Human Female Hair Follicles Are a Direct, Nonclassical Target for Thyroid-Stimulating Hormone

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Pituitary thyroid-stimulating hormone (TSH) regulates thyroid hormone synthesis via receptors (TSH-R) expressed on thyroid epithelial cells. As the hair follicle (HF) is uniquely hormone-sensitive and, hypothyroidism with its associated, increased TSH serum levels clinically can lead to hair loss, we asked whether human HFs are a direct target for TSH. Here, we report that normal human scalp skin and microdissected human HFs express TSH-R mRNA. TSH-R-like immunoreactivity is limited to the mesenchymal skin compartments *in situ*. TSH may alter HF mesenchymal functions, as it upregulates α-smooth muscle actin expression in HF fibroblasts. TSH-R stimulation by its natural ligand in organ culture changes the expression of several genes of human scalp HFs (for example keratin K5), upregulates the transcription of classical TSH target genes and enhances cAMP production. Although the functional role of TSH in human HF biology awaits further dissection, these findings document that intracutaneous TSH-Rs are fully functional *in situ* and that HFs of female individuals are direct targets for nonclassical, extrathyroidal TSH bioregulation. This suggests that organ-cultured scalp HFs provide an instructive and physiologically relevant human model for exploring nonclassical functions of TSH, in and beyond the skin.

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INTRODUCTION

Hair follicles (HFs) represent one of the most hormone-sensitive tissue interaction systems in the mammalian body (Slominski and Wortsman, 2000; Slominski *et al.*, 2000; Stenn and Paus, 2001; Tobin and Kauser, 2005; Ohnemus *et al.*, 2006; Paus *et al.*, 2006) and are exquisitely thyroid hormone-sensitive (Safer *et al.*, 2001; Stenn and Paus, 2001). As numerous endocrine abnormalities are associated with hair loss (alopecia, effluvium) or unwanted hair growth (hirsutism), it is clinically important to comprehensively investigate the entire spectrum of potential (neuro)endocrine controls of human HF growth and pigmentation—well beyond the well-recognized impact of other hormones such as androgens, estrogens, and retinoids on human hair growth (Freinkel and Freinkel, 1972; Billoni *et al.*, 2000; Messenger, 2000; Stenn and Paus, 2001; Larsen *et al.*, 2003; Bravermann and Utiger, 2005; Slominski *et al.*, 2005; Ohnemus *et al.*, 2006).

It has been known for decades that thyroid disorders that lead to elevated or decreased thyroid hormone serum levels are associated with altered human skin and hair structure as well as function (Freinkel and Freinkel, 1972; Messenger, 2000; Safer, 2005a, b). This includes, for example a higher telogen rate (Freinkel and Freinkel, 1972), altered hair diameter (Jackson *et al.*, 1972), dry, brittle, coarse hair (Safer, 2005a), reduced hair bulb cell proliferation (Schell *et al.*, 1991), and hair loss (effluvium/alopecia) in hypothyroidism, as well as increased hair bulb cell proliferation (Schell *et al.*, 1991) and hair loss in hyperthyroidism (Safer, 2005b). However, it is as yet entirely unknown whether thyroid disorder-associated hair abnormalities are exclusively due to altered thyroid hormone levels (which may directly impact on the thyroid hormone-receptor expressing human scalp (HF Billoni *et al.*, 2000)), or whether other endocrine players along the hypothalamic–pituitary–thyroid axis are also involved in mediating any related hair effects. As thyroid abnormalities are typically associated with major fluctuations

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Abbreviations: ACTA, α-actin; BV, blood vessel; CTGF, connective tissue growth factor; CTS, connective tissue sheath; DP, dermal papilla; FLNA, filamin Aα; GPX3, glutathione peroxidase 3; HFs, hair follicles; HS, hair shaft; IVL, involucrin; KRT5, keratin 5; MK, matrix keratinocytes; MTCO1, cytochrome c oxidase 1; N, nerves; NBs, nerve bundles; PKM2, pyruvate kinase; SWG, sweat gland; TBS, Tris-buffered saline; TG, thyroglobulin; TSH, thyroid-stimulating hormone (thyrotropin); TSH-R, thyrotropin-receptor; TTF-1, thyroid transcription factor-1

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in the serum level of thyroid-stimulating hormone (TSH) (Larsen *et al.*, 2003; Bravermann and Utiger, 2005), it is particularly interesting to study the direct effects of TSH, the major pituitary regulator of thyroid hormone synthesis, on human hair growth.

Thyroid-stimulating hormone operates by activating a cognate receptor (TSH-R) expressed on thyroid epithelial cells (Larsen *et al.*, 2003; Bravermann and Utiger, 2005). However, recent evidence also suggests that there are extrathyroidal target cells for TSH stimulation (Drvota *et al.*, 1995; Klein, 2003; Scofield *et al.*, 2005; Garrity and Bahn, 2006), including adipose tissue (Bell *et al.*, 2000; Sorisky *et al.*, 2000). Moreover, both cultured human HF keratinocytes and human dermal papilla fibroblasts express TSH-R mRNA *in vitro* (Slominski *et al.*, 2002), and human skin fibroblasts reportedly express functional TSH-R proteins *in vitro* (Agretti *et al.*, 2005). Therefore, it is intriguing to ask whether normal human skin and/or skin appendages may also serve as a direct target for TSH.

To clarify this is important in the context of our ongoing endeavor to characterize the complex neuroendocrine controls of human HF biology (Kobayashi *et al.*, 2005; Ito *et al.*, 2005a; Kauser *et al.*, 2006; Peters *et al.*, 2006; Slominski *et al.*, 2007). Clinically, such studies may carry added importance, as the TSH-R is one of the most common targets for autoimmunity (Grave's disease), whereas thyroid autoimmunity classically is associated with skin abnormalities (for example myxedema, altered pigmentation and hair texture, effluvium, skin inflammation), whose pathogenesis is poorly understood (Leonhardt and Heymann, 2002; Bravermann and Utiger, 2005; Rose and Mackay, 2006). As it has long been controversially debated whether extrathyroidal TSH-Rs (including intracutaneous TSH-R) are important in the establishment of anti-TSH-R autoimmune responses (Kohn *et al.*, 2000; Rapoport *et al.*, 2000; Daumerie *et al.*, 2002; Slominski *et al.*, 2002; Agretti *et al.*, 2005), we wanted to clarify whether or not normal human skin expresses these (apparently rather immunogenic) autoantigens. There are conflicting reports as to whether TSH-R is expressed in healthy human pretibial and periorbital skin (Rapoport *et al.*, 2000; Daumerie *et al.*, 2002; Agretti *et al.*, 2005), and the published immunohistological evidence remains unpersuasive.

Therefore, we investigated whether human skin in general, and human scalp HFs in particular, express functional TSH-Rs and are direct, nonclassical, extrathyroidal targets for TSH-R-mediated signaling. Owing to the clinical importance of scalp HF, the availability of excess human scalp skin from routine female facelift surgery, and the establishment of optimally suited human HF organ culture assays (Philpott *et al.*, 1990; Foitzik *et al.*, 2006), we opted for studying this region of the integument. TSH-R expression was examined by reverse transcriptase (RT)–PCR and immunohistology, whereas TSH-R-mediated signaling was assessed by cAMP measurement, DNA microarray, and qPCR analysis of normal, microdissected, organ-cultured human scalp HF. In addition, we examined the functional effects of TSH on organ-cultured human scalp HFs.

RESULTS

TSH receptor mRNA and protein are expressed in human scalp skin and hair follicle mesenchyme

RT–PCR and immunohistology were performed in order to clarify whether normal human scalp skin and HFs express TSH-R on the gene and protein level *in situ*. We were able to demonstrate specific transcripts at the expected base-pair length (582 bp) in RNA extracts prepared from freshly microdissected human scalp HFs (the total number of follicles studied per group was 54–60, derived from three separate individuals) in anagen VI (Figure 1a). These transcripts corresponded to specific, TSH-R-like protein immunoreactivity (IR) in human scalp skin and its appendages. Thyroid gland sections were used as positive and negative controls and TSH-R IR was confined to the expected regions (Figure 2).

Intriguingly, and in striking contrast to the classical TSH-R expression by thyroid *epithelial* cells, within the detection limits of our standard immunohistology protocol (ABC peroxidase), TSH-R expression in human scalp skin appeared to be largely restricted to defined mesenchymal compart-

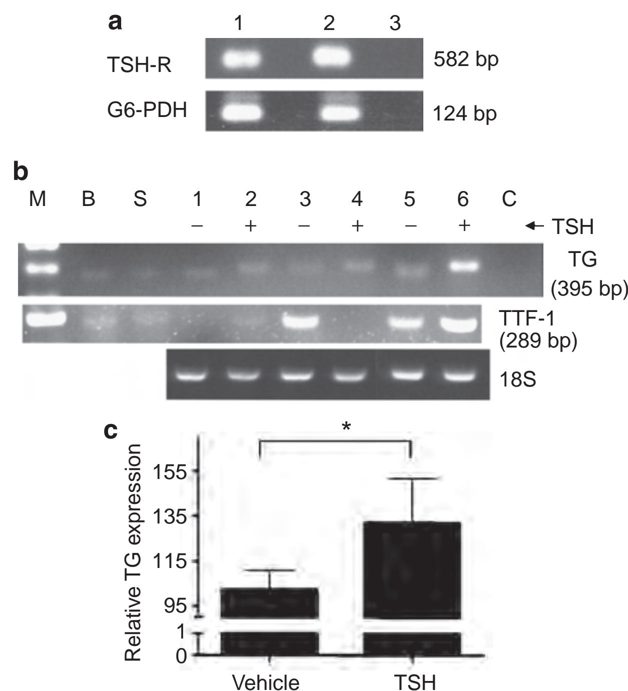


Figure 1. TSH receptor (TSH-R) (a) and thyroglobulin (TG) (b) mRNA is expressed in human scalp skin and hair follicles. TSH administration upregulates TG mRNA expression in hair follicle organ culture (b, c). (**a**) Detection of TSH-R transcripts in human HFs. 1: HFs (30 HFs per group), 2: thyroid gland, 3: negative control; (**b**) 18–20 microdissected hair follicles from three different female patients (1, 2; 3, 4; 5, 6) were treated with either vehicle (1, 3, and 5) or with TSH (2, 4, and 6). A semiquantitative PCR was performed for thyroglobulin (TG) and thyroid transcription factor-1 (TTF-1). M: marker, B: brain (positive control), S: full skin, C: negative control; (**c**) the intensity of TG bands was quantified. Data of TG expression were normalized to the expression of a housekeeping gene (S18, 18S rRNA subunit) of the same sample; average value from three different patients is shown. There was a significant difference ($P<0.05$ by Mann–Whitney *U*-test) found between vehicle and treated groups. Mean ± SEM, the total number of follicles studied per group was 54–60, derived from three separate individuals.

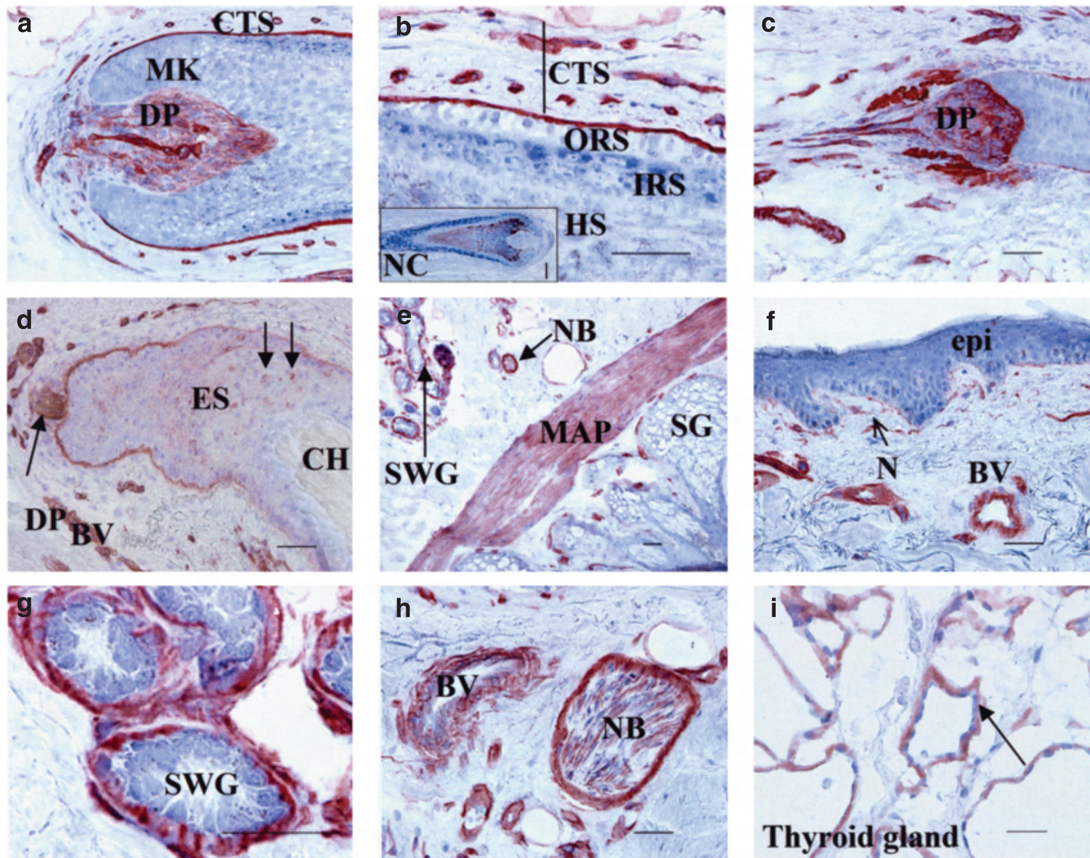


Figure 2. Human scalp hair follicles express thyroid stimulating hormone receptor (TSH-R) protein only in their mesenchyme. (**a**) TSH-R immunoreactivity is restricted to mesenchymal compartments of scalp anagen VI HFs (dermal papilla, DP, and connective tissue sheath, CTS) *in situ*. (**b**) Follicular keratinocytes of scalp skin (IRS: inner root sheath keratinocytes, ORS: outer root sheath, HS: hair shaft) do not express the receptor. NC: negative control (by omitting of the primary antibody). (**c**) Catagen scalp HFs show a similar staining pattern, but a slightly more intensive immunoreactivity in the DP and retracting basal membrane. (**d**) TSH-R IR on telogen hair follicles of buttock skin was predominantly localized (similarly to anagen phase) to mesenchymal compartments (DP, CTS) in comparable intensity. However, we observed few single positive cells in the HF epithelium (s. arrows). ES: epithelial strand, CH: club hair. (**e**) Positive staining was also found on the arrector pili muscle (MAP), nerve bundles (NBs), periglandular myoepithelial cells around the sweat (SWG) and sebaceous glands (SGs). (**f**) Cutaneous nerves (N) and blood vessels (BV). (**g**) Higher magnification of positively stained sweat glands (SWG). (**h**) Higher magnifications of BVs and NBs. (**i**) The expected TSH-R immunostaining of the basolateral site of thyroid gland epithelial cells served as positive control, whereas the absence of TSH-R immunoreactivity on the apical site of the cells provides an internal negative control (Bravermann and Utiger, 2005). Scale bars = 50 μm.

ments of the pilosebaceous unit (namely, to the follicular dermal papilla (DP), the connective tissue sheath (CTS), and the arrector pili muscle), but was not seen in interfollicular dermal fibroblasts (Figure 2a–c and e). Furthermore, also isolated, CD90-positive DP fibroblasts expressed TSH-R *in vitro* (Figure 5e). In addition, large cutaneous nerve bundles (NBs), blood vessels (BVs), and periglandular myoepithelial cells around sweat (SWG) and sebaceous glands also showed prominent TSH-R-like IR (Figure 2d–h) *in situ*. The intensity of TSH-R IR did not exhibit noticeable interindividual variations in the 13 female individuals whose scalp skin and HFs were examined.

Next we investigated whether TSH-R expression shows hair-cycle-dependent changes *in situ*. The localization of TSH-R IR in catagen scalp HFs showed a rather similar staining pattern as that of anagen VI scalp HFs ones (mesenchymal localization), but a slightly more intensive staining in the DP and in the retracting HF basement membrane (Figure 2c). As we had only access to telogen HFs from human gluteal skin, we performed TSH-R staining on these skin samples, which may exhibit regional differences compared to human scalp skin. Similar to anagen VI HFs, TSH-R IR was predominantly localized to mesenchymal compartments (DP, CTS) in comparable intensity. However, in these telogen samples, we also observed few single TSH-R-IR cells in the HF epithelium (Figure 2d). Thus, there appear to be only minor TSH-R expression changes during human HF cycling, and the restriction of TSH-R expression to the HF mesenchyme seen in terminal scalp HFs may not hold true for all other human skin HF populations and locations.

TSH does not significantly modulate human hair growth and pigmentation *in vitro*

To study direct, functional effects of TSH on classical hair biology parameters (that is hair shaft (HS) growth, follicle cycling, and pigmentation of isolated human HFs), 1–100 mU TSH ml^{-1} was administered to the serum-free culture medium of microdissected, organ-cultured human

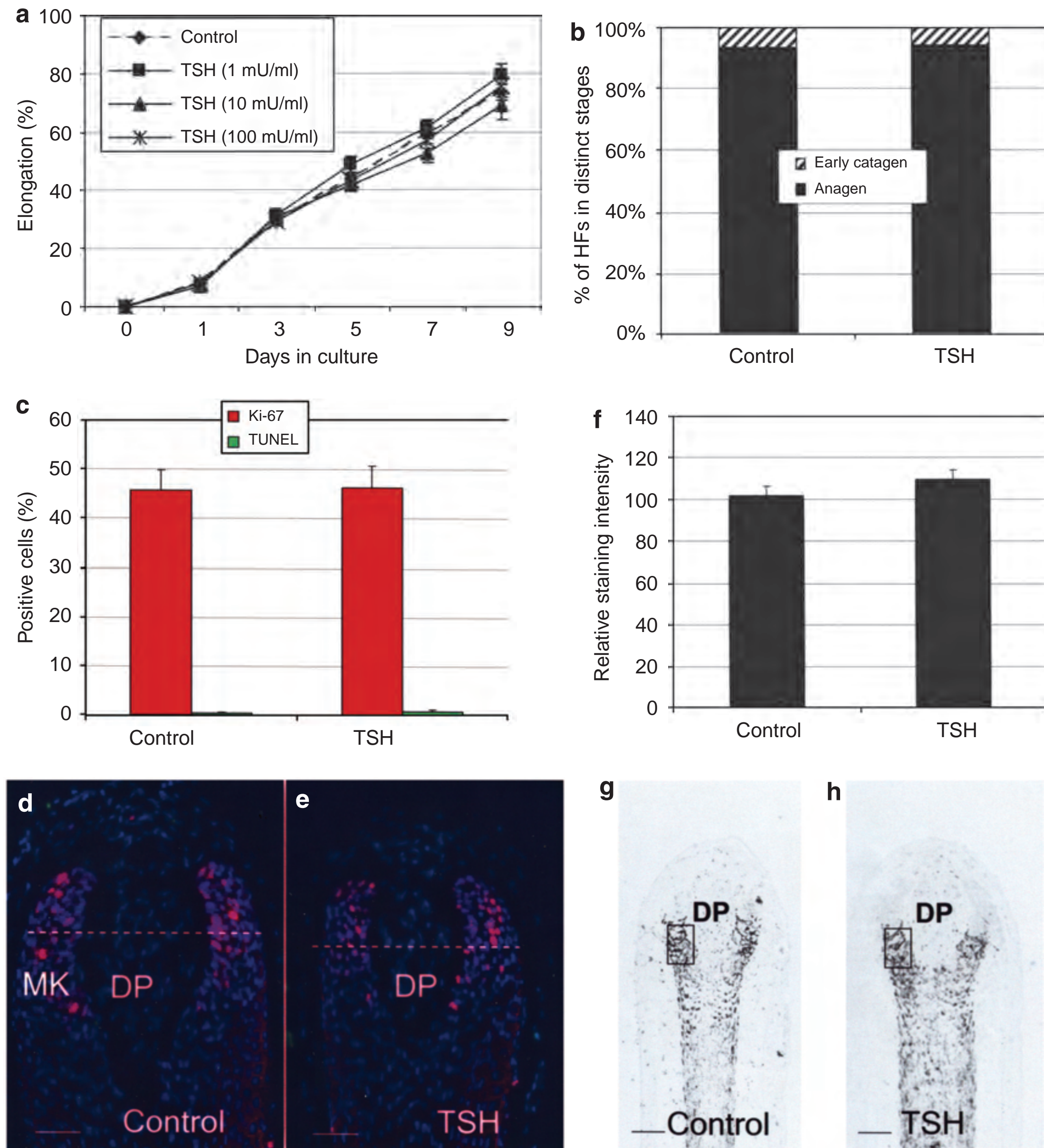


Figure 3. High-dose TSH does not significantly modulate human hair shaft elongation (a), HF cycling (b), HF keratinocyte proliferation/apoptosis (c–e) and pigmentation (f–h) *in vitro*. (**a**) Anagen VI HFs from human scalp skin were treated with three different TSH concentrations, and hair shaft elongation was measured every second day. (**b**) Quantitative hair cycle staging of vehicle- or TSH-treated HFs (shown are only the data for HFs treated with 100 mU ml^{-1} TSH; the results for 1 and 10 mU ml^{-1} TSH were very similar). The percentage of anagen VI and early catagen follicles was determined by morphological analysis (quantitative hair cycle histomorphometry). (**c**–**e**) Ki-67 (proliferation—red)/TUNEL (apoptosis—green) double immunofluorescence shows no significant differences (**c**) between (**d**) vehicle- and (**e**) TSH-treated groups upon quantitative immunohistomorphometry. Positive cells were counted only in the hair matrix below Auber's level (dotted white line). (**f**–**h**) Melanin (produced by the HF pigmentary unit) was visualized by Masson-Fontana histochemistry and was quantitatively compared the ImageJ software (NIH) in the indicated reference area on 20 test and control HFs. DP: dermal papilla. All these experiments were repeated three times using approximately 20 HFs from three different patients. Representative images are shown. Scale bars = 50 μm, mean ± SEM, N = 15–20.

anagen VI scalp hair bulbs. These doses were selected as they are within the range of customarily employed TSH doses in cell culture studies and as the lowest dose tested here lies only moderately above the elevated TSH serum levels in severely hypothyroid patients, which can reach up as high as 0.5 mU ml^{-1} (Bravermann and Utiger, 2005).

However, as shown in Figure 3a, neither HS elongation (which occurs in this physiologically and clinically highly relevant assay system at almost the same speed as on the living human scalp Philpott *et al*., 1990), nor the rate of hair matrix keratinocyte (MK) proliferation or apoptosis (Figure 3c–e) were significantly altered by high-dose TSH administration. We also asked whether TSH influences the anagen–catagen transition, the only segment of human HF cycling that can currently be studied *in vitro* (Philpott *et al*., 1990; Philpott, 1999; Jarrousse *et al*., 2001; Paus and Foitzik, 2004;

Ito *et al.*, 2005b). Likewise, TSH did not significantly alter human HF cycling *in vitro*, as measured by quantitative hair cycle histomorphometry (Foitzik *et al.*, 2006; Bodó *et al.*, 2005) (Figure 3b). TSH also failed to significantly alter intrafollicular melanin production, as assessed by quantitative Masson-Fontana histochemistry (Figure 3f–h).

TSH does not induce marked changes in human HF mesenchyme proliferation *in situ*

It had previously been proposed that pretibial and periocular skin dermal fibroblasts may be stimulated by TSH by cognate receptors (Porcellini *et al.*, 2003). As our own analyses had revealed very prominent TSH-R expression in the fibroblasts of the CTS of human scalp HFs, we specifically checked for differences in the number of Ki-67-positive cells in this mesenchymal HF compartment between vehicle- and TSH-treated HFs. However, by quantitative immunohistomorphometry, the number of proliferating cells/microscopic field did not differ significantly between test and control HFs *in situ* (vehicle: 2.03 ± 1.21 SEM, TSH: 3.36 ± 2.59 SEM, $P > 0.05$). Immunohistologically, TSH did not substantially up- or downregulate the IR intensity or pattern of its cognate receptor in organ-cultured human scalp HFs (Figure S1A–D, Supplementary Materials and Methods).

TSH administration stimulates cAMP secretion into the culture medium

These disappointing results had left us without any evidence that the prominently expressed TSH-R proteins in human scalp skin and HFs are functionally active. Therefore, we next analyzed whether engagement of the TSH-R by its high-affinity ligand is associated with any change in the cAMP level, as cAMP upregulation is the classical second messenger of TSH-R activation (Povey *et al.*, 1976; Bravermann and Utiger, 2005). As shown in Figure 4a, this is indeed the case: $30.02 \pm 2.38\,\text{pmol ml}^{-1}$ cAMP was secreted into the culture medium after TSH treatment (in the presence of isobutil-1-methylxanthine, a potent inhibitor of cAMP degradation to AMP), a three times higher cAMP level than found in the supernatant of vehicle-treated control HFs. This indicates that TSH-Rs expressed in the CTS of human scalp HF engage in their expected signaling activity. This finding is in agreement with the previous demonstration that TSH stimulates cAMP production in HaCaT keratinocytes and melanoma cells in a dose-dependent manner (Slominski *et al.*, 2002). This again supports the above conclusion.

TSH treatment of human HFs does not induce measurable thyroid hormone secretion, but upregulates the transcription of classical TSH target genes (thyroglobulin, thyroid transcription factor-1)

Next we also explored whether this TSH-R signaling activity alters the transcription of classical TSH target genes. As the most prominent function of TSH in the thyroid gland is the stimulation of thyroid hormone synthesis and secretion (Bravermann and Utiger, 2005), we first asked whether human scalp HFs exhibit the enzymatic apparatus to produce thyroid hormones. However, we were not able to detect the key enzyme for thyroid hormone production, thyroid peroxidase (Bravermann and Utiger, 2005), either at mRNA (data not shown), or at protein levels (Figure S1E and F). Furthermore, we searched for the presence of thyroid hormones in the supernatant of cultured human scalp HFs by electrochemiluminescence immunoassay and did not find any evidence for measurable thyroid hormone above background levels in the culture medium (van Beek *et al.*, 2008). Thus, human scalp HFs do not seem to be able to produce thyroid hormones, constitutively or in response to TSH stimulation.

Subsequently, we studied whether TSH stimulation of organ-cultured human scalp HFs modulates classical target genes of TSH-R stimulation. TSH upregulates expression of the chief matrix glycoprotein for thyroid hormone synthesis and storage, thyroglobulin (TG), by specific transcription factors, for example thyroid transcription factor-1 (TTF-1), (Suzuki *et al.*, 1998, 1999; Bravermann and Utiger, 2005). Therefore, the level of intrafollicular TTF-1 and TG transcription with and without TSH stimulation was assessed by RT–PCR (18–20 follicles per group from 3 different patients were studied).

Although the TG mRNA was detected in keratinocytes and some melanoma cells cultured *in vitro* by RT–PCR (Slominski *et al.*, 2002), extrathyroidal TG transcription in human skin *in situ*, has not been reported before. Thus, both RNA extracted from freshly microdissected human scalp HF (not shown), or from organ-cultured, vehicle-treated HF contained TG transcripts of the expected length (395 bp) as shown by RT–PCR (cp. Figure 1b). The specificity of the amplification products was confirmed by sequence analysis, which revealed full agreement with the DNA sequence of the most recently published sequence of human TG (GenBank accession number NM_003235) (Figure S2, Supplementary Materials and Methods). Moreover, TSH stimulation was indeed able to upregulate the steady-state level of TG transcripts (Figure 1b and c). However, employing different primary antibodies and distinct secondary detection systems, we failed to obtain convincingly specific immunohistological evidence for the translation of TG transcripts into TG protein in human scalp skin (that is the IR patterns observed were not convincingly and reproducibly above background; data not shown). However, we cannot yet rule out definitively that TG mRNA is translated.

In the same HF samples, we also detected specific transcripts for TTF-1 (289 bp RT–PCR fragment), whose mRNA steady-state levels were also upregulated after TSH administration in 2 of 3 tested patients (Figure 1b). This TSH-induced upregulation of TG and TTF-1 transcripts suggests that the TSH-Rs of human scalp HFs are indeed functionally active and that the intrafollicular stimulation of TG transcription by TSH occurs in the recognized manner (that is by TTF-1) (Suzuki *et al.*, 1998, 1999).

TSH stimulation alters the gene expression profile of human scalp hair follicles and reveals previously unknown extrathyroidal TSH target genes

To obtain additional evidence for TSH-R functionality, the expression of extrathyroidal target genes of TSH stimulation was investigated in two independent sets of organ-cultured

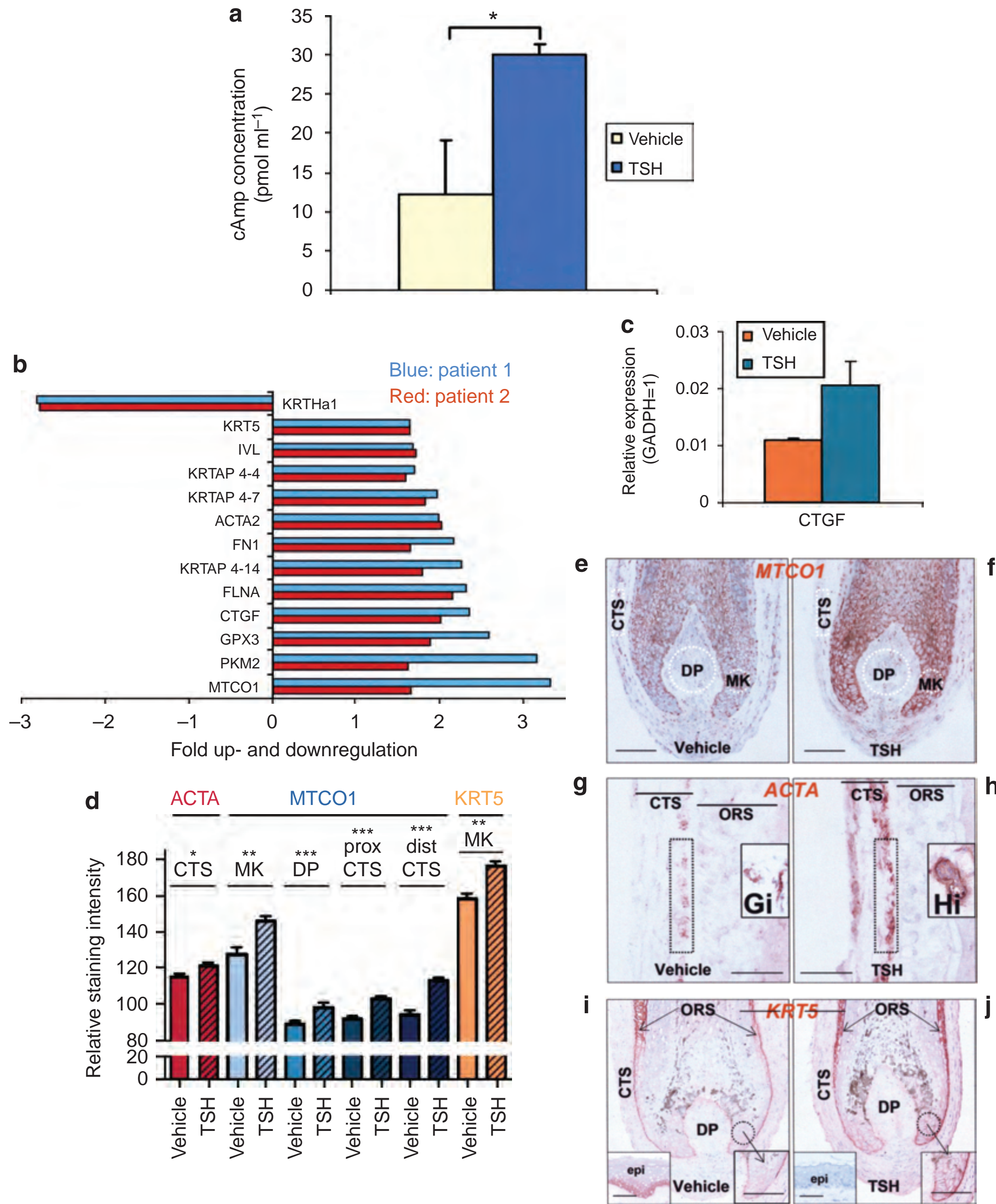


Figure 4. TSH administration increases cAMP secretion into the culture medium and causes differential gene expression changes. (**a**) HFs were treated with TSH (100 mU ml^{-1}) and cAMP were measured using a competitive assay by R&D Biosystems. TSH induced a threefold increase of cAMP concentration compared to the control. (**b**) Approximately 30 HFs of two different female donors (patient 1 in blue and patient 2 in red) were treated with vehicle or TSH (100 mU ml^{-1}) and microarray analysis (Human Whole Genome Microarray; Miltenyi, Cologne, Germany; expression differences visualized by Agilent technique) was performed. When only genes with equidirectional changes in both individuals were included, where—in addition—*P*-value was <0.0001 and fold changes were >1.5, 13 differentially regulated genes were identified: acidic hair keratin 1 (Ha1, now K31 gene: KRTHa1, now KRT31), keratin K5 (KRT5), involucrin (IVL), keratin associated proteins KAP 4-4 (KRTAP 4-4), KAP 4-7 (KRTAP 4-7), KAP 4-14 (KRTAP 4-14), α-actin 2 (ACTA2), fibronectin 1 (FN1), filamin Aα (FLNA), connective tissue growth factor (CTGF), glutathione peroxidase 3 (GPX3), pyruvate kinase (PKM2), cytochrome *c* oxidase 1 (MTCO1) (see also Table 1). (**c–j**) Confirmation of selected TSH target candidate genes. (**c**) Relative expression of CTGF by real-time quantitative-PCR. TSH treatment upregulates (**f**) MTCO1, (**h**) ACTA, and (**j**) KRT5 protein expressions compared to the vehicle-treated follicles (**e**, **g**, **i**, respectively). Staining intensity was measured (**d**) in previously defined, standardized regions (indicated by circles or quadrangles). (Gi, Hi): positive controls of the α-MSA staining: immunoreactivity in dermal blood vessels and sweat glands, respectively. CTS, connective tissue sheath; DP, dermal papilla; MK, matrix keratinocytes; ORS, outer root sheath keratinocytes; epi, epidermis; prox, proximal; dist, distal. Mean ± SD, ****P*<0.001, ***P*<0.01, **P*<0.5. Scale bars = 50 μm.

human scalp HFs (derived from two different, healthy women). The DNA microarray was performed from samples that had been treated with TSH (100 mU ml^{-1}) or vehicle. Rigid selection criteria were employed to single-out ''differentially expressed'' genes by accepting only equidirectional expression changes in HF RNA extracts from both examined individuals with a *P*-value of <0.0001, and fold changes of >1.5 as strong indications for ''differential gene expression'' after HF stimulation with TSH. By these selection criteria and possible skin/HF-relevance further genes were selected: KRTHa1, keratin 5 (KRT5), involucrin (IVL), keratin-associated protein 4-4, keratin-associated protein 4-7, α-actin 2 (ACTA2), fibronectin 1, keratin-associated protein 4-14, filamin Aα (FLNA), connective tissue growth factor (CTGF), glutathione peroxidase 3 (GPX3), pyruvate kinase (PKM2), cytochrome *c* oxidase 1 (MTCO1), respectively (Figure 4b; Table 1).

For selected genes, TSH-induced gene expression was confirmed by examining HFs from a third and fourth, distinct patient by quantitative real-time PCR (for CTGF, GPX3, and PKM2, from HF homogenate) and/or by immunhistochemistry (KRT5, IVL, ACTA (α-isoform, but not aorta-specific ACTA2 was investigated), FN (isoform not specified), FLNA, and MTCO1). It has to be noted that, to our knowledge there are no reliable, well-established antibodies to ACTA2 and FN1 available. Therefore a closely related protein of the same protein family was investigated.

As shown in Figure 4c, we could confirm by real-time PCR that TSH upregulates CTGF transcription, whereas the transcription level of GPX3 and PKM2 remained unaltered (Figure S1G). By quantitative immunohistomorphometry, significantly altered protein expression *in situ* was evident for ACTA, MTCO1, and KRT5. As shown in Figure 4g, ACTA immunohistochemistry showed an intensive staining in human CTS fibroblasts *in vitro*, which further increased after TSH stimulation. TSH-like IR also localized to SWG myofibroblasts and BV smooth muscle *in situ* (inserts in Figure 4g and h), serving as positive control. The ACTA immunostaining intensity of human CTS fibroblasts *in situ* was significantly enhanced (*$P<0.05$) in TSH-treated follicles (Figure 4d, g and h). Furthermore, after TSH stimulation of organ-cultured human HFs, MTCO1 was upregulated in several compartments of the HF (MKs, DP, proximal and distal CTS) (Figure 4d–f). Also, TSH stimulated significantly K5-IR in the hair matrix (Figure 4d, i and j).

These data convincingly document that the TSH-Rs expressed by normal human scalp HF *in situ* are functionally active, and suggest several (direct or indirect) target genes for TSH-R-mediated signaling in extrathyroidal human tissues such as the skin, namely CTGF, MTCO1, ACTA, and KRT5.

Cultured human dermal papilla fibroblasts are direct targets of TSH-R-mediated signaling

As all TSH effects described here so far had been seen in complex miniorgans, which are composed from several cell populations of different origin and function, we finally checked whether TSH-R expressing, isolated human HF cell populations also respond to TSH stimulation. Therefore we performed a DP cell culture, employing passage 1 and 2 cells (Figure 5). By immunofluorescence microscopy analysis, TSH-R was identified on cultured DP fibroblasts (Figure 5a), which also were immunoreactive for the fibroblast marker CD90, as expected (Figure 5a). TSH treatment significantly and dose-dependently increased the secretion of cAMP by DP cells into the culture medium (Figure 5b). Similar to organ-cultured HF, treatment of cultured DP fibroblasts with TSH prominently and dose-dependently upregulated the expression of specific transcript steady-state levels: As seen in Figure 5c, the mRNA steady-state levels for PKM2, GPX3, CTGF, and MTCO1 were 16- to 75-fold higher in DP cells treated with 100 mU ml^{-1} TSH than in vehicle-treated control DP cells. The upregulation of MTCO1 was also verified by immunofluorescence (Figure 5d). These data underscore that TSH-R expressing cellular key constituents of the human HF mesenchyme (that is DP cells) retain their responsiveness to the cognate ligand even upon isolation, culture, and repeated passage.

DISCUSSION

Here, we show that human scalp HFs are direct targets for TSH, and identify TSH-R-mediated signaling as a to our knowledge this is previously unreported neuroendocrine pathway in human skin, besides the well-recognized endo-

Table 1. TSH causes differential gene expression changes in human scalp HFs

Name	Microarray	Confirmed by qPCR	Confirmed by IH
Cytochrome *c* oxidase I	↑	×	Yes
Connective tissue growth factor	↑	Yes	×
Actin, α2, smooth muscle	↑	×	Yes
Keratin-associated protein KAP 4-4	↑	×	×
Keratin-associated protein KAP 4-7	↑	×	×
Keratin-associated protein KAP 4-14	↑	×	×
Keratin K5	↑	×	Yes
Keratin, hair, acidic, 1Ha1K31	↓	×	×

Abbreviations: ↑, upregulated; ↓, downregulated; ×, not investigated; yes, microarray could be confirmed.

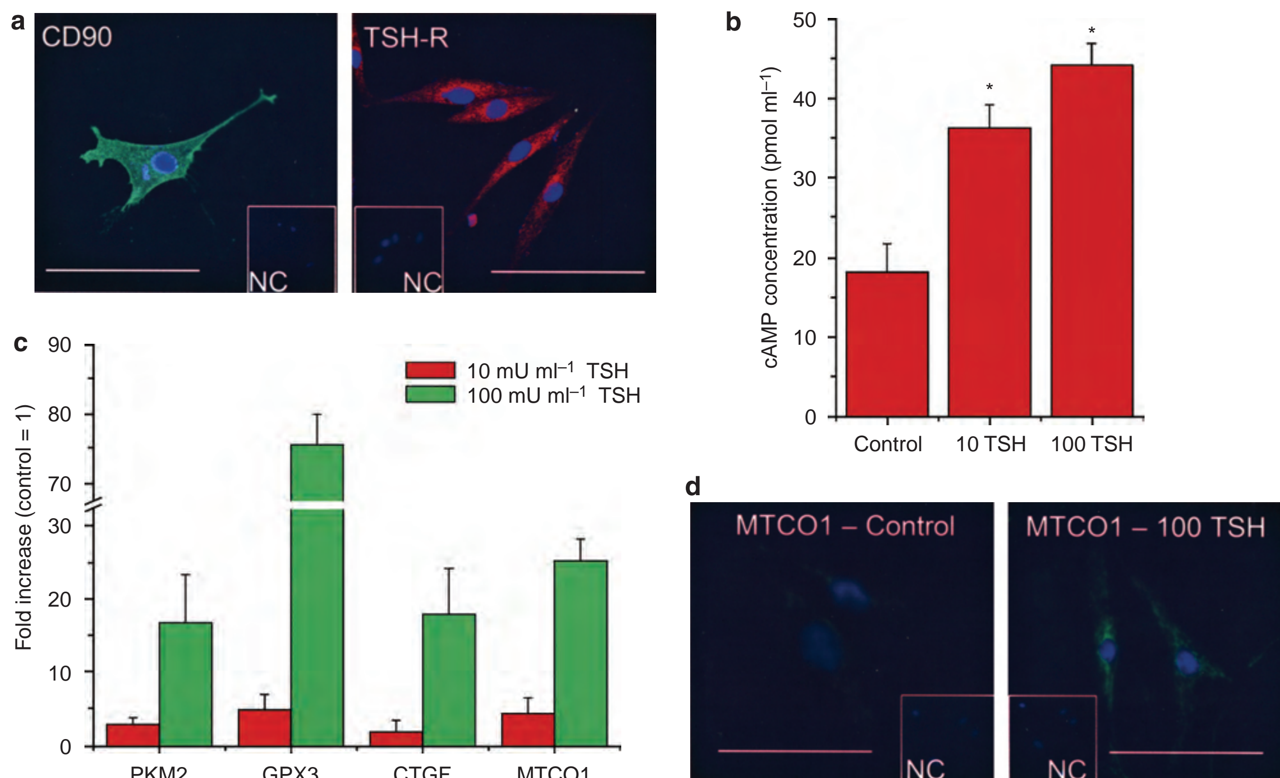


Figure 5. Cultured dermal papilla fibroblasts serve as functional, direct target for TSH signaling. (**a**) Cultured, CD90-positive dermal papilla cells express TSH-R. NC, negative control (by omitting the primary antibody). (**b**) Dermal papilla cells were treated with TSH (10 and 100 mU ml^{-1}) and cAMP concentration was measured in the culture supernatant. (**c**) Treatment of cultured DP fibroblasts with TSH upregulated the expression of some specific mRNA transcripts in a dose-dependent manner. Pyruvate kinase (PKM2), glutathione peroxidase (GPX3), connective tissue sheath growth factor (CTGF), cytochrome *c* oxidase 1 (MTCO1) transcription was investigated by quantitative real-time PCR. (**d**) The upregulation of MTCO1 was also verified by immunofluorescence. NC, negative control (by omitting the primary antibody). Scale bars = 50 μm.

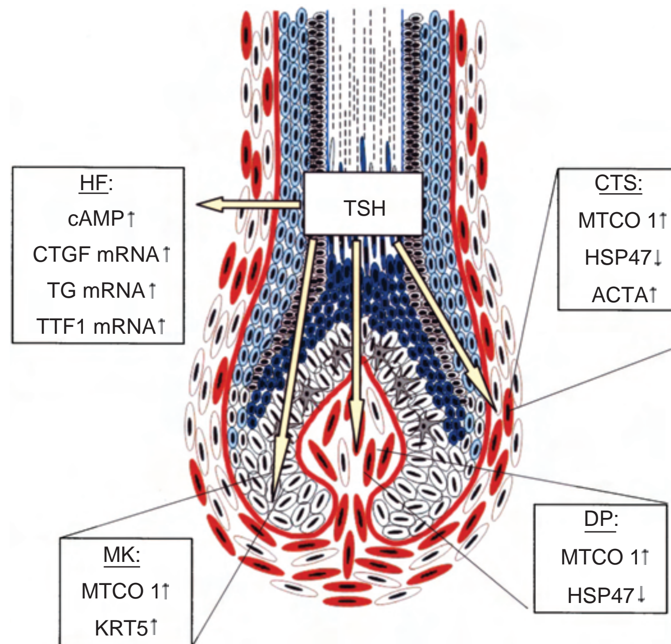
crine modulation of human skin and HF biology, for example by thyroid hormones, androgens, and estrogens (Freinkel and Freinkel, 1972; Billoni *et al.*, 2000; Messenger, 2000; Stenn and Paus, 2001; Larsen *et al.*, 2003; Bravermann and Utiger, 2005; Ohnemus *et al.*, 2006).

However, TSH failed to show significant effects on human HS elongation, anagen duration, and hair matrix proliferation in female human scalp HF organ culture. Despite the presence of functional TSH-Rs in human scalp HFs, the elevated levels of TSH seen in hypothyroid patients, therefore, do not likely reflect direct TSH effects, but rather result from thyroid hormone effects: Indeed, we have just shown that both tetraiodothyronine and thyroxin prolong the duration of anagen in organ-cultured human HFs (van Beek *et al.*, 2008).

If intracutaneous TSH-R expression plays any role in antithyroid autoimmunity at all, our data also suggest that CTS and DP fibroblasts—rather than interfollicular dermal fibroblasts—may present the autoantigens against which TSH-R autoantibodies are generated (Kohn *et al.*, 2000; Leonhardt and Heymann, 2002; Bravermann and Utiger, 2005; Rose and Mackay, 2006). By immunhistochemistry, TSH-R expression was almost exclusively observed in the mesenchymal compartment of scalp skin HFs, whereas interfollicular dermal fibroblasts of normal human scalp and buttock skin did not show TSH-R IR *in situ*. Intriguingly, the human HF mesenchyme, appears to enjoy some (relative) protection from immune rejection (Paus *et al.*, 2006), even upon allotransplantation across the gender barrier (Reynolds *et al.*, 1999). One wonders, therefore, whether the development of anti-TSH-R autoimmunity and subsequent thyroid disease may be causally linked to a prior collapse of immune sequestration of/tolerance to HF mesenchyme-associated TSH-R antigens.

The mesenchymal expression of TSH-R in human scalp HFs also may explain why TSH stimulation did not alter intraepithelial hair growth parameters (that is HS elongation, hair matrix proliferation/apoptosis, and HF pigmentation), as the HF epithelium and the HF pigmentary unit embedded in it (Tobin and Paus, 2001) apparently do not express TSH-R during anagen VI or catagen. However, the fact that the expression of clearly intraepithelial genes and/or gene products (such as keratin 5, hair-keratin Ha1/K31 gene (KRTHa1, now KRT31 Langbein and Schweizer, 2005; for new keratin nomenclature see Schweizer *et al.*, 2006)) was also altered by TSH suggests that the stimulation of cognate receptors in HF mesenchyme exerts indirect signaling effects that profoundly impact on HF epithelial functions.

Thus, it is conceivable that TSH stimulation of cognate receptors in the CTS of the HF upregulates production and



Observation/effect	Found in HF
TSHR mRNA expression	Yes
TSHR protein expression	Yes
TSH → TSHR → cAMP↑	Yes
TSH → TSHR → TGmRNA↑	Yes
TSH → TSHR → TTF1 mRNA↑	Yes
TSH → TSHR → CTGF mRNA↑	Yes
TSH → TSHR → ACTA mRNA and protein↑	Yes
TSH → TSHR → MTCO 1 mRNA and protein↑	Yes
TSH → TSHR → KRT5 mRNA and protein↑	Yes
TSH → TSHR → HSP47 protein↑	Yes

Figure 6. TSH regulates the expression of several epithelial and mesenchymal factors by functional TSH-R expressed in the scalp HF mesenchyme. TSH-R expression in human scalp skin was exclusively observed in the mesenchyme of the HFs (in red). TSH-R activation enhanced cAMP production, connective tissue growth factor (CTGF), thyroglobulin (TG), and thyroid transcription factor-1 (TTF1) transcription in organ-cultured HFs. TSH treatment stimulated MTCO1 mRNA and protein expression in the dermal papilla (DP), connective tissue sheath fibroblasts (CTS), and matrix keratinocytes (MK). CTS fibroblasts upregulated the molecular chaperone HSP47 (heat-shock protein 47), whereas downregulated α-smooth muscle actin (ACTA) expression after TSH treatment. We observed enhanced keratin K5 (KRT5) mRNA and protein expression in the MK.

secretion of diffusible factors—such as CTGF (Table 1; Figure 4b and c). These HF-mesenchyme-derived factors may then alter specific functions of the HF epithelium (such as the production of selected hair keratin and keratin-associated proteins; Table 1) in a paracrine manner. Central, and possibly peripheral, TSH secretion may thus trigger complex regulatory loops that recruit, for example mesenchymal factors (such as CTGF) to indirectly modulate HF epithelial functions (Figure 6). The characteristics of these epithelial–mesenchymal interactions may slightly differ between integumental regions and hair cycle stages, as indicated by our results on TSH-R IR in human buttock telogen HFs, where a few TSH-R-positive cells were identified even inside the HF epithelium. In addition, we cannot exclude a direct action of TSH on epidermal keratinocytes, as TSH-R is transcribed in adult and neonatal keratinocytes, and as TSH stimulation increased cAMP in HaCaT keratinocytes *in vitro* (Slominski *et al.*, 2002).

That two classical TSH target genes, *TG* and *TTF-1*, are upregulated in human scalp HFs after TSH treatment *in vitro* at the transcriptional level (Figure 1), serves as further evidence for the functionality of the intrafollicularly

expressed TSH-R receptors, and blends in well with the demonstration that TSH activates the cAMP signaling pathway (Figure 4) that is characteristically employed by the TSH-R (Povey *et al*., 1976; Bravermann and Utiger, 2005). Although we had previously found TG transcripts in a human keratinocyte line (HaCaT cells) and in some human melanoma cell lines *in vitro* (Slominski *et al*., 2002), TG transcripts had never before been detected in normal human skin *in situ*. Thus, our findings provide the evidence that TG is transcribed in human scalp skin and HFs, and suggest that HFs utilize the same transcription factor for upregulating TG expression that is employed by thyroid epithelial cells (that is TTF-1) (Suzuki *et al*., 1998, 1999).

Furthermore, we show that human scalp HF offer an easily handled, physiologically relevant discovery tool for identifying previously unreported, nonclassical TSH target genes. This unconventional, but instructive new model provides important pointers to previously unknown TSH functions well beyond skin and hair biology. Our differential gene expression analyses identify several TSH target genes in human scalp HFs that have not previously been considered in the context of TSH/TSH-R biology (for example keratin associated protein genes KAPs, CTGF, ACTA), which—directly or indirectly—are regulated by TSH. Gene and/or protein expression changes for CTGF, ACTA, MTCO1, and K5 were independently confirmed by quantitative PCR and/or quantitative immunohistomorphometry in HFs derived from additional patients, while the differential transcription of other genes (FLNA, IVL) identified by microarray analysis could not be confirmed in this manner.

Although the functional significance of these TSH target genes in human skin and HF biology requires systematic further dissection, our data indicate that, in extrathyroidal tissues, TSH exerts functions well beyond its classical spectrum (that is stimulation of thyroid hormone production and thyroid growth; regulation of thyroid enzyme production (Kohrle, 1990; Larsen *et al*., 2003; Bravermann and Utiger, 2005)): For example, FLNA is an important element of normal connective tissue function in human skin (Gomez-Garre *et al*., 2006), whereas CTGF modulates WNT signaling and interacts with the WNT receptor complex (Mercurio *et al*., 2004; Mou *et al*., 2006)—key regulators of HF morphogenesis and cycling (Schmidt-Ullrich and Paus, 2005). The observed stimulation of follicular MTCO1 expression *in situ* by TSH raises the possibility that TSH also impacts on HF energy metabolism by upregulating, for example MTCO1 (Sheehan *et al*., 2004). TSH also directly upregulates PKM2, glutathione peroxidase, and mitochondrial cytochrome *c* oxidase in human DP fibroblasts *in vitro*, further supporting a role for TSH signaling in HF metabolism and oxidation processes.

That TSH upregulates the myofibroblast marker ACTA, in turn, may suggest that TSH can promote the transformation of normal fibroblasts to myofibroblasts, as it typically occurs in wound healing (Tomasek *et al*., 2002; Werner and Grose, 2003). We speculate that downregulation of HSP47 and the simultaneous upregulation of ACTA may indicate a transformation of HF-associated fibroblasts towards a myoepithelial phenotype (Goldberg *et al*., 2007; Hinz, 2007; Lygoe *et al*., 2007). As the follicular CTS is also prominently involved in wound healing (Gharzi *et al*., 2003) and as HF organ culture goes along with substantial wounding (that is microdissection), human HF organ culture may serve as an unconventional, but instructive model for exploring the role of TSH in cutaneous wound healing.

These examples illustrate that the human HF invites exploitation as a uniquely instructive, clinically relevant model system for dissecting previously unreported nonclassical, peripheral activities of TSH in a prototypic neuroectodermal–mesodermal interaction system (Paus and Cotsarelis, 1999; Lindner *et al*., 2000; Stenn and Paus, 2001; Ito *et al*., 2004, 2005a; Kobayashi *et al*., 2005; Schmidt-Ullrich and Paus, 2005; Slominski, 2005; Foitzik *et al*., 2006).

MATERIALS AND METHODS

Hair follicle microdissection, organ culture, and hair shaft elongation

Anagen VI HFs were isolated from normal human scalp skin obtained after written informed patient consent from healthy females undergoing routine face-lift surgery for cosmetic purposes as described (Philpott *et al*., 1990), adhering to Helsinki guidelines and with permission from the ethics committe of the University of Lübeck. Buttock skin was also obtained from plastic surgery from female patient. Isolated HFs were either snap-frozen immediately (for RT–PCR analysis) or organ-cultured as previously described (Bodó *et al*., 2005; Ito *et al*., 2005a; Foitzik *et al*., 2006), following the basic technique pioneered by Philpott *et al*. (1990). Bovine TSH (which activates also human TSH-R Clark *et al*., 1982) (1, 10, 100 mU ml^{-1}) (from Sigma-Aldrich, Taufkirchen, Germany) or vehicle (distilled water) were administered once for each change of culture medium (that is every 48 h). HFs (15–20) were used per group and that all experiments where repeated twice, each experiment using HFs from one (different) individual.

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

Dermal papilla cell culture

After microdissection of anagen VI HF from scalp skin biopsies, isolation of DP fibroblasts from HF was established according to Magerl *et al*. (2002). Outgrowing cells were then cultured in cell growth basal medium supplemented with 4% fetal bovine serum, 5 μg ml^{-1} insulin, and 1 ng ml^{-1} basic fibroblast factor (all from PromoCell, Heidelberg, Germany). The passages of 1–2 were used.

Semiquantitative RT-PCR

Total RNA was isolated from cultured HFs using the RNeasy kit (Qiagen, Hilden, Germany). Total RNA (0.5 μg) was reverse-transcribed with SuperScript First-Strand Synthesis System (Applied Biosystems, Foster City, CA). For the PCR analysis of TSHR standardization of the cDNA samples was performed by amplification of the housekeeping gene G6-PDH using undisclosed primer pairs from Roche (Mannheim, Germany). PCR amplification (94 °C for 2 minutes; 40 cycles of 94 °C for 30 seconds, 58 °C (G6-PDH), or 50 °C (TSH-R) for 30 seconds, 68 °C for 30 seconds) was performed with the following primers (MWG Biotech, Ebersberg,

Germany): TSH-R forward: ATGAGGCGATTTCGGAGGATGGA; TSH-R reverse: ATGCATGACTTGGAATAGTTCTC.

For the semiquantitative PCR analysis of TG and TTF-1 (TTF) the quality and quantity of cDNA in all samples were standardized by the amplification of housekeeping gene 18S rRNA subunit. PCR amplification (95 °C for 2.5 minutes; 25 (18S), 35 (TG), or 30 (TTF1) cycles of 94 °C for 30 seconds, 60 °C (18S), 64 °C (TG), or 58 °C (TTF1) for 30 seconds, 72 °C for 30 seconds; 72 °C for 5 minutes) was performed with the following primers (Integrated DNA Technology Inc., Corallville, IA): 18S forward: TTCGGAACTGAGGCCATGAT, 18S reverse: TTTCGCTCTGGTCCGTCTTG, TG forward: CCGCCG TCATCAGCCATGAG, TG reverse: TGAGTCCTCGCCACCCAGA GAA, TTF1 forward: CAGTGTCTGACATCTTGAGT, TTF1 reverse: AGCGCTGTTCCGCATGGTGT. PCR products were visualized on a 0.7% (TSH-R) or 2% (TG, TTF-1) agarose gel with ethidium bromide and the photographed bands were quantified by the ImageJ software (National Institutes of Health, Bethesda, MD). Data of TG expression were normalized to the expression of 18S of the same sample. For TG transcripts, brain extracts served as positive control. Nontemplate control (by omitting RNA) was used as negative control.

DNA sequence analysis

The specificity of the amplification of the TG cDNA was assessed by a melting curve analysis on a Light Cycler (Roche). In addition, the sequence of the TG cDNA obtained after PCR amplification was analyzed following the dideoxy method using the forward and reverse PCR primer, respectively. DNA sequences were analyzed by 377 DNA sequence analyzer (Applied Biosystems) (Sanger, 1981).

Quantitative immunhistochemistry and immuncytochemistry

For the detection of TSH-R, alpha-smooth muscle actin (ACTA) protein, keratin 5 and cytochrome *c* oxidase I, peroxidase-based ABC (avidin–biotin complex, Vectastain; Vector, Wertheim, Germany) (Foitzik *et al.*, 2006) immunohistology was performed (Table 2). Aceton-fixed, 8 μm thick human scalp skin/HF frozen sections were blocked with H_2O_2 (3% in phosphate-buffered saline, 15 min). After 20 min pretreatment with goat normal serum (10% in phosphate-buffered saline), slides were incubated with the primary antibodies (for dilutions see Table 2, antibodies were diluted in Tris-buffered saline (TBS) percent goat normal serum) over night at 4 °C, followed by a secondary biotinylated goat anti-mouse/rabbit/guinea pig antibody (1:200; Jackson ImmunoResearch Laboratories, Cambridgeshire, UK), respectively. Subsequently, streptavidin-conjugated horseradish peroxidase (Vectastain ABC kit; Vector; 30 min) and its substrate (peroxidase substrate kit, AEC; Vector) were applied. Cell nuclei were counterstained with hematoxylin (Sigma-Aldrich). For TSH-R immunohistology, frozen human thyroid sections were used as positive and internal negative controls, and primary antibody was omitted as an additional negative control. Human skin served as positive control for ACTA, and keratin 5 stainings: ACTA is expressed by the myoepithelial cells of SWGs (Schon *et al.*, 1999) and BVs (Scofield *et al.*, 2005), whereas basal layer of epidermal and outer root sheath keratinocytes express keratin 5 (Langbein *et al.*, 2005). The intensity of these immunostainings was quantified by ImageJ software (National Institutes of Health). Staining intensity of defined reference regions in the HF (see figure legends) was measured and compared between control and TSH-treated groups.

For the immunodetection of TSH-R on cultured DP fibroblasts, acetone-fixed (5 min, −20 °C) CTS cells were preincubated with 10% normal goat serum in TBS followed by the overnight incubation with the TSH antibody (1:100 in TBS + 2% goat normal serum). For the detection of the primary antibody, the Rhodamine Red-conjugated goat anti-mouse antibody was used (1:200 in TBS; Jackson ImmunoResearch Laboratories), finally slides were counterstained with DAPI.

For the detection of the fibroblast marker CD90 as well as MTCO1, similar labeling procedures were performed using mouse anti-CD90 (1:100 in TBS; Dianova, Hamburg, Germany) and anti-MTCO1 (1:50 in TBS; Mitosciences, Eugene, OR) primary antibodies, respectively. In both cases, visualization was performed using goat anti-mouse FITC-conjugated (1:200 in TBS; Vector) secondary antibodies.

Quantitative histomorphometry and hair cycle analysis

For HF cycle staging hematoxylin–eosin (Sigma-Aldrich, Taufkirchen, Germany) staining, and for histochemical visualization of melanin, routine Masson-Fontana staining was performed on frozen sections. Quantitative Masson-Fontana histochemistry was employed as described (Ito *et al.*, 2005a) (this simple histochemical method is a very sensitive and reliable indicator of changes in intrafollicular melanin synthesis, as recently shown by standard tyrosinase expression and enzyme activity assays (Kauser *et al.*, 2006), which have confirmed our previous, quantitative Masson-Fontana histo-

Table 2. Antibodies used for immunhistochemistry

Name	Host	Dilution	Method	Source	Clone
TSH-R[1] (Costagliola *et al.*, 1998)	Mouse	1:200, 1:1,000, 1:100	ABC, TSA, indirect IF	InVivo BioTech Services, Henningsdorf, Germany	BA-8
MHC class II (Falini *et al.*, 1984)	Mouse	1:100	Direct IF	DAKO, Glostrup, Denmark	CR3/43
MTCO1[2]	Mouse	1:50	ABC	Mitosciences, Eugene, OR, USA	1D6E1A8
SMA (Skalli *et al.*, 1986)	Mouse	1:50	ABC	Sigma-Aldrich, Taufkirchen, Germany	1A4
Keratin K5 (Langbein *et al.*, 2005)	Guinea pig	1:500	ABC	PROGEN, Heidelberg, Germany	—

TSH-R, thyrotropin-receptor; MTCO1, cytochrome *c* oxidase 1.
[1]The TSHR antibody was directed against the TSH non-binding region of the outer membrane TSH-R domain and was generously provided by InVivo BioTech Services (Costagliola *et al.*, 1998).
[2]For reference staining with the MTCO antibody see www.mitosciences.com/ms404.html.

chemistry-based finding that CRH stimulates melanogenesis in human scalp HFs14). Staining intensity was analyzed in a defined reference region of the HF pigmentary unit, using the ImageJ software (National Institute of Health). Quantitative hair cycle histomorphometry (that is HF cycle staging) was carried out according to well-defined morphological criteria, and the percentage of HFs in anagen or catagen was determined, as described (Bodó *et al*., 2005; Foitzik *et al*., 2006).

Determination of cAMP concentration in culture supernatant

HFs or DP cells were treated for 24 hours with vehicle or TSH ($100\,mU\,ml^{-1}$; Sigma-Aldrich) in the presence of 0.45 mM 3-isobutil-1-methylxanthine (Sigma-Aldrich), a potent inhibitor of camp phosphodiesterase. Culture supernatant was collected for the quantitative determination of cAMP concentration, using Parameter Cyclic AMP Assay (R&D Systems, Wiesbaden-Nordenstadt, Germany).

Microarray and q-PCR analysis of selected candidate genes

Gene expression analysis of HFs from two different individuals (30 HFs per patient) using Human Whole Genome Oligo Microarray (44K) was performed by Miltenyi Biotech GmbH (Bergisch-Gladbach, Germany) as a commercial service. Freshly isolated HFs (20 per group, all derived from a single donor) were treated with vehicle/TSH ($100\,mU\,ml^{-1}$) for 24 hours, total RNA was isolated according to standard protocols (Trizol; Sigma-Aldrich). Quality of total RNA was controlled via the Agilent 2100 Bioanalyzer System. Linear amplification of RNA and hybridization of whole genome oligo microarray was performed according to the manufacturer's protocols.

Candidate genes were selected according to the following criteria: equidirectional expression changes in both individuals, *P*-value <0.0001, >1.5-fold changes. Given the usually exceptionally high congruence of differential gene expression results obtained with Human Whole Genome Oligo Microarray with independent, confirmatory quantitative PCR assays (manufacturer's information), due to the extreme difficulty to obtain sufficient additional human scalp HF samples for such confirmatory analyses, and due to the unavailability of sufficient corresponding funding, microarray results were not confirmed with qPCR. They can therefore be considered only as exploratory. In compensation for this shortcoming, very strict selection criteria were employed to identify only genes that were modulated in a substantial and interindividually reproducible manner.

Selected genes were then subjected to qPCR analysis performed by the company Abiol Biotech (Debrecen, Hungary) as a commercial service of separate RNA extracts derived from a third female individual, using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) and the 5′ nuclease assay (Bodó *et al*., 2005). In addition, expression of selected genes was also defined on cultured DP fibroblasts. From control and TSH-treated HFs (20 per group), and cultured fibroblasts total RNA was isolated using Trizol (Invitrogen). Then, 3 μg of total RNA were reverse transcribed into cDNA by using 15 units of AMV reverse transcriptase (Promega, Madison, WI) and 0.025 μg/μl random primers (Promega). PCR amplification was carried out by using the TaqMan primers and probes (recognizing the following human genes: Assay ID hs00170014_m1 for CTGF, hs00173566_m1 for GPX3, hs00987255_m1 for PKM2), (Assay ID) hs02596864_g1 for MTCO1 using the TaqMan Universal PCR Master Mix Protocol (Applied Biosystems). As internal controls, transcripts of glyceraldehyde 3-phosphate dehydrogenase were determined (Assay ID: Hs99999905_m1 for human glyceraldehyde 3-phosphate dehydrogenase).

Statistical analysis

Data were analyzed using a two-tailed Student's *t*-test for unpaired samples, and $P<0.05$ values were regarded as significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary Materials and Methods

Figure S1. TSH treatment does not influence significantly follicular TSH-R expression.

Figure S2. DNA sequence analysis of human thyroglobulin expressed in hair follicles.

REFERENCES

Agretti P, De Marco G, De Servi M, Marcocci C, Vitti P, Pinchera A *et al.* (2005) Evidence for protein and mRNA TSHr expression in fibroblasts from patients with thyroid-associated ophthalmopathy (TAO) after adipocytic differentiation. *Eur J Endocrinol* 152:777–84

Bell A, Gagnon A, Grunder L, Parikh SJ, Smith TJ, Sorisky A (2000) Functional TSH receptor in human abdominal preadipocytes and orbital fibroblasts. *Am J Physiol Cell Physiol* 279:C335–40

Billoni N, Buan B, Gautier B, Gaillard O, Mahe YF, Bernard BA (2000) Thyroid hormone receptor beta1 is expressed in the human hair follicle. *Br J Dermatol* 142:645–52

Bodó E, Bíró T, Telek A, Czifra G, Griger Z, Tóth BI *et al.* (2005) A hot new twist to hair biology: involvement of vanilloid receptor-1 (VR1/TRPV1) signaling in human hair growth control. *Am J Pathol* 166:985–98

Bravermann LE, Utiger RD (2005) The normal thyroid (part I). In: *The Thyroid, A Fundamental and Clinical Text* (Bravermann LE, Utiger RD, eds). 9th ed, Philadelphia: Lippincott Williams & Wilkins, 3–305

Clark OH, Gerend PL, Nissenson RA (1982) Thyrotropin binding and adenylate cyclase activation in normal and neoplastic human thyroid tissue: lack of effect of thyroglobulin. *J Clin Endocrinol Metab* 54:1157–63

Costagliola S, Rodien P, Many MC, Ludgate M, Vassart G (1998) Genetic immunization against the human thyrotropin receptor causes thyroiditis and allows production of monoclonal antibodies recognizing the native receptor. *J Immunol* 160:1458–65

Daumerie C, Ludgate M, Costagliola S, Many MC (2002) Evidence for thyrotropin receptor immunoreactivity in pretibial connective tissue from patients with thyroid-associated dermopathy. *Eur J Endocrinol* 146:35–8

Drvota V, Janson A, Norman C, Sylven C, Haggblad J, Bronnegard M *et al.* (1995) Evidence for the presence of functional thyrotropin receptor in cardiac muscle. *Biochem Biophys Res Commun* 211:426–31

Falini B, Martelli MF, Tarallo F, Moir DJ, Cordell JL, Gatter KC *et al.* (1984) Immunohistological analysis of human bone marrow trephine biopsies using monoclonal antibodies. *Br J Haematol* 56:365–86

Foitzik K, Krause K, Conrad F, Nakamura M, Funk W, Paus R (2006) 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* 168:748–56

Freinkel RK, Freinkel N (1972) Hair growth and alopecia in hypothyroidism. *Arch Dermatol* 106:349–52

Garrity JA, Bahn RS (2006) Pathogenesis of graves ophthalmopathy: implications for prediction, prevention, and treatment. *Am J Ophthalmol* 142:147–53

Gharzi A, Reynolds AJ, Jahoda CA (2003) Plasticity of hair follicle dermal cells in wound healing and induction. *Exp Dermatol* 12:126–36

Goldberg MT, Han YP, Yan C, Shaw MC, Garner WL (2007) TNF-alpha suppresses alpha-smooth muscle actin expression in human dermal fibroblasts: an implication for abnormal wound healing. *J Invest Dermatol* 127:2645–55

Gomez-Garre P, Seijo M, Gutierrez-Delicado E, Castro del Rio M, de la Torre C, Gomez-Abad C *et al.* (2006) Ehlers-Danlos syndrome and periventricular nodular heterotopia in a Spanish family with a single FLNA mutation. *J Med Genet* 43:232–7

Hinz B (2007) Formation and function of the myofibroblast during tissue repair. *J Invest Dermatol* 127:526–37

Ito N, Ito T, Kromminga A, Bettermann A, Takigawa M, Kees F *et al.* (2005a) Human hair follicles display a functional equivalent of the hypothalamic–pituitary–adrenal axis and synthesize cortisol. *FASEB J* 19:1332–4

Ito T, Ito N, Bettermann A, Tokura Y, Takigawa M, Paus R (2004) Collapse and restoration of MHC class-I-dependent immune privilege: exploiting the human hair follicle as a model. *Am J Pathol* 164:623–34

Ito T, Ito N, Saathoff M, Bettermann A, Takigawa M, Paus R (2005b) Interferon-gamma is a potent inducer of catagen-like changes in cultured human anagen hair follicles. *Br J Dermatol* 152:623–31

Jackson D, Church RE, Ebling FJ (1972) Hair diameter in female baldness. *Br J Dermatol* 87:361–7

Jarrousse F, Boisnic S, Branchet MC, Beranger JY, Godeau G, Breton L *et al.* (2001) Identification of clustered cells in human hair follicle responsible for MMP-9 gelatinolytic activity: consequences for the regulation of hair growth. *Int J Dermatol* 40:385–92

Kauser S, Slominski A, Wei ET, Tobin DJ (2006) Modulation of the human hair follicle pigmentary unit by corticotropin-releasing hormone and urocortin peptides. *FASEB J* 20:882–95

Klein JR (2003) Physiological relevance of thyroid stimulating hormone and thyroid stimulating hormone receptor in tissues other than the thyroid. *Autoimmunity* 36:417–21

Kobayashi H, Kromminga A, Dunlop TW, Tychsen B, Conrad F, Suzuki N *et al.* (2005) A role of melatonin in neuroectodermal-mesodermal interactions: the hair follicle synthesizes melatonin and expresses functional melatonin receptors. *FASEB J* 19:1710–2

Kohn LD, Napolitano G, Singer DS, Molteni M, Scorza R, Shimojo N *et al.* (2000) Graves' disease: a host defense mechanism gone awry. *Int Rev Immunol* 19:633–64

Kohrle J (1990) Thyrotropin (TSH) action on thyroid hormone deiodination and secretion: one aspect of thyrotropin regulation of thyroid cell biology. *Horm Metab Res Suppl* 23:18–28

Langbein L, Rogers MA, Praetzel S, Cribier B, Peltre B, Gassler N *et al.* (2005) Characterization of a novel human type II epithelial keratin K1b, specifically expressed in eccrine sweat glands. *J Invest Dermatol* 125:428–44

Langbein L, Schweizer J (2005) Keratins of the human hair follicle. *Int Rev Cytol* 243:1–78

Larsen K, Melmed S, Polonsky KS (2003) Thyroid. In: *Williams Textbook of Endocrinology* 10th ed. Philadelphia: Saunders, 331–490

Leonhardt JM, Heymann WR (2002) Thyroid disease and the skin. *Dermatol Clin* 20:473–81

Lindner G, Menrad A, Gherardi E, Merlino G, Welker P, Handjiski B *et al.* (2000) Involvement of hepatocyte growth factor/scatter factor and met receptor signaling in hair follicle morphogenesis and cycling. *FASEB J* 14:319–32

Lygoe KA, Wall I, Stephens P, Lewis MP (2007) Role of vitronectin and fibronectin receptors in oral mucosal and dermal myofibroblast differentiation. *Biol Cell* 99:601–14

Magerl M, Kauser S, Paus R, Tobin DJ (2002) Simple and rapid method to isolate and culture follicular papillae from human scalp hair follicles. *Exp Dermatol* 11:381–5

Mercurio S, Latinkic B, Itasaki N, Krumlauf R, Smith JC (2004) Connective-tissue growth factor modulates WNT signalling and interacts with the WNT receptor complex. *Development* 131:2137–47

Messenger A (2000) Thyroid hormone and hair growth. *Br J Dermatol* 142:633–4

Mou C, Jackson B, Schneider P, Overbeek PA, Headon DJ (2006) Generation of the primary hair follicle pattern. *Proc Natl Acad Sci USA* 103:9075–80

Ohnemus U, Uenalan M, Inzunza J, Gustafsson JA, Paus R (2006) The hair follicle as an estrogen target and source. *Endocr Rev* 27:677–706

Paus R, Cotsarelis G (1999) The biology of hair follicles. *N Engl J Med* 341:491–7

Paus R, Foitzik K (2004) In search of the ''hair cycle clock'': a guided tour. *Differentiation* 72:489–511

Paus R, Theoharides TC, Arck PC (2006) Neuroimmunoendocrine circuitry of the ''brain–skin connection''. *Trends Immunol* 27:32–9

Peters EM, Ericson ME, Hosoi J, Seiffert K, Hordinsky MK, Ansel JC *et al.* (2006) Neuropeptide control mechanisms in cutaneous biology: physiological and clinical significance. *J Invest Dermatol* 126:1937–47

Philpott M (1999) *In vitro* maintenance of isolated hair follicles: current status and future development. *Exp Dermatol* 8:317–9

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

Porcellini A, Messina S, De Gregorio G, Feliciello A, Carlucci A, Barone M *et al.* (2003) The expression of the thyroid-stimulating hormone (TSH) receptor and the cAMP-dependent protein kinase RII beta regulatory subunit confers TSH-cAMP-dependent growth to mouse fibroblasts. *J Biol Chem* 278:40621–30

Povey PM, Smith BR, Davies TF, Hall R (1976) Thyrotrophin receptor binding, intracellular cyclic AMP levels and iodine release in isolated thyroid cells. *FEBS Lett* 72:251–5

Rapoport B, Alsabeh R, Aftergood D, McLachlan SM (2000) Elephantiasic pretibial myxedema: insight into and a hypothesis regarding the pathogenesis of the extrathyroidal manifestations of Graves' disease. *Thyroid* 10:685–92

Reynolds AJ, Lawrence C, Cserhalmi-Friedman PB, Christiano AM, Jahoda CA (1999) Trans-gender induction of hair follicles. *Nature* 402:33–4

Rose NR, Mackay IR (2006) Thyroid desease. In: *The Autoimmun Disease* (Weetman AP, ed). 4th ed. Elsevier Academic Press, New York, 467–83

Safer JD (2005a) The skin and connective tissue in hypothyroidism. In: *The Thyroid, A Fundamental and Clinical Text* (Bravermann LE, Utiger RD, eds). Philadelphia: Lippincott Williams & Wilkins, 769–73

Safer JD (2005b) The skin in thyrotoxicosis. In: *The Thyroid, A Fundamental and Clinical Text*. (Bravermann LE, Utiger RD, eds). Philadelphia: Lippincott Williams & Wilkins, 553–8

Safer JD, Fraser LM, Ray S, Holick MF (2001) Topical triiodothyronine stimulates epidermal proliferation, dermal thickening, and hair growth in mice and rats. *Thyroid* 11:717–24

Sanger F (1981) Determination of nucleotide sequences in DNA. *Biosci Rep* 1:3–18

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* 182:23–6

Schmidt-Ullrich R, Paus R (2005) Molecular principles of hair follicle induction and morphogenesis. *Bioessays* 27:247–61

Schon M, Benwood J, O'Connell-Willstaedt T, Rheinwald JG (1999) Human sweat gland myoepithelial cells express a unique set of cytokeratins and reveal the potential for alternative epithelial and mesenchymal differentiation states in culture. *J Cell Sci* 112:1925–36

Schweizer J, Bowden PE, Coulombe PA, Langbein L, Lane EB, Magin TM *et al.* (2006) New consensus nomenclature for mammalian keratins. *J Cell Biol* 174:169–74

Scofield VL, Montufar-Solis D, Cheng E, Estes MK, Klein JR (2005) Intestinal TSH production is localized in crypt enterocytes and in villus ''hotblocks'' and is coupled to IL-7 production: evidence for involvement of TSH during acute enteric virus infection. *Immunol Lett* 99:36–44

Sheehan TE, Kumar PA, Hood DA (2004) Tissue-specific regulation of cytochrome *c* oxidase subunit expression by thyroid hormone. *Am J Physiol Endocrinol Metab* 286:E968–74

Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillessen D, Gabbiani G (1986) A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol* 103: 2787–96

Slominski A (2005) Neuroendocrine system of the skin. *Dermatology* 211:199–208

Slominski A, Wortsman J (2000) Neuroendocrinology of the skin. *Endocr Rev* 21:457–87

Slominski A, Wortsman J, Kohn L, Ain KB, Venkataraman GM, Pisarchik A *et al.* (2002) Expression of hypothalamic-pituitary-thyroid axis related genes in the human skin. *J Invest Dermatol* 119:1449–55

Slominski A, Wortsman J, Luger T, Paus R, Solomon S (2000) Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. *Physiol Rev* 80:979–1020

Slominski A, Wortsman J, Plonka PM, Schallreuter KU, Paus R, Tobin DJ (2005) Hair follicle pigmentation. *J Invest Dermatol* 124:13–21

Slominski A, Wortsman J, Tuckey RC, Paus R (2007) Differential expression of HPA axis homolog in the skin. *Mol Cell Endocrinol* 265–266:143–9

Sorisky A, Bell A, Gagnon A (2000) TSH receptor in adipose cells. *Horm Metab Res* 32:468–74

Stenn KS, Paus R (2001) Controls of hair follicle cycling. *Physiol Rev* 81:449–94

Suzuki K, Lavaroni S, Mori A, Ohta M, Saito J, Pietrarelli M *et al.* (1998) Autoregulation of thyroid-specific gene transcription by thyroglobulin. *Proc Natl Acad Sci USA* 95:8251–6

Suzuki K, Mori A, Saito J, Moriyama E, Ullianich L, Kohn LD (1999) Follicular thyroglobulin suppresses iodide uptake by suppressing expression of the sodium/iodide symporter gene. *Endocrinology* 140:5422–30

Tobin DJ, Kauser S (2005) Hair melanocytes as neuro-endocrine sensors—pigments for our imagination. *Mol Cell Endocrinol* 243:1–11

Tobin DJ, Paus R (2001) Graying: gerontobiology of the hair follicle pigmentary unit. *Exp Gerontol* 36:29–54

Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA (2002) Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 3:349–63

van Beek N, Bodó E, Kromminga A, Gáspár E, Meyer K, Zmijewski MA *et al.* (2008) Thyroid hormones directly alter human hair follicle functions: Anagen prolongation, and stimulation of both hair matrix keratinocyte proliferation and hair pigmentation. *J Clin Endocrinol Metab* 11:4381–8

Werner S, Grose R (2003) Regulation of wound healing by growth factors and cytokines. *Physiol Rev* 83:835–70