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NEW MECHANISMS IN THE BIOLOGICAL PROCESSES OF HUMAN HAIR GROWTH REGULATION

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ABSTRACT

In recent years, research has shown that numerous neuro-immuno-endocrine mechanisms can significantly regulate fundamental biological processes in the hair follicles of mice and humans. The sudden development of this area is due to the opportunity of researchers to study the fundamental processes of hair follicles in human hair follicle organ cultures *in vitro*. The present work is based on the results obtained from this model.

Our workgroup has successfully demonstrated the role of the cellular receptor TRPV1 or capsaicin receptor – a member of the transient receptor potential (TRP) channel vanilloid subfamily – in the control of growth in mouse and human hair follicles. In recent years the focus of attention has shifted to another member of the TRP family, the warm-sensitive TRPV3. Examination revealed that a mutation in the coding region of the *trpv3* gene caused the hairless phenotype in some "naked" rodent strains. In the present work our aim was to explore the role of TRPV3 in the biological processes of human hair follicles.

In-depth examination of the aforementioned model revealed a previously unknown significance of hair follicles in endocrinology. It has been shown that the human hair follicle is not only a target, but also a source for many classical hormones. In addition, the human pilosebaceous unit is a functional peripheral counterpart of the hypothalamic-pituitary-adrenal axis. Since the direct role of the thyroid-stimulating hormone (TSH) has not been investigated in the physiological processes of the human hair follicles, in the second half of our work we aimed to study the effects of the TSH on isolated human organ cultured hair follicles.

INTRODUCTION

The hair cycle

The hair follicle is unique among other mammalian organs, because of its lifelong alternation of regression and regeneration. These cyclical variations are called hair cycling, which typically has three distinguished stages. During the *anagen* phase of active growth and development the hair follicle reaches its maximum level of development; in the *catagen* phase growth stops, and regressional processes occur; the resting *telogen* phase is the preparation for the upcoming anagen phase.

Once the first hair follicle is established during embryogenesis the first signs of regression in the hair bulb appear quickly, thereby starting the first catagen phase. In this stage, which lasts for an average of 2-3 weeks, apoptotic cellular processes start leading to hair follicle regression. This programmed cell death can be observed in the outer and inner root sheath (ORS and IRS) layers, the matrix keratinocytes and melanocytes as well. Accordingly, the bulb gets increasingly thinner and rises closer to the epidermis during the catagen phase. The dermal papilla (DP) descends towards the proximal direction and then in the late catagen phase moves away from the non-growing club hair with an epithelial handle.

After the relatively short catagen stage, the follicle enters the resting telogen phase, which has an average length of 3 months. In essence, this period prepares the hair follicles for entering the first anagen phase, starting the first hair cycle. At the beginning of the anagen phase the hair follicle is increasingly extended and is pushed deeper. The very rapid proliferation of the matrix keratinocytes creates the hair shaft and the bordering layers of IRS, while the DP is increasingly surrounded by the keratinocytes of the bulb, and the nutritious layers of ORS are reborn as well. At the last, sixth stage of the anagen phase the hair follicle attains the characteristic onion shape, creating a new growing hair,

which is bounded by the IRS, ORS and connective tissue sheath (CTS). In the next 2-6 years the hair follicles of the human scalp stay at this stage until the catagen transformation once again starts leading to regression.

The hair follicle as an "independent" neuroendocrine system?

The latest research suggests that the hair follicle is the most prominent target tissue of major hormonal pathways. These include sexual steroids, retinoids, glucocorticoids, thyroid hormones, adrenocorticotropic hormone (ACTH), corticotrophin-releasing hormone (CRH), melanocortins, vitamin D3, insulin, prolactin, substance P, melatonin, acetylcholine, endovanilloids, endocannabinoids, prostaglandins and catecholamines, which can influence fundamental biological processes of the hair follicle, i.e. hair growth, hair cycle progression, pigmentation, hair development and the release of growth factors, cytokines and hormones.

It has been discovered that the pilosebaceous unit is a non-classical, peripheral hormone-producing area for a surprisingly wide range of steroid hormones, neuropeptides and neurotrophins. In addition, it is a sensitive target organ and producer of stress response mediators.

Last but not least, the hair follicle expresses a fully functional peripheral hypothalamic-pituitary-adrenal (HPA) axis, which is characterized by the presence of the regulatory feedback loops of the central HPA axis (CRH, ACTH and cortisol). It is not an exaggeration to say that the pilosebaceous unit is an active, autoregulated neuroendocrine organ.

The transient receptor potential vanilloid 3 (TRPV3) channel in the skin

The transient receptor potential vanilloid (TRPV) family is composed of various heat-sensitive non-selective cation channels which are able to detect several environmental factors (e.g. temperature, pH and osmolarity changes). Among them the TRPV3 channel is activated by a moderately warm temperature range (31-39°C). The description and molecular characterization of

the protein on keratinocytes instead of sensory neurons (where the other members of the family where first described) anticipated the potential relationship of the channel and the skin. It has been shown that temperatures raised between 23-36ºC activated a non-specific cationic current in a cell line derived from BALB/c mouse strain and human *trpv3* transfected cells. TRPV3 overexpressing cells can also be activated by terpenoids known to be more or less skin irritating, such as eugenol the primary active ingredient of clove, thymol from thyme, camphor isolated from the camphor tree and carvacrol, a significant component of oregano oil. Furthermore, examining cells originating from the skin, the first synthetic agonist of TRPV3 - 2-aminoethoxydiphenyl borate (2-APB) - has been also described.

Animal studies, analyzing the function of TRPV3 led to the most exciting result, since the examination of hairless rodent strains (DS-*Nh* mice and WBN/Kob-*Ht* rats) revealed that the hairless phenotype (*Nh* - no hair, *Ht* - hairless type) of these animals is caused by a single "gain-of-function" mutation in the coding region of *trpv3* gene. This research suggests that the TRPV3 mutation stopped hair growth in early anagen phase.

Various pro-inflammatory mediators and hormones (e.g. dihydrotestosterone) have also been recently reported to increase the activity and expression of TRPV3. We, however, do not have any data about the functional role of TRPV3 in human skin, despite the fact that the presence of the TRPV3 protein have also been reported in human epidermal keratinocytes.

Thyroid-stimulating hormone (TSH) and hair growth

It has been known for decades that thyroid-related disorders are associated with structural and functional lesions of human skin and hair. Several clinical and *in vitro* observations reported a high telogen ratio, altered hair diameter, dry, fragile and coarse hair shafts, decreased follicular proliferation and hair loss

(e.g. effluvium, alopecia) in case of hypothyroid gland function; and increased cell proliferation in the hair bulb in spite of hair loss during hyperthyroidism.

Since thyroid disorders typically involve high serum TSH level fluctuations, the study of the impact of TSH on human hair growth is paticularly interesting.

Objectives

The present study was designed to gain deeper insight into the regulation of the biological processes of hair follicles in the following two issues:

1. As shown above, some of the TRPV3 mutations in mice led to the expression of constitutively active TRPV3 channels and the formation of a hairless phenotype. So far, however, we have no knowledge on the presence and biological role of TRPV3 in human hair follicles. In the first part of our experiments, in order to enlighten this phenomenon the following objectives were formulated:

- To examine the expression of TRPV3 in human hair follicles or hair follicle derived ORS keratinocyte cultures (at gene and protein levels).
- To examine the effects of TRPV3 activation on the biological processes of isolated human hair follicles *in vitro* (elongation, cell proliferation and apoptosis, anagen-catagen transition).
- To examine the effects and specificity of TRPV3 activation on the biological processes of primary cultures of hair follicle derived ORS keratinocytes *in vitro* (channel function, proliferation, apoptosis, examination of the specificity by RNAi technology).

2. In recent years, the presence of many hormonal signaling pathways were studied in human hair follicles. In the second part of the present study we examined the direct effects of TSH in the regulation of biological processes in the human hair follicle. Our objectives were as follows:

- To examine the expression of the TSH receptor in human scalp and isolated hair follicles (at gene and protein levels).
- To examine the effects of TSH treatment on the biological processes of isolated human hair follicles *in vitro* (elongation, cell proliferation and apoptosis, anagen-catagen transition, pigmentation).
- To identify the TSH receptor associated signal transduction of isolated human hair follicles and hair follicle derived DP fibroblasts *in vitro* (cAMP release, identification of TSH target genes).

MATERIALS AND METHODS

Isolation and culture of human hair follicles, histomorphometric studies

Anagen human hair follicles, the basis of our experiments, were isolated from plastic surgery from hairy temporal (female) and occipital (male) regions. Telogen hair follicles were obtained from gluteal skin samples from cosmetic operations. The samples were used in accordance with the Helsinki Declaration of the WHO. First the scalp was cut into approximately 0.5x1 cm pieces, slit parallel to the skin surface, then the epidermis and dermis were removed, and the hair follicles were raised from the subcutis using tweezers. Subsequently, intact anagen VI hair follicles in growth phase were placed into 24 well plates as triplets, and bred for 9 days at 37°C, 5% CO_2 atmosphere in Williams' E medium supplemented with 2 mM L-glutamine, 10 ng/ml hydrocortisone, 10 ng/ml insulin and penicillin-streptomycin mix. Afterwards the hair follicles were embedded in freezing medium, and then sections were made from them with a Cryotome.

After that hematoxylin-eosin staining and histomorphometric analysis was used for the separation of various stages of the hair cycle. The Fontana-Masson histologic technique was used for the visualization of melanin content.

Evaluation of proliferating and apoptotic cells in the hair follicles

For the simultaneous detection of proliferating and apoptotic cells in the cultured follicles the Ki-67/TUNEL double staining was used. Information on the presence of the proliferating cells is provided by fluorescent immunohistochemical detection of Ki-67 nuclear marker; DNA fragmentation (free 3'ends) occurred during apoptotic events indicated by TUNEL nick end labeling kit.

Culturing of outer root sheath (ORS) and HaCaT keratinocytes

ORS keratinocytes were isolated from anagen VI hair follicles removed from the eyebrows using tweezers. Hair follicles were washed in PBS, and then digested in 0.1% trypsin-0.2% EDTA solution for one hour. After digestion the ORS cells were seeded onto a human dermal fibroblast (HDF) feeder layer treated with 0.4 mg/ml mitomycin-C to inhibit their proliferation. The culture medium was a 1:3 mixture of Ham's F12 and Dulbecco's Modified Eagle's media supplemented with 10% Fetal Clone II, 0.1 nM choleratoxin, 5 mg/ml insulin, 0.4 mg/ml hydrocortisone, 2.43 mg/ml adenine, 2 nM triiodothyronine, 10 ng/ml epidermal growth factor, 1 mM ascorbyl-2-phosphate and penicillin-streptomycin mix.

The HaCaT keratinocytes were cultured in Dulbecco's Modified Eagle's medium containing 10% FBS, 2 mM L-glutamine, and antibiotics.

Establishment of dermal papilla (DP) derived primary fibroblast cultures

Freshly isolated anagen VI hair follicles were used for the development of DP fibroblast cultures. The hair follicles were grabbed parallel with their longitudinal axis, CTS were cut through at the proximal end with a scalpel and the emerging DP were transferred into culture dishes containing the appropriate culture medium (follicular DP cell medium supplemented with 4% FBS, 5

mg/ml insulin, 1 ng/ml fibroblast growth factor). Collected DPs were kept in the medium till DP fibroblast cells arose from the DP.

Semi-quantitative reverse transcriptase PCR (RT-PCR) and product sequence analysis

First, total RNA fraction was isolated from cultured hair follicles using RNeasy Kit, and 0.5 μg of the RNA was reverse transcribed using SuperScript reverse transcriptase according to manufacturer's specifications. The resulting cDNA was used for polymerase chain reaction (PCR) as a template with the following targets: TSH receptor, thyroglobulin (TG), thyroid transcription factor-1 (TTF-1); and the internal controls: glucose-6-phosphate dehydrogenase (G6PDH) and 18S ribosomal RNA. The PCR products were separated in ethidium bromide-containing agarose gel, and then the photographed bands were analyzed using ImageJ software.

Melting curve analysis, was performed using Light Cycler instrument for testing the specificity of the TG cDNA amplification. In addition, the sequence of TG band were analysed with a 377 DNA sequence analyzer device.

Microarray analysis

Microarray analysis of gene expression was preformed in hair follicle samples of two individuals using Human Whole Genome Oligo Microarray ® (44k) chip. The study was conducted by Miltenyi Biotech GmbH. Alterations observed in both subjects were considered as significant expression changes in case of at least 1.5 fold uni-directional changes if the p value <0.0001.

RNA isolation, reverse transcription, quantitative PCR (q-PCR)

The total RNA content of cells was isolated using TRIzol reagent. 3 mg of total RNA was reverse transcribed with AMV reverse transcriptase and random primers. The obtained cDNA was used for q-PCR with TaqMan Universal PCR Master Mix protocol. Transcripts were determined using the following TaqMan

primers and probes: TRPV3, connective tissue growth factor (CTGF), glutathione peroxidase-3 (GPX3), muscle-type pyruvate kinase-2 (PKM2), cytochrome-C oxidase-1 (MTCO1); internal controls: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ciklofilin-A (PPIA).

Immunohistochemistry and -cytochemistry

Fluorescent immunohistochemistry (IHC-F) and immunocytochemistry (F-ICC) methods were applied to the following proteins: TRPV3, keratin 7 (KRT7), a fibroblast marker CD90 and MTCO1 detection (Table 1). Avidin-biotin-peroxidase complex (ABC) based immunohistochemistry was used for the detection of TSH receptor, alpha smooth muscle actin (ACTA2), MTCO1 and KRT5 proteins (Table 1). Changes in TSH receptor expression levels in the isolated hair follicles during TSH treatment was observed by tyramide substrate amplification (TSA) (Table 1). Recordings of the immuno stainings were made with Nikon Eclipse E600 fluorescent and Zeiss LSM 510 confocal microscopes.

Target protein	*Origin*	*Species*	*Dilution*	*Method*
ACTA2	Sigma-Aldrich,	Mouse	1:50	ABC
CD90	Dianova	Mouse	1:100	F-ICC
KRT5	PROGEN	Guinea pig	1:500	ABC
KRT7	Novus Biologicals	Mouse	1:100	F-ICC
Ki-67	DAKO	Mouse	1:20	F-IHC
MTCO1	Mitosciences	Mouse	1:50, 1:50	ABC, F-ICC
TRPV3	Abcam	Rabbit	1:200	F-IHC, F-ICC
TSH receptor	InVivo BioTech Services	Mouse	1:200, 1:1000, 1: 100, 1:100	ABC, TSA, F-IHC, F-ICC

Table 1: Primary antibodies used for immunolabelling

Abreviations of methods: ABC: avidin-biotin complex, F-ICC: fluorescent immunocytochemistry F-IHC: fluorescent immunohistochemistry, TSA: tyramide substrate amplification.

Western blot

The cells were harvested in lysis buffer supplemented with protease inhibitor, followed by ultrasonic excavation performed on ice. After the determination of the protein level of the cell lysate the samples were denatured and equal quantities of the protein samples were electrophoresed using an SDS-polyacrylamide gel. Subsequently, the proteins were transferred to a nitrocellulose membrane form the gel, and the non-specific binding sites of the membrane were blocked. The membrane was then incubated with the above-mentioned anti-TRPV3 antibody followed by a horseradish peroxidase-conjugated anti-rabbit secondary antibody. Finally, chemiluminescent ECL signals were detected using Kodak Gel Logic 1500 Imaging System.

Determination of intracellular calcium concentration ($[Ca^{2+}]_{IC}$)

ORS keratinocyte cells (20,000 cells per well) were seeded on a HDF feeder layer (900 cells per well with inhibited proliferation) in black-walled clear-bottomed 96 well plates. The following day the attached cells were loaded with 2 μM Fluo-4 AM (in Hank solution with probenecid and albumin). The Ca^{2+} signal was measured with II^{384} FlexStation fluorescence microplate reader (λ_{EX}: 480 nm, λ_{EM}: 520 nm).

Patch-clamp

For the patch-clamp measurements ORS keratinocytes were seeded on thin coverslips. A Multiclamp-700A type amplifier was used in whole-cell configuration for the measurements. Borosilicate pipettes with 2-4 MOhm resistance were filled up with K-aspartate internal solution (pH 7.2). The coverslips were perfused with normal Ringer solution (pH 7.4). In the case of each cell measured ionic currents were normalized to the capacity of the cell. The electrical signals were recorded at a frequency of 10 kHz (Digidata 1322) and the analysis was performed using pClamp 9.0 software.

Determination of viability

The growth of the cells was determined by CyQuant cell proliferation kit. ORS keratinocyte cells (10,000 cells per well) were seeded on HDF feeder layer (900 cells per well with inhibited proliferation) in black-walled clear-bottomed 96 well plates, cultured and treated for 72 hours. On the day of the measurement the cells were lysed, and incubated for 5 min with CyQuant dye. The fluorescence of the dye was measured using FlexStation II^{384} (λ_{EX}: 480 nm, λ_{EM}: 520 nm).

Examination of the mitochondrial membrane potential

The loss of mitochondrial membrane potential is one of the earliest hallmarks of apoptosis, which we investigated using MitoProbe™ DiIC$_1$(5) Assay Kit. The ORS keratinocytes were cultured and treated as mentioned above. After the aspiration of the supernatants the cells were incubated with DiIC$_1$(5) working solution diluted according to the manufacturer's instructions. After the removal of the working solution the cells were washed, and fluorescence was measured using FlexStation II^{384} (λ_{EX}: 630 nm, λ_{EM}: 670 nm).

Deretmination of cytotoxicity

SYTOX Green fluorescent dye was used to monitor cytotoxic processes characterized by a damaged cell membrane, since it is only able to get into cells with ruptured membranes and then binds to DNA. To do this, the cells were cultured and treated as mentioned above, and then incubated with 1 μM SYTOX Green dye. The fluorescence was measured using FlexStation II^{384} (λ_{EX}: 480 nm, λ_{EM}: 520 nm).

RNA interference (RNAi)

ORS keratinocytes were transfected with TRPV3-specific small interfering RNA (siRNA) using the Neon Transfection Kit. The effectiveness of RNAi-mediated gene silencing was checked daily with Western blot and Ca^{2+}-

imaging techniques for four days. In the case of two siRNA oligonucleotide the TRPV3 gene silencing efficiency was more than 70% 2 days after transfection , so the experiments were started 2 days after transfection.

cAMP level determination in supernatants

For the quantitative measurement of cAMP levels in the supernatants of hair follicle and DP fibroblast cultures a competitive ELISA assay were performed according to manufacturer's specifications.

Statistical analysis

Two-sample unpaired t-test (paired t-test for patch-clamp measurements) with 5% significance level was used for the evaluation and comparison of statistical data.

RESULTS

TRPV3 is expressed in human hair follicles and primary cultures of outer root sheath (ORS) keratinocytes

Previous data from the literature shows that TRPV3 is expressed in the interfollicular keratinocytes of the human epidermis. In our present experiments, we wanted to determine the expression of TRPV3 in human hair follicles isolated from healthy scalp skin (from routine plastic surgeries). Immunohistochemistry techniques showed that TRPV3 was expressed in anagen hair follicles: the presence of the protein was mainly confined to the ORS and partly to the layers of matrix keratinocytes. In accordance with these observations significant positive TRPV3 immunoreactivity was shown in primary cultures of KRT7 positive ORS keratinocytes with both immunocytochemistry and Western blot techniques.

RNA was extracted from freshly isolated hair follicles and cultures of ORS keratinocytes and q-PCR experiments were performed to verify the

intraepithelial presence of TRPV3 transcripts. The TRPV3-specific mRNA segments were clearly identified in hair follicles from healthy scalp skin, and ORS keratinocytes derived from hair follicles.

Another important result of these experiments was that TRPV3 immunoreactivity and specific mRNA was not detected in the mesenchymal DP fibroblasts of hair follicles and HDF feeder layer cells used during the breeding of ORS keratinocytes.

The stimulation of TPRV3 inhibits elongation and proliferation of hair matrix keratinocytes and causes apoptosis-driven early regression

Having confirmed the presence of TRPV3 in human hair follicles, we aimed to investigate the functional consequences of activation of the protein. In order to ascertain the effects of TRPV3 agonists on human hair follicles organ cultured anagen VI follicles isolated from human scalp skin were investigated, the closest *in vitro* model to physiological conditions. During the eight days of treatment hair elongation were measured daily in both the control and treated groups, and a growth curve was plotted using the measured data. We found that plant derived substances, such as eugenol, thymol, carvacrol and the synthetic agonist 2-APB significantly inhibited the elongation of hair follicles in a time- and dose-dependent manner.

Afterwards we examined the effects of TRPV3 activation on the fundamental biological processes of hair follicles. Hair follicle sections from control and treated groups were assayed for the detection of proliferating cells (Ki-67 staining), and visualization of the apoptotic events (TUNEL reaction). The results were in line with the previously observed inhibitory effect on hair growth: the treatment of organ cultured hair follicles with herbal and synthetic TRPV3 activators for five days significantly reduced the proportion of Ki-67 positive keratinocytes in the bulb of anagen hair follicles. It is also important to note that the aforementioned drugs significantly increased the number of

TUNEL positive keratinocytes, which indicates the onset of apoptosis caused by TRPV3 stimulation.

To elucidate the impact of TRPV3 agonists on the hair cycle the anagen-catagen transition of hair follicles was investigated. While the vast majority of hair follicles in the control group (> 80%) remained in anagen VI stage after 8 days the hair follicles of the eugenol and 2-APB treated groups entered catagen phase (60-100%). However, it should be noted that the TRPV3 agonists are mainly able to induce premature catagen transformation; a very small number of hair follicles have undergone a late catagen regression after the application of these drugs.

TRPV3 is a functional Ca^{2+}-channel in the ORS keratinocytes

Although the above data alludes to the expression of "active" TRPV3 in the keratinocytes of the human hair follicle, we wanted to further analyze the functionality of the TRPV3 channel. To do this, we performed cellular experiments with primary human ORS keratinocytes, which, as already mentioned earlier, express the TRPV3 channel at both mRNA and protein levels. Using a FLIPR-based Ca^{2+}-imaging technique we found that TRPV3 agonists significantly increased the $[Ca^{2+}]_{IC}$ of the cells in a dose-dependent manner. In addition, the reduction of $[Ca^{2+}]_{EC}$ (from 1.8 to 0.02 mM), as well as administration of 10 µM ruthenium red (a non-selective TRP channel blocker) almost completely prevented the$[Ca^{2+}]_{IC}$ increasing effect of TRPV3 agonists.

To further analyze channel function, patch-clamp technique in whole cell configuration with ramp protocol was applied. In the case of vehicle-treated ORS keratinocytes (Control) outward rectifier currents were measured with a reversal potential of –12,6±1,5 mV (mean±SEM, n=7). Currents were measured at four points of the ramp (–90, –40, +40 and +90 mV), all data were always normalized to the current capacity of membrane (–9,5±2,5, –3,5±1,0, 6,2±1,0 and 15,9±3,5 pA/pF respectively) (mean ± SE for all data values).

Treatment with 100 μM 2-APB substantially and significantly ($p < 0.05$) increased both inward and outward currents, this effect was found to be reversible. In the presence of 2-APB the currents measured at –90, –40, +40 and +90 mV were the following: –31,9±14,2, –13,8±6,4, 17,3±6,1 and 57,7±16,6 pA/pF respectively (mean±SEM, n=7). In average, the TRPV3 activator 2-APB caused about three-fold increase in both inward and outward currents at all tested potentials.

These data suggest that the ORS keratinocytes of human hair follicles indeed express functional TRPV3 channels, which act as Ca^{2+}-permeable cation channels on the cell surface.

TRPV3 activation inhibits proliferation and induces cell death in cultured ORS keratinocytes

Because TRPV3 stimulation inhibited hair growth, intrafollicular proliferation, and induced apoptosis in organ cultured human hair follicles, we aimed to explore the impact of TRPV3 activation on cell growth and survival of ORS keratinocytes. In good agreement with the previous data eugenol and 2-APB reduced the proliferation of ORS keratinocytes in a dose-dependent manner indicated by DNA synthesis (CyQuant assay). In addition, TRPV3 activation significantly reduced the mitochondrial membrane potential, which is one of the earliest markers of apoptosis. Finally, the highest concentrations of these substances significantly increased the accumulation of SYTOX Green dye, which is a sensitive indicator of necrotic and cytotoxic processes.

It has been also shown that the growth inhibitory and cell death inducing effects of TRPV3 activators are significantly inhibited by the lowered Ca^{2+} content of the media (form 1.8 to 0.02 mM), or application of 10 μM ruthenium red. These phenomena support the hypothesis that the observed cellular processes in the ORS keratinocytes are due to the openinig of TRPV3 ion channels on the cell surface followed by Ca^{2+}-influx.

To ensure that the listed cellular responses are convincingly TRPV3 specificic, RNAi technique was used. In perfect accordance with the above data we found that the silencing of TRPV3 significantly prevented the growth inhibiting and apoptosis-inducing effects of eugenol and 2-APB, which clearly demonstrates the specific role of TRPV3 in the biological processes of ORS keratinocytes.

The TSH receptor is expressed in the mesenchymal areas of human scalp and hair follicles at mRNA and protein levels

The following pages present the results of our TSH receptor studies. RT-PCR and immunohistochemistry methods were performed in order to clarify whether the normal human scalp hair follicles express the TSH receptor mRNA and protein *in situ*. The presence of TSH receptor-specific transcript in freshly isolated anagen VI follicles of human skalp was successfully demonstrated. In accordance with this finding TSH receptor-specific immunoreactivity was observed in the human scalp as well, within appendices, largely restricted to the mesenchymal areas of the pilosebaceous unit (e.g. DP, CTS and arrector pili muscle). The TSH receptor staining of anagen VI hair follicles showed similar intensity and was predominantly restricted to mesenchymal elements (DP, CTS). In addition samples of telogen hair follicles from the gluteal region displayed a few TSH receptor-positive cells in the epithelium.

Furthermore, the large nerve bundles, blood vessels, the myoepithel cells around sweat and sebaceous glands of the skin also showed TSH receptor immunopositivity *in situ*.

TSH treatment did not significantly affect the hair growth, the percentage of proliferating and apoptotic cells and pigmentation

In order to study the direct functional effects of the TSH on hair follicles the aforementioned classical biological parameters were studied in control and 1-100 mIU/ml TSH-treated groups of organ cultured anagen VI hair follicles.

None of the TSH concentrations affected the elongation and proliferation of hair matrix keratinocytes and did not significantly change the proportion of apoptotic cells. Similarly, TSH neither affected the proportion of anagen and catagen staged follicles nor the human hair cycle directly *in vitro*. Furthermore, TSH did not significantly change the intrafollicular melanin production, which was established by Masson-Fontana histochemistry staining.

The use of TSH stimulated cAMP secretion

In the following step of our experiments, we examined whether the binding of the high-affinity ligand of TSH receptor cause any change in cAMP levels, since the TSH receptor activation classically followed by a rise in cAMP. Our results suggest that secreted cAMP levels of the hair follicles in the medium were three times higher after TSH treatment than in the solvent control.

TSH treatment did not induce measurable thyroid hormone secretion, but stimulated the transcription of classical TSH target genes in the human hair follicles

Subsequently, we examined whether the activation of TSH receptor-coupled signaling pathways alter the transcription of the classical TSH target genes. In our experiments, we were unable to detect either any thyroid hormone production or the transcription of thyroid peroxidase, the key enzyme in their production, on mRNA or protein levels. At the same time there is no evidence that the supernatants of human organ cultured scalp hair follicles contain measurable amounts of thyroid hormone.

Furthermore, the present experiments on RNA samples obtained from both freshly isolated and solvent-treated control hair follicles showed the presence of mRNA transcripts of thyroglobulin (TG). They also showed that TSH stimulation was able to increase the level of TG transcripts in isolated follicle culture. In addition, thyroid transcription factor-1 (TTF-1) specific

transcripts were detected in these samples, the steady-state mRNA levels of which increased during TSH treatment.

TSH stimulation alters gene expression patterns in human hair follicles

To ensure that the TSH receptor is functional in human hair follicles and to find previously unknown extra-thyroid target genes that are regulated by TSH stimulation, DNA Microarray analysis was carried out on control and TSH-treated samples. Based on a strict selection criteria one down- and twelve up-regulated genes were identified: acidic hair keratin-1 (KRTHa1), keratin 5 (KRT5), involucrin (IVL), keratin-associated protein (KRTAP) 4-4, KRTAP 4-7, 4 KRTAP -14, alpha-actin 2 (ACTA2), fibronectin 1 (FN1), alpha-filamin A (FLNA), connective tissue growth factor (CTGF), glutathione peroxidase 3 (GPX3), pyruvate kinase (PKM2), cytochrome-C oxidase-1 (MTCO1).

Using quantitative PCR we demonstrated that TSH treatment was capable of upregulating CTGF transcription, whereas the GPX3 and PKM2 transcription levels remained unchanged. The MTCO1, ACTA and KRT5 immunohistomorphometry clearly confirmed quantitative changes in protein expression levels *in situ*. Nevertheless, MTCO1 upregulation after TSH stimulation was observed in several areas of the isolated human hair follicles. Furthermore the immunohistochemistry of human CTS fibroblasts showed intense ACTA staining *in vitro*, which was further increased by TSH stimulation. In addition, the TSH-treatment significantly increased the immunostaining of KRT5 in layers of matrix keratinocytes of the hair. All these experimental data clearly show that healthy human scalp hair follicles express functionally active TSH receptors.

Cultured human DP fibroblasts are direct targets for the TSH receptor-mediated signal transduction

Whereas all the previously described TSH responses are from the investigation of a complex miniorgan model which is built of diverse cell

populations with different a functions, our aim was to use primary human DP fibroblast cultures hereafter. TSH receptor-specific immunoreactivity was found in CD90 fibroblast marker positive cultured DP fibroblasts under confocal microscope. It was also shown that TSH treatment of DP fibroblast cells significantly and dose-dependently increased the secretion of cAMP into the medium. TSH treatment significantly and dose-dependently upregulated the levels of certain mRNA transcripts (PKM2, GPX3 and CTGF) in DP fibroblasts, just as in hair follicle cultures. The upregulation of MTCO1 was checked by immunofluorescence as well.

DISCUSSION

The role of TRPV3 in the biological processeses of human hair follicles

Studies revealed that in certain nude rodent strains (DS-*Nh* mice and WBN/Kob-H rat) the mutation responsible for their hairless phenotype is found in the coding region of the *trpv3* gene. Further studies of DS-*Nh* mice have highlighted the importance of TRPV3 in mice skin functions. TRPV3 has been shown to be essential for the proper function and normal development of hair follicles, and it is also involved in the development of a spontaneous itching dermatitis that associated with hairlessness.

Previous data from literature shows that TRPV3 is expressed in human epidermal keratinocytes, but its presence in the human hair follicles has not been revealed yet. The characterization of the role of TRP channels in the physiological and pathological processes of human skin is far from satisfactory. The exploration of the regulatory roles of these molecules in non-neuronal cells and tissues are currently in progress. In this context the present study provides the first evidence that TRPV3 is an inhibitor of human hair growth *in vitro*, by suppressing the hair follicle keratinocyte proliferation and inducing apoptosis-driven catagen regression. Given that these effects are gained from an accepted model for human organ culture which allows the testing of the essential

functions of hair follicles in a quasi-*in vivo* system, these findings were considered to be relevant both physiologically and clinically. Therefore we can say that based on the results presented here TRPV3 is emerging as a newly recognized regulatory molecule in human hair growth and a hair cycle control, which is perfectly consistent with previous data obtained from mouse models.

The presence of functional TRPV3 channels in the epithelial areas of human hair follicles is substantiated by examination of primary cultures of ORS keratinocytes obtained from human hair follicles, i.e. FLIPR-based Ca^{2+}-imaging, patch-clamp measurements, viability and cell death tests. In addition, currents and rise in $[Ca^{2+}]_{IC}$ induced by TRPV3 activators were inhibited by $[Ca^{2+}]_{EC}$ reduction, or ruthenium red administration (which is a general TRP channel blocker). The functional role of TPRV3 is convincingly corroborated by siRNA-mediated gene silencing experiments, which successfully inhibit the cellular effects of TRPV3 activators (eg, proliferation inhibition, apoptosis induction). Taken together, these data argue that there is a signaling pathway in the human hair follicle, in which the TRPV3 channel opening induces Ca^{2+}-influx into the cells, thereby preventing the proliferation of ORS keratinocytes causing hair growth inhibition and apoptosis induction.

Previously, the functional expression of TRPV3 has been mainly described in mouse epidermal keratinocytes. In fact, TRPV3-specific membrane current and increase in $[Ca^{2+}]_{IC}$ were also experienced after heat-activation and pharmacological stimuli in these cells. In addition, the activation of TRPV3 in keratinocytes led to the release of various mediators (e.g. IL-1α, prostaglandin E2, ATP), which are "intercellular messengers" of pain- and/or itching-sensitive sensory afferents. Because the proinflammatory impact on the skin of these molecules are known, the keratinocytes expressing TRPV3 channels may play a role in skin inflammatory processes as well. Indeed, overexpression of the "gain-of-function" mutation *trpv3* gene in epidermal keratinocytes caused not only itch, but severe dermatitis in DS-*Nh* mice. In addition, our preliminary data

suggests that the activation of TRPV3 significantly reduces the growth of human epidermal keratinocytes. These findings, together with our present results emphasize the relevance of TRPV3 channels and the related (so far unspecified) endogenous ligands, with related signaling pathways in physiological and pathophysiological regulation of human and mouse hair growth.

A very similar regulatory mechanism to that described here was identified in the skin in case of another TRP channel. The activation of TRPV1 could inhibit human and mouse hair growth and proliferation, promote early catagen regression of the human hair follicle, induce apoptosis in human epidermal and ORS keratinocytes and also induce release of inflammatory cytokines. It seems that TRPV3 and TRPV1-related signaling pathways (in principle) are able to synergistically regulate the growth of human hair, the hair cycle, and possibly other key biological processes of the skin.

Summarizing the results of our experiments we can state that the TRPV3 expressed in isolated human hair follicles has an important role in the regulation of hair growth and the hair cycle. In the present study we presented that TRPV3 activation effectively inhibits the growth of human hair, which implies that TRPV3 agonists could be used in the clinical treatment of unwanted hair growth (e.g. hypertrichosis, hirsutism) and provide an opportunity to develop a variety of new well-tolerated agents (either in combination with TRPV1 activators, or as stand-alone effects). The future examination of TRPV3 antagonists has been justified now in the treatment of different forms of hair loss (e.g. alopecia, effluvium). Finally, our data highlight the importance of systematic assessment of TRPV3-signaling pathways in the treatment of pro-inflammatory, anti-proliferative and pruritogen diseases such as psoriasis, certain dermatitis or even skin cancer in the near future. Based on the above, the possibilities of clinical applications of substances causing activation and/or sensitization of TRPV3 (eg camphor) should be considered.

The TSH receptor regulates biological processes in the human hair follicle

The present study also aimed to show that the human scalp hair follicle can serve as direct target for TSH, the central upregulator of primary thyroid hormone synthesis and the controller of the hypothalamic-pituitary-thyroid (HPT) axis. As a result of this a new TSH receptor-mediated neuroendocrine signaling pathway was identified in human skin. It is possible that these processes are the causes for well-known effects of endocrine modulators in human skin and hair follicles, as it has been demonstrated in the cases of thyroid hormones, androgens and estrogens.

The lesions in hair growth observed in hypothyroid patients are more likely to be results of thyroid hormone influence since (experimentally) elevated TSH levels showed no significant direct effects on human hair growth, hair cycle, or on the proliferation of matrix keratinocytes in organ cultured hair follicles from female scalp skin. This idea was recently confirmed in an article also demonstrating that both triiodothyronine and thyroxin are able to modulate the length of anagen phase in organ cultured human hair follicles.

Based on immunohistochemical studies the TSH receptor expression was only observed in the mesenchymal areas of scalp hair follicles, whereas interfollicularis dermal fibroblasts of human head and gluteal skin showed no specific TSH receptor immunoreactivity *in situ*. Because the mesenchyme of human hair folliclea seems to be protected against the rejection reaction in a certain extent, allogeneic transplantation is possible even for different-sex patients.. The possibility arises, therefore, that the emerging autoimmunity against the TSH receptor, and the resulting thyroid diseases may be causally related to the isolation of hair follicle mesenchyme and/or to the prior collapse of immune tolerance.

The strictly mesenchymal expression of the TSH receptor in human hair follicles also explains why the TSH stimulation did not affect hair growth, pigmentation, proliferation and apoptosis of the hair matrix keratinocytes, since

the intrafollicular epidermis and pigmentary unit does not appear to show TSH receptor expression in anagen VI and catagen phases. In fact, the expression of intraepithelial target genes and/or gene products (eg KRT5, KRTHa1 which is currently KRT31) are also clearly modified by TSH in isolated human hair follicle cultures. This finding suggests that stimulation of the receptors in the scalp hair follicle mesenchyme chiefly affects the functions of the hair follicle cells in an indirect way. Thus, it is possible that stimulation of TSH receptors in the hair follicle CTS induces production and secretion of certain diffusible factors, such as CTGF. These factors from the hair follicle mesenchyme are probably capable of influencing the different tasks of the hair follicle epithelium (such as hair keratin production, as shown by the up-regulation of KRT5 KRTAPs) in a paracrine way. Consequently, central and peripheral TSH secretion may be able to activate the complex regulatory loops through the recruitment of mesenchymal factors (such as CTGF) which indirectly modulate functions in the epithelial cells of hair follicles. These epithelial-mesenchymal interactions are likely to vary slightly in the different areas of the integument and at various stages of the hair cycle, as our results show some TSH receptor-positive epithelial cells also appeared in human hair follicles obtained from the gluteal region. At the same time we can not rule out the possibility of the direct effect of TSH on epidermal keratinocytes, whereas the TSH receptor is expressed in newborn and adult keratinocytes as well, and its activation caused cAMP increase in human keratinocytes.

The fact that the transcriptional levels of two classical TSH target genes, TG and TTF-1, in human scalp hair follicles were up-regulated after TSH treatment *in vitro*, provides further evidence of the TSH receptor functionality expressed intrafollicularly. In addition, TSH caused activation of the cAMP signaling pathway. which corresponds to the classical TSH receptor-mediated processes. Although the presence of TG transcripts in human keratinocytes (HaCaT) and human melanoma cell lines was previously shown *in vitro*, their

presence has never before been described in normal human skin *in situ*. Accordingly, our observations provide the first evidence that the transcription of TG is up-regulated in human scalp skin and hair follicles by the same transcription factor (e.g. TTF-1) activated in thyroid epithelial cells.

We have also shown that human hair follicles from the scalp are an easy to use, physiologically relevant tool for new non-classical analysis of TSH target genes. This unusual but well-studied model may be the key to understanding previously uncharted new features of TSH, which goes well beyond the recognition of TSH in the role of biological processes of the skin and hair. In addition, a number of new TSH target genes were identified in the follicles of human scalp by the analysis of gene expression, which had not been taken into account in the investigation of the biological relationships between TSH/TSH receptor (e.g. KRTAPs, CTGF, ACTA). Changes in mRNA and/or protein expression level were independently confirmed in several of the newly identified TSH target genes (CTGF, ACTA, MTCO1 and KRT5) using samples from additional patients (quantitative immunohistomorphometry and q-PCR techniques), while expression changes of other factors (FLNA, IVL) that were shown to be different in the two patients by Microarray analysis could not be reinforced in this way.

Although the understanding of the significance of these new target genes in the biological processes of human skin and hair follicles require further investigation, based on the above results, we can say that the new effects of TSH are far beyond the range of classically known processes (e.g. production of thyroid hormone stimulation, thyroid growth and regulation of enzyme production in the thyroid gland). For example FLNA, the expression of which is regulated by TSH is an important component of connective tissue in the normal operation of the human skin, whereas CTGF (in collaboration with the "wingless-type protein (WNT) receptor complex) modulates the WNT signaling pathway, that is one of the principal regulators of hair follicle morphogenesis

and hair cycle. The follicular up-regulation of MTCO1 observed after TSH stimulation *in situ* raises the theoretical possibility that the TSH may be able to directly influence the energy metabolism of the hair follicle. Finally, up-regulation of PKM2, GPX3, MTCO1 and CTGF was observed in human DP fibroblasts (target cells of TSH receptor-mediated direct stimulation) after administration of TSH. This suggests that TSH signaling may play a direct role in the regulation of metabolism and oxidation processes in the DP fibroblasts of hair follicles.

However, TSH up-regulated the immunoreactivity of the myofibroblast marker ACTA in the skin, which suggested that TSH is able to facilitate the transdifferentiation of fibroblasts into myofibroblasts. This transformation typically occurs during wound healing and closure in normal skin when fibroblasts change their typical appearance into smooth muscle like phenotype, i.e. expressing contractile proteins and as well as changing orientation. We suspect that the up-regulation of ACTA suggests that the hair follicle derived fibroblasts undergo transformation into a myoepithelial phenotype. Since the follicular CTS also plays a prominent role in wound healing, and since the human hair follicles were exposed to significant injury during the establishment of organ cultures via microdissection this model serves as an unconventional, but instructive model for further discoveries of TSH impact on wound healing, scarring and skin renewal.

SUMMARY

In the first part of our experiments the presence of transient receptor potential vanilloid-3 (TRPV3), at both protein (immunohistochemistry) and mRNA levels (q-PCR), was identified in isolated human hair follicle cultures. We have shown that different TRPV3 activators inhibited hair growth, reduced the amount of the proliferating cells (Ki-67, nuclear proliferation marker), and stimulated apoptosis (TUNEL reaction, DNA fragmentation) in the hair bulb, and induced catagen transformation in a dose-dependent manner. The functional presence of TRPV3 in ORS keratinocyte cultures has also been successfully demonstrated; since the activation of the channel induced membrane currents and elevated Ca^{2+} levels, which lead to the suppression of cell proliferation and the induction of apoptosis. These have proven to be TRPV3-specific on ORS keratinocytes, since TRPV3-specific gene silencing antagonized these actions. Based on these results, we can conclude that certain cells of the human hair follicles express TRPV3 channels, which play a significant role in the regulation of follicular growth and hence the processes of hair cycling.

The effects of thyroid-stimulating hormone (TSH) were then investigated on the human hair follicle, which – based on recent studies – is a uniquely hormone sensitive tissue. The presence of the TSH receptor specific mRNA and immunoreactivity was detected in the mesenchymal areas of human skin. In addition, the stimulation of TSH receptor resulted in an increase in cAMP levels in cultures of hair follicles and DP fibroblasts, and led to a change in the expression levels of certain classical and so far unknown TSH target genes as well. Although further studies are required to fully understand of the exact function of TSH in the biological processes of the human hair follicle, our novel findings introduce the hair follicles as an interesting, new extra-thyroid target for TSH.

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List of publications related to the dissertation

1. **Borbíró, I.**, Lisztes, E., Tóth, I.B., Czifra, G., Oláh, A., Szőllősi, A., Szentandrássy, N., Nánási, P.P., Paus, R., Kovács, L., Bíró, T.: Activation of transient receptor potential vanilloid-3 inhibits human hair growth.
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