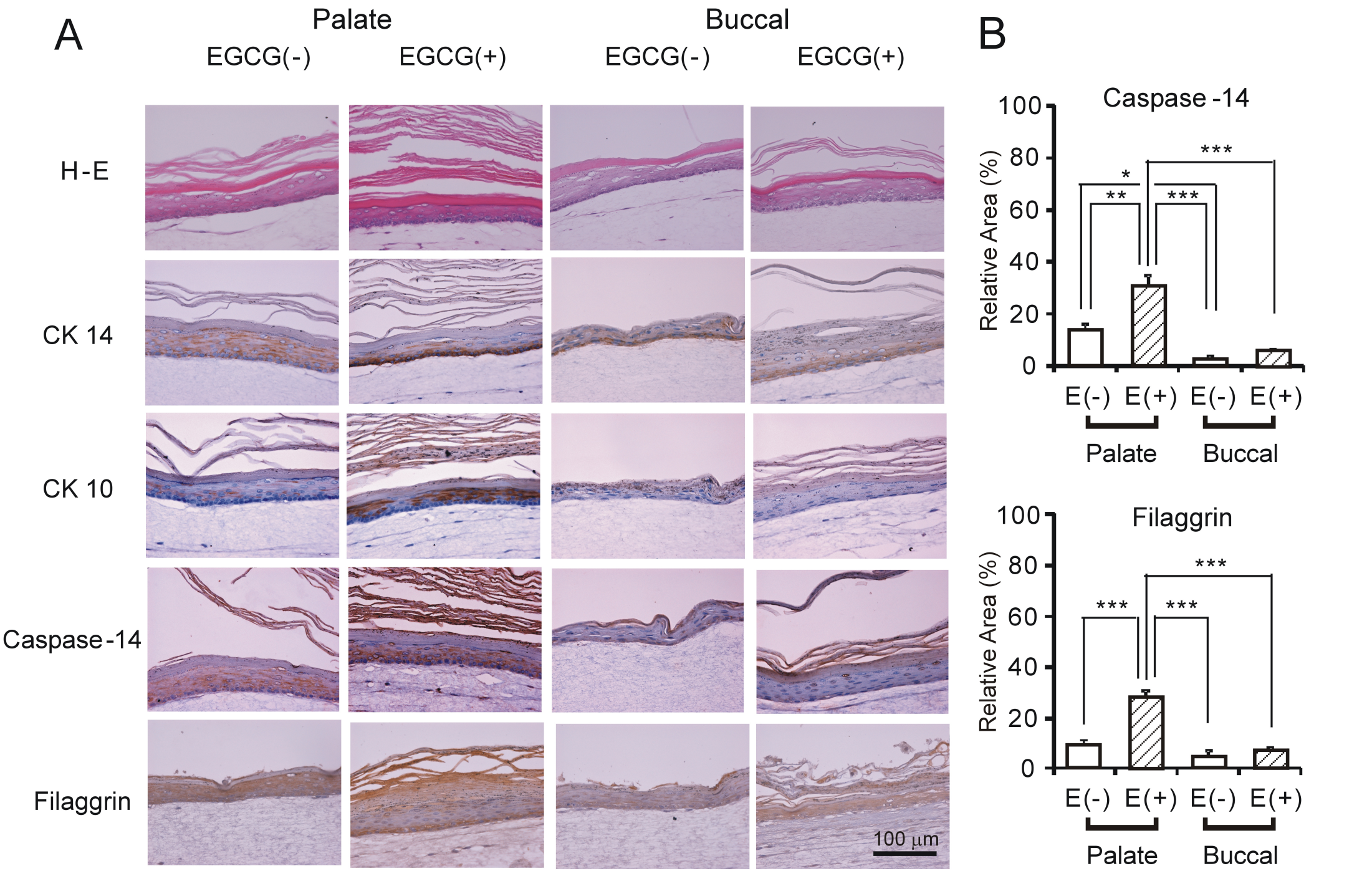

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