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Differential effects of caffeine on hair shaft elongation, matrix and outer root sheath keratinocyte proliferation, and TGF-β2-/IGF-1-mediated regulation of hair cycle in male and female human hair follicles *in vitro*

Running head: Caffeine-mediated regulation of human hair follicles (53 characters including space)

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Author's contribution: T.W.F. designed all experiments, performed biopsies, data analysis, interpretation of data and wrote and revised the manuscript. W.F. performed face-lift biopsies. E.H.-L. and T.B. performed all experiments and data evaluation with outer root sheath keratinocytes. T.B.

and D.Z. revised and approved the final version of the manuscript. R.P. designed experiments, performed interpretation of data and revised and approved the final version of the manuscript.

What´s already known about this topic?

- Caffeine stimulates hair growth in androgen-sensitive testosterone-suppressed male human hair follicles (HFs) *in vitro*.

What does the study add?

- First evidence for caffeine-stimulated growth of female human HFs; more caffeine-sensitive than male HFs.
- Proliferation increase by caffeine in human HF matrix keratinocytes *in situ* and HF-derived outer root sheath keratinocytes (ORSK).
- Down-regulation of catagen-inducer TGF-β2 and up-regulation of anagen-promoting factor IGF-1 in human HFs *in situ* and in ORSK.

Abstract

Background Caffeine reportedly counteracts the suppression of hair shaft production by testosterone in organ-cultured male human hair follicles (HFs).

Objectives We aimed at investigating the impact of caffeine a) on additional key hair growth parameters, b) on major hair growth-regulatory growth factors and c) on male versus female HFs in the presence of testosterone.

Methods Microdissected male and female human scalp HFs were treated in serum-free organ culture for 120 h with testosterone alone (0,5 µg/ml) or in combination with caffeine (0.005–0.0005%), and effects on hair shaft elongation, HF cycling (i.e. anagen–catagen transition), hair matrix keratinocyte proliferation and expression of a key catagen inducer, transforming growth factor β2 (TGF-β2), and anagen-prolonging insulin-like growth factor 1 (IGF-1) were evaluated by quantitative (immuno-) histomorphometry. Caffeine effects were further investigated in human outer root sheath keratinocytes (ORSK).

Results Caffeine enhanced hair shaft elongation, prolonged anagen duration and stimulated hair matrix keratinocyte proliferation. Female HFs showed higher sensitivity to caffeine compared to male HFs. Caffeine counteracted testosterone-enhanced TGF-β2 protein expression in male HFs. In female HFs, testosterone failed to induce TGF-β2 expression, while caffeine reduced it. In male and female HFs, caffeine enhanced IGF-1 protein expression. In ORSK, caffeine stimulated cell proliferation, inhibited apoptosis/necrosis, up-regulated IGF-1 gene expression and protein secretion, while TGF-β2 protein secretion was down-regulated.

Conclusions This study reveals new growth-promoting effects of caffeine on human hair follicles of both genders at different (molecular, cellular and organ) levels.

Keywords: apoptosis, Ki67, gene expression, hair organ culture, androgenetic alopecia

Introduction

Caffeine is a well-known stimulant contained in coffee, whose effects mainly are mediated through inhibition of phosphodiesterase. This leads to increased intracellular adenylate cyclase activity and enhanced cyclic 3´,5´-adenosine mono-phosphate (cAMP) levels,

therefore providing higher energy levels to promote increased metabolic activity and cell proliferation[1]. Little is as yet known about the effects of caffeine on human hair follicle (HF) growth, but it has been hypothesiszed that caffeine may counteract HF miniaturization in patiens with androgenetic alopecia (AGA)[2]. Although approximately 50% of men at the age of 50 years suffer from AGA[3-5], there are still only two FDA-approved drugs available for AGA management treatment, namely finasterid and minoxidil[3]. Therefore, development of additional effective treatment strategies seems to be a reasonable need.

Dihydrotestostone (DHT) is the testosterone-derived, AGA-promoting androgen that causes a continuous shortening of hair growth cycles (anagen) in favor of longer resting phases (telogen), along with HF miniaturization of genetically predetermined areas in the fronto-temporal and vertex region of men affected with AGA[6-9]. Since testosterone inhibits adenylate cyclase activity in human HFs[6], we hypothesized that caffeine may counteract the DHT-induced growth inhibition of human androgen-sensitive scalp HFs.

So far, caffeine has been shown to reverse the inhibitory effect of testosterone on keratinocyte proliferation in a male skin organ culture model (MSOCM)[7] and to normalize the testosterone-induced inhibition of hair shaft elongation and to stimulate hair matrix keratinocyte proliferation in organ-cultured human HFs[2]. In the current study, we have extended this line of research by examining whether caffeine also impacts on human HF cycling, hair matrix apoptosis and the expression of two major antagonistic hair growth-regulatory growth factors, i.e., transforming growth factor β2 (TGF-β2) and insulin-like growth factor 1 (IGF-1) as well as its effects on isolated human HF-derived outer root sheath keratinocytes (ORSK). In addition, we have compared the effects of caffeine on male versus female HFs in the presence of testosterone.

Materials and methods

Human hair follicle culture

Whole human HFs (anagen VI) were microdissected from biopsies from women undergoing face-lift surgery, and male HFs were obtained from electively taken biopsies (0.5×1.5 cm) from the balding vertex region in the border area of the dense to the shedding area (androgen-sensitive) of men affected with AGA in moderate stage (Norwood-Hamilton stage III vertex and IV[5]). The study was approved by the Ethics Committee of the University of Lübeck (reference 06-109) and written informed consent was obtained from the patients in accordance with the "Helsinki Declaration".

HF extraction and cultivation in the established HF organ culture model was performed as previously described[2,8,9]. After 24 h recovery time in serum-free, supplemented William's E medium, experiments were started by replacing normal medium with fresh medium containing a) testosterone alone or b) testosterone in combination with caffeine or c) normal growth medium as control. Control and treatment media were changed every other day and total culture time was 120 h. Testosterone was used in the concentration of 0.5 µg/ml and caffeine at concentrations of 0.005% and 0.001% in order to permit comparison with earlier work[2]. After the first experiments with female HFs, the concentration of caffeine was lowered to 0.0005% due to observed higher responsiveness of female HFs to this concentration. Hair shaft elongation was measured every 24 h by using a scaled microscopic eyepiece. After 120 h, follicles were frozen at –80° C, and 6 µm cryosections were processed for immunohistochemistry and immuno-fluorescence as described previously[8,10].

Hair follicle immunohistochemistry and immunofluorescence microscopy

HFs were stained with hematoxylin-eosin for assessment of HF morphology and hair cycle phase (anagen, catagen)[10]. The hair cycle score was calculated as described earlier[11].

Proliferation and apoptosis were assessed by Ki67/TUNEL double-immunostaining as reported before[12].

To detect the *in situ* protein expression of the key-catagen inductor TGF-β2 and one major growth factor IGF-1, immunofluorescence with tyramide signal amplification (TSA) was performed using the polyclonal rabbit anti-TGF-β2 IgG (1:4000) and the polyclonal goat anti-IGF-1 IgG (1:500) antibody, respectively (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) (for staining protocol details see supplementary file 1).

Proliferation and apoptosis/necrosis in human HF-derived outer root sheath keratinocytes

Human plucked eyebrow HFs of several male healthy donors were obtained after written informed consent adhering to Helsinki guidelines and after Institutional Research Ethics Committee permission. Outer root sheath keratinocytes (ORSK) were then isolated and cultured under optimized conditions as previously described[13-15] (for detailed description see also supplementary file 1). ORSK were then treated with caffeine (0.00001%, 0.0001%, 0.001%; Azelis, Antwerpen, Belgium) and positive stimulatory reference agents such as IGF-1 (100 ng/ml) and minoxidil (0.1 µM)[8] as well as the negative regulatory reference substance tretinoin (1 µM) (all from Sigma-Aldrich, St. Louis, MO, USA) for 24, 48, 72 and 96 h[15]. Cellular proliferation under these different treatment conditions was quantified by using CyQUANT Cell Proliferation Assay Kit (Invitrogen, Paisley, UK) and additionally assessed by immunocytochemical labeling of the proliferation nuclear marker Ki67 as described previously[13,16] (see also supplementary file 1).

Caffeine was further investigated for protective effects against apoptosis- and necrosis-inducing agents such as catagen inducers which, *in vivo*, may induce clinically relevant hair loss. For this purpose, two strong catagen inducers, TGF-β2 (50 ng/ml) (Sigma-Aldrich)[17] and anandamide (AEA; 30 µM) (Cayman, Ann Arbor, MI, USA)[18] were applied for 24 or 48 h in ORSK culture. In parallel,

three concentrations of caffeine or catagen-inhibitory agents, i.e. IGF-1 (100 ng/ml) and keratinocyte growth factor (KGF; 20 ng/ml) (both from Sigma-Aldrich)[19,20] were applied for 1 h to induce anti-apoptotic/-necrotic effects. Mitochondrial membrane potential reduction as early marker of apoptosis was determined using a MitoProbe DiIC1 (5) Assay Kit (Invitrogen) following previously optimized protocols[16,21] (see supplementary file 1). Necrotic cell death was determined by Sytox Green nucleic acid staining (Invitrogen) as described previously[16,21] (see supplementary file 1).

TGF-β2 and IGF-1 gene expression (Q-PCR) and protein secretion (ELISA) in ORSK

To determine the quantitative gene expressions of TGF-β2 and IGF-1 from cell lysates of ORSK after 120 h stimulation with caffeine (0.00001%, 0.0001%, 0.001%) and positive stimulatory reference agents (IGF-1: 100 ng/ml; minoxidil: 0.1 µM) as well as the negative regulatory reference substance tretinoin (1 µM), Q-PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) using the 5' nuclease assay as detailed in our previous reports[16,22,23] (see also supplementary file 1). The supernatants of ORSK treated with the above mentioned substances were taken at 24, 72 and 120 h for quantitative determination of TGF-β2 and IGF-1 protein levels using specific ELISA kits (R&D Systems, Minneapolis, MN, USA) following the protocol of the manufacturer.

Statistical analysis

Data were expressed as means ±SEM of pooled data from independent experiments for each study parameter. Values were normalized and expressed as percentage of control. All data were analyzed with GraphPad Prism 5.02 software (La Jolla, CA, USA) using Student's *t*-test for independent samples. A *p*-value of <0.05 was considered statistically significant.

Results

Male and female hair shaft elongation is differently stimulated by caffeine

Hair shaft elongation of male HFs revealed the same concentrations of 0.005% and 0.001% caffeine to be stimulatory effective as shown in previous experiments[2] (data not shown). For the first female HFs experiments, the same concentrations failed to promote hair shaft elongation. Therefore, the caffeine concentration was reduced to 0.0005% in the following experiments. This significantly counteracted the testosterone-induced inhibition of hair shaft elongation at 120 h (p<0.05) **(Fig. 1)**.

Hair matrix keratinocyte proliferation is enhanced by caffeine

Caffeine (0.001%) significantly increased the number of Ki67-positive hair matrix keratinocytes compared to testosterone- or vehicle-treated control male HFs (*p<0.05; **p<0.01) **(Fig. 2A,B).** In female HFs, vehicle-treated control HFs revealed 39% Ki67-positive matrix keratinocytes, the testosterone-treated hair follicles showed only 19% (p<0.05) and caffeine (0.0005%) increased the percentage of Ki67-positive matrix keratinocytes up to 30% **(Fig. 2C,D)**.

Caffeine stimulates cellular proliferation in ORSK

Over 96 h, a significant pro-proliferative effect of caffeine was observed in ORSK by CyQUANT assay. This effect was most prominent at 24 h and exerted by all applied caffeine concentrations (0.00001, 0.0001, 0.001%) reaching up to 160% compared to 100% control (p<0.05) **(Fig. 3A)**. The positive control IGF-1 (100 ng/ml) lead also to a significant, but less expressed increase of proliferation compared to control (ca. 140%; p<0.05). Minoxidil, the second positive control, lead to comparable growth enhancement (ca. 140%; p<0.05), but again, less expressed than caffeine. The growth-inhibitory negative standard tretinoin (1 µM) did not lead to any change of proliferation. At 48 h,

caffeine at 0.00001% and 0.0001% lead to stimulation of proliferation (ca. 120 and 130%, respectively), however, only 0.0001% reached significance (p<0.05) **(Fig. 3B)**. IGF-1 significantly stimulated proliferation as well (ca. 130%; p<0.05), but minoxidil not. At 72 h, only IGF-1 showed a significant pro-proliferative effect (p<0.05) **(Fig. 3C)**, while at 96 h there was no effect of any substance **(Fig. 3D).**

Immunofluorescence-cytochemical labeling of proliferating Ki67-positive ORSK confirmed the data assessed by CyQUANT. Namely, incubation of ORS keratinocytes with caffeine for 24 h leads to a significantly higher, caffeine dose-dependent percentage of Ki67-positive cells as compared to vehicle-treated control. **(Fig. 4A,B)**.

Caffeine inhibits apoptosis and necrosis in ORSK

In TGF-β2-treated ORSK, there was a biologically relevant and significant induction of apoptosis, indicated by decreased DilC1 (5) signal, and necrosis, as shown by Sytox Green increase, as well (p<0.05). This was significantly counteracted after 48 h incubation with caffeine at the concentration of 0.0001% (only necrosis) and 0.001% as well as by IGF-1 and KGF (p<0.05) **(Fig. 5A, right columns).** In anandamide-treated ORSK, similar protective effects of caffeine, IGF-1 and KGF were observed; however significant suppression of apoptosis and necrosis by caffeine were exclusively observed at the concentration of 0.001% **(Fig. 5B, right columns)**.

Anagen-stage suppression by testosterone is counteracted by caffeine in male and partly in female hair follicles

As expected, testosterone reduced the percentage of male HFs in anagen VI to 39% compared to 56% in controls after 120 h of HF organ culture. Co-incubation with caffeine (0.001%) strikingly

raised the percentage of anagen HF percentage up to 70% **(Fig. 6A)**. A similar, though less pronounced effect of caffeine was also seen in female HFs at the concentrations 0.001% and 0.0005%, respectively **(Fig. 6B)**. These findings were independently corroborated for male HFs by analysing the hair cycle score **(Fig. 6C)**. In female HFs, the hair cycle score showed higher values in testosterone treated HFs, while HFs co-cultivated with caffeine 0.0005% showed no decrease **(Fig. 6D)**.

Caffeine differentially modulates intrafollicular TGF-β2 and IGF-1 protein expression

TGF-β2-immunoreactivity (IR) *in situ* was not only detected in the outer root sheath (ORS) of the HF[18], but also in the Henle-layer of the inner root sheath (IRS). In male human HFs, testosterone significantly up-regulated intrafollicular protein expression of the catagen-promoting growth factor, TGF-β2 ($p<0.05$) **(Fig. 7A,B)**. Caffeine 0.001% significantly reduced the TGF-β2 expression down to normal (control) levels (98%; $p<0.01$). In female HFs, testosterone did not significantly up-regulate TGF-β2 protein expression, while co-culture with caffeine (0.0005%) significantly reduced IGF-1 IR ($p<0.01$) **(Fig. 7C,D).**

IGF-1 IR was detected in both the ORS and IRS **(Fig. 8A).** Protein expression of IGF-1 in male HFs was significantly reduced by testosterone ($p<0.05$), whereas co-culture with caffeine (0.001%) significantly up-regulated IGF-1 IR compared to testosterone- ($p<0.001$) or vehicle-treated control HFs ($p<0.01$) **(Fig. 8B).** In female HFs, IGF-1 was not consistently regulated by testosterone (data not shown), but in HFs treated with caffeine alone, there was a stimulation of IGF-1 expression which was significant for the concentrations of 0.005% and 0.001% ($p<0.001$ and $p<0.01$, respectively) **(Fig. 8C,D)**. At the concentration 0.0005%, caffeine did not modulate IGF-1 expression (data not shown).

TGF-β2 and IGF-1 gene expression and protein secretion in male ORSK is partly modulated by caffeine

TGF-β2 mRNA transcripts were detected in ORSK from all 6 male donors, however, no gene regulation by caffeine in any concentration was observed (data not shown).

Specific mRNA transcripts of IGF-1 were below the detection limit in ORSK from three out of six male donors (data not shown), whereas in the other three donors, IGF-1 was detected (albeit at very low levels) **(Fig. 9)**. Interestingly, caffeine (applied for 120 h) exerted mixed effects: in two donors, caffeine (0.001%) significantly up-regulated IGF-1 gene expression ($p<0.05$) **(Fig. 9A,C)**, while in the third donor, IGF-1 gene expression was not regulated by caffeine at any concentration **(Fig. 9B)**.

Analysis of cellular TGF-β2 and IGF-1 secretion assessed by ELISA from cell supernatants detected both substances. Intriguingly, caffeine did not modify levels of TGF-β2 up to 72 h **(Fig. 10A,B).** Yet, in one out of three donors at 120 h incubation time, the concentration of 0.001% significantly suppressed TGF-β2 secretion compared to control ($p<0.05$) **(Fig. 10C)**. Corresponding to known effects of tretinoin, this standard catagen inducer significantly up-regulated TGF-β2 secretion at 72 and 120 h in two donors ($p<0.05$) **(Fig. 10B,C)**.

Regarding IGF-1 detection, caffeine at the concentration of 0.001% (identical in all 3 male donors) lead to increased IGF-1 secretion at all time-points investigated (24, 72, 120 h) **(Fig. 10D,E,F)**. IGF-1 itself, added externally to the HF culture as an internal control (100 ng/ml), could also be detected at all investigated time points (24, 72, 120 h) at high levels and significantly different from the control ($p<0.05$).

Discussion

The current study reveals potent, differential hair growth-stimulatory effects on scalp HFs from both genders that substantially extend beyond the previous literature[11,12] and examines for the first time also caffeine effects on female HFs. Caffeine enhances hair shaft elongation, prolongs anagen duration and stimulates hair matrix keratinocyte proliferation, with female HFs showing a higher sensitivity to caffeine than male HFs. Caffeine counteracts testosterone-enhanced TGF-β2 protein expression in male HFs and reduces TGF-β2 expression in female HFs, while it enhances intrafollicular IGF-1 protein expression in both genders. That these caffeine effects can essentially also be seen in isolated ORSK further supports the overall concept that caffeine may be a genuine hair growth-stimulatory agent.

The current data are well in line with earlier reports that show caffeine counteracting the growth-suppressive effect of testosterone on epidermal keratinocyte proliferation[7] and testosterone-induced human skin barrier function *in vivo*[24]. That the most effective caffeine concentration in male HFs was 0.001%, independently confirms our previously published results[2], while the increased caffeine sensitivity of female scalp HFs is revealed here for the first time. It remains rather speculative whether or not this is due to gender-dependent differences in putative caffeine target molecules[25].

The influence of caffeine on two key hair growth-regulatory factors, TGF-β2 (catagen promotion)[11,17] and IGF-1 (anagen maintenance)[9,17,20,26,27] demonstrated here for the first time, corresponds well to related caffeine effects described in other systems: Caffeine exerts protective effects in a rat cirrhosis model by reducing TGF-β2 serum levels[28,29] and inhibits TGF-β-stimulated connective tissue growth factor expression in hepatocytes[30]. Caffeine also reduces oxidized glutathione and TGF-β1 protein expression during experimentally induced rat liver injury[31].

In our study, caffeine stimulated also the *in situ* protein expression, gene expression and protein secretion of IGF-1 in human HFs and/or ORSK. This is in line with the observation in pancreatectomized diabetic rats that showed long-term caffeine supplementation to potentiate an insulin/IGF-1 signaling cascade leading to an alleviation of insulin resistance by improved glucose metabolism[32]. Given that serum-free human HF organ culture requires insulin supplementation of the medium[9], increased cellular insulin sensitivity might also underlie the caffeine-induced stimulation of IGF-1 expression, along with hair growth stimulation, observed in the current study. IGF-1 stimulation by caffeine may even counteract human HF aging as represented by reduced hair diameter and density[33,34], since relative IGF-1 deficiency may occur with increasing age as a result of reduced systemic IGF-1 levels[35].

Here we show that caffeine increases proliferation and reduces apoptosis of human ORSK. Similar caffeine effects have been described in murine vascular smooth muscle cells revealing concentration-dependent stimulatory effects on cell proliferation, protection against apoptosis and reduction of reactive oxygen species[36]. Since caffeine also reduces reactive nitrogen species and serum levels of the oxidatively damaged DNA adduct 8-oxo-guanine in fat-fed ApoE(-/-) mice *in vivo*[36], and caffeine-containing extracts from roasted coffee protect against lipid peroxidation and protein oxidation by directly scavenging free radicals and reducing ferrous ions[37], caffeine may well exert complex antioxidative protective properties also in human HFs. Finally, mitochondrial function is also increased by caffeine[38] which may further protect against HF aging and generally support HF growth.

Given that dermal papilla fibroblasts from men with AGA display a higher sensitivity to oxidative stress than those from non-balding men[39], caffeine might counteract AGA progression in men on several different levels, not only by enhancing intrafollicular cAMP and IGF-1 levels and reducing TGF-β2 expression, but also by reducing oxidative stress in genetically predisposed balding HFs. Our

study suggests that these putative AGA-protective effects of caffeine may also be clinically relevant in women.

In summary, the *in vitro* data in ORSK and human whole HFs presented are just opening a new chapter of the complex and obviously pluripotent role of caffeine in human hair biology. They strongly support the concept that caffeine is a credible hair growth-promoting and HF-protective substance that deserves further *in vitro* as well as *in vivo* exploration.

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References

1 Green H. Cyclic AMP in relation to proliferation of the epidermal cell: a new view. *Cell* 1978; **15**: 801-11.

2 Fischer TW, Hipler UC, Elsner P. Effect of Caffeine and Testosterone on the Proliferation of Human Hair Follicles in vitro. *Int J Dermatol* 2007; **46**: 27-35.

3 Fischer TW. Alopecia - Diagnostic and Therapeutic Management. *Akt Dermatol* 2008; **34**: 209-25.

4 Hamilton JB. Patterned loss of hair in man; types and incidence. *Ann N Y Acad Sci* 1951; **53**: 708-28.

5 Norwood OT. Male-pattern baldness. Classification and incidence. *South Med J* 1975; **68**: 1359-70.

6 Adachi K, Kano M. Adenyl cyclase in human hair follicles: its inhibition by dihydrotestosterone. *Biochem Biophys Res Commun* 1970; **41**: 884-90.

7 Tsianakas A, Hüsing B, Moll I *et al.* An ex vivo model of male skin - caffeine conteracts testosterone effects. *Arch Dermatol Res* 2005; **296**: 450.

8 Philpott MP, Green MR, Kealey T. Human hair growth in vitro. *J Cell Sci* 1990; **97 (Pt 3)**: 463-71.

9 Philpott MP, Sanders D, Westgate GE *et al.* Human hair growth in vitro: a model for the study of hair follicle biology. *J Dermatol Sci* 1994; **7 Suppl**: S55-72.

10 Kloepper JE, Sugawara K, Al-Nuaimi Y *et al.* Methods in hair research: how to objectively distinguish between anagen and catagen in human hair follicle organ culture. *Exp Dermatol* 2010; **19**: 305-12.

11 Peters EM, Hansen MG, Overall RW *et al.* Control of human hair growth by neurotrophins: brain-derived neurotrophic factor inhibits hair shaft elongation, induces catagen, and stimulates follicular transforming growth factor beta2 expression. *J Invest Dermatol* 2005; **124**: 675-85.

12 Foitzik K, Krause K, Conrad F *et al.* Human scalp hair follicles are both a target and a source of prolactin, which serves as an autocrine and/or paracrine promoter of apoptosis-driven hair follicle regression. *Am J Pathol* 2006; **168**: 748-56.

13 Bodo E, Biro T, Telek A *et al.* A hot new twist to hair biology: involvement of vanilloid receptor-1 (VR1/TRPV1) signaling in human hair growth control. *Am J Pathol* 2005; **166**: 985-98.

14 Havlickova B, Biro T, Mescalchin A *et al.* Towards optimization of an organotypic assay system that imitates human hair follicle-like epithelial-mesenchymal interactions. *Br J Dermatol* 2004; **151**: 753-65.

15 Havlickova B, Biro T, Mescalchin A *et al.* A human folliculoid microsphere assay for exploring epithelial- mesenchymal interactions in the human hair follicle. *J Invest Dermatol* 2009; **129**: 972-83.

16 Borbiro I, Lisztes E, Toth BI *et al.* Activation of transient receptor potential vanilloid-3 inhibits human hair growth. *J Invest Dermatol* 2011; **131**: 1605-14.

17 Soma T, Tsuji Y, Hibino T. Involvement of transforming growth factor-beta2 in catagen induction during the human hair cycle. *J Invest Dermatol* 2002; **118**: 993-7.

18 Telek A, Biro T, Bodo E *et al.* Inhibition of human hair follicle growth by endo- and exocannabinoids. *FASEB J* 2007; **21**: 3534-41.

19 Jang JH. Stimulation of human hair growth by the recombinant human keratinocyte growth factor-2 (KGF-2). *Biotechnol Lett* 2005; **27**: 749-52.

20 Rudmann SM, Philpott MP, Thomas GA *et al.* The role of IGF-1 in human skin and its appendages: morphogen as well as mitogen? *J Invest Dermatol* 1997; **109**: 770-7.

21 Toth BI, Dobrosi N, Dajnoki A *et al.* 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* 2011; **131**: 1095-104.

22 Dobrosi N, Toth BI, Nagy G *et al.* Endocannabinoids enhance lipid synthesis and apoptosis of human sebocytes via cannabinoid receptor-2-mediated signaling. *FASEB J* 2008; **22**: 3685-95.

23 Toth BI, Geczy T, Griger Z *et al.* Transient receptor potential vanilloid-1 signaling as a regulator of human sebocyte biology. *J Invest Dermatol* 2009; **129**: 329-39.

24 Brandner JM, Behne MJ, Huesing B *et al.* Caffeine improves barrier function in male skin. *Int J Cosmet Sci* 2006; **28**: 343-7.

25 Rubtsov AM, Murphy AJ. Caffeine interaction with the Ca-release channels of heavy sarcoplasmic reticulum. Evidence that 170 kD Ca-binding protein is a caffeine receptor of the Ca-channels. *Biochem Biophys Res Commun* 1988; **154**: 462-8.

26 Hibino T, Nishiyama T. Role of TGF-beta2 in the human hair cycle. *J Dermatol Sci* 2004; **35**: 9-18.

27 Su HY, Hickford JG, Bickerstaffe R *et al.* Insulin-like growth factor 1 and hair growth. *Dermatol Online J* 1999; **5**: 1.

28 Arauz J, Zarco N, Segovia J *et al.* Caffeine prevents experimental liver fibrosis by blocking the expression of TGF-beta. *Eur J Gastroenterol Hepatol* 2013; **Epub ahead of print**.

29 Arauz J, Moreno MG, Cortes-Reynosa P *et al.* Coffee attenuates fibrosis by decreasing the expression of TGF-beta and CTGF in a murine model of liver damage. *J Appl Toxicol* 2013; **33**: 970-9.

30 Gressner OA, Lahme B, Rehbein K *et al.* Pharmacological application of caffeine inhibits TGF-beta-stimulated connective tissue growth factor expression in hepatocytes via PPARgamma and SMAD2/3-dependent pathways. *J Hepatol* 2008; **49**: 758-67.

31 Furtado KS, Prado MG, Aguiar ESMA *et al.* Coffee and caffeine protect against liver injury induced by thioacetamide in male Wistar rats. *Basic Clin Pharmacol Toxicol* 2012; **111**: 339-47.

32 Park S, Jang JS, Hong SM. Long-term consumption of caffeine improves glucose homeostasis by enhancing insulinotropic action through islet insulin/insulin-like growth factor 1 signaling in diabetic rats. *Metabolism* 2007; **56**: 599-607,.

33 Ellis RA. Aging of the human scalp. In: *The Biology of Hair Growth* (Ellis RA, ed), Vol. 1. New York: Academic Press. 1958.

34 Barman JM, Astore I, Pecoraro V. The Normal Trichogram of the Adult. *J Invest Dermatol* 1965; **44**: 233-6.

35 Zouboulis CC, Makrantonaki E. Hormonal therapy of intrinsic aging. *Rejuvenation Res* 2012; **15**: 302-12.

36 Mercer JR, Gray K, Figg N *et al.* The methyl xanthine caffeine inhibits DNA damage signaling and reactive species and reduces atherosclerosis in ApoE(-/-) mice. *Arterioscler Thromb Vasc Biol* 2012; **32**: 2461-7.

37 Yen WJ, Wang BS, Chang LW *et al.* Antioxidant properties of roasted coffee residues. *J Agric Food Chem* 2005; **53**: 2658-63.

38 Dragicevic N, Delic V, Cao C *et al.* Caffeine increases mitochondrial function and blocks melatonin signaling to mitochondria in Alzheimer's mice and cells. *Neuropharmacology* 2012; **63**: 1368-79.

39 Bahta AW, Farjo B, Philpott MP. Premature senescence of balding dermal papilla cells in vitro is associated with p16INK4a expression. . *J Invest Dermatol* 2008; **128**: 1088-94.

Legends to Figures

Fig. 1:

Hair shaft elongation in female hair follicles at 120 h of hair follicle culture.

Testosterone (T) significantly suppressed hair shaft elongation in female human hair follicles down to 77%, and co-cultivation with caffeine (T + C 0.0005%) significantly enhanced hair shaft elongation up to 96% compared to 100% control. Data are presented as mean ±SEM. **p*<0.05, ***p*<0.01.

Fig. 2:

Hair matrix keratinocyte proliferation

Testosterone showed a slight inhibition of Ki67-positive matrix keratinocytes in male hair follicles assessed by immuno-fluorescence (A), and caffeine 0.001% significantly increased the number of Ki67-positive matrix keratinocytes compared to testosterone and to control (B). Proliferation in female hair follicles showed a significant suppression of the number of Ki67-positive matrix keratinocytes by testosterone, and caffeine (0.0005%) induced a higher percentage of proliferating Ki67 matrix keratinocytes in hair follicles co-cultured with caffeine (C,D). #1, #2 and #3 are exemplary images of the different treatment conditions from the HF from three different individuals. Data are presented as mean ±SEM. **p*<0.05.

Fig. 3:

Effect of caffeine on the proliferation of ORSK - CyQUANT assay

ORSK from 3 male donors were incubated with caffeine at different concentrations (0.00001%, 0.0001%, 0.001%) and IGF-1 (100 ng/ml), minoxidil (0.1 µM) and tretinoin (1 µM) for 24, 48, 72 and

96 h, respectively. After 24 h incubation, caffeine significantly stimulated ORSK proliferation in all three concentrations, while after 48 h incubation only at the concentration of 0.0001%, and at later time points at no concentration. The effect size of stimulation exerted by caffeine at 24 h was superior to IGF-1 and minoxodil. IGF-1 significantly stimulated proliferation at 24, 47 and 72 h incubation time. Minoxidil stimulated ORSK proliferation only at 24 h incubation time and was inferior to the caffeine effect. The cell proliferative activity was evaluated by fluorimetric CyQUANT assay. Data are expressed as means ±SEM in comparison to the vehicle-treated control (=100%, solid line).

Fig. 4:

Effect of caffeine on the proliferation of ORSK - Ki67 immunofluorescence-cytochemistry.

ORSK, obtained from 3 male donors, were treated by vehicle (Control) or various concentrations of caffeine for 24 hrs and showed a significant dose-dependent stimulation of proliferation by immunolabeling with the proliferation marker Ki67. A) Representative images of Ki67-positive ORSK of control and caffeine-treated (0.001%) samples. B) Evaluation of Ki67-positive keratinocyte percentage of keratinocytes in culture after 24 h caffeine incubation or vehicle treatment. The number of Ki67-positive cells was determined in 10 independent visual fields in each of the four groups of cell culture (control, caffeine), and the average mean ±SEM values are presented in comparison to the control (=100%, solid line). *p<0.05.

Fig. 5:

Effects of caffeine on preventing the cell death-promoting actions of TGF-β2 (A) and anandamide (B).

Combined fluorimetric DilC1(5)/SYTOX Green assays performed after 48 hrs treatment. Apoptosis is induced when the DilC1(5) signal intensity decreases whereas necrosis is induced when the Sytox Green signal intensity increases. Caffeine significantly counteracted apoptosis and necrosis in TGF-β2 and AEA treated ORSK at the concentration of 0.0001% (only necrosis) and 0.001%. Data are expressed as mean ±SEM. *p<0.05 in comparison to the vehicle-treated controls (100%, dotted red lines). #p<0.05 in comparison to the TGF-β2 (A) or AEA (B) treated controls (solid blue lines). Experiments using cells from 2 other donors yielded similar results.

Fig. 6:

Hair cycle analysis of male and female hair follicles.

The rate of anagen phase HFs was reduced by testosterone to 39% in male hair follicles and enhanced up to 70% by caffeine at 0.001% (A). In female HF, testosterone suppressed the rate of anagen phase HF to 55%, and caffeine enhanced anagen rate up to 65% (0.001%) and 63% (0.0005%) (B). Hair cycle score in male (C) and female (D) hair follicles: each HF was ascribed an arbitrary value corresponding to its histomorphologically detected hair cycle stage (anagen: 100; late anagen: 200; early catagen: 300; catagen: 400). The sum of values within one experimental condition was divided by the number n of evaluated hair follicles (control: n=37; testosterone: n=29; testosterone and caffeine: n=28). In male HF, a significant shift of hair cycle score to catagen was observed by testosterone and a back-shift towards anagen by caffeine 0.001%. In female HF, only testosterone induced a catagen-shift, while no effect of caffeine was observed. Data represent results from independent experiments from three different individuals and are presented as mean values ±SEM; *p<0.05; ** p<0.01.

Fig. 7:

Modulation of in situ TGF-β2 protein expression by caffeine

In male human hair follicles, testosterone induced a significant up-regulation of the catagen-inductor TGF-β2 up to 122.7% compared to 100% control (**p*<0.05) (A,B). Caffeine 0.001% significantly reduced the TGF-β2 expression down to normal (control) levels (97.59%; ***p*<0.01). #1, #2 and #3 are exemplary images of the different treatment conditions from the HF from one representative experiment; mean ±SEM; n=9-11. In female hair follicles, there was no relevant up-regulation of TGF-β2 by testosterone, but a significant decrease induced by caffeine 0.0005% from 102.5% to 89.24% (*p*<0.01) (C,D). #1 and #2 show images of the different treatment conditions from the HF from two different individuals and are presented as mean values ±SEM; n=17-32; ORS: outer root sheath; He: Henle layer;

Fig. 8:

Modulation of in situ IGF-1 protein expression by caffeine

IGF-1 IF-reactivity was detected in the outer root sheath (ORS), but also in the inner root sheath (IRS) (A,C). In male hair follicles, testosterone significantly reduced the expression of the growth factor IGF-1 (*p*<0.05), whereas co-cultivation with caffeine lead to a significant up-regulation of IGF-1 expression compared to testosterone-incubated hair follicles (*p*<0.001), but also to hair follicles cultivated under normal conditions (control) (*p*<0.01) (B).

#1, #2 and #3 are exemplary images of the different treatment conditions from the HF from two different individuals. Values are represented as means ±SEM; n=21-27. In female hair follicles, there was a stimulation of IGF-1 expression by the concentrations of 0.005% and 0.001% caffeine (C;D).

Data represent results from one experiment. Values are represented as means ±SEM; n=6-16; ORS: outer root sheath; IRS: inner root sheath; DP: dermal papilla;

Fig. 9:

Effects of caffeine, IGF-1, minoxidil and tretinoin on IGF-1 gene expression of ORSK.

Q-PCR analysis of ORSK of 3 donors. Caffeine at the concentration 0.001% significantly up-regulated IGF-1 gene expression of ORSK in 2 donors and minoxidil (0.1 µM) significantly up-regulated IGF-1 gene expression in all three donors ($p<0.05$) **(Fig. 9A,B,C)**. The negative standard tretinoin (1 µM) had no influence on IGF-1 gene expression in any donor. Data are expressed as mean ±SEM. *$p<0.05$ in comparison to the vehicle-treated control.

Fig. 10:

Effects of caffeine, IGF-1, minoxidil and tretinoin on TGF-β2 and IGF-1 protein secretion from ORSK.

Caffeine significantly stimulated TGF-β2 protein secretion from ORSK after 120 h incubation with the concentration 0.001% (C), and the catagen inducer tretinoin enhanced TGF-β2 secretion at 72 and 120 h (B,C). IGF-1 protein secretion was enhanced by caffeine at the concentration of 0.001% and by IGF-1 at all incubation times, 24 h (D), 72 h (E) and 120 h (F). Minoxidil had only a stimulatory effect on IGF-1 protein secretion at 120 h (F). Data are expressed as mean ±SEM. *$p<0.05$ in comparison to the vehicle-treated control. Experiments using cells from 2 other donors yielded similar results.

Fig. 1

Hair shaft elongation in female human hair follicles

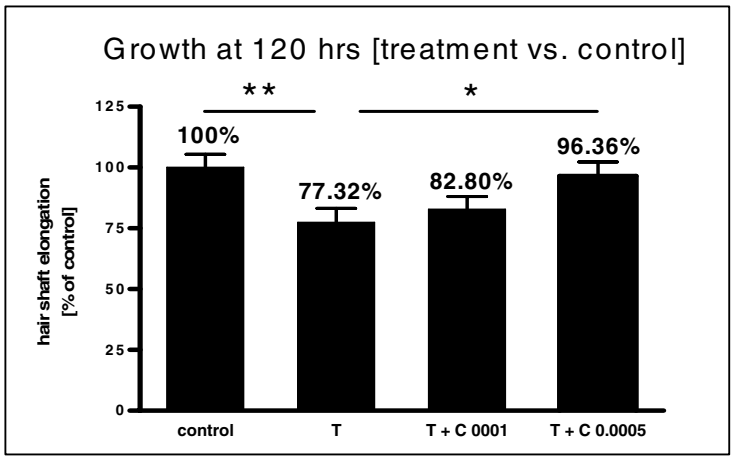


Fig 2

Hair matrix keratinocyte proliferation

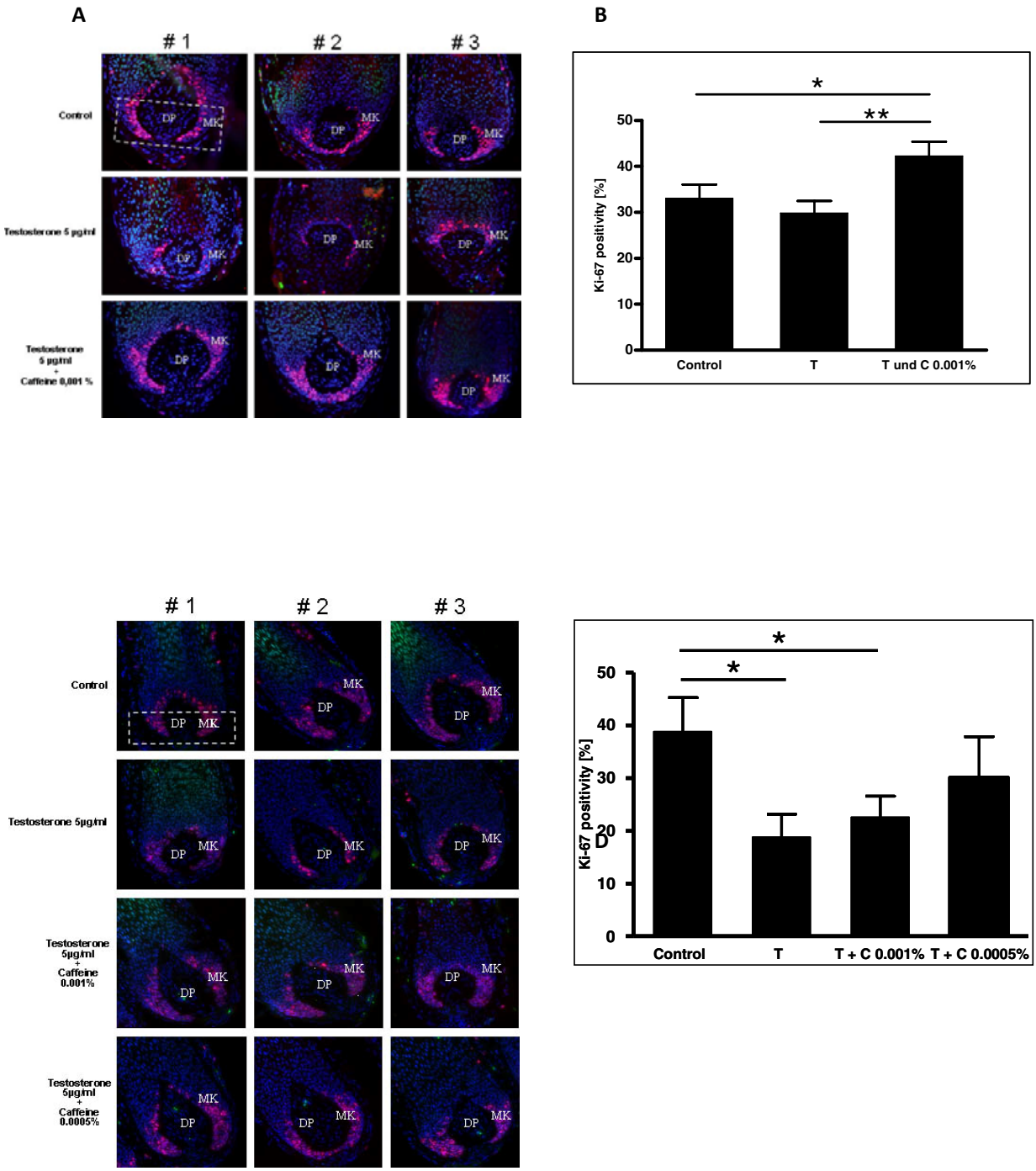
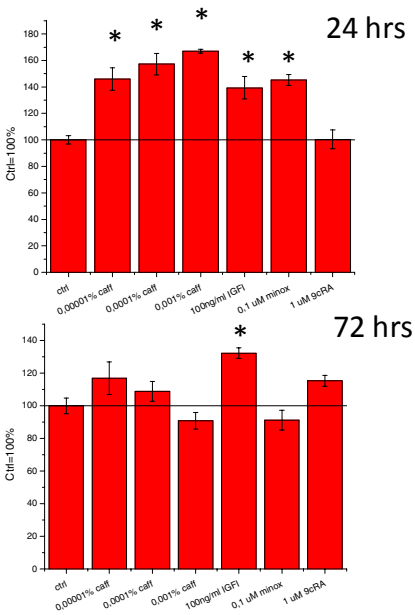


Fig 3

Determination of cellular proliferation

A



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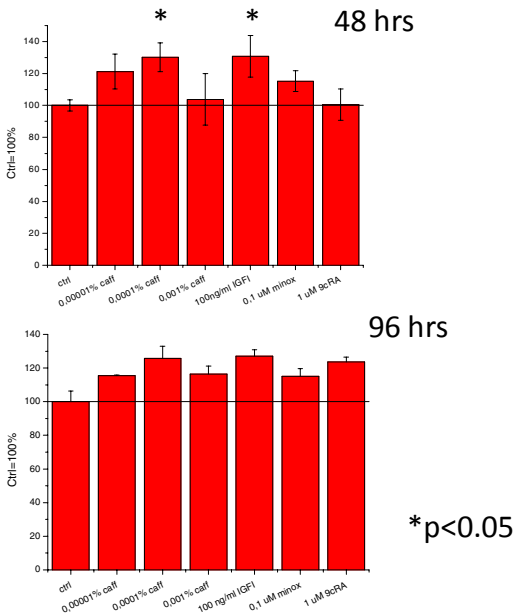


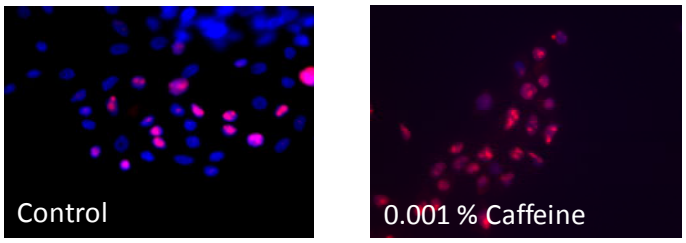
C

D

Fig 4

Immunofluorescence-cytochemical labeling of proliferation

A



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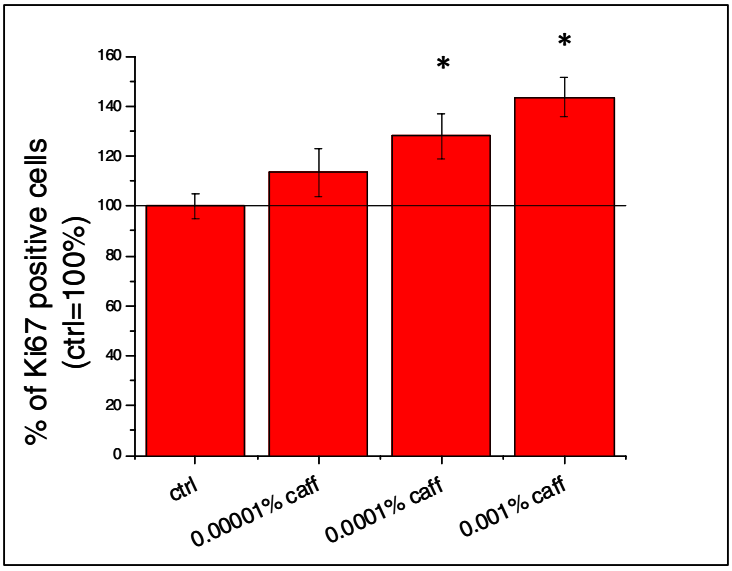
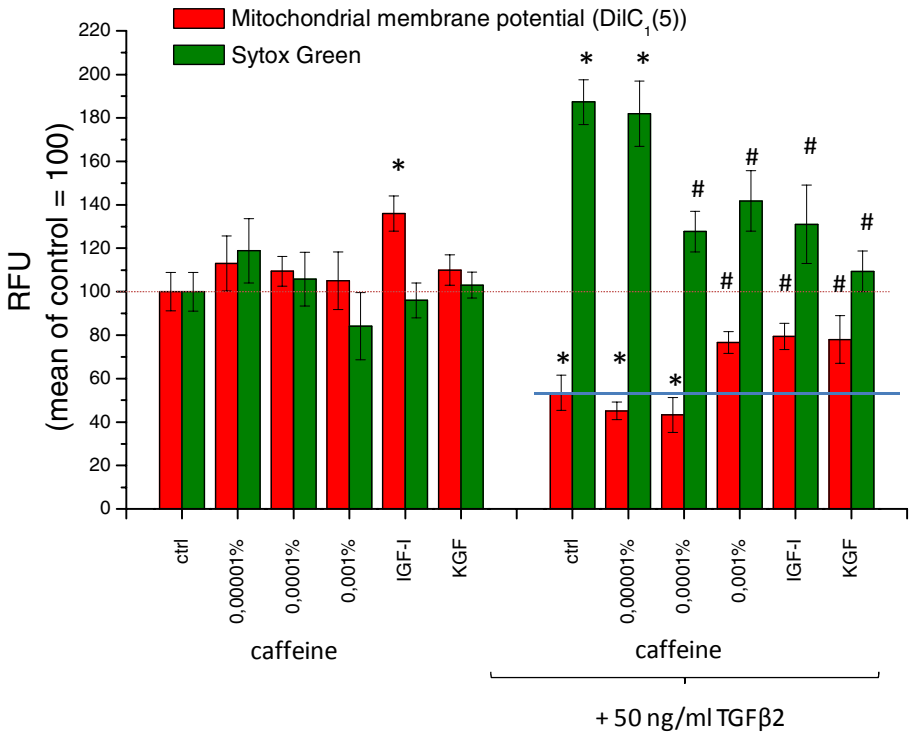


Fig 5

Determination of apoptosis and necrosis

A



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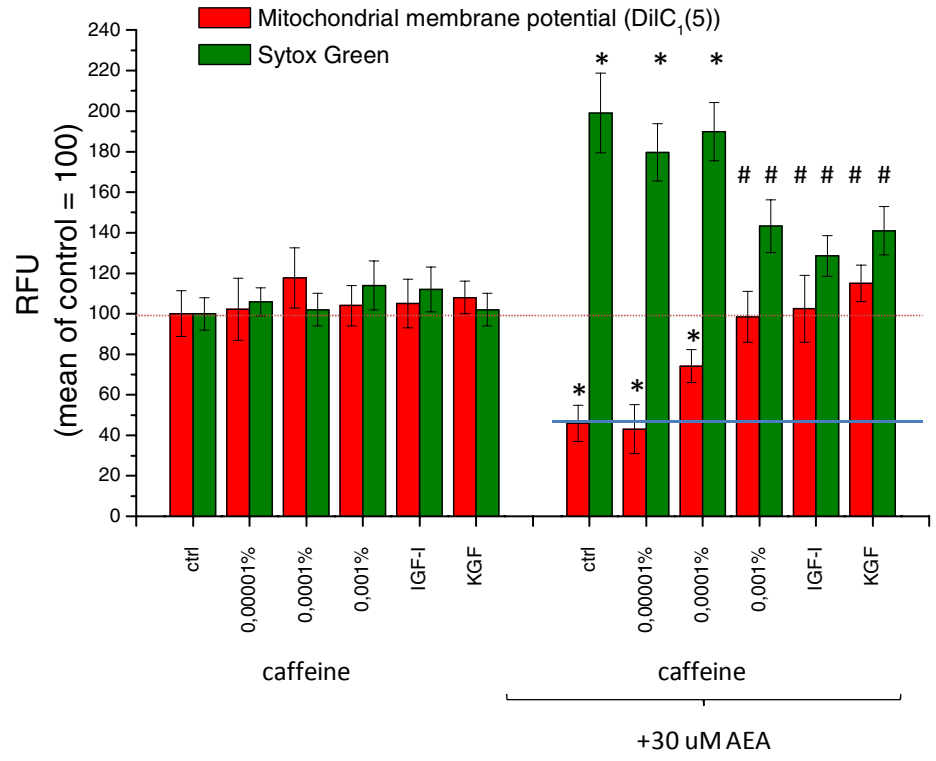


Fig 6

Hair cycle analysis

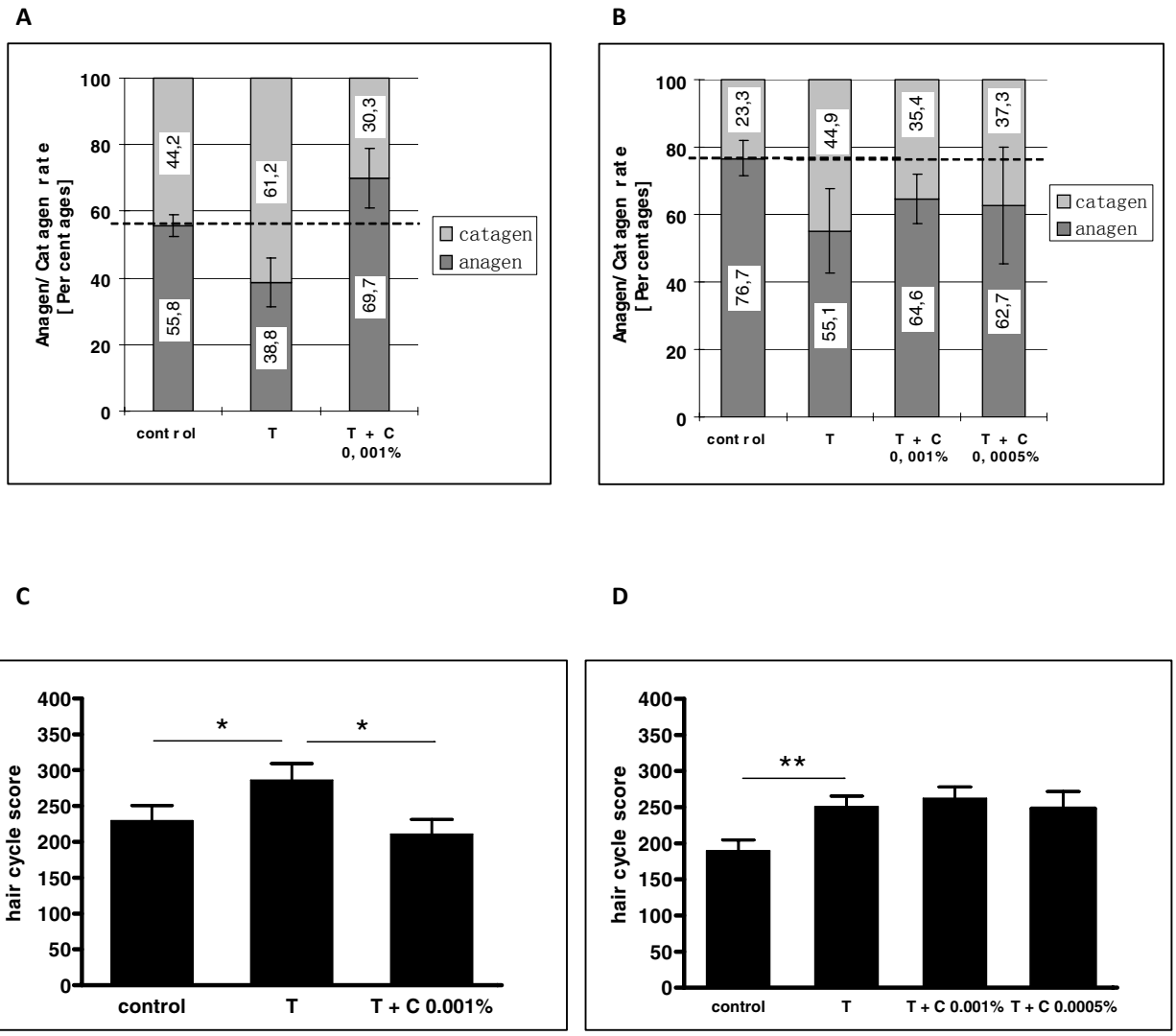


Fig 7

Modulation of TGF-β2 protein expression in situ

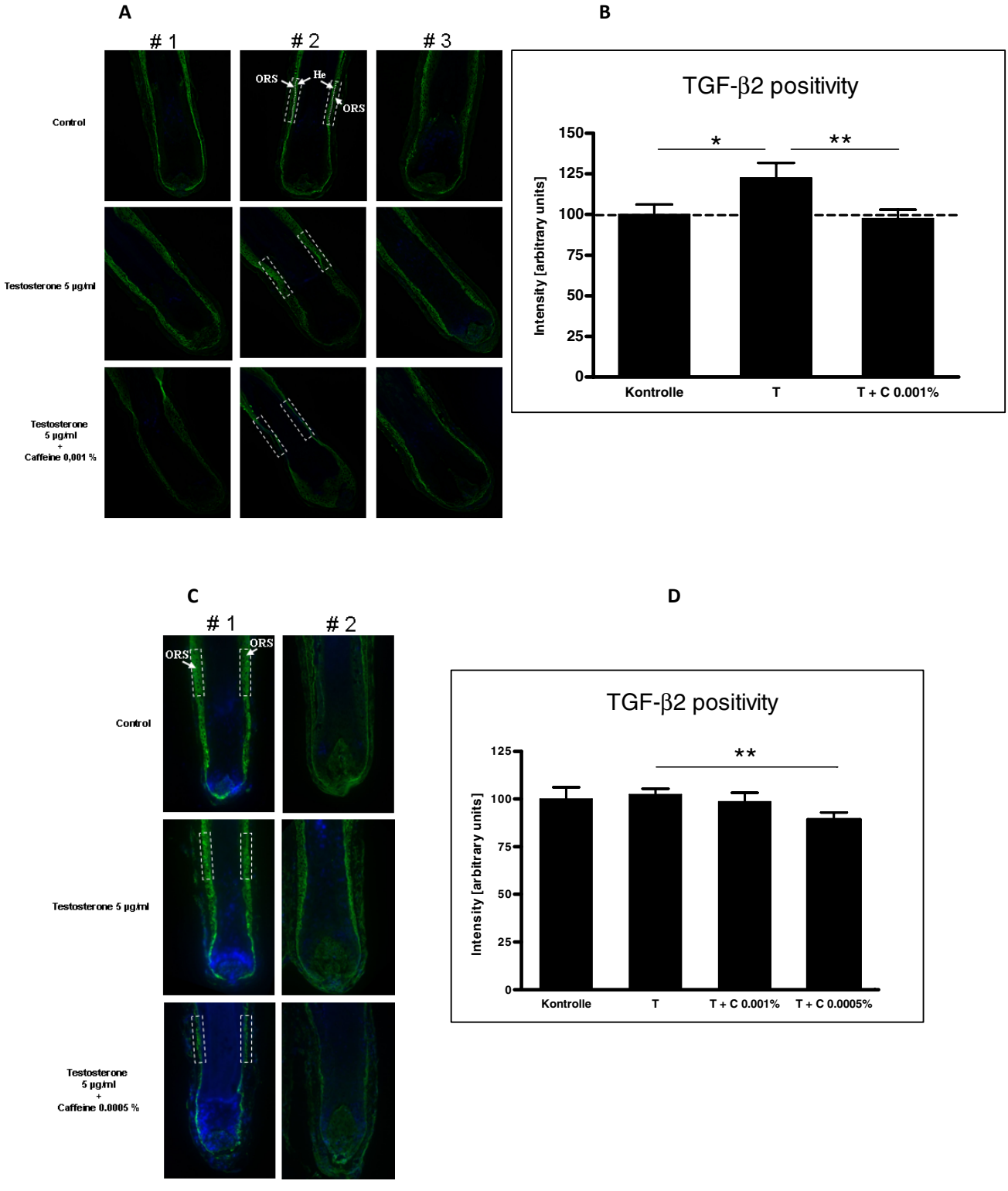


Fig 8

Modulation of IGF-1 protein expression in situ

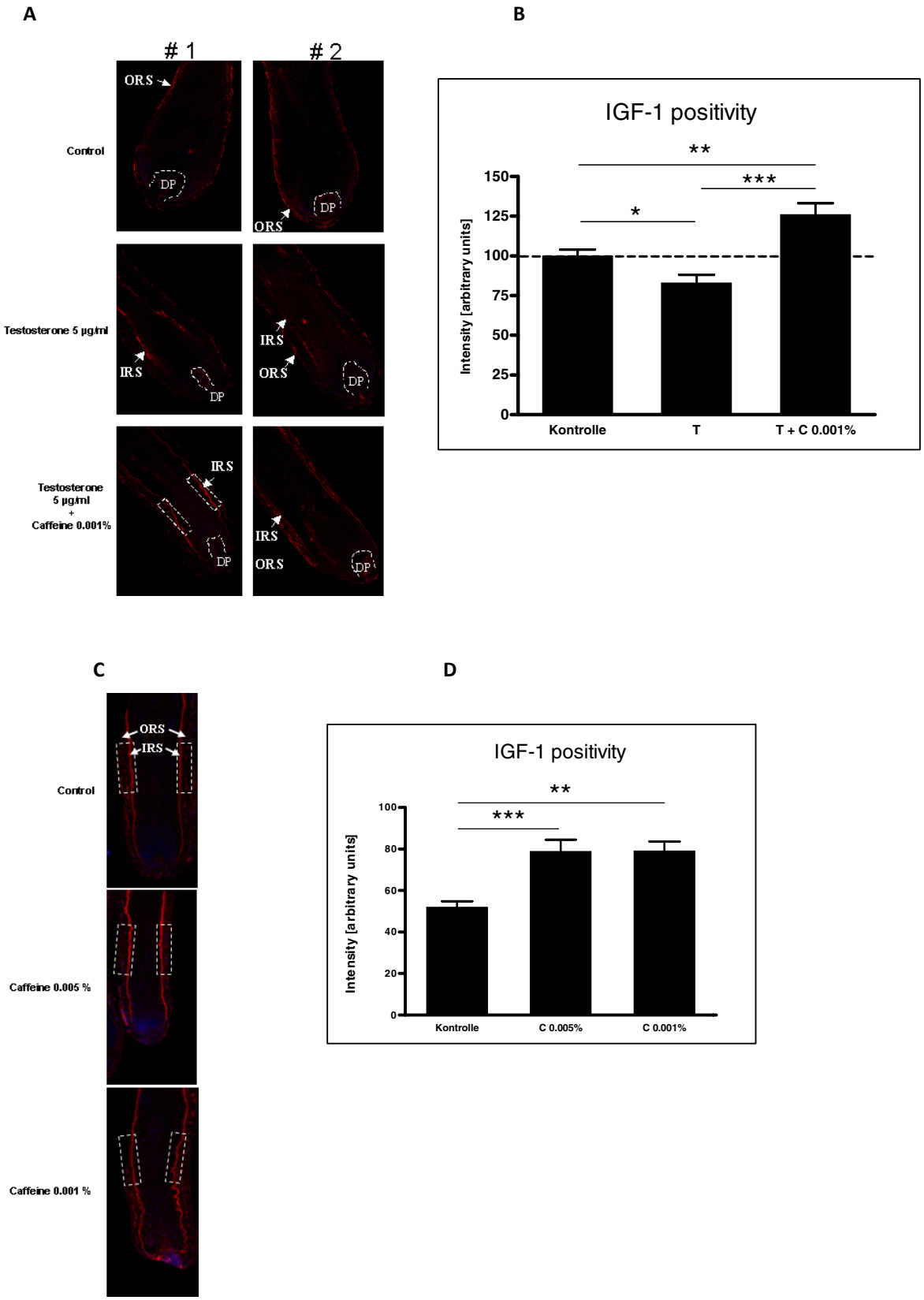
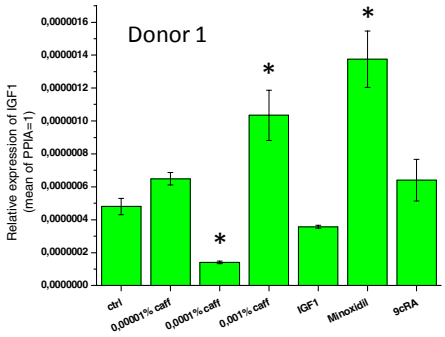


Fig 9

IGF-1 gene expression

A



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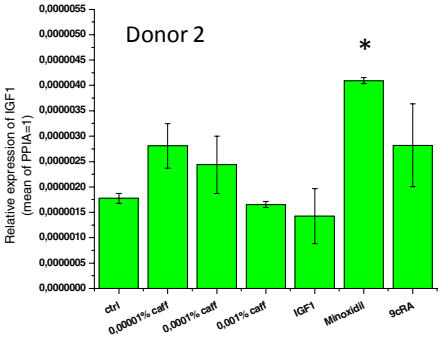

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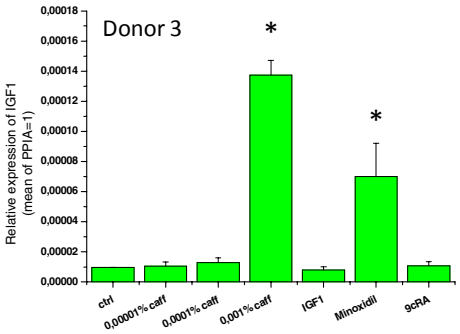

Fig 10

TGF-β2 (A-C) and IGF-1 (D-F) protein secretion