Letter to the Editor

TSH is a novel neuroendocrine regulator of selected keratins in the human hair follicle

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Keratins and keratin-associated proteins (KAPs) constitute the major structural protein components of the hair. Regulation of their expression is critical for proper hair follicle (HF) structure and function [1]. Therefore, it is important to fully elucidate the controls that regulate keratin expression. Although it is accepted that the expression of selected keratin genes underlies endocrine controls [1,2], our understanding of the complex regulation of keratin expression remains rather fragmentary. To better elucidate this regulation, human skin and HF organ culture offer an instructive, physiologically relevant research tool [1,3].

In particular, very little is known on the neuroendocrine controls of keratin transcription. The importance of the latter is highlighted by the recent discovery that the "pituitary" neuropeptide hormone, prolactin, which is also expressed by human HFs, potently regulates the expression of selected human keratins on the gene and protein level [3]. Moreover, microarray analyses had provided first clues that thyroid stimulating hormone (TSH) and its proximal regulator in the hypothalamic-pituitary-thyroid axis, thyrotropin-releasing hormone (TRH), might operate as previously unsuspected modulators of human hair keratin and KAP gene transcription *in situ* [4-6]. Also, we had recently found that TSH upregulates keratin K5 gene expression and protein synthesis as well as K14 transcription in human epidermis [4].

Therefore, we have asked whether TSH operates as a novel neuroendocrine regulator of human keratins *in situ*, using microdissected, organ-cultured human scalp HFs as a physiologically and clinically relevant assay systems [3-5]. This was complemented by studying the effect of TSH on keratin expression in cultured human outer root sheath (ORS) keratinocytes (KCs).

Anagen VI HFs were isolated from normal frontotemporal scalp skin obtained after written informed consent from three healthy females undergoing routine face-lift surgery, as

previously described [4,5], adhering to Helsinki guidelines and under a licence from the ethics committee of the University of Lübeck. HF mRNA extracts from one female patient were subjected to quantitative real time PCR (qPCR) for selected hair keratin genes after 24 hrs treatment with TSH (100 mU ml^{-1}) or vehicle. For immunohistochemical analysis, isolated HFs from additional two female patients were organ cultured for 6 days as described previously [3,4], and expression of keratins K6, K14, K17, K31, K32, K85 and MSX-2 was studied with our previously published basic immunohistology protocols [3,4] (Table 1). For epithelial keratin qPCR experiments, human ORS KCs were obtained from an additional female patient, as previously described [3]. These were treated for 24h with TSH (100 mU ml^{-1}) or vehicle.

Our previous microarray results had suggested that TSH administration significantly downregulates expression of K31 [4], thus suggesting a role for TSH in the regulation of hair keratins. To further explore this microarray lead, we performed qPCR on carefully selected hair keratins that are expressed in the hair shaft-generating HF epithelium, representing three major compartments of the HF epithelium: the hair matrix/precortex and cuticle (K35), the hair cortex (K31) and the hair cuticle (K32) [2]. Particular emphasis was placed on studying *KRT85* and *KRT35* transcription, since both keratins are the earliest ones to be expressed in the precortical hair matrix and early cuticle [2].

These qPCR analyses demonstrated that TSH downregulated transcription of *KRT31* and *KRT32* genes (Fig. 1A). In these qPCR analyses, *KRT35* transcription was largely unaffected by TSH. Instead, transcription of its type II counterpart keratin gene, *KRT85* [2], was downregulated (Fig. 1A).

Immunohistochemical studies on TSH treated HFs confirmed the downregulation of the hair keratins also at the protein level (Fig. 2A-C): K31 immunoreactivity, localized to the hair cortex (Fig. 2A), K85 immunoreactivity, which is expressed in the hair cortex and in the precortical hair matrix (Fig. 2B) and K32 immunoreactivity, which serves as a marker for the hair cuticle (Fig. 2C), were significantly downregulated following TSH administration. Since MSX2 serves as a major transcription factor that regulates hair keratin expression [7], analysis of MSX2 expression was also performed. Indeed, TSH downregulated *MSX2* transcription in human anagen HFs *in situ* (Fig. 1A). MSX2 immunoreactivity was found to correspond to the expression pattern in the mouse, the hair matrix and cortex (Fig. 2D) [8]. In line with the qPCR results, TSH downregulated MSX2 immunoreactivity (Fig. 2D).

Several epithelial keratins are widely expressed in the HF ORS, where they are thought to play an important role in maintaining structural integrity and homeostasis [1]. We have previously confirmed that TSH has a regulatory effect on K5 [4], which is prominently expressed in the ORS [1]. We have therefore further explored this finding, by studying the effects of TSH on selected epithelial keratins which are constitutively expressed in the ORS [2]. HF treatment with TSH significantly downregulated K6, K14 and K17 immunoreactivity (Fig. 2E-G). In order to confirm these findings in isolated HF KCs in situ, TSH treated ORS KCs were analyzed by qPCR. This showed a significant downregulation of *KRT6* and *KRT17* transcription, but no effect on *KRT14* transcription (Fig. 1B).

Exploiting the human HF as a discovery tool for keratin research, the current pilot study identifies TSH as a novel neuroendocrine regulator of keratin expression in human skin. The data provided here also suggest that the hair shaft abnormalities seen in patients with hyper- or hypothyroidism [9] may result not only from perturbations in the thyroid hormone blood

levels, but also from associated neuroendocrine changes in TSH levels in the blood, or even in the skin [6,10].

The regulation of hair keratin gene expression is tightly controlled by a complex mechanism involving several upstream mediators. These include members of the Wnt/β-catenin pathway and of the TGF-β family which control MSX2, FOXN1 and HOXC13 activity [1,2,7,11,12]. Since MSX2 is a major regulator of hair shaft differentiation, our observation that TSH reduces *MSX2* expression on the gene and protein level encourages one to pursue the hypothesis that TSH may act at least in part via inhibiting *MSX2* expression.

While *KRT35* transcription was not inhibited by TSH, TSH downregulated *KRT85* expression. Such a unilateral keratin regulation has been documented before for the cuticle keratin K82 [13]. It is conceivable that the TSH-induced upregulation of KAPs transcription previously observed by microarray analysis [4] may compensate for the downregulation of one type of keratin, as an attempt to maintain structural stability of the hair shaft.

In summary, our pilot study reveals that the expression of selected HF keratins underlies novel neuroendocrine controls, possibly as part of the unfolding hypothalamus-pituitary-thyroid axis equivalent present in human skin [4-6,10]. As shown for prolactin [3] and TSH, human HF organ culture offers an excellent research tool to further dissect ”novel” neuroendocrine *in situ*-controls that drive keratin expression in the human system under clinically relevant conditions on the gene and protein level.

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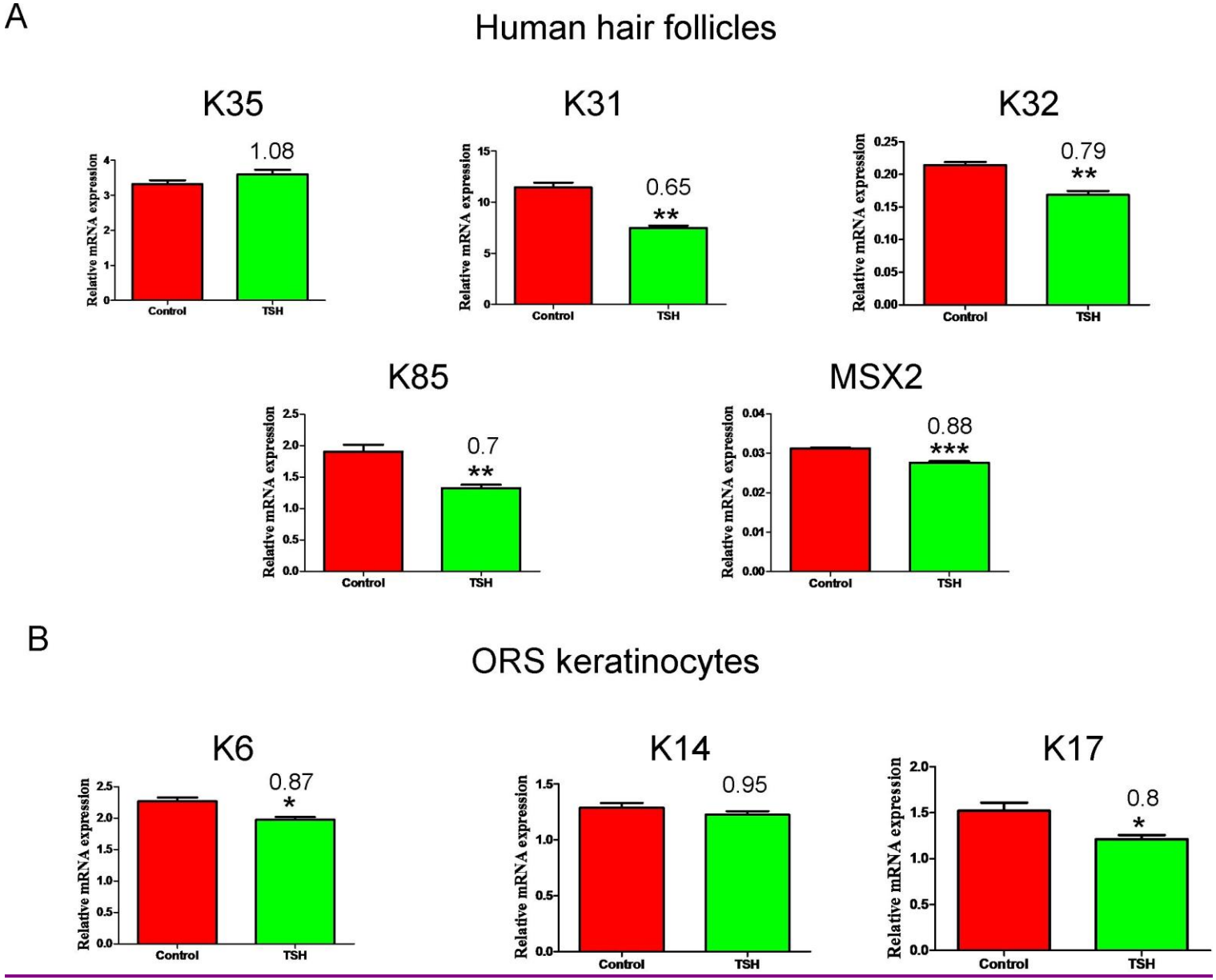
Figure legends

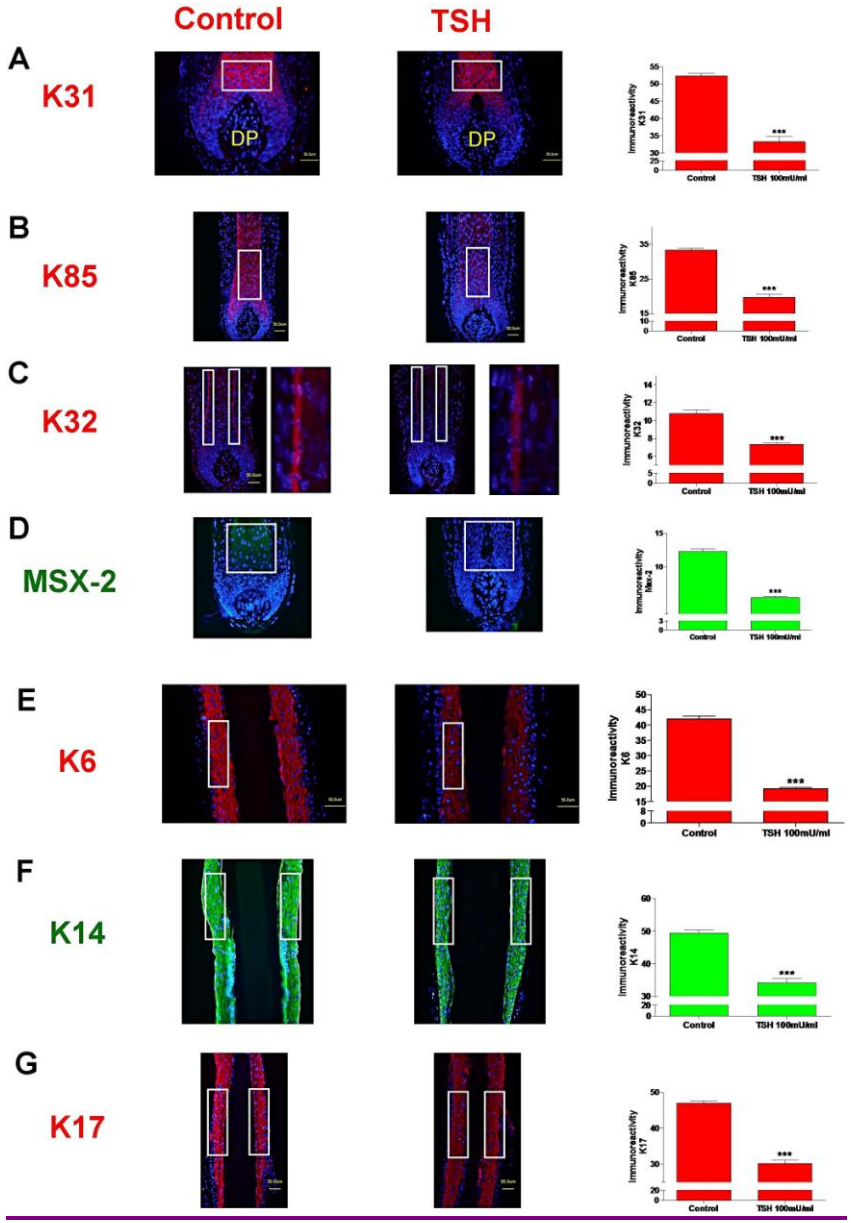
Fig 1. A. Relative mRNA expression of *KRT35, KRT31, KRT32, KRT85* and *MSX2* genes following treatment with TSH (100 mU ml^{-1}). This dose was selected since it is within the range of customarily employed TSH concentrations in cell culture studies, and because this dose has been previously shown by microarray analysis to regulate the expression of several genes in organ-cultured human hair follicles [4]. Results represent triplicate determinations of samples. Total RNA was pooled from 20 HFs. B. Relative mRNA expression of *KRT6, KRT14* and *KRT17* following administration of TSH to ORS KCs in culture, extracted from HFs of an additional female patient. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$; mean ± SEM. PCR amplification was carried out by using the TaqMan primers and probes (recognizing the following human genes: Assay ID: Hs01699178_gH for *KRT6*, Assay ID: Hs00265033_m1 for *KRT14*, Assay ID: Hs00356958_m1 for *KRT17*, Assay ID: Hs00605539_m1 for *KRT31*, Assay ID: Hs00605543_g1 for *KRT32*, Assay ID: Hs00605557_g1 for *KRT35*, Assay ID: Hs00158558_m1 for *KRT85*, Assay ID: Hs00741177_m1 for *MSX2*) using the TaqMan Universal PCR Master Mix Protocol (Applied Biosystems). As internal controls, transcripts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined (Assay ID: Hs99999905_m1 for human *GAPDH*).

Fig. 2. TSH (100 mU ml^{-1}) downregulates immunoreactivity of K31 (A), K85 (B), K32 (C), MSX-2 (D), K6 (E), K14 (F) and K17 (G) in microdissected, organ-cultured normal, human scalp skin HFs, after 6 days of administration. Densitometric measurements of staining intensities in defined reference areas (quantitative immunohistomorphometry) were performed using ImageJ software (NIH, Bethesda, MD, USA; http://rsbweb. nih.gov/ij/) as described previously [3,4]. Columns represent means±SEM ; n=15–18 HFs/group; cumulative results of two different experiments. ***$P<0.001$.

TABLE 1. Primary antibodies used

Name	Host	Dilution	Positive control	Method	Source	Clone
Keratin 6	Mouse	1:10	Suprabasal layers of the ORS; suprabasal layers of wounded skin [2,14]	Indirect IF	PROGEN, Heidelberg, Germany	Ks6.KA12
Keratin 14	Mouse	1:50	Skin epidermis, basal layer; basal and suprabasal layers of the ORS [2,14]	Indirect IF	Sigma-Aldrich, Taufkirchen, Germany	CKB1
Keratin 17	Mouse	1:50	Suprabasal layers of the ORS [14]	Indirect IF	PROGEN, Heidelberg, Germany	Ks17.E3
Keratin 31	Guinea Pig	1:7000	Precortex region [2]	Indirect IF	Lutz Langbein, DKFZ, Heidelberg, Germany	hHa1 prot.1
Keratin 32	Guinea Pig	1:2000	Hair cuticle [10,14]	Indirect IF	Lutz Langbein, DKFZ, Heidelberg, Germany	Ha2.1
Keratin 85	Guinea Pig	1:1000	Hair matrix, cortex and cuticle [14]	Indirect IF	Lutz Langbein, DKFZ, Heidelberg, Germany	hHb 5co.2
Msx-2	Goat	1:100	Hair matrix and cortex [8]	Indirect IF	Santacruz, CA, USA	-


A
Human hair follicles
K35
1.08
Relative mRNA expression
Control
TSH
K31
0.65
**
K32
0.79
**
K85
0.7
**
MSX2
0.88
***
B
ORS keratinocytes
K6
0.87
*
K14
0.95
K17
0.8
*


Control
TSH
A
K31
DP
DP
Immunoreactivity K31
Control
TSH 100mU/ml
B
K85
Immunoreactivity K85
Control
TSH 100mU/ml
C
K32
Immunoreactivity K32
Control
TSH 100mU/ml
D
MSX-2
Immunoreactivity Msx-2
Control
TSH 100mU/ml
E
K6
Immunoreactivity K6
Control
TSH 100mU/ml
F
K14
Immunoreactivity K14
Control
TSH 100mU/ml
G
K17
Immunoreactivity K17
Control
TSH 100mU/ml