Re-Evaluating Cyclosporine A as a Hair Growth–Promoting Agent in Human Scalp Hair Follicles

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TO THE EDITOR

Cyclosporine A (CsA) has long been recognized as a potent hair growth stimulator in both humans and rodent. The induction of a dose-dependent hypertrichosis is one of the most frequent adverse effects of long-term CsA therapy (Lutz, 1994). However, it is unclear how this immunosuppressant induces hypertrichosis in patients or stimulates hair growth in human scalp skin transplanted on nude mice (Gilhar *et al.*, 1988; Gilhar *et al.*, 1991).

Previous murine studies have shown that CsA potently modulates hair follicle (HF) cycling by inducing anagen in telogen HFs and by suppressing the development of catagen in anagen HFs (Paus *et al.*, 1989, 1994, 1996). CsA directly targets human HFs, promoting hair shaft production and potentially prolonging anagen in organ-cultured human scalp HFs (Taylor *et al.*, 1993).

CsA is thought to act by forming a complex with cyclophilin A, which in turn binds to and inhibits calcineurin (CN), preventing the dephosphorylation of target proteins, which include ion channels, receptors, and transcription factors (Wu *et al.*, 2007; Li *et al.*, 2011). Among the latter, members of the nuclear factor of activated T-cells (NFAT) family, which controls a wide range of cellular functions that include proliferation, apoptosis, and differentiation, are thought to be most important (Wu *et al.*, 2007; Li *et al.*, 2011). The hypothesis that CN inhibition has a role in the hair cycle–modulatory effects of CsA is supported by work in mice (Gafter-Gvili *et al.*, 2003). It was also shown that NFATc1 is activated in murine epithelial HF stem cells (HFSCs) where it maintains HF quiescence (Horsley *et al.*, 2008). Conversely, NFATc2 inhibits the proliferation of murine hair matrix (HM) keratinocytes in a cyclin G2 and $p21^{waf/cip1}$ – dependent manner (Gafter-Gvili *et al.*, 2003; Fujimura *et al.*, 2011). In mice, CsA releases the inhibition of HFSCs by NFATc1, thus promoting anagen (Horsley *et al.*, 2008). Catagen development in rats is prevented by CsA suppressing NFATc2 activity in the anagen HM (Fujimura *et al.*, 2011).

However, these concepts as well as the intrafollicular expression pattern of NFAT family members are derived from rodent studies, and remain to be confirmed in human HFs.

As such, we initially sought to clarify whether CsA inhibits catagen development in human HFs. To investigate this, normal human scalp HFs were stimulated with CsA (6 days), applying standardized quantitative (immuno-) histomorphometry (Kloepper *et al.*, 2010). We used 10^{-7} M CsA, replicating a previous study and imitating the therapeutic CsA plasma level (Taylor *et al.*, 1993). In addition, we analyzed the NFAT family expression and localization in human scalp HFs in response to CsA. HF samples were harvested with written informed patient consent and with approval under an institutional ethics license.

CsA stimulation for 6 days significantly enhanced hair shaft production in normal human scalp HFs *in vitro*, indicating that CsA-treated HFs retain their (anagen-restricted) capacity to generate a hair shaft (Figure 1a). This was independently confirmed through a series of histological analysis (Figures 1b–g). Masson-Fontana histochemistry demonstrated significantly higher melanin content in CsA-treated HFs (melanogenesis is shut-off upon catagen entry) (Figures 1b and c). Next, we performed Ki-67/TUNEL dual immunofluorescence (Figure 1d). HM keratinocyte proliferation (Ki-67) was significantly higher following CsA treatment (Figure 1e), whereas the number of DAPI+ HM cells below Auber's line was also significantly greater compared with vehicle-treated HFs (Figure 1f).

The number of nuclei in the dermal papilla (DP) stalk was assessed as an indicator of fibroblast emigration from the DP (Kloepper *et al.*, 2010), a phenomenon occurring during catagen development, which reduces DP volume (Tobin *et al.*, 2003). CsA significantly reduced the number of DAPI+ nuclei in the stalk (Figure 1g), indicating that fewer DP fibroblasts have emigrated and suggesting prolonged anagen duration following CsA treatment. We also hypothesize that CsA can promote hypertrichosis by retaining inductive fibroblasts within the DP, thus enlarging DP volume and thereby HM volume (Tobin *et al.*, 2003). This is supported by a recent study demonstrating that CsA enlarges the DP in mice (Lan *et al.*, 2015).

We could not detect any significant differences in the expression of apoptosis markers (TUNEL/Caspase-3) between CsA and control HFs after 6 days of treatment (Supplementary Figure S1 online). However, we show a significant decrease in caspase-3 mRNA following 6-hour CsA incubation (Figure 1h). Collectively, these results provide compelling evidence that CsA inhibits spontaneous catagen development in human HFs.

Next, intrafollicular NFATc1 and NFATc2 proteins and gene expression were assessed. In line with the reported distribution in murine HFs (Horsley *et al.*, 2008), nuclear NFATc1 immunoreactivity (IR) was observed in the

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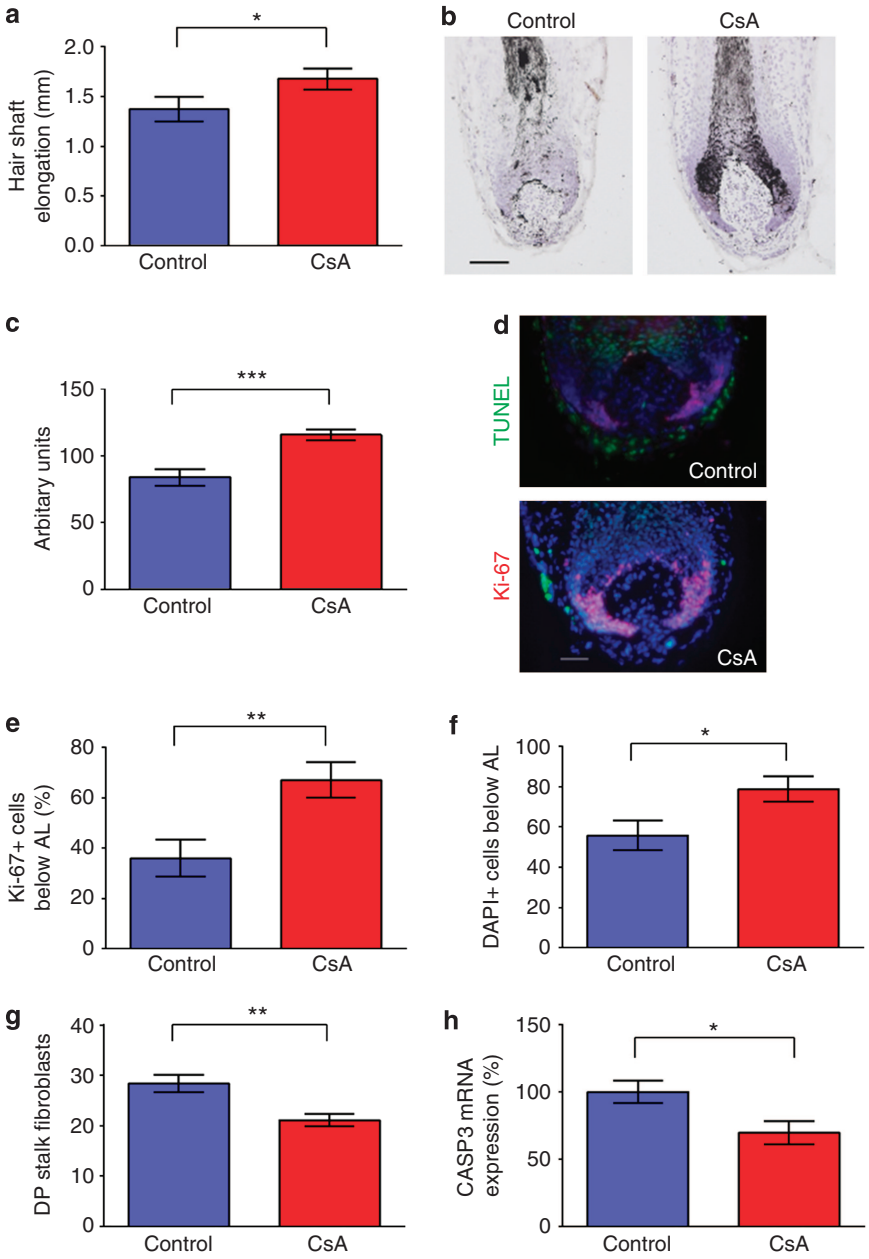

Figure 1. Cyclosporine A (CsA) treatment inhibits catagen development. Human hair follicles (HFs) were cultured for 6 days with either CsA (10^{-7} M) or vehicle control. CsA-treated HFs had significantly greater hair shaft elongation on day 6 ($n = 39$ HFs control, 43 HFs CsA; pooled from 4 male patients); (**a**). HF sections were stained with Masson-Fontana ($n = 19$ HFs control, 20 HFs CsA; pooled from three patients); (**b**). CsA-treated HFs had significantly higher melanin content (**c**). HF sections were stained with Ki-67/TUNEL to asses additional parameters ($n = 13$ HFs control, 15 HFs CsA; pooled from three patients); (**d**–**g**). CsA significantly increased proliferation (**e**) and total cell number (**f**), whereas significantly decreased fibroblast migration within the DP stalk (**g**). CsA significantly decreased CASP3 mRNA (6 hours incubation; $n = 6$ patient samples) (**h**). AL, Auber's line; CASP3, caspase-3; DP, dermal papilla; Data expressed as mean ± SEM; unpaired *t*-test was performed for **a**, **c**, **e**–**g**; one sample *t*-test was performed for **g**; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Scale bar = 100 μm (**b**) and 50 μm (**d**).

human HF bulge, suggesting that NFATc1 is constitutively activated in human HFSCs *in situ* during anagen (Supplementary Figure S2 online). Conversely, NFATc2 IR in human HFs showed a very different expression pattern than in rat HFs (Fujimura *et al.*, 2011), being localized to the cytoplasm of the basal outer root sheath (Supplementary Figure S2 online). Notably, NFATc2 IR was absent from the human anagen hair bulb (Supplementary Figure S2 online). NFATc1 nuclear translocation was blocked following CsA-treatment (Figure 2a), whereas NFATc2 remained cytoplasmic (Figure 2b). In addition, only NFATc3 gene expression was significantly modulated by CsA treatment (Figure 2c). Cytoplasmic (inactive) NFATc3 IR was detected in the bulge region of anagen HFs (Figure 2d).

To further examine the potential involvement of NFATc2, we measured the expression of the recognized

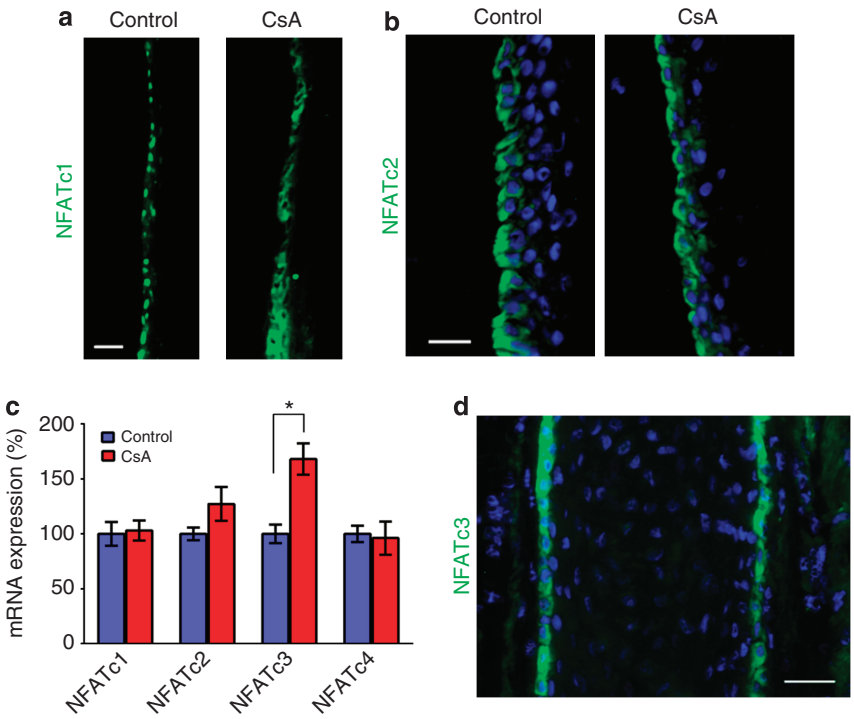

Figure 2. Cyclosporine A (CsA) effect on the NFAT family within the human hair follicle. HFs were cultured for 6 days with CsA (10^{-7} M) or vehicle control, and the cellular distribution of NFATc1 (**a**) and NFATc2 (**b**) was investigated. NFATc1 nuclear translocation was blocked with CsA (one patient) (**a**). NFATc2 remained cytoplasmic when treated with CsA (two patients) (**b**). The mRNA expression of the NFAT family was analyzed in response to CsA after 6-hour incubation ($n = 6$ patient samples) (**c**). NFATc3 mRNA expression was significantly increased (**c**). Cytoplasmic NFATc3-positive cells can be detected in the bulge region of anagen HFs (**d**). NFAT, nuclear factor of activated T-cells; Data expressed as mean ± SEM; One sample *t*-test was used for statistical analysis (**c**); $^*P < 0.05$. Scale bars = 20 μm (**a**), 15 μm (**b**), and 30 μm (**d**).

NFATc2 target genes, *CCNG2* and *CDKN1A*, which encode for cyclin G2 and p21$^{waf/cip1}$, respectively (Fujimura *et al.*, 2011). Following 6-hour CsA treatment, no difference in *CCNG2* and *CDKN1A* mRNA levels was found (Supplementary Figure S3 online).

Our data provide definitive evidence that CsA is capable of inhibiting catagen development in human HFs. The mechanism for CsA-induced anagen induction identified in mice may also apply to the human HF, given our demonstration that activated NFATc1 is prominently expressed in the human bulge. However, our observation that the human anagen bulb does not express NFATc2 protein strongly suggests that other, NFATc2-independent mechanisms underlie the catagen-inhibitory properties of CsA. This is further supported by our data demonstrating that the transcription of classical NFATc2 target genes are unaltered in response to CsA.

The current results caution against extrapolating the mechanism for CsA-dependent hair growth modulation in the human HF from corresponding work in rodents. Elucidating the mechanisms of CsA-induced hypertrichosis in human skin is of major translational relevance, as the key genes and pathways may be targeted directly for the therapeutic modulation of human hair growth, thus avoiding use of this potent immunosuppressant.

CONFLICT OF INTEREST

RP serves as a consultant for Giuliani S.p.A. The company has no commercial interest in CsA. The remaining authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Identification of Two Loci Associated with Generalized Pustular Psoriasis

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TO THE EDITOR

Generalized pustular psoriasis (GPP; OMIM 614204) is a severe type of psoriasis characterized by widespread erythematous skin and sterile pustules that affects up to 1.3% of psoriasis patients (Takahashi *et al.*, 2011). The acute onset of GPP is typically associated with high-grade fever, rigor, and toxicity. Acitretin is considered to be first-line therapy for GPP, and it appears to provide better efficacy as a single agent in the treatment of pustular psoriasis compared with psoriasis vulgaris (PV; OMIM 177900) as a single agent treatment (Sbidian *et al.*, 2011). Using homozygosity mapping and direct sequencing in consanguineous Tunisian multiplex families with autosomal recessive GPP, Marrakchi *et al.* (2011) first reported the association between IL36RN and GPP. A variety of missense and nonsense changes in IL36RN have since been described in European and Asian patients. Individuals with an IL36RN mutation show upregulated IL1 expression, which likely accounts for the occurrence of systemic inflammation and peripheral neutrophilia, two cardinal features of GPP (Onoufriadis *et al.*, 2011). However, the percentages of IL36RN-negative patients have been reported to range from 51% (Li *et al.*, 2013) to 84% (Setta-Kaffetzi *et al.*, 2013), implying t hat additional risk loci, genetic interactions, and other factors account for the remaining GPP occurrence. Recently, Jordan *et al.* (2012) identified the mutation c.413A>C (p.Glu138Ala) in CARD14 in a child with sporadic, early-onset GPP, which indicates that CARD14 is another candidate gene responsible for GPP.

Compared with GPP, PV has been intensively studied, and numerous risk-associated variants within 44 susceptibility loci have been discovered (Tang *et al.*, 2014). Among these susceptibility loci, IL36RN was not reported to be associated with PV. Furthermore, HLA-Cw6, the major susceptibility determinant for PV, is not associated with GPP (Griffiths and Barker, 2010).

To identify the relationship between GPP and PV, further our understanding of GPP, and detect other susceptibility genes/loci, we performed a candidate loci study in GPP patients. GPP cases and healthy controls were recruited from multiple hospitals in the central areas of China (Shanghai and Shandong provinces). The patients were isolated individuals of Chinese origin with typical clinical presentations or pathologic features. A total of 238 patients and 421 controls were included in the analysis. The clinical characteristics of the cases and controls are indicated in Supplementary Table S1 online. The study was approved by the Ethics Committee of Fudan University Huashan Hospital and was conducted according to the principles of the Declaration of Helsinki. All the participants signed informed consent about this experiment. For the selection of the single-nucleotide polymorphisms (SNPs), we searched published genome-wide association study (GWAS) on PV from the GWAS catalog and summarized the reported susceptibility loci. Using HapMap data (Han Chinese in Beijing, China), we identified the most significant SNPs of the linkage disequilibrium (LD) region and excluded redundant and monomorphic SNPs in Chinese population; in total, 50 SNPs were ultimately selected (Supplementary Table S2 online). Allele detection was performed using matrix-assisted laser desorption/ionization time of flight mass spectroscopy. The mass spectrograms were analyzed with MassARRAY TYPER software (Sequenom, San Diego, CA). Minor allele frequency was >1% with $P>0.01$ for Hardy–Weinberg equilibrium in the controls, and SNPs with call rates higher than 95% in the cases or controls were used in accordance with the SNP quality criteria. For the association testing, the Armitage trend test was employed to detect allelic

Abbreviations: GPP, generalized pustular psoriasis; GWAS, genome-wide association study; IPA, ingenuity pathway analysis; LD, linkage disequilibrium; OR, odds ratio; PV, psoriasis vulgaris; RAR, retinoic acid receptor; SNP, single-nucleotide polymorphism

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