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Is thyrotropin-releasing hormone (TRH) a novel neuroendocrine modulator of keratin expression in human skin?

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Keywords:	human hair follicle, keratins, skin, epidermis, TRH

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Dr. Tanya O. Bleiker
Editor, British Journal of Dermatology

Re: "Is thyrotropin-releasing hormone (TRH) a novel neuroendocrine modulator of keratin expression in human skin?"

Enclosed, please find our manuscript entitled "**Is thyrotropin-releasing hormone (TRH) a novel neuroendocrine modulator of keratin expression in human skin**" for your consideration, which we submit for publication as a **Concise Communication** in the British Journal of Dermatology. The manuscript has not been published or submitted for publication elsewhere. All authors have contributed significantly and are in agreement with the content of the manuscript.

In this report, we describe novel effects of the neurohormone, TRH, on the expression of selected human hair and epidermal keratins. While selected nuclear receptor hormones are appreciated to regulate specific keratins, very little is known about the neuroendocrine control of human keratin expression. Since preliminary microarray evidence from my lab had suggested that TRH may regulate keratin gene transcription, we have followed these pointers up in a pilot study, using organ cultures of microdissected, female scalp hair follicles and full-thickness human skin.

The current study suggests that TRH is indeed a novel modulator of the expression of human hair and epithelial keratins *in situ*, thus identifying the neuroendocrine regulation of keratin expression as an exciting new research frontier in skin biology. Together with the recently identified stimulatory activity of TRH on mitochondrial activity, hair pigmentation and hair growth, the potent keratin expression-modulating effects of TRH revealed here may serve as a basis for novel therapeutic strategies that recruit neurohormones to modulate keratin expression in human skin and appendages.

We thank you for your consideration of our manuscript and look forward to your reply.

Best regards,

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Is thyrotropin-releasing hormone (TRH) a novel neuroendocrine modulator of keratin expression in human skin?

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Running title: TRH modulates keratin expression

Keywords: human hair follicle, keratins, skin, epidermis, TRH

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Conflict of interest: None.

What's already known about this topic?

- Although keratins are one of the major structural components of the hair fiber and skin epithelium, little is known about their hormonal influences. Since the role of neuropeptide hormones in the control of keratin expression remains largely obscure, we have followed preliminary microarray-based evidence which had suggested that thyrotropin-releasing hormone (TRH) may modulate keratin expression in human hair follicles.

What does this study add?

- The current study provides evidence that the neuropeptide hormone TRH can act as a novel modulator of the expression of human hair and epithelial keratins *in situ*, thus identifying the neuroendocrine regulation of keratin expression as an exciting new research frontier in skin biology.
- Together with the recently identified stimulatory activity of TRH on mitochondrial activity, hair pigmentation and hair growth, the potent keratin expression-modulating effects of TRH revealed here may serve as a basis for novel therapeutic strategies that recruit neurohormones to modulate keratin expression in human skin and appendages.

Summary

Background Hair and epithelial keratins constitute the major structural components of the skin and its appendages, including the hair fiber. While selected steroid hormones are appreciated to regulate specific keratins, little is known about the neuroendocrine control of human hair keratin expression. Preliminary evidence had suggested that thyrotropin-releasing hormone (TRH) may regulate keratin gene transcription.

Objective We wanted to clarify whether TRH operates as a novel neuroendocrine regulator of human hair and epithelial keratin expression under physiologically relevant conditions *in situ*.

Methods Microdissected human female scalp hair follicles (HFs) and female scalp skin were treated in serum-free organ culture for 12h – 6d with 100ng/ml TRH or vehicle. Both quantitative immunohistomorphometry and RT-qPCR were utilized to assess expression of selected keratins.

Results TRH significantly increased expression of the hair keratins K31 and K32, while that of K85 and K86, and of the epithelial keratins K14 and K17 was reduced. In the interfollicular epidermis, TRH stimulated expression of K6, K14 and K17, both at the mRNA and protein levels. Stimulation of the same keratins was also evident in the eccrine sweat and sebaceous glands.

Conclusions Selected human hair and epithelial keratins are modulated *in situ*. This may be relevant to explain hair shaft growth-promoting effects of TRH. Our pilot study suggests that the neuroendocrine controls that regulate the expression of human keratins deserve more systematic exploration and that these may be harnessed therapeutically.

Keratins are intermediate filaments, which exert vital functions in maintaining the homeostasis of the skin and its appendages.[1] Their main role is to provide structural stability to the tissue, but recently it has been recognized that they might have a much wider spectrum of functions.[2] Therefore, it is crucial to understand their regulatory mechanisms. While it is appreciated that nuclear receptor hormones such as thyroid hormones, glucocorticoids, calcitriol, and retinoids profoundly impact the expression of keratins and keratin-associated proteins in human skin,[3] little is known on the neuroendocrine modulation of keratin expression in human skin, namely of neuropeptide hormones known to be generated in the skin itself.

Interest in the neuroendocrine control of keratin expression in human skin has been invigorated by recent evidence that the neuropeptide hormone, prolactin, profoundly impacts on the expression of keratins such as K5, K6, K14, K15, K19 and K31 in human hair follicles (HFs).[4] Thyroid stimulating hormone (thyrotropin, TSH) was also found to modulate keratin gene expression in the epidermis and HFs.[5,6] This was underscored by preliminary gene profiling evidence that another neuropeptide hormone, thyrotropin-releasing hormone (TRH), which is also produced by HFs, may interfere and regulate selected intrafollicularly expressed keratins and keratin-associated proteins.[7]

Since TRH is a potent stimulator of human hair growth *in situ*,[7] and also stimulates hair pigmentation,[8] we wanted to elucidate whether TRH acts as a novel regulator of keratin expression *in situ*. This was studied on microdissected, organ-cultured female scalp HFs[8,9] and interfollicular skin[10] under serum-free conditions in the presence of insulin and hydrocortisone.[11]

Materials and methods

Excess human scalp skin was obtained after written informed consent from 6 healthy females during cosmetic face lift surgery (after the University of Luebeck ethics committee approval), as previously described.[5,7] HF mRNA extracts from two females were subjected to quantitative real-time PCR (RT-qPCR) for selected hair keratin genes after 12h or 24h treatment with TRH (100ng/ml) or vehicle. For immunohistochemical analysis, isolated HFs from an additional female volunteer were organ cultured for 6 days as described previously,[4,7] and expression of selected keratins and Msx2 (muscle segment homeobox-like 2), a major transcription factor that regulates hair keratin expression[12], was studied using the staining protocols described in Table 1. For RT-qPCR evaluation of epithelial keratins, total RNA was isolated from enzymatically separated human epidermis from an additional female volunteer, after 12h culture with 100ng/ml TRH or vehicle. For immunohistochemical analysis of epidermal keratins, skin from two additional different females was organ cultured for 4d with 100 ng/ml TRH or vehicle.

Results

Treatment with 100 ng/ml TRH for 6d significantly increased *in situ* the immunostaining intensity of the hair keratins K31 (hair cortex) and of K32 (hair cuticle). In contrast, staining intensity of the hair keratins K85 (precortical hair matrix and cuticle), and K86 (mid-to-upper hair cortex) was reduced by TRH. TRH also decreased staining intensity *in situ* of the epithelial keratins K14 and K17 in the outer root sheath (ORS) of the female anagen scalp HFs (Figure 1, A-F).

K31 transcription was decreased while K17 mRNA levels were increased by treatment with 100ng/ml TRH for 12h, as assessed by RT-qPCR (Figure 1, G). Since according to our previous TRH microarrays,[7] K31 and K32, which represent the hair cortex and hair cuticle,[13] respectively, were both found to be differentially regulated, and considering the importance of K85 as the earliest keratin expressed in the precortical hair matrix and early cuticle,[13] we also checked their transcription in an additional patient (24h culture period). While mRNA levels of both K32 and K85 were decreased, K31 transcription was unchanged (Figure 1, H and Supplementary Table 1).

In the epidermis, TRH profoundly increased staining intensity for keratins K14, K6 and K17 after 4d of serum-free organ culture in the presence of 100ng/ml TRH (Figure 2, A-C). TRH also significantly stimulated K14 and K6 transcription, while a trend towards slightly increased K17 mRNA expression was observed, but did not reach significance (Figure 2, A-C). Finally, TRH also appeared to enhance K14, K6 and K17 expression in the secretory and ductal parts of the eccrine sweat glands, including myoepithelial cells, and in the secretory part of the sebaceous glands (Figure 2, D-F).

Discussion

Our pilot study suggests that TRH is a potent, novel modulator of keratin expression in defined epithelial compartments of human skin. This confirms earlier preliminary gene profiling evidence,[7] and suggests that keratins, namely K14, K17, K31, K32, K85 and K86, are (direct or indirect) targets of TRH regulation. Moreover, this study underscores that human skin and HF organ culture are instructive, clinically relevant

discovery tools for dissecting the neuroendocrine controls of human keratin expression *in situ*. However, especially in the HF, the puzzling mRNA and protein expression discrepancies observed after TRH stimulation in our pilot study warrant systematic follow-up work to clarify the exact effect of TRH on human keratin expression *in situ* (for additional discussion, see supplementary text 1).

The mechanism(s) by which TRH affects expression of these keratins remains presently unclear. Msx2, i.e. one reasonable candidate transcription factor that might have been involved in mediating these effects[12] did not respond to TRH stimulation on the mRNA and protein levels (see Supplementary Table 1). Moreover, prominent TRH receptor (TRH-R) protein expression in human anagen HFs is restricted to the inner root sheath.[7,8] Therefore, it is conceivable that the keratin expression-regulatory effects of TRH outside of the inner root sheath are not directly mediated via TRH-R stimulation. Since TRH upregulates intraepidermal TSH expression in human epidermis,[10] some of the keratin effects of TRH may actually be mediated by promoting the intraepithelial production of TSH. In fact, TSH administration to cultured human HFs modulates expression of several keratins in a somewhat comparable manner as TRH, such as K14, K17 and K85.[6]

Following the recent development of novel TRH analogs, which are metabolically stable, and are currently in different stages of preclinical or clinical development,[14] it becomes clinically important to understand whether such analogs might have an effect on skin, and on possible endocrine interactions between intracutaneous production of neurohormones and external TRH application.

In summary, our pilot study reveals yet another important frontier in the ongoing quest to explore of the hypothalamus-pituitary-thyroid axis equivalent of human

skin:[15] the differential neuroendocrine regulation of selected sets of keratins in distinct epithelial tissue compartments by TRH.

Figure legends

Figure 1. TRH (100 ng/ml) regulates expression of K31 (A), K32 (B), K85 (C), K86 (D), K14 (E) and K17 (F) in microdissected, organ-cultured, normal female scalp skin HFs, after 6d of administration, as documented by immunostaining intensity. Microarray analysis has previously shown that several genes are regulated in organ-cultured human hair follicles at this time point and dose.[7,9] The staining pattern of selected keratins precisely corresponds to the previously reported pattern; see Table 1 for details. Staining intensities were measured in defined reference areas by quantitative immunohistomorphometry using ImageJ software (NIH, Bethesda, MD, USA; http://rsbweb.nih.gov/ij/) as previously described. Columns represent means±SEM ; n=15–18 HFs/group. $P<0.05$ vs. Control, $^{**}P<0.01$, $^{***}P<0.001$; unpaired two-tailed Student's t-test. Scale bars: 50 μm. For recognized standard keratin expression patterns in human HFs see Langbein et al., 2005[13] and Moll et al., 2007.[1] (G, H) Relative mRNA expression following administration of TRH to HFs in culture for 12h (G) or 24h (H), extracted from HFs of additional two patients. TRH did not significantly alter mRNA levels of the other keratins (K14, K32, K85 and K86) evaluated during the short 12h incubation time (Supplementary Table 1). In addition, MSX2 expression, a transcription factor important for keratin regulation,[6,12] was not affected by TRH, both at protein and mRNA levels (Supplementary Table 1). Results represent triplicate determinations of samples. Total RNA was pooled from 20 HFs. $^{**}P<0.01$; mean ±SEM. Statistical analysis was performed by two-tailed

Student's t-test. PCR amplification was carried out by using the TaqMan primers and probes (Assay IDs: Hs00356958_m1 for K17, Hs00605539_m1 for K31, Hs00605543_g1 for K32 and Hs00158558_m1 for K85) 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 GAPDH).

Figure 2. TRH (100 ng/ml) upregulates expression of K14 (A, D), K6 (B, E) and K17 (C, F) in several skin compartments, including epidermis (semi-quantitative evaluation using ImageJ), sweat glands and sebaceous glands (visual evaluation). Columns represent means±SEM; n = 2 different experiments. Statistical analysis was performed by two-tailed Student's t-test. Relative mRNA expression of *KRT6*, *KRT14* and *KRT17* in enzymatically dissected epidermal cells after 12h organ culture with TRH (100 ng/ml), showed increased mRNA levels of K6 and K14, but no statistically significant change in K17 mRNA levels (A-C). ***P<0.001; mean ±SEM. PCR amplification was carried out by using the TaqMan primers and probes (Assay IDs: Hs01699178_g1 for K6, Hs00265033_m1 for K14, and Hs00356958_m1 for K17) 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 GAPDH).

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TABLE 1. Primary antibodies used

Protein	Clone/ antiserum	Source	Dilution	Host	Expression site
Keratin K6	Ks6.KA12	PROGEN, Heidelberg, Germany	1:10	Mouse	Inner layers of the ORS; companion layer; suprabasal layers of wounded skin; ductal luminal cells of eccrine sweat glands[1,6,13]
Keratin K14	CKB1	Sigma-Aldrich, Taufkirchen, Germany	1:50	Mouse	Skin epidermis, basal layer; all layers of the ORS[1,6,13]
Keratin K17	Ks17.E3	PROGEN, Heidelberg, Germany	1:50	Mouse	Inner layers of the ORS, companion layer; suprabasal layers of wounded skin, sweat glands, sebaceous glands[1]
Keratin K31	hHa1 prot.1	Lutz Langbein, DKFZ, Heidelberg, Germany	1:7000	Guinea Pig	Hair fiber precortex/cortex [6,13]
Keratin K32	Ha2.1	Lutz Langbein, DKFZ, Heidelberg, Germany	1:2000	Guinea Pig	Hair fiber cuticle[6]
Keratin K85	hHb 5co.2	Lutz Langbein, DKFZ, Heidelberg, Germany	1:1000	Guinea Pig	Hair fiber matrix, cortex and cuticle[1,6]
Keratin K86	hHb 6-1	Lutz Langbein, DKFZ, Heidelberg, Germany	1:2000	Guinea Pig	Hair fiber cortex[1]
Msx-2	-	Santacruz, CA, USA	1:100	Goat	Hair fiber matrix and cortex[6]

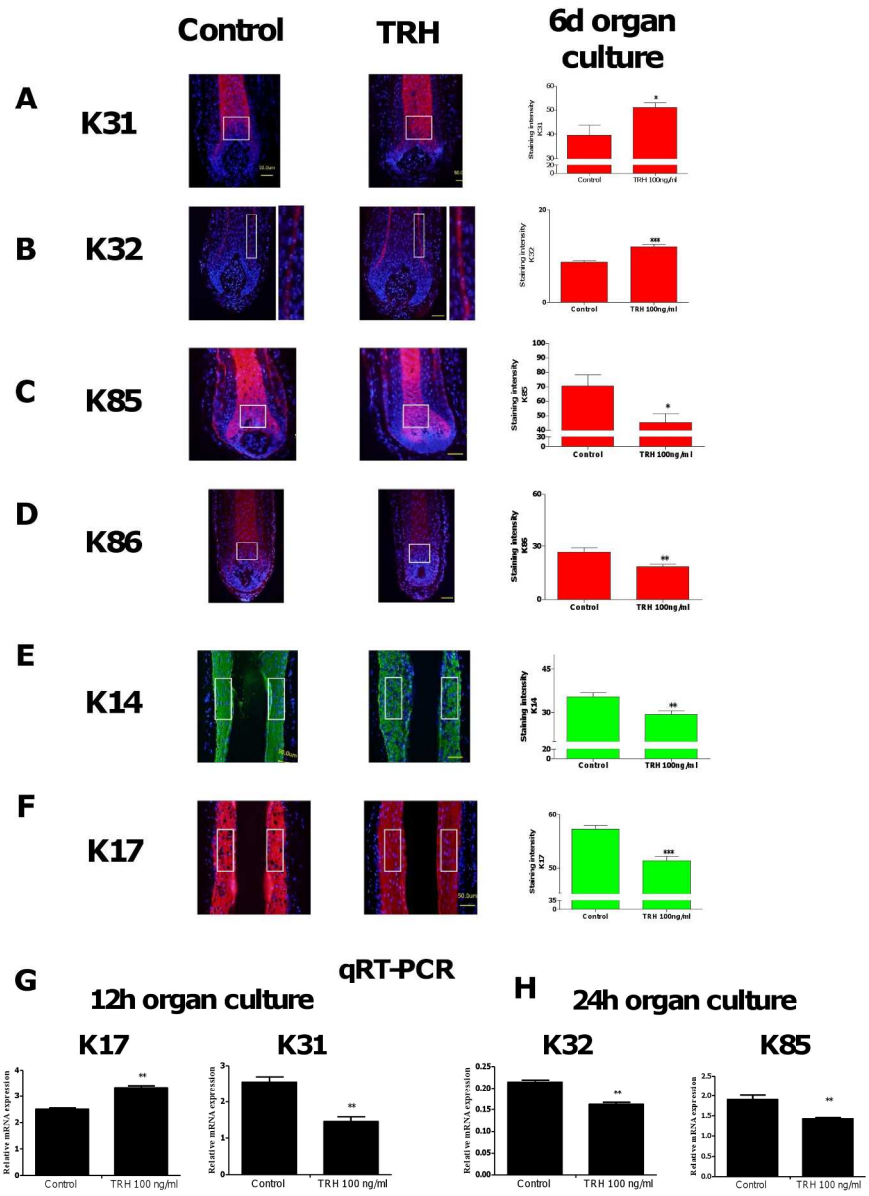

Figure 1. TRH (100 ng/ml) regulates expression of K31 (A), K32 (B), K85 (C), K86 (D), K14 (E) and K17 (F) in microdissected, organ-cultured, normal female scalp skin HFs, after 6d of administration, as documented by immunostaining intensity. Microarray analysis has previously shown that several genes are regulated in organ-cultured human hair follicles at this time point and dose.7,9 The staining pattern of selected keratins precisely corresponds to the previously reported pattern; see Table 1 for details. Staining intensities were measured in defined reference areas by quantitative immunohistomorphometry using ImageJ software (NIH, Bethesda, MD, USA; http://rsbweb.nih.gov/ij/) as previously described. Columns represent means±SEM ; n=15–18 HFs/group. P<0.05 vs. Control, **P<0.01, ***P<0.001; unpaired two-tailed Student's t-test. Scale bars: 50 μm. For recognized standard keratin expression patterns in human HFs see Langbein et al., 200513 and Moll et al., 2007.1 (G, H) Relative mRNA expression following administration of TRH to HFs in culture for 12h (G) or 24h (H), extracted from HFs of additional two patients. TRH did not significantly alter mRNA levels of the other keratins (K14, K32, K85 and K86) evaluated during the short 12h incubation time (Supplementary Table 1). In addition, MSX2 expression, a transcription factor important for keratin

regulation,6,12 was not affected by TRH, both at protein and mRNA levels (Supplementary Table 1). Results represent triplicate determinations of samples. Total RNA was pooled from 20 HFs. **P<0.01; mean ±SEM. Statistical analysis was performed by two-tailed Student's t-test. PCR amplification was carried out by using the TaqMan primers and probes (Assay IDs: Hs00356958_m1 for K17, Hs00605539_m1 for K31, Hs00605543_g1 for K32 and Hs00158558_m1 for K85) 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 GAPDH).
190x254mm (300 x 300 DPI)

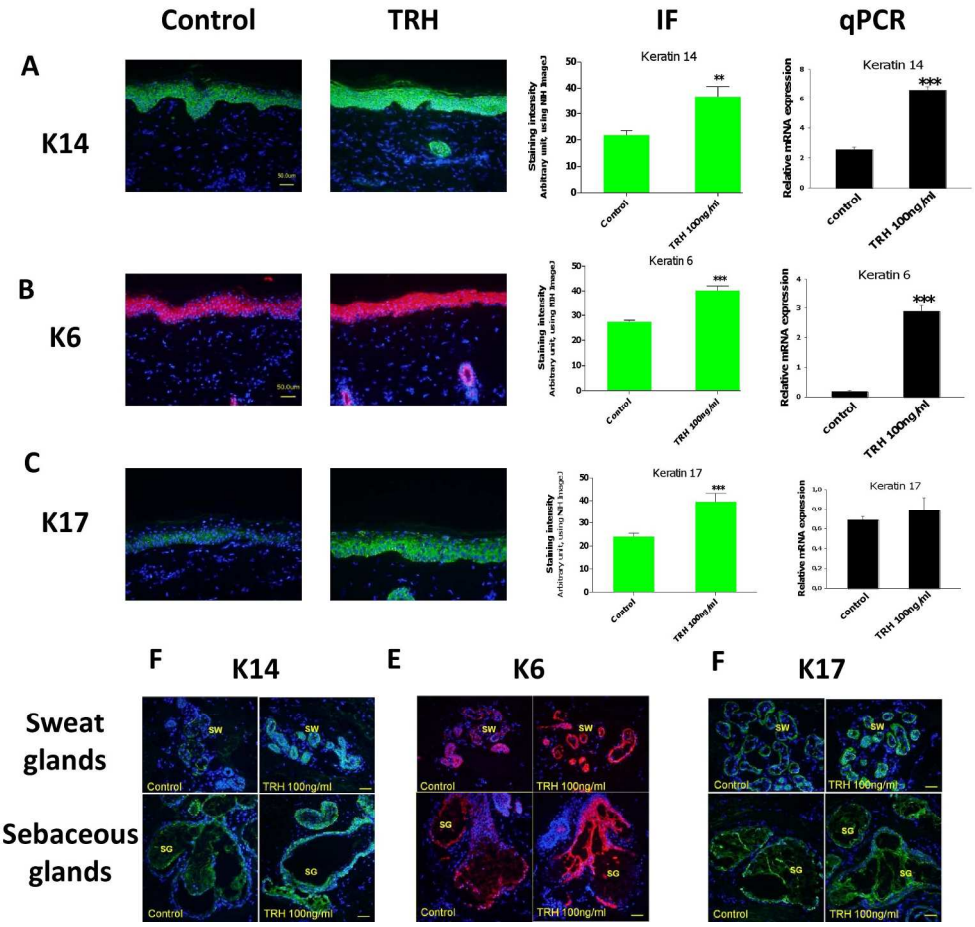

Figure 2. TRH (100 ng/ml) upregulates expression of K14 (A, D), K6 (B, E) and K17 (C, F) in several skin compartments, including epidermis (semi-quantitative evaluation using ImageJ), sweat glands and sebaceous glands (visual evaluation). Columns represent means±SEM; n = 2 different experiments. Statistical analysis was performed by two-tailed Student's t-test. Relative mRNA expression of KRT6, KRT14 and KRT17 in enzymatically dissected epidermal cells after 12h organ culture with TRH (100 ng/ml), showed increased mRNA levels of K6 and K14, but no statistically significant change in K17 mRNA levels (A-C). ***P<0.001; mean ±SEM. PCR amplification was carried out by using the TaqMan primers and probes (Assay IDs: Hs01699178_g1 for K6, Hs00265033_m1 for K14, and Hs00356958_m1 for K17) 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 GAPDH).
205x189mm (300 x 300 DPI)

Supplementary text 1:

That TRH potently stimulates hair shaft elongation[1] is supported in our pilot study by showing that TRH stimulates expression of the important, proximally expressed hair keratins K31 and K32 (Figure 1, B and C). Intriguingly, ORS keratins and more distally expressed hair keratins were found to be downregulated by TRH. This suggests that TRH exerts complex, differential regulatory effects on distinct subsets of human hair and epithelial keratins *in situ*, which deserve systematic exploration. In this context, it is notable that human skin and ORS keratinocytes *in situ*, express TRH at the mRNA and protein levels.[1,2] Therefore, while these remain to be formally demonstrated, auto- and paracrine regulatory effects of TRH on the expression of selected keratins should be conceivable.

In normal human epidermis, TRH strongly stimulated K14, expressed in basal and lower supra-basal keratinocytes, and of K6 and K17, expressed in hyperproliferative epidermis, companion layer and ORS keratinocytes. All of these, including keratin 6, are up-regulated/overexpressed or functionally important under wound healing conditions.[3,4] Moreover, wound healing and the hair follicle cyling anagen phase share a number of common important biological features,[5] and both are stimulated by TRH.[1] Therefore, these keratin-upregulatory effects of TRH are in line with the anagen- and wound healing promoting properties of this intraepithelially produced tripeptide neurohormone, which may serve as an important neuroendocrine coordinator of epithelial tissue remodelling and regeneration. This is also consistent with the upregulation of the epidermal keratins, suggesting an overall stimulatory effect of TRH on epidermal cells, which recruits the mitochondria for, among others, upregulating expression of selected keratins.

While TRH regulation of keratins K6, K14, and K85 staining intensity *in situ* was confirmed on the transcript level, keratins K17 (in the HF), K31 and K32 transcription showed an opposite trend. A summary of available data on TRH regulatory effects at the mRNA and protein levels on selected keratins and MSX2 is reported in Supplementary Table 1. This discrepancy between the mRNA and protein data could be related to the greatly different lengths of organ culture (protein expression: 4 or 6 days; qRT-PCR: 12 or 24 hours) as well as to individual characteristics of protein and/or mRNA processing and turnover for these specific keratins as cytoskeletal proteins. In addition, in the current pilot study, only a very limited number of human skin fragments was available for organ culture so that definitive conclusions on how TRH regulates expression of any of these keratins on the gene and protein level *in situ* should be reserved until independent repeat experiments with tissue from additional patients has been performed (since there is rapid RNA degradation in HF organ cultures, qRT-PCR was performed at 12 or 24 hours). The discrepancy between protein and transcription makes it difficult to draw definitive conclusions on whether TRH up- or downregulates intrafollicular expression of these keratins. However, our data available from this pilot study suggest that these keratins are regulated by TRH. It is also evident that there are large inter-individual differences in response to TRH, which in part might be related to age differences between the subjects examined (see Supplementary Table 1), and the protein and qRT-PCR data are never from the same patient.

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Supplementary Table 1. TRH modifies keratin and MSX2 gene expression in human female scalp HFs

Analysis	**Microarray 1**	**Microarray 2**	**qRT-PCR 1**	**qRT-PCR 2**	**IF**
Age	55 years	62 years	47 years	49 years	66 years
Location on scalp	Fronto-temporal	Fronto-temporal	Fronto-temporal	Fronto-temporal	Fronto-temporal
Treatment time	24 hours	24 hours	12 hours	24 hours	4 days
K6	**NC**	**NC**	**NC**	**-**	**-**
K14	↓	↓	**NC**	**-**	↓
K16	↓	↓	-	-	-
K17	↓	**NC**	↑	**-**	↓
K31[a]	↓	↓	↓	**NC**	↑
K32	↓	↓	**NC**	↓	↑
K33a	↓	↓	-	-	-
K33b	↓	NC	-	-	-
K34	↓	↓	-	-	-
K35[a]	↓	↓	**NC**	**-**	**-**
K36	NC	NC	-	-	-
K37	↓	↓	**NC**	**-**	**-**
K81	↓	NC	-	-	-
K82	NC	↓	-	-	-
K83	↓	NC	-	-	-
K84	NC	NC	-	-	-
K85	↓	**NC**	**NC**	↓	↓
K86[a]	↓	↓	**-**	**-**	↓
MSX2	**NC**	**NC**	**NC**	**-**	**NC**

[a]Data appear in Gaspar *et al*., 2010, FASEB J.

IF, immunofluorescence; NC, not changed; -, not investigated.

In **bold,** microarray genes that were evaluated in at least one additional method.