Differential effects of caffeine on hair shaft elongation, matrix and outer root sheath keratinocyte proliferation, and transforming growth factor-β2/insulin-like growth factor-1-mediated regulation of the hair cycle in male and female human hair follicles *in vitro*

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Summary

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Conflicts of interest
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Background Caffeine reportedly counteracts the suppression of hair shaft production by testosterone in organ-cultured male human hair follicles (HFs).
Objectives We aimed to investigate the impact of caffeine (i) on additional key hair growth parameters, (ii) on major hair growth regulatory factors and (iii) on male vs. 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^{-1}) or in combination with caffeine (0·005–0·0005%). The following effects on hair shaft elongation were evaluated by quantitative (immuno)histomorphometry: HF cycling (anagen–catagen transition); hair matrix keratinocyte proliferation; expression of a key catagen inducer, transforming growth factor (TGF)-β2; and expression of the anagen-prolonging insulin-like growth factor (IGF)-1. Caffeine effects were further investigated in human outer root sheath keratinocytes (ORSKs).
Results Caffeine enhanced hair shaft elongation, prolonged anagen duration and stimulated hair matrix keratinocyte proliferation. Female HFs showed higher sensitivity to caffeine than 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 ORSKs, caffeine stimulated cell proliferation, inhibited apoptosis/necrosis, and upregulated IGF-1 gene expression and protein secretion, while TGF-β2 protein secretion was downregulated.
Conclusions This study reveals new growth-promoting effects of caffeine on human hair follicles in subjects of both sexes at different levels (molecular, cellular and organ).

What's already known about this topic?

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

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What does this study add?

- The first evidence is presented for caffeine-stimulated growth of female human HFs, which are more caffeine sensitive than male HFs.
- Proliferation is increased by caffeine in human HF matrix keratinocytes *in situ* and in HF-derived outer root sheath keratinocytes (ORSKs).
- The catagen inducer transforming growth factor-β2 is downregulated, and the anagen-promoting factor insulin-like growth factor-1 is upregulated in human HFs *in situ* and in ORSKs.

Caffeine is a well-known stimulant contained in coffee. Its effects are mediated mainly through inhibition of phosphodiesterase. This leads to increased intracellular adenylate cyclase activity and enhanced cyclic 3′,5′-adenosine monophosphate (cAMP) levels, therefore providing higher energy levels to promote increased metabolic activity and cell proliferation.[1] Little is currently known about the effects of caffeine on human hair follicle (HF) growth, but it has been hypothesized that caffeine may counteract HF miniaturization in patients 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 Food and Drug Administration-approved drugs available for AGA treatment, namely finasteride and minoxidil.[3] Therefore, there seems to be a reasonable need for the development of additional effective treatment strategies.

Dihydrotestosterone (DHT) is the testosterone-derived, AGA-promoting androgen that causes a continuous shortening of hair growth cycles (anagen) in favour of longer resting phases (telogen), along with HF miniaturization of genetically predetermined areas in the frontotemporal and vertex regions of men affected with AGA.[6–9] As 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,[7] 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 factors: transforming growth factor (TGF)-β2 and insulin-like growth factor (IGF)-1. We also examined its effects on isolated human HF-derived outer root sheath keratinocytes (ORSKs). In addition, we have compared the effects of caffeine on male vs. female HFs in the presence of testosterone.

Materials and methods

Human hair follicle culture

Whole human female HFs (anagen VI) were microdissected from biopsies taken from women undergoing facelift surgery, and male HFs were obtained from electively taken biopsies (0·5 × 1·5 cm) from the balding vertex region in the border between the dense and shedding areas (androgen sensitive) of men affected with AGA in the 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 Declaration of Helsinki.

HF extraction and cultivation in the established HF organ culture model was performed as previously described.[2,8,9] After a 24-h recovery time in serum-free, supplemented William's E medium, experiments were started by replacing normal medium with fresh medium containing (i) testosterone alone, (ii) testosterone in combination with caffeine, or (iii)

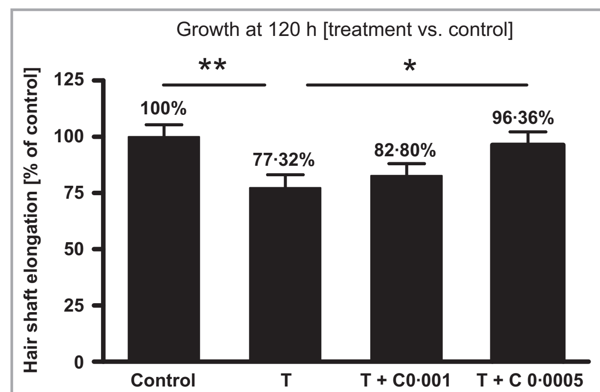

Fig 1. Hair shaft elongation in female hair follicles at 120 h of hair follicle culture. Testosterone (T) significantly suppressed hair shaft elongation in female human hair follicles to 77%, and cocultivation with caffeine (T + C 0·0005%) significantly enhanced hair shaft elongation to 96% compared with control. Data are presented as mean ± SEM. *P < 0·05, **P < 0·01.

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normal growth medium as control. Control and treatment media were changed every other day and the total culture time was 120 h. Testosterone was used at a concentration of 0·5 μg mL^{-1} 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 using a scaled microscopic eyepiece. After 120 h, follicles were frozen at −80 °C, and 6-μm cryosections were processed for immunohistochemistry and immunofluorescence as described previously.[8,10]

Hair follicle immunohistochemistry and immunofluorescence microscopy

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

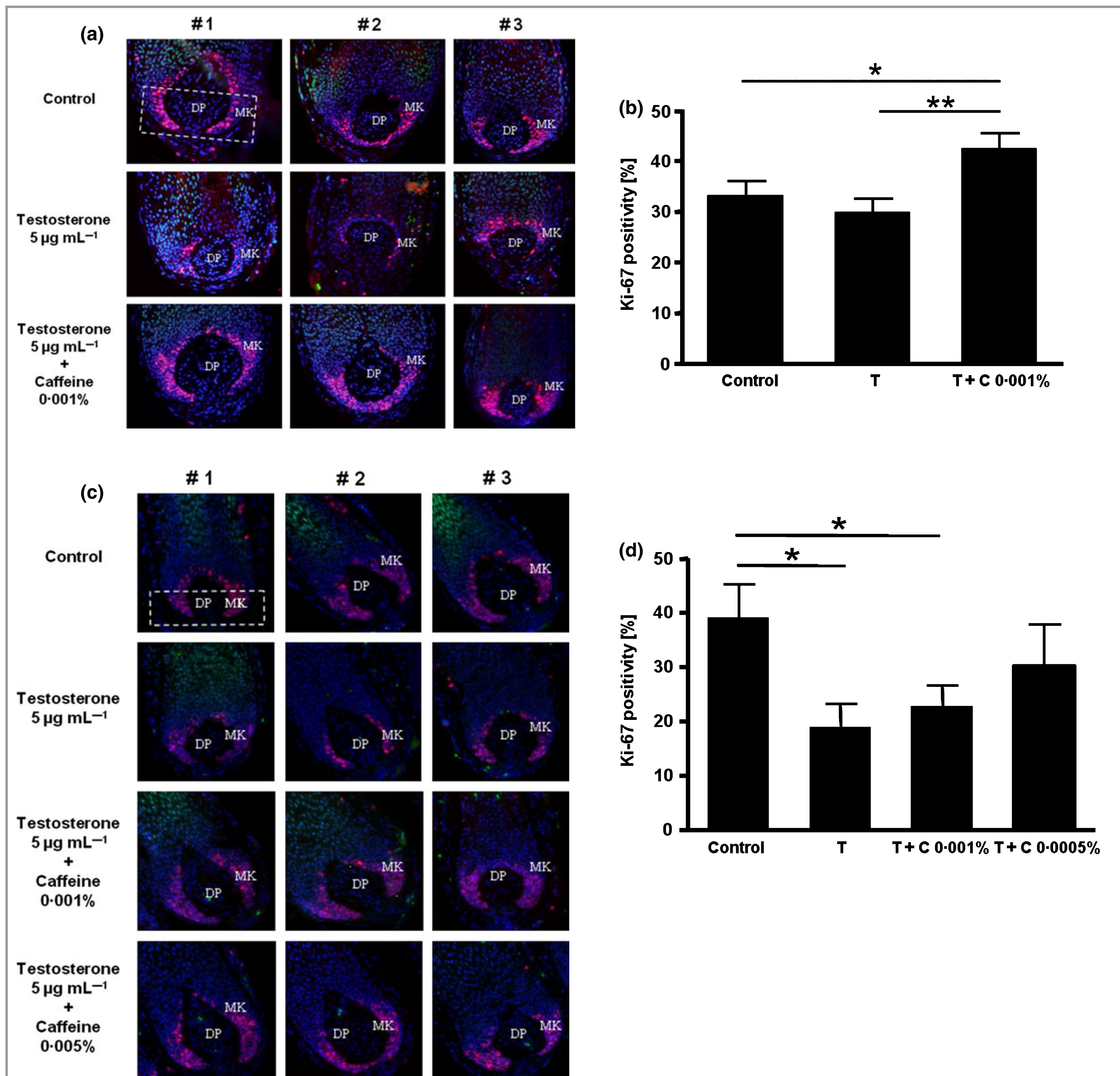

Fig 2. Hair matrix keratinocyte (MK) proliferation. (a) Testosterone showed a slight inhibition of Ki67-positive MKs in male hair follicles assessed by immunofluorescence, and (b) caffeine 0·001% significantly increased the number of Ki67-positive MKs compared with both control and testosterone alone. (c,d) Proliferation in female hair follicles showed a significant suppression of the number of Ki67-positive MKs by testosterone, and caffeine (0·0005%) induced a higher percentage of proliferating Ki67 MKs in hair follicles cocultured with caffeine. #1, #2 and #3 are exemplar images of the different treatment conditions from the hair follicles of three different individuals. Data are presented as mean ± SEM. *$P < 0·05$, **$P < 0·01$. DP, dermal papilla.

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Proliferation and apoptosis were assessed by Ki67/TUNEL double-immunostaining as reported previously.[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 was performed using polyclonal rabbit anti-TGF-β2 IgG (1 : 4000) and polyclonal goat anti-IGF-1 IgG (1 : 500) antibodies, respectively (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.). Staining protocol details are provided in File S1 (see Supporting Information).

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

Human plucked eyebrow HFs of several male healthy donors were obtained after written informed consent. ORSKs were then isolated and cultured under optimized conditions as previously described[13–15] (for a detailed description see File S1). ORSKs were then treated with caffeine (0·00001%, 0·0001% or 0·001%; Azelis, Antwerp, Belgium) and positive stimulatory reference agents such as IGF-1 (100 ng mL^{-1}) and minoxidil (0·1 μmol L^{-1}),[8] as well as the negative regulatory reference substance isotretinoin (1 μmol L^{-1}) (all from Sigma-Aldrich, St. Louis, MO, U.S.A.) for 24, 48, 72 and 96 h.[15] Cellular proliferation under these different treatment conditions was quantified using the CyQUANT Cell Proliferation Assay Kit (Invitrogen, Paisley, U.K.) and additionally assessed by immunocytochemical labelling of the proliferation nuclear marker Ki67 as described previously[13,16] (see also File S1).

Caffeine was further investigated for protective effects against apoptosis- and necrosis-inducing agents such as

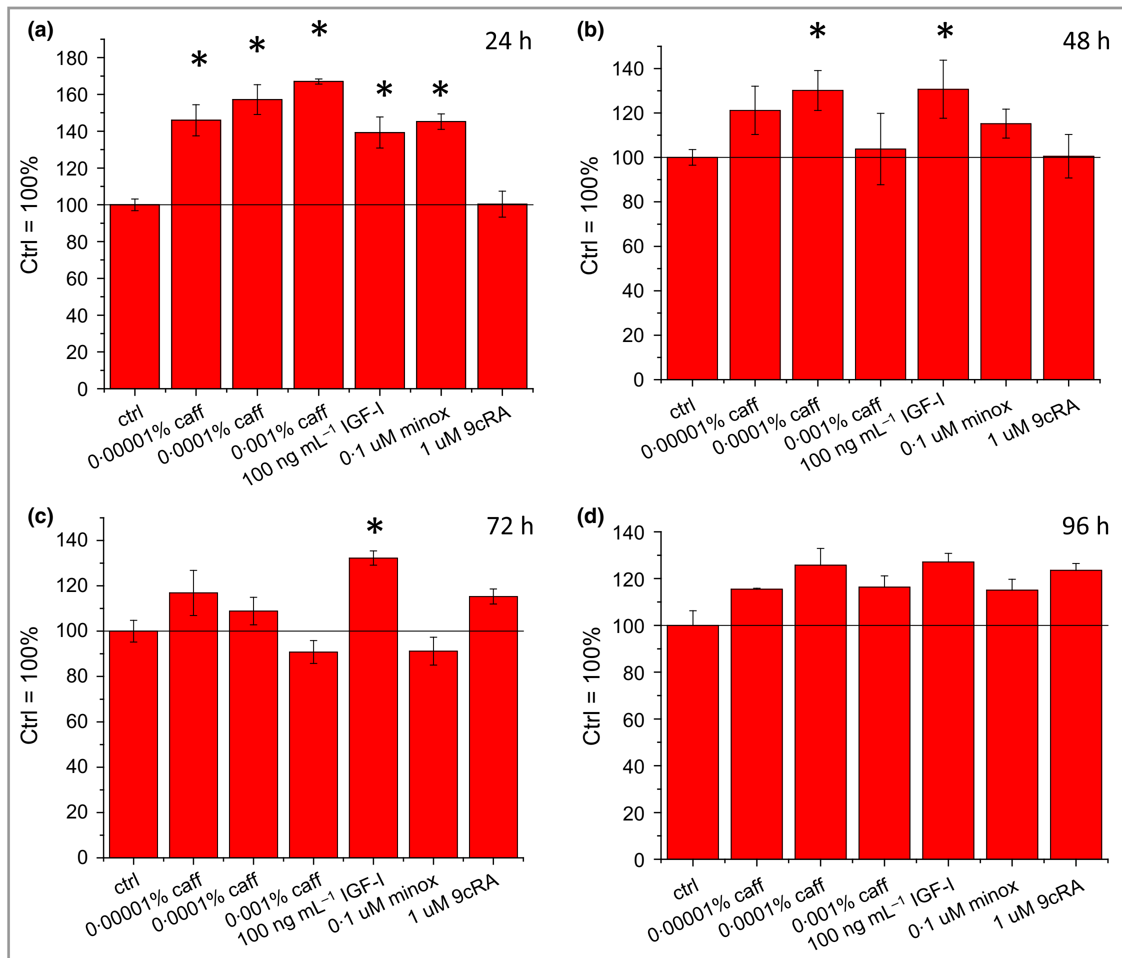

Fig 3. Effect of caffeine (caff) on the proliferation of outer root sheath keratinocytes (ORSKs) – CyQUANT assay. ORSKs from three male donors were incubated with caffeine at different concentrations (0·00001%, 0·0001%, 0·001%) and insulin-like growth factor (IGF)-1 (100 ng mL^{-1}), minoxidil (minox) (0·1 μmol L^{-1}) and isotretinoin (9cRA) (1 μmol L^{-1}) for 24(a), 48(b), 72(c) and 96(d) h, respectively. After 24 h incubation, caffeine significantly stimulated ORSK proliferation at all three concentrations, while after 48 h incubation this was seen only at 0·0001%, and it was not seen at all at later time points. The effect size of stimulation exerted by caffeine at 24 h was superior to that of IGF-1 and minoxidil (minox). IGF-1 significantly stimulated proliferation at 24, 48 and 72 h incubation time. Minoxidil stimulated ORSK proliferation only at 24 h and was inferior to caffeine. The cell proliferative activity was evaluated by fluorometric CyQUANT assay. Data are expressed as mean ± SEM vs. vehicle-treated control (100%, solid line). 9cRA, 9-*cis*-retinoic acid. *$P < 0·05$.

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catagen inducers, which, *in vivo*, may induce clinically relevant hair loss. For this purpose, two strong catagen inducers, TGF-β2 (50 ng mL^{-1}) (Sigma-Aldrich)[17] and anandamide (30 μmol L^{-1}) (Cayman, Ann Arbor, MI, U.S.A.)[18] were applied for 24 or 48 h in ORSK culture. In parallel, three concentrations of caffeine or catagen inhibitory agents – IGF-1 (100 ng mL^{-1}) and keratinocyte growth factor (KGF, 20 ng mL^{-1}) (both from Sigma-Aldrich)[19,20] – were applied for 1 h to induce antiapoptotic/antinecrotic effects. Mitochondrial membrane potential reduction as an early marker of apoptosis was determined using a MitoProbe DilC1(5) Assay Kit (Invitrogen) following previously optimized protocols[16,21] (see File S1). Necrotic cell death was determined by Sytox Green nucleic acid staining (Invitrogen) as described previously[16,21] (see File S1).

Transforming growth factor-β2 and insulin-like growth factor-1 gene expression and protein secretion in outer root sheath keratinocytes

To determine the quantitative gene expressions of TGF-β2 and IGF-1 from cell lysates of ORSKs after 120 h stimulation with caffeine (0·00001%, 0·0001%, 0·001%) and positive stimulatory reference agents (IGF-1, 100 ng mL^{-1}; minoxidil, 0·1 μmol L^{-1}), as well as the negative regulatory reference substance isotretinoin (1 μmol L^{-1}), quantitative polymerase chain reaction was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.) using the 5′-nuclease assay as detailed in our previous reports[16,22,23] (see also File S1). The supernatants of ORSKs 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 enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, U.S.A.) following the protocol of the manufacturer.

Statistical analysis

Data were expressed as mean ± SEM of pooled data from independent experiments for each study parameter. Values were normalized and expressed as a percentage of control. All data were analysed with GraphPad Prism 5.02 software (GraphPad, La Jolla, CA, U.S.A.) using Student's t-test for independent samples. A P-value $< 0·05$ was considered statistically significant.

Results

Male and female hair shaft elongation is differently stimulated by caffeine

Hair shaft elongation of male HFs revealed 0·005% and 0·001% caffeine to be stimulatory, as shown in previous experiments[2] (data not shown). For the first female HF 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 with testosterone- or vehicle-treated control male HFs (Fig. 2a,b). In female HFs, vehicle-treated control HFs revealed 39% Ki67-positive matrix keratinocytes; the testosterone-treated HFs showed only 19% ($P < 0·05$) and caffeine (0·0005%) increased the percentage of Ki67-positive matrix keratinocytes to 30% (Fig. 2c,d).

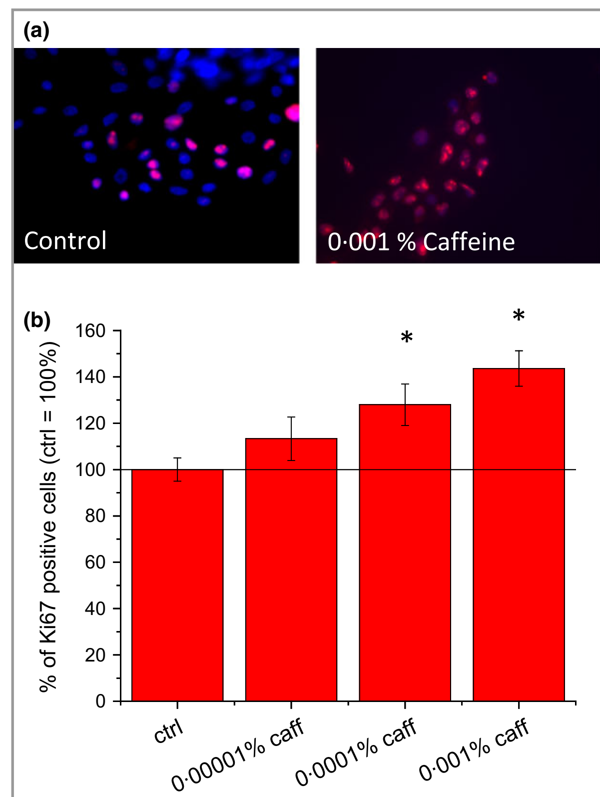

Fig 4. Effect of caffeine (caff) on the proliferation of outer root sheath keratinocytes (ORSKs): Ki67 immunofluorescence cytochemistry. ORSKs, obtained from three male donors, were treated with vehicle (control, ctrl) or various concentrations of caffeine for 24 h, and showed a significant dose-dependent stimulation of proliferation by immunolabelling with the proliferation marker Ki67. (a) Representative images of Ki67-positive ORSKs of control and caffeine-treated (0·001%) samples. (b) Evaluation of percentages of Ki67-positive 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 vs. control (100%, solid line). *$P < 0·05$.

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Caffeine stimulates cellular proliferation in outer root sheath keratinocytes

Over 96 h, a significant proliferative effect of caffeine was observed in ORSKs by CyQUANT assay. This effect was most prominent at 24 h and was exerted by all applied caffeine concentrations (0·00001%, 0·0001%, 0·001%), reaching up to 160% of the control value ($P < 0{\cdot}05$) (Fig. 3a). The positive control IGF-1 (100 ng mL^{-1}) also led to a significant but smaller increase of proliferation compared with control (~140%; $P < 0{\cdot}05$). Minoxidil, the second positive control, led to comparable growth enhancement (~140%; $P < 0{\cdot}05$), but again to a lower degree than caffeine. The growth inhibitory negative standard isotretinoin (1 μmol L^{-1}) did not lead to any change of proliferation. At 48 h, caffeine at 0·00001% and 0·0001% led to stimulation of proliferation (~120% and 130%, respectively); however, only the 0·0001% values reached significance ($P < 0{\cdot}05$) (Fig. 3b). IGF-1 also significantly stimulated proliferation (~130%; $P < 0{\cdot}05$), but minoxidil did not. At 72 h, only IGF-1 showed a significant proliferative effect ($P < 0{\cdot}05$) (Fig. 3c), while at 96 h there was no significant effect of any substance (Fig. 3d).

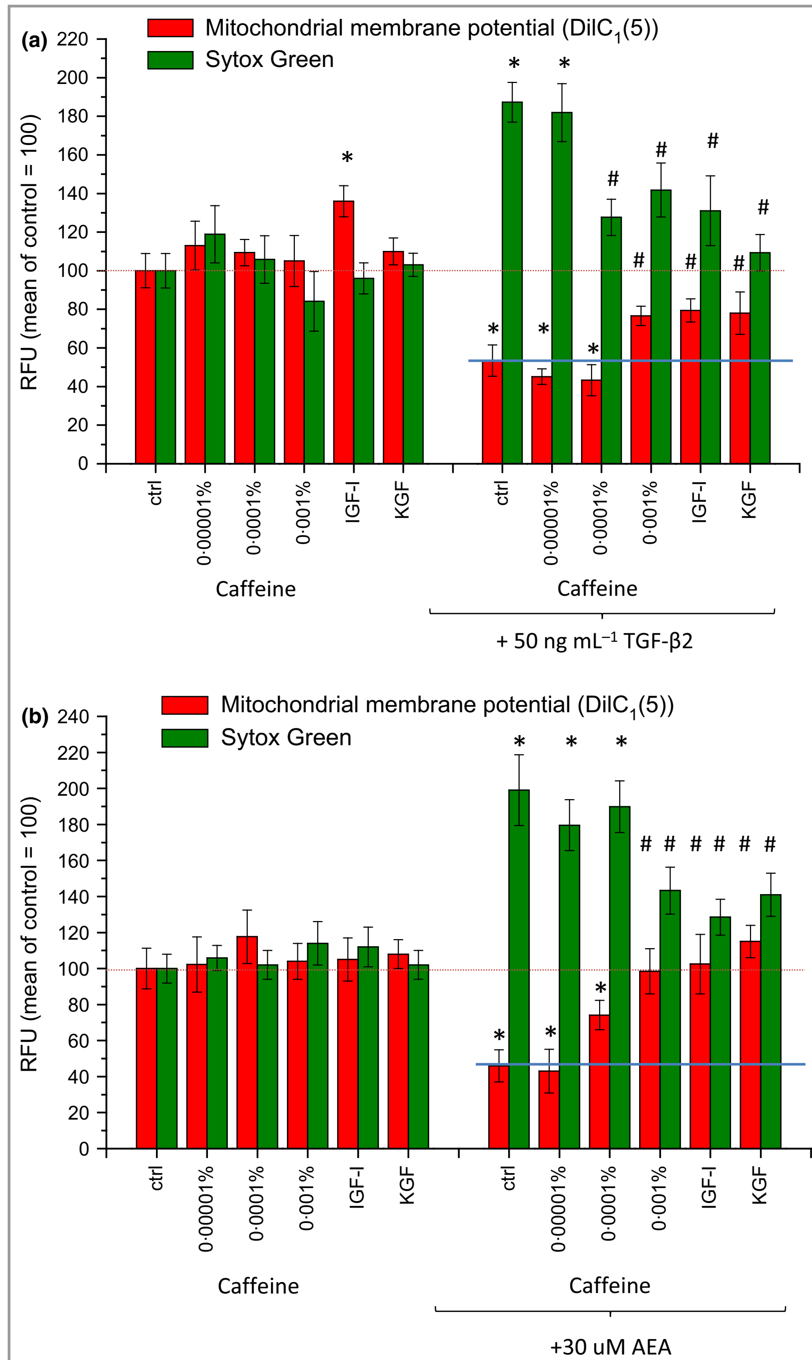

Fig 5. Effects of caffeine on preventing the cell death-promoting actions of transforming growth factor (TGF)-β2 (a) and anandamide (AEA) (b). Combined fluorometric DilC1(5)/Sytox Green assays performed after 48-h treatment. Induction of apoptosis is represented by a decrease in DilC1(5) signal intensity, whereas necrosis is shown by an increase in Sytox Green signal intensity. Caffeine significantly counteracted apoptosis and necrosis in TGF-β2- and AEA-treated ORSKs at 0·0001% (only necrosis) and 0·001%. Data are expressed as mean ± SEM. *$P < 0{\cdot}05$ vs. vehicle-treated controls (100%, dotted red lines). #$P < 0{\cdot}05$ vs. (a) TGF-β2- or (b) AEA-treated controls (solid blue lines). Experiments using cells from two other donors yielded similar results. ctrl, control; IGF, insulin-like growth factor; KGF, keratinocyte growth factor; RFU, relative fluorescence units.

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Immunofluorescence–cytochemical labelling of proliferating Ki67-positive ORSKs confirmed the data assessed by CyQUANT. Namely, incubation of ORSKs with caffeine for 24 h lead to a significantly higher, caffeine-dose-dependent percentage of Ki67-positive cells compared with vehicle-treated control (Fig. 4).

Caffeine inhibits apoptosis and necrosis in outer root sheath keratinocytes

In TGF-β2-treated ORSKs, there was a biologically relevant and significant induction of apoptosis, indicated by decreased DilC1(5) signal, and also necrosis, as shown by Sytox Green increase ($P < 0{\cdot}05$). This was significantly counteracted after a 48-h incubation with caffeine at the concentration of 0·0001% (only necrosis) and 0·001%, as well as by IGF-1 and KGF ($P < 0{\cdot}05$) (Fig. 5a, right columns). In anandamide-treated ORSKs, 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 hair follicles of male subjects, and partly in female subjects

As expected, testosterone reduced the percentage of HFs in anagen VI in men to 39%, compared with 56% in controls after 120 h of HF organ culture. Coincubation with caffeine (0·001%) strikingly raised the percentage of anagen HFs to 70% (Fig. 6a). A similar, although less pronounced effect of caffeine was also seen in female HFs at the concentrations 0·001% and 0·0005% (Fig. 6b). These findings were independently corroborated for male HFs by analysing the hair cycle score (Fig. 6c). In HFs from women, the hair cycle score showed higher values in testosterone-treated HFs, while HFs cocultivated with caffeine 0·0005% showed no decrease (Fig. 6d).

Caffeine differentially modulates intrafollicular transforming growth factor-β2 and insulin-like growth factor-1 protein expression

TGF-β2 immunoreactivity *in situ* was detected not only in the outer root sheath of the HF,[18] but also in the Henle layer of the inner root sheath. In male human HFs, testosterone significantly

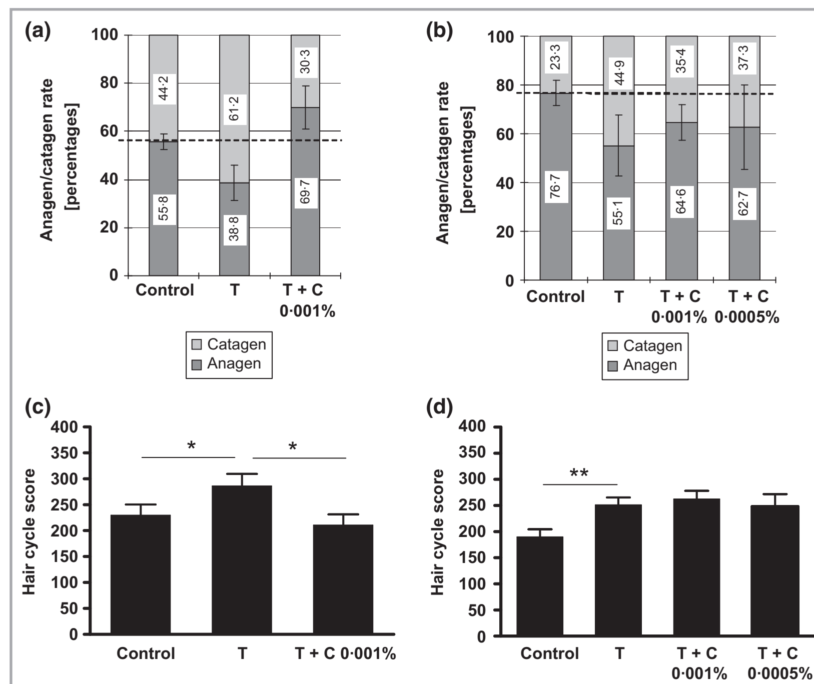

Fig 6. Hair cycle analysis of male and female hair follicles (HFs). (a) The rate of anagen-phase HFs was reduced by testosterone (T) to 39% in male hair follicles and enhanced to 70% by caffeine (C) 0·001%. (b) In female HFs, testosterone suppressed the rate of anagen-phase HFs to 55%, and caffeine enhanced the anagen rate to 65% (0·001%) and 63% (0·0005%). Hair cycle score in (c) male and (d) female 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 of evaluated hair follicles (control $n = 37$, testosterone $n = 29$, testosterone plus caffeine $n = 28$). In male HFs, 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 HFs, 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 ± SEM; $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$.

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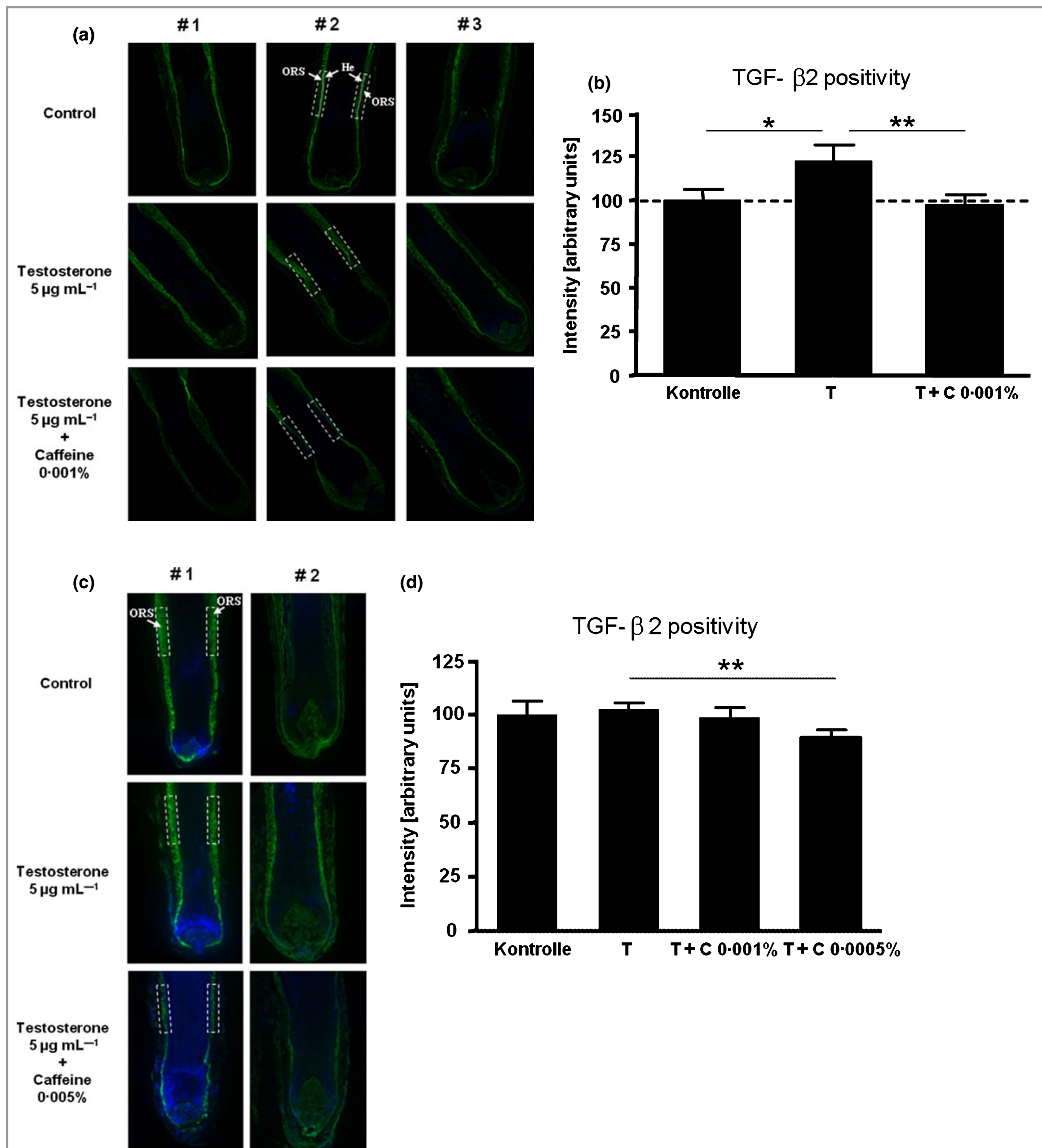

Fig 7. Modulation of *in situ* transforming growth factor (TGF)-β2 protein expression by caffeine (C). (a,b) In male human hair follicles, testosterone (T) induced a significant upregulation of the catagen inductor TGF-β2 to 122·7% vs. control (*$P < 0{\cdot}05$). Caffeine 0·001% significantly reduced the TGF-β2 expression to below normal (control) levels (97·6%; **$P < 0{\cdot}01$). #1, #2 and #3 are exemplar images of the different treatment conditions from the hair follicle of one representative experiment; mean ± SEM; n = 9–11. (c,d) In female hair follicles, there was no relevant upregulation of TGF-β2 by testosterone, but there was a significant decrease induced by caffeine 0·0005% from 102·5% to 89·2% ($P < 0{\cdot}01$). #1 and #2 show images of the different treatment conditions from the hair follicles of two different individuals and are presented as mean ± SEM; n = 17–32. ORS, outer root sheath; He, Henle layer.

Fig 8. Modulation of *in situ* insulin-like growth factor (IGF)-1 protein expression by caffeine (C). IGF-1 immunofluorescence reactivity was detected in the outer root sheath (ORS), and also in the inner root sheath (IRS) (a,c). In male hair follicles, testosterone (T) significantly reduced the expression of the growth factor IGF-1 (*$P < 0{\cdot}05$), whereas cocultivation with caffeine led to a significant upregulation of IGF-1 expression compared with both testosterone-incubated hair follicles (**$P < 0{\cdot}001$) and hair follicles cultivated under normal conditions (control) ($P < 0{\cdot}01$) (b). #1, #2 and #3 are exemplar images of the different treatment conditions from the hair follicles of two different individuals. Values are represented as mean ± SEM; n = 21–27. (c,d) In female hair follicles, there was stimulation of IGF-1 expression by caffeine 0·005% and 0·001%. Data represent results from one experiment. Values are represented as mean ± SEM; n = 6–16. *$p < 0.05$, **$p < 0.01$, ***$P < 0{\cdot}001$. DP, dermal papilla.

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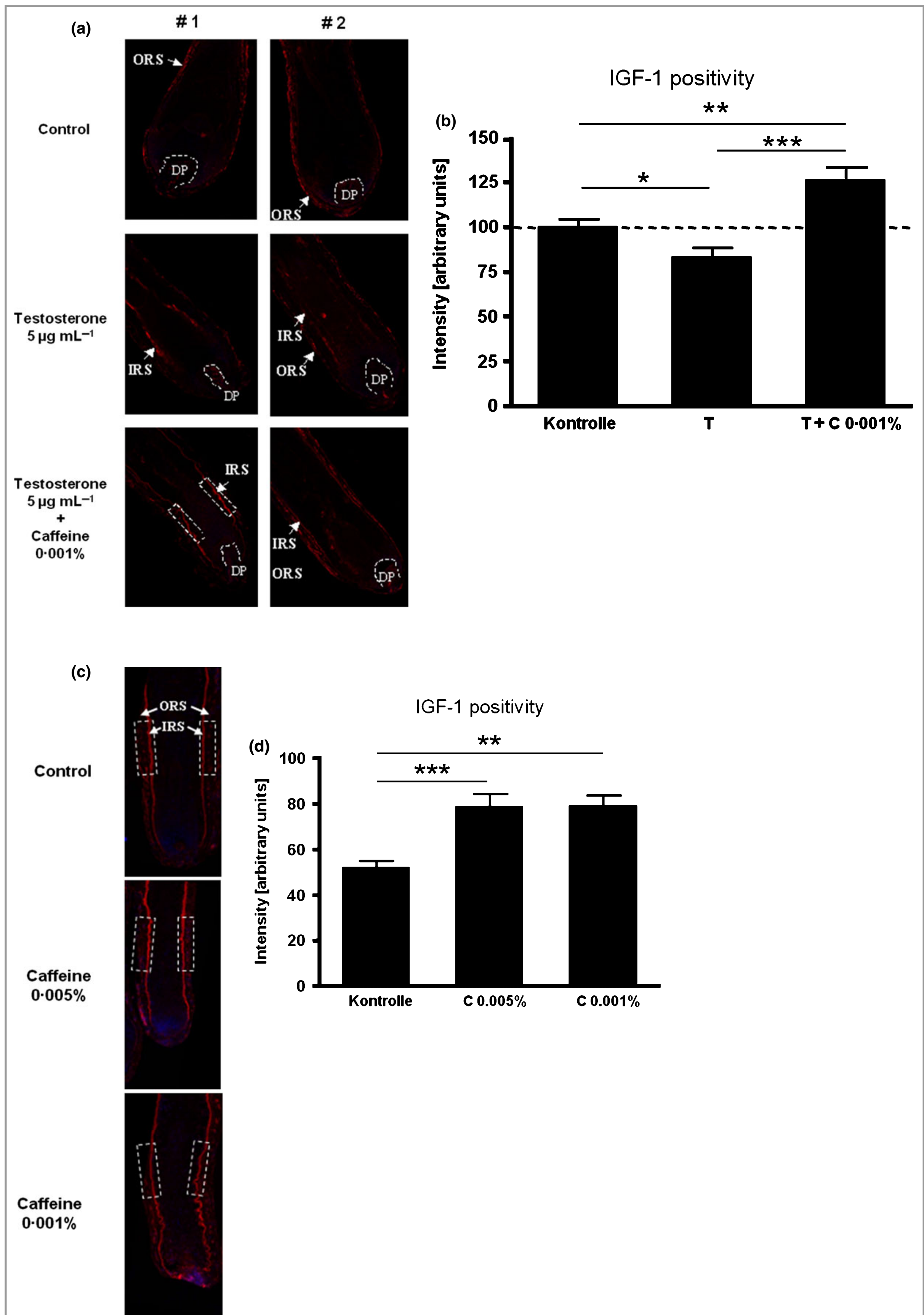
(a)
1
2
Control
Testosterone 5 µg mL⁻¹
Testosterone 5 µg mL⁻¹ + Caffeine 0·001%
ORS
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upregulated intrafollicular protein expression of the catagen-promoting growth factor TGF-β2 ($P < 0{\cdot}05$) (Fig. 7a,b). Caffeine 0·001% significantly reduced the TGF-β2 expression down to normal (control) levels (98%; $P < 0{\cdot}01$). In HFs of female subjects, testosterone did not significantly upregulate TGF-β2 protein expression, while coculture with caffeine (0·0005%) significantly reduced IGF-1 immunoreactivity ($P < 0{\cdot}01$) (Fig. 7c,d).

IGF-1 immunoreactivity was detected in both the outer and inner root sheath (Fig. 8a). Protein expression of IGF-1 in male HFs was significantly reduced by testosterone ($P < 0{\cdot}05$), whereas coculture with caffeine (0·001%) significantly upregulated IGF-1 immunoreactivity compared with testosterone- ($P < 0{\cdot}001$) or vehicle-treated control HFs ($P < 0{\cdot}01$) (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% ($P < 0{\cdot}001$ and $P < 0{\cdot}01$, respectively) (Fig. 8c,d). At 0·0005% concentration, caffeine did not modulate IGF-1 expression (data not shown).

Transforming growth factor-β2 and insulin-like growth factor-1 gene expression and protein secretion in outer root sheath keratinocytes of male subjects are partly modulated by caffeine

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

Specific mRNA transcripts of *IGF1* were below the detection limit in ORSKs from three of the six male donors (data not shown), whereas in the other three donors *IGF1* 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 upregulated *IGF1* expression ($P < 0{\cdot}05$) (Fig. 9a,c), while in the third donor, *IGF1* 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 the 72-h time point (Fig. 10a,b). However, in one of the three donors at the 120-h incubation time, caffeine 0·001% significantly sup-

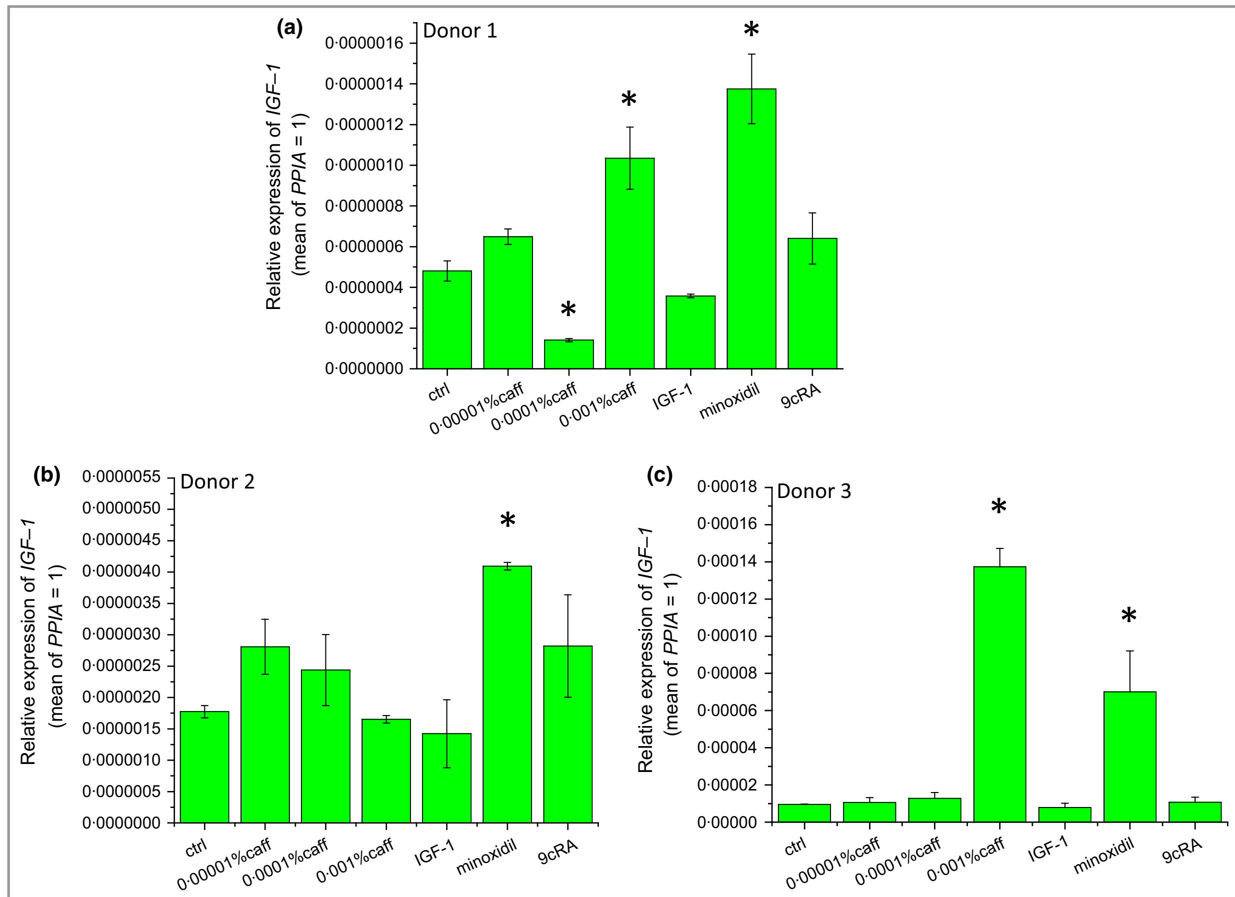

Fig 9. Effects of caffeine (caff), insulin-like growth factor (IGF)-1, minoxidil and isotretinoin (9cRA) on *IGF1* gene expression in outer root sheath keratinocytes (ORSKs). Quantitative polymerase chain reaction analysis of ORSKs of three donors. (a–c) Caffeine 0·001% significantly upregulated *IGF1* expression of ORSK in two donors, and minoxidil 0·1 μmol L^{-1} significantly upregulated *IGF1* expression in all three donors ($P < 0{\cdot}05$). The negative standard isotretinoin (9cRA) (1 μmol L^{-1}) had no influence on *IGF1* expression in any donor. Data are expressed as mean ± SEM. *$P < 0{\cdot}05$ vs. vehicle-treated control (ctrl). 9cRA, 9-*cis*-retinoic acid/isotretinoin.

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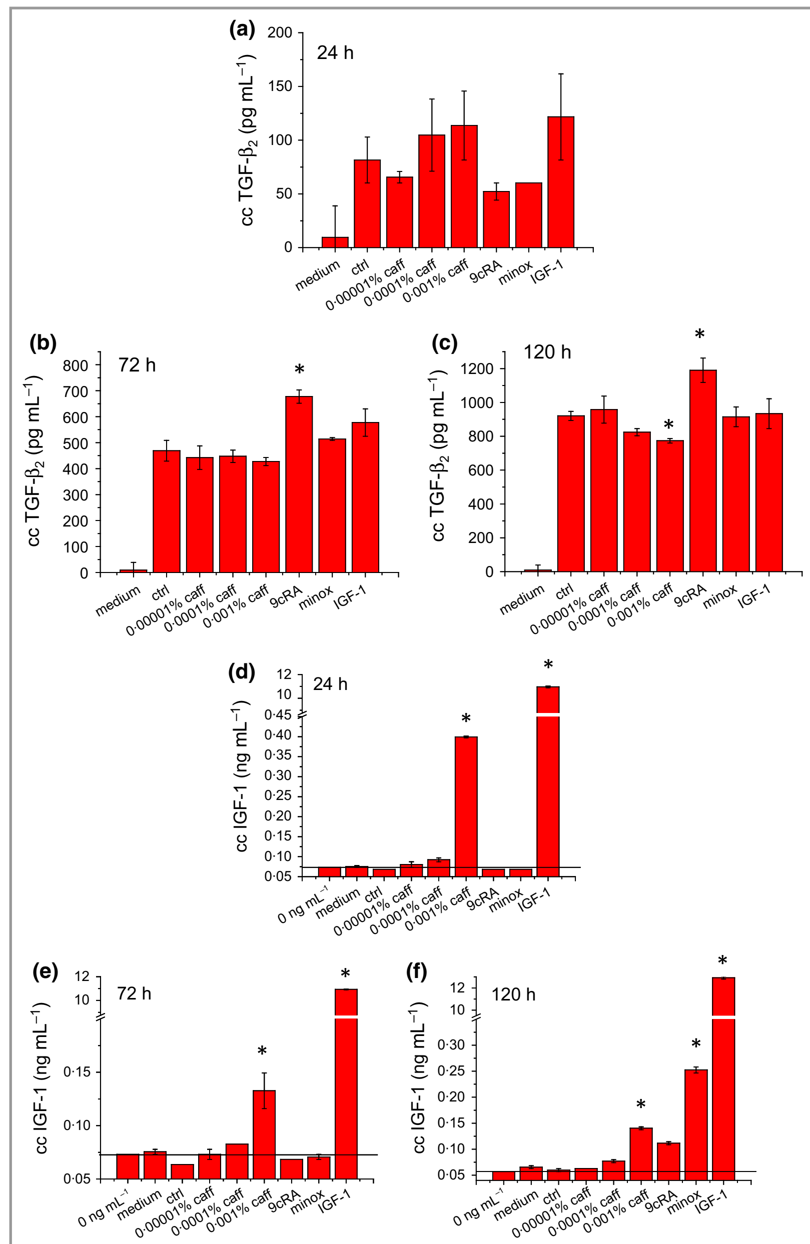

Fig 10. Effects of caffeine (caff), insulin-like growth factor (IGF)-1, minoxidil (minox) and isotretinoin (9cRA) on (a–c) transforming growth factor (TGF)-β2 and (d–f) IGF-1 protein secretion from outer root sheath keratinocytes (ORSKs). Caffeine 0·001% significantly suppressed TGF-β2 protein secretion from ORSKs after 120-h incubation (c), and the catagen inducer isotretinoin (9cRA) enhanced TGF-β2 secretion at 72 h and 120 h (b,c). IGF-1 protein secretion was enhanced by caffeine 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. *$P < 0{\cdot}05$ vs. vehicle-treated control. Experiments using cells from two other donors yielded similar results. ctrl, control; 9cRA, 9-*cis*-retinoic acid.

pressed TGF-β2 secretion compared with control ($P < 0{\cdot}05$) (Fig. 10c). The effects of isotretinoin as a catagen inducer are known, and this substance upregulated TGF-β2 secretion at 72 and 120 h in two donors ($P < 0{\cdot}05$) (Fig. 10b,c).

Regarding IGF-1 detection, caffeine 0·001% (identical in all three male donors) led to increased IGF-1 secretion at all time points investigated (24, 72, 120 h) (Fig. 10d–f). IGF-1 itself, added externally to the HF culture as an internal control (100 ng mL^{-1}), could also be detected at all investigated time points at high levels, and significantly differently from the control ($P < 0{\cdot}05$).

Discussion

The current study reveals potent, differential hair growth stimulatory effects on scalp HFs from both sexes that substantially extend beyond the previous literature.[11,12] We also examine for the first time the effects of caffeine on HFs in women. 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 sexes. The fact that these caffeine effects can essentially also be seen in isolated ORSKs 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

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testosterone-induced human skin barrier function *in vivo*.[24] The most effective caffeine concentration in male HFs was 0·001%, independently confirming 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 sex-dependent differences in putative caffeine target molecules.[25]

The influence of caffeine on two key hair growth regulatory factors, TGF-β2 (catagen promotion)[11,17,26] and IGF-1 (anagen maintenance),[9,17,20,27] 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 also stimulated the *in situ* protein expression, gene expression and protein secretion of IGF-1 in human HFs and/or ORSKs. This is in line with the observation in pancreatectomized diabetic rats that long-term caffeine supplementation potentiates an insulin/IGF-1 signalling cascade, leading to 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 ageing as represented by reduced hair diameter and density,[33,34] as 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 ORSKs. 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] 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] Therefore, 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 ageing and generally support HF growth.

Given that dermal papilla fibroblasts from men with AGA display a higher sensitivity to oxidative stress than those from nonbalding 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 HFs of genetically predisposed balding men. 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 ORSKs and human whole HFs presented here 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 exploration both *in vitro* and *in vivo*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

File S1. Supplementary methods.

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