NF-κB-regulated transcriptional control of CLCA in a differentiated mouse keratinocyte line

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Running title: Transcriptional control of CLCA

Keywords: CLCA, keratinocyte, NF-κB, differentiation

The authors have no conflict of interest to declare.

Text, 5793 words (excluding this page); abstract, 250 words; number of references, 30; number of tables, 1; number of figures, 5; suppl. figures, 6

Funding sources: A Grant-in-Aid for Challenging Exploratory Research (Grant Number 26670104) (J.Y.) and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology-Supported Program for the Strategic Research Foundation at Private Universities (S1001059) (J.Y.).

Abstract

Background: CLCA was postulated to be a calcium-activated chloride channel accessory protein. Recent reports indicate that CLCA isoforms are likely to be expressed in different layers of the stratified epithelium of the skin. *Objective:* The present study investigated the transcriptional mechanism by which murine CLCA2 (mCLCA2) is expressed in the transformed keratinocyte line Pam212 that can differentiate. Methods: A luciferase reporter assay, chromatin immunoprecipitation (ChIP) assay, reverse transcription-PCR, and immunocytochemistry were performed using Pam212 cells. *Results:* Promoter activity of mCLCA2 was inhibited profoundly by site-directed mutagenesis of a putative nuclear factor-κB (NF-κB) binding site and by treatment with siRNA against p65. ChIP and transcription factor assays showed the specific association of endogenously activated p65 protein with the NF-κB binding domain. As confirmed by the nuclear translocation of p65, tumor necrosis factor α and caffeic acid phenethyl ester (CAPE) increased and decreased mCLCA2 promoter activity, respectively, but exhibited modest effects on endogenous mCLCA2 expression in cells in culture medium containing 0.05 mM Ca^{2+}. When the Ca^{2+} concentration was raised to 1.0 mM, the mRNA and protein levels of mCLCA2 increased as well as those of the differentiation markers keratin 1 (K1) and K10. CAPE profoundly suppressed only the Ca^{2+}-triggered expression of mCLCA2, not K1 or K10. Immunohistochemistry of native skin and organotypic 3D cultures confirmed the distribution of the CLCA2 homolog in differentiated cells. *Conclusion:* The present study revealed for the first time that basal NF-κB activity is involved in the Ca^{2+}-dependent regulation of mCLCA2 expression in a mouse keratinocyte line.

1. Introduction

CLCA was postulated to be a calcium-activated chloride channel accessory protein that modulates the channel pore [1,2]. Previously, we identified a rat CLCA homolog (rCLCA, rCLCA2) that is responsible for modulating Ca^{2+}-dependent Cl^- transport in salivary ductal cells of the rat submandibular gland [3]. More recently, we have shown that isoforms of rCLCA exhibit specific localization and function on epithelial cells in rat skin [4].

Among the 8 mouse *Clca* genes located on chromosome 3, mCLCA2, an isoform sharing a 83% amino acid identity with rCLCA, was shown to be expressed in lactating and involuting mammary glands, suggesting its involvement in stage-specific organogenesis [5,6]. Another mouse isoform, mCLCA1, shares a 95% amino acid sequence identity with mCLCA2, but its tissue distribution is quite distinct [7]. These observations suggest that specific transcriptional regulation occurs for the distinct expression and function of CLCA isoforms [6].

Several CLCA isoforms are reportedly expressed in the stratified epithelium. Human CLCA2 (hCLCA2) is localized along the basal membrane of basal epithelial cells of the cornea and skin and its expression is elevated during epithelial stratification [8,9]. Porcine CLCA2 was detected in the granular layer [10]. Among the mouse isoforms, mCLCA5 was shown to be expressed in the granular layer of the skin and oral cavity, especially in keratohyalin granules [11]. We demonstrated the expression of the full-length isoform of rCLCA in granular and spinous layers of the skin [4]. Recently, Bart *et al.* have shown that rCLCA and mCLCA2 are novel target genes of ultraviolet (UV) radiation and may play a role in epidermal differentiation [12]. Although these CLCA isoforms are likely to be expressed in

keratinocytes, specific regulation of their gene expression has not yet been elucidated.

To clarify the transcriptional mechanism underlying the expression of these CLCA isoforms in differentiated epidermis, we searched for a suitable cell line to achieve this purpose. In a preliminary experiment, we found the expression of mCLCA2, which is highly homologous to rCLCA, in the transformed mouse keratinocyte line Pam212 [13]. In addition, this cell line is reportedly an appropriate model for keratinocyte differentiation. Like normal keratinocytes and other cell lines [14,15], Pam cell lines have the capacity to grow in culture medium containing a low Ca^{2+} concentration (0.02–0.09 mM) and demonstrate the differentiation characteristics of normal keratinocytes when the Ca^{2+} concentration is raised to 1.4 mM. The present study revealed for the first time that basal nuclear factor-κB (NF-κB) activity is involved in the Ca^{2+}-dependent regulation of mCLCA2 expression in a mouse keratinocyte line.

2. Materials and Methods

2.1. Reagents and cell culture

The primary antibodies used were rabbit monoclonal anti-p65 (D14E12; Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal anti-keratin 10 (K10; Dako, Glostrup, Denmark), mouse monoclonal anti-RNA pol II (Active Motif, Carlsbad, CA, USA), rabbit polyclonal anti-CLCA [4,12], and mouse monoclonal anti-β-actin (sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Caffeic acid phenethyl ester (CAPE) and tumor necrosis factor α (TNF-α) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Calbiochem (San Diego,

CA, USA), respectively. The other compounds were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

The Pam212 cell line derived from mouse keratinocytes was a kind gift from Dr. Yuspa (Center for Cancer Research, National Cancer Institute, MD, USA) [13]. They were grown in Joklik modification of minimum essential medium Eagle supplemented with 10% Ca^{2+}-stripped fetal bovine serum (FBS) in low Ca^{2+} concentration (0.05 mM) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The cells were seeded at a density of 5.0×10^4 cells/cm^2 and cultured for 48 h before the assay. Cells were switched from a medium with low Ca^{2+} to one with 1.0 mM Ca^{2+} and cultured for 24 h before the assay to obtain a differentiated phenotype.

2.2. Reverse transcription (RT)-PCR

Pam212 cells were homogenized in an RNA extraction reagent (Isogen; Nippon Gene, Tokyo, Japan). Total RNA was isolated and the mRNA was reverse transcribed into cDNA using a PrimeScript II 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. PCR was performed with a pair of primers (Table 1) specific to mCLCA2, mCLCA1, mCLCA5, keratin 1 (K1), p65 (RelA), and hypoxanthine phosphoribosyltransferase (Hprt) and Taq polymerase (Ex-Taq; Takara) under the following thermal cycling conditions for 40 cycles: 94 °C for 30 s; 54 °C for 30 s; and 72 °C for 60 s. PCR products were analyzed on an ethidium bromide-stained agarose (1.5%) gel.

2.3. Luciferase assay

Fragments of the mCLCA2 5′-flanking region were cloned into a luciferase reporter (pGL3-Basic vector; Promega, Madison, WI, USA). Pam212 cells were transiently transfected with pGL3-Basic or the mCLCA2 reporter construct and with pRL-SV40 (transfection efficiency control) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). The cells were harvested at 24 h after transfection, and luciferase activity was determined with a Dual-Luciferase Reporter Assay System (Promega). Activity was shown as x-fold activity relative to the value for the pGL3-Basic control vector.

2.4. RNA interference

A small interfering RNA (siRNA) to target the mouse p65 gene was purchased (#6337; Cell Signaling). Scrambled RNA control was used as a negative control (#6568; Cell Signaling). The cells were lysed at 48 h after transfection with Lipofectamine 2000 (Life Technologies). The western blot procedure used to confirm the ability of the siRNA to suppress expression is described in the Supplemental Materials.

2.5. Chromatin immunoprecipitation (ChIP)

Nuclear protein and DNA complexes were cross-linked in the culture medium containing 1% formaldehyde for 10 min at 21–25 °C, and were then homogenized in NP-40 buffer for 5 min at 21–25 °C. After centrifugation, the pellet was resuspended in SDS lysis buffer, followed by a 5-fold dilution in ChIP dilution buffer. Ten micrograms of soluble sheared chromatin were incubated overnight at 4 °C with the anti-p65 or anti-Pol II antibody, or control rabbit IgG bound to protein G magnetic

beads (#9006; Cell Signaling). After washing, immune complexes were eluted by incubation for 20 min at 65 °C with ChIP direct elution buffer, and then the cross-links were reversed by an overnight incubation at 65 °C. DNA was purified using a GenElute PCR Clean-up Kit (Sigma-Aldrich), and PCR was performed for DNA amplification specific to the mCLCA2 promoter. The buffers used for ChIP are listed in the Supplemental Materials.

2.6. Transcription factor assays

A nuclear extract was obtained from Pam212 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. For the enzyme-linked immunosorbent assay (ELISA)-based detection of transcription factor activation, 10 μg nuclear extract incubated with 20 pmol oligonucleotides of the mCLCA2 promoter region (−134 to −101) including a putative NF-κB binding sequence (wild, 5′-GGAAAGTCCC-3′) and its mutated sequence (mutated, 5′-GGATAGTATC-3′) were added to a 96-well plate in which oligonucleotides containing the NF-κB site had been immobilized (TransAM NF-κB p65; Active Motif). The binding activity of p65 was quantified colorimetrically by using the anti-p65 antibody and the HRP-conjugated secondary antibody.

2.7. Immunofluorescence

The cells were cultured on type I collagen-coated glass slides. The cells were fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.1% Triton X-100 for 5 min. They were incubated with 10% goat serum for 30 min,

followed by a 2-h incubation with an anti-K10 (1:100), -CLCA2 (1:200), or -p65 (1:100) antibody, and a 1-h incubation with an anti-rabbit or anti-mouse IgG antibody (1:800) conjugated with Alexa Fluor 488 or 594 (Molecular Probes, Inc., Eugene, OR, USA). Fluorescence was observed using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). p65 activity was estimated by examining the number (%) of cells with p65 localized in the nucleus. Quantification of the relative intensity of immunostaining (in arbitrary units [a.u.]) was performed with the aid of ImageJ software (NIH, Bethesda, MD, USA).

2.8 Construction of 3D cultures

All animal experiments were approved by the Animal Research Committee of Fukuoka Dental College. Two-day- and 4-week-old Wistar rats and 4-week-old ddY mice were killed by inhalation of the anesthetic isoflurane. To make an organotypic skin model, keratinocytes and dermal fibroblasts were dissociated from 2-day-old rat dorsal skin, and the 3D culture model was constructed as reported previously [16]. Briefly, fibroblasts were embedded in collagen gel poured into a culture insert (2.5-cm diameter, Millicell CM; Millipore, Temecula, CA, USA) and keratinocytes were overlaid on the gel 2 days later. The culture was performed using DMEM and Ham's F-12 medium (3:1 volume) 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; HKGS Kit; Life Technologies), and 250 μM ascorbic acid (Sigma-Aldrich Co.), and incubated at 37 °C in 5% CO_2. After the keratinocytes grew to confluency, the gel surface was transferred to the air-liquid interface to produce differentiated cell

layers. New culture medium was added every other day to make the organotypic model after 7 days.

2.9 Immunohistochemistry

Paraffin-embedded 5-μm-thick cross-sections of 4-week-old male ddY mouse and Wistar rat skin and 3D cultures were mounted on glass slides, and were used for 3,3′-diaminobenzidine visualization of immunostaining using anti-CLCA2 (1:300), anti-K10 (1:100), and anti-p65 (1:100) antibodies. The sections were counterstained with hematoxylin.

2.10 Statistical analysis

All values are presented as means ± standard deviation (*n*, number of observations). Statistical analysis was performed using one-way analysis of variance followed by a *post hoc* Dunnett's or Scheffe's *t*-test. A grouped *t*-test was employed when two groups were compared. A P value less than 0.05 was considered to indicate statistical significance ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

3. Results

3.1. Analysis of the mCLCA2 promoter

We maintained the mouse keratinocyte line Pam212 in 0.05 mM Ca^{2+}-containing medium in which the cells underwent substantial proliferation. To investigate the regulatory elements of the mCLCA2 promoter, different length segments of the promoter were cloned into luciferase reporter plasmids. Their ability to promote transcription was examined in Pam212 cells transfected with the

reporter plasmid. The luciferase activity of the constructs containing the mCLCA promoter region was greater than that of the control pGL3 plasmid (Fig. 1A). The highest activity was obtained for the plasmid pCLG2-478, which included the promoter region from −478 to the TSS. The upstream region from −1000 to −479 demonstrated decreased reporter activity (Fig. 1A).

Then, we examined the nucleotide sequence of the proximal mCLCA2 promoter region (−478 to TSS) for the presence of putative transcription factor binding sites (Suppl. Fig. 1A). According to *in silico* analysis using a transcription element search program (JASPAR; http://jaspar.binf.ku.dk/), the region contained the putative binding sites of several transcription factors such as NF-κB (GGAAAGTCCC, −122 to −113) and GATA (ACAGATAAGG, −183 to −174). These binding sites were also located in the proximal rCLCA promoter region (Suppl. Fig. 1B). In contrast, mCLCA5 contained none of these binding sites in its proximal promoter region (Suppl. Fig. 2) while mCLCA1 only possessed a putative NF-κB binding site in its distal promoter region (−960 to −950; Suppl. Fig. 3). NF-κB is known to be involved primarily in inflammation and other biological processes in keratinocytes [17]. GATA-3 is reportedly involved in lipid biosynthesis and differentiation [18]. Here, we examined the putative binding sites of these transcription factors in the proximal mCLCA2 promoter region.

To determine whether NF-κB and/or GATA binding sites contribute to the regulation of mCLCA2 promoter activity, we performed a luciferase reporter assay using plasmids containing a mutated NF-κB or GATA binding site. Site-directed mutagenesis of the putative NF-κB binding site profoundly inhibited promoter activity by 80%, although mutation of the putative GATA binding site did not alter

promoter activity (Fig. 1B). Mutagenesis of both GATA and NF-κB binding sites decreased promoter activity to the level observed for mutagenesis of only the NF-κB binding site.

To investigate the role of NF-κB in mCLCA2 transcriptional regulation, promoter activity was examined in Pam212 cells treated either with CAPE, an inhibitor of the NF-κB pathway, or TNF-α, a strong activator of the NF-κB pathway. CAPE (10–50 μg/mL) decreased promoter activity in a concentration-dependent manner, suggesting the involvement of NF-κB signaling (Fig. 1C). In contrast, TNF-α (10 ng/mL) slightly increased luciferase activity, which was significantly inhibited by CAPE (25 μg/mL) (Fig. 1D). These findings suggest that basal NF-κB activity induces a considerable level of mCLCA2 promoter activity and that additional activation of the NF-κB pathway elicits a modest further increase of activity in Pam212 cells.

3.2. Association of p65 with the mCLCA2 promoter in Pam212 cells

To determine whether p65 interacts with the mCLCA2 promoter, a ChIP assay was performed. Anti-p65, anti-Pol II, or negative control IgG antibodies were used to immunoprecipitate protein-DNA complexes from Pam212 nuclear extracts. Amplification of the mCLCA2 promoter region with a pair of PCR primers surrounding the GATA and NF-κB binding sites (Suppl. Fig. 1) revealed the presence of p65-associated cDNA fragments as well as the Pol II-associated fragments used as a positive control (Fig. 2A).

Next, using Pam212 nuclear extracts, we performed an ELISA-based transcription factor assay to test the association of endogenously activated NF-κB

dimers, including p65, with the putative NF-κB binding site in the mCLCA2 promoter region. Spectrophotometry to quantify p65 proteins attached to the immobilized NF-κB consensus sequence revealed the presence of activated NF-κB dimers including p65 in the Pam212 extracts and their association with the consensus sequence (Fig. 2B). Oligonucleotides containing the putative NF-κB binding motif competitively inhibited the association of endogenous p65 with the immobilized consensus sequence (wild, Fig. 2B). Conversely, mutated oligonucleotides failed to inhibit this association (mutated, Fig. 2B). These results confirmed the specific association of endogenously activated p65 protein with the NF-κB binding domain within the mCLCA2 promoter region.

To determine whether p65 is required for the basal activation of mCLCA2 promoter activity, a luciferase reporter assay was performed in Pam212 cells in which p65 had been knocked down. Western blot analysis confirmed the effectiveness of the siRNA used in the present study. Treatment with siRNA against p65 significantly suppressed the promoter activity of mCLCA2 (Fig. 2C).

3.3. NF-κB-dependent mCLCA2 expression in Pam212 cells

We examined whether activation of the NF-κB pathway affects endogenous mCLCA2 expression and the expression of the differentiation markers K1 and K10 using RT-PCR and immunocytochemistry (Fig. 3). Firstly, we confirmed that treatment with TNF-α (10 ng/mL) results in the nuclear translocation of p65 within 20 min (Fig. 3A). CAPE (25 μg/mL, 24 h) decreased p65 nuclear localization in control and TNF-α-stimulated cells.

TNF-α slightly increased mCLCA2 mRNA and protein levels after 24 h,

1 although it failed to alter K1 mRNA and K10 protein expression (Fig. 3B & C).
2 CAPE decreased mCLCA2 levels modestly, while it exhibited a slight increase in K1
3 and K10 expression (Fig. 3B & C). Western blot analysis also showed changes in
4 mCLCA2 protein expression (~82 kDa), similar to the immunocytochemistry
5 findings (Suppl. Fig. 4). The expression of the proliferation markers Ki-67 and
6 proliferating cell nuclear antigen (PCNA) were negatively correlated with NF-κB
7 activity; that is, TNF-α decreased the number of Ki-67- and PCNA-positive cells,
8 whereas CAPE increased it (Suppl. Fig. 5). Each of these compounds had a small
9 effect on mCLCA1 and mCLCA5 expression (Fig. 3B). These results indicate that
10 NF-κB activation is moderately linked to mCLCA2 expression but not to the
11 expression of the differentiation markers.
12
13 *3.4. Enhancement of mCLCA2 expression in Pam212 cells maintained in the*
14 *differentiated phenotype*
15 At 24 h after the culture medium was switched to one containing 1.0 mM Ca^{2+},
16 mCLCA2 mRNA levels were increased in Pam212 cells. In contrast, mCLCA1
17 mRNA was expressed at a lower level, even though Ca^{2+}-induced regulation was
18 seen. mCLCA5 mRNA expression levels were almost the same in both high and low
19 Ca^{2+} conditions (Fig. 4A). In the medium containing 1.0 mM Ca^{2+}, K1 mRNA levels
20 increased (Fig. 4B) and the culture became more compact (Fig. 4C), suggesting cell
21 differentiation. Concurrently, elevated Ca^{2+} decreased the expression of the
22 proliferation markers Ki-67 and PCNA (Suppl. Fig. 5).
23 We then tested whether mCLCA2 expression during Ca^{2+}-induced
24 differentiation was dependent on the NF-κB pathway (Fig. 4B & C). Increased

mCLCA2 expression in 1.0 mM Ca^{2+} was accompanied by an increase in K1 and K10 expression, but inversely associated with a decrease in p65 nuclear translocation. Notably, CAPE inhibited p65 translocation without changing its mRNA levels, and strongly attenuated mCLCA2 mRNA and protein levels in 1.0 mM Ca^{2+} (Fig. 4B & C). However, CAPE failed to inhibit K1 and K10 expression. Western blot analysis also showed changes in mCLCA2 protein expression that were similar to the immunocytochemistry findings (Suppl. Fig. 4). These results suggest that Ca^{2+}-induced mCLCA2 expression, but not the expression of the differentiation markers, is highly dependent on basal NF-κB activation.

3.5 Expression of mCLCA2 and rat homolog in dorsal skin and in 3D cultures using rat skin cells or Pam212 cells

To confirm the localization of mCLCA2 in the stratified epithelium of the mouse epidermis, we performed immunohistochemistry using mouse dorsal skin and the same anti-CLCA antibody. Adult mouse epidermal layers appeared to be 2–3 cells thick, and the signal was localized in both basal and suprabasal cells (Fig. 5A). p65 was mainly detected in the cytoplasm of cells, but some cells possessed nuclear localization.

Previously, we reported a more obvious distribution pattern of the CLCA2 homolog in the epidermis of adult rat [4]. As reported, CLCA2 was expressed mainly in the spinous layer of rat skin. p65 was detected in the cytoplasm of cells throughout the layers, but some cells possessed a faint nuclear localization together with a cytoplasmic distribution (Fig. 5B). The rat organotypic 3D skin model revealed CLCA2 expression mostly in the K10-positive suprabasal layers. A few

basal cells possessed weak reactivity to the CLCA2 antibody. p65 was detected mainly in the cytoplasm of most cells, while a few cells had a p65-positive nucleus in the basal and suprabasal layers (Fig. 5C).

We produced 3D cultures made of Pam212 cells (Suppl. Materials). Cell proliferation was perpendicular to the bottom of the inner dish and formed an irregular structure that was 4–6 cells thick. Most cells were rounded, and flattened cells were found rarely. Differentiation appeared to be poor, judging from cell shape and the localization of K10. This 3D structure resulted in an irregular distribution of CLCA2 with scarce expression of p65 (Suppl. Fig. 6).

4. Discussion

The present study revealed for the first time the NF-κB-regulated transcriptional control of mCLCA2 expression in a transformed mouse keratinocyte line that can differentiate. NF-κB is a transcription factor that orchestrates inflammation and other biological processes in keratinocytes [17]. The classical NF-κB activation pathway is presumed to play a role in the transcriptional control of mCLCA2 in Pam212 cells because of the abundant expression of p65 in these cells. The luciferase assay for mCLCA2 revealed the pivotal role of p65 in its promoter activity (Fig. 1). ChIP and transcription factor assays showed direct evidence of the interaction between p65 and the mCLCA2 promoter (Fig. 2). GATA-3 is expressed in the spinous layer of the epidermis [19] and is implicated in lipid biosynthesis and differentiation in the skin [18]. Although a putative GATA-3 binding domain is located near the NF-κB site of the mCLCA2 promoter, the present study showed that this factor is unlikely to be involved in the endogenous transcriptional

regulation of mCLCA2 in Pam212 cells.

Only a few studies have demonstrated the transcriptional regulation of *Clca* genes. hCLCA1 and its mouse homolog mCLCA3 were shown to be upregulated by signal transducer and activator of transcription 6 and were implicated in mucous cell metaplasia and airway hyperreactivity [20,21]. hCLCA2 and its mouse homolog mCLCA5 are reportedly p53 target genes that regulate the p53-induced apoptotic pathway [22]. Transcriptional regulation of mCLCA2 gene expression seemed to be subtype-specific because CAPE induced no significant change in mCLCA1 and mCLCA5 expression, consistent with the absence of an NF-κB domain in their proximal promoter regions.

Seitz *et al.* [23] have shown that NF-κB is expressed in the nucleus of suprabasal cells, while it is found in the cytoplasm of basal cells and plays an inhibitory role in the growth of stratified epithelium. Furthermore, NF-κB activation in the suprabasal layers prevents premature apoptosis to allow terminal keratinocyte differentiation [24]. In another report [25], although NF-κB was expressed predominantly in the cytoplasm of keratinocytes in normal epidermis, which was similar to our results, the conclusion about the inhibitory role of NF-κB in keratinocyte proliferation was drawn from the fact that TNF-α was shown to activate NF-κB and inhibit proliferation. We confirmed this relationship by counting Ki-67- and PCNA-positive Pam212 cells in the present study. Conversely, loss or gain of NF-κB function was shown not to change the expression of the differentiation genes K10, involucrin, transglutaminase-I, and filaggrin in stratified epithelium [23]. In fact, we could not find a relationship between NF-κB activation and K1 and K10 expression (differentiation markers) (Fig. 3). These results suggest

that no causal relationship exists between endogenous mCLCA2 expression and K1 and K10 expression in terms of NF-κB-dependency (Figs. 3 and 4).

In stratified epithelium, intracellular Ca^{2+} forms a steep gradient within the epidermis, with the highest concentration observed in the granular layer and the lowest concentrations in the basal and spinous layers [26]. This transepidermal Ca^{2+} gradient is an important trigger of keratinocyte differentiation involved in regulating the formation of stratified layers and epidermal barrier [27]. A sustained increase in Ca^{2+} results in the expression of differentiation markers such as K1, K10, involucrin, transglutaminase-I, loricrin, and filaggrin. A number of these genes have response elements, such as activator protein 1 (AP-1) sites, to which members of the Fos/Jun families bind following protein kinase C activation [28]. Keratinocytes exposed to a low extracellular Ca^{2+} concentration remained proliferative, while differentiation was initiated by elevated Ca^{2+} concentrations *in vitro* [13], which was assessed in the present study using Ki-67, PCNA, and K10 staining. We found that mCLCA2 was expressed to a greater extent when Pam212 cells were differentiated by Ca^{2+} (Fig. 4) A further study is required to investigate the roles of the AP-1 binding regions in CLCA promoter activity, although the deletion of the most proximal AP-1 region is unlikely to change mCLCA2 promoter activity (Fig. 1, Suppl. Fig. 1).

Previously, keratinocyte proliferation was shown to be controlled by two distinct pathways—NF-κB-dependent (e.g., TNF-α) and -independent (e.g., Ca^{2+})—because increasing concentrations of Ca^{2+} inhibited proliferation potently without any activation of NF-κB in keratinocytes [25]. The tests in the present study suggest that the Ca^{2+}-induced expression of differentiation markers in Pam212 cells is

unlikely to correlate positively with NF-κB activity. In contrast, mCLCA2 levels in the differentiated state were more sensitive to CAPE than in the undifferentiated one (Fig. 4). As synergism of heterologous transcription factors including NF-κB has been reviewed extensively [29], it is assumed that basal NF-κB activation is a prerequisite for mCLCA2 expression during Ca^{2+}-induced differentiation, in which another mechanism triggered by Ca^{2+} is involved synergically.

Previously, we reported that two variants of rat CLCA2 are localized specifically in epidermal layers and are likely to be related to the differentiation state [4]. In the present study, CLCA2 homologs were located mainly in K10-positive cells of native skin and in the organotypic culture (Fig. 5). In contrast, the 3D structure made of Pam212 cells, which did not differentiate correctly, failed to express mCLCA2 in the regular distribution. A recent paper revealed that CLCA2 is strongly down-regulated by UV radiation and suggested that it may play a role in epidermal differentiation and UV-dependent skin malignancies [12]. Some of the other CLCA members are also reportedly associated with epidermal differentiation [10,11]. In addition to such relationships, based on the *in vitro* findings of p65-induced CLCA regulation, the possible roles of NF-κB activity *in vivo* should be taken into consideration since some populations of keratinocytes exhibited nuclear distribution of p65 in the basal and suprabasal layers of native skin and in the organotypic culture. The present study showed, at least in part, NF-κB-mediated transcriptional regulation of CLCA expression, which should be one of the complicated transcriptional mechanisms involved in epidermal differentiation. NF-κB is known to serve as a key regulator of inflammation and is a crucial mediator in the pathogenesis of psoriasis, an inflammatory dermatosis [17]. Dysregulation of the

NF-κB system observed in skin pathology, for example, sunburn reactions, inflammatory processes, and carcinogenesis [30], is therefore likely to alter CLCA expression and might disturb epidermal differentiation. In our previous study, the expression of an rCLCA splicing variant was found to be more intense in the suprabasal layers of wounded skin than in normal skin [4]. Thus, CLCA2 expression may also be linked with the pathophysiology of wounded skin.

Overall, the findings made in the present study suggest that basal NF-κB activity is involved in the control of the Ca^{2+}-dependent transcriptional regulation of mCLCA2 expression in a mouse keratinocyte line. Given its possible function and transcriptional regulation, alterations in CLCA expression owing to dysregulation of the NF-κB system may be involved in skin diseases and might be a therapeutic target. Further research is needed to deepen our understanding of the roles of CLCA in the physiological regulation of the skin. It would be interesting to investigate further any cross-talk between NF-κB-based transcriptional control and other regulatory elements in differentiated keratinocytes.

Acknowledgements

The authors are grateful to Dr. H. Murakami for support during the revision of the manuscript. This study was supported by a Grant-in-Aid for Challenging Exploratory Research (Grant Number 26670104) (J.Y.) and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology-Supported Program for the Strategic Research Foundation at Private Universities (S1001059) (J.Y.).

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Figure legends

Figure 1. Promoter analysis of the 5′-end of the *mClca2* gene in Pam212 cells. A. Luciferase activity in Pam212 cells transfected with a pGL3 construct containing different length mCLCA2 promoter segments. The numbers correspond to the position of the first nucleotide of the segment relative to the TSS. B. Analysis of site-specific mutations of the putative NF-κB (GGATAGTATC) and GATA (ACACTTAAGG) binding sites in the mCLCA2 promoter region (pGL3-CLCA-302). The mutations are indicated by crosses. C and D. Effects of TNF-α and CAPE on luciferase activity. pGL3-CLCA-302 was transfected. Each compound was added immediately after transfection ($n = 4$).

Figure 2. Role of p65 in mCLCA2 promoter activity in Pam212 cells. A. ChIP assay using anti-p65, anti-Pol II, and control mouse IgG antibodies and nuclear extract from Pam212 cells. B. ELISA-based detection of p65 activation. The nuclear extract was incubated with oligonucleotides of the sequence containing the putative NF-κB binding motif in the mCLCA2 promoter region (wild) or with a mutated binding motif (mutated), or without any oligonucleotides (control) ($n = 4$). C. Effect of p65-siRNA on luciferase activity ($n = 4$). Upper: western blot showing p65 and β-actin protein expression. (–) denotes the proteins obtained from untransfected cells. Markers (M) denote 80 kDa and 60 kDa for p65, and 50 kDa and 40 kDa for β-actin.

Figure 3. Expression of mCLCAs and keratins in Pam212 cells in medium containing 0.05 mM Ca^{2+}. A. Effect of TNF-α (10 ng/mL, 20 min) and CAPE (25 μg/mL, 24 h) on NF-κB activity by examining nuclear localization of p65 (arrows) (n = 3). B & C. Effects of TNF-α and CAPE on the expression of endogenous mCLCAs, K1 and K10. Intensity: n = 6. Each compound was added at 24 h before the analysis. Markers (M) denote 100 and 200 bp. Bars = 50 μm.

Figure 4. Endogenous mCLCA2, keratins, and p65 mRNA and protein expression in Pam212 cells in medium with different Ca^{2+} concentrations. Markers (M) denote 100 and 200 bp. A. RT-PCR analysis of mCLCAs and Hprt expression in medium with different Ca^{2+} concentrations. B. CAPE inhibition (24 h) of mCLCA2 mRNA expression was enhanced by 1.0 mM Ca^{2+}. C. Immunocytochemistry study showing inhibition of mCLCA2 expression by CAPE in medium with 1.0 mM Ca^{2+}. Intensity: n = 4; nuclear localization: n = 3. Arrows denote the nuclear localization of p65. Bars = 50 μm.

Figure 5. Immunohistochemical assessments of mouse and rat skin and 3D cultures. A and B. Immunoreactivity to the CLCA2 antibody was seen in the basal and suprabasal cells of the dorsal skin of a male ddY mouse and in the suprabasal layers of the dorsal skin of a male Wistar rat. Some cells showed faint nuclear staining of p65 (arrowheads). C. Organotypic 3D reconstruction model constructed using native keratinocytes and fibroblasts derived from rat skin, showing the distribution of CLCA2 in a similar pattern to that of K10 in the suprabasal layers. Interestingly, some cells showed CLCA2-positive staining in the basal layer (arrows). Nuclear

1 staining of p65 was observed (arrowheads). Counterstaining: hematoxylin. Bar = 50
2 μm.
3

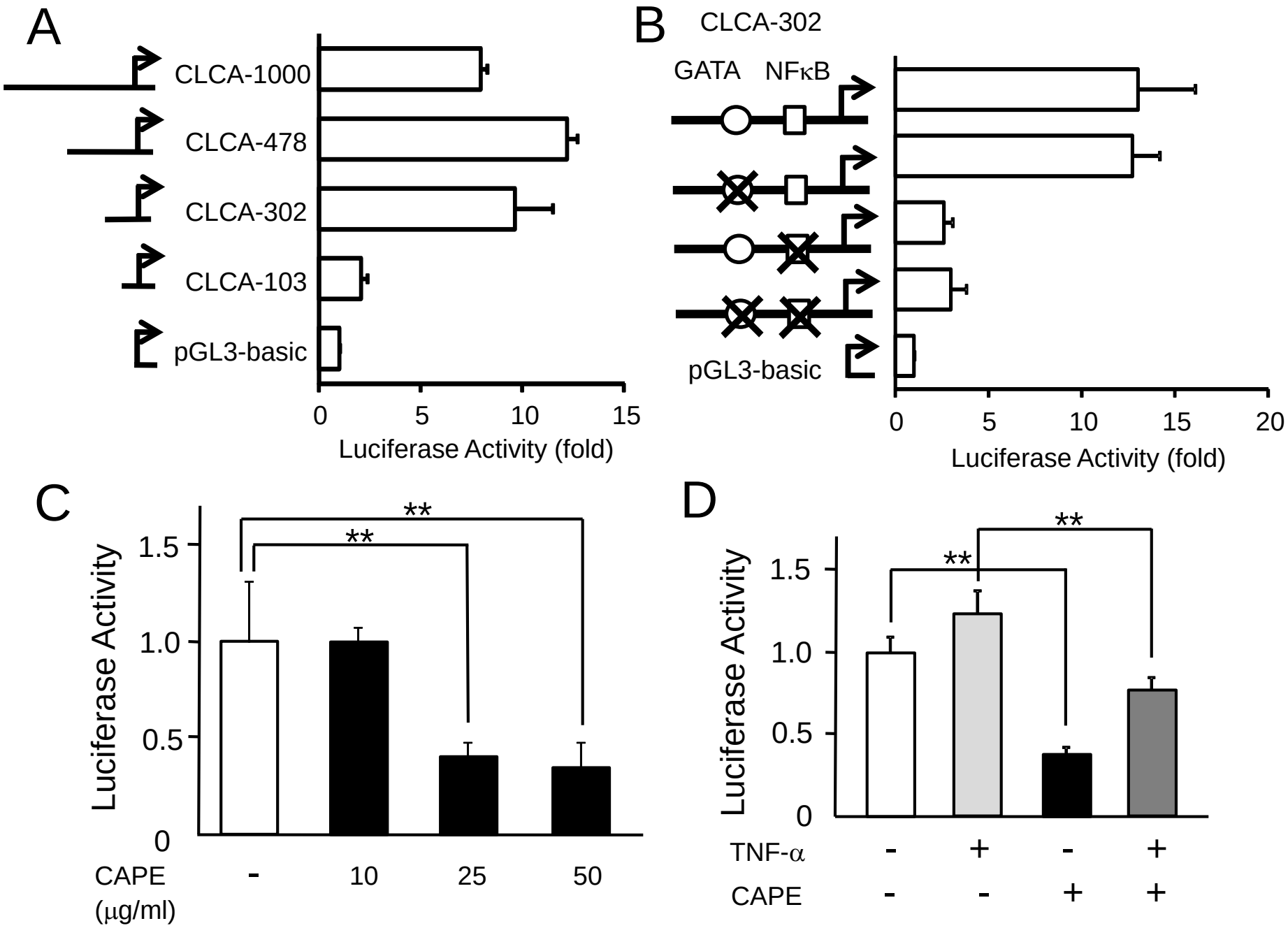


Fig.1 Hiromatsu *et al.*

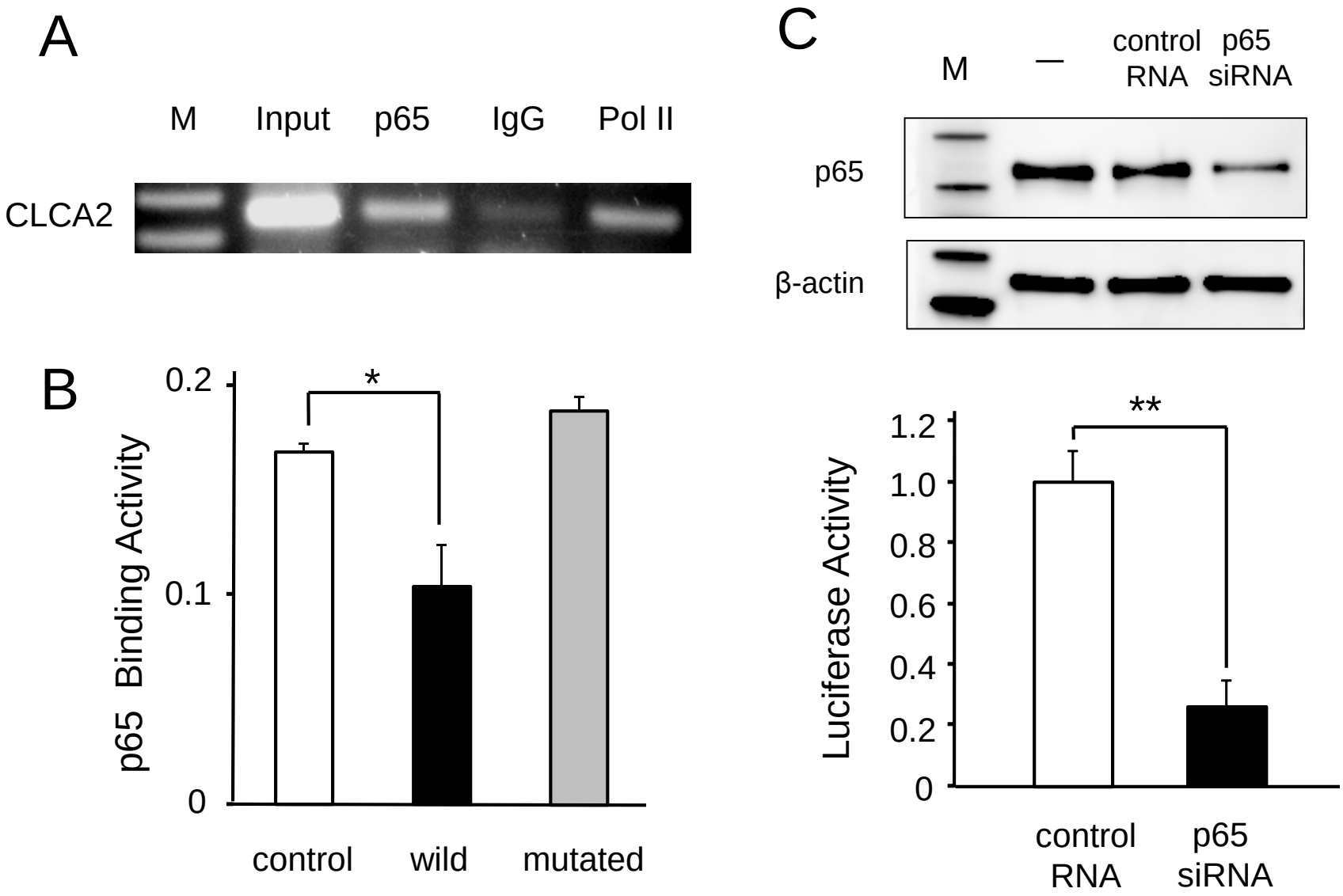
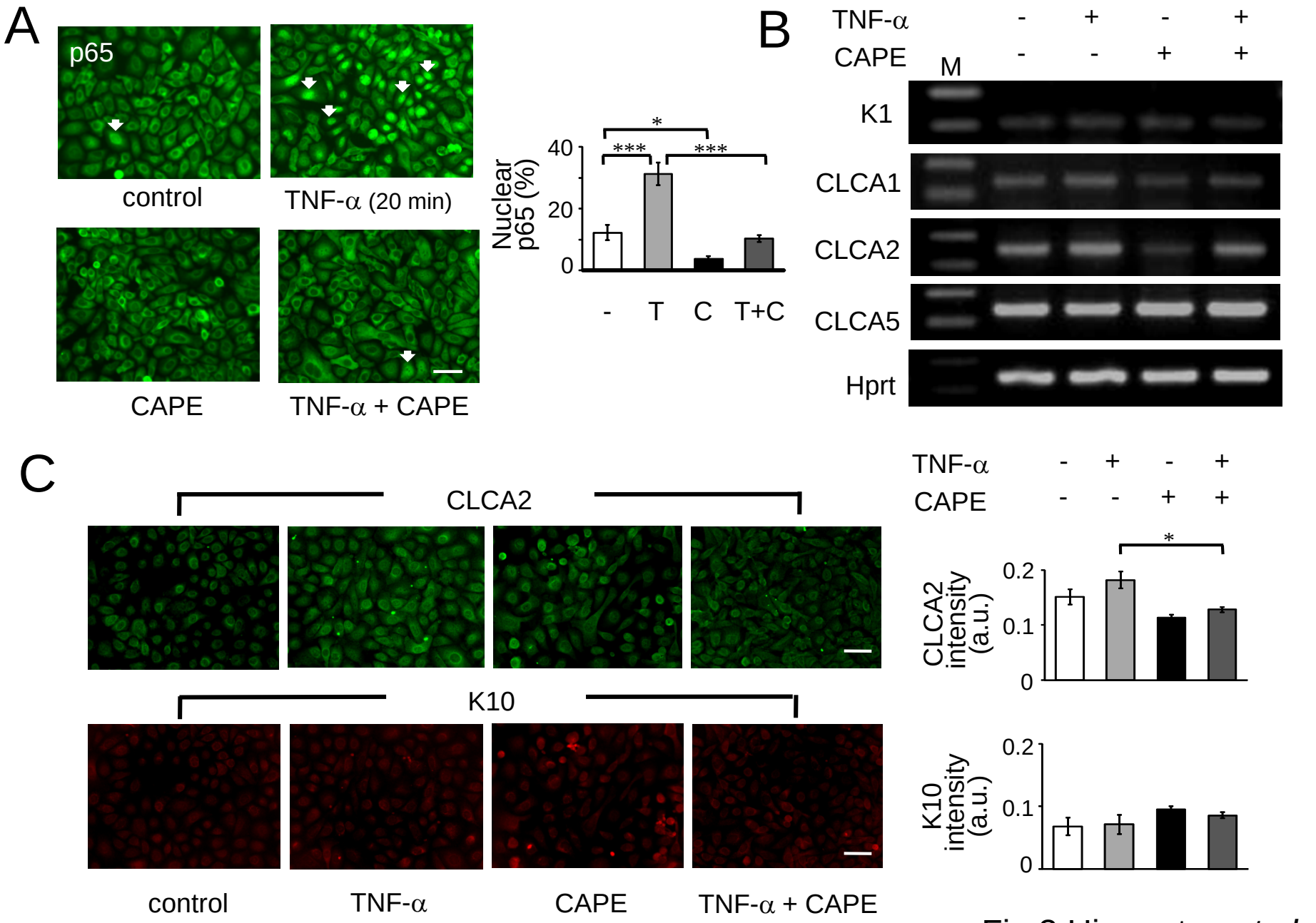


Fig.2 Hiromatsu *et al.*



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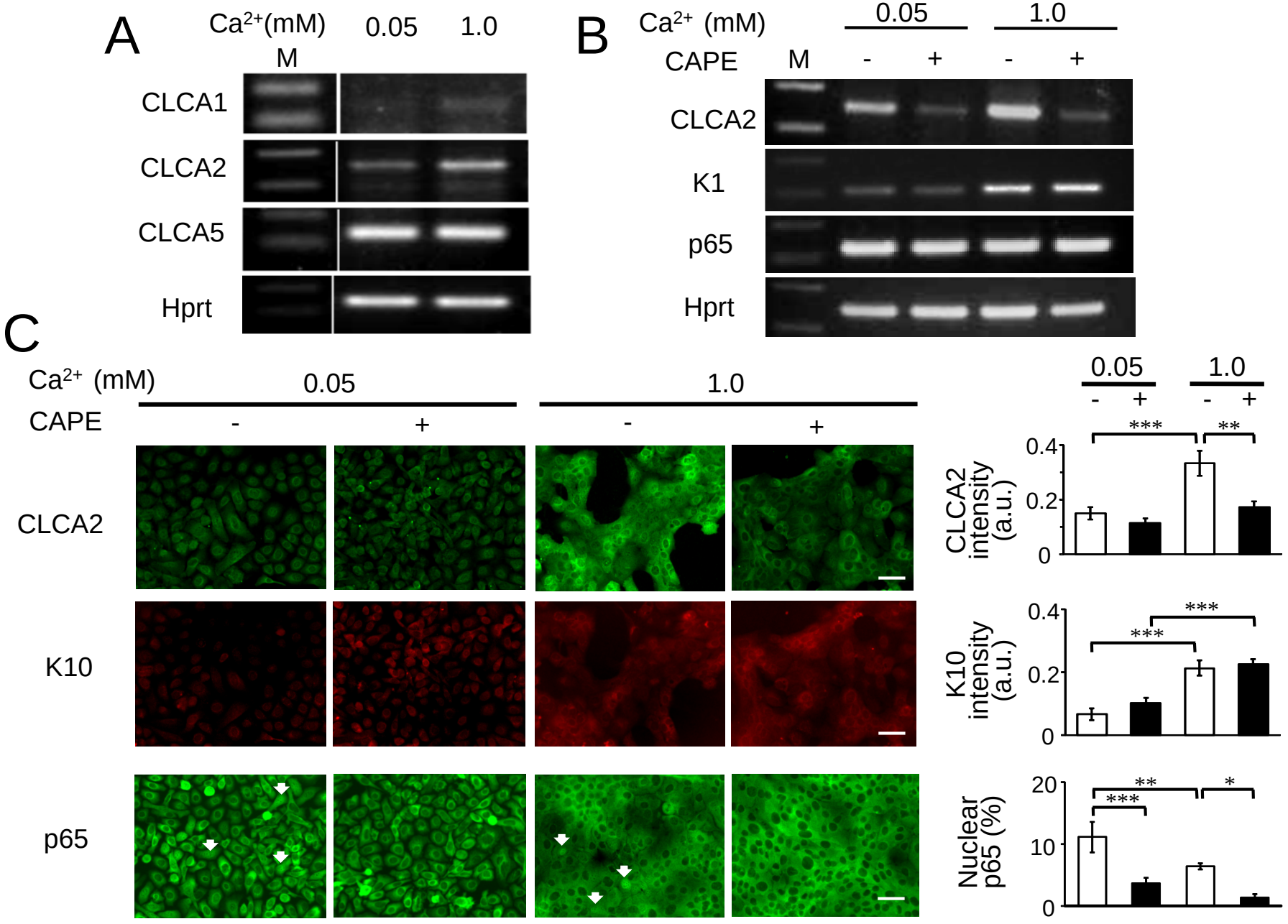

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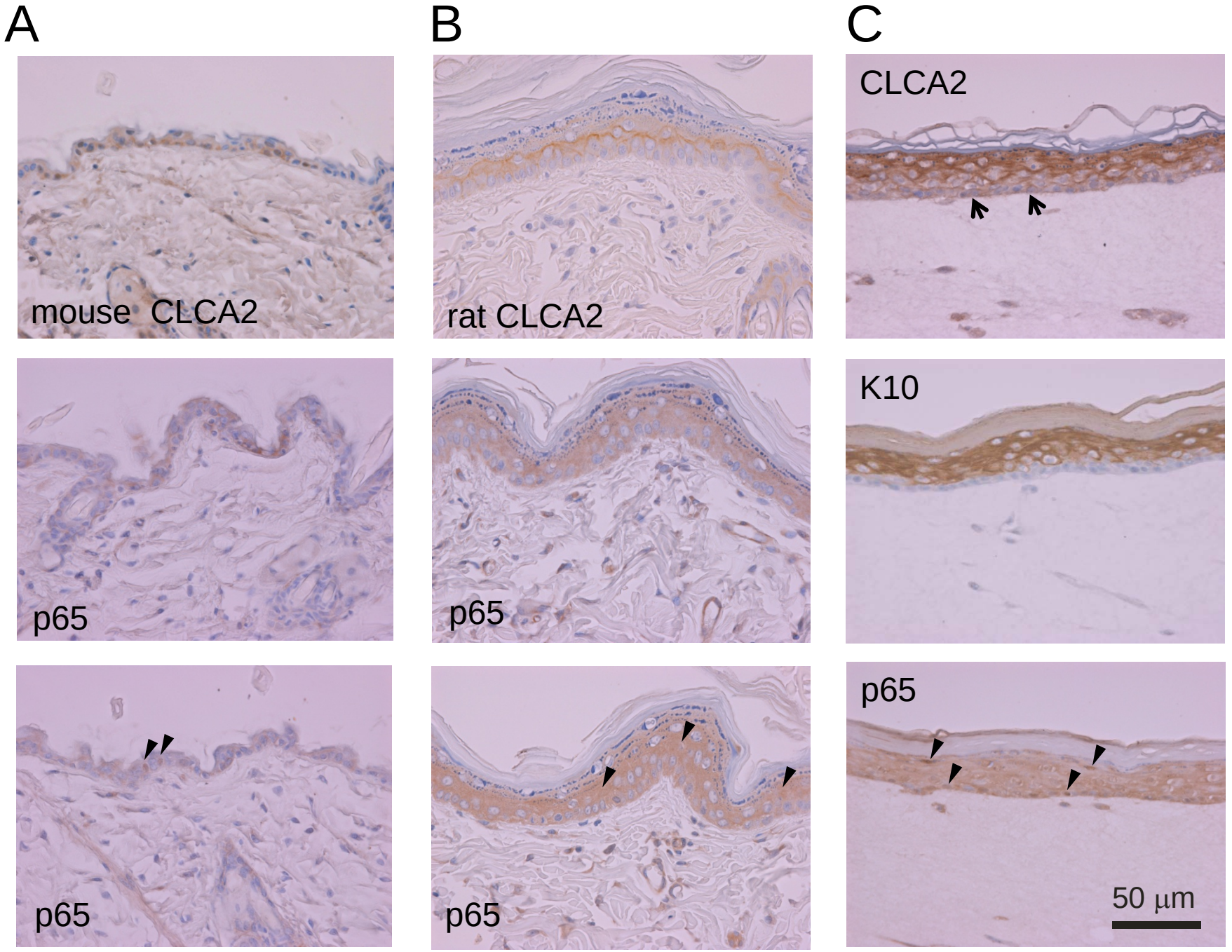

Fig.5 Hiromatsu *et al.*

Suppl. Materials

NF-κB-regulated transcriptional control of CLCA in a differentiated mouse keratinocyte line

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Supplemental Methods

Buffers used for chromatin immunoprecipitation (ChIP)

NP-40 buffer: 10 mM Tris-HCl pH 8.0, 10 mM NaCl, and 0.5% NP-40.

SDS lysis buffer: 50 mM Tris-HCl pH 8.0, 1% SDS, and 10 mM EDTA.

ChIP dilution buffer: 50 mM Tris-HCl pH 8.0, 167 mM NaCl, 1.1% Triton X-100, and 0.11% sodium deoxycholate.

ChIP direct elution buffer: 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% SDS.

Western blot analysis

The cells were homogenized at 4 °C in RIPA buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 5 mM EDTA, 1% sodium deoxycholate). Twenty microliters of the total protein extract were used for SDS-polyacrylamide gel (12%) electrophoresis. Immunoblotting was performed using an anti-p65, anti-β-actin, or anti-CLCA (CN1) [4] antibody, followed by incubation with a horseradish peroxidase-conjugated anti-IgG antibody (Jackson ImmunoResearch Lab., West Grove, PA, USA). Specific bands were detected using ECL Prime

Western Blotting Detection Reagent (GE Healthcare Life Sciences, Buckinghamshire, UK).

Construction of 3D culture with Pam212 cells

To prepare a 3D Pam212 cell culture, the cells were grown to confluence on a type I and III collagen-coated Transwell insert (12 mm diameter, 0.4 μm pore; Transwell-COL, Corning, NY, USA) in JMEM supplemented with Ca^{2+}-deprived serum. Then, the surface was transferred to the air-liquid interface and grown in JMEM supplemented with normal serum and 1.0 mM Ca^{2+} plus growth factor cocktail. New culture medium was added every other day, resulting in a 3D culture after 7 days.

Supplemental Figure Legends

Suppl. Fig. 1. Nucleotide sequence of the mCLCA2 (A) and rCLCA (B) promoter. *In silico* analysis revealed the putative binding sites of transcription factors, indicated by the lines above the sequence. The broken line shows the predicted AP-1 site with a relatively low JASPAR score. The arrow denotes the transcriptional start site (TSS). The arrows with broken lines show the pair of PCR primers used for the amplification of the promoter region.

Suppl. Fig. 2. Nucleotide sequence of the mCLCA5 promoter. *In silico* analysis revealed the putative binding sites of transcription factors, indicated by the lines above the sequence. The arrow denotes the TSS.

Suppl. Fig. 3. Nucleotide sequence of the mCLCA1 promoter. *In silico* analysis revealed the putative binding sites of transcription factors, indicated by the lines above the sequence. The arrow denotes the TSS.

Suppl. Fig. 4. Western blotting using total lysate obtained from Pam212 cells. Effects of TNF-α and CAPE on the expression of endogenous mCLCA2 with different Ca^{2+} concentrations. Each compound was added 24 h before the analysis. A. A representative result. Protein loading was 20 μg per lane. The 82 kDa band was detected using a CLCA2 antibody (CN1). An anti-β-actin antibody was used as a loading control (45 kDa). Markers (M) denote 80 kDa for mCLCA2, and 50 kDa and 40 kDa for β-actin. B. Densitometric analysis of the blot is shown (mCLCA2 / β-actin; $n = 3$).

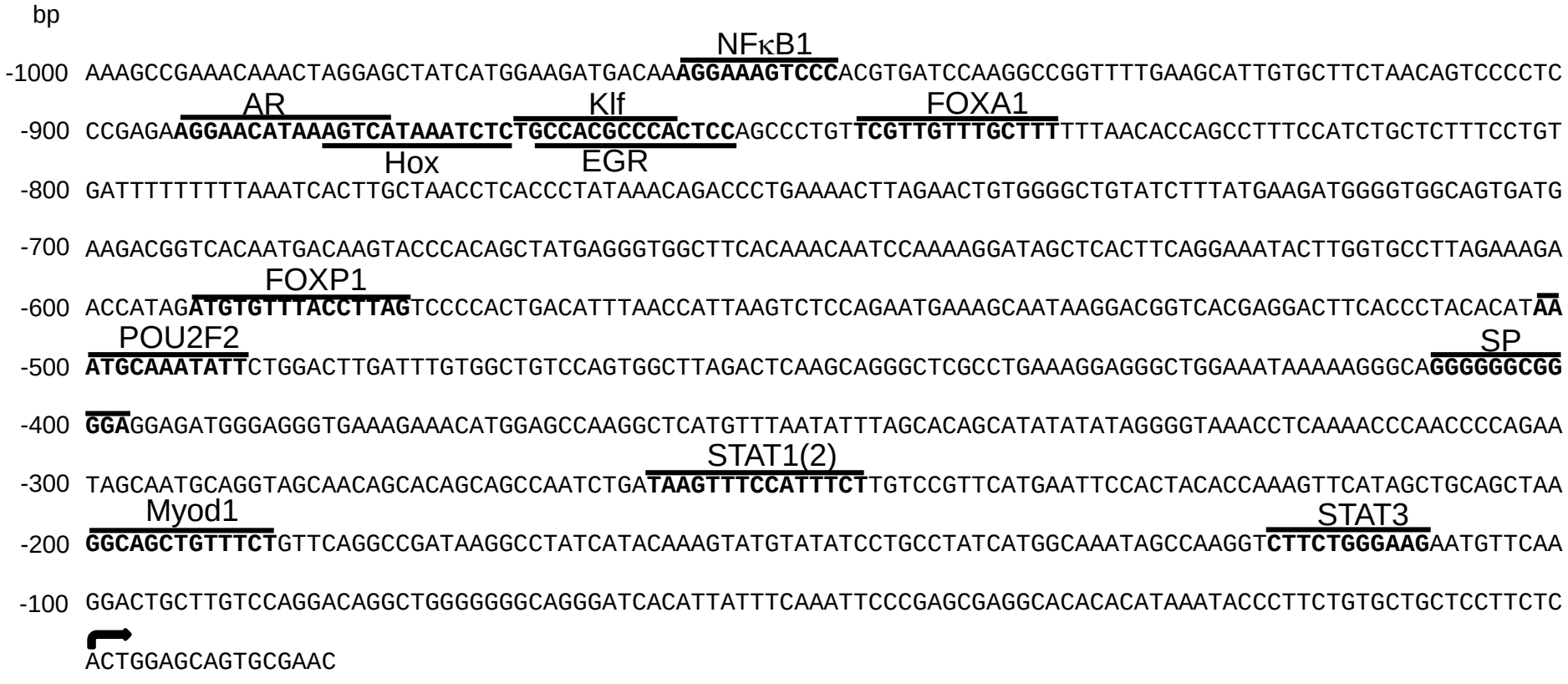
Suppl. Fig. 5. Detection of Pam212 cell growth in the presence or absence of TNF-α or CAPE in media with different Ca^{2+} concentrations by immunocytochemistry with the proliferation markers Ki-67 (anti-Ki-67, 1:100, MIB-5; Dako, Glostrup, Denmark) and PCNA (anti-PCNA, 1:400, PC10; Dako). A. Typical nuclear staining of Ki-67 and PCNA (red) in the control is shown (arrowheads). Blue: nuclear staining using 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies). Percentages of the marker-positive cells are shown in B. Bar = 50 μm.

Suppl. Fig. 6 Immunohistochemical assessments of 3D culture using Pam212 cells. The irregular distribution of CLCA2 and K10 was observed. A nucleus was positive for p65 (arrowhead). * denotes non-specific staining. Counterstaining was performed with hematoxylin. Bar = 50 μm.

Suppl. Fig.1A Hiromatsu *et al.*

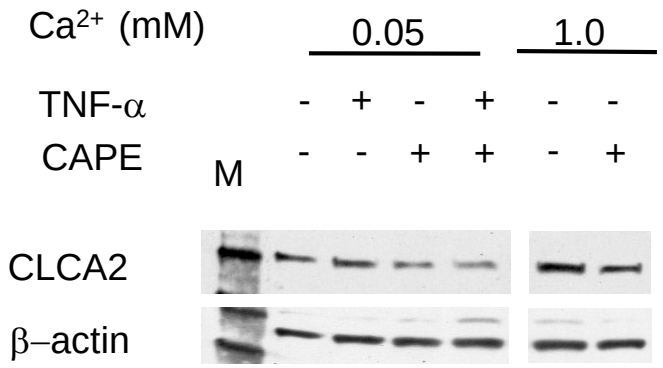
Suppl. Fig.1B Hiromatsu *et al.*

Suppl. Fig.2 Hiromatsu *et al.*



Suppl. Fig.3 Hiromatsu *et al.*

A



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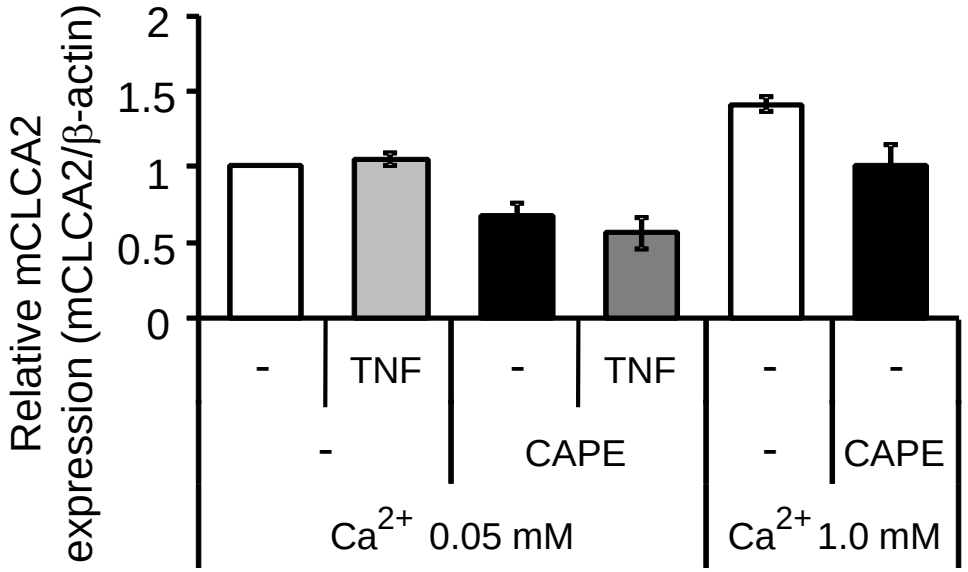
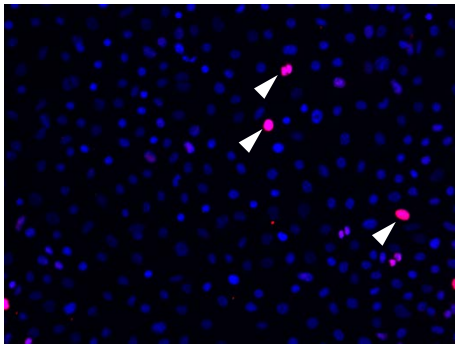
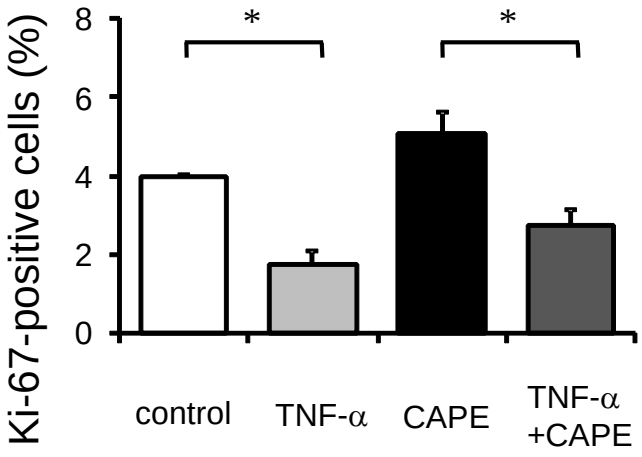


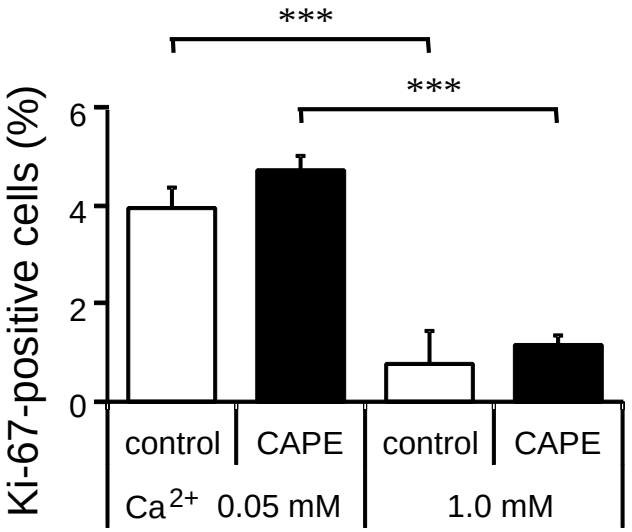
Suppl. Fig.4 Hiromatsu *et al.*

A



Ki-67





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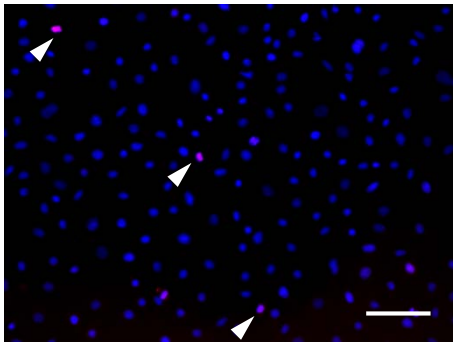

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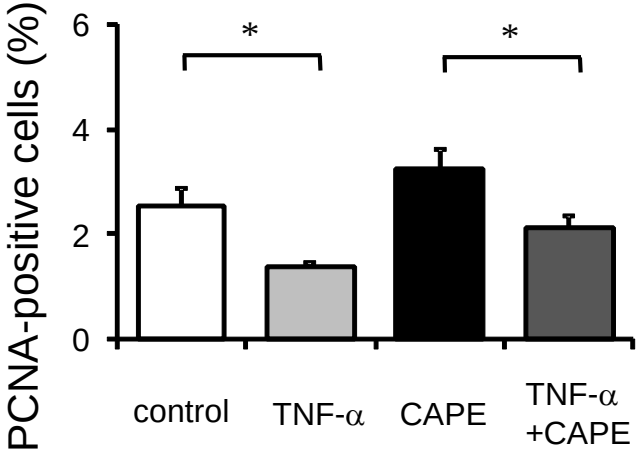

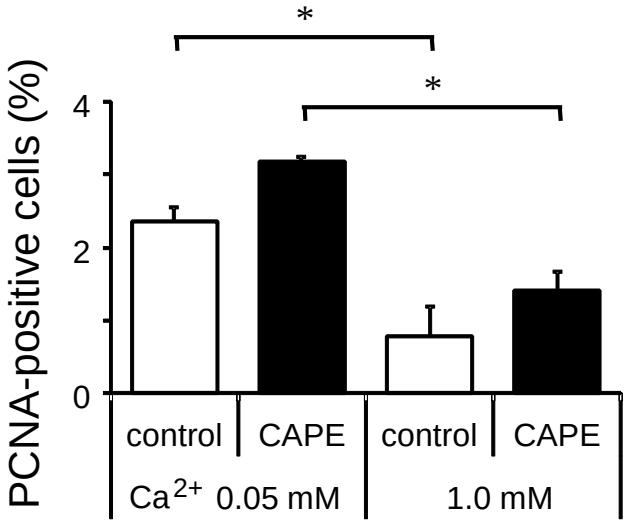

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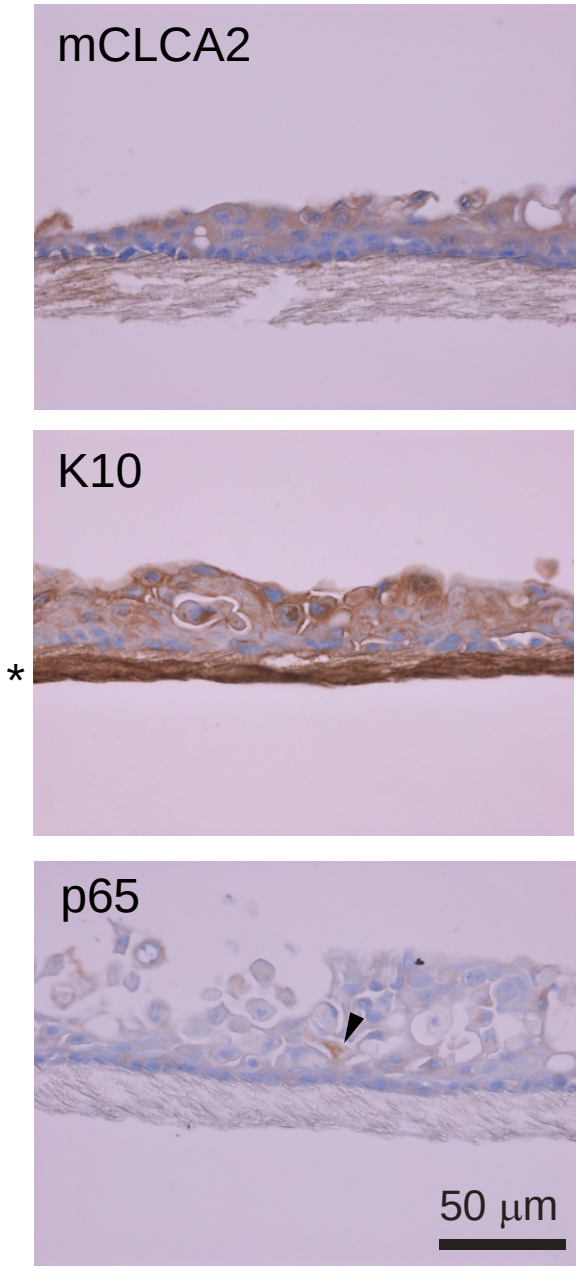

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