EPIGENETIC CONTROL OF SKIN DIFFERENTIATION GENES BY PHYTOCANNABINOIDS

Mariangela Pucci [1§], Cinzia Rapino [1§], Andrea Di Francesco [1]

Enrico Dainese [1, 2], Claudio D'Addario [1*], Mauro Maccarrone [2, 3*]

[1] Department of Biomedical Sciences, University of Teramo, Teramo, Italy; [2] European Center for Brain Research (CERC)/Santa Lucia Foundation, Rome, Italy; [3] Center of Integrated Research, Campus Bio-Medico University of Rome, Rome, Italy

[§] *Equal contribution.*

* *Corresponding authors.*

Dr. Claudio D'Addario
Department of Biomedical Sciences
University of Teramo
Piazza Aldo Moro 45, 64100 Teramo
Tel/Fax: +390861266877
E-mail: cdaddario@unite.it

Prof. Mauro Maccarrone
Center of Integrated Research
Campus Bio-Medico University of Rome
Via Alvaro del Portillo 21, 00128 Rome
Tel: +39 06 2254 19169
Fax: +39 06 2254 1456
E-mail: m.maccarrone@unicampus.it

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bph.12309

Summary

Background and purpose: A role for endocannabinoid signaling has been reported in the control of epidermal physiology, whereby anandamide is able to regulate the expression of skin differentiation genes through DNA methylation. Here, we have investigated the possible epigenetic regulation of these genes by selected phytocannabinoids, plant-derived cannabinoids holding potential as novel therapeutics for various human diseases.

Experimental approach: The effects of cannabidiol, cannabigerol and cannabidivarin were investigated in human keratinocytes (HaCaT cells) on the expression of the skin differentiation genes keratins 1 and 10, involucrin and transglutaminase 5, as well as on DNA methylation of keratin 10 gene. Moreover, changes induced by phytocannabinoids in global DNA methylation and in the activity and expression of four major DNA methyltransferases (DNMT1, 3a, 3b and 3L) were studied.

Key results: Treatment of differentiated HaCaT cells with cannabidiol or cannabigerol significantly reduced the expression of all genes tested via increased DNA methylation for keratin 10 gene; instead, cannabidivarin was ineffective. Remarkably, cannabidiol reduced keratin 10 mRNA through a type-1 cannabinoid (CB_1) receptor-dependent mechanism, whereas cannabigerol did not engage CB_1 nor type-2 cannabinoid (CB_2) receptors of HaCaT cells. In addition cannabidiol, but not cannabigerol, increased global DNA methylation levels by selectively enhancing DNMT1 expression, without affecting DNMT 3a, 3b or 3L.

Conclusions and Implications: These findings identify the phytocannabinoids cannabidiol and cannabigerol as transcriptional repressors that can control cell proliferation and differentiation, suggesting (especially for cannabidiol) a possible exploitation as lead compounds to be used in the development of novel therapeutics for skin diseases.

Keywords*:* Phytocannabinoids; endocannabionid system; gene expression; DNA methylation; skin.

Introduction

Endocannabinoids (eCBs) are lipid mediators derived from membrane precursors and are implied in multiple regulatory functions, both in health and disease (Di Marzo and Petrosino, 2007). The two most important eCBs are *N*-arachidonylethanolamine (‘‘anandamide’’, AEA) and 2-arachidonoylglycerol (2-AG), that elicit their activity via at least two G-protein–coupled cannabinoid receptors (CB_1 and CB_2), both widely distributed throughout the body (Rodríguez de Fonseca et al., 2005). AEA and 2-AG can also activate non-CB_1/non-CB_2 receptors and/or a purported ‘‘CB_3’’ (or GPR55) receptor (Baker et al., 2006); yet, there is controversy about the actual involvement of GPR55 in eCBs signaling (Pertwee et al., 2010). Furthermore AEA, but not 2-AG, behaves as a ligand to type-1 vanilloid receptor (transient receptr potential vanilloid 1, TRPV1) channels (Pertwee et al., 2010). Several enzymes are involved in eCBs synthesis and degradation: AEA is synthesized mainly by *N*-acyl-phosphatidylethanolamines-specific phospholipase D (NAPE-PLD), and is degradated by fatty acid amide hydrolase (FAAH); 2-AG is mainly synthesized by an *sn*-1-specific diacylglycerol lipase (DAGL), and is degraded by a specific monoacylglycerol lipase (MAGL) (Ahn et al., 2008; Di Marzo, 2008; Ueda et al., 2011). Within the central nervous system and in peripheral tissues, eCBs, their target receptors and metabolic enzymes, along with the proteins responsible for their transport and intracellular trafficking, form the endocannabinoid system (ECS) (Maccarrone et al., 2010).

Recently, a role for the ECS has been reported in the control of skin physiology (Birò et al., 2009; Pasquariello et al., 2009), and has been suggested a potential exploitation of ECS

elements as new targets for future therapies in dermatology (Kupczyk et al., 2009; Paus et al., 2006; Karsak et al., 2007; Petrosino et al., 2010).

The epidermis is the outer layer of the skin serving as a physical and chemical barrier to the environment, provided by terminally differentiated keratinocytes (Nemes and Steinert, 1999; Kalinin et al., 2001). Epidermal differentiation begins with the migration of keratinocytes from basal layer, composed of proliferating cells, and ends with the formation of the cornified cell envelope, an insoluble protein structure found in differentiated keratinocytes (Candi et al., 2005).

All major ECS components have been found to be active in human epidermis, where CB_1 cannabinoid receptor expression is higher in more differentiated (i.e., granular and spinous) layers of skin (Casanova et al., 2003; Stander et al., 2005). Also immortalized and normal epidermal keratinocytes have a fully and functional ECS (Berdyshev et al., 2000; Maccarrone et al., 2003; Oddi et al., 2005). In these cells, AEA mediates transcriptional effects associated with epidermal differentiation and skin development, through a CB_1-dependent mechanism (Maccarrone et al., 2003). In line with this, in spontaneously immortalized human keratinocytes (HaCaT cells) and in normal human epidermal keratinocytes (NHEK cells) induced to differentiate *in vitro* by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) plus calcium, AEA levels were reduced due to enhanced degradation by FAAH (Maccarrone et al., 2003). Moreover, in HaCaT cells exposed to AEA it has been observed a reduction in the formation of cornified envelopes (Maccarrone et al., 2003) and a reduction in the expression of keratins 1 (K1) and 10 (K10), involucrin and transglutaminase 5 (TGase 5) genes, which were all up-regulated during cornification (Paradisi et al., 2008).

Gene expression is controlled by epigenetic mechanisms, that cause heritable but potentially reversible changes in DNA methylation, histone modification, and RNA-associated silencing (Jaenisch and Bird, 2003). Epigenetics is thus the study of molecular mechanisms by which

the environment controls gene activity independently of DNA sequence. It is well-established that complex diseases generally are caused by both genetic and environmental factors, but even if the role of genetic abnormalities in the pathogenesis of many skin diseases has been deeply investigated (for review see Zhang, 2012), studies on the importance of epigenetics in altering the course of these diseases are still a few (Millington, 2008; Lopez et al., 2009; Chen et al., 2008).

Variations in global DNA methylation have been reported between differentiated and undifferentiated cells (Ehrlich, 2003; Lyon et al., 1987), and in particular an hypomethylation in differentiated *versus* undifferentiated keratinocytes has been documented (Veres et al., 1989). Moreover, inhibition of DNA methylation and histone deacetylation has been shown to promote keratinocyte differentiation (Rosl et al., 1988; Schmidt et al., 1989; Staiano-Coico et al., 1989), and an inverse correlation between DNA methylation and the expression of differentiating genes has been demonstrated in human keratinocytes (Engelkamp et al., 1993; Elder et al., 2002). It has been also suggested that inhibition of differentiation by AEA occurs through changes in chromatin methylation patterns (Paradisi et al., 2008; Pasquariello et al., 2009), and that AEA induces DNA methylation of keratinocyte-differentiating genes by increasing DNA methyltransferase (DNMT) activity via a CB_1-dependent involvement of p38 and p42/p44 mitogen-activated protein kinases (MAPK) (Paradisi et al., 2008).

Based on these findings, here we have investigated the possible epigenetic regulation of skin differentiation genes by selected phytocannabinoids, that are plant-derived cannabinoids which mimic the natural eCBs, thus holding potential as novel therapeutics for human diseases (Hill et al., 2012a).

Phytocannabinoids are known to have anti-inflammatory properties (Klein, 2005) and to inhibit growth of proliferating cancerogenic cells (Kogan, 2005). These compounds are lipophilic, and hence readily absorbed through the skin. In particular, it has been documented

that CBD accumulates only in the stratum corneum, without penetrating into the deeper layers (Lodzki et al., 2003). However, the therapeutic potential of cannabinoid-based preparations for skin diseases has not been yet investigated. Up to date, just one study reported the inhibition of human keratinocyte proliferation by phytocannabinoids, suggesting that the latter substances could be beneficial in the treatment of psoriasis (Wilkinson and Williamson, 2007).

In this study we investigated the effects of three major non-psychoactive components of *Cannabis sativa* (Izzo et al., 2009): cannabidiol (CBD) and its precursor cannabigerol (CBG), that are with Δ9-tetrahydrocannbinol (THC) the most abundant phytocannabinoids (Hill et al. 2012b); and cannabidivarin (CBDV), a propyl analogue of CBD which shares with its congener anti-convulsant properties (Jones et al., 2010, Hill et al., 2012a).

The understanding of the epigenetic regulation of keratinocyte differentiation by phytocannabinoids may pave the way to the development of new drugs for skin diseases, by analogy with other human disorders like multiple sclerosis (Rog, 2010), bowel disease (Lal et al., 2011), and cancer (Solinas et al., 2012).

Methods

Nomenclature of all drug/molecular targets described in this study conforms to BJP's Guide to Receptors and Channels (Alexander et al., 2011).

Materials

Chemicals were of the purest analytical grade. Anandamide (AEA) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *S*-Adenosyl-L-[methyl-^{3}H]methionine was from Amersham Biosciences (Buckinghamshire, UK). Cannabidiol (CBD), cannabigerol (CBG) and cannabidivarin

(CBDV) were kind gifts of GW Pharma Ltd (Sittingbourne, United Kingdom). Capsazepine (*N*-[2-(4-chlorophenyl) ethyl]-1,3, 4, 5-tetrahydro-7, 8-dihydroxy-2H-2-benzazepine-2-carbothioamide, CPZ) was from Calbiochem (San Diego, CA,USA). *N*-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole carboxamide (SR141716) and *N*-[(1)-endo-1,3,3-trimethy-1-bicyclo [2.2.1]-heptan-2-yl]5-(4-chloro-3-methyl-phenyl)-1-(4-methyl-benzyl)-pyrazole-3-carboxamide (SR144528) were from Sanofi-Aventis Recherche (Montpellier, France). Goat anti-DNMT1 and anti-Lamin A polyclonal antibodies, and rabbit anti-goat antibody conjugated to horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and treatment

HaCaT cells were grown in a 1:1 mixture of minimum essential medium and Ham's F-12 medium (Invitrogen, Berlin, Germany), supplemented with 10% fetal calf serum and 1% non-essential amino acids, at 37°C in a 5% CO_2 humified atmosphere. Cell differentiation was induced by treating HaCaT cells with TPA (10 ng/ml) plus $CaCl_2$ (1.2 mM) for 5 days (Candi et al., 2001).

AEA, CBD, CBG, CBDV were dissolved in methanol; SR141716, SR144528, and CPZ were dissolved in DMSO; these compounds were added at the indicated concentrations directly to the serum-free culture medium, at the same time as TPA plus calcium (Paradisi et al., 2008). Culture medium containing vehicles alone was added to controls under the same conditions. After each treatment, cell viability was determined by Trypan Blue dye exclusion, as reported (Paradisi et al., 2008).

Normal human epidermal keratinocytes (NHEK) (Lonza Group Ltd, Basel, Switzerland) were grown at 37°C in a humidified 5% CO_2 atmosphere in KGM-Gold™ growth medium (Lonza Group Ltd), according to the manufacturer's instructions. NHEKs were treated for 5

days with AEA, CBD and CBG at the indicated concentrations, as described above for HaCaT cells.

Quantitative real-time RT-PCR

RNA was extracted using RNeasy extraction kit (Qiagen, Crawley, UK) from proliferating and differentiated HaCaT cells, following the manufacturer's instructions. RT-PCR reactions were performed using the QuantiTect Reverse Transcription Kit (Qiagen). The relative abundance of each mRNA species was assessed by quantitative real-time RT-PCR (qRT-PCR), using QuantiFast Multiplex PCR Kit (Qiagen) on a DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA). The primers used for PCR amplification are shown in Table 1. Actin was used as housekeeping gene for quantity normalization (D'Addario et al., 2008). One µl of the first strand cDNA product was used for amplification in triplicate in 20 µl reaction solution, containing 10 µl of QuantiFast Multiplex PCR Kit (Qiagen) and 10 pmol of each primer. The following PCR program was used: 95°C for 10 min, followed by 50 amplification cycles of 95°C for 10 sec, and 60°C for 30 sec.

Genomic methylation level

A modification of the methyl-accepting assay (Broday et al., 1999) was used to determine the methylation level of DNA isolated from HaCaT cells. DNA (200 ng) was incubated with 4 units of SssI methylases (New England Biolabs, Ipswich, MA) in the presence of 1.5 mM *S*-adenosyl-L-[methyl-[^{3}H]methionine and 1.5 mM nonradioactive S-adenosylmethionine (New England Biolabs). The reaction mixtures (20 µl) were incubated at 37°C for 4 h in the manufacturer's buffer containing 0.1 µg of RNase A. The reactions were terminated by adding 300 µl of stop solution (1% sodium dodecyl sulfate, 2 mM EDTA, 5% 2-propyl

alcohol, 125 mM NaCl, 1 mg of proteinase K per ml, 0.25 mg of carrier DNA per ml) for 1 h at 37°C. DNA was extracted with phenol-chloroform and was ethanol-precipitated. The recovered DNA was resuspended in 30 µl of 0.3 M NaOH and incubated for 30 min at 37°C. DNA was spotted on Whatman GF/C filter discs, dried, and then washed five times with 5% (w/v) trichloroacetic acid followed by 70% (v/v) ethanol. Filters were placed in scintillation vials and incubated for 1 h at 60°C with 500 µl of 0.5 M perchloric acid. Then, 5 ml of scintillation mixture was added, and tritium incorporation was determined in a Tri-Carb 2810 TR liquid scintillation analyzer (Perkin Elmer, Waltham, MA) . Higher levels of [3H]methyl group incorporated into DNA were indicative of lower levels of genomic DNA methylation (Paradisi et al., 2008).

Assay of DNA methyltransferase activity

Cell extracts were prepared in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 10% glycerol, 0.01% sodium azide, 10% Tween-80, 100 µg/ml RNase A, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). *De novo* methyltransferase activity was measured in cell extracts (30 µg proteins per test), that were incubated in the presence of 3 µg double-stranded oligonucleotides and 2.4 µCi of *S*-adenosyl-L-(methyl-^{3}H)methionine (Amersham Biosciences), at 37°C for 1 h. The reaction was terminated by adding 90 µl of stop solution (1% sodium dodecyl sulfate, 2 mM EDTA, 3% (wt/vol) 4-amino salicylate, 5% butanol, 0.25 mg/ml calf thymus DNA, and 1 mg/ml proteinase K), followed by incubation at 37°C for 45 min. The reaction mixture was then spotted on a Whatman GF/C filter paper disc (Sigma). Filters were washed twice with 5% trichloroacetic acid, rinsed in 70% ethanol, and dried at 56°C for 20 min. Finally, filters were submerged in UltimaGold (Packard, Meriden, CT, USA) scintillation solution, and radioactivity was measured in a Tri-Carb 2810 TR liquid scintillation analyzer (Perkin Elmer). A blank control reaction was done simultaneously using

cell extracts that were heated to 80°C for 15 min to inactivate DNMT. The results were expressed as counts per min (cpm), and were corrected by background subtraction.

Analysis of DNA methylation by methylation-specific primer real-time PCR

Genomic DNA was isolated from HaCaT cells using DNeasy kit (Qiagen). After DNA extraction, DNA (2 µg) was treated with bisulfite, using the Methyl Detector Bisulfite Modification Kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Relative abundance of each mRNA species was assessed by real-time qRT-PCR, using QuantiFast Multiplex PCR Kit (Qiagen) on a DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research). The amplification program was as follow: 95°C for 2 min, 50 cycles at 95°C for 10 s, and 60°C for 30s. PCR was also performed for the non-CpG-containing region of myoD, that served as control gene (D'Addario et al., 2012). One µl of bisulfite-treated DNA was used for amplification in triplicate in a 20 µl reaction solution containing 10 µl of QuantiFast SYBR Green PCR (Qiagen) and 10 pmol of each primer. The DNA methylation level was calculated as $(1/1+2^{-\Delta Ct})$, where $\Delta Ct = Ct_U - Ct_M$ (Lu et al., 2007). The data were reported as fold induction over proliferating cells (Prol=1). The primers used for PCR amplification for both gene expression and K10 DNA methylation levels are shown in Table 1.

Immunochemical analysis

The nuclear extracts content was determined by the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). For Western blotting, equal amounts of protein (25 µg/lane) were loaded onto 8% sodium dodecyl sulfate–polyacrylamide gels, and were electroblotted onto polyvinylidene fluoride sheets (GE-Healthcare, Pollards Woods, UK). Membranes were blocked with 3% bovine serum albumin for 2 h, and then were incubated with anti-DNMT1

(1:500), or Lamin A (1:1000) antibodies. Then, membranes were rinsed and incubated with HRP-conjugated secondary antibody (diluted 1:1000) in blocking solution. Membranes were washed with TBS-T buffer and incubated with a 1:1000 dilution of HRP–conjugated secondary antibodies (Sigma, St Louis, MO, USA), for 1 h at room temperature. After washing with TBS-T buffer, proteins ware visualized using the HRP substrate ECL Prime (GE-Healthcare, Pollards Woods, UK).

Statistical analysis

The data reported are the mean ± S.E.M. of at least three independent determinations, each performed in triplicate. Statistical analysis was performed by unpaired t-test or One-Way ANOVA test, as appropriate. *Post hoc* comparisons between pairs of groups were performed by Bonferroni test, using GraphPAD Software for Science (San Diego, CA, USA).

Results

The effects of the three major phytocannabinoids CBD, CBG and CBDV were tested in proliferating and differentiated HaCaT cells, and were compared to those of the endogenous cannabinoid AEA as a control (Maccarrone et al., 2003; Paradisi et al., 2008). In a preliminary set of dose-response experiments on K10 gene expression levels (Figure 1), the lowest effective dose of CBD ($p < 0.001$) and CBG ($p < 0.05$) was found to be 0.5 μM, whereas CBDV was ineffective up to 1.0 μM, previously found to be the lowest effective dose of AEA (Paradisi et al., 2008). Therefore, CBD and CBG were used at 0.5 μM, and CBDV and AEA at 1.0 μM in all subsequent experiments. By using qRT-PCR analysis, we showed a significant reduction of the expression of K10 and TGase5 genes upon treatment of differentiated HaCaT cells with 0.5 μM CBD ($p < 0.001$) or CBG ($p < 0.05$ for K10; $p <$

0.001 for TGase5), as well as with 1.0 μM AEA ($p < 0.001$) (Figure 2). Also the expression of involucrin and K1 genes was significantly inhibited by CBD but not by CBG, under the same experimental conditions, once again resembling the effect of AEA; instead, 1.0 μM CBDV did not change the expression of any gene tested (Figure 2). Based on these findings, we chose to perform further analyses on K10 only, as we did previously to test the effects of AEA (Paradisi et al., 2008). We have also extended to primary NHEKs the analysis of the most relevant effects of AEA, CBD and CBG, showing a consistent down-regulation of K10 gene expression upon all the treatments (see supplementary Table S1). Unfortunately, the difficulty to grow NHEKs prevented a further extension of our analyses to these primary cells. We sought to check the molecular mechanism by which CBD and CBG affect K10 gene expression, and we found that the effect of 0.5 μM CBD was reversed by 0.05 μM SR141716 ($p < 0.05$; Figure 3), a selective CB_1 antagonist (Pertwee, 2010), but not by 0.05 μM SR144528, a selective antagonists of CB_2 (Pertwee, 2010). In addition 0.5 μM CPZ, a selective antagonist of TRPV1 (De Petrocellis and Di Marzo, 2010), was ineffective, in keeping with the absence of TRPV1 in HaCaT cells (Maccarrone et al., 2003) (Figure 3). Collectively, these data suggest that CBD triggered a CB_1-dependent mechanism that resembled that already observed for AEA (Paradisi et al., 2008). Instead, the effect of CBG on K10 mRNA levels was not counteracted by any of the three selective receptor antagonists, supporting the involvement of a distinct transduction pathway (Figure 3). Incidentally, SR141716 and SR144528 were used at concentrations previously shown to block their specific targets in HaCaT cells (Maccarrone et al., 2003; Paradisi et al., 2008). Next, we assessed the methylation status of K10 gene, using a bisulfite-based MSP assay. Indeed, it is known that gene expression is regulated by epigenetic mechanisms such as DNA methylation. As shown in Figure 4, the methylation level of K10 significantly decreased ($p < 0.001$) upon differentiation of proliferating HaCaT cells with TPA plus calcium.

Interestingly, both CBD and CBG significantly increased ($p < 0.001$ for CBD; $p < 0.05$ for CBG) K10 gene methylation in differentiated cells (Figure 4), thus resembling the effect of AEA (Paradisi e al., 2008). The effect of CBD was due to a CB_1-dependent mechanism, because it was prevented by SR141716 ($p < 0.05$; Figure 4). Instead, CBG did not trigger CB_1 signaling, because SR141716 did not counteract the effect of this phytocannabinoid on K10 gene (Figure 4). In addition, the overall methylation levels were measured in human keratinocytes by using an SssI methylase assay. Firstly, differentiation of HaCaT cells led to a significant reduction ($p < 0.05$, Figure 5A) of global DNA methylation; secondly, AEA ($p < 0.01$) and CBD ($p < 0.05$), but not CBG, increased DNA methylation levels in differentiated cells, up to those of proliferating cells (Figure 5A). Once again, the effect of CBD was reversed by SR141716 ($p < 0.05$), indicating a CB_1-dependent mechanism, in contrast the effect of CBG was independent of the same receptor (Figure 5A). We also tested whether CBD and CBG could affect genomic DNA methylation through the regulation of DNMT activity. Similarly to AEA, CBD induced a slight increase ($p = 0.4156$) of DNMT activity in differentiating cells, whereas CBG induced a small (yet not significant; $p = 0.1043$) decrease of the enzyme activity in the same cells (Figure 5B). Finally, we demonstrated selective alterations of DNMTs gene expression in differentiated HaCaT cells, either untreated or upon exposure to AEA, CBG and CBD (Table 2). In particular, we found that DNMT1 gene expression was significantly reduced ($p = 0.0039$) in differentiated cells and, even if without reaching statistical significance, was up-regulated by AEA ($p = 0.1014$), CBD ($p = 0.3290$), or CBG ($p = 0.0520$). Consistently, densitometric analysis of DNMT1 levels revealed a reduction of the enzyme protein in differentiated cells, and a recovery towards proliferating cells after any treatment (Table 2). Gene expression of all other DNMTs analysed (DNMT3a, DNMT3b, DNMT3L) was not affected by any compound tested under the same experimental conditions (Table 2).

Discussion and Conclusions

In this report, we show that the expression of epidermal differentiation genes (i.e., keratins, involucrin and transglutaminase) is regulated by the phytocannabinoids CBD and CBG, but not by CBDV, through distinct mechanisms. Indeed, the effect of CBD is dependent on CB_1 cannabinoid receptors, similar to what was previously reported for AEA (Paradisi et al., 2008), whereas CBG does not engage neither this nor the other AEA-binding receptor subtype, the CB_2 cannabinoid receptor. Moreover, CBG does not affect the transcription of involucrin and K1, but it down-regulates that of K10 and TGase 5. In this context, it should be recalled that CBD and CBG also inhibit dose-dependently keratinocyte proliferation (Wilkinson and Williamson, 2007), though at an effective dose (>1 μM) higher than the optimal dose (0.5 μM) found here to reduce the differentiation markers K10 and TGase5. Additionally, we suggest that inhibition of epidermal differentiation elicited by CBD shares with AEA the same CB_1-dependent mechanism of action. This seems remarkable, because CBD is generally reported to have a very low affinity (in the micromolar range) for CB_1 and CB_2 cannabinoid receptors, though independent investigations have recently shown that it can also behave as an inverse agonist or antagonist at the same receptors (Thomas et al., 2007; Castillo et al., 2010). Moreover, it should be recalled that CBD might enhance the biological activity of endogenous cannabinoids like AEA also by increasing their release and/or by inhibiting their degradation. Such an "entourage effect" (Ben-Shabat et al., 1998; Ligresti et al., 2006) may represent an additional indirect mechanism by which CBD might modulate CB_1/CB_2 signaling. On the other hand, the effects of CBG on K10 gene expression were not mediated by CB_1 or CB_2 cannabinoid receptors. In vitro studies have shown that CBG is also an α_2-adrenoceptor agonist, and an antagonist at type 1A 5-hydroxytryptamine ($5HT_{1A}$)

(Cascio et al., 2010) and TRPV1 (De Petrocellis et al., 2008; De Petrocellis et al., 2011) receptors. Moreover, the possibility that these receptors as well as other eCBs targets like peroxisome proliferator-activated receptor-γ (PPAR-γ), that plays roles in skin biology (Kuenzli and Saurat, 2004; Bhagavathula et al., 2004; Ellis et al., 2000), or GPR55 might be triggered by CBG remains to be explored. In this context, it should be mentioned that SR141716 also behaves as an agonist at GPR55 (Kapur et al., 2009), though data on a possible involvement of this receptor in the epidermis are still missing. It should be also noted that recent findings have shown that the barrier recovery is delayed in CB_1 KO mice, while it is accelerated in CB_2 KO mice (Roelandt et al., 2012). Additionally, CB_1 activation in human keratinocytes by high doses (2.5 and 10 μM) of arachidonoylcyclopropylamide for 24 hours increased the mRNA level of K10 at high Ca^{2+} concentrations, while reducing K10 protein level under the same conditions (Roelandt et al., 2012). On the one hand, it can be proposed that KO animals might have developed different compensatory mechanisms, that do not fully reflect the physiology of normal (wild-type) keratinocytes. On the other hand, the opposite effects of arachidonoylcyclopropylamide on human keratinocytes (so called "cannabinoid paradox") at doses well-above those used here might be due to complex mechanisms, that may be related to eCBs signaling mechanisms that inhibit mRNA translation (Roelandt et al., 2012), as well as to reduced cell viability and proliferation induced by eCBs and phytocannabinoids at concentrations > 1 μM (Siegmund et al., 2006; Wilkinson and Williamson, 2007; Tóth et al., 2011; Pucci et al., 2012). At any rate, consistently with what we previously reported for AEA (Paradisi et al., 2008), here we show that changes in K10 gene expression induced by CBD but not CBG are due to increased methylation of genomic DNA. It is noteworthy that an inverse correlation between DNA methylation and the expression of differentiating genes has been already identified in human

keratinocytes (Elder and Zhao, 2002; Engelkamp et al., 1993), although a role for phytocannabinoids in this process is unprecedented.

We also observed an overall reduction of DNA methylation in differentiating keratinocytes, in agreement with an early study showing that DNA methylation in human keratinocytes varies depending on the differentiation state, whereby there is a lower methylcytosine content in the DNA of differentiated *versus* undifferentiated cells (Veres et al., 1989). CBD was able to revert these changes and to increase global DNA methylation in differentiated cells, thus suggesting a broader effect, not restricted to K10 gene only.

Finally, we evaluated the effect of CBD and CBG, as well as of AEA, on the expression of DNMTs, the enzymes that catalyse DNA methylation (Baylin and Herman, 2000), in order to better dissect the role of DNA methylation on the modulation of epidermal differentiation by phytocannabinoids. We observed that the induction of HaCaT cell differentiation for 5 days determined a selective and significant reduction of DNMT1 gene expression. Consistently, DNMT1 was previously found to be down-regulated during epidermal differentiation (Sen et al., 2010). CBG and CBD, like AEA, were able to revert these changes, and thus to induce an up-regulation, even if not in a significant manner, of DNMT1 in line with the observed increase in DNA methylation and reduction in mRNA levels. It is important to point out that these changes were selective, since we did not observe any alteration of DNMT3a, DNMT3b and DNMT3L gene expression whatever the treatment, nor upon cell differentiation alone. Consistently, DNMT3a and DNMT3b are known to mediate methylation-independent gene repression (Bachman et al., 2001). Overall, our data confirm that DNA methylation is altered during cell differentiation, and that DNMT1 is required to maintain a progenitor state. In addition, we might also suggest changes of cellular maintenance but not *de novo* methyltransferase activity, because DNMT3a and 3b can methylate unmethylated DNA, and

are thus referred to as *de novo* DNMTs; instead, DNMT1 primarily functions to maintain DNA methylation by preferentially methylating hemimethylated DNA (Dodge et al., 2005). Taken together, present data clearly identify the phytocannabinoids CBD and CBG as transcriptional repressors, further suggesting a role for eCBs signaling in the control of cell proliferation and differentiation (Maccarrone et al., 2003; Galve-Roperh et al, 2006; Aguado et al., 2006; Matias et al., 2006; Ofek et al., 2006; Telek et al., 2007; Laezza et al., 2006; Cavuoto et al., 2007).

In conclusion, understanding the nature of genetic and epigenetic interactions in the regulation of epidermal differentiation, and clarifying how phytocannabinoids could possibly modulate these effects represent a major challenge in the skin biology arena. Our data might pave the way to the development of preventive strategies, for example aimed at reducing allergic inflammation, or to the design of new and more effective therapeutics for the management of skin cancer. Plant-derived cannabinoids that are devoid of psychoactive effects can be proposed as good candidates for these purposes. More in general, our findings suggest that phytocannabinoids might act through epigenetic mechanisms also in other human diseases (e.g., multiple sclerosis), where their administration has been proven to be beneficial (Rog, 2010). Yet, major differences in signaling mechanisms triggered by different phytocannabinoids, that might act through CB_1-dependent (CBD), CB_1-independent (CBG), or might not act at all (CBDV), call for a careful investigation into their activity before any therapeutical exploitation.

Finally, we believe that the importance of our findings goes beyond the role in keratinocyte differentiation that we have shown here. In fact, DNA methylation is an epigenetic mechanism involved in the regulation of different cellular processes, including: embryonic development, transcription, chromatin structure, X chromosome inactivation, genomic imprinting, and chromosome stability. A reduction in DNA methylation has been

demonstrated in different human diseases, most notably cancer (Robertson, 2005). Therefore, natural compounds that act as DNA methyltransferase enhancers, like phytocannabinoids, may be exploited well-beyond skin biology.

Acknowledgements: We are grateful to GW Pharmaceuticals for financial support. The authors declare that there are no conflicts of interest.

References

Aguado T, Palazuelos J, Monory K, Stella N, Cravatt B, Lutz B, et al. (2006). The endocannabinoid system promotes astroglial differentiation by acting on neural progenitor cells. J Neurosci 26: 1551-61.

Ahn K, McKinney MK, Cravatt BF (2008). Enzymatic pathways that regulate endocannabinoid signaling in the nervous system. Chem Rev 108: 1687-1707.

Alexander SPH, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th edition (2011). Br J Pharmacol 164 (Suppl. 1): S1–S324.

Bachman KE, Rountree MR, Baylin SB (2001). Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin. J Biol Chem. 276: 32282-7.

Baker D, Pryce G, Davies WL, Hiley CR (2006). In silico patent searching reveals a new cannabinoid receptor. Trends Pharmacol Sci 27:1-4.

Baylin SB, Herman JG (2000). DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends Genet 16: 168-74.

Ben-Shabat S, Fride E, Sheskin T, Tamiri T, Rhee MH, Vogel Z, et al. (1998). An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity. Eur J Pharmacol 353: 23-31.

Berdyshev EV, Schmid PC, Dong Z, Schmid HH (2000). Stress-induced generation of N-acylethanolamines in mouse epidermal JB6 P+ cells. Biochem J 346: 369-74.

Bhagavathula N, Nerusu KC, Lal A, Ellis CN, Chittiboyina A, Avery MA, et al. (2004). Rosiglitazone inhibits proliferation, motility, and matrix metalloproteinase production in keratinocytes. J Invest Dermatol 122: 130-9.

Bíró T, Tóth BI, Haskó G, Paus R, Pacher P (2009). The endocannabinoid system of the skin in health and disease: novel perspectives and therapeutic opportunities. Trends Pharmacol Sci 30: 411-20.

Broday L, Lee YW, Costa M (1999). 5-azacytidine induces transgene silencing by DNA methylation in Chinese hamster cells. Mol Cell Biol 19: 3198-3204.

Candi E, Oddi S, Terrinoni A, Paradisi A, Ranalli M, Finazzi-Agro` A, Melino G (2001). Transglutaminase 5 cross-links loricrin, involucrin, and small proline-rich proteins in vitro. J Biol Chem 276: 35014-23.

Candi E, Schmidt R, Melino G (2005). The cornified envelope: a model of cell death in the skin. Nat Rev Mol Cell Biol 6: 328-40.

Casanova ML, Blázquez C, Martínez-Palacio J, Villanueva C, Fernández-Aceñero MJ, Huffman JW, et al. (2003). Inhibition of skin tumor growth and angiogenesis in vivo by activation of cannabinoid receptors. J Clin Invest 111: 43-50.

Cascio MG, Gauson LA, Stevenson LA, Ross RA, Pertwee RG (2010). Evidence that the plant cannabinoid cannabigerol is a highly potent alpha2-adrenoceptor agonist and moderately potent 5HT1A receptor antagonist. Br J Pharmacol. 159: 129-41.

Castillo A, Tolón MR, Fernández-Ruiz J, Romero J, Martinez-Orgado J (2010). The neuroprotective effect of cannabidiol in an in vitro model of newborn hypoxic-ischemic brain damage in mice is mediated by CB(2) and adenosine receptors. Neurobiol Dis 37: 434-40.

Cavuoto P, McAinch AJ, Hatzinikolas G, Cameron-Smith D, Wittert GA (2007). Effects of cannabinoid receptors on skeletal muscle oxidative pathways. Mol Cell Endocrinol 267: 63-6.

Chen M, Chen ZQ, Cui PG, Yao X, Li YM, Li AS, et al. (2008). The methylation pattern of p16INK4a gene promoter in psoriatic epidermis and its clinical significance. Br J Dermatol 158: 987-93.

D'Addario C, Di Francesco A, Arosio B, Gussago C, Dell'Osso B, Bari M, et al. (2012). Epigenetic regulation of fatty acid amide hydrolase in Alzheimer disease. PLoS One 7: e39186.

D'Addario C, Ming Y, Ogren SO, Terenius L (2008). The role of acetaldehyde in mediating effects of alcohol on expression of endogenous opioid system genes in a neuroblastoma cell line. FASEB J 22: 662-70.

De Petrocellis L, Di Marzo V (2010). Non-CB1, non-CB2 receptors for endocannabinoids, plant cannabinoids, and synthetic cannabimimetics: focus on G-protein-coupled receptors and transient receptor potential channels. J Neuroimmune Pharmacol. 5: 103-21.

De Petrocellis L, Ligresti A, Moriello AS, Allarà M, Bisogno T, Petrosino S, et al. (2011). Effects of cannabinoids and cannabinoid-enriched Cannabis extracts on TRP channels and endocannabinoid metabolic enzymes. Br J Pharmacol 163: 1479-94.

De Petrocellis L, Vellani V, Schiano-Moriello A, Marini P, Magherini PC, Orlando P, et al. (2008). Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8. J Pharmacol Exp Ther 325: 1007-15.

Di Marzo V (2008). Endocannabinoids: synthesis and degradation. Rev Physiol Biochem Pharmacol 160:1-24.

Di Marzo V, Petrosino S (2007). Endocannabinoids and the regulation of their levels in health and disease. Curr Opin Lipidol 18: 129-40.

Dodge JE, Okano M, Dick F, Tsujimoto N, Chen T, Wang S, et al. (2005) Inactivation of Dnmt3b in mouse embryonic fibroblasts results in DNA hypomethylation, chromosomal instability, and spontaneous immortalization. J Biol Chem. 280: 17986-91.

Ehrlich M (2003). Expression of various genes is controlled by DNA methylation during mammalian development. J Cell Biochem 88: 899-910.

Elder JT, Zhao X (2002). Evidence for local control of gene expression in the epidermal differentiation complex. Exp Dermatol 11: 406–12.

Ellis CN, Varani J, Fisher GJ, Zeigler ME, Pershadsingh HA, Benson SC et al. (2000). Troglitazone improves psoriasis and normalizes models of proliferative skin disease: ligands for peroxisome proliferator-activated receptor-gamma inhibit keratinocyte proliferation. Arch Dermatol 136: 609-16.

Engelkamp D, Schafer BW, Mattei MG, Erne P, Heizmann CW (1993). Six S100 genes are clustered on human chromosome 1q21: identification of two genes coding for the two previously unreported calcium-binding proteins S100D and S100E. Proc Natl Acad Sci USA 90: 6547-51.

Galve-Roperh I, Aguado T, Rueda D, Velasco G, Guzman M (2006). Endocannabinoids: a new family of lipid mediators involved in the regulation of neural cell development. Curr Pharm Des 12: 2319-25.

Hill AJ, Williams CM, Whalley BJ, Stephens GJ (2012a). Phytocannabinoids as novel therapeutic agents in CNS disorders. Pharmacol Ther 133: 79-97.

Hill AJ, Mercier MS, Hill TD, Glyn SE, Jones NA, Yamasaki Y, Futamura T, Duncan M, Stott CG, Stephens GJ, Williams CM, Whalley BJ (2012b). Cannabidivarin is anticonvulsant in mouse and rat. Br J Pharmacol 167: 1629-1642.

Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R (2009). Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. Trends Pharmacol Sci 30: 609.

Jaenisch R, Bird A (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 33: 245-54.

Jones NA, Hill AJ, Smith I, Bevan SA, Williams CM, Whalley BJ, Stephens GJ (2010). Cannabidiol displays antiepileptiform and antiseizure properties in vitro and in vivo. J Pharmacol Exp Ther 332: 569-77.

Kalinin A, Marekov LN, Steinert PM (2001). Assembly of the epidermal cornified cell envelope. J Cell Sci 114: 3069-70.

Kapur A, Zhao P, Sharir H, Bai Y, Caron MG, Barak LS, Abood ME (2009). Atypical responsiveness of the orphan receptor GPR55 to cannabinoid ligands. J Biol Chem 284: 29817-827.

Karsak M, Gaffal G, Date R, Wang-Eckhardt L, Rehnelt J, Petrosino S, et al. (2007). Attenuation of allergic contact dermatitis through the endocannabinoid system. Science 316: 1494-97.

Klein TW (2005). Cannabinoid-based drugs as anti-inflammatory therapeutics. Nat Rev Immunol 5: 400-11.

Kogan NW (2005). Cannabinoids and cancer. Mini Rev Med Chem 5: 941-52.

Kuenzli SS, Saurat JH (2004). Peroxisome proliferator-activated receptors as new molecular targets in psoriasis. Curr Drug Targets Inflamm Allergy 3: 205-11.

Kupczyk P, Reich A, Szepietowski JC (2009). Cannabinoid system in the skin - a possible target for future therapies in dermatology. Exp Dermatol 18: 669-79.

Laezza C, Pisanti S, Crescenzi E, Bifulco M (2006). Anandamide inhibits Cdk2 and activates Chk1 leading to cell cycle arrest in human breast cancer cells. FEBS Lett. 580: 6076-82.

Lal S, Prasad N, Ryan M, Tangri S, Silverberg MS, Gordon A, Steinhart H (2011). Cannabis use amongst patients with inflammatory bowel disease. Eur J Gastroenterol Hepatol 23: 891-6.

Ligresti A, Moriello AS, Starowicz K, Matias I, Pisanti S, De Petrocellis L, et al. (2006). Antitumor activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. J Pharmacol Exp Ther 318: 1375-87.

Lodzki M, Godin B, Rakou L, Mechoulam R, Gallily R, Touitou E (2003). Cannabidiol-transdermal delivery and anti-inflammatory effect in a murine model. J Control Release 93: 377-87.

Lopez J, Percharde M, Coley HM, Webb A, Crook T (2009). The context and potential of epigenetics in oncology. Br J Cancer 100: 571-77.

Lu L, Katsaros D, de la Longrais IA, Sochirca O, Yu H (2007). Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis. Cancer Res 67: 10117-22.

Lyon SB, Buonocore L, Miller M (1987). Naturally occurring methylation inhibitor: DNA hypomethylation and hemoglobin synthesis in human K562 cells. Mol Cell Biol 7: 1759-63.

Maccarrone M, Dainese E, Oddi S (2010). Intracellular trafficking of anandamide: new concepts for signaling Trends Biochem Sci 35: 301-8.

Maccarrone M, Di Rienzo M, Battista N, Gasperi V, Guerrieri P, Rossi A, Finazzi-Agrò A (2003). The endocannabinoid system in human keratinocytes. Evidence that anandamide inhibits epidermal differentiation through CB1 receptor-dependent inhibition of protein kinase C, activation protein-1, and transglutaminase. J Biol Chem 278: 33896-903.

Matias I, Gonthier MP, Orlando P, Martiadis V, De Petrocellis L, Cervino C, et al (2006). Regulation, function, and dysregulation of endocannabinoids in models of adipose and beta-pancreatic cells and in obesity and hyperglycemia. J Clin Endocrinol Metab 91: 3171-80.

Millington GW (2008). Epigenetics and dermatological disease. Pharmacogenomics 9: 1835-50.

Nemes Z, Steinert PM (1999). Bricks and mortar of the epidermal barrier. Exp Mol Med 31: 5-19.

Oddi S, Bari M, Battista N, Barsacchi D, Cozzani I, Maccarrone M (2005) Confocal microscopy and biochemical analysis reveal spatial and functional separation between anandamide uptake and hydrolysis in human keratinocytes. Cell Mol Life Sci 62: 386-95.

Ofek O, Karsak M, Leclerc N, Fogel M, Frenkel B, Wright K, et al. (2006) Peripheral cannabinoid receptor, CB2, regulates bone mass. Proc Natl Acad Sci USA 103: 696-701.

Paradisi A, Pasquariello N, Barcaroli D, Maccarrone M (2008). Anandamide regulates keratinocyte differentiation by inducing DNA methylation in a CB1 receptor-dependent manner. J Biol Chem 283: 6005-12.

Pasquariello N, Oddi S, Malaponti M, Maccarrone M (2009). Regulation of gene transcription and keratinocyte differentiation by anandamide. Vitam Horm 81: 441-46.

Paus R, Schmelz M, Bíró T, Steinhoff M (2006). Frontiers in pruritus research: scratching the brain for more effective itch therapy. J Clin Invest 116: 1174-86.

Pertwee RG, Howlett AC, Abood ME, Alexander SP, Di Marzo V, et al. (2010) International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB1 and CB2. Pharmacol Rev 62: 588-631.

Petrosino S, Cristino L, Karsak M, Gaffal E, Ueda N, Tüting T, et al. (2010). Protective role of palmitoylethanolamide in contact allergic dermatitis. Allergy 65: 698-11.

Pucci M, Pirazzi V, Pasquariello N, Maccarrone M (2011) Endocannabinoid signaling and epidermal differentiation. Eur J Dermatol 2: 29-34.

Robertson KD (2005) DNA methylation and human disease. Nat Rev Genet 6: 597-610.

Rodríguez de Fonseca F, Del Arco I, Bermudez-Silva FJ, Bilbao A, Cippitelli A, Navarro M (2005). The endocannabinoid system: physiology and pharmacology. Alcohol Alcohol 40: 2-14.

Roelandt T, Heughebaert C, Bredif S, Giddelo C, Baudouin C, Msika P, Roseeuw D, Uchida Y, Elias PM, Hachem JP (2012). Cannabinoid receptors 1 and 2 oppositely regulate epidermal permeability barrier status and differentiation. Exp Dermatol 21: 688-93.

Rog DJ (2010). Cannabis-based medicines in multiple sclerosis-a review of clinical studies. Immunobiology 215: 658-72.

Rosl F, Durst M, Zur Hausen H (1988). Selective suppression of human papillomavirus transcription in non-tumorigenic cells by 5-azacytidine. EMBO J 7: 1321-28.

Schmidt R, Cathelineau C, Cavey MT, Dionisius V, Michel S, Shroot B, Reichert U (1989). Sodium butyrate selectively antagonizes the inhibitory effect of retinoids on cornified envelope formation in cultured human keratinocytes. J Cell Physiol 140: 281-7.

Sen GL, Reuter JA, Webster DE, Zhu L, Khavari PA (2010). DNMT1 maintains progenitor function in self-renewing somatic tissue. Nature 463: 563-7.

Siegmund SV, Seki E, Osawa Y, Uchinami H, Cravatt BF, Schwabe RF (2006) Fatty acid amide hydrolase determines anandamide-induced cell death in the liver. J Biol Chem. 281: 10431-8.

Solinas M, Massi P, Cantelmo A, Cattaneo M, Cammarota R, Bartolini D, et al. (2012) Cannabidiol inhibits angiogenesis by multiple mechanisms. Br J Pharmacol 167: 1218-31.

Staiano-Coico L, RE Helm RE, McMahon CK, Pagan-Charry I, LaBruna A, Piraino V, Higgins PJ (1989). Sodium-N-butyrate induces cytoskeletal rearrangements and formation of cornified envelopes in cultured adult human keratinocytes. Cell Tissue Kinet 22: 361-75.

Stander S, Schmelz M, Metze D, Luger T, Rukwied R (2005). Distribution of cannabinoid receptor 1 (CB1) and 2 (CB2) on sensory nerve fibers and adnexal structures in human skin. J Dermatol Sci 38: 177-88.

Telek A, Biro T, Bodo E, Toth BI, Borbiro I, Kunos G, Paus R (2007). Inhibition of human hair follicle growth by endo- and exocannabinoids. FASEB J 21: 3534-41.

Thomas A, Baillie GL, Phillips AM, Razdan RK, Ross RA, Pertwee RG (2007). Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonist. Br J Pharmacol 150: 613-23.

Tóth BI, Dobrosi N, Dajnoki A, Czifra G, Oláh A, Szöllosi AG, et al. (2011) Endocannabinoids modulate human epidermal keratinocyte proliferation and survival via the sequential engagement of cannabinoid receptor-1 and transient receptor potential vanilloid-1. J Invest Dermatol 131 :1095-104.

Ueda N, Tsuboi K, Uyama T, Ohnishi T (2011). Biosynthesis and degradation of the endocannabinoid 2-arachidonoylglycerol. Biofactors 37: 1-7.

Veres DA, Wilkins L, Coble DW, Lyon SB (1989). DNA methylation and differentiation of human keratinocytes. J Invest Dermatol 93: 687-90.

Wilkinson JD, Williamson EM (2007). Cannabinoids inhibit human keratinocyte proliferation through a non-CB1/CB2 mechanism and have a potential therapeutic value in the treatment of psoriasis. J Dermatol Sci 45: 87-92.

1 Zhang X (2012). Genome-wide association study of skin complex diseases. J Dermatol Sci 6:
2 89-97.
3

Table 1. Primer sequences used for reverse transcription–polymerase chain reaction.

Human Gene	Forward (5' → 3')	Reverse (3' → 5')
K10	ACGAGGAGGAAATGAAAGAC	GGACTGTAGTTCTATCTCCAG
K1	AGAAAGCAGGATGTCTGG	AAACAAACTTCACGCTGG
Involucrin	CTCTGCCTCAGCCTTACT	GCTGCTGATCCCTTTGTG
TGase 5	TCAGCACAAAGAGCATCCAG	TTCAGGGAGACTTGCACCAC
β-actin	TGACCCAGATCATGTTTGAG	TTAATGTCACGCACGATTTCC
DNMT1	CCCCTGAGCCCTACCGAAT	CTCGCTGGAGTGGACTTGTG
DNMT3a	TATTGATGAGCGCACAAGAGAGC	GGGTGTTCCAGGGTAACATTGAG
DNMT3b	GGCAAGTTCTCCGAGGTCTCTG	TGGTACATGGCTTTTCGATAGGA
DNMT3L	GGCTCTGGTTTCGGAAGAA	TCTCTTAGGGGGAGAAAGCA
GAPDH	CAGCCTCAAGATCATCAGCA	TGTGGTCATGAGTCCTTCCA
M K10	AGTTTTCGTTTTCGTAGTCGTC	CGAATATAACCTCACCCCG
U K10	GGAGTTTTTGTTTTTGTAGTTGTT	AACCAAATATAACCTCACCCCA
myoD	CCAACTCCAAATCCCCTCTCTAT	TGATTAATTTAGATTGGGTTTAGAGAAGGA

Table 2. Effect of AEA, CBD and CBG on DNMTs gene expression, and on DNMT1 protein levels.

mRNA level	Prol[a]	Diff[b]	Diff + AEA	Diff + CBD	Diff + CBG
DNMT1	0.99 ± 0.11	0.44 ± 0.06[c]	1.92 ± 0.76	1.24 ± 0.55	1.46 ± 0.47
DNMT3a	1.03 ± 0.20	1.40 ± 0.40	2.45 ± 1.12	1.77 ± 0.61	1.25 ± 0.35
DNMT3b	1.10 ± 0.32	1.07 ± 0.24	2.91 ± 1.45	2.65 ± 1.00	0.85 ± 0.27
DNMT3L	1.10 ± 0.28	1.82 ± 0.31	2.94 ± 1.23	2.48 ± 0.50	1.01 ± 0.28
DNMT1 (protein level)[d]	1.00 ± 0.11	0.74 ± 0.08	0.96 ± 0.12	1.13 ± 0.11	1.30 ± 0.15

[a] Prol, Proliferating keratinocytes.

[b] Diff, Differentiated keratinocytes.

[c] $p < 0.01$ *versus* Prol.

[d] Protein levels were quantified by densitometric analysis of three independent Western blots (see Supplementary Figure 1S for a representative blot).

Legends to Figures:

Figure 1.: Expression of K10 gene in HaCaT cells. Keratinocytes were induced to differentiate by treatment with TPA plus calcium for 5 days. Differentiated HaCaT cells were treated with 1 μM AEA and different amounts (0.1 - 0.5 - 1.0 μM) of CBD, CBG and CBDV. K10 was detected by quantitative RT-PCR, under condition and with primers found in the Methods section. For the quantitation of gene expression, β-actin was used as housekeeping gene. The results are shown as fold induction over proliferating cells of three independent experiments. Prol, proliferating cells; Ctrl, differentiated cells. ***, $p<0.001$ *vs* Prol; ###, $p<0.001$ *vs* Ctrl; ##, $p<0.01$ *vs* Ctrl; #, $p<0.05$ *vs* Ctrl.

Figure 2.: Expression of epidermal differentiation-related genes in HaCaT cells. Differentiated HaCaT cells were treated with 1 μM AEA, 0.5 μM CBD, 0.5 μM CBG or 1.0 μM CBDV. K10 (A), involucrin (B), TGase 5 (C) and K1 (D) were detected by quantitative RT-PCR, under condition and with primers found in the Methods section. The results are shown as fold induction over proliferating cells of three independent experiments. Prol, proliferating cells; Diff, differentiated cells. ***, $p<0.001$ *vs* Prol; ###, $p<0.001$ *vs* Diff; ##, $p<0.01$ *vs* Diff; #, $p<0.05$ *vs* Diff.

Figure 3.: Effect of AEA (1.0 μM), CBD and CBG (both used at 0.5 μM), alone or in the presence of 0.05 μM SR141716, 0.05 μM SR144528 or 0.5 μM capsazepine (CPZ), on K10 gene expression in HaCaT cells. SR141716, SR144528 and CPZ were ineffective when used

alone. Prol, proliferating cells; Diff, differentiated cells.***, p<0.001 *vs* Prol, ###, p<0.001 *vs* Diff; #, p<0.05 *vs* Diff; $$, p<0.01 *vs* Diff + AEA; &, p<0.05 *vs* Diff + CBD.

Figure 4.: Methylation-specific primed PCR. DNA methylation levels of K10 gene in differentiated HaCaT cells treated with CBD and CBG (both used at 0.5 μM), alone or in the presence of SR141716 (0.05 μM). SR141716 was ineffective when used alone. The methylation status of K10 gene was analyzed as described in the Methods section. Prol, proliferating cells; Diff, differentiated cells. ***, p<0.001 *vs* Prol; ###, p<0.001 *vs* Diff, #, p<0.05 *vs* Diff; &, p<0.05 *vs* Diff + CBD.

Figure 5.: A) Methylation levels of genomic DNA were measured by methyl-accepting assay with CpG methylase SssI, in the presence of *S*-adenosyl-L-[methyl-^{3}H]methyonine (see Methods for details). Higher levels of [^{3}H]methyl group incorporated into DNA indicated lower level of genomic DNA methylation. Prol, proliferating cells; Diff, differentiated cells. *, p<0.05 *vs* Prol; ##, p<0.01 *vs* Diff, #, p<0.05 *vs* Diff; &, p<0.05 *vs* Diff + CBD. B) Proliferating and differentiated keratinocytes treated with 1 μM AEA, 0.5 μM CBD or 0.5 μM CBG were lysed, and DNA methyltransferase activity was measured as described in the Methods section. Prol, proliferating cells; Diff, differentiated cells. **, p<0.01 *vs* Prol.

Figure 1

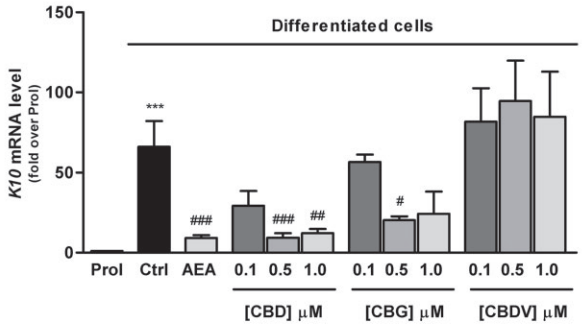

Figure 2

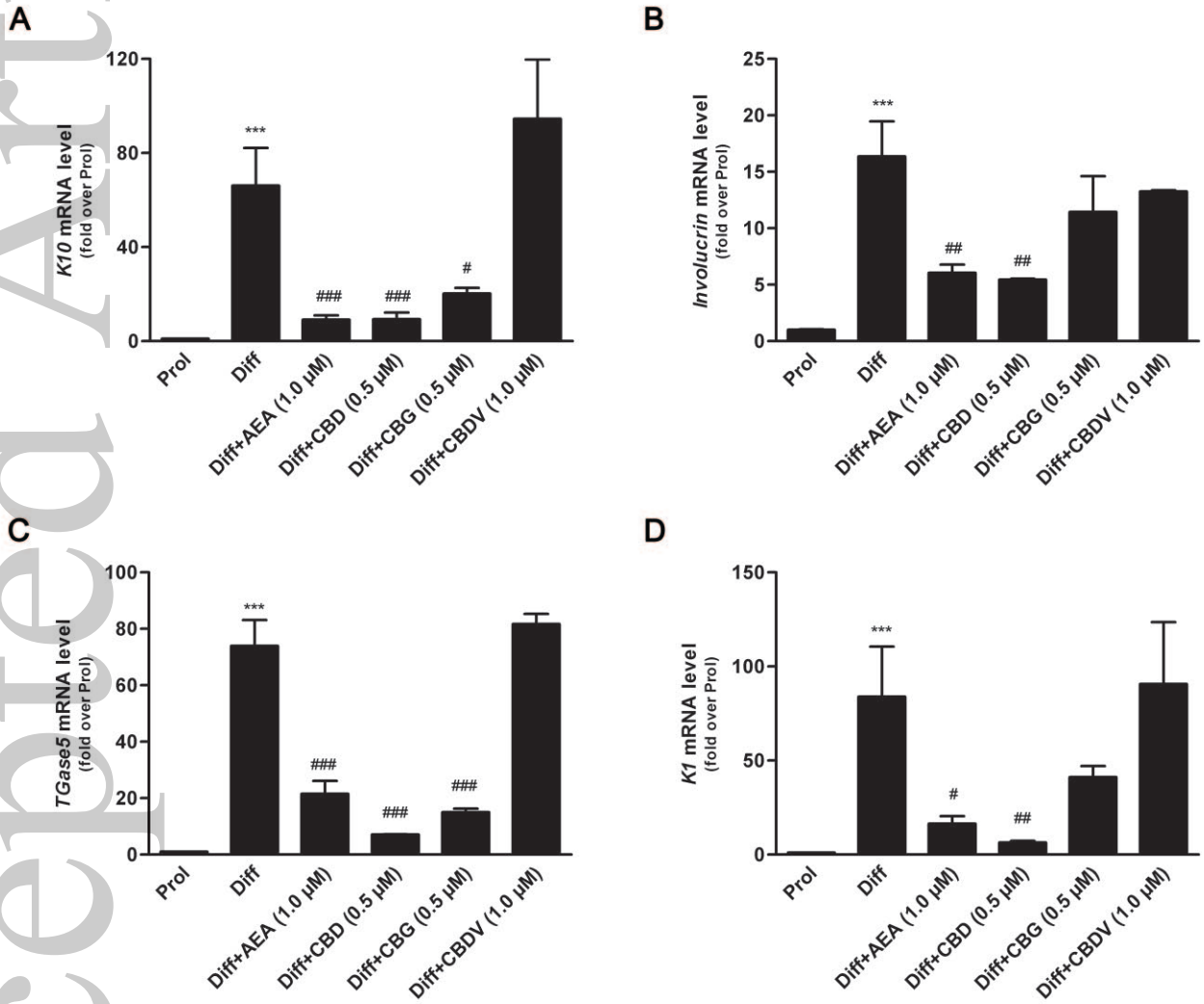

Figure 3

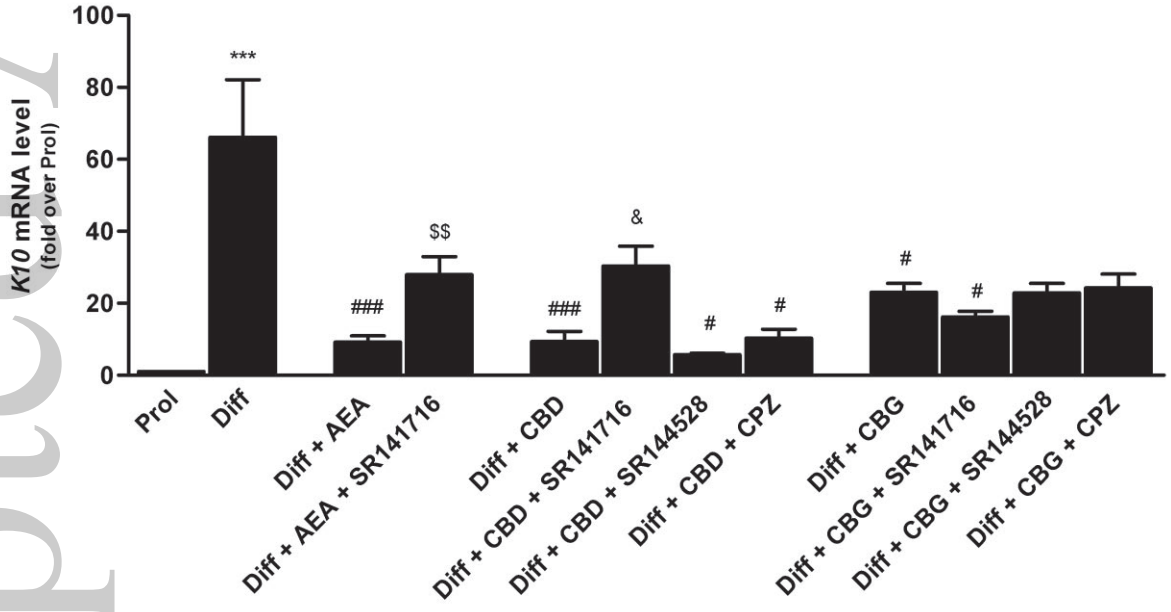

Figure 4

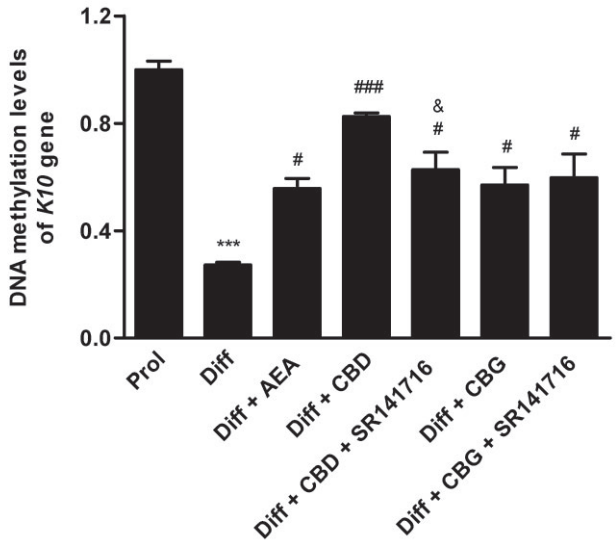

Figure 5