Human epithelial stem cell survival within their niche requires "tonic" cannabinoid receptor 1-signalling-Lessons from the hair follicle

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Human epithelial stem cell survival within their niche requires “tonic” cannabinoid receptor 1-signaling – Lessons from the hair follicle

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Regular Article

*Human epithelial stem cell survival within their niche requires
“tonic” cannabinoid receptor 1-signaling – Lessons from the hair
follicle*

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Running title: Epithelial stem cells require CB1 signaling

ABSTRACT

The endocannabinoid system (ECS) regulates multiple aspects of human epithelial physiology, including inhibition/stimulation of keratinocyte proliferation/apoptosis, respectively. Yet, how the ECS impacts on human adult epithelial stem cell (eSC) functions remains unknown. Scalp hair follicles (HFs) offer a clinically relevant, prototypic model system for studying this directly within the native human stem cell niche. Here, we show in organ-cultured human HFs that, unexpectedly, selective activation of cannabinoid receptor-1 (CB1)-mediated signaling via the MAPK (MEK/Erk 1/2) and Akt pathways significantly increases the number and proliferation of cytokeratin CK15+ or CK19+ human HF bulge eSCs *in situ*, and enhances CK15 promoter activity *in situ*. In striking contrast, CB1-stimulation promotes apoptosis in the differentiated progeny of these eSC (CK6+ HF keratinocytes). Instead, intrafollicular *CB1* gene-knockdown or CB1 antagonist treatment significantly reduces human HF eSCs numbers and stimulates their apoptosis, while CB1 knockout mice exhibit a reduced bulge eSCs pool *in vivo*. This identifies “tonic” CB1 signaling as a required survival stimulus for adult human HF eSCs within their niche. This novel concept must be taken into account whenever the human ECS is targeted therapeutically.

Key words: cannabinoid receptor/epithelial stem cell/hair follicle/proliferation/survival

INTRODUCTION

The endocannabinoid system (ECS) plays a significant role in the establishment and maintenance of cutaneous physiology^[1-4]. It encompasses cannabinoid receptors, endocannabinoids produced locally on-demand, and enzymes and transports involved in the metabolism of endocannabinoids that critically regulate the intra-tissue endocannabinoid “tone”^[5-8]. CB1 and/or CB2 cannabinoid receptors are expressed on numerous cell populations of human and mouse skin, whose stimulation controls multiple aspects of skin physiology. These range from the stimulation of epidermal keratinocyte differentiation and apoptosis^[9,10] and sebum production^[11,12] via epidermal barrier homeostasis^[9,13] to robust anti-inflammatory activities^[14-16]. Yet, the role of the ECS in controlling the functions of human hair follicle (HF) adult epithelial stem cells (eSCs) remains unknown (for details, see **Supplementary Introduction**).

Characterizing the role of the ECS in eSC physiology is important not only because eSCs are fundamental to skin homeostasis, repair and regeneration^[17-19], but also since the number ECS-targeting trials and applications in clinical medicine has increased exponentially over the last decade. Indeed, novel agents that raise the endocannabinoid tone or stimulate CB signaling are currently being explored in human skin, for e.g. pro-inflammatory and hyperproliferative skin diseases like atopic dermatitis and allergic contact dermatitis and for melanoma therapy, while systemic CB1 antagonists are explored in the management of multiple pathological conditions^[3,4,8,16,20-23].

Therefore, it has become a matter of urgency to learn how ECS-modulatory agents impact on the physiology of adult human eSCs, so as not to negatively impinge on their regenerative capacity^[19,24,25]. For example, if CB1 agonists inhibit eSC proliferation and stimulate the

differentiation and/or apoptosis of human eSCs, as they do in their differentiated progeny (HF and epidermal keratinocytes) or in skin carcinoma and melanoma cells^[9,10,26,27], this could eventually cause a depletion of the cutaneous eSC pool in human skin with serious clinical consequence, such as skin atrophy and defective wound healing.

To explore this under clinically maximally relevant conditions and simultaneously elucidate whether and how the ECS regulates human eSC biology, the organ culture of micro-dissected human scalp HFs provides an excellent preclinical assay system: here, the physiology of adult human eSCs and their progeny can be studied in a prototypic human (mini-)organ within their main physiological stem cell niche, the bulge, in the presence of their native extracellular matrix (ECM) milieu and neuroectodermal-mesodermal tissue habitat^[28-31]. As the bulge and the basal layer of the outer root sheath (ORS) of the human HF epithelium are richly endowed with cytokeratin CK15+ and/or CK19+ adult eSCs, HF organ-culture permits one to compare the CB stimulation response of relatively quiescent human eSCs to their epithelial cell progeny, the rapidly proliferating CK6- hair matrix keratinocytes and differentiated, CK6+ ORS keratinocytes^[28,29,32-36].

Moreover, we have already shown that the ECS is functionally active in human scalp HFs^[37]: these produce substantial amounts of the prototypic endocannabinoid, anandamide, *selectively* transcribe and translate fully functional CB1 receptors, but not CB2, whose stimulation in HF organ culture inhibits the proliferation and induces apoptosis of hair matrix keratinocytes while promoting apoptosis-driven HF regression (catagen) *ex vivo*^[37]. Moreover, CB1 can be effectively silenced in human HF organ culture^[38]. Furthermore, growing (anagen) scalp HFs cultured in serum-free, defined medium temporarily continue their hair shaft production and cycling activity *ex vivo* almost as they do *in vivo*^[28] and that live-imaging of CK15 promoter

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activity is possible^[39,40]. Taken together, this makes human scalp HF organ culture uniquely suited as an accessible, experimentally tractable and clinically relevant preclinical research model for interrogating the physiological role of CB1-mediated signaling in primary, unmanipulated adult human eSCs directly within their native, immunologically privileged stem cell niche(s), which have been well-studied in mice but are under-investigated in human skin [19,41,42].

Using this exceptional *ex vivo* model system for human eSC research, we have asked how CB1-mediated signaling impacts on the proliferation, survival, CK15 expression and promoter activity of adult human eSCs, compared to that of their immediate progeny, i.e. CK6+ ORS keratinocytes. This was complemented by analyzing CD34+ HF eSC in the HF bulge of CB1 knockout mice *in vivo*^[38,43]. Based on our previous findings on CB1 stimulation in the anagen hair bulb^[37], we hypothesized that CB1 stimulation in human HF eSCs would promote their apoptosis and/or inhibit proliferation and possibly also shut down CK15 expression. Unexpectedly, quite the opposite turned out to be the case, thus identifying an important novel neuroendocrine control mechanism of human eSCs within their stem cell niche, and the principle that the stimulation of the same CB receptor expressed on directly neighboring epithelial cells can have diametrically opposed effects on eSC and their progeny.

METHODS

Detailed methods and additional information are available in the **Supplementary Information**.

Human skin and HF organ culture

For adult human scalp HF organ culture, 426 full-length anagen VI HF^s^[28,45-47] were isolated from excess normal occipital and temporal scalp skin obtained from fifteen healthy individuals (male: 6, female: 9) undergoing routine face-lift surgery (mean age = 50.6) after informed consent and ethics approval during routine facelift surgery and further microdissected and organ-cultured as described previously^[37,38,44-47] or processed for quantitative Real-Time PCR (Q-PCR).

Isolated HF^s were first maintained in supplemented serum-free William's E medium overnight to adapt to culture conditions^[28]. On the following day the medium was replaced and vehicle or test substances were added, were appropriate together with ACEA (30 μ M in dimethyl sulfoxide (DMSO)), AM251 (1 μ M in DMSO), AM6545 (1 μ M in DMSO), T1117 (1 μ M in DMSO) or LPI (5 μ M in DMSO) for 24 hrs, or for 6 days (in the latter experiment, medium was exchanged every 48 hrs and fresh test compound[s] added at this time). HF^s were then cryoembedded and prepared for histology and immunohistochemistry.

CB1 gene knockdown *in situ*

CB1 knock-down *in situ* followed our previously published protocol^[38]. All reagents required for transfection (human CB1 siRNA (sc-39910), control (scrambled, SCR) siRNA (sc-37007), siRNA transfection reagent (sc-29528) and siRNA transfection Medium (sc-36868)) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). HF transfection was performed according to the manufacturer's protocol. Briefly, after the careful wash, HF^s were applied to

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each well (3 HFs per well) and incubated with only transfection reagent, control siRNA or CB1 siRNA at 37°C in a CO2 incubator for 6 hours. Then the medium was replaced with supplemented William’s E medium and the HFs were organ cultured for additional 18 hrs. Afterward, the HFs were cryoembedded. When *CB1* gene knockdown was performed for human HF organ cultures, scrambled siRNA (SCR) and only transfection reagent treated (TFE) HFs are also organ cultured and compared.

Transfection of *CK15*-promoter-GFP into human scalp HFs

GFP-vector subcloning of the entire human genomic *CK15* promoter region was performed as previously reported^[39]. Transient transfection of the human *CK15*-promoter-GFP construct into reconstituted dispase (Invitrogen) pre-treated and subsequently microdissected human scalp HF epithelium was performed by using lipofectamine 2000 reagent (Invitrogen) described previously^[39]. Transient transfection of the *CK15*-promoter-GFP construct into primary human HF-ORS cell culture was also performed as described previously^[39].

Cell isolation and culture

Human ORS keratinocyte culture essentially followed our previously published protocol^[45-48]. For isolation of human HF bulge ORS keratinocytes, dispase-pretreated full-length HF epithelium was dissected and as described above, and HF bulge ORS-enriched keratinocytes were obtained by dividing the HF epithelium into fifths across its full length, and only, the distal third of the HF epithelium (which included the bulge region) was picked out with tweezers and used for subsequent trypsin-EDTA dissociation and further cell culture.

Quantitative Real-Time PCR (Q-PCR)

Quantitative real-time PCR for *CB1* mRNA was performed as described previously^[37,38], and the same protocol was adopted for *GPR55* gene expression measurement.

Statistical analyses

Data were analyzed using Mann-Whitney test or Student's *t*-test for unpaired samples or 1-way ANOVA Bonferroni multiple comparison test or Dunn's multiple comparisons test using Prism 4.0 software (GraphPad Prism Program, GraphPad, San Diego, CA). *p* values <0.05 were regarded as significant. All data in the figures are expressed as mean ± SEM. * *p*<0.05, ** *p*<0.01, *** *p*<0.001 for the indicated comparisons.

Study approval

Human skin tissue collection and handling was performed according to Helsinki guidelines, after Institutional Research Ethics approval (University of Lübeck, where these studies were initiated) and informed patient consent.

Data availability

All primary data, detailed protocols, and non-commercially available materials can be requested from the corresponding author.

RESULTS

Human HF epithelial SCs express functional CB1 receptors in situ

We already knew that CB1 is mostly expressed by ORS keratinocytes in the human HF epithelium^[28], but its expression in the human bulge was unclear. Immunofluorescence microscopy of freshly isolated human scalp skin and double-immunolabeling for the human bulge eSC marker, CK15^[29-34] and CB1 protein demonstrated that most CK15+ cells in the bulge co-express CB1 protein (**Fig 1A**). (For verifying the specificity of the immunostaining, we employed an extensive range of positive and negative controls^[37,38], see **Supplementary Results SR1 and Supplementary Fig S1**). Functionality of these CB1 proteins on CK15+ eSCs was shown by demonstrating specific binding of the fluorescence-conjugated CB1 ligand, Tocrifluor T1117^[49] in micro-dissected, organ-cultured human scalp HFs^[28,44]: this specific CB1 ligand selectively bound to bulge CK15+ eSCs (note the almost complete overlap of the two fluorescence signals) as well as to other CK15+ cells throughout the whole length of the ORS, i.e. the immediate progeny of bulge eSCs^[29,32] (**Fig 1B**).

CB1 stimulation increases eSC number and proliferation ex vivo

CB1 activation by the selective CB1 agonist, arachidonoyl-2 chloroethanolamine (ACEA)^[7,50], inhibits the proliferation of human HF matrix keratinocytes in the anagen hair bulb^[37], i.e. the rapidly proliferating and relatively undifferentiated progeny of HF eSCs^[32]. Therefore, we next probed our working hypothesis that CB1-mediated signaling acts similarly on adult human HF epithelial eSCs by implementing a variation of the standard HF organ culture method: anagen VI terminal scalp HFs were not amputated below the bulge as usual; instead, micro-dissected HFs were cultured *in toto* so as to fully preserve the intact HF eSC niche and its surrounding mesenchyme *ex vivo*^[29,31,32,51].

Unexpectedly, the selective CB1 agonist (ACEA, at 30 μ M, 24 hrs) significantly *increased* the number of CK15+ cells (**Fig 2A and B**) (for ACEA concentration selection, see **Supplementary Results SR2, Supplementary Fig S2A, and Supplementary Discussion SD1**). Most importantly, ACEA significantly stimulated the proliferation of CK15+ human HF epithelial progenitor cells *in situ*, both in the bulge region (**Fig 2C and D**) and in the lower ORS (**Fig 2E and F**), but did not significantly modulate apoptosis of these cells *in situ/ex vivo* (**Supplementary Fig S2B-E**). Thus, selective activation of CB1 enhances both the proliferation of human HF epithelial progenitor cells *ex vivo*.

CB1 stimulation also increases CK15 protein expression and promoter activity *ex vivo* and *in vitro*

Moreover, ACEA stimulation also significantly increased the overall CK15 immunofluorescence intensity in the ORS of human HFs *in situ* (**Fig 2G**). To independently validate this striking observation (an effect of CB1-mediated signaling on this key eSC marker had not previously been identified), we also transiently transfected a construct encoding a GFP-tagged CK15-promoter via lipofectamine, thus specifically targeting eSCs and hence permitting the live imaging of CK15 promoter activity within these cells in the human HF epithelium *ex vivo*^[39,40]. This demonstrated that the CB1 agonist ACEA (30 μ M, 24 hrs), significantly increased CK15-promoter controlled GFP expression in human HFs *in situ*, as visualized by both, increased levels of GFP protein (red fluorescence) (**Fig 3A**) and by direct GFP fluorescence intensity (**Fig 3B**) detected in the bulge area of HFs transfected with CK15-promoter driven GFP^[39]. Further, ACEA also stimulated CK15-promoter activity in isolated and cultured CK15-GFP+ human HF eSCs/progenitor cells *in vitro*^[39,40] (**Fig 3C**).

CB1 signaling also promotes the proliferation of isolated human HF eSCs *in vitro*

To probe whether the observed unexpected proliferation effects were only indirectly mediated through other neighboring cell populations, such as prominently CB1+ mast cells in the HF mesenchyme^[38], or were dependent on ECM signals arising from the bulge eSC niche, CK15-GFP+ eSCs were isolated and cultured from human scalp HF epithelium as previously described^[39]. This showed that CB1-stimulation by 1 μ M ACEA for 24 hrs also significantly enhanced the proliferation of isolated human (Fig 3D and E), but did not induce apoptosis (Supplementary Fig S2G). This strongly suggests that the effects of CB1 stimulation seen *ex vivo* result from a direct stimulation of CB1-expressing CK15+ human HF eSCs and do not require input from neighboring cells or eSC niche-specific ECM signals.

CB1 stimulation also increases the intrafollicular number of CK19+ progenitor cells *ex vivo*

CK19 protein expression is another characteristic marker of human HF epithelial progenitor cells, whose distribution partially overlaps, but is not identical with that of CK15+ cells in human HF epithelium, since CK19+ cells likely represent a slightly more committed progenitor cell population within human HF epithelium^[29-32,52,53]. Moreover, differential effects of eSC-modulatory agents on human HF CK15+ versus CK19+ cells are recognized^[53]. Therefore, we examined whether CB1 stimulation by ACEA (30 μ M, 24 hrs) also impacts on CK19+ cells *ex vivo*. This showed that CB1 stimulation also up-regulated the intrafollicular number of CK19+ human HF epithelial progenitor cells *in situ* (Fig 3F and G). These data independently corroborate that CB1-mediated signaling targets at least two distinct human eSCs/progenitor cell populations within their natural tissue habitat, i.e. human scalp HF epithelium, and expands this heterogeneous progenitor cell population *ex vivo*, independent of whether these progenitor cells are located within the bulge or along the ORS.

eSC effects of ACEA are abrogated by AM251 and thus CB1-specific

Next, we explored the CB1 specificity/selectivity of the above findings by (co-)administration of ACEA with the well-recognized CB1 antagonist/inverse agonist, AM251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide)^[37,38,54,55]. This showed that AM251 (1 μ M, 24 hrs) abrogated the stimulatory effects of ACEA on the number and proliferation of CK15+ (**Fig 2B-D**) as well as on the number of CK19+ cells *in situ* (**Fig 3G**). This corroborates that the effects of ACEA reported above were indeed specifically/selectively mediated by CB1.

CB1 signaling “tonically” controls human HF eSCs biology

We have previously shown that the “tonic” activation of CB1-signaling profoundly regulates mast cell activation and local maturation from resident progenitor cells in the human HF mesenchyme *ex vivo*^[38]. To assess whether this phenomenon also applies to human HF eSC growth and maintenance, we first applied CB1 antagonists alone (i.e. without prior stimulation of the receptor with ACEA). Short-term application of the CB1 antagonist/inverse agonist AM251 (1 μ M) alone for 24 hrs resulted in insignificant alterations in the number of CK15+ (**Fig 2B**) or CK19+ cells (**Fig 3G**), or of *CK15*-promoter activity in the epithelium of organ-cultured human HFs (**Supplementary Fig S2H**). Likewise, 24 hrs treatment with AM251 did not significantly affect proliferation or apoptosis of HF eSCs (**Fig 2C and Supplementary Fig S2B and C**).

However, in striking contrast, treatment of organ-cultured HFs with 1 μ M AM251 alone for 6 days significantly reduced the number of CK15+ progenitor cells in the bulge region and along the ORS compared to vehicle controls (**Fig 4A and Supplementary Fig S3A-C**). (For additional confirmatory experiments with AM251, see **Supplementary Results SR3**,

Supplementary Fig S3, and Supplementary Discussion SD2). To further validate this finding, treatment of full-length human HF organ cultures with another inhibitor, namely AM6545 (at 1 μ M, 24 hrs), a neutral antagonist of CB1^[55,56], also significantly decreased CK15+ stem/progenitor cells numbers throughout the HF epithelium compared to controls (**Fig 4B**). These experiments not only provide the first proof-of-principle that CB1 signaling can effectively and selectively be targeted pharmacologically in adult human eSC *in situ*, but also suggest that HF eSCs are “tonically” controlled by CB1-coupled signaling.

CB1 silencing reduces the number of human eSCs and promotes their apoptosis within their stem cell niche

To further probe this hypothesis and to exclude potential non-selectivity and off-target signaling issues with synthetic CB1 ligands^[6-8,20,23] that might have confounded the results reported above, we next specifically knocked down *CB1* gene function in human HF organ culture by lipofectamin-mediated siRNA transfection (6 hrs for transfection, and organ culture for additional 18 hrs) as described^[38]. As expected, this reduced both *CB1*-mRNA levels and CB1 protein expression (**Supplementary Figs. S4A-C**). Perfectly in line with our CB1 antagonist data, *CB1*-knock-down significantly decreased the number of CK15+ progenitor cells in the human HF *ex vivo* (**Fig 4C and D**). Inversely, CB1 silencing significantly increased the percentage of apoptotic (TUNEL+) CK15+ progenitor cells within the ORS (**Fig 4E and F**), without affecting their proliferation (**Supplementary Fig S4D**).

This independently confirms the CB1 agonist and antagonist data reported above. Most importantly, these CB1 silencing experiments also indicate that continuous (“tonic”) engagement of CB1 signaling by *endogenous*, intrafollicularly generated CB1 ligands (e.g. the

endocannabinoid anandamide)^[37], is *required* to maintain the pool of human CK15+ HF eSC cells and to protect them from apoptosis, at least under *ex vivo* conditions.

CB1 deletion reduces the number of murine HF eSCs in vivo

Next, we explored whether and how the constitutive absence of functional CB1-signaling impacts on adult mammalian HF eSCs *in vivo* by comparing the percentage of murine epithelial CD34+ HF eSCs^[57,58] in the isthmus/bulge region of *CB1*-knockout mice^[38,43] with that of age- and gender-matched WT controls. CD34 rather than CK15 was used as well-recognized standard marker for murine HF eSCs^[59], since the former yielded more robust results and much lower background immunostaining than the latter in our hands. These experiments revealed that the percentage of CD34+ epithelial SCs in the bulge of murine *CB1*^{-/-} HFs was significantly reduced *in vivo*, compared to WT control HFs (**Fig 4G and H**).

This not only further corroborates the novel principle that continued “tonic” CB1-signaling is required for eSC maintenance in the human HF, but also that it is evolutionarily conserved between these mammalian species. In addition, this important finding documents that the dependence of HF eSCs on “tonic” CB1 signaling also applies to CD34+ cells. Thus, different mammalian eSC/progenitor cell populations in the HF depend on continuous CB1 stimulation, even across species boundaries, and this not only under the relatively stressful conditions of HF organ culture^[28], but also in unmanipulated mouse skin *in vivo*.

CB1 inhibits proliferation and induces apoptosis of differentiated HF keratinocytes

CB1 activation by the endocannabinoid anandamide inhibits human hair shaft production, decreases the proliferation of hair matrix keratinocytes, promotes catagen development, and induces apoptosis in the bulb region *ex vivo*^[37]. Therefore, we also checked how CB1-mediated

signaling impacts on the differentiated progeny of eSCs within the same epithelial HF compartment, the ORS, i.e. neighboring adult, CK15-, but CK6+, human HF ORS keratinocytes^[29,36,53,60].

This showed that CB1 activation by ACEA significantly *reduced* the proliferation of human differentiated ORS keratinocytes, both within the ORS *in situ* (below the isthmus part) in full-thickness human scalp skin organ culture (**Fig 5A and B**) and in isolated human ORS-keratinocytes *in vitro* (**Fig 5C and D**). The proliferation of CK15-*negative* differentiated ORS keratinocytes in organ-cultured amputated HFs was also reduced (**Fig 5E and G**). In contrast, apoptosis of human CK15-*negative* ORS keratinocytes was up-regulated by the CB1 agonist treatment in organ-cultured amputated human HFs *in situ* (**Fig 5F and H**) and in cultured ORS keratinocytes *in vitro* (**Fig 5I and J**). Finally, in organ cultures of full-length human HFs, ACEA did not induce the proliferation of CK15-*negative* ORS cells outside the bulge region (**Fig 5K**); however, as expected (**Fig 2C and D**), within the same experimental setting, CB1 stimulation significantly increased the proliferation of CK15-*positive* eSCs/progenitor cells of the bulge region within the ORS compartment (**Fig 5K**).

Thus, CK15+ human HF epithelial SCs and their more differentiated, distant progeny within the ORS exhibit a diametrically opposed, differentiation-dependent response to stimulation of the same receptor (CB1) by the same agonist, despite their close spatial proximity within the same epithelial HF compartment.

CB1 signaling in human HF eSCs operates via the MEK/Erk 1/2 and Akt pathways

Finally, we began to probe the relevant intracellular signaling pathways that may mediate the observed CB1 stimulation phenomena in human HF eSCs. For this, we focused on key

downstream signaling cascades recognized to be recruited by CB1 stimulation, namely the mitogen-activated protein kinase (MAPK) family (Erk1/2, p42/44) and Akt signaling pathways^[6-8,20].

As expected, in isolated human HF bulge cells, which co-expressed CK15 and CB1 (**Fig 6A**), ACEA-treatment (1 μ M, 24hrs) significantly upregulated CK15 expression (**Fig 6B**). Furthermore, in organ-cultured HFs, the specific MAPK kinase (MEK) inhibitor, PD98059 (50 μ M, 24 hrs), significantly abrogated the ACEA-induced increase in the intrafollicular number of CK15+ progenitor cells (**Fig 6C and D**), while the p38-MAPK inhibitor, SB20358 (30 μ M, 24 hrs), failed to show this effect (**Fig 6C and D**).

Lastly, we asked whether the strikingly differential effects of CB1 agonists on the proliferation and apoptosis of eSCs versus differentiated ORS keratinocytes can be also identified at the downstream signaling level. To probe this, we isolated both bulge cells (eSCs) and ORS cells from human HFs and cultured them with ACEA (at 1 μ M) for 24 hrs. Importantly, in cultured bulge cells, CB1 stimulation significantly up-regulated both p-Erk 1/2 and p-Akt immunofluorescence intensity, reflecting increased activities of these signaling enzymes (**Fig 6E and F, Supplementary Fig S5A and B**). In contrast, in cultured (non-bulge) ORS keratinocytes, albeit ACEA treatment also significantly up-regulated p-Erk 1/2 expression, there was no significant change in p-Akt immunofluorescence intensity (**Fig 6E and F, Supplementary Fig S5A and B**). These results suggest that CB1 stimulation selectively increases CK15+ cell proliferation, at least in part, via engagement of the MAPK/MEK/Erk 1/2 signaling cascade and the Akt pathway.

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3 **DISCUSSION**
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5 Here, we have used human HF organ culture as a prototypic, clinically relevant, accessible, and
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7 experimentally tractable preclinical model system for studying adult human eSC physiology
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9 and pathology *in situ/ex vivo*^[28,30,31,61]. In this model, we provide the first evidence that,
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11 unexpectedly, CB1-mediated signaling significantly increases the number and proliferation of
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13 human HF bulge eSCs, while *CB1* gene-knockdown and selective CB1 antagonists reduce their
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15 number and promote their apoptosis – in striking contrast to the differentiated progeny of human
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17 eSCs, i.e. HF keratinocytes in the ORS (**Supplementary Fig S6**) (for further details, see
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19 **Supplementary Discussion SD2**). This identifies CB1 signaling as a key survival factor for
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21 adult human skin eSCs *in situ*, whose maintenance requires “tonic” CB1-mediated signaling.
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23 Mechanistically, our data suggest that these eSC effects are mediated, at least in part, by CB1-
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25 dependent engagement of the MAPK (MEK/Erk 1/2) pathway.
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33 This strongly suggests that eSC in the human HF depend on the generation and maintenance of
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35 a minimal level of locally produced endocannabinoids such as anandamide or 2-AG^[5-7] and that
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37 the continuous enzymatic degradation of endocannabinoids (e.g. of anandamide by fatty acid
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39 amide hydrolase, FAAH^[5-7,21,62,63]) must be tightly and differentially controlled within distinct
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41 HF compartments (e.g. bulge versus bulb), in order to maintain the eSC pool. Thus, the
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43 surprisingly high endocannabinoid level of human scalp HFs, which approximates that of the
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45 brain^[37], likely multiple critical, but diverse purposes in human skin appendage physiology: not
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47 only does the endocannabinoid “tone” negatively regulate hair matrix^[37] and ORS keratinocyte
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49 growth through apoptosis induction (**Fig 5F, H, I and J**) as well as excessive perifollicular mast
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51 cell activities^[38], but it also preserves the functionally most important eSC/progenitor pool of
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53 human skin, i.e. CK15+ and CK19+ cells^[30,31] (**Supplementary Fig S6**). Moreover, the
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55 dependence of adult eSCs in mammalian skin on “tonic” CB1 stimulation uncovered here also
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transcends to other mammalian species and is relevant *in vivo*, as documented in the bulge of CB1^{-/-} mice (**Fig 4G and H**) (albeit further flow cytometry analysis would be optimal to complement our current immunohistomorphometry data). (In the **Supplementary Discussion SD3**, we detail those mechanisms and factors which most probably contribute to the observed opposite effects of CB1 signaling on the two cell populations.)

Colloquially phrased, our findings raise the clinically important and biologically intriguing, provocative question: Do adult human eSCs have to be continuously “stoned” for optimal maintenance of the SC pool? This novel concept now needs to be taken into account and systematically interrogated whenever the human ECS is targeted therapeutically, namely when pharmacological CB1 inhibition is being considered so as not to risk depleting eSC pools, namely in human skin. Obviously, it remains to be explored, next, whether the novel principle uncovered here, i.e. the diametrically opposed regulation of eSCs versus their more differentiated progeny by signaling through the same receptor (CB1) within the same epithelial tissue and by the same ECS (**Supplementary Fig S6**), also applies to the epithelial compartment of other human organs, such as gut, lung, or liver.

In order to generate data of maximal physiological and clinical relevance we advocate to study this primarily in available organ culture systems of intact human organ fragments that permit the characterization and experimental manipulation of primary eSC functions within their natural SC niches and in the presence of physiological signals arising from their immediate epithelial, mesenchymal and ECM tissue milieu, rather than in cultured eSCs that have been isolated through highly disruptive enzymatic digestion, have been ripped out of their eSC physiology-dictating specific niches, and are cultured in isolation from neighboring cell and ECM signals under highly non-physiological conditions. (While we have shown that SC niche

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3 signals are not required for the CB1-mediated regulation of human eSC proliferation, apoptosis,
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5 and CK15 expression, they likely are important in the control of other eSC functions^[64,65].
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10 This novel regulation of human eSC function in their physiological niche has important
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12 translational implications. On the one hand, CB1-antagonistic drugs, such as the CB1
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14 antagonist/inverse agonist, rimonabant, have undergone extensive clinical testing for the
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16 management of obesity^[22,66]. While the published clinical trials with CB1 antagonists have not
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18 reported relevant hair or skin alterations, appropriate read-out parameters were not specifically
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20 assessed. Our new data suggest that patients treated with CB1 antagonists should be
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22 systematically (re-)screened by obtaining (minimally invasive) HF/skin biopsies so as to
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24 specifically search for eSC-related effects *in vivo*.
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30 On the other hand, insufficient CB1 signaling may also contribute to eSC pathology in human
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32 skin, notably in lichen planopilaris (LPP) or frontal fibrosing alopecia (FFA), the two most
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34 frequent eSC pathologies of human HFs that lead to scarring alopecia through eSC apoptosis
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36 and pathological EMT induction^[61,67]. Re-analysis of our previously published microarray
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38 results (Affymetrix®) of laser capture-microdissected HF bulge cells from five patients with
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40 LPP or FFA indeed showed a >2-fold significantly reduced expression of CB1 in the bulge
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42 epithelium of lesional LPP/FFA HFs compared to that of non-lesional HFs from the same
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44 patient (full microarray data are available at Array Express [www.ebi.ac.uk/arrayexpress/],
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46 reference number: E-MEXP-3859). Urged by these intriguing data, we performed a pilot CB1
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48 immunolabeling on paraffin-embedded sections of a LPP patient (along with non-LPP
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50 controls). Most importantly, as shown in **Supplementary Fig S7**, CB1 protein expression *in*
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52 *situ* was also significantly reduced in lesional LPP HF bulge region, compared to those of
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54 healthy individuals (CB1 immunoreactivity within the bulge area is marked by a black rectangle
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in the figure). This renders it plausible that insufficient CB1-mediated signaling may contribute to the depletion of bulge eSCs in scarring alopecia.

If this working hypothesis is confirmed in future CB1 studies of LPP and FFA, raising the intrafollicular “ECS tone” in affected patients (e.g., by elevating the level of CB1-agonistic endocannabinoids via pharmacological FAAH inhibition^[62,63]) could promote bulge eSC survival in these patients – the cardinal therapeutic challenge in the management of irreversible scarring alopecias^[67]. Given the anti-inflammatory properties of endocannabinoids and CB1 stimulation^[14-16], that “tonic” CB1 signaling suppresses pro-inflammatory activities of perifollicular human mast cells and their excessive local maturation from resident progenitor cells in the human HF mesenchyme^[38], and the key role of bulge immune privilege collapse in LPP and FFA^[67,68], elevation of the “HF ECS tone” promises multilevel benefits in eSC pathology-based pro-inflammatory alopecias like LPP and FFA.

CB receptor activation can engage a multitude of intracellular signaling cascades, often in a biased manner. The pattern, activities and functional outcomes of CB-coupled signaling markedly depend on cell type, cell cycle and differentiation status, the local tissue milieu, and cross-signaling/cross-regulation with other receptor such as certain transient receptor potential (TRP) ion channels (e.g. TRPV1) which are considered as ionotropic cannabinoid receptor^[4-8,20-22,69]. This makes it fundamentally important to study CB1 signaling physiology within a defined tissue context, since studies on isolated cells cannot recapitulate the signaling complexity *in vivo* and risk to be conceptually misleading. Yet, this also renders it extremely challenging to dissect the main mechanism(s) of action that underlie the observed CB1-mediated signaling outcome.

When probing at least some of the recognized post-CB1 receptor signaling pathways^[4-8], p-Erk 1/2 and p-Akt expressions were up-regulated by CB1 stimulation exclusively on undifferentiated bulge cells but not on differentiated ORS keratinocytes where only p-Erk 1/2 upregulation was observed (**Fig 6E and F**). Moreover, only the tested MEK inhibitor was able to antagonize the eSC effect of the CB1 antagonist (increase the number of CK15+ HF cells) (**Fig 6C and D**). Our initial findings therefore strongly argue in favor of a dominant involvement of the MAPK and Akt pathways in mediating the CB1-mediated effects on human CK15+ eSCs within their HF niches. Our current dissection of signaling pathways with quantitative immunohistomorphometry obviously can only be a first step, which should be followed up and substantiated with Western blots (especially after short-term CB1 engagement), and a variety of inhibitor assays. For example, the MEK/Erk and Akt pathways identified here may be downstream of CB1 signaling (especially after a relatively long incubation period of 24h), and might reflect secondary events such as cell proliferation, and might not necessarily result directly from of CB1 receptor stimulation/inhibition.

That the observed eSC stem cell effects of a CB1-targeting antagonist (**Fig 2B-D, Fig 3F**) were fully recapitulated by highly selective CB1 silencing (**Fig 4C-F**) and were confirmed in CB1^{-/-} mice *in vivo* (**Fig 4 G and H**) documents a previously unknown key role of CB1 signaling itself for the survival of bulge HF eSC, which also prominently express functional CB1 binding sites (**Fig 1B**). This encourages one to systematically explore, next, the entire ECS and its controls in human eSC biology, and to elucidate details of endocannabinoid synthesis and breakdown within eSC niches, the enzymatic machinery that controls of the endocannabinoid tone^[4-8], the role of other ECS metabotropic and ionotropic cannabinoid receptors, and the modulation of endocannabinoid transports (for details, see **Supplementary Discussion SD4**).

Taken together, our study identifies the ECS and specifically CB1-mediated signaling as a novel, drugable control system of human eSC physiology and pathology.

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AUTHOR CONTRIBUTIONS

K.S. performed human HF organ cultures, immunohistochemistry, cell cultures, statistical analysis, data analysis and summarized the findings. N.Z. assisted with human HF cultures, immunohistochemistry and cell cultures, and figure generation. S.T. performed transfection of CK15-promoter-GFP into human scalp HFs and isolation of human HF bulge and ORS cells. T.P. performed human HF organ cultures and quantitative immunohistomorphometry. M.H. performed immunolabeling of LPP samples. T.B. provided input into experimental design, data analysis, and manuscript editing, and performed quantitative Real-Time PCR. D.T. assisted with data interpretation and manuscript editing. R.P. designed and supervised the project, interpreted the results. R.P. and T.B. wrote the manuscript, which was edited by all co-authors.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists. However, for the record, R.P. is founder & CEO of a skin research CRO that engages in HF organ culture (www.monasteriumlab.com), while T.B. consults for this CRO and for another company engaged in ECS targeting therapeutics (Phytects, INC., Los Angeles, CA, USA).

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FIGURE LEGENDS

Fig 1. Human HF eSCs express CB1

A. CK15 (green) and CB1 (red) double immunofluorescence of the HF-bulge region of a freshly isolated human scalp skin sample. CK15+ bulge cells co-express CB1 in situ. Scale bar: 10 μ m.

SG: sebaceous gland. APM: arrector pili muscle. HF: hair follicle.

B. Representative image of organ cultured human HFs treated with the CB1-selective ligand, Tocrifluor T1117 (1 μ M, 24 hrs). The incubation of HFs with Tocrifluor T1117 (red) showed specific fluorescent bindings in CK15+ (green) progenitors/eSCs within the HF's outer root sheath (ORS). Note the almost complete overlap of the two fluorescence signals resulting in a merged yellow signal. Tocrifluor T1117 (red), CK15 (green). Scale bar: 10 μ m.

Fig 2. CB1 stimulation increases the proliferation of human CK15+ HF eSCs ex vivo

A. Left two panels: Representative images of CK15+ ORS cells (green) within organ cultured human HFs (middle to lower part of HFs) treated with either vehicle or ACEA (30 μ M, 24 hrs) detected by fluorescence microscope (FM). Scale bar: 50 μ m. **Right two panels:** Representative images of CK15+ ORS cells (green, indicated by arrows) within organ-cultured human HFs detected by confocal microscope (CM). Scale bar: 10 μ m. Dotted lines represent basal membrane of the HFs. Arrows indicate CK15+ cells. ORS: outer root sheath.

B. Number of CK15+ cells within the organ cultured human HFs (middle to lower part of HFs) which were organ cultured with vehicle, ACEA (30 μ M), AM251 (1 μ M) or ACEA+AM251 for 24 hrs.

C. Representative images of cells expressing CK15 (green) and Ki-67 (red) within the HF-bulge area of vehicle, ACEA, AM251 or ACEA+AM251 treated organ cultured full-length human HFs for 24 hrs. Scale bar: 100 μ m. Arrows indicate cells that co-express the two markers. Dotted lines represent basal membrane of the HFs.

D. Percentage of cells co-expressing CK15 and Ki-67 within the HF-bulge area of vehicle, ACEA, AM251 or ACEA+AM251 treated organ cultured full-length human HF for 24 hrs.

E. Representative images of cells expressing CK15 (green) and Ki-67 (red) within organ cultured human HF treated with either vehicle or ACE for 24 hrs. Scale bar: 100 μ m. Yellow arrows indicate cells Ki-67+ (proliferating) cells whereas white arrows indicate cells that co-express the two markers. ORS: outer root sheath.

F. Percentage of Ki-67+ (proliferating) cells of CK15+ ORS cells (per HF) within organ cultured human HF treated with either vehicle or ACEA for 24 hrs.

G. Quantitative CK15 immunohistomorphometry (per HF) of either vehicle or ACEA treated human HF (middle to lower part of HF).

ACEA: CB1 specific agonist, AM251: CB1 antagonist/inverse agonist. Data are means $\pm$ SEM.

*p<0.05, **p<0.01, N.S.=not significant.

Fig 3. CB1 stimulation increases the proliferation of human CK15+ HF eSCs in the CK15 promoter-GFP system and of CK19+ HF eSCs ex vivo

A. Upper: CK15-promoter activity assessed by GFP immunofluorescence (red). HF were treated with either vehicle or ACEA (30 μ M, 24 hrs). Lower: Mean GFP immunofluorescence intensity per HF was measured and compared. Dotted lines represent basal membrane of the HF. Arrows indicate GFP+ cells. Scale bar: 50 μ m.

B. Quantification of CK15 promoter-GFP-fluorescence intensity within transfected organ cultured human HF. HF were treated with either vehicle or ACEA (30 μ M, 24 hrs). Each GFP fluorescence intensity was measured as arbitrary unit and compared per HF. Scale bar: 100 μ m.

C. Quantitative analysis of CK15-promoter-GFP fluorescence intensity of isolated CK15 promoter-GFP cells treated with either vehicle or ACEA (1 μ M, 24 hrs). GFP fluorescence

intensity per each cell was measured and compared. GFP fluorescence intensity per each cell is expressed as arbitrary unit.

D. Percentage of Ki-67 positive (proliferating) cultured CK15-promoter-GFP cells (per visual field) treated with either vehicle or ACEA (1 μ M, 24 hrs).

E. Representative images of Ki-67 positive (proliferating, red), indicated by arrows, cultured CK15-promoter-GFP cells treated with either vehicle or ACEA (1 μ M, 24 hrs). Scale bar: 50 μ m.

F. Representative images of CK19 immunofluorescence (green) of organ cultured human HF (middle to lower part of HF) treated with either vehicle or ACEA (30 μ M, 24 hrs). The image was obtained by confocal microscope. Arrows denote CK19+ ORS cells.

G. Number of CK19+ cells within organ cultured human HF (per HF) treated with vehicle, ACEA (30 μ M), AM251 (1 μ M) or ACEA+AM251 (24 hrs).

Data are means $\pm$ SEM. * p <0.05, ** p <0.01.

Note that the fluctuations of the arbitrary unit of the Y-axes are due to the features and auto-settings of the NIH Image J software as no analysis parameter was artificially and subjectively set.

Fig 4. CB1 blockade by CB1 antagonists or gene silencing reduces the number of CK15+ progenitor cells in human HF. Likewise, CB1 knockout reduces the number of CD34+ eSCs in the bulge of murine HF in vivo

A. Number of CK15+ cells of either vehicle or CB1 selective antagonist/inverse agonist AM251 (1 μ M) treated organ cultured human full-length HF for 6 days (middle to lower part of HF).

B. Number of CK15+ cells number of either vehicle or CB1 selective neutral antagonist, AM6545 (1 μ M) treated organ cultured human full-length HF for 24 hrs.

C. Representative images of CK15+ ORS cells (Green) within organ cultured human HF (middle to lower part of HF) treated with TFE, SCR or CB1-siRNA. Yellow arrows indicate CK15+ ORS cells. The basal membrane of ORS is shown by white dotted line. Scale bar: 10 μ m.

D. Number of CK15 + progenitor cells per visual field within the ORS of HF treated with TFE, SCR, or CB1-siRNA.

E. Representative images of CK15 (Red) and TUNEL (Green) double immunofluorescence within organ cultured human HF treated with SCR or CB1-siRNA. Yellow arrows indicate TUNEL (nuclear staining) and CK15 (cell membrane and cytoplasm) double-positive cells. The basal membrane of ORS is shown by white dotted line. Scale bar: 50 μ m.

F. Number of cells demonstrating CK15+/TUNEL+ co-expression within organ cultured human HF.

ORS: outer root sheath, TFE: transfection reagent treated, SCR: scrambled siRNA treated.

G. Representative images of CD34 immunofluorescence (green) of wild type (WT) and CB1-knockout (CB1 KO) mice, including the bulge zone (red arrows). Scale bar: 50 μ m. The basal membrane of ORS is shown by white dotted lines.

H. Percentage of CD34+ cells within the isthmus/bulge area of murine late anagen HF (post-natal day 32, only anagen VI HF were analyzed and compared between CB1+/+ and CB1-/- mice). 49 HF from wild type (n=6) and 78 HF from CB1 knockout (n=4) were analyzed.

Data are means $\pm$ SEM. *p<0.05, **p<0.01.

Fig 5. CB1 stimulation inhibits proliferation and promotes apoptosis of human HF-derived differentiated ORS keratinocytes ex vivo and in vitro

A. Representative images of Ki-67 (red) immunofluorescence of HF (below isthmus part) within organ cultured human scalp skin samples treated with either vehicle or ACEA (30 μ M,

24 hrs). Yellow arrows indicate Ki-67 positive ORS cells. The basal membrane of ORS is shown by white dotted lines. Scale bar: 50 μ m.

B. Percentage of Ki-67+ human HF-ORS cells within the ORS (below isthmus part, per visual field) of organ cultured human scalp skin samples treated with either vehicle or ACEA (30 μ M, 24 hrs).

C. Representative images of Ki-67+ primary cultured human ORS keratinocytes treated with either vehicle or ACEA (1 μ M, 24 hrs). Scale bar: 50 μ m.

D. Percentage of Ki-67+ primary cultured human ORS keratinocytes (per visual field) treated with either vehicle or ACEA (1 μ M, 24 hrs).

E. Representative images of cells expressing CK15 (green) and Ki-67 (red) within organ cultured human amputated HF treated with either vehicle or ACEA (30 μ M, 24 hrs). Scale bar: 100 μ m. Yellow arrows indicate cells Ki-67+ CK15-negative cells whereas white arrows indicate CK15+ cells. ORS: outer root sheath.

F. Representative images of cells expressing CK15 (red) and TUNEL (green) within organ cultured human amputated HF treated with either vehicle or ACEA (30 μ M, 24 hrs). Scale bar: 100 μ m. Yellow arrows indicate cells TUNEL+ CK15-negative cells whereas white arrows indicate CK15+ cells. ORS: outer root sheath.

G. Percentage of Ki-67+ CK15-negative ORS cells of organ-cultured amputated human HF treated with either vehicle or ACEA (30 μ M, 24 hrs).

H. Percentage of TUNEL+ CK15-negative ORS cells of organ cultured human amputated HF treated with either vehicle or ACEA (30 μ M, 24 hrs).

I. Representative images of cleaved caspase-3+ primary cultured ORS keratinocytes (indicated by arrows) treated with either vehicle or ACEA (1 μ M, 24 hrs). Scale bar: 50 μ m.

J. Percentage of cleaved caspase-3+ primary cultured ORS keratinocytes (per visual field) treated with either vehicle or ACEA (1 μ M, 24 hrs).

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K. Percentage of Ki-67+, CK15-negative ORS keratinocytes and Ki-67 positive, CK15+ bulge ORS cells within the organ cultured human full-length HF treated with ACEA (30 μ M, 24 hrs).

ACEA: CB1 specific agonist. Data are means $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$. N.S.=not significant.

Fig 6. CB1 stimulation upregulates CK15 expression in isolated human HF bulge cells

A. Representative image of CK15 (green) and CB1 (red) double-immunofluorescence with isolated primary human HF bulge ORS cells.

B. CK15 immunofluorescence intensity of isolated human bulge cells treated with either vehicle or ACEA (1 μ M, 24 hrs). CK15 immunofluorescence intensity was expressed as arbitrary unit. Data are means $\pm$ SEM. *** $p < 0.001$.

C. Representative images of CK15+ cells in organ cultured human HF treated with vehicle (control), ACEA (30 μ M), ACEA+PD98059 (50 μ M), or ACEA+SB20358 (30 μ M) for 24 hrs.

Scale bar: 100 μ m.

D. Number of CK15+ cells of organ cultured human HF treated with vehicle (control), ACEA (30 μ M), ACEA+PD98059 (50 μ M), or ACEA+SB20358 (30 μ M) for 24 hrs.

E. p-Erk 1/2 immunofluorescence intensity of primary cultured cells from either lower part of the ORS (ORS cells) or the bulge area (Bulge cells) of human HF and treated with vehicle (Control) or ACEA (1 M, 24 hrs).

F. p-Akt immunofluorescence intensity of primary cultured cells from either lower part of the ORS (ORS cells) or the bulge area (Bulge cells) of human HF and treated with vehicle (Control) or with ACEA (1 M, 24 hrs).

ACEA: CB1 specific agonist, PD98059: MEK inhibitor, SB20358: p38 MAPK inhibitor. Data are means $\pm$ SEM. * $p < 0.05$, *** $p < 0.001$.

A

CK15

CB1

Merge

B

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

A

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E

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Figure 6

*Human epithelial stem cell survival within their niche requires
“tonic” cannabinoid receptor 1-signaling – Lessons from the hair
follicle*

Sugawara et al.

SUPPLEMENTARY INFORMATION

For Review Only

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SUPPLEMENTARY INTRODUCTION

Albeit the we lack information on how the ECS controls epithelial SC biology, it was shown to be involved in the regulation of other stem/progenitor cells^[S1]. Indeed, CB1 stimulation promoted the survival of isolated murine embryonic stem cells (SCs) *in vitro*^[S2,S3] and also enhanced mouse neural precursor cell proliferation *via* PI3K/Akt pathways^[S4]. In addition to CB1, CB2 was also found to exert a critical role in the proliferation of neural SCs^[S5], and the CB2 agonist, AM1241 promoted the proliferation and differentiation of human neural SCs^[S6]. In a murine stroke model, CB2 stimulation drove neuroblast migration and was suggested to be required for post-stroke neurogenesis^[S7]. Reportedly, CB1 stimulation also promoted rat mesenchymal SC survival and differentiation *in vitro*^[S8] and enhanced the osteo/dentinogenic differentiation ability of periodontal ligament stem cells via p38 MAPK and JNK in an inflammatory environment^[S9]. Likewise, CB2 signaling was found to be important in the osteogenic differentiation of bone marrow-derived mesenchymal SCs^[S10]. CB1 has been implicated in experimental mouse liver fibrosis, presumably by promoting the homing of bone marrow-derived mesenchymal SCs during the repair of damaged liver tissue^[S11].

SUPPLEMENTARY RESULTS

Supplementary Results SR1

As an additional control, both CB1 immunoreactivity and immunofluorescence intensity were significantly reduced within the ORS of organ cultured human HF treated with CB1 siRNA compared to controls^[37,38]. Moreover, CB1 antibodies from two different vendors showed similar immunostaining reactivity (**Supplementary Fig S1A**). Using an anti-CB1 antibody (H-150, Cat-Nr: sc-20754, Santa Cruz), CB1 immunoreactivity in the epidermis and ORS of HF within unmanipulated human scalp skin corresponded to the previously reported pattern^[37,38] (**Supplementary Fig S1B**). In addition, as expected, CB1 immunoreactivity was reduced within skin samples from CB1 knockout mice when a primary antibody was used that recognizes both mouse and human CB1 (**Supplementary Fig S1C**).

Supplementary Results SR2

Though the scarce availability of human HF for study precluded comprehensive dose-response studies, we tested a range of additional ACEA concentrations (300 nM, 3 μ M, 30 μ M and 50 μ M, for 24 hrs) in full-length human HF organ culture. Among these, treatment with 30 μ M ACEA reproducibly resulted in the greatest increase in the number of CK15+ HF epithelial SCs within the isthmus area (**Supplementary Fig S2A**), and was therefore used in all subsequent *ex vivo* experiments.

Supplementary Results SR3

AM251 may also act as an agonist for GPR55, another receptor of the endocannabinoid system^[5-7,S12,S13]. Therefore, we also assessed the potential role of GRP55 by applying Tocrifluor T1117, a fluorescent AM251 derivative that selectively binds CK15+ cells in human HF *in situ* (**Fig 1B**) and is considered as a ligand for GPR55^[S14,S15] as well as another

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recognized GPR55 ligand, lysophosphatidylinositol (LPI)^[S12,S13,S16]. Neither T1117 (1 μ M) nor LPI (5 μ M), applied for 6 days, altered the number of CK15+ bulge cells or CK15 immunofluorescence intensity *in situ* (**Supplementary Fig S3A-C**). In contrast, in this independent experiment, the CB1 antagonist/inversed agonist AM251 significantly decreased the number of CK15+ bulge cells and CK15 immunofluorescence intensity within the HF bulge (**Fig 4A and Supplementary Fig S3A-C**). Of further importance, GPR55 gene expression in the bulge area was close to or below the detection limit (**Supplementary Fig S3D**), suggesting that, if GPR55 is transcribed in the bulge at all, its expression level is extremely low. These finding collectively and markedly reduce the likelihood that GPR55-mediated signaling and endogenous ligands of GPR55 impact significantly on epithelial SC functions in human scalp HFs.

SUPPLEMENTARY DISCUSSION

Supplementary Discussion SD1

Although the concentrations of ACEA used in this study were considerably higher than the previously published dissociation constant (K_d) values that had been determined by membrane fraction binding assays^[S17], we have previously shown the suitability of these higher concentrations under physiological conditions, i.e. in human skin, HF and nasal polyp organ culture studies^[37,38,60,S18]. This was further confirmed by our full-length HF organ culture with an extended range of ACEA concentrations (300 nM to 50 μ M). This revealed that 30 μ M ACEA had the strongest effect on the CK15+ cell number (**Supplementary Fig S2A**). In addition, there are several types of endocannabinoids including AEA, 2-AG, noladin ether, N-arachidonoyldopamine, N-oleoyl dopamine, oleamide and virodhamine, all of which can interact with CB1^[5-8,S19]. As these endocannabinoids are reported to be produced on demand^[5,6], physiological concentrations of endogenous CB1 ligands in a given tissue location could be much higher than previously assumed, thus further justifying the high dose of ACEA tested in our study. Therefore, in future studies, it certainly will be interesting to examine whether different concentrations of distinct endocannabinoids or their metabolites activate other receptors besides CB1.

Supplementary Discussion SD2

Although neither ACEA nor AM251 are completely selective for CB1 (e.g., AM251 can also interact with GPR55), these compounds have routinely employed as CB1-specific ligands^[5-8,S19]. The CB1-specificity of our CB1 antagonist data (AM251) is underscored by our demonstration that the GPR55 agonists, LPI and Tocrifluor, by themselves had little or no impact on human HF epithelial stem/progenitor cells *ex vivo*. In addition, the CB1 neutral antagonist, AM6545, which does not bind to GPR55, also reduced the number of human CK15+

cells *in situ*. Finally, GPR55 gene and protein expression in the bulge area of human HFs is minimal, and human HFs do not express CB2^[37]. Nevertheless, *CB1*-selective knock-down experiments clearly verified that human HF eSCs indeed depend on “tonic” CB1 stimulation *ex vivo*. Taken together, this set of data strongly supports that the human epithelial SC effects described here are indeed CB1-mediated. This concept is further strongly supported by the reduction in the epithelial HF SC pool seen in CB1 knock-out mice *in vivo*.

Supplementary Discussion SD3

To the best of our knowledge, our current findings are the first to describe a diametrically opposite effects of CB1 signaling on stems cells and their differentiated progeny within the same tissue or mini-organ. Therefore, only further studies could identify those mechanisms and/or factors that determine whether exogenous CB1 activators or endogenous CB1 ligands – such as e.g. anandamide which, as we have showed previously, is locally produced in the human hair follicle^[37] – exert pro-survival (on K15+ eSCs) or pro-apoptotic (on K15- ORS keratinocytes) actions. Below, we highlight those potential factors which might underline the observed opposite affects, and which should be investigated in further experiments.

It was previously shown that stimulation of the same receptor in distinct cell populations of the human HF may target very different genes whose products then cause opposite biological effects. The classical example of such opposite actions is the follicular effects of androgens (e.g. testosterone, dihydrotestosterone). Indeed, androgens promote the synthesis of insulin-like growth factor-I, one of the most potent hair growth stimulators, in androgen-dependent beard HFs, thereby promoting the growth of these follicles. In contrast, in androgen-insensitive HFs, androgen treatment upregulated the production of transforming growth factor β (TGF β), one of the key catagen inducer molecules, thereby inducing hair growth inhibition^[S20]. In this context

it is worth mentioning that, in an ongoing separate study, we found that the activation of CB1 on cultured human ORS keratinocytes by the application of high (10-30 μ M) anandamide concentration (which was sufficient to inhibit proliferation and apoptosis of the ORS cells) markedly upregulated TGF β synthesis (unpublished observation) – similar to the effect of activation of another anandamide receptor, TRPV1, in organ-culture human HF, as we had shown previously^[45]. Therefore, although further, most optimally, genomics and/or proteomics analyses are to be performed to uncover the effects of CB1 stimulation on gene and protein expression patterns of eSCs and the differentiated ORS cells, we hypothesize that one of the key factors that determine the opposite effects of CB1 signaling could be the differential modulation of gene and protein expression profiles on the two distinct cells populations.

It is also well known that activation of CB1 engages multiple signaling pathways in the target cells^[2,5-7]. Further, cell type and context-dependent receptor-coupled signaling as well as biased agonism have also been described for CB1^[5-8,20,23]. Therefore, it is also tempting to propose that, besides the above proposal, different signaling mechanisms could be additionally involved in mediating the opposite cellular effects of CB1 activation on the eSCs and the differentiated ORS cells. Actually, our current presentation that, on bulge cells, CB1 stimulation results in the activation of MEK/Erk 1/2 and Akt pathways whereas, on ORS keratinocytes, only the engagement of MEK/Erk 1/2 signaling was observed (**Fig6** and **Supplementary Fig S5**) also supports this proposal.

Another exciting possibility, which also should be extensively studied in the future, could be the different epigenetics and promoter/enhancer properties of the two cell populations upon CB1 activation. Indeed, increasingly more evidence suggests the key role of epigenetic regulation of human hair biology^[S21]. Although we lack published data on the involvement of the ECS or

CB1 signaling in such processes within the HF, it is worth mentioning that cannabidiol, a non-psychoactive phytocannabinoid from the *Cannabis* plant, markedly decreased keratin 10 mRNA expression and increased DNA methylation of the gene in a CB1-dependent manner mechanism on cultured human epidermal keratinocytes. In addition, cannabidiol increased global DNA methylation levels by selectively enhancing DNMT1 expression, thereby proposing the possible role of cannabinoid signaling in epigenetic regulation of skin and keratinocyte functions^[S22].

Albeit we did not investigate the effects of endogenous anandamide in our current study, we finally propose another possibility for explaining the diametrical differences. As mentioned before, we have previously showed that the human HF is able produce surprisingly high amount of anandamide, which approximates that of the brain^[37]. Yet, we still do not know whether the relatively high amount of anandamide is produced by exactly what cell type(s) of the HF and, further, whether it is equally “distributed” among the different cellular compartments of the HF (most probably, not). Since multiple previous findings suggest that the cellular consequences of CB1 activation strongly depends on the actual concentration of the CB1 ligand – e.g. high concentrations of anandamide significantly decreased functionality of cultured neurons, whereas lower concentrations significantly enhanced the frequency of synaptic activity^[S23] –, one might speculate that the observed differences on eSCs and ORS cells are (additionally) due to different local endocannabinoid concentrations, hence the different quantitative and qualitative engagement of CB1 activation and the coupled signaling mechanisms.

Supplementary Discussion SD4

Prototypic endocannabinoids (such as e.g. anandamide and 2-AG) bind to both CB1 and CB2^[5-8]. Since we have previously shown that 2-AG does not affect human HF cycling, hair shaft

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3 production, hair matrix cell proliferation or apoptosis^[37], it appears unlikely that the 2-AG
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5 pathway is functionally important in the control of human HF epithelial SC biology; however,
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7 this remains to be systematically investigated. Since anandamide is not only a key endogenous
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9 CB1 agonist, but also stimulates the transient receptor potential ion channel TRPV1^[5-8,67]; and
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11 since both endo- and exocannabinoids can exhibit the phenomenon of “biased agonism”^[5-8,20,23],
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13 such an extended future analysis of the ECS in human SC and HF biology will also have to take
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15 into account signaling through ionotropic cannabinoid receptors such as certain TRP ion
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17 channels (besides TRPV1, TRPV3 and TRPV4 should also be considered) whose importance
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19 in the control of human (and murine) HF growth and cycling have previously been documented
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24 by our workgroups^[45,68,69,S24].
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SUPPLEMENTARY METHODS

Agents

All reagents for human skin/HF organ cultures including, ACEA (Catalog number <Cat-Nr>: A9719), AM251 (Cat-Nr: A6226), AM6545 (Cat-Nr: A1987), LPI (Cat-Nr: L7635), wortmannin (Cat-Nr: W1628), SB203580 (Cat-Nr: S8307) and PD98059 (Cat-Nr: P215) were purchased from Sigma-Aldrich (Taufkirchen, Germany). 5-carboxytetramethylrhodamine (5-TAMRA) conjugated AM251, Tocrifluor (Cat-Nr: 2540) was from Tocris Bioscience (Bristol, UK).

Immunocytochemistry

For the detection of CB1 and CK15 in cultured human HF bulge ORS cells, double immunocytochemistry was performed. Cells were first rinsed with PBS and then fixed with ice-cold acetone for 10 min. After the wash with PBS, cells were incubated with primary antibodies against CB1 (1:200) and CK15 at (1:100) overnight at 4 °C. After washing with PBS, cells were incubated with rhodamine conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:200) and FITC conjugated goat anti-mouse antibody (Jackson ImmunoResearch, 1:200) for 45 min at RT. Cells were then washed with PBS and their nuclei were counterstained by DAPI for 1 min and mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL). Intracellular detection of p-Erk 1/2, p-Akt, cleaved caspase-3, or Ki-67 immunostaining was performed. Briefly, after washing with PBS, cells were fixed with 4% paraformaldehyde in PBS for 15 min at RT, followed by the incubation in 0.1% Triton-X100 in PBS for 10 min at RT. After the wash with PBS, cells were incubated with anti-p-Erk 1/2 antibody (1:200), anti-p-Akt (1:200), cleaved caspase-3 (1:200) (all Cell Signaling Technology, Inc, Danvers, MA), Ki-67 (1:20) (DAKO, Hamburg, Germany) at 4 °C for overnight. FITC (p-ERK1/2 and p-AKT) or rhodamine (cleaved caspase-3) conjugated anti-rabbit IgG antibody

(Jackson ImmunoResearch), or FITC conjugated anti-mouse IgG antibody (Jackson ImmunoResearch) was used as a secondary antibody (1:200, RT, 45 min).

Immunohistochemistry

For the detection of CK15 and CB1, the highly sensitive tyramide signal amplification (TSA) technique (Perkin Elmer, Boston, MA) was applied. Briefly, cryosections were fixed with 1% paraformaldehyde in PBS for 10 min at RT and incubated with ethanol-acetic acid (2:1) for 5 min at -20°C. After the block of endogenous peroxidase by 3% H₂O₂ solution and the subsequent incubation with avidin and biotin solution (Vector Laboratories Inc, Burlingame, CA), sections were incubated overnight at 4°C with primary antibodies, either rabbit anti-human CB1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA or Cayman Chemical Company, Ann Arbor, MI) at 1:400, or mouse anti-human CK15 (Merck Millipore, Temecula, CA) at 1:200 diluted in TNB (Tris, NaOH, Blocking reagent, TSA kit; Perkin-Elmer). Thereafter, the cryosections were incubated with biotinylated goat antibodies against rabbit or mouse IgG (Jackson ImmunoResearch Laboratories) at 1:200 in TNB for 45 min at RT. Sections were then treated with streptavidin-conjugated horseradish peroxidase (HRP) (1:100, 30 min, TSA kit) and were finally incubated with fluorescein isothiocyanate (FITC) or rhodamine conjugated tyramide (1:50, TSA kit). The TSA method was applied according to the manufacturer's protocol.

Specificity of the employed CB1 antibody was confirmed by running the positive (murine brain samples as well as human skin samples) and negative controls (omission of the primary antibody) reported before^[37,38]. In addition, both CB1 immunoreactivity and immunofluorescence intensity were significantly reduced within ORS of organ cultured human HF treated with CB1 siRNA compared to appropriate controls^[37,38].

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In addition, specificity of CB1 antibody was further documented by showing that CB1 antibodies from two different vendors (Santa Cruz and Cayman) showed similar immunostaining reactivity and by demonstrating only a very faint CB1-like signal in CB1 knockout mice, which contrasted with the strong signal obtained in WT mice (**Supplementary Fig S1**).

Double-immunostaining for CK15 and CB1 was performed by the combination of the TSA technique. Cryosections were incubated overnight at 4°C with a primary antibody against CK15 followed by biotinylated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) (1:200 in TNB, 45 min, RT). Sections were then incubated with streptavidin-conjugated HRP (1:100, 30 min, TSA kit) and finally incubated with fluorescein isothiocyanate (FITC) conjugated tyramide (1:50, TSA kit). After careful washing with TNT wash buffer (0.1 M Trizma hydrochloride, 0.15 M NaCl and 0.05% Tween 20), sections were incubated overnight with rabbit anti-human CB1 antibody (Santa Cruz) at 4°C followed by incubation with biotinylated goat antibody against rabbit IgG (Jackson ImmunoResearch Laboratories) (1:200 in TNB, 45 min, RT). After incubating with streptavidine-conjugated HRP (1:100, 30 min, TSA kit), sections were incubated with tetramer rhodamine conjugated tyramide (1:50, TSA kit).

To study the proliferation of the CK15+ cells, double-immunostaining of CK15 and Ki-67 was performed. After the staining for CK15 using a TSA kit (FITC) as described above, sections were incubated overnight at 4°C with a mouse anti-human Ki-67 antibody (DAKO) at 1:20 in PBS. Sections were then washed with PBS, followed by incubation with rhodamine conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) (1:200 in PBS, 45 min) at RT.

To evaluate the apoptosis of CK15+ cells, CK15 immunostaining and terminal dUTP nick-end labeling (TUNEL) was performed on the same sections. Briefly, after the immunostaining for CK15 using TSA kit (rhodamine) as described above, sections were incubated with a digoxigenin-deoxy-UTP (ApopTag fluorescein in situ apoptosis detection kit; Millipore Corp., Billerica, MA) in the presence of terminal deoxynucleotidyl transferase (60 min) at 37°C. After the incubation with stop/wash buffer (ApopTag kit) (10 min, RT) and the additional wash with PBS, TUNEL-positive cells were visualized by an antidigoxigenin fluorescein isothiocyanate-conjugated antibody (ApopTag kit) (30 min, at RT).

To evaluate the immunoreactivity of CB1 in *CB1*-siRNA-treated HF, CB1 immunohistochemistry was performed using cryosections as previously reported^[37,38].

For detecting CK19 in organ cultured human HF, indirect immunofluorescence using anti-human CK19 (Progen Biotechnik GmbH, Heidelberg, Germany) at 1:10 in PBS as a primary antibody and FITC conjugated goat anti-mouse IgG at 1:200 in PBS as a secondary antibody has been performed.

For detecting GFP immunoreactivity in *CK15* promoter-GFP transfected organ cultured human scalp HF, indirect immunofluorescence method was applied using mouse anti-GFP (Merck Millipore) at 1:50 in PBS as a primary antibody and rhodamine conjugated goat anti-mouse IgG at 1:200 in PBS as a secondary antibody has been applied.

The immunoreactivity of CB1, CK15, CK19, GFP, p-Ekt 1/2 or p-Akt in defined reference areas was assessed by quantitative immunohistomorphometry^[37,38,45,68,69] using the ImageJ software (National Institutes of Health, Bethesda, MD).

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For detection of CD34 in mice skin samples, we performed CD34 immunofluorescence by using cryosections of murine wild type skin samples and CB1 knockout mice^[38]. Murine skin samples were air-dried and incubated with 1% Triton-X100/PBS for 10 min at RT and then fixed with ice-cold acetone for 2 min. After the wash with PBS (5 min, 3 times), slides were blocked with 5% goat normal serum in PBS for 1 hr. Afterwards, the samples were incubated in primary antibody against CD34 (Hycult biotech Inc, Plymouth Meeting, PA) 1:40 at 4°C for overnight. After the wash with PBS, samples were incubated with FITC conjugated goat anti-rat antibody as secondary antibody at RT for 45 min (at 1:200). After the wash with PBS, sections were counter-stained with DAPI and slides were mounted.

High magnification images of CB1 and CK15 were taken by laser scanning confocal microscopy (Fluoview 300, Olympus Tokyo, Japan) running Fluoview 2.1 software (Olympus).

Quantitative immunohistomorphometry, cell counting

When performing quantitative immunohistomorphometry, after immunolabeling on HF sections or cell cultures, the number of immune-positive cells per visual field was counted by either the Image J program or by visual counting of the cells. In the latter case, at least 2 individuals of the team independently analyzed the same sections/visual fields, and the resulted data were verified by the KS or NZ. More details of the methods can be found in our numerous previous publications^[28-34,37-41,45-47].

Transfection of isolated HFs

For transient transfection of HF_s, 4 µg of the *K15* promoter GFP expression construct were used to generate DNA-cationic lipid reagent complexes according to the manufacturer's protocol (Invitrogen-Gibco) as described previously^[39].

Isolation, selection and culture of human K15-GFP⁺ cells

Positively transfected HF_s were processed as described previously^[39]. To test the influence of ACEA (Catalog number <Cat-Nr>: A9719, Sigma-Aldrich, Taufkirchen, Germany) and/or AM251 (Cat-Nr: A6226, Sigma-Aldrich) were added to the cell culture with a donor age adjusted physiological concentration (mean donor age: 50 years; concentrations used for cell culture: ACEA at 1 µM and AM251 at 1 µM).

Cell isolation and culture

For human ORS keratinocyte isolation, scalp skin samples were dissected into approximately 0.5 cm² pieces, which were washed with PBS (37°C) to remove cell debris. Skin pieces were incubated overnight in William's E Medium, supplemented with 0.01% weight/volume reconstituted Dispase at 4°C. After washing with PBS, the core of, full-length HF-epithelium was carefully plucked out by gripping with sterile forceps. The HF-epithelium was collected in a 60-mm bacteriological dish containing 5 mL PBS and the remaining distal keratinized hair shaft was discarded. The bulbar part and the distal fifth of the total follicular length (corresponding to the infundibular part) were removed. The HF_s were covered by a minimal volume of trypsin-EDTA solution (0.1 %) and incubated at 37°C until detachment of the outermost ORS cells became visible. Further release of ORS cells still adhering to the HF_s was achieved by vigorous pipetting. The suspension was then diluted with K-SF medium (Gibco) (final volume in mL corresponds to the number of HF_s prepared). The cells were incubated at 37°C (5% CO₂). Spreading of ORS cells occurs after 2-4 days. At this time, the ORS cells

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display the typical epithelioid morphology, with a well-discernible nucleus and a large cytoplasm. Colonies of ORS cells developed over time. The first medium change was not done before culture day 7. Thereafter, medium was changed three times/week before special experimental procedures as described above.

Quantitative Real-Time PCR (Q-PCR)

Briefly, on a Stratagene Mx3005p sequence detection system (Agilent Technologies Inc., Santa Clara, CA, USA) by using 5' nuclease assay. Total RNA was isolated using TRIzol reagent (Life Technologies Corporation, Foster city/Carlsbad, CA, USA) according to manufacturer's protocol and digested with recombinant RNase-free DNase-1 (Life Technologies) according to the manufacturer's protocol. After isolation, 1 µg of total RNA were reverse-transcribed into cDNA by using High Capacity cDNA kit (Life Technologies) following the manufacturer's protocol. PCR amplification was performed by using specific TaqMan primers and probes (Life Technologies, assay IDs: Hs00271662_s1 for human G protein-coupled receptor 55 (GPR55). The expression of β-actin was determined as internal housekeeping gene control (Assay ID: Hs999999903). The amount of the above mentioned transcript was normalized to the control gene using the ΔCT method. As positive controls, human prostate cancer-derive LNCaP cells were used which reportedly express GPR55^[10].

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Fig S1. Controls of immunolabeling for CB1

A. Representative images of CB1 immunohistochemistry with murine skin samples using different antibodies against CB1 (Santa Cruz and Cayman). Yellow arrows indicate CB1 positive immunoreactivity.

B. Representative image of CB1 immunofluorescence with human scalp skin sample. Yellow arrows indicate CB1 positive immunoreactivity.

C. Representative images of CB1 immunofluorescence with wild type and CB1 knockout mouse skin samples. Yellow arrows indicate CB1 positive immunoreactivity.

Supplementary Fig S2. CB1 stimulation induces human HF eSCs proliferation but does not induce apoptosis

A. Number of CK15+ eSCs within the bulge area of organ cultured human full-length HF with different concentrations of ACEA (300 nM to 50 μ M, 24 hrs).

B. Representative images of cells expressing CK15 (red) and TUNEL (green) within the HF-bulge area of vehicle, ACEA, AM251 or ACEA+AM251 treated organ cultured full-length human HF for 24 hrs. Scale bar: 50 μ m. Arrows indicate TUNEL+ cell. Dotted lines represent basal membrane of the HF.

C. Percentage of cells co-expressing CK15 and TUNEL within the HF-bulge area of vehicle, ACEA (30 μ M), AM251 (1 μ M) or ACEA+AM251 treated 24 hrs organ cultured full-length human HF.

D. Representative images of cells expressing CK15 (red) and TUNEL (green) within organ cultured human HF treated with either vehicle or ACE for 24 hrs. Scale bar: 100 μ m. ORS: outer root sheath.

E. Percentage of TUNEL+ (apoptotic) cells of CK15+ ORS cells (per HF) within organ cultured human HF's treated with either vehicle or ACEA (30 μ M, 24 hrs).

F. Representative images of cleaved caspase-3 positive (apoptotic) cultured CK15-promoter-GFP cells treated with either vehicle or ACEA (1 μ M, 24 hrs).

G. Percentage of cleaved caspase-3 positive (apoptotic) cultured CK15-promoter-GFP cells (per visual field) treated with either vehicle or ACEA (1 μ M, 24 hrs).

H. Quantitative analysis of CK15 promoter-GFP fluorescence intensity of organ cultured human HF's treated with either vehicle or AM251 (1 μ M, 24 hrs). GFP-fluorescence intensity per HF was measured as arbitrary unit and compared.

Data are means $\pm$ SEM. * p <0.05, ** p <0.01. N.S.=not significant.

Supplementary Fig S3. GPR55 is not involved on human HF SCs

A. Representative images of CK15+ ORS cells within the organ cultured human full-length HF's treated with vehicle, LPI (5 μ M), T1117 (1 μ M) or AM251 (1 μ M) for 6 days.

B. Number of CK15+ cells within organ cultured human HF's treated with vehicle, LPI, T1117 or AM251.

C. CK15 immunofluorescence intensity of bulge cells of organ cultured human HF's treated with vehicle, LPI, T1117 or AM251.

D. Relative GPR55 gene expression within the isthmus part of isolated human full-length HF's (from 3 individuals, LNCaP cells are regarded as control). GPR55 expression in the bulge and isthmus area was assessed by quantitative RT-PCR, using the mRNA extracted from bulge and isthmus region by microamputation with a scalpel, in relation to the GRP55-expressing human prostate adenocarcinoma cell line, LNCaP, as a positive control [10].

Data are means $\pm$ SEM. ** p <0.01.

Supplementary Fig S4. CB1 gene silencing in organ cultured human HF's

A. RT-PCR for CB1 gene of organ cultured human HF's treated with TFE (transfection reagent treated), SCR (scrambled siRNA treated) or CB1-siRNA. GAPDH was used for internal control gene.

B. Representative images of CB1 immunohistochemistry with TFE, SCR and CB1 siRNA treated human HF's (24 hrs). Arrows denote positive CB1 immunoreactivity within the ORS of HF's. **Reduced CB1 protein expression was not only seen in K15+ cells within the ORS, but also in the bulge (not shown).**

C. Quantitative determination of CB1 immunoreactivity of organ cultured human HF's (middle to lower part of HF's) treated with TFE, SCR or CB1-siRNA.

D. Number of CK15/Ki-67+ cells per visual field of organ cultured human HF's (middle to lower part of HF's) treated with TFE, SCR or CB1-siRNA.

TFE: transfection reagent treated, SCR: scrambled siRNA treated. Data are means±SEM.

*p<0.05, **p<0.01. N.S.=not significant.

Supplementary Fig S5. CB1 upregulates the expressions of p-Erk 1/2 and p-Akt on isolated human HF bulge cells

A. Representative images of p-Erk 1/2 immunofluorescence on primary cultured human HF ORS cells and Bulge cells treated with either vehicle (Control) or ACEA (1 µM, 24 hrs).

B. Representative images of p-Akt immunofluorescence on primary cultured cells human HF ORS cells and Bulge cells treated with either vehicle (Control) or ACEA (1 µM, 24 hrs).

Supplementary Fig S6. A schematic representation of the effects of CB1 stimulation on various types of epithelial cells within the human HF

See text for details.

Supplementary Fig S7. Down-regulation of CB1 immunoreactivity in the bulge region of

HF s from patients with LPP

Representative images of CB1 immunohistochemistry (red) of the HF bulge region of either non-LPP individual (A) or LPP patient (F). The insertion of arrector pili muscles (APM, indicated by dotted lines) which demarcates HF bulge area is indicated by black rectangle. Scale bar: 50 μ m.

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Supplementary Figure S1

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Supplementary Figure S3

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A: p-Erk 1/2

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Supplementary Figure S6

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