Thyroid Hormones Directly Alter Human Hair Follicle Functions: Anagen Prolongation and Stimulation of Both Hair Matrix Keratinocyte Proliferation and Hair Pigmentation

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Context: Both insufficient and excess levels of thyroid hormones (T_3 and T_4) can result in altered hair/skin structure and function (*e.g.* effluvium). However, it is still unclear whether T_3 and T_4 exert any direct effects on human hair follicles (HFs), and if so, how exactly human HFs respond to T_3/T_4 stimulation.

Objective: Our objective was to asses the impact of T_3/T_4 on human HF *in vitro*.

Methods: Human anagen HFs were isolated from skin obtained from females undergoing facelift surgery. HFs from euthyroid females between 40 and 69 yr (average, 56 yr) were cultured and treated with T_3/T_4.

Results: Studying microdissected, organ-cultured normal human scalp HFs, we show here that T_4 up-regulates the proliferation of hair matrix keratinocytes, whereas their apoptosis is down-regulated by T_3 and T_4. T_4 also prolongs the duration of the hair growth phase (anagen) *in vitro*, possibly due to the down-regulation of TGF-β2, the key anagen-inhibitory growth factor. Because we show here that human HFs transcribe deiodinase genes (D2 and D3), they may be capable of converting T_4 to T_3. Intrafollicular immunoreactivity for the recognized thyroid hormone-responsive keratins cytokeratin (CK) 6 and CK14 is significantly modulated by T_3 and T_4 (CK6 is enhanced, CK14 down-regulated). Both T_3 and T_4 also significantly stimulate intrafollicular melanin synthesis.

Conclusions: Thus, we present the first evidence that human HFs are direct targets of thyroid hormones and demonstrate that T_3 and/or T_4 modulate multiple hair biology parameters, ranging from HF cycling to pigmentation. (***J Clin Endocrinol Metab* 93: 4381–4388, 2008**)

Clinically, it has long been observed that patients with thyroid dysfunction may show prominent hair abnormalities (1–4) and several *in vivo* studies have demonstrated (partially conflicting) hair growth-modulatory effects of thyroid hormone (TH) in sheep, rats, and mice (5–8). In humans, hypothyroidism can be associated with telogen effluvium, along with the presentation of dry, brittle, and dull hair shafts (2–4). Confusingly, hyperthyroid states can also lead to effluvium, together with thinned hair shaft diameter and brittle, greasy hair (1, 9–11), despite an apparently increased hair matrix proliferation (3). Hair shafts of patients with hyperthyroidism also show substantially reduced tensile strength (10). Early graying has been claimed to be related to autoimmune thyroid disease, hypothyroidism, and hyperthyroidism (11, 12), whereas darkening of

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Abbreviations: CK, Cytokeratin; DAPI, 4′,6-diamidino-2-phenylindole; fT_3, free T_3; HF, hair follicle; IR, immunoreactivity; TBS, Tris-buffered saline; TH, thyroid hormone; TR, TH receptor; TRE, TH-responsive element; TUNEL, terminal dUTP nick-end labeling.

gray/white hair may occur in some patients after TH administration (13).

Although these hair effects may well reflect a direct modulatory influence of TH on human hair follicle (HF) cycling (2, 4) and/or on hair keratin expression (14), this remains to be documented. Also, it remains to be conclusively shown that TH affects human HF pigmentation. Because thyroid dysfunction is associated with multiple secondary endocrine abnormalities, *e.g.* up-regulation of serum TSH as a defining feature of hypothyroidism (15) and changes of insulin serum level (16, 17), these may have caused the clinically observed abnormalities of hair growth and pigmentation. Thus, evidence is still missing that TH directly alter human HF growth, pigmentation, and/or cycling.

Human HFs underlie a lifelong cyclic regression and regeneration, the so-called hair cycle (18). After completion of their morphogenesis, HFs enter the so-called catagen (organ involution driven by controlled apoptosis) phase, followed by a stage of relative rest (telogen) (18). After telogen, HFs enter the active growth phase (anagen), which is associated with pigmented hair shaft formation (18).

That human scalp HFs do express TH receptors (TR) on the gene and protein level (TRβ1) and that T_3 reportedly prolongs the survival of microdissected, organ-cultured human scalp HFs (19) encourages one to exploit this model (20) to further dissect the direct effects of TH on human HFs. Therefore, we have employed this as an excellent, physiologically relevant preclinical test system for probing the effects of TH on human HF growth, cycling, and/or pigmentation.

This model also allows us to dissect differences between T_3 and T_4, which is actively transported into cells and then transformed into T_3 by intracellular deiodinases (21), with respect to human HF biology. Because the expression of TRs in human HFs had already been convincingly documented by others (19, 22), we have studied here only deiodinase expression (23).

This was complemented with an analysis of keratin immunoreactivity (IR) *in situ*, because the keratins cytokeratin (CK) 14 and CK6 genes display a TH-responsive element (TRE) that upon ligand binding to TR reportedly down-regulated transcription of these keratin genes (24–26). Finally, TGF-β2 is recognized as a central hair cycle-modulatory growth factor for human HFs, operating as a key terminator of anagen (27–29). Previously, we had shown that other steroid hormones (*i.e.* retinoic acid) exploit TGF-β2 to mediate, at least in part, their hair growth-inhibitory, anagen-shortening effects (30). Given that retinoic acid is a heterodimerization partner of THs (31), we concluded our analyses by also assessing whether or not T_3/T_4 treatment alters the intrafollicular IR for TGF-β2.

Materials and Methods

HF microdissection and organ culture

The study was approved by the Institutional Research Ethics Committee and adhered to Declaration of Helsinki guidelines. Human anagen HFs were isolated from skin obtained from females undergoing facelift surgery. We cultured HFs from euthyroid females between 40 and 69 yr (average, 56 yr). Isolated HFs were maintained in 24-multiwell plates in serum-free Williams' E medium (Biochrom, Cambridge, UK) supplemented with 2 mmol/liter I-glutamine (Invitrogen, Paisley, UK), 10 ng/ml hydrocortisone (Sigma-Aldrich, Taufkirchen, Germany), 10 μg/ml insulin (Sigma), and antibiotics (Sigma). After 24 h preincubation, HFs were treated with vehicle (Williams' E medium)/THs (Sigma) T_3 (concentrations of 1 pM, 100 pM, or 10 nM)/T_4 (concentrations of 10 nM, 100 nM, or 1 μM) for either 5 or 9 d. Normal T_3 and T_4 serum levels are 0.92–2.7 nM and 56–154 nM, respectively (32).

Hair shaft elongation, quantitative hair cycle histomorphometry, and histology

Hair shaft length measurements of vehicle/T_3/T_4-treated HFs were performed every second day on individual HFs using a Zeiss inverted binocular microscope with an eyepiece measuring graticule.

Seven-micrometer-thick cryostat sections of cultured HFs were fixed in acetone, air dried, and processed for histology. Masson-Fontana histochemistry was used for studying HF morphology as well as visualizing melanin pigment. HF cycle staging was carried out according to previously defined morphological criteria, and the percentage of HFs in anagen and early, mid, or late catagen was determined. Densitometric measurement of melanin staining intensity was performed with ImageJ software (National Institutes of Health, Bethesda, MD).

Quantitative immunohistochemistry (Ki67/TUNEL, CK14, CK6, and TGF-β2)

To evaluate apoptotic cells in colocalization with a proliferation marker Ki-67, a Ki-67/terminal dUTP nick-end labeling (TUNEL) double-staining method was used. Cryostat sections were fixed in paraformaldehyde and ethanol-acetic acid (2:1) and labeled with a digoxigenin-deoxy-UTP (ApopTag fluorescein *in situ* apoptosis detection kit; Intergen, Purchase, NY) in the presence of terminal deoxynucleotidyl transferase, followed by incubation with a mouse anti-Ki-67 antiserum (1:20 in PBS overnight at 4 C; Dako, Glostrup, Denmark). TUNEL-positive cells were visualized by an antidigoxigenin fluorescein isothiocyanate-conjugated antibody (ApopTag kit), whereas Ki-67 was detected by a rhodamine-labeled goat antimouse antibody (Jackson ImmunoResearch, West Grove, PA). Negative controls were performed by omitting terminal deoxynucleotidyl transferase and the Ki-67 antibody. Counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI) (Roche Molecular Biochemicals GmbH, Mannheim, Germany). Proliferating matrix/epidermal keratinocytes of normal human skin and frozen sections of murine spleen were used as positive control tissues for the Ki-67/TUNEL reaction, respectively.

Quantitative immunohistomorphometry was performed as described previously; Ki-67-, TUNEL-, or DAPI-positive cells were counted in a previously defined reference region of the HF matrix, and the percentage of Ki-67/TUNEL-positive cells was determined.

For the detection of CK14, the peroxidase-based avidin-biotin complex method (Vector Laboratories, Burlingame, CA) was used. After fixation in acetone, blocking of endogenous peroxidases (3% H_2O_2) and preincubation with goat serum [10% in Tris-buffered saline (TBS); Dako] cryosections were incubated with monoclonal mouse antihuman CK14 antibody (1:200 in TBS overnight at 4 C; Sigma). Cryosections were stained with biotinylated goat antimouse IgG (1:200 for 45 min at room temperature; Beckmann Coulter, Marseille, France) as secondary antibody and then with an avidin-biotin kit (Vector) followed by 3-amino-9-ethylcarbazole substrate-chromogen system (Dako). As negative controls, the primary antibodies were omitted, and human skin (epidermis) was used as a positive control. Counterstaining was performed with Meyer's hematoxylin.

To investigate CK6 and TGF-β_2 expression, acetone-fixed cryosections were pretreated with goat serum (10% in TBS; Dako; only for CK6). Cryoslides were incubated first with the primary antibodies against CK6 (mouse antihuman, 1:10, overnight at 4C; PROGEN, Heidelberg, Germany) and TGF-β_2 (rabbit antihuman, 1:50, overnight at 4 C; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and then with rhodamine-conjugated goat antimouse (for CK6, 1:200 in TBS for 45 min at room temperature; Jackson ImmunoResearch) and fluorescein

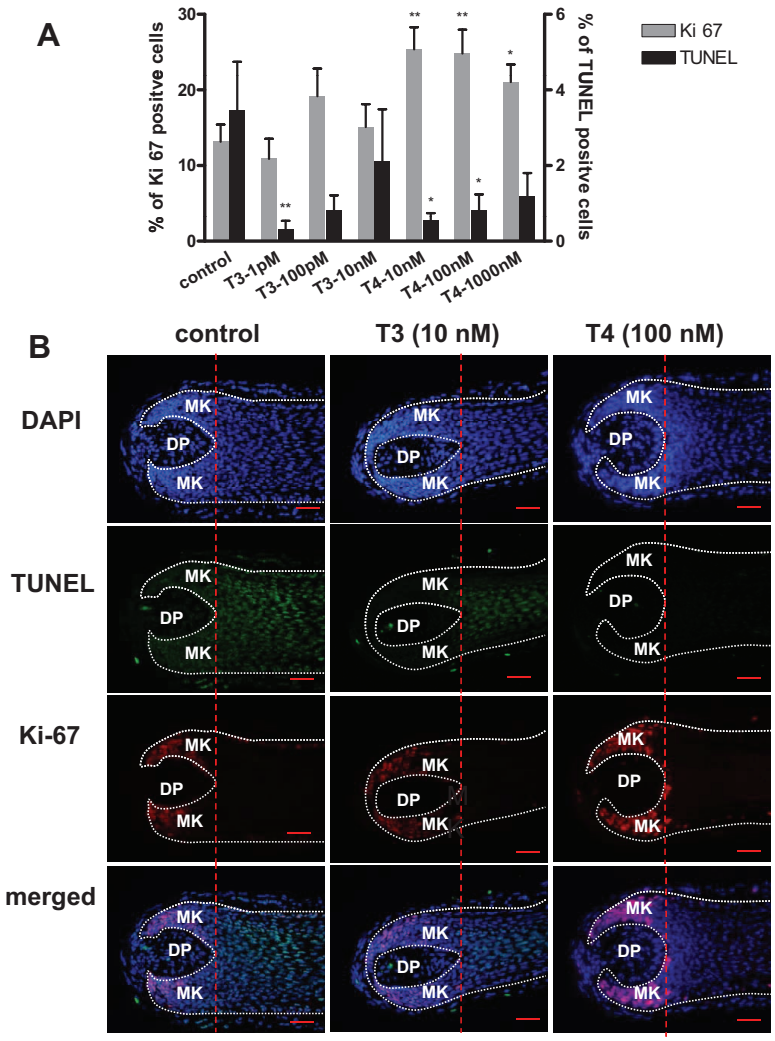

FIG. 1. T_4 stimulates human hair matrix keratinocyte proliferation, and both T_3 and T_4 inhibit their apoptosis. A, Percentage of positive cells was compared between vehicle- and T_3/ T_4-treated follicles. *, $P < 0.05$; **, $P \leq 0.01$ (mean ± SEM). B, Cryosections of cultured, vehicle-, and 10 nM T_3/100 nM T_4-treated HFs were double labeled with Ki-67 (*red*)/ TUNEL (*green*) staining. Ki-67/TUNEL-positive cells were counted below a line marking the end of the dermal papilla (indicated in *red*). DP, Dermal papilla; MK, matrix keratinocytes.

isothiocyanate-labeled goat antirabbit (for TGF-β_2, 1:200 in TBS for 45 min at room temperature; Jackson ImmunoResearch) secondary antibodies. Counterstaining was performed with DAPI (Roche Molecular Biochemicals).

Densitometric measurement of staining intensities were performed using the Image J software (National Institutes of Health).

Semiquantitative RT-PCR for deiodinases type 2 (D2) and 3 (D3)

For the semiquantitative PCR analysis of expression of D2 (accession no. AF093774) and D3 (accession no. NM 001362), total RNA was isolated from microdissected HFs using the RNA easy kit (QIAGEN, Hilden, Germany), and 0.5 μg of total RNA was reverse-transcribed with SuperScript First-Strand Synthesis System (Applied Biosystems, Foster City, CA). The quality and quantity of cDNA in all samples were standardized by the amplification of housekeeping gene GAPDH as described previously (33). The PCR conditions for D2 and D3 amplification were as follows: initial denaturation at 95 C for 2 min followed by 30 cycles of denaturation at 94 C for 30 sec, annealing at 60 C for 30 sec, and elongation 72 C for 30 sec. The final elongation step was at 72 C for 5 min. The primers used for amplification were described previously (34) and purchased from Integrated DNA Technology Inc., (Coralville, IA). For detection of D2, PCR was repeated with a second pair of primers and 0.5 μl of reaction mixture from the first round. PCR products were visualized on 2% agarose gel with ethidium bromide. Data of D2 and D3 expression were normalized to the expression of GAPDH of the same sample. Nontemplate control (by omitting RNA) was used as negative control.

Free T_3 (fT_3) and fT_4 immunoassay

For investigating whether HFs are able to produce T3 and whether possible endogenous T3 production can be stimulated by TSH, furthermore whether HFs are able to convert T4 to T3, HFs were washed several times and treated for 48 hours with T4 (100 nM) and TSH (100 mU/ml, Sigma-Aldrich). The THs fT_3 and fT_4 was measured by an electrochemiluminescent immunoassay on an automated Modular Analytics E170 according to the recommendation of the manufacturer (Roche Diagnostics, Mannheim, Germany). HFs were washed several times and cultured for 48 h. Serum-free supernatant was collected for the analysis. The sensitivity of the assays was 0.260 pg/ml (0.400 pmol/liter) for fT_3 and 0.23 pg/ml (0.30 pmol/liter) for fT_4.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney *U* Test and/ or two-tailed paired *t* test. *P* values <0.05 were regarded as significant differences.

Results

T_4 stimulates human hair matrix keratinocyte proliferation, whereas both T_3 and T_4 inhibit apoptosis of these cells

First we studied by quantitative immunohistomorphometry of key proliferation and apoptosis parameters (Ki67/TUNEL) whether T_3 (1 pM, 100 pM, and 10 nM) and/or T_4 (10, 100, and 1000 nM) modulate human hair matrix keratinocyte proliferation and apoptosis when added directly to the serum-free medium of microdissected, organ-cultured normal human scalp HFs that were in the stage of maximal growth during HF cycling (anagen VI). As shown in Fig. 1, A–D, the proliferation of hair matrix keratinocytes was significantly stimulated by T_4, whereas the proliferation-modulatory effects of the T_3 concentrations tested did not reach the level of significance. However, both T_3 and T_4 significantly reduced the number of TUNEL-positive matrix keratinocytes in defined reference areas (Fig. 1, A–D).

T_3 and T_4 do not significantly alter human hair shaft formation *in vitro*

However, during the relatively short HF organ culture period (9 d), these effects on hair matrix keratinocyte proliferation/ apoptosis did not result in marked alterations of actual hair shaft

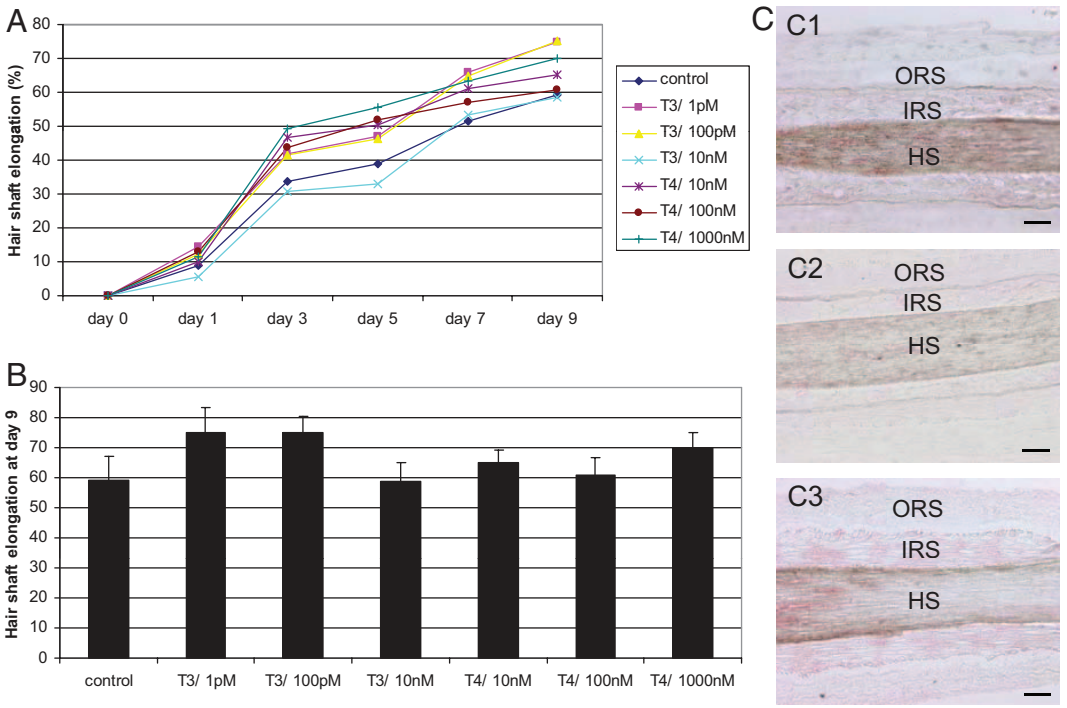

FIG. 2. Hair shaft elongation and structure did not alter after T_3/T_4 treatment. A, Percentage of hair shaft elongation measured every second day and compared with d 0; B, final percentage of hair shaft elongation after 9 d culturing compared with d 0 (mean ± SEM), all tested concentrations (T_3/ T_4) not significant; C, hair shaft morphology: C1, control; C2, T_3 1 pM; C3, T_4 1000 nM. *Scale bars*, 50 μm. HS, Hair shaft; IRS, inner root sheath; ORS, outer root sheath.

morphology (Fig. 2) or hair shaft production: When measuring the rate of hair shaft elongation *in vitro*, this remained essentially unaltered by TH. Compared with vehicle controls, significant, reproducible, hair shaft growth-promoting effects of either T_3 or T_4 (Fig. 2) were not detectable.

Both T_3 and T_4 prolong anagen duration

Clinically, the most important effect that hair loss-inhibitory/hair growth-promoting agents can have is to prolong the duration of anagen, which is indistinguishable from an inhibition of catagen development (18). Therefore, we next tested by quantitative hair cycle histomorphometry whether TH-treated human HFs showed any evidence of such an effect. Indeed, this was the case. Both T_3 and T_4 increased the number of anagen and decreased the number of catagen HFs after either 5 (Fig. 3A) or 9 d in organ culture (Fig. 3B). This provides the first direct evidence that THs are potent modulators of human scalp HF cycling, at least *in vitro*.

TGF-β2 IR is down-regulated by THs

Next we tested whether the anagen prolongation by TH is associated with an altered intrafollicular expression of TGF-β, the best-studied endogenous human hair growth inhibitor (27–29). Although substantial interindividual variations in intrafollicular TGF-β2 IR intensity and pattern were noted (data not shown), quantitative immunohistomorphometry revealed that TH treatment results in a discrete but significant reduction of TGF-β2 IR in the proximal hair bulb epithelium ($P < 0.05$) (Fig. 3, C–E). This suggests that TH down-regulates *in situ* protein expression for a key hair growth-inhibitory endogenous growth factor in the proliferatively most active epithelial hair shaft factory, the anagen hair bulb.

T_3 and T_4 differentially modulate intrafollicular keratin expression

Next, we assessed TH actions on the intrafollicular protein expression for two cytokeratins with recognized TH-responding elements (24–26), namely human CK14 and CK6. Interestingly, CK6 IR is significantly increased after treatment with 1 pM T_3 and 10 nM T_4 and 1 μM T_4 ($P < 0.05$) (Fig. 4, A–C and H), whereas quantitative immunohistomorphometry for CK14 IR showed its expression to be decreased in all TH-treated groups ($P < 0.001$) (Fig. 4, D–G).

T_3 and T_4 stimulate HF pigmentation

As revealed by quantitative Masson-Fontana histochemistry, both T_3 and T_4 significantly stimulate human HF melanin synthesis, with supraphysiological concentrations of T_4 showing the strongest stimulation of HF melanogenesis (Fig. 5, A and B1–D1). Although high-power magnification suggested that TH synthesis not only stimulates the total hair bulb melanin content but also appeared to stimulate HF melanocyte dendricity (Fig. 5, B2, C2, and D2), the strength of the melanin-associated histochemical signals hindered definitive confirmation of this intriguing observation.

D2 and D3 are transcribed by microdissected human HFs

In the current study, both T_4 and T_3 exerted (partially differential) effects on treated HFs. This already suggests that T_4 can indeed be transported into human HF cells in organ culture and is here intracellularly deiodinated to T_3. To confirm the intrafollicular presence of deiodinases, we finally studied by RT-PCR whether D2 (which transforms T_4 into T_3 by outer ring deiodinization) (21) and/or whether D3 (which transforms T_4 into inactive, rT_3) (21) are transcribed in human scalp HFs. As

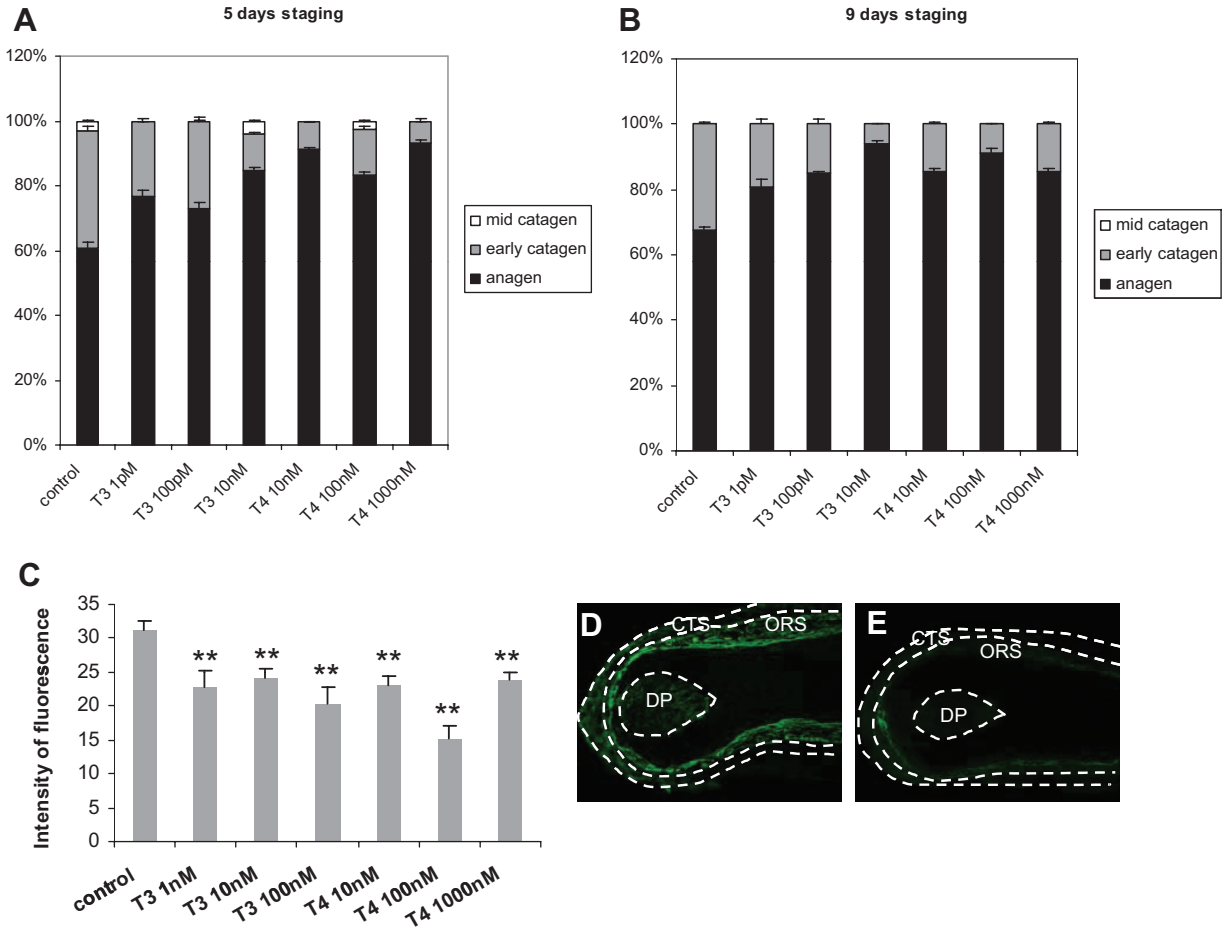

FIG. 3. Both T_3 and T_4 prolong anagen duration possibly via TGF-β2 down-regulation. Quantitative histomorphometry shows the percentage of HFs in distinct HF stages (anagen, early catagen, midcatagen). A, Staging of HFs cultured for 5 d; B, staging of HFs cultured for 9 d; C, quantitative histomorphometry of TGF-β2 IR, **, $P < 0.01$; D and E, representative staining pattern of control (D) and T_4-treated (E) follicles. Results are shown as mean in percent ± SEM. CST, Connecting tissue sheath; DP, dermal papilla; ORS, outer root sheath.

shown in Fig. 6A, specific mRNA for both D2 and D3 are indeed expressed in microdissected human HFs derived from three different individuals. To test whether T_4 deiodination occurs in HF, we measured fT_3 in culture supernatant after 48 h T_4 treatment (Fig. 6B). Compared with the vehicle (which has a value at the detection or baseline limit, suggesting that HFs are not able to produce detectable levels of T_3), we observed a significantly higher fT_3 level after T_4 treatment. This indirect result indicates that D2 may be functionally active. We also asked whether the main regulator of TH synthesis (TSH) may influence T_3 levels in HFs, but we could not detect any significant alteration (Fig. 6B). Together with the functional data obtained with T_4 stimulation listed above, this further supports the concept that human scalp HFs have indeed the enzymatic capacity to transform T_4 into T_3.

Discussion

Following in the footsteps of Billoni *et al.* (19), who had already demonstrated TH receptor transcript and protein expression in human HFs, here we provide evidence that these receptors are functional and show that human scalp HFs are indeed direct targets of TH; T_3 and T_4 modulate multiple important HF functions, ranging from HF epithelial cell proliferation, apoptosis, and keratin expression via HF cycling to HF pigmentation. T_4 up-regulates the proliferation of hair matrix keratinocytes, whereas their apoptosis is down-regulated by both T_3 and T_4. T_4 also prolongs the duration of the hair growth phase (anagen) *in vitro*, possibly due to the down-regulation of TGF-β2, the key anagen-inhibitory endogenous growth factor. THs also modulate the intrafollicular protein expression for recognized TH-responsive keratins and, importantly, stimulate intrafollicular melanin synthesis in normal human scalp HFs *in vitro*.

Because both T_3 and T_4 alter key parameters of human HF biology in organ culture and because HFs transcribe deiodinase genes, it is likely that human HFs can convert T_4 to T_3 (just like all TH-sensitive target tissues) (21), in line with the previous demonstration of D2 and D3 transcripts in cultured human skin fibroblasts, melanocytes. and keratinocytes (34). However, the subtle differences observed here between both THs in the human HF biology parameters that are significantly modulated by T_3 *vs.* T_4 (see *e.g.* Figs. 1 and 5) raise the possibility that T_4 also unfolds separate activities that are independent of its conversion to T_3 (35). However, TSH stimulation of organ-cultured

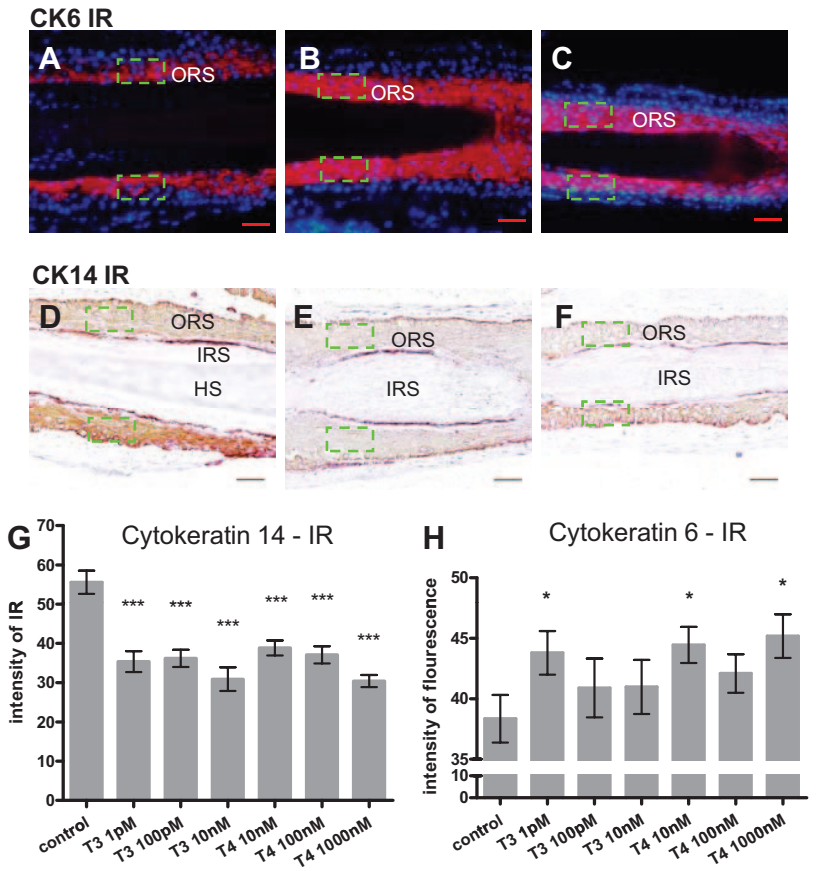

FIG. 4. T_3 and T_4 modulate CK14 and CK6 expression. A–C and H, CK6 expression is significantly altered by T_4 as well as by T_3: A, control; B, T_3 1 pM; C, T_4 10 nM. D–G, T_3 and T_4 down-regulate CK14 significantly: D, control; E, T_3 100 pM; F, T_4 10 nM. *, $P \leq 0.05$; ***, $P \leq 0.001$ (mean ± SEM). *Scale bars*, 50 μm. HS, Hair shaft; IRS, inner root sheath; ORS, outer root sheath. Reference area is indicated by *rectangles*.

HFs did not result in a significant alteration of the new fT_3 level detectable in the medium.

Because the promoter regions of CK6 and CK14 host TRE (24–26), our finding that T_3 and T_4 modulate the intraepithelial protein expression of these TH-responsive keratins CK6 and CK14 in a differential manner (Fig. 4) suggests that the effects of TH on human HFs are mediated via the classical pathway characterized by intranuclear interaction of TH-TR complexes with TRE-containing promoters (24–26). However, the very low TH doses at which some effects were seen (see Figs. 1, 3, and 4) raise the question whether they were actually mediated by recently demonstrated (35) TH membrane-bound receptors. In view of the increasing awareness of nuclear TR-independent, nongenomic signaling mechanisms of TH (whose investigation clearly was outside the scope of the current study), (36) it now also needs to be systematically investigated whether T_4 and T_3 differ not only in their stimulation of classical intrafollicular nuclear TR but also in the nongenomic, TR-independent signaling events each of these TH elicits/modulates in human scalp HFs.

Although the paucity of available human scalp HFs precluded systematic dose-response studies, our findings suggest that the predominant direct effect of both physiological and supraphysiological T_3 and/or T_4 concentrations in human hair growth control is that of a hair growth-promoting agent: 1) THs prolong anagen duration (*i.e.* retard spontaneous catagen development) (Fig. 3), T_4 stimulates hair matrix keratinocyte proliferation (Fig. 1, A and D), and 3) matrix keratinocyte apoptosis is inhibited by T_3 and T_4 (Fig, 1, A, C, and D).

The anagen prolongation documented here may correspond to the prolonged HF survival previously seen by Billoni *et al.* (19). Interestingly, the hair growth-promoting effects of TH were not associated with significant stimulatory effects of either T_3 or T_4 on hair shaft growth. This confirms corresponding findings of Billoni *et al.* (19) and might simply be explained by the relatively short culture period, which may be too short to allow detection of subtle TH effects on hair shaft formation.

In this context, it needs to be kept in mind that the assay employed here (re)creates a severely hypothyroid state *in vitro*, because HF organ culture is performed with serum-free William's E medium supplemented only with insulin and hydrocortisone (20). Thus, HFs could only have been stimulated by residual quantities of endogenous TH already bound within the HFs before microdissection and organ culture. Such residual endogenous TH likely are further reduced with every change of medium (every 2 d) and are expected to quickly lose activity with increasing culture time. Nevertheless, even in the absence of extrafollicular TH, as long as human scalp HFs remain in anagen, their hair shaft production *in vitro* progresses at almost the normal speed seen *in vivo* (37, 38), as can be seen here in the vehicle control groups (Figs. 2 and 3). This suggests that, despite the modulatory effects of TH on CK6 and CK14, extrafollicular THs are dispensable for normal hair shaft production under assay conditions. Naturally, this does not exclude a long-term role for TH in human hair shaft production *in vivo*, especially because hair shaft abnormalities have been clinically reported in both hypo- and hyperthyroid patients (1, 9, 10).

Our preclinical data help to explain the previously unclear pathogenesis of the telogen effluvium observed in hypothyroid patients, where a relative TH deficiency has been proposed to cause premature catagen induction and reduced hair matrix proliferation (2–4). Our demonstration that addition of exogenous TH to human HFs cultured in the absence of systemic TH prolongs anagen and stimulates hair matrix keratinocyte proliferation and inhibits their apoptosis provides the first direct evidence for the validity of this old postulate. Our findings also fit well with the reportedly increased hair matrix proliferation in hyperthyroid patients *in vivo* (3). Unfortunately, amputated, organ-cultured human scalp anagen VI hair bulbs do not run through full cycles *in vitro* and decay shortly after catagen development (30, 37–39). Therefore, this assay cannot clarify another old, as

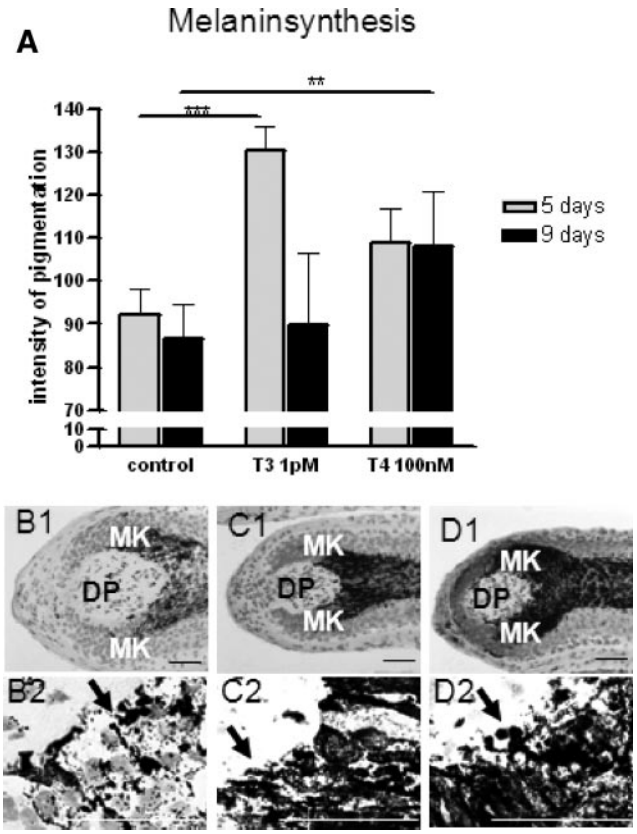

FIG. 5. T_3 and T_4 stimulate HF pigmentation. A–D, T_3 and T_4 stimulate HF pigmentation: A, significantly stronger pigmentation of T_3 1 pM and T_4 100 nM (**, $P \leq 0.01$; ***, $P \leq 0.001$; mean ± SEM); B1 and B2, control; C1 and C2, T_3 1 pM; D1 and D2, T_4 100 nM. *Scale bars*, 50 μm. DP, Dermal papilla; MK, matrix keratinocytes. *Arrows* indicate melanocytes.

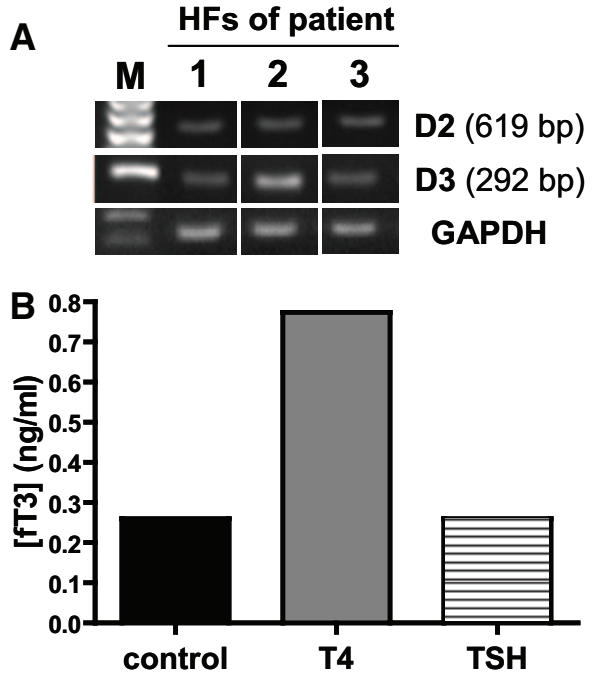

FIG. 6. Microdissected human anagen VI HFs express D2 and D3. A, HFs from three different female patients (HF 1–3) were microdissected and analyzed by RT-PCR. GAPDH served as housekeeping gene. M, DNA standard. B, HFs were treated with T_4 (100 nM) or TSH (10 mU/ml) for 48 h. fT_3 level was measured by electrochemiluminescent immunoassay in organ culture medium. GAPDH, Glycerinaldehyd-3-phosphate-dehydrogenase.

yet unconfirmed hypothesis that, conversely, hyperthyroidism may also lead to effluvium due to the induction of faster HF cycling (1, 9–11).

The current study provides at least one important pointer to the molecular mechanisms by which TH may exert their anagen-prolonging effects: THs down-regulate the intrafollicular protein expression of TGF-β2 (Fig. 3C), one of the recognized key catagen inducers during human HF cycling, which also inhibits proliferation and stimulates apoptosis of human hair matrix keratinocytes *in situ* (27). Suppression of intrafollicular TGF-β2-mediated signaling by THs, therefore, constitutes at least one conceivable pathway by which THs may prolong anagen, stimulate proliferation, and inhibit apoptosis in the human hair matrix. Such an inhibitory effect of THs on intrafollicular TGF-β2 expression would be in striking contrast to that of another important, hair growth-modulatory steroid hormone, all-*trans* retinoic acid, which we had previously shown exerts its hair growth-inhibitory, catagen-promoting effects on organ-cultured human HFs at least in part via up-regulating TGF-β2 (30). However, given the many direct TH target genes that display a TRE in their promoter region and the numerous additional, TRE-negative genes that nevertheless are regulated by TH (40) as well as nonclassical, receptor-independent TH activities (35, 36), multiple additional pathways and molecular targets by which TH may impact on human HF biology must now be considered and systematically explored.

Finally, we show that both T_3 and T_4 significantly stimulate intrafollicular melanin synthesis (Fig. 5, A–D). Because quantitative Masson-Fontana histochemistry was compared only between anagen VI HFs from test and control groups (intrafollicular melanogenesis is strictly coupled to anagen, and HF pigmentation declines sharply early during the anagen-catagen transformation of HFs (41, 42), this pigmentation-stimulatory effect cannot have been a simple reflection of the anagen-prolonging effects of TH. Thus, this is the first evidence that THs can directly alter human HF pigmentation and, to the best of our knowledge, also the first indication that THs can stimulate melanogenesis in the mammalian system *in situ*. These findings are in line with the recently reported darkening of gray/white hair in patients with increased exogenous T_3 (13). Although the underlying mechanism remains to be dissected, this further illustrates how well-suited organ-cultured human scalp HFs are as a clinically relevant, highly instructive, yet still under-exploited discovery tool for the identification of new functions for ancient steroid hormones in cutaneous endocrinology.

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References

1. **Messenger AG** 2000 Thyroid hormone and hair growth. Br J Dermatol 142: 633–634
2. **Freinkel RK, Freinkel N** 1972 Hair growth and alopecia in hypothyroidism. Arch Dermatol 106:349–352
3. **Schell H, Kiesewetter F, Seidel C, von Hintzenstern J** 1991 Cell cycle kinetics of human anagen scalp hair bulbs in thyroid disorders determined by DNA flow cytometry. Dermatologica 182:23–26
4. **Ebling F, Patricia A, Randall V** 1991 Hormones and hair growth. In: Goldsmith L, ed. Physiology, biochemistry and molecular biology of the skin. New York: Oxford University Press; 660–696
5. **Ferguson K, Wallace A, Lindner H** 1965 Hormonal regulation in wool growth. In: Lyne AG Short BF, eds. Biology of the skin and hair growth. Sydney: Angus and Robertson; 655–677
6. **Hale PA, Ebling FJ** 1975 The effects of epilation and hormones on the activity of rat hair follicles. J Exp Zool 191:49–62
7. **Safer JD, Fraser LM, Ray S, Holick MF** 2001 Topical triiodothyronine stimulates epidermal proliferation, dermal thickening, and hair growth in mice and rats. Thyroid 11:717–724
8. **Hale PA, Ebling FJ** 1979 The effect of a single epilation on successive hair eruptions in normal and hormone-treated rats. J Exp Zool 207:49–71
9. **Fistarol SK** 2002 Skin and hair, marker organs for thyroid diseases. Schweiz Rundsch Med Prax 91:1019–1028
10. **Stüttgen G** 1974 Funktionelle Dermatologie. Berlin: Springer
11. **Kaminski C** 1997 Alopecia in systemic diseases. In: Camacho F, Montagna W, eds. Trichology: Diseases of the pilosebaceus follicle. Madrid: Anca Media Group; 500–505
12. **Lerner AB** 1971 On the etiology of vitiligo and gray hair. Am J Med 51: 141–147
13. **Redondo P, Guzman M, Marquina M, Pretel M, Aguado L, Lloret P, Gorrochategui A** 2007 Repigmentation of gray hair after thyroid hormone treatment. Actas Dermosifiliogr 98:603–610
14. **Schweizer J, Langbein L, Rogers MA, Winter H** 2007 Hair follicle-specific keratins and their diseases. Exp Cell Res 313:2010–2020
15. **Sawin CT, Bigos ST, Land S, Bacharach P** 1985 The aging thyroid. Relationship between elevated serum thyrotropin level and thyroid antibodies in elderly patients. Am J Med 79:591–595
16. **Velija-Asimi Z, Karamehic J** 2007 The effects of treatment of subclinical hypothyroidism on metabolic control and hyperinsulinemia. Med Arh 61:20–21
17. **Dimitriadis G, Mitrou P, Lambadiari V, Boutati E, Maratou E, Panagiotakos DB, Koukkou E, Tzanela M, Thalassinos N, Raptis SA** 2006 Insulin action in adipose tissue and muscle in hypothyroidism. J Clin Endocrinol Metab 91: 4930–4937
18. **Paus R, Cotsarelis G** 1999 The biology of hair follicles. N Engl J Med 341: 491–497
19. **Billoni N, Buan B, Gautier B, Gaillard O, Mahe YF, Bernard BA** 2000 Thyroid hormone receptor β1 is expressed in the human hair follicle. Br J Dermatol 142:645–652
20. **Philpott MP, Green MR, Kealey T** 1990 Human hair growth in vitro. J Cell Sci 97:463–471
21. **Köhrle J** 2000 The deiodinase family: selenoenzymes regulating thyroid hormone availability and action. Cell Mol Life Sci 57:1853–1863
22. **Ahsan MK, Urano Y, Kato S, Oura H, Arase S** 1998 Immunohistochemical localization of thyroid hormone nuclear receptors in human hair follicles and in vitro effect of L-triiodothyronine on cultured cells of hair follicles and skin. J Med Invest 44:179–184
23. **Bianco AC, Larsen PR** 2005 Intrecellular pathways of iodothyronine metabolism. In: Braverman LE, Utiger RD, eds. The thyroid: a fundamental and clinical text. Philadelphia: Lippincott Williams & Wilkins; 109–133
24. **Radoja N, Diaz DV, Minars TJ, Freedberg IM, Blumenberg M, Tomic-Canic M** 1997 Specific organization of the negative response elements for retinoic acid and thyroid hormone receptors in keratin gene family. J Invest Dermatol 109:566–572
25. **Freedberg IM, Tomic-Canic M, Komine M, Blumenberg M** 2001 Keratins and the keratinocyte activation cycle. J Invest Dermatol 116:633–640
26. **Blumenberg M, Connolly DM, Freedberg IM** 1992 Regulation of keratin gene expression: the role of the nuclear receptors for retinoic acid, thyroid hormone, and vitamin D3. J Invest Dermatol 98:42S–49S
27. **Hibino T, Nishiyama T** 2004 Role of TGF-β2 in the human hair cycle. J Dermatol Sci 35:9–18
28. **Itami S, Inui S** 2005 Role of androgen in mesenchymal epithelial interactions in human hair follicle. J Investig Dermatol Symp Proc 10:209–211
29. **Soma T, Tsuji Y, Hibino T** 2002 Involvement of transforming growth factor-β2 in catagen induction during the human hair cycle. J Invest Dermatol 118:993–997
30. **Foitzik K, Spexard T, Nakamura M, Halsner U, Paus R** 2005 Towards dissecting the pathogenesis of retinoid-induced hair loss: all-trans retinoic acid induces premature hair follicle regression (catagen) by upregulation of transforming growth factor-β2 in the dermal papilla. J Invest Dermatol 124:1119–1126
31. **Leid M, Kastner P, Lyons R, Nakshatri H, Saunders M, Zacharewski T, Chen JY, Staub A, Garnier JM, Mader S** 1992 Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell 68:377–395
32. **Molina PE** 2006 Thyroid gland. In: Endocrine Physiology. 2nd ed. New York: McGraw-Hill
33. **Zmijewski MA, Sharma RK, Slominski AT** 2007 Expression of molecular equivalent of hypothalamic-pituitary-adrenal axis in adult retinal pigment epithelium. J Endocrinol 193:157–169
34. **Slominski A, Wortsman J, Kohn L, Ain KB, Venkataraman GM, Pisarchik A, Chung JH, Giuliani C, Thornton M, Slugocki G, Tobin DJ** 2002 Expression of hypothalamic-pituitary-thyroid axis related genes in the human skin. J Invest Dermatol 119:1449–1455
35. **Davis PJ, Davis FB, Cody V** 2005 Membrane receptors mediating thyroid hormone action. Trends Endocrinol Metab 16:429–435
36. **Davis PJ, Leonard JL, Davis FB** 2008 Mechanisms of nongenomic actions of thyroid hormone. Front Neuroendocrinol 29:211–218
37. **Philpott MP, Sanders D, Westgate GE, Kealey T** 1994 Human hair growth in vitro: a model for the study of hair follicle biology. J Dermatol Sci 7(Suppl): S55–S72
38. **Philpott MP, Sanders DA, Kealey T** 1994 Effects of insulin and insulin-like growth factors on cultured human hair follicles: IGF-I at physiologic concentrations is an important regulator of hair follicle growth in vitro. J Invest Dermatol 102:857–861
39. **Ito T, Ito N, Saathoff M, Bettermann A, Takigawa M, Paus R** 2005 Interferon-γ is a potent inducer of catagen-like changes in cultured human anagen hair follicles. Br J Dermatol 152:623–631
40. **Shen X, Li QL, Brent GA, Friedman TC** 2004 Thyroid hormone regulation of prohormone convertase 1 (PC1): regional expression in rat brain and in vitro characterization of negative thyroid hormone response elements. J Mol Endocrinol 33:21–33
41. **Slominski A, Wortsman J, Plonka PM, Schallreuter KU, Paus R, Tobin DJ** 2005 Hair follicle pigmentation. J Invest Dermatol 124:13–21
42. **Slominski A, Paus R** 1993 Melanogenesis is coupled to murine anagen: toward new concepts for the role of melanocytes and the regulation of melanogenesis in hair growth. J Invest Dermatol 101:90S–97S