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Thyrotropin releasing hormone (TRH) selectively stimulates human hair follicle pigmentation

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der Universität zu Lübeck
- Aus der Sektion Medizin -

vorgelegt von
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Table of contents

Abbreviations

AC:	Adenyl cyclase
ACTH:	Adrenocorticotropin hormone
APM:	Arrector pili muscle
ASP:	Agouti-Signaling protein
ATP:	Adenosine triphosphate
B-AMc:	Bulbar amelanotic melanocyte
BM:	Basal membrane
B-MMc:	Bulber melanogenic melanocyte
BSA:	Bovine serum albumin
cAMP/ AMPc:	Cyclic adenosine monophosphat
CLIP:	Corticotropin-like intermediate peptide
CRH:	Corticotropin-releasing hormone
CRH-R:	Corticotropin-releasing hormone receptor
CTS:	Connective tissue sheath
D:	Dermis
DAPI:	4´,6´-diamidin-2´-phenylindol-dihydrochlorid
DMSO:	Dimethylsulfoxid
DOPA:	Dihydroxyphenylalanin
DP:	Dermal papilla
EDTA:	Ethylenediaminetetraacetic acid
EMU:	Epidermal melanin unit
Epi-Mc:	Epidermal melanocyte
FITC:	Fluorescein isothiocyanate
Gs:	G signal protein
Gp100:	Glycoprotein 100
HCl:	hydrochloric acid
HF:	Hair follicle
HFPU:	Hair follicle pigmentary unit
HM:	Hair matrix
HPT:	Hypothalamic-pituitary-thyroid
HS:	Hair shaft

IF-Mc:	Infundibulum melanocyte
IRS:	Inner root sheath
MC-1R:	Melanocortin 1 receptor
MITF:	Microphthalmia-associated transcription factor
MX:	Matrix
NaCl:	sodium chloride
NaOH:	Sodium hydroxide
NGS:	Normal goat serum
ORS:	Outer root sheath
ORS-AMc:	Outer root sheath amelanogenic melanocyte
PBS:	Phosphate buffered saline
POMC:	Proopiomelanocortin
PPIA:	Peptidylprolyl isomerase A (cyclophilin A)
RT-PCR:	Real-Time Polymerase Chain Reaction
SEM:	Standard error of mean
SG:	Sebaceous gland
ß-MSH:	Beta-melanocyte-stimulating hormone
T3:	Tri-iodothyronine
T4:	Thyroxine
TBS:	Tris buffered saline
TNT:	Tris-NaCl-Tween
TRH:	Thyrotropin releasing hormone
TRH-Rx:	Thyrotropin-releasing hormone receptor
TSA:	Tyramide Signal Amplification
TSH:	Thyroid-stimulating hormone
αMSH:	Alpha-melanocyte-stimulating hormone
γ-LPH:	Gamma-Lipotropin
γ-MSH:	Gamma-melanocyte-stimulating hormone

List of figures

List of tables

Preface and project overview

This MD thesis project explores a novel neuroendocrine control of human pigmentation by focusing on a - previously unknown – regulatory role for the neurohormone, thyrotropin-releasing hormone (TRH), in human hair follicles. Experimental work for this project was started by the candidate in October 2009, and finished by September 2011. Soon thereafter, the data generated by the candidate were integrated into and published in the context of a corresponding extensive study in the #1 skin research journal*, J Invest Dermatol* (Gaspar et al. 2011), in which the candidate served as second author, owing to her substantial contributions to the overall study.

Due to clinical training and family planning/child care priorities, formal write-up of this work as an MD thesis could only commence recently, explaining the long hiatus between the publication of these data and submission of the current thesis.

In the following INTRODUCTION, the relevant literature is therefore primarily being reviewed from a perspective that reflects the background literature, thought process, and working hypothesis that had led to the conception of the current work in the laboratory of the thesis supervisor (Prof. Paus). However, where particularly relevant, selected citations from the literature after 2011 are listed from completeness, as well.

The RESULTS presented subsequently reflect the candidate's specific contributions to Gaspar et al. *J Invest Dermatol* 2011.

At the end of the DISCUSSION, it is then explicitly delineated and discussed how the field of neuroendocrine pigmentation biology and controls of human hair follicle pigmentation have progressed between 2011 and 2016.

1 INTRODUCTION

During the course of evolution, various forms of natural camouflage have developed that assist animals in hiding from predators and thus ensure the survival of the species. Some animals, especially certain fish and amphibians, often have the remarkable ability to change skin color. (**Figure 1.1**) (Garcia and Sih 2003;Stuart-Fox and Moussalli 2009; Rossiter et al., 2012;(Burgdorf and Hoenig 2015). For example, the Peron´s Tree Frog can change its skin color very quickly, shifting between gray and brown in the shade and white when on a bright background, all in less than one hour (Richard B. King 1994) .One key question is how do they accomplish this?

Previous investigators have identified a small tripeptide neurohormone, thyrotropin releasing hormone (TRH), which is best known as the neuroendocrine key regulator of thyroid functions in vertebrates (Fliers et al., 2014; Joseph-Bravo et al., 2015; Joseph-Bravo et al., 2016; Ortiga-Carvalho et al., 2016), as one of the agents that facilitates rapid amphibian skin color changes and thus contributes to camouflage (Jackson and Reichlin 1977; Vaudry et al., 1999; Jenks et al., 2007; Galas et al., 2009; Jenks et al., 2010; Haslam et al., 2014) [discussed below in detail]. This suggested that TRH might operate as an important “molecular bodypainting artist” in lower vertebrate species.

Figure 1. 1: An example of nature´s best camouflage.

It could be possible, that people cannot recognize, that there is a frog in some of the pictures, because of the perfect camouflage technique (from website lifewithinme.com July 24. 2011).

The goal of this thesis project was to determine if any of these pigmentation-modulatory properties of TRH have been conserved in human skin, especially in the human hair follicle organ (HF). This was inspired by clinical observations, the most impressive of which is the phenomenon of “overnight graying” or “sudden whitening” (**Figure 1. 2**) (Jelinek 1972; Plinck et al., 1993; Paus 2011; Tan and Weller 2012; (Nahm et al., 2014), given that TRH has been implicated in neuroendocrine changes in connection with postnatal and other psychoemotional stress responses as well as in the cold stress response (Michalaki et al., 2009; Nillni 2010; Helmreich and Tylee 2011; Joseph-Bravo et al., 2015; Jaimes-Hoy et al., 2016; Joseph-Bravo et al. 2016). One wonders, if TRH-related HF pigmentation changes in response may be an atavistic relic of a neuro-endocrinologically mediated „camouflage“ response. Of

course, at the time when this thesis projected originated, this was a completely unproven hypothesis. Yet, first preliminary unpublished data generated by a previous MD student in the Paus Lab (C. Hardenbicker) had suggested, but not yet convincingly documented that TRH might indeed impact on human HF pigmentation, albeit rather as a stimulator than as an inhibitor.

Figure 1. 2: A case of real sudden graying.

"A women with very long, full hair that had suddenly turned grey, apparently shortly after her Jewish spouse had been arrested by the Gestapo, the dreaded Nazi secret police." Courtesy of Professor Wolfram Sterry, from the photoarchives of the Dept. of Dermatology, Charite´ University Medicine, Berlin, Germany (Paus 2011).

Just before the project was started, the Paus Lab had discovered that human HFs not only express TRH mRNA and peptide, but also respond strongly to stimulation with exogenous TRH by prolongation of HF growth phase (anagen) and by enhanced hair shaft production (Gáspár et al., 2010). Since this was also associated with increased HF pigmentation, the first question that arose was if such HF pigmentation effects were only related to hair growth (anagen)-coupled pigmentation (HF melanogenesis) (see below), or rather if TRH exerted hair cycle-independent effects on intrafollicular melanogenesis and/or other HF melanocyte functions.

Furthermore, in light of the recognized impact of TRH on frog skin pigmentation (Jackson and Reichlin 1977; Vaudry et al. 1999; Jenks et al. 2007; Galas et al. 2009; Jenks et al. 2010) and the prior human HF study from our laboratory (Gaspar et al. 2010), a second question arose: Does TRH also impact on human epidermal melanocyte functions *in situ* ? These questions were pursued in the current doctoral thesis project, using established human HF and skin organ culture assays.

1.1 The human skin and hair follicle

The skin is the largest organ of the human body, which accounts for about 15% of the total adult body weight (Judah et al., 2012; Slominski et al., 2016). The skin has multiple biological functions such as protection against microbes, external physical, chemical aggressions and regulation of the body temperature. Furthermore, the skin has a major role on the perceptions of beauty, health and age (Ning and Andl 2012; Ramot and Paus 2014). The skin has three layers: the epidermis, the dermis and the hypodermis. The basal layer of the epidermis is the location of specialized cells called melanocytes, which create the skin´s color (Kanitakis 2002). (**Figure 1.3**).

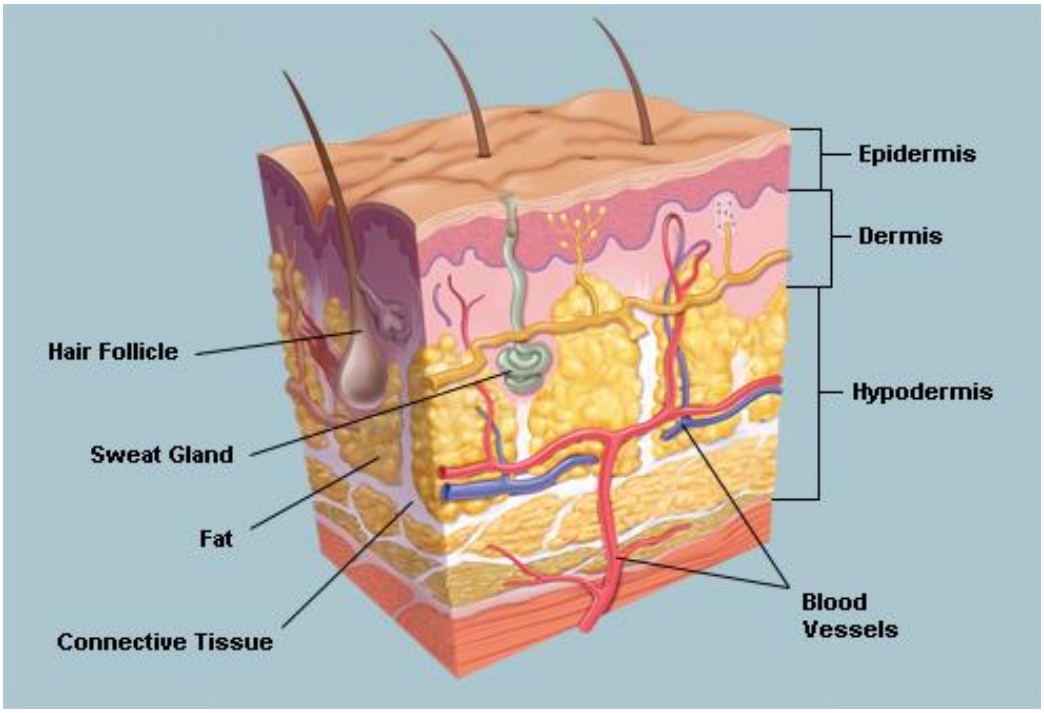

Figure 1. 3: The structure of human skin

The human skin is composed of three layers the epidermis, the dermis and the hypodermis. It contains a vast network of nerve endings, blood vessels and connective tissue. The skin colour is created by the special cells called melanocytes, which are located in the basal layer of the epidermis. (from the website webmd.com/skin-problems-and-treatments/picture-of-the-skin March 30.2016).

Hair is not as simple as it seems. In addition to a wide range of biological functions such as protection against environmental stressors, sensory activity, and thermoregulation, the most important function of hair in human society is as a physical instrument of social communication (Paus and Cotsarelis 1999; Stenn and Paus 2001; Tobin 2008, 2011)

1.2 The hair life cycle

HF development is completed during the fetal period. Hence, the number of HFs is already determined before birth. After birth each HF undergoes amazing life-long cycles of growth (anagen), involution (catagen) and quiescence (telogen) (Fuchs 2007; Rabbani et al., 2011; Gilhar et al., 2012; Chang et al., 2013; Won Oh et al., 2015) (**Figure 1.4**).

The anagen phase, which has six sub-stages during which the root divides rapidly, is recognized as the growing phase of the HF. About 80 - 90 percent of the scalp HFs are in this anagen phase, which can last for three and more years. In this stage the hair shaft grows continuously and the hair bulb [discussed in detail in the section below] produces hair pigment (Tobin 2008; Rabbani et al. 2011). The hair shaft looks thick and nourished (Stenn and Paus 2001). About 2 - 3 % of the scalp hairs are in the catagen phase, which last for 2 to 4 weeks. In this phase the hair bulb stops producing the melanin, the pigment that provides hair shaft with color (Tobin 2011).

The final phase of the hair growth cycle is the “resting phase”, known as telogen , after which the hair shaft will fall out by an actively regulated process (“exogen”) (Paus and Cotsarelis 1999; Stenn and Paus 2001; Paus and Foitzik 2004; Slominski et al., 2005; Tobin 2011; Hardman et al., 2015). Around 10 - 15 % of the scalp HFs are in this phase, which can last for three months. Physiologically 50 - 100 hair shafts will be shed daily during telogen. However, most recently, the traditionally assumed relative distribution between anagen, catagen and telogen HFs has been questioned, and the percentage of scalp HFs in catagen may actually be higher, and that of telogen HF lower than generally assumed (Oh et al. 2015). Moreover, it is increasingly appreciated that telogen is not really as quiescent a hair cycle phase as it morphologically appears to be (Geyfman et al., 2012; Geyfman et al., 2015).

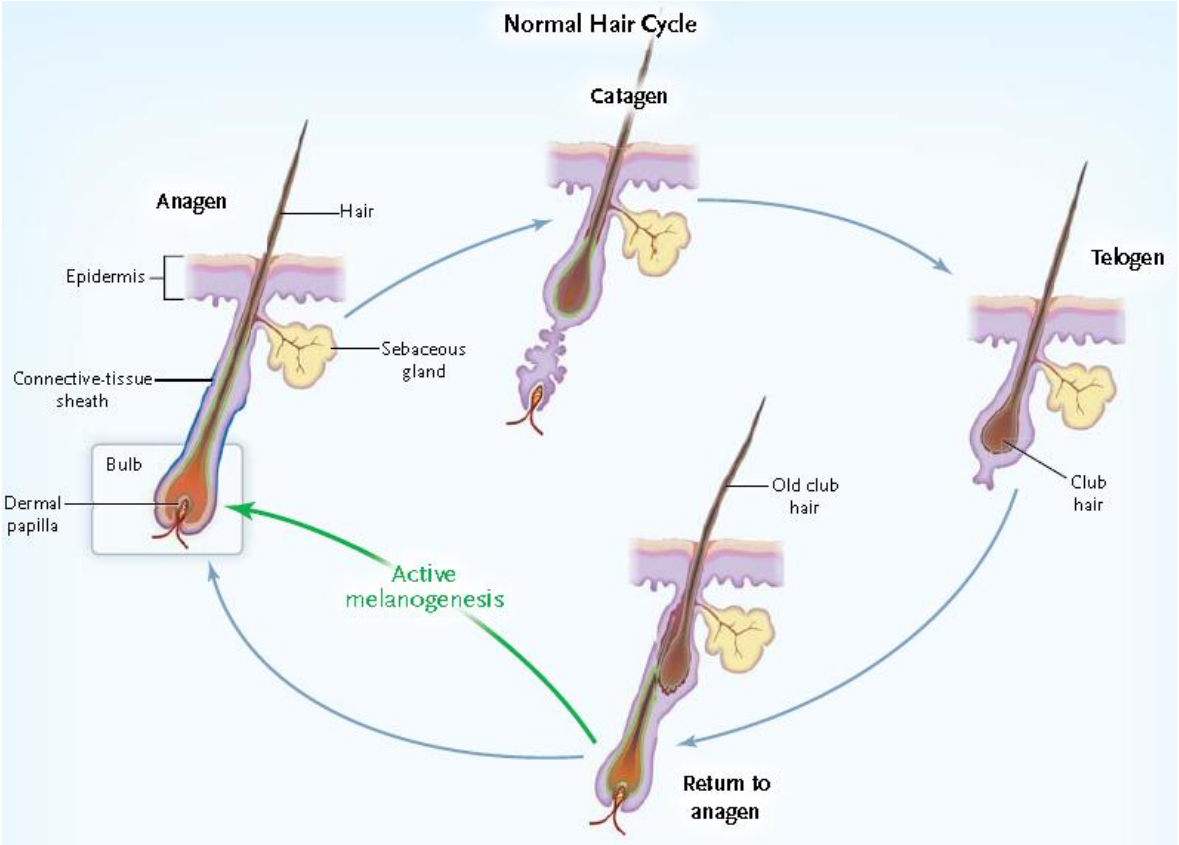

Figure 1. 4: Schematic illustration of the hair life cycle.

The hair follicles progress to the destructive (catagen) phase, during which the lower two-thirds of the follicle undergo apoptosis and regress. After the resting (telogen) phase, factors activate hair follicle stem cells to regrow the hair in the anagen phase, in which the pigmented hair shaft I generated. (Gilhar et al. 2012)

1.3 Functional hair follicle anatomy

The anatomy of the HF is best shown in the anagen phase. The HF is comprised of the upper segment (infundibulum), the middle segment (isthmus), and the lower segment (bulb and suprabulb) (**Figure 1.5A**).

The infundibulum, which is the most distal part of the HF, extends from the epidermal surface to the insertion point of the HF associated sebaceous gland. The isthmus begins at the attachment point of the arrector pili muscle (APM) and goes to the entrance of the sebaceous gland duct to the follicular orifice (Schneider et al., 2009).

The suprabulb region starts from the hair bulb and ends at the isthmus of the HF and consists of components of the hair shaft, inner root sheath (IRS), outer root sheath (ORS) and others (**Figure 1.5 B**).

The hair bulb, which acts as the actual “hair shaft factory”, consists of the dermal papilla (DP) and the surrounding hair matrix (HM). The dermal papilla is fed by very small blood vessels and contains papillary fibroblasts, which interact directly with the follicular hair matrix and are supposed to play an important role in follicular development and cycling (Matsuzaki and Yoshizato 1998; Samuelov et al., 2015).

The hair matrix is composed of rapidly proliferating keratinocytes and is responsible for hair shaft production. During their differentiation, keratinocytes receive melanosomes from the melanocytes of the HF pigmentary unit [see below] for hair shaft pigmentation (Paus and Foitzik 2004; Slominski et al. 2005; Paus 2007; Schneider et al. 2009).

The outermost mesenchymal layer of the HF, the connective tissue sheath (CTS) forms the interface between the HF and the dermis/subcutis and is contiguous with the DP. There is a substantial change in fibroblasts between the CTS and the DP, which modifies the diameter of the DP and thereby influences the activity of matrix keratinocytes, and the number of morphogen-secreting DP fibroblasts greatly impacts on HF cycling, hair quality and length (Tobin et al., 2003; Ohyama et al., 2010; Morgan 2014).

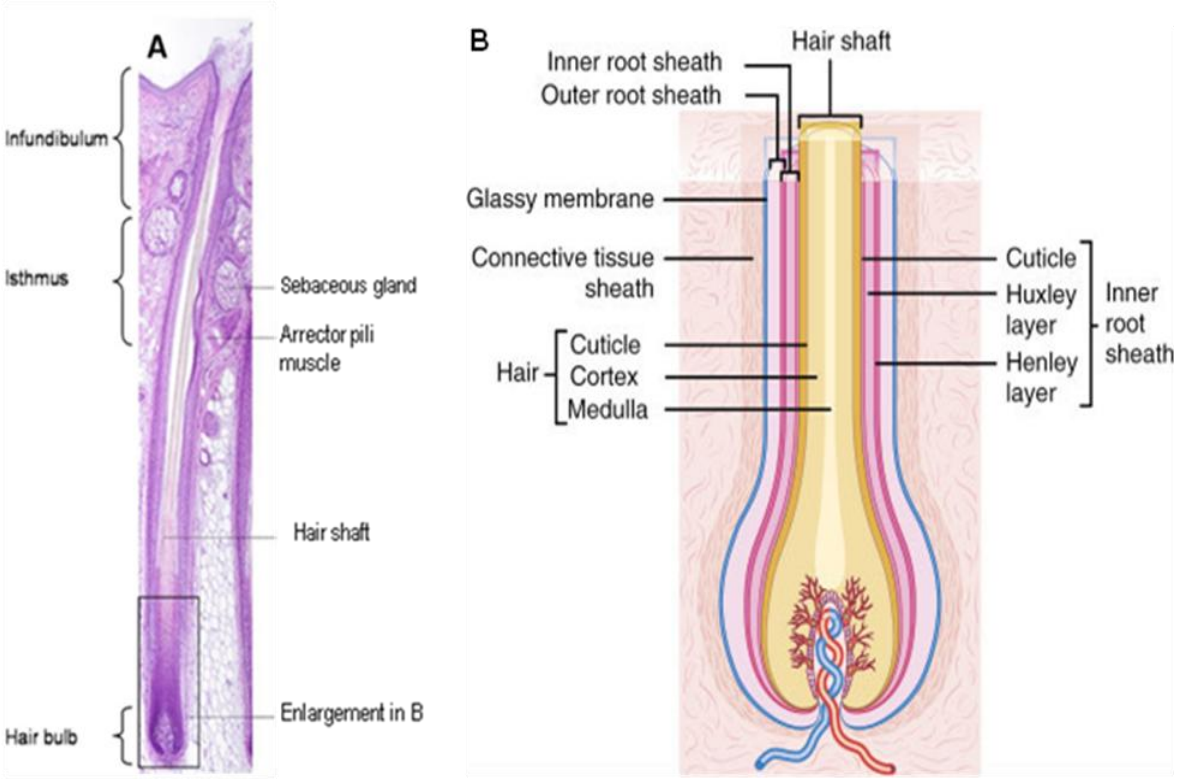

Figure 1. 5: The anatomy of the hair follicle.

As shown a histogical longitudinal section of an anagen VI HF with the different anatomic structures (A) (HF photograph generously provided by Dr. Jennifer Kloepper, Lübeck). Enlargement of the proximal part of the HF gives a detailed view of the different compartments within the human HF (B). (from website http://medicine.academic.ru/135206/Henle_layer March 30.2016)

1.4 Melanogenesis in the human skin and human hair follicle

1.4.1 The pigmentary unit

The main determinant of differences in skin and hair shaft colour is melanin, the major product of melanocytes. Both epidermal and follicular melanocytes are derived from immature melanocytes, which migrate from the neural crest (Aoki et al., 2011; Rabbani et al. 2011; Djian-Zaouche et al., 2012; Wu and Hammer 2014; Mort et al., 2015). Epidermal melanocytes are located in the bottom layer, the stratum basale, of

the skin's epidermis (Miot et al., 2009). Here a melanocyte exists in a symbiotic relationship with a pool of associated keratinocytes in a high melanocyte-to-kerationcyte ratio (1:25-40). This symbiotic relationship is called the epidermal melanin unit (EMU) (Tobin and Paus 2001; Lin and Fisher 2007; Yamaguchi and Hearing 2009; Weiner et al., 2014; Tobin 2015; Bastonini et al., 2016). (**Figure 1.6**).

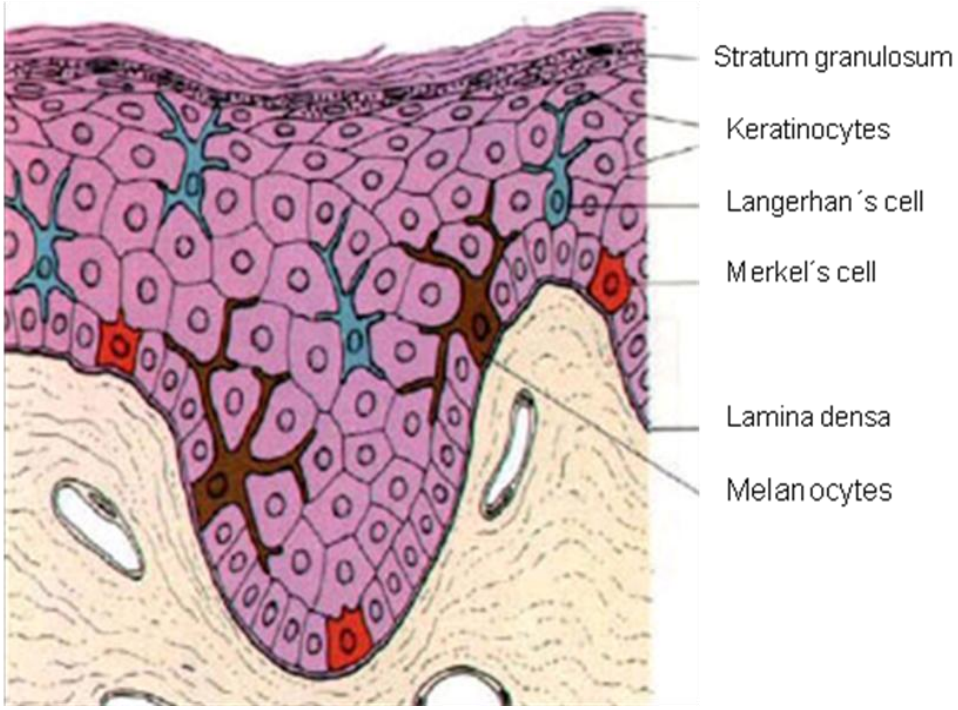

Figure 1. 6: Schematic representation of melanocytes in the epidermis and their inter-relation with keratinocytes

There are four types of cells in the epidermis: keratinocytes, Langerhan´s cells, Merkel´s cells and melanocytes. A melanocyte gains a symbiotic relationship with a pool of associated keratinocytes with a high melanocyte-to-kerationcyte ratio (1:25-40) and this symbiotic relationship is called as the epidermal melanin unit (EMU) (Miot et al. 2009).

It is intriguing how many hair colours are found in the world e.g. the deep brown–black hair colour of humans of African origin, while the humans in northern Europe have a bewildering array of hair colours that range from white blonde, yellow blonde, auburn to red and all shades in between (Tobin 2008, 2011; Commo et al., 2012).
In the hair follicle five different melanocyte subpopulations can be detected (Tobin 2008). There are infundibular melanocytes, sebaceous gland melanocytes, ORS melanocytes, melanogenic bulbar melanocytes, amelanotic bulbar melanocytes (Glover et al., 2015; Purba et al., 2015; Savkovic et al., 2015)(**Figure 1.7**).

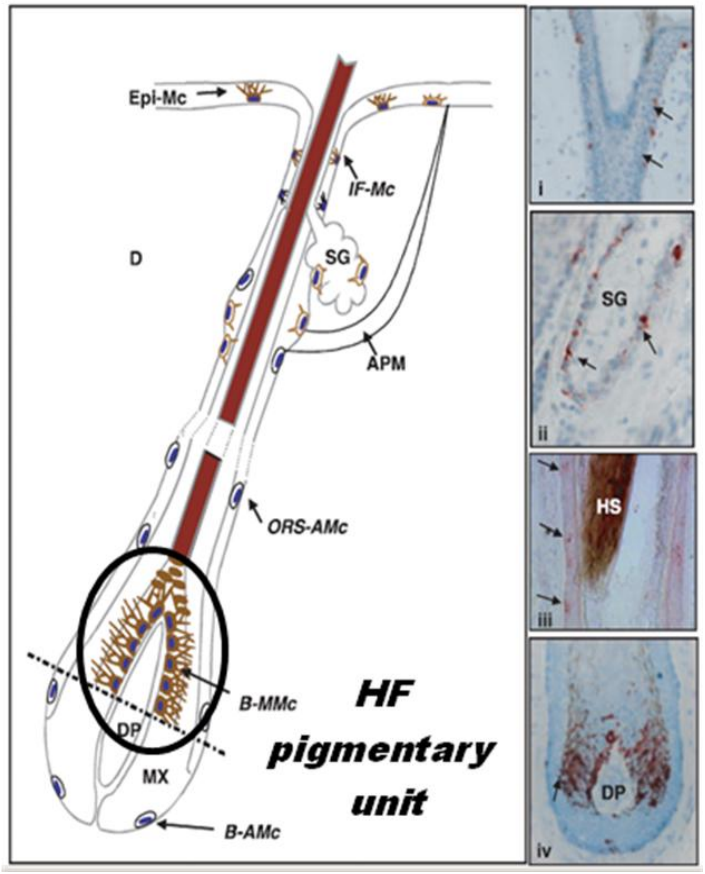

Figure 1. 7: Schematic and immunohistologic representations of the distribution of melanocyte subpopulations in the different regions of the human anagen hair follicle.

Melanocytes in frozen scalp sections were detected using the antibody to glycoprotein 100 (gp100); (i) hair follicle infundibulum; (ii) sebaceous gland; (iii) mid outer root sheath; (iv) hair bulb. B-AMc Bulbar amelanotic melanocyte; B-MMc, bulbar melanogenic melanocyte; DP, follicular dermal papilla; HS, hair shaft; MX, matrix; Epi-Mc, epidermal melanocyte; IF-Mc, Infundibulum melanocyte; ORS-AMc, outer root sheath amelanogenic melanocyte; SG, sebaceous gland; D, dermis. Magnification ·125. (Tobin 2008)

The melanogenically active melanocytes can be found in three follicular compartments i.e. the infundibulum, sebaceous gland, and the hair bulb around and above the DP. The follicular melanocytes, keratinocytes, and DP fibroblasts constitute the hair follicle pigmentary unit (HFPU)(Tobin 2015).

The pigment production for the hair shaft is only formed in the hair bulb (Slominski et al. 2005). The follicular melanocytes are quite distinct from those of the epidermis. They are larger, display more and longer dendrites, have more extensive Golgi, a

rough endoplasmic reticulum, and a substantially lower keratinocyte-to-melanocyte ratio (1:1-1:4) than the EMU (1:25-40) (Tobin and Paus 2001; Slominski et al. 2005; Tobin 2008; Plonka et al., 2009; Tobin 2011).
The follicular melanogenesis is closely connected to the hair growth cycle (anagen stage III-VI), while the melanogenesis in the epidermis is continuous (Slominski and Paus 1993; Tobin and Paus 2001; Slominski et al. 2005).

1.4.2 Melanogenesis

Melanogenesis can be mostly conveniently divided into 1) melanosome biogenesis and 2) the biochemical pathway that converts tyrosine into the melanin (d'Ischia et al., 2015).

Melanosomes are specific organelles, which are produced by melanocytes (Lee and Fisher 2015). In the melanosomes, the melanin pigment is synthesized and deposited. To form skin and hair colour, the melanosomes are transferred from melanocytes to neighbouring keratinocytes (Ando et al., 2011; Aydin et al., 2012; Weiner et al. 2014; Wu and Hammer 2014). Structurally, the melanosomes are assembled via a process resembling lysosome biogenesis (Marks and Seabra 2001). Their development and maturation proceeds morphologically via four stages: Stage I pre-melanosomes are spherical vesicles produced at the peri-nuclear area of melanogenically active melanocytes, this stage is characterized by early matrix organization. The stage II melanosomes are formed by the elongation of the stage I vesicle. At this stage, the melanosome matrix is now completed and contains the cleaved fragments of the structural protein Silv/gp100/Pmel 17 (pre-melanosomal protein 17) and melanin synthesis can begin. Melanin is deposited in stage III melanosomes and fills the melanosome in stage IV completely (Slominski et al. 2005; Singh et al., 2008; Aspengren et al., 2009; Aydin et al. 2012) (**Figure 1.8**).

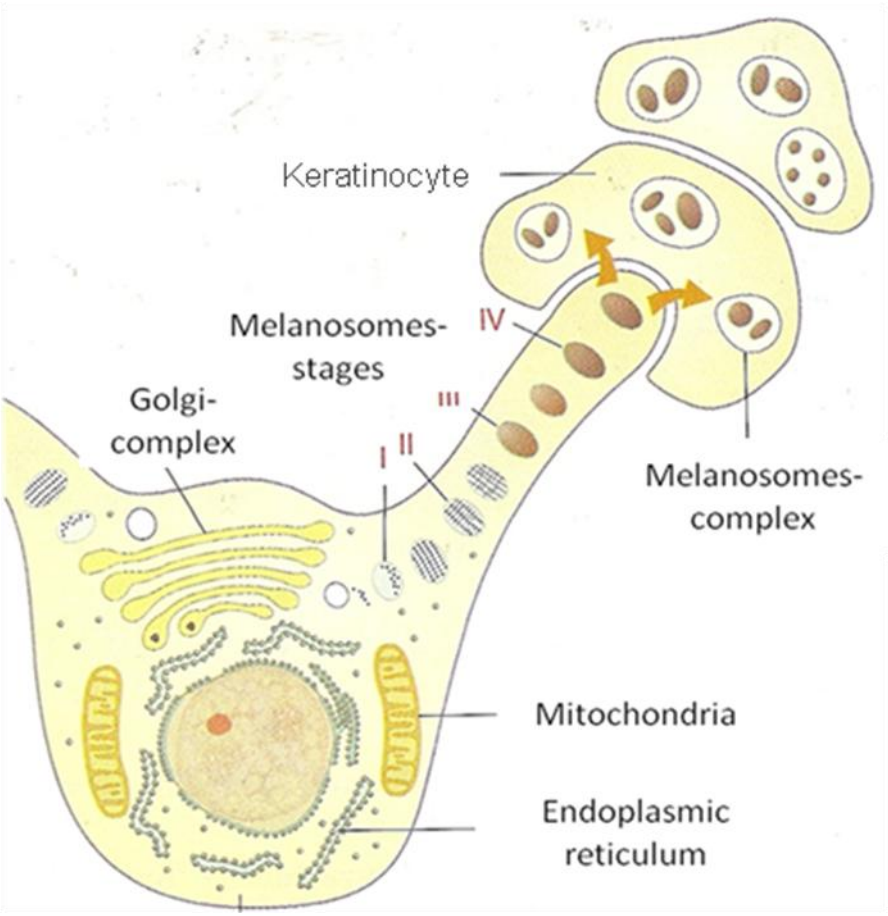

Figure 1. 8: Schematic representation of the melanosomes prosessing.

The Melanosomes are produced by melanocytes. Their development and maturation proceeds morphologically via four stages. In the melanosomes, the melanin pigment is synthesized and deposited. To form the skin and hair colour the melanosmes are transferred from melanocytes to neighboring keratinocytes.(slightly modified after (Fritsch 2004).

The substrates needed for melanin synthesis, the amino acid tyrosine and the enzyme tyrosinase, are generated in the melanocyte ribosome and packaged into melanosomes, where the biosynthesis of the polymerized product melanin occurs. The first two steps of the melanogenesis, the oxidation of tyrosine to dihydroxyphenylalanin (DOPA) and further to DOPAchinon, are catalyzed by tyrosinase, all later steps are not enzyme-catalyzed. Therefore, tyrosinase is the rate-limiting enzyme of melanin synthesis (Slominski et al. 2005; Simon et al., 2009; Paterson et al., 2015). There are two forms of melanin, the brown-black eumelanin

and the red-yellow pheomelanin, that can be produced. (Ito 2003; Park et al., 2009; Commo et al. 2012) (**Figure 1.9**).

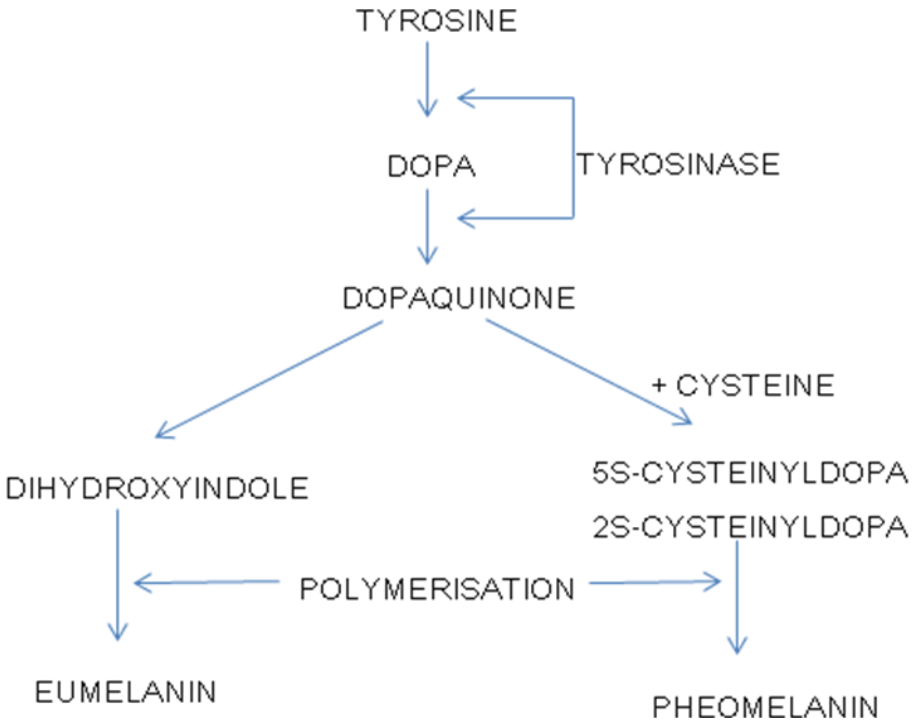

Figure 1. 9: The melanin biosynthesis (in simplified terms)**.**

The frist two steps of the melanin biosynthesis are catalyzed by the rate-limiting enzyme tyrosinase. As products there are two forms of melanin, the brown-black eumelanin and the red-yellow pheomelanin (Fritsch 2004).

Eumelanin is synthesized and deposited into elliptical melanosomes, whereas pheomelanin is sythesized in smaller round melanosomes (Ito 2003; Slominski et al. 2005; Kadekaro and Abdel-Malek 2007; Simon et al. 2009). These products deposited in the melanosomes are transported from melanocytes to cortical and medulla keratinocytes to generate hair shaft pigmentation or to epidermal keratinocytes to generate skin pigmentation (Ando et al. 2011; Peters et al., 2011; Wu and Hammer 2014).

The relative amounts of eumelanin and pheomelanin are an important determinant of constitutive epidermal and follicular pigmentation (Kadekaro and Abdel-Malek 2007; Paterson et al. 2015). The ratio of eumelanin to pheomelanin is substantially higher

in melanocytes in dark skin than in melanocytes in lightly pigmented skin (Kadekaro et al., 2003) (**Table 1**).

Black-hair follicle melanocytes contain the largest number of eumelanosomes, brown-hair bulb eumelanosomes are somewhat smaller, blonde hair follicles have poorly melanized melanosomes, and red-hair contain pheomelanosomes (Slominski et al. 2005). The great variation in hair and skin colour derives from the different amount and composition of melanosomes, whereas the number of melanocytes typically stays constant.

	African	Oriental	Caucasian
Type of Melanin Mixture	Pheomelanine  Eumelanine	Pheomelanine  Eumelanine	Pheomelanine  Eumelanine
Proportion of Free Melanin Grains in the Epidermis	Complexed  Free	Complexed  Free	Complexed  Free
Melanine Grain Morphology			
Melanine Grain Size (nm)	1 x 0.5	0.6 x 0.3	0.5 x 0.3

Table 1: The determinant factors of skin pigmentation.

The different amounts and compositions of melanosomes and the ratio of eumelanin to pheomelanin determine the variation of skin types among African, Oriental and Caucasian. The number of melanocytes typically stays constant. (from website http://www.coverderm.com/lumin_prot/index.html March 30. 2016)

1.4.3 The regulation of melanin synthesis

Skin and hair colour are principally genetically determined. More than 120 genes have been identified and seem to regulate pigmentation, but only two genes, *Extension* and *Agouti*, play a key role (Ollmann et al., 1998; Dessinioti et al., 2011; Rodrigues et al., 2015).

The melanocortin-1-receptor (MC1R), which is the most important regulator of pigmentation, is encoded by the *Extension* locus. Gain-of-function MC1R mutations cause exclusive production of eumelanin, whereas loss-of-function MC1R mutations cause exclusive production of pheomelanin (Miot et al., 2009; Branicki et al., 2011; Garcia-Borron et al., 2014). In 1995, Valverde et al. reported the presence of MC1R gene sequence variants in humans. Variant gene sequences of MC1R gene were found in over 80% of individuals with red hair and light skin, in less than 20% of individuals with brown or black hair, and in less than 4% of those who showed a good tanning response. These findings underscore the significance of MC1R in regulating human pigmentation (Valverde et al., 1995; Commo et al. 2012).

The MC1R is a seven-transmembrane G protein-coupled receptor and is expressed on the surface of melanocytes (Kauser et al., 2005; Rousseau et al., 2007). The activaton of this receptor is regulated by the α-melanocyte-stimulating hormone (αMSH) and adrenocorticotropin hormone (ACTH), which are derived from proopiomelanocortin (POMC) (Slominski et al., 2004). After the binding of these peptide hormones to MC1R, adenyl cyclase is activated and this increases the intracellular cyclic adenosine monophosphate (cAMP) concentration, which in turn activates protein kinase A. This signal transduction results in the increased transcription of microphthalmia-associated transcription factor (MITF), which controls melanogenesis by activation of melanogenic enzymes (tyrosinase), which modulate the amounts of eumelanin and pheomelanin that are produced (Rouzaud et al., 2006; Dessinioti et al. 2011; Djian-Zaouche et al. 2012; Hartman and Czyz 2015) (**Figure 1.10**).

The genetic locus *agouti,* which encodes a paracrine-signaling molecule, the agouti-signaling protein (ASP), also controls the relative amounts of eumelanin and pheomelanin pigments produced (Baxter and Pavan 2013; Jirtle 2014; Wolf Horrell et al., 2016). ASP is a high-affinity antagonist of the MC1R. ASP blocks the binding of

melanocortins to the MC1R and inhibits the stimulatory effects of α–MSH on cAMP accumulation, tyrosinase activity, and cell proliferation, thus resulting in the subterminal band of pheomelanin often visible in hair follicles (Lu et al., 1994; Ollmann et al. 1998; Branicki et al. 2011).

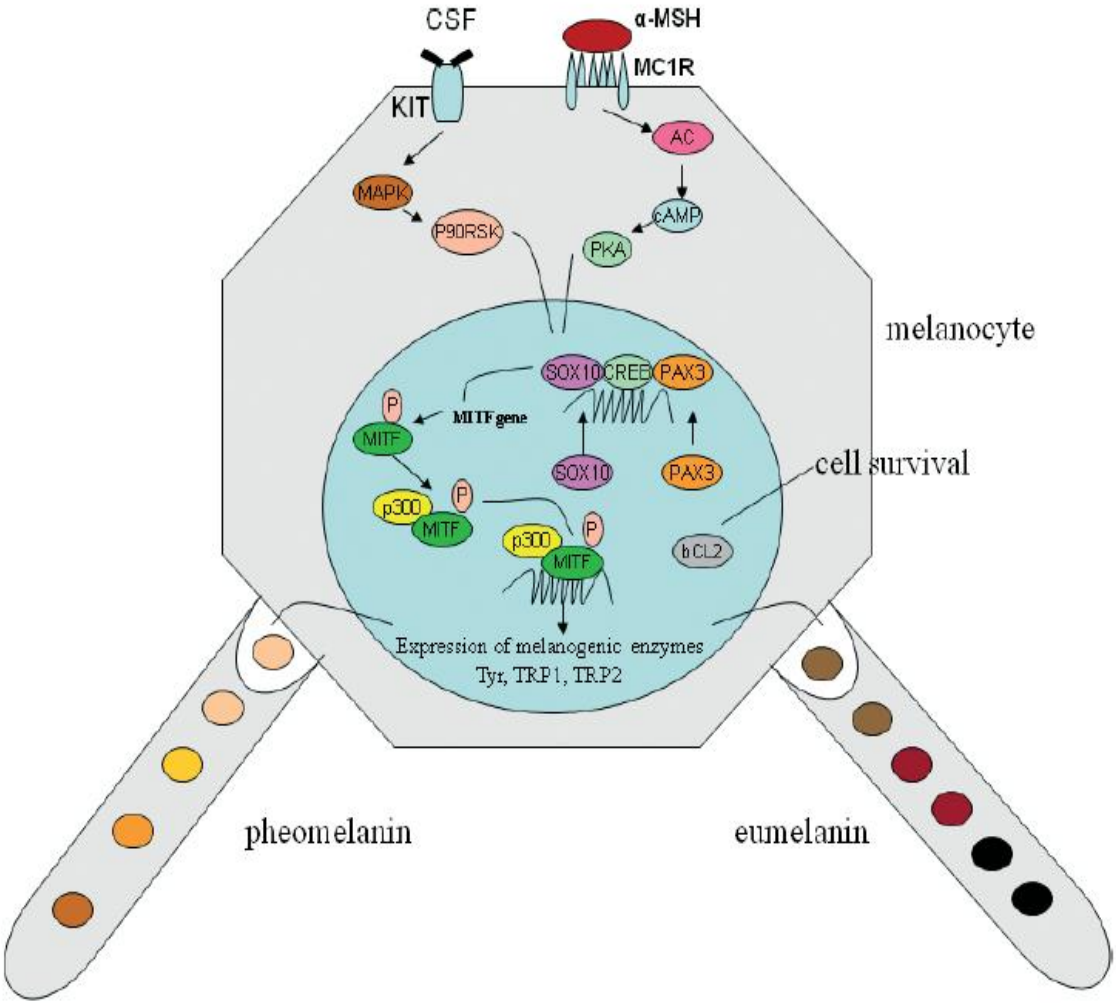

Figure 1. 10: Signaling pathway of a-MSH via MC1R.

The binding of α-MSH to MC1R on melanocytes activates adenylate cyclase (AC) and increases cyclic adenosine monophosphate (cAMP) formation. The increase in cAMP leads to activation of protein kinase A (PKA), which in turn leads to increased transcription of microphthalmia transcription factor (MITF). MITF controls melanogenesis by activating melanogenic enzymes, namley tyrosinase (Tyr), and tyrosinase-related protein 1 and 2 (TRP1, TRP2), which modulate the amounts of eumelanin and pheomelanin; SCF: stem cell factor; SOX 10:SRY-box factor, PAX3: the paired box transcription factor (Dessinioti et al. 2011).

1.4.4 Known neuroendocrine stimulators of human hair follicle and skin pigmentation

The regulation of skin and hair pigmentation has been investigated from several perspectives, e.g. a) the biochemistry of melanin formation in terms of enzymes, pH, and cysteine levels (Ito and Wakamatsu 2011; Slominski et al., 2012), b) oxidative stress and other redox issues, which are also related to the regulation of follicular pigmentation (Schallreuter et al., 2010; Daulatabad et al., 2015), or c) neuroendocrine regulation, e.g. dopamine (Langan et al., 2012), which stimulates human HF pigmentation.

Slominski et al. found that human skin, including the HF, expresses the genes and proteins for corticotropin-releasing hormone (CRH) and the receptors for CRH (CRH-R) (Slominski et al., 2000; Slominski et al. 2016). Moreover, *in vitro* CRH can modulate the melanocyte phenotype by upregulating melanogenesis, dendricity, and proliferation (Tobin and Kauser 2005).

Proopiomelanocortin (POMC) is a polypeptide hormone precursor that is expressed in the anterior and intermediate lobes of the pituitary and in peripheral tissues, including the skin (Bicknell 2008; Cyr et al., 2013). Tissue-specifically, the POMC is cleaved by prohormone convertases enzymes (PC1 and PC2) during post-translational processing. (Benjannet et al., 1991; Raffin-Sanson et al., 2003; Cyr et al. 2013). (**Figure 1.11**). The enzymatically cleaved products of POMC, e.g. α-MSH, adrenocorticotropin hormone (ACTH) and β-endorphin play an important role in the regulation of melanin production. The α-MSH and ACTH stimulate HF pigmentation via binding to MC1R, whereas β -endorphin stimulates pigmentation via binding to the μ-opiate receptor (Tobin and Kauser 2005; Rousseau et al. 2007; Rodrigues et al. 2015).

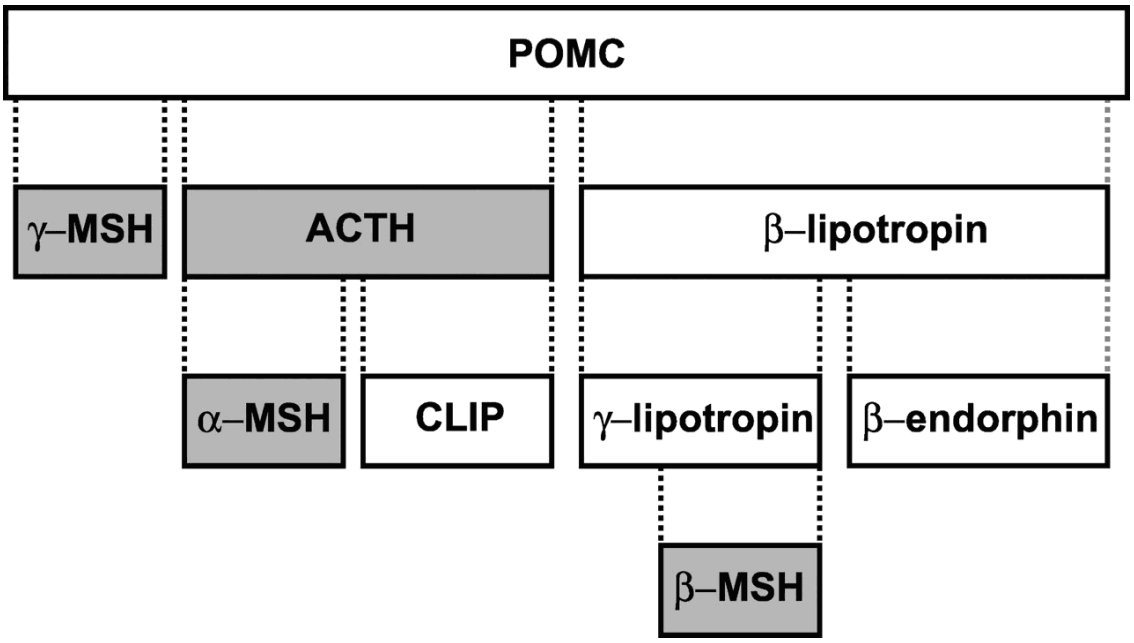

Figure 1. 11: The proopiomelanocortin (POMC) processing.

The POMC precursor is cleaved by prohormone convertases during post-translational processing (Cyr et al. 2013). Its enzymatically cleaved products e.g. α-MSH, adrenocorticotropin hormone (ACTH) and ß-Endorphin play an important role in the regulation of melanin production (Tobin and Kauser 2005; Rousseau et al. 2007) (from the website http://de.academic.ru/pictures/dewiki/80/POMC.png January 08. 2016).

In amphibians, the regulation of melanin synthesis by TRH has been more intensely investigated. Here the thyrotropin-releasing hormone (TRH) stimulates skin melanomorphes by inducing secretion of α-MSH in the pituitary gland. However, it is unknown whether this tripeptide neurohormone exerts any effects on human melanocytes under physiological conditions (see below for details).

1.5 Thyrotropin releasing hormone

In October 1977 a Nobel Prize in Physiology or Medicine was awarded to Guillemin and Schally. These two independent groups had first isolated, sequenced and synthesized the hypothalamic hormone, “thyrotropin-releasing hormone” (TRH) (Boler et al., 1969; Burgus et al., 1969), which was also the first hypothalamic

hormone that had been identified. With these landmarkdiscoveries Guillemin and Schally opened the new field of hypothalamic research (Reichlin 1989)

TRH (synonyms: thyroliberin, thyrotropin releasing factor) is a tripeptide neuro-hormone that is best-known as the hypothalamic neuroendocrine stimulus for thyrotropin (TSH) release from the pituitary gland (Fliers et al., 1998; El Yamani et al., 2013; Fliers et al. 2014; Joseph-Bravo et al. 2015; Joseph-Bravo et al. 2015; Joseph-Bravo et al. 2016). TRH reaches the anterior pituitary gland via the hypophyseal portal system to induce the release of thyroid-stimulating hormone (TSH) by pituitary "thyrotrope" cells.(Lechan RM 2003; Chiamolera and Wondisford 2009; Carbone et al., 2012). (**Figure 1.12**).

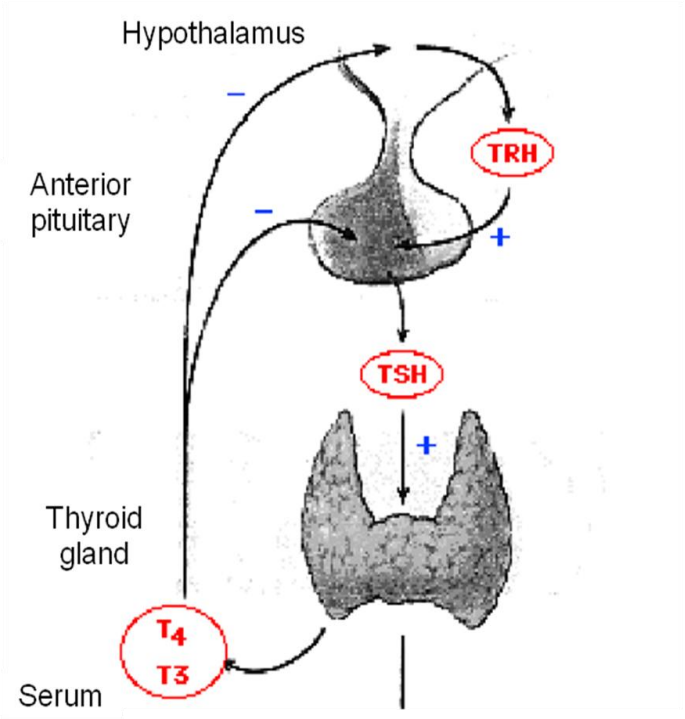

Figure 1. 12: Central hypothalamus-pituitary-thyroid axis.

TRH, which is synthezied in the paraventricular nucleus of the hypothalamus, releases the hypothalamus via the hypophyseal-portal system to stimulate the biosynthesis and secretion of the thyroid stimulating hormone (TSH) from the pituitary. TSH in turn stimulates biosynthesis of the thyroid hormones tri-iodothyronine (T3) and thyroxine (T4). The negative feedback of thyroid hormones suppress the TRH expression and TSH secretion (Chiappini et al., 2013; El Yamani et al. 2013) (from website http://www.drharper.ca/images/HPT%20Axis.gif, February 08.2016).

As the most proximal regulatory element of the hypothalamic-pituitary-thyroid (HPT) axis, TRH is initially synthesized in medial neurons of the paraventricular nucleus of the hypothalamus as a 242 amino acid precursor, which is proteolytically processed to the mature TRH molecule composed of three amino acids in a special fashion (pyro)Glu-His-Pro-(NH_2) (Lechan et al., 1986; Schaner et al., 1997; Nillni 2010; Chiappini et al. 2013; Cyr et al. 2013) (**Figure 1.13**).

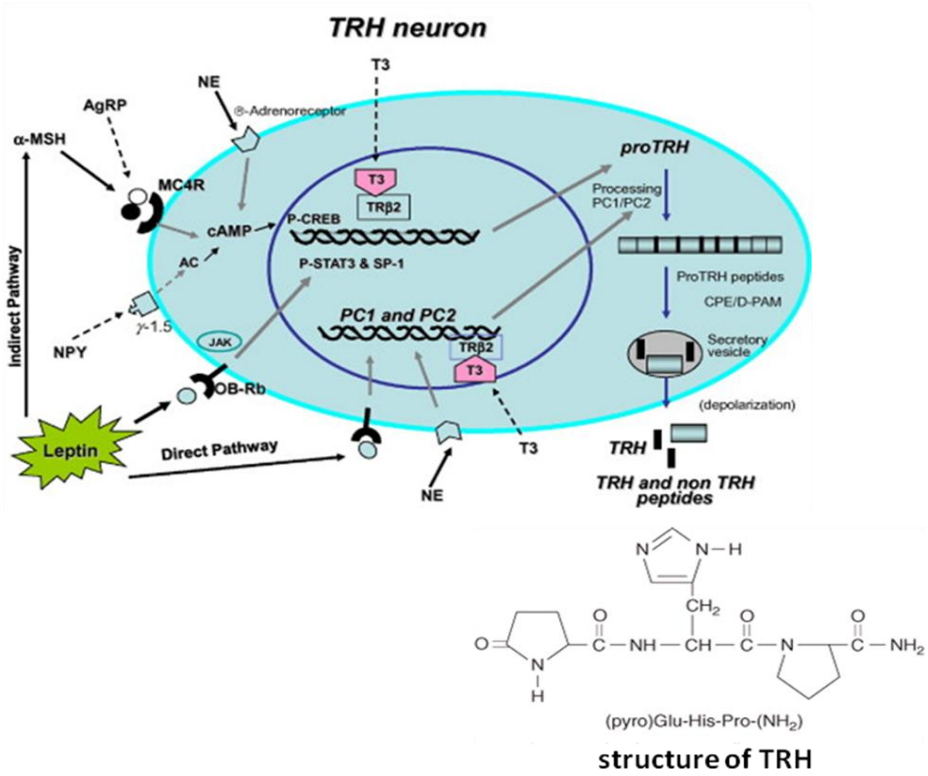

Figure 1. 13: Schematic representation of the gene expression and synthesis of TRH and its regulation.

The TRH neuron in the paraventricular nucleus of the hypothalamus receives multiple signals from other regions of the brain as well as from the peripheral circulation. These different inputs regulate the gene expression and synthesis of TRH. The biosynthesis of TRH begins with the transcription of the complementary DNA of TRH into corresponding mRNA, which is translated by ribosomal units into the preprohormone TRH (prepro TRH). The preproTRH contains 242 amino acids and generates six copies of the prohormone TRH (proTRH). The proTRH undergoes a series of proteolytic cleavages by the action of two prohormone convertases (PC-1 and PC-2) and carboxyl peptidase E and D (CPE, CPD) . Finally, the immediate precursor to TRH is amidated at its carboxyl terminus by action of peptidylglycine α-amidating monooxygenase enzyme (PAM) to yield mature TRH (Nillni 2010). AgRP: agouti-related peptide; α-MSH: melanocortin stimulating hormone; NPY: neuropeptide Y; NE: norepinephrine; ObRb: leptin receptor; T3: triiodothyronine.

However, the brain is not the only site of TRH expression and synthesis. For example, the gastrointestinal system and pancreatic islet cells have been reported to constitute extra-hypothalamic sites of TRH production (Martino et al., 1978; Leduque et al., 1989; Chiappini et al. 2013). This has greatly raised interest in the full range of *non-classical* TRH functions beyond its effects on thyroid function such as the following ones: "cytokine-induced sickness behaviour" (Kamath et al., 2009; Kamath 2013); control of energy homeostasis, including in human epidermal and HF keratinocytes (Gáspár et al., 2010; Cyr et al. 2013; Vidali et al., 2014) and via central effects on feeding behaviour (Sarvari et al., 2012; Gotoh et al., 2013), thermogenesis, locomotor activation, autonomic nervous regulation (Lechan and Fekete 2006; Gertig et al., 2012; Thirunarayanan et al., 2012; Volkoff 2012; Zhang and van den Pol 2012); preservation of pancreatic islet cell function (Luo and Jackson 2007); regulation of hepatic cAMP (Yoneda et al., 2005); modulation of cardiovascular functions (Garcia and Pirola 2005); multifunctional hypophyseotropic modulation (Galas et al. 2009); modulation of several central nervous activities (Khomane et al., 2011). It was also recently published that patients with Alzheimer's disease have significantly lower levels of TRH (Yong-Hong et al., 2013) and that ingested (oral) TRH inhibits experimental autoimmune encephalomyelitis (EAE) (Brod and Bauer 2013). Despite multitude of non-classical functions described or postulated or TRH and leads from lower vertebrates (see above), mammalian pigment biology research had not investigated whether TRH impats on normal mammalian skin melanocytes and their melanogenic activity.

Similar to the widespread distribution of TRH, the receptors for TRH, which belong to the G protein-coupled receptor family and as such members have seven transmembrane-spanning helices, are also fairly widespread (Joseph-Bravo et al. 2016; Ortiga-Carvalho et al. 2016). TRH receptors are found throughout the central and peripheral nervous system, as well as in other organs and tissues (Sun et al., 2003; Mulla et al., 2009; Gehret and Hinkle 2012). Two receptor isoforms, TRH receptor R1 (TRH-R1) and TRH receptor R2 (TRH-R2) have been identified (Gershengorn and Osman 1996; Thirunarayanan et al. 2012). The TRH-R1 is found in different vertebrates, including rats, mice, chickens and humans (Matre et al., 1993). The TRH-R2 however has not yet been seen in humans (Sun et al. 2003). It

has only been found in rats (Cao et al., 1998; Itadani et al., 1998; O'Dowd et al., 2000), mice (Harder et al., 2001) and the fish *Catostomus commersoni* (Harder et al. 2001). Recently, a third type of TRH receptor (TRH-R3) was cloned in *Xenopus laevis* (Bidaud et al., 2004).

TRH binding to this cell-surface receptor results in the activation of the phospholipase C (PLC) signaling pathway. PLC is a hydrolytic enzyme that cleaves phosphatidylinositol 4,5-P2 (PIP2) to form inositol 1,4,5-triphosphosphate (InsP3) and 1,2-diacylglycerol (DAG). This results in an InsP3-mediated increase in cytosolic Ca^2,which in conjunction with the DAG, activates Protein Kinase C (PKC) (Jones et al., 2007; Gehret and Hinkle 2012; Thirunarayanan et al. 2012). The activation of PKC regulates the interaction of several transcription factors with the promoter regions of various target genes to induce gene transcription and, ultimately, to affect protein synthesis, e.g. TSH and prolactin (Carr et al., 1989; Mijiddorj et al., 2012) **(Figure 1.14).**

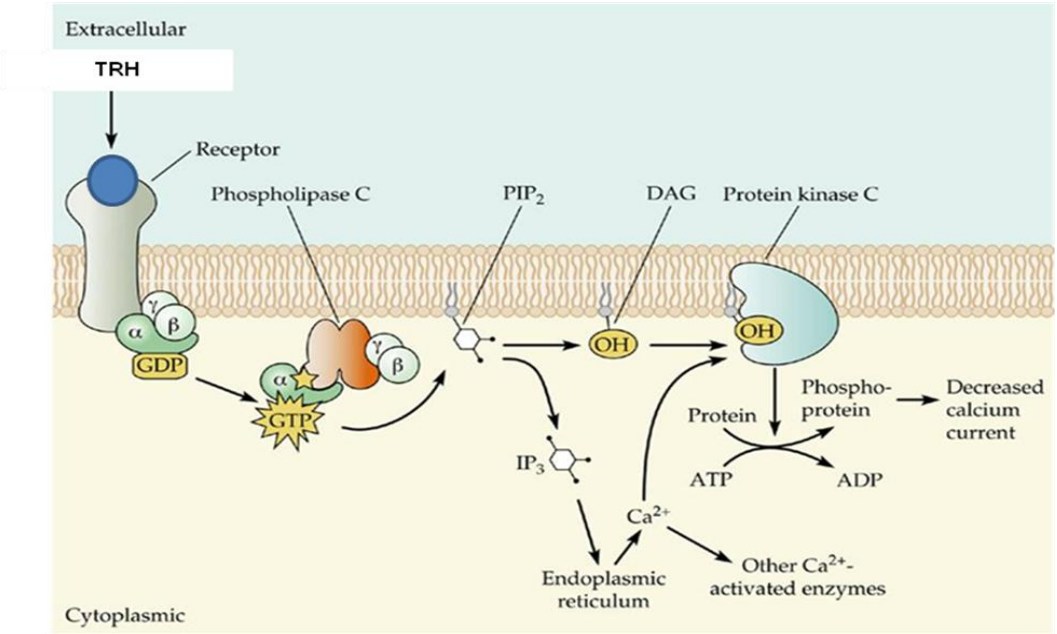

Figure 1. 14: TRH receptor-mediated intracellular signaling pathways.

IP3: inositol 1,4,5-tripohosphate; PIP2: phosphatidylinositol 4,5-biphosphate;. DAG: 1,2-diacylglycerol; GDP: guanosine diphosphate; GTP: guanosine triphosphate; ATP: Adenosine triphosphate; ADP: Adenosine diphosphate. (from the website neuroamer.files.wordpress.com/2011/09/pip2-pathway.jpg April 19.2016)

TRH-R signaling can be rapidly regulated via ligand-dependent endocytosis, a specific pathway by which the TRH-TRH-receptor complexes are internalized and the TRH-receptor is recycled to the plasma membrane (Ashworth et al., 1995; Jones et al. 2007).

Moreover, it has been reported that TRH not only can bind to TRH receptors, but can also bind to and act as a selective agonist for the MC1-receptor in isolated COS-1 (CV-1 Origin, SV 40) cells *in vitro* (Schiöth et al., 1999), which plays a key role in regulating pigmentation (see above).

1.6 The role of TRH in pigment biology

Amphibians such as frogs have developed the remarkable ability to adapt to their backgrounds by changing their skin colour in response to the colours present in a new environment. TRH plays a crucial role in this type of camouflage. (Vaudry et al. 1999).

Frog skin contains surprisingly high amounts of TRH (110µg/g protein, which is much higher than that in the hypothalamus (17µg/g protein)) (Jackson and Reichlin 1977; Bolaffi and Jackson 1979; Vaudry et al. 1999; Vazquez-Martinez et al., 2003; Meier et al., 2013). Interestingly, the dorsal skin of *Rana pipiens, Rana ridibunda,* and of *Xenopus laevis,* which is more intensely pigmented than the ventral skin, contains the highest amount of TRH (Bolaffi and Jackson 1979; Ravazzola et al., 1979; Vaudry et al. 1999) (**Figure 1.15**).

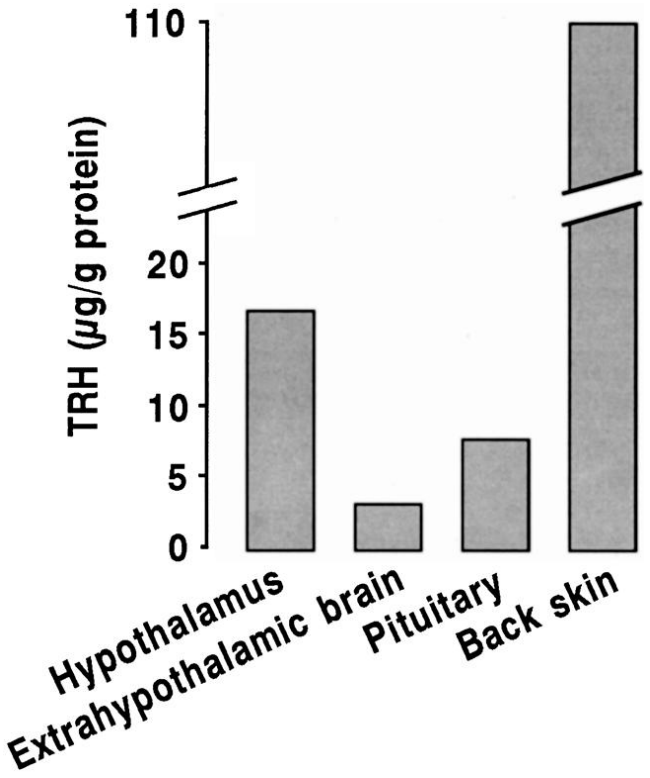

Figure 1. 15: TRH cencentration in different tissues of the frog *Rana pipens*.

The highest concentrations of TRH have been found in the back skin, which is more intensely pigmented than ventral skin. (Vaudry et *al. 1999)*

Skin-derived TRH can also have hypophyseotropic functions (e.g. it stimulates pituitary TSH; prolactin, which regulates water and ionic transport through the skin; and growth hormone release in adult frogs) that may extend to other vertebrates (Tonon et al., 1980; Vaudry et al. 1999; Jenks et al. 2007; Galas et al. 2009; Jenks et al. 2010). TRH is also recognized as a potent stimulator of POMC expression and α-MSH secretion in frogs (Vaudry et al. 1999; Jenks et al. 2007; Jenks et al. 2010).

TRH, derived from either the hypothalamus or the skin (Jackson and Reichlin 1977) (Vaudry et al. 1999; Galas et al. 2009) is thought to induce amphibian skin darkening *indirectly* via stimulating α-MSH-release by the pituitary gland, which then induces rapid skin darkening via melanophore stimulation (Jackson and Reichlin 1977; Vaudry et al. 1999; Vazquez-Martinez et al. 2003; Jenks et al. 2007; Jenks et al. 2010; Swope et al., 2012). In *Xenopus laevis,* the TRH-evoked α-MSH secretion is mediated through TRH-R3 (Galas et al. 2009) (**Figure 1.16**).

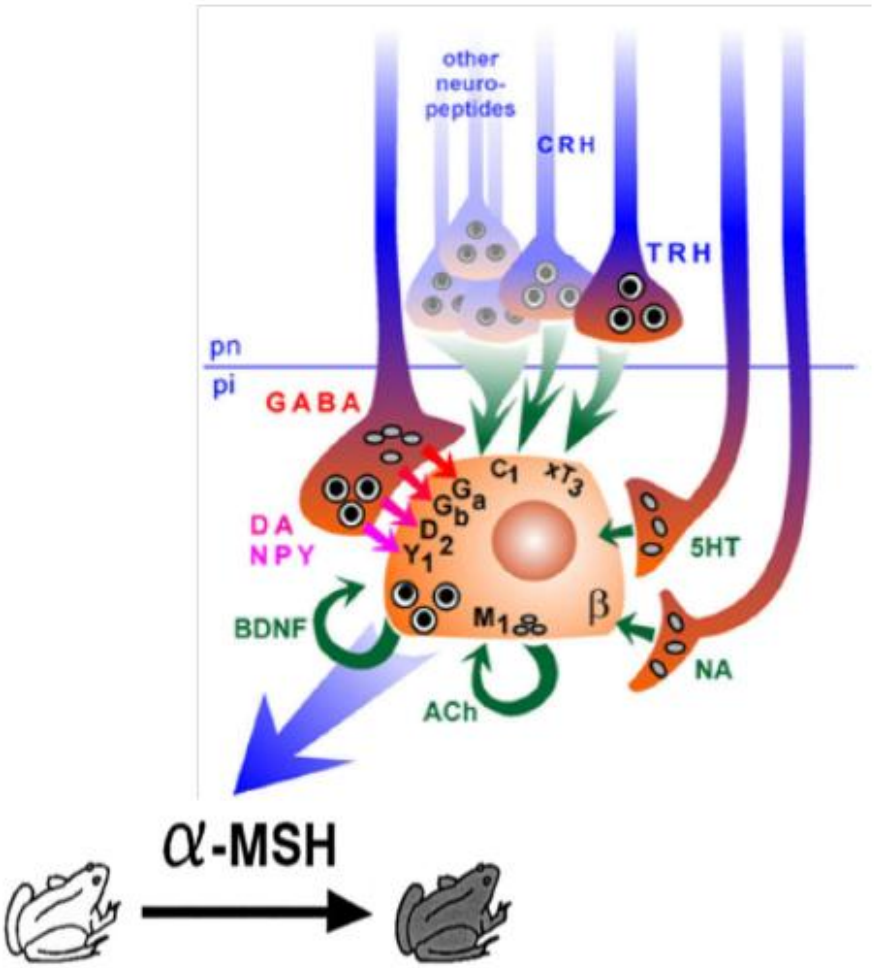

Figure 1. 16: Schematic representation of the various neuroendocrine factors that may participate in the regulation of *Xenopus laevis* melanotrope cell activity in pituitary pars intermedia (pi).

TRH from melanotropes cells in the pars nervosa (pn) activated the TRH-R3 (xT3) and induced a secretion of alpha-melanocyte stimulating hormone (α-MSH), which causes skin darkening. Other stimulatory factors (ACh, acetylcholine; BDNF,brain-derived neurotropic factor; CRH, corticotrophin-releasing hormone; NA, noradrenaline; 5HT, serotonin; other neuropeptides) and inhibitory factors (GABA, γ-amonobytyric acid; DA, dopamine; NPY, neuropeptide Y) control also α-MSH secretion. ß, ß-adrenergic receptor; C_1, CRF-R1 receptor; G_a, $GABA_a$ receptor; G_b, $GABA_b$ receptor; D_2, dopamine D_2 receptor; M_1, muscarinic acetylcholne M1 receptor; Y_1 , NPY-Y1 receptor. (Vaudry et al. 1999; Galas et al. 2009)

However, it is not known yet whether TRH has any pigmentation-modulatory effects on amphibian skin melanophores and/or on normal melanocytes of any mammalian species in the absence of a pituitary gland.

Intriguingly, not only is amphibian skin itself is a major source of TRH peptides (Jackson and Reichlin 1977; Bolaffi and Jackson 1979; Vaudry et al. 1999), but also human skin and its cultured resident cell populations transcribe hypothalamus-pituitary-thyroid axis-related genes, such as *TRH, TSH* and their *receptors* (*TRH-R*, *TSH-R*) (Slominski et al., 2002; Bodo et al., 2009; Gáspár et al. 2010; Paus 2010; Knuever et al., 2012; Paus et al., 2014). Isolated neonatal human epidermal keratinocytes reportedly express TRH mRNA *in vitro* (Slominski et al. 2002). It is also known that normal human scalp HFs are yet another extra-hypothalamic source of TRH production (Gáspár et al. 2010). TRH not only is expressed constitutively by human HFs at the gene and protein level, but also stimulates hair shaft production and HF keratinocyte proliferation in isolated, organ-cultured human scalp HFs (Gáspár et al. 2010).

At the time the current thesis project was started, it had also just been discovered by the Paus Lab that normal human scalp HFs are yet another extra-hypothalamic source of TRH production:TRH not only is expressed constitutively by human HFs at the gene and protein level, but also stimulates hair shaft production and HF keratinocyte proliferation in isolated, organ-cultured human scalp HFs (Gáspár et al. 2010). In the meantime, TRH has also been shown to be an important regulator of human epidermal and HF mitochondrial energy metabolism (Knuever et al. 2012; Paus et al. 2014; Ramot and Paus 2014; Vidali et al. 2014) keratin gene and protein expression in human skin (Ramot et al., 2013; Ramot and Paus 2014) and of the re-epithelialisation of experimentally wounded frog and human skin *ex vivo* (Meier et al. 2013).

1.7 Working hypothesis and specifical question addressed

Given that TRH exerts pigmentation-modulatory effects on amphibian skin melanophores (Jackson and Reichlin 1977; Vaudry et al. 1999; Vazquez-Martinez et al., 2001; Vazquez-Martinez et al. 2003; Jenks et al. 2007; Galas et al. 2009; Jenks et al. 2010) and given that both human skin and the human HF express TRH, which has multiple central and peripheral functions in human tissues (Slominski et al. 2002;

Bodo et al., 2010; Gáspár et al. 2010; Paus 2010; Knuever et al. 2012; Ramot et al., 2013), we hypothesized that TRH may have regulatory functions in the human pigmentary system.

To investigate this working hypothesis, the following specific questions were addressed in this study:

1. Does TRH stimulate human HF pigmentation directly, i.e. in the absence of a pituitary gland *in situ* and *in vitro*?
2. What exactly are the effects of TRH on the human HF pigmentary unit (HFPU)?
3. Does TRH have similar effects on human epidermal melanocytes *in situ*?
4. How does TRH stimulate human HF pigmentation?

1.8 Experimental design

These questions were addressed primarily by using a well-established HF ex vivo assay, in which microdissected human scalp HFs in the anagen VI stage of the hair cycle are organ-cultured in supplemented, see-free medium, i.e. in the absence of any systemic (e.g. neural, vascular and endocrine signals, incl. thyroid hormones) (Philpott et al., 1990; Langan et al., 2015). This highly instructive and physiologically relevant assay had been further developed into an assay for human pigmentary *in situ*-research, in which the responses of human HFPU melanocytes to experimental manipulation can be studied within their natural tissue habitat (Bodo et al., 2007; van Beek et al., 2008), i.e. under full preservation of the specific interactions between human HF melanocytes, hair matrix keratinocytes, and the HF mesenchyme, which control melanogenesis and other activities of HFPU melanocytes (Slominski et al. 2005; Paus 2011; Tobin 2011; Samuelov et al., 2013).

Pigmentary effects of TRH were evaluated by melanin histochemistry and immunohistochemistry for relevant pigmentary markers by quantitative (immune-) histomorphometry, including use of an immunofluorescent assay that permits one to quantify tyrosinase activity *in situ* (Han et al., 2002). This was complemented by qRT-PCR analyses for selected pigmentation-relevant gene transcripts (see below).

In order to explore where TRH exerts differential or similar effects on epidermal versus HF melanocytes, HF organ culture was complemented with human full-thickness skin organ culture (Lu et al. 2007), and isolated, cultured human melanocytes were investigated as well.

Mechanistically, it was re-examined whether the HFPU really does not express TRH receptor protein, as had been suggested by previous work from the Paus Lab (Gaspar et al. 2010). Also, the possibility that TRH may signal non-classically via the melanocortin-1 receptor (MC-1R) (Ollmann et al. 1998; Schiöth et al. 1999) was probed by co-administering the MC-1R antagonist, agouti-signaling protein, with TRH, and by studying whether TRH impacted on the intrafollicular expression of ACTH, a key pigmentation-promoting melanocortin (Paus 2011).

2 MATERIALS AND METHODS

2.1 Human tissue collecting

This study adhered to the Declaration of Helsinki guidelines and was approved by the Institutional Research Ethics Committee of the University of Lübeck (No. 06-109). Informed consent was obtained from all patietns. The anagen HFs were microdissected from human scalp skin obtained from five healthy adult females undergoing routine cosmetic surgery.

After obtaining informed written patient consent, collaborating plastic surgeons from Bonn, Augsburg, and Munich collected skin samples from healthy patients undergoing elective cosmetic surgery and couriered them overnight under sterile conditions in William´s E Medium (Biochrom, Cambridge, UK) at 4°C to Lübeck. The collected samples of scalp skin from the temporal and occipital area from five randomly selected females, aged 43 to 54 (means age 48 years). The skin samples were used for HF isolation and cultureHF melanocyte isolation and culture, or for full-thickness skin culture.

2.1.1 Hair follicle isolation

Only the skin samples obtained within 24 hours after surgery were used for HF isolation by microdissection. The procedure used for human HF organ culture was according to that of Philpott et al (Philpott et al. 1990; Philpott et al., 1991; Philpott et al., 1994; Philpott et al., 1996).

The isolation medium consisted of William´s E medium (Biochrom, Cambridge, UK) with 1% antibiotic/antifungal mixture B (Gibco, Karlsruhe, Germany), containing penicillin G (Sigma, St Louis, Mo, USA), streptomycin (Sigma) and amphotericin B (Gibco, Karlsruhe, Germany). The skin samples were transferred into a petri dish with isolation medium and were cut into approximately 10 mm x 5 mm – sized pieced with a sterile scalpel. The cutis was separated from the subcutis at the dermo-subcutaneous fat interface to make the HF microdissection easier. The aim of the

microdissection was to obtain a complete HF with an intact outer root sheath (ORS) and connective tissue sheath (CTS) and without any damage. The intact HF was carefully removed by gripping the HF and pulling it out from the subcutaneous tissue under a binocular stereo microscope (Zeiss).

The isolated HFs were maintained free floating in the sterile culture medium, which was constituted of William`s E Medium (Biochrom, Cambridge, UK), 2 mmol/L L-glutamine (Invitrogen, Paisley, UK), 10 µg/ml insulin (Sigma-Aldrich, Taufkirchen, Germany), 10 ng/ml hydrocortisone (Sigma-Aldrich, Taufkirchen, Germany) and 1 % penicillin/streptomycin (Gibco, Karlsruhe, Germany) (**Figure 2.1**).

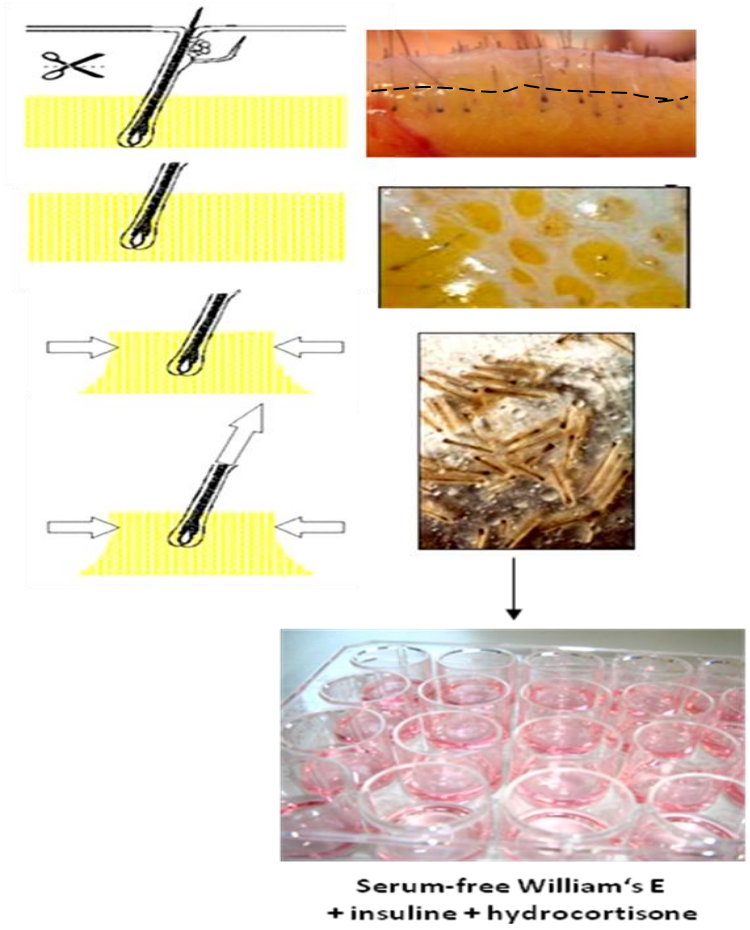

Figure 2. 1: Schematic illustration of the hair follicle isolation steps.

The cutis was separated from the subcutis at the dermo-subcutaneous fat interface, and then carefully removed by gripping the HF and pulling it out from the subcutaneous tissue. The isolated HFs were pre-incubated in culture medium for 24h in an incubator to recover from the mechanical stress of the isolation process (AG Paus, University of Luebeck).

2.1.2 Hair follicle organ culture

For the study only pigmented anagen VI HFs were selected and then randomly allocated into 24-well plates with three HFs per well in 50 µl fresh culture medium. To recover from the mechanical stress of the isolation process, the HFs were pre-incubated in culture medium for 24h in an incubator at 37°C with an atmosphere of 5% CO_2, 95% air.(Langan et al. 2015)

After the 24h pre-incubation phase, the HFs were treated with different concentrations of TRH (Bachem Ltd., St.Helens, UK) for six days. The HFs were divided into four experimental groups with different TRH treatments (control, TRH 1, 5, 10, 100 ng/ml ≙ 0, 3, 15, 30, 300 nM). The TRH concentrations were selected on the basis of Trouslard´s experiment (Trouslard et al., 1989).

Given that every HF is an autonomous mini-organ with individual characteristics and that each donor provided at least 50 HFs for analysis, for every experimental group 15 -18 HFs per donor were used. This ensured that the “n” of HFs was sufficient for analysis.The culture medium was exchanged in all experimental groups every second day, with the appropriate amount of fresh TRH also being added. After six days of incubation with TRH, the HFs were embedded and processed for longitudinal cryosection. All three HFs of a given culture well were carefully positioned in a parallel fashion on a metal plate, then covered with Shandon Cryomatrix (Pittsburg, PA, USA), and finally snap-frozen with liquid nitrogen. The embedded HFs were stored at -80°C until use.

Using a Leica CM 3500 cryostat, the deep-frozen HFs were cut into 6 µm thick cryocestions, which were placed on superfrost plus slides (Menzel GmbH & Co KG, Braunschweig, Germany). Only complete longitudinal sections (approximately five per embedded HF) with the dermal papilla, including hair bulb and hair shaft, were used for analysis. Until further processing sections were stored at -80°C.

2.1.3 Human HF melanocyte culture

Melanocytes from the ORS of human anagen VI scalp HFs were isolated and cultured as described by Tobin et al. (Tobin et al., 1995), with some modifications.
Using sterile technique, human skin samples were cut with a scalpel into 5 mm^2 pieces and incubated overnight at 4°C in 10 ml 0.1 w/v DispaseR (Gibco). HFs were separated by using forceps. Anagen hair bulbs were cut off and digested in 0.1 % trypsin-EDTA (Sigma-Aldrich) to obtain a cell suspension. Fetal Bovine Serum (FBS) (Sigma-Aldrich) was added to neutralize the trypsin. The cell suspension was centrifuged at 200g for 5 minutes. Cells were resuspended in melanocyte culture medium (M2, Promocell) and seeded at a density of 1x 10^5 cells per flask. The primary culture consisted primarily of keratinocytes with a small percentage of melanocytes and fibroblasts. After 7–14 days in the culture medium, with M2 re-feeds every 3–4 days, a large number of melanocytes were evident among the keratinocyte colonies. These melanocytes were harvested by exposing to 0.1% w/v trypsin and 0.02% w/v EDTA for 30 seconds to 1 minute, followed by serum-free trypsin inhibitor (soybean trypsin inhibitor) (Invitrogen). Although no keratinocytes were evident in the melanocyte cultures after 1–2 trypsin splits, fibroblasts were often present. The fibroblasts were removed by adding 100g/ml geneticin (Sigma-Aldrich) to the medium for 3 days. Melanocytes were used between passages 2 and 6. Melanocytes were transferred onto coverslips and were treated with different concentrations of TRH (control, TRH 1, 5, 10, 100 ng/ml ≙ 0, 3, 15, 30, 300nM) (Bachem Ltd., St.Helens, UK) for 6 days. The culture medium was exchanged in all experimental groups every second day, along with the appropriate amount of fresh TRH. After six days of incubation with TRH, the melanocytes were fixed in 4% Formaldehyde and stored at -20°C until use.

2.1.4 Human scalp skin organ culture

Full-thickness human scalp skin was washed in isolation medium for 5 minutes. The outgrowing hair shafts were shaved down to the level of the epidermis. At an oblique angle, parallel to the direction of hair growth, 4 mm biopsies of intact scalp skin were

then punched out, using an Acu-puncher (STIEFEL, Offenbach am Main, Germany). These pieces of human scalp tissue were carefully placed into a 6-well plate with 5ml culture medium, with the epidermis facing up at the air/liquid interface and the dermis/subcutis facing down. After the 24h pre-incubation phase, the skin pieces were treated with different concentrations of 3–300nM TRH (Bachem Ltd., St.Helens, UK) or vehicle for six days. The culture medium was changed every other day (Lu et al., 2007) (**Figure 2.2**).

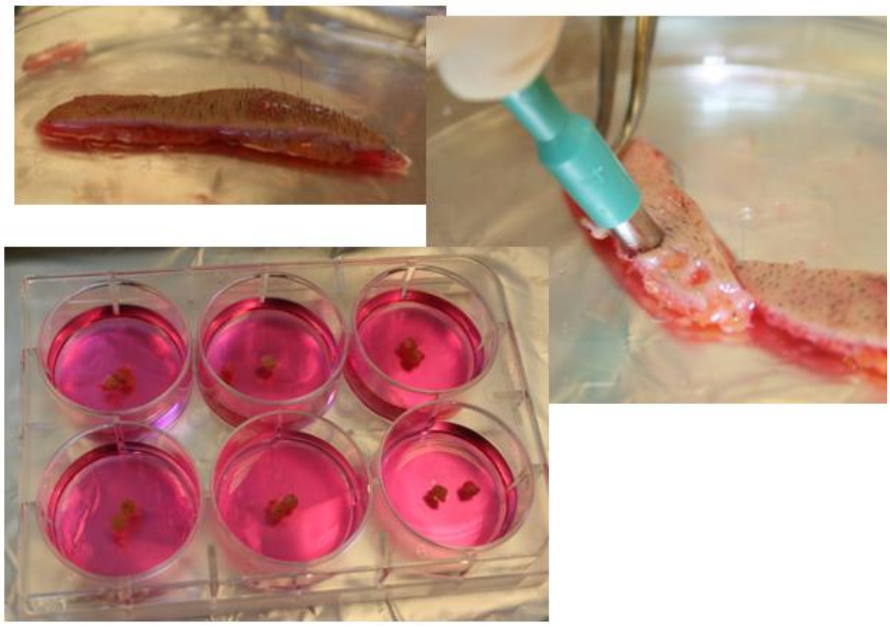

Figure 2. 2: Full-thickness human scalp skin punch biopsies.

Parallel to the direction of hair growth 4 mm biopsies of intact scalp skin were punched out, using an Acu-puncher. The punch biopsies were organ-cultured in 6-well plates for 6 days.

Finally, the skin or HF samples were embedded in Shandon cryomatrix (Thermo Scientific, Pittsburg, PA, USA) and snap-frozen in liquid nitrogen for cryosections. For analysis, the samples were cut into 7µm thick sections using a Leica CM 3500 cryostat and devolved on superfrost plus slides (Menzel GmbH & Co KG, Braunschweig, Germany). The cryosections produced were vertically oriented, showing the epidermis at one pole and the subcutis at the other pole. Sections were stored at -80°C until use.

2.2 Immuno-and histochemistry

2.2.1 Primary antibodies

Primary antibody	Origin	Vendor	Dilution	Positive control	Secondary detection system	References
NKI/beteb (anti-gp 100)	mouse	Monosan, Uden, Netherlands	1:30	human skin	IF/Rh IF/FITC	(Singh et al. 2008)
Anti-Ki-67	rabbit	Zymed Laboratories South San Francisco, CA	1:100	human skin	IF/FITC	(van Beek et al. 2008; Gáspár et al. 2010)
Anti-ACTH	rabbit	Chemico International, Inc., USA	1:30	human skin	IF/FITC	(Kauser et al. 2005; Rousseau et al. 2007)
Anti-MITF	mouse	Novo-Castra Laboratories Ltd, UK	1:50	human skin	IF/FITC	(Nishimura et al., 2005; Vachtenheim and Borovansky 2010)
Anti-TRH-R1	rabbit	Acris, Hidden-hausen, Germany	1:500	human pituitary	TSA/FITC	(Zhao et al., 2008)

Table 2: Primary antibodies and secondary detection systems.

Antibodies used for immunohistochemistry in this study are listed and described in detail. ACTH: Adrenocorticotropic hormone, FITC: Fluorescein isothiocyanate IF: Immunofluorescence, MITF: Microphthalmia transcription factor, TRH-R1: Thyrotrophin-releasing-hormone-receptor-1, Rh: Rhodamine; TSA: Thyramide signal amplification.

2.2.2 Masson-Fontana histochemistry

Masson–Fontana histochemistry was used to visualize the melanin content in cryosections. This method relies upon the melanin granules to reduce solutions of ammoniacal silver nitrate to a visible metallic silver (demarcated as black dots), without the use of an external reducing agent.

Procedure:

The HFs cryosections which have been stored at -80°C were first dried in the air for 10 minutes and then were fixed in ethanol/acetic acid (2/1) at -20°C for 10 minutes. The slides were washed three times for 5 minutes in tris buffered saline (TBS) and 10 minutes in distilled water.

For preparation of TBS: 6.1 g tris-base and 8.8 g sodium chloride (NaCl) were diluted in 800 ml distilled water. 1N hydrochloric acid (HCl) was used to adjust the pH to 7.6. Finally the solution was topped up to 1000ml with distilled water.

The slides were kept in ammoniac 10% silver nitrate dilution (Merck, Darmstadt, Germany) for 40 minutes at 56°C in the dark.

For the preparation of the ammoniac 10% silver nitrate solution: 1g silver nitrate was diluted in 10 ml distilled water. While stirring 5 drops of 40% sodium base solution were added. A dark brown precipitate of silver oxide was formed. Next, ammoniac was carefully added drop by drop until the precipitate was completely dissolved and the solution was clear again. Finally, the solution was topped up to 20 ml with distilled water.

After being washing three times in distilled water for 5 minutes, the slides were fixed in sodiumthiosulfate (5% in distilled water; Merck, Darmsatdt, Germany) for 1 minute and followed by washing in tap water for 3 minutes. The sections were counterstained with hematoxylin and were rapidly dehydrated in 70/96/100% ethanol. Finally slides were cleared with xylene and mounted in Eukitt (O.Kindler GmbH, Freiburg, Germany). As a positive control, the strong pigmentation of anagen HFs was used (Kloepper et al., 2010).

2.2.3 NKI/beteb immunofluorescence

The structural protein Silv/gp100/Pmel17 is a 100 kDa pre-melanosomal glycoprotein and is essential for the transition of stage I (pre-) melanosomes to stage II melanosomes. Furthermore, gp100 is cleaved into several fragments, which form the fibrillar matrix of the organelle (Singh et al. 2008).
For this study, NKI/beteb (gp100) antibody was used to identify the cleaved formed of gp100 present in stages III/IV melanosomes and to detect melanosome formation and melanocyte dendricity in the HF pigmentary unit (HFPU) and in isolated and cultured melanocytes (Singh et al. 2008; Samuelov et al. 2013; Hardman et al. 2015).

Cryosections stored at -80°C were first air dried for 10 minutes, fixed in acetone at -20°C for 10 minutes, and then washed three times for 5 minutes in phosphate buffered saline (PBS).
For preparation of the PBS, 1.8 g sodium dihydrogenphosphate monohydrate and 8.0 g NaCl were diluted in 800 ml distilled water. To adjust the pH to 7.2, sodium hydroxide (NaOH) was used. Finally, the solution was topped up to 1000 ml with distilled water.
The sections were incubated with the monoclonal NKI/beteb primary antibody (Monosan, Uden, Netherlands) at a dilution 1:30 in 0.3% Triton X in PBS or in Antibody-Diluent (DAKO, Glostrup, Denmark) overnight at 4°C.
After washing three times for 5 minutes in PBS, sections were stained with rhodamin labelled or FITC labelled (1:200 Jackson ImmunoResearch, West Grove, USA) goat anti-mouse secondary antibodies. After 40 minutes incubation at room temperature, the slides were washed three times for 5 minutes in PBS. Counterstaining was performed with DAPI (4',6´-diamidin-2'-phenylindol-dihydrochlorid) (Boehringer Mannheim GmbH, Mannheim, Germany) for 1 minute. DAPI is a nuclear fluorescent stain that binds selectively to DNA and forms strongly fluorescent DNA-DAPI complexes.
Finally, the sections were mounted in Fluoromount (Southern Biotechnologies, Birmingham, USA) after washing three times in PBS.
Negative controls include omission of primary antibodies and their replacement with Antibody-Diluent.

2.2.4 Ki-67/ NKI/beteb (gp100) double-immunofluorescence

To evaluate the proliferation rate of HF melanocytes *in vitro* the Ki-67/NKI-beteb(gp100) doublestaining method was used.
The Ki-67 is a nuclear protein, which is present during all active phases of the cell cycle (G1, S, G2 and M-Phase), but is absent from resting cells (G0-Phase) (Gerdes et al., 1983). Ki-67antigen expression, therefore, is used as a marker for proliferation and has become a standard proliferation assessment tool in human HF research (Langan et al. 2015; Purba et al., 2016).

Human HF melanocytes, which were isolated and cultured as described by Tobin et al (Tobin et al. 1995), grown on coverslips fixed in 4% formalin were first air dried for 10 minutes and then washed three times for 5 minutes in PBS. The coverslips were preincubated with 5% goat serum (DAKO, Glostrup, Denmark) in PBS with 0.3 % Triton for 60 minutes. Then, the goat serum was drained off the coverslips without washing. The coverslips were subsequently incubated with mixture of primary antibodies from mouse anti-NKI/beteb (Monosan, Uden, Netherlands, 1:30 in 0.3% Triton X in PBS) and rabbit-anti-Ki-67 (Zymed Laboratories South San Francisco, CA, 1:100 in 0.3% Triton X in PBS) overnight at 4°C.
After washing three times for 5 minutes in PBS, the coverslips were stained with a mixture of secondary antibodies for 90 minutes at room temperature. The mixture consisted of rhodamin labelled goat anti-mouse antibody (1:200 Jackson ImmunoResearch, West Grove, USA) and FITC labelled goat anti-rabbit antibody (1:200 Jackson ImmunoResearch, West Grove, USA).
After that the coverslips were washed three times for 5 minutes in PBS. Counterstaining was performed with DAPI (Boehringer Mannheim GmbH, Mannheim, Germany) for 1 minute. Finally the sections were mounted in Fluoromount (Southern Biotechnologies, Birmingham, USA) after washing three times in PBS.
Negative controls include omission of primary antibodies and their replacement with 0.3% Triton X in PBS.

2.2.5 Adrenocorticotropin hormone (ACTH) immunofluorescence

ACTH plays an important role in the pigmentation of human skin and hair (Kauser et al. 2005; Rousseau et al. 2007; Paus 2011). The expression of ACTH at the protein level was detected via immunofluorescence staining as described by Kauser et al. 2005 with some modifications, which is analogous to the NKI/beteb immunofluorescence staining steps. The sections were incubated with rabbit anti-human ACTH polyclonal antibody (Chemicon Internaltional, Inc., USA 1:30 in Antibody-Diluent (DAKO, Glostrup, Denmark)) as the primary antibody for 60 minutes at 4°C. As the secondary antibody, the goat anti-rabbit labelled with FITC (1:200 in PBS, Jackson ImmunoResearch, Cambridgeshire, UK) was used.

2.2.6 Microphthalmia transcription factor (MITF) immunofluorescence

MITF is a key transcription factor that regulates the development and survival of melanocytes and that is also crucially involved in regulating the transcription of genes that code for key pigmentation-related enyzmes, incl. tyrosinase, i.e. the rate-limiting enzyme of melanogenesis (Nishimura et al. 2005; Slominski et al. 2005; Vachtenheim and Borovansky 2010; Bell and Levy 2011; Hartman and Czyz 2015). The anti-MITF antibody used here recognizes a nuclear protein which is expressed in human melanocytes (King et al., 1999)

The immunostaining steps were analogous to the NKI/beteb immunofluorescence staining steps described above.

These sections were incubated with mouse anti-human MITF monoclonal antibody (Novo-Castra, Laboratories Ltd, UK or DAKO, Glostrup, Denmark, 1:50) as a primary antibody overnight at 4°C. As the secondary antibody, the goat anti-mouse labelled with FITC (1:200 in PBS, Jackson ImmunoResearch, Cambridgeshire, UK) was used.

2.2.7 Tyrosinase enzyme activity-Tyramide signal amplification technique

In situ the tyrosinase enzyme activity was measured by the tyramide-based tyrosinase assay, in which tyrosinase reacts with biotinyl tyramide, thus causing the substrate to deposit near the enzyme. These biotinylated deposits are then visualized with streptavidin conjugated to a fluorescent dye (Han et al. 2002).

Cryosections stored at -80°C were first air dried and fixed in methanol/acetone (1/1) at -20°C for 10 minutes. The sections were washed once for 5 minutes in PBS. Then endogenous peroxidase was blocked by washing with 3% H_2O_2 (Merck, Darmstadt, Germany) in PBS for 15 minutes, followed by washing once in PBS for 5 minutes.

The preincubation was performed with a blocking kit (Vector Laboratories, Inc, Burlingame, CA, USA) which consists of avidin and of biotin in convenient dropper bottles and with 5% bovine serum albumin (BSA) in PBS. The avidin was applied first, followed by biotin for 15 minutes and then 5% BSA in PBS for 30 minutes, with a washing step in-between (one time in PBS for 5 minutes).

For the preparation of the Biotinyl Tyramide Amplification Reagent stock solution, the solid was prepared with 1.2 ml DMSO (Dimethylsulfoxide). To make the working solution, the stock solution was diluted 1:50 using Amplification Diluent (PerkinElmer,Inc, USA).

After preincubation, the sections were washed, then tipped to drain, and finally incubated with the Biotinyl Tyramide Amplifikation Reagent working solution for 30 minutes at room temperature.

Next, the sections were washed three times in a detergent 0.1% Igepal/PBS (Fluka, Buchs, Switzerland) for 5 minutes, followed by the administration of streptavidin-Cy3 (Sigma-Aldrich, Steinheim, Germany) 1:600 in 5% BSA/PBS for 60 minutes at room temperature.

After washing once in the detergent 0.1% Igepal/PBS, the sections were counterstained with DAPI (Boehringer Mannheim, Germany) for 1 minute. Finally they were washed in 0.1% Igepal/PBS and in distilled water and mounted with Flouromount (Southern Biotechnologies, Birmingham, USA).

As negative control Kojic acid, a tyrosinase inhibitor, (Sigma-Aldrich, Steinheim, Germany) was dissolved in DMSO at 500 mg/ml. This stock solution was added to

the Amplifikation Diluent to make the working solution with a final concentration of 15 mg/ml.

2.2.8 TRH-R1- Tyramide signal amplification technique

The Tyramide Signal Ampflification (TSA) technique was also used to detect the TRH-R1 antigen. After air drying and standard fixation in acetone at -20°C, the cryosections were washed three times for 5 minutes using the Tris-NaCl-Tween buffer **(**TNT buffer). TNT buffer consists of 0.1 mol/l Tris-HCl, 0.15 mol/l NaCl and 0.05% Tween 20, pH 7.5). To quench the peroxidase activity, all sections were treated with 3% H2O2 (Merck, Darmstadt, Germany) in PBS for 15 minutes, followed by washing three times in TNT buffer for 5 minutes.

Preincubation was performed with avidin and biotin for 15 minutes (Vector Laboratories, Inc, Burlingame, CA, USA) and 5% goat normal serum (DAKO, Glostrup, Denmark) in TNT for 30 minutes with washing steps in-between (three times for 5 minutes in TNT). The sections were incubated with the polyclonal rabbit anti-TRH-receptor as primary antibody (Acris, Hiddenhausen, Germany), which was diluted 1:500 in TNT + 2% normal goat serum (NGS), overnight at 4°C. The primary antibody was then removed by washing three times with TNT buffer for 5 minutes. The biotinylated goat anti-rabbit secondary antibody (1:200 in TNT buffer + 2% NGS; Jackson ImmunoResearch, Cambridgeshire, UK) was added for 45 minutes at room temperature. After the washing steps, three times for 5 minutes, the sections were incubated with streptavidin conjugated horseradish peroxidase (1:100 in TNT buffer; TSA kit; Perkin-Elmer, USA) for 30 minutes at room temperature. Next, the sections were washed and amplified by FITC-tyramide (TSA kit; 1:50 in amplification dilutent) for 5 minutes. After washing three times in TNT buffer for 5 minutes, the cryosections were counterstained with DAPI (Boehringer Mannheim, Germany; 1 µg/ml) for 1 minute and then mounted in Flouromount-G (Southern Biotechnologies, Birmingham, USA).

The human pituitary “thyrotrope” cells in the anterior lobe of pituitary (Sun et al. 2003) were used as a positive control. For the negative control, the primary antibody was omitted.

2.2.9 RT-PCR for tyrosinase and POMC mRNA

Normal human scalp skin HFs were microdissected and cultured in the absence or presence of TRH (15-300nM) for 8h. Total RNA was isolated from 20 HFs/experimental group as described earlier. Quantitative RT-PCR analysis was carried out on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) by using the 5' nuclease assay and the TaqMan Universal PCR Master Mix Protocol and TaqMan primers and probes recognizing the human *tyrosinase* (Assay ID Hs00165976_m1) and *POMC* (Assay ID Hs00174947_m1) mRNAs (Applied Biosystems). As internal controls, transcripts of *β-actin* and *cyclophilin A* (*PPIA*) were used. (Assay ID: Hs99999903_m1 and Hs99999904_m1, respectively). This experiment was conducted with assistance from Tamás Bíró at the Department of Physiology, Research Center for Molecular Medicine, University of Debrecen, Debrecen, Hungary.

2.3 Hair follicle histomorphometry

A number of previously developed and published morphometric techniques was used in order to quantitatively assess various relevant read-out parameters within human HFs *in situ*.

2.3.1 Quantitative melanin histomorphometry

The slides were photographed using an Olympus BH-2 microscope (Olympus Optical Co. Hamburg) at 200x magnification.

The melanin staining intensity of anagen VI HFs was analyzed in precisely defined, selected two reference areas of the HFPU by using the ImageJ software (http://rsbweb.nih. gov/ij/download.html). These two references areas (lateral hair matrix) were selected in order to assess melanin production changes in the hair follicle pigmentary unit with maximal accuracy. About 15-18 different HFs per TRH concentration per each experiment were analyzed. (**Figure 2.3**)

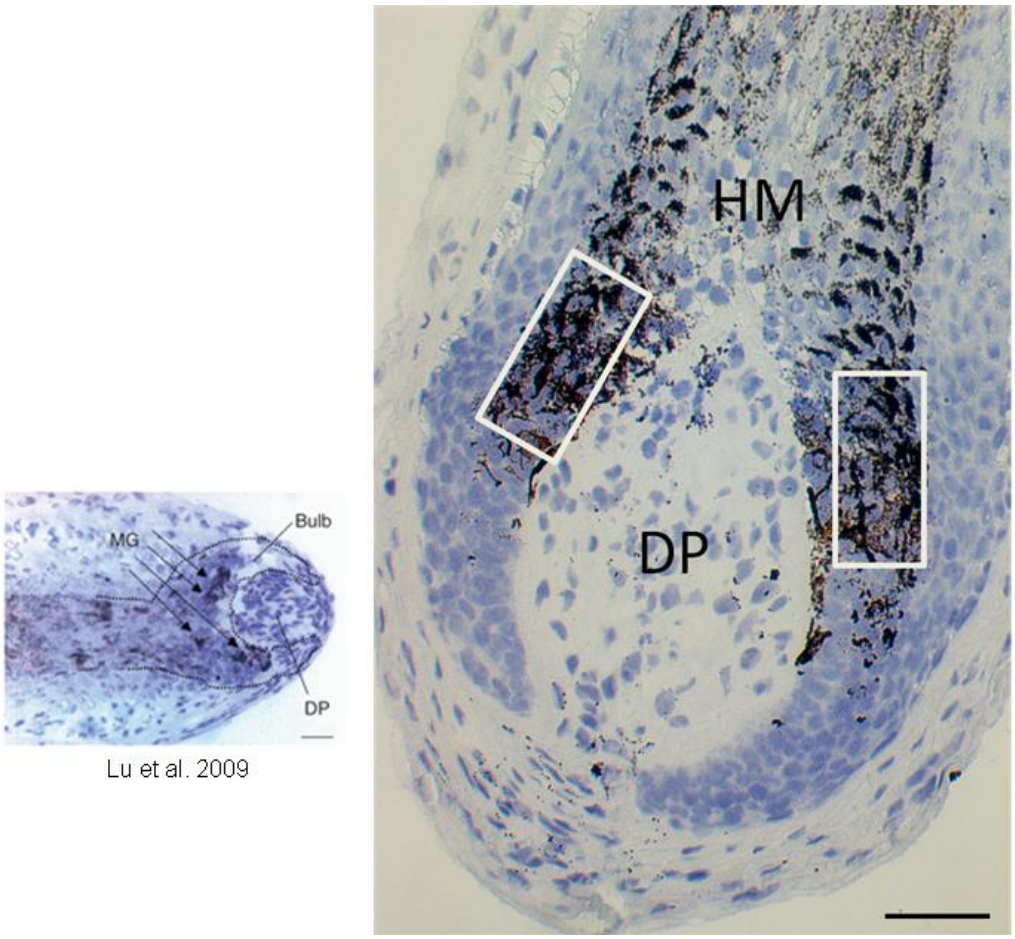

Figure 2. 3: Measurement of the melanin staining intensity in hair follicle.

The melanin content was measured in two reference areas in the hair follicle pigmentary unit (HFPU) to calculate a mean value (Samuelov et al. 2013). An example of Masson-Fontana staining, which (Lu et al., 2009) have published before (left image); DP: dermal papilla; HM: hair matrix; MG: melanin granula; scale bar: 50 µm.

2.3.2 Quantitative immunohistomorphometry

Slides were photographed using the Keyence all-in-one microscope (Keynce, USA) at 200x magnification. Only anagen VI HFs were measured for quantitative immunohistochemistry in two selected and precisely defined, reference areas of the HFPU by using the ImageJ software (http://rsbweb.nih. gov/ij/download.html). Approximately 15-18 different HFs per TRH concentration, per each experiment were analyzed.

2.3.2.1 Assessment of gp100 (NKI/beteb) immunostaning intensity

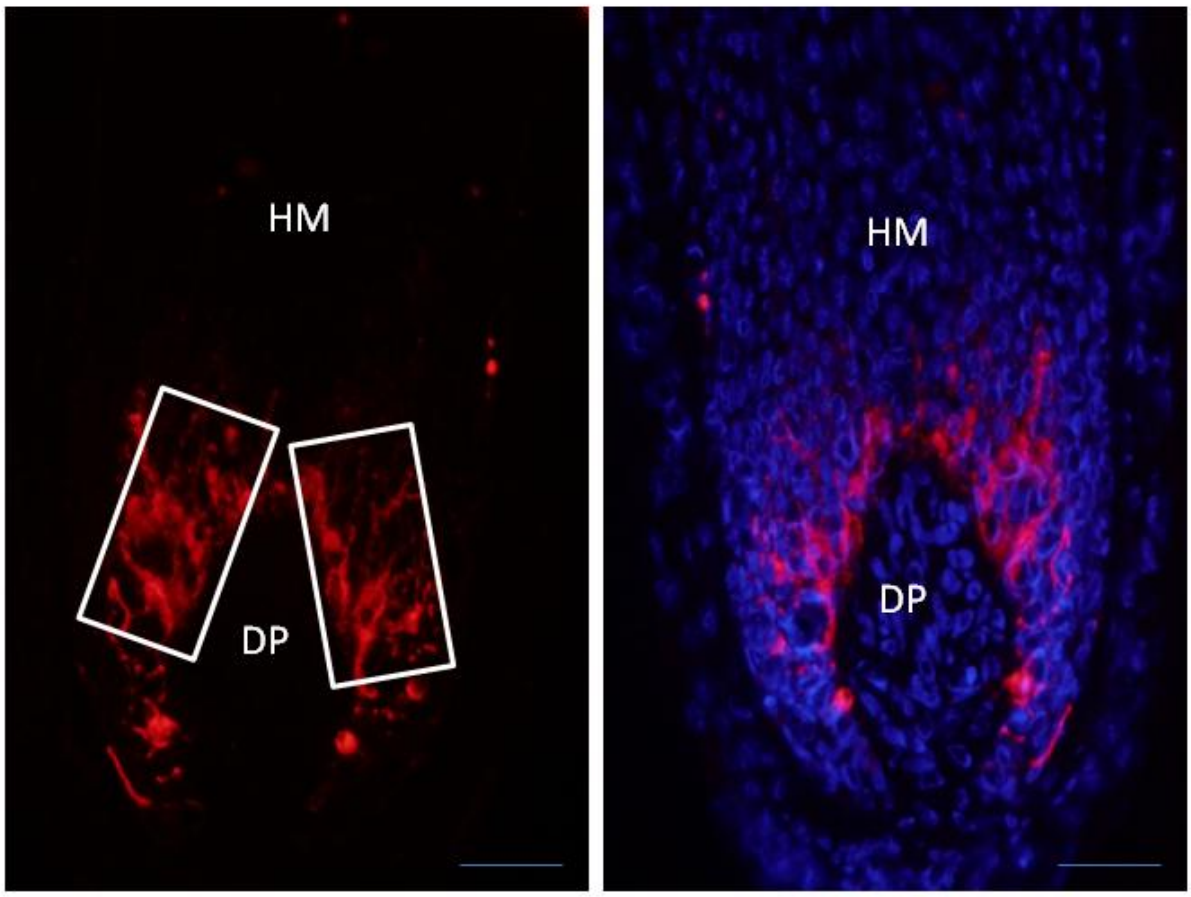

Figure 2. 4: Measurement of the immunostaining intensity in hair follicle.

The staining intensity was measured in two reference areas in the hair follicle pigmentary unit (HFPU) to calculate a mean value. (A) Assessment of the gp100 (NKI/beteb) immunostaining intensity. (B) NKI/beteb with DAPI overlay. DP: dermal papilla; HM: hair matrix; scale bar: 50 µm.

2.3.2.2 Assessment of tyrosinase immunostaning intensity

The measurement of tyrosinase immunnostaning intensity was analogous to gp100 (NKI/beteb).

2.3.2.3 Assessment of ACTH immunostaning intensity

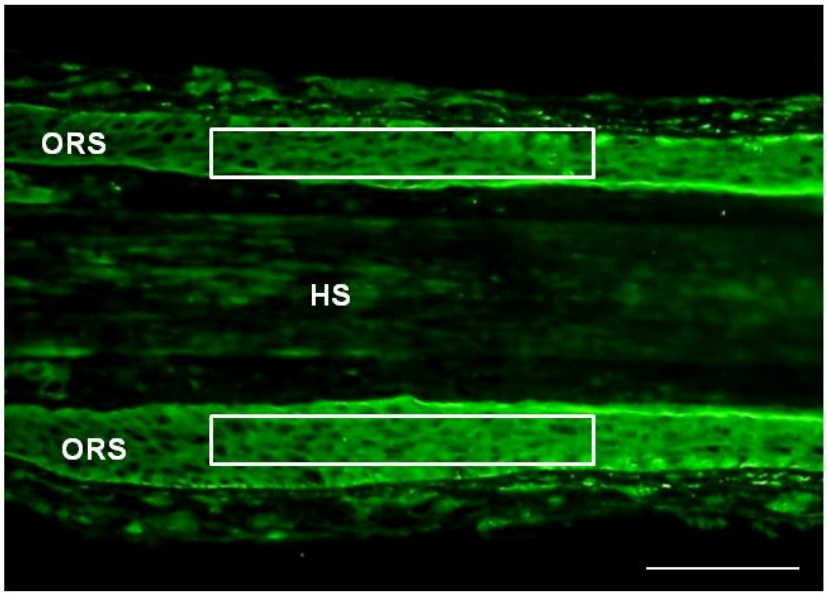

Figure 2. 5: Measurement of the ACTH immunostaining intensity in hair follicle.

The staining intensity of two reference areas in the ORS was measured to calculate a mean value. ORS: outer root sheet; DP: dermal papilla; scale bar: 50µm.

2.3.2.4 Assessment of MITF immunostaning intensity

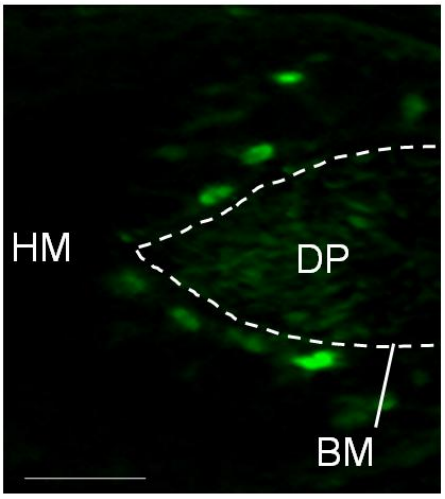

Figure 2. 6: Measurement of the MITF immunostaining intensity in melanocytes.

The Anti-MITF recognizes a nuclear protein, which is expressed in melanocytes. The positive nuclear (green) was counted. HM:Hair matrix; DP: Dermal papilla; BM: basal membrane; scale bar: 50µm.

2.4 Histomorphometry in isolated melanocytes

The slides were photographed using the Keyence all-in-one microscope (Keynce, USA) at 200x magnification. 20-25 melanocytes per donor were measured for quantitative immunohistochemistry in precisely defined, selected reference areas of the HFPU by using the ImageJ software (http://rsbweb.nih. gov/ij/download.html).

2.4.1 Assessment of gp100 (NKI/beteb) immunostaning intensity

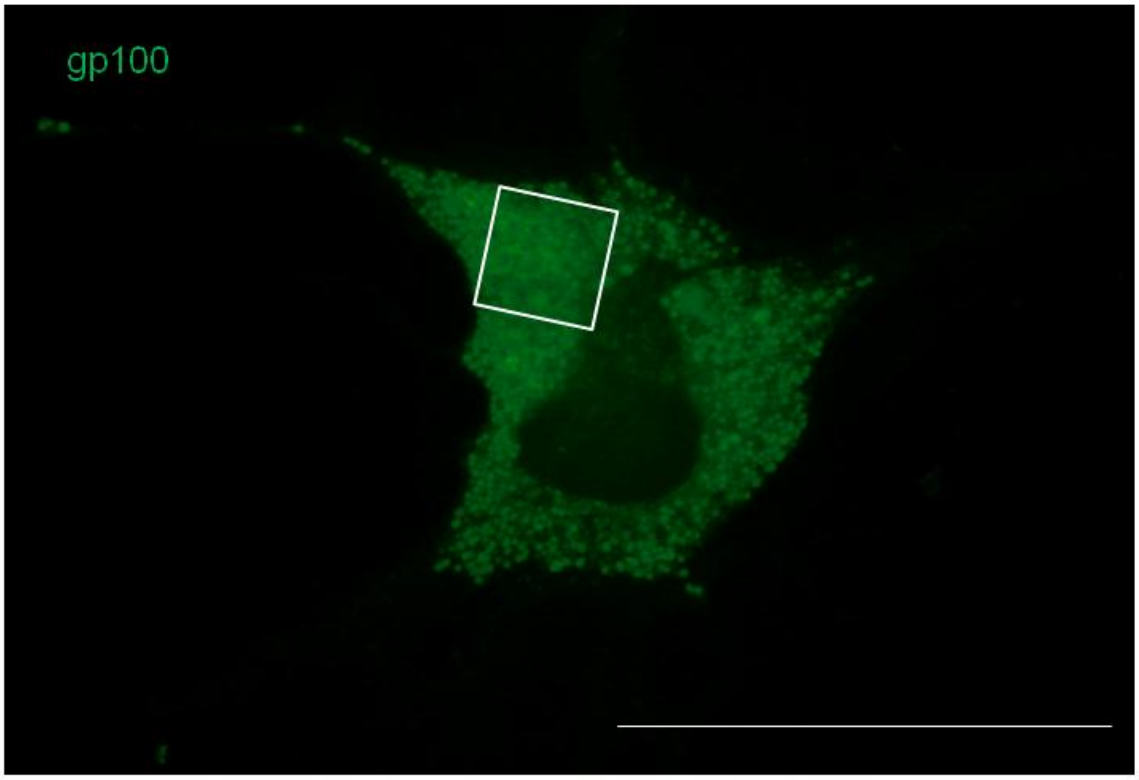

Figure 2. 7: Measurement of the immunostaining intensity in isolated melanocytes.
The staining intensity was measured in the reference area in the melanocytes. Scale bar: 50 µm.

2.4.2 Assessment of tyrosinase immunostaning intensity

The measurement of tyrosinase immunostaining intensity in melanocytes was analogous to NKI/beteb.

2.4.3 Assessment of melanocytes dendricity in vitro and in situ

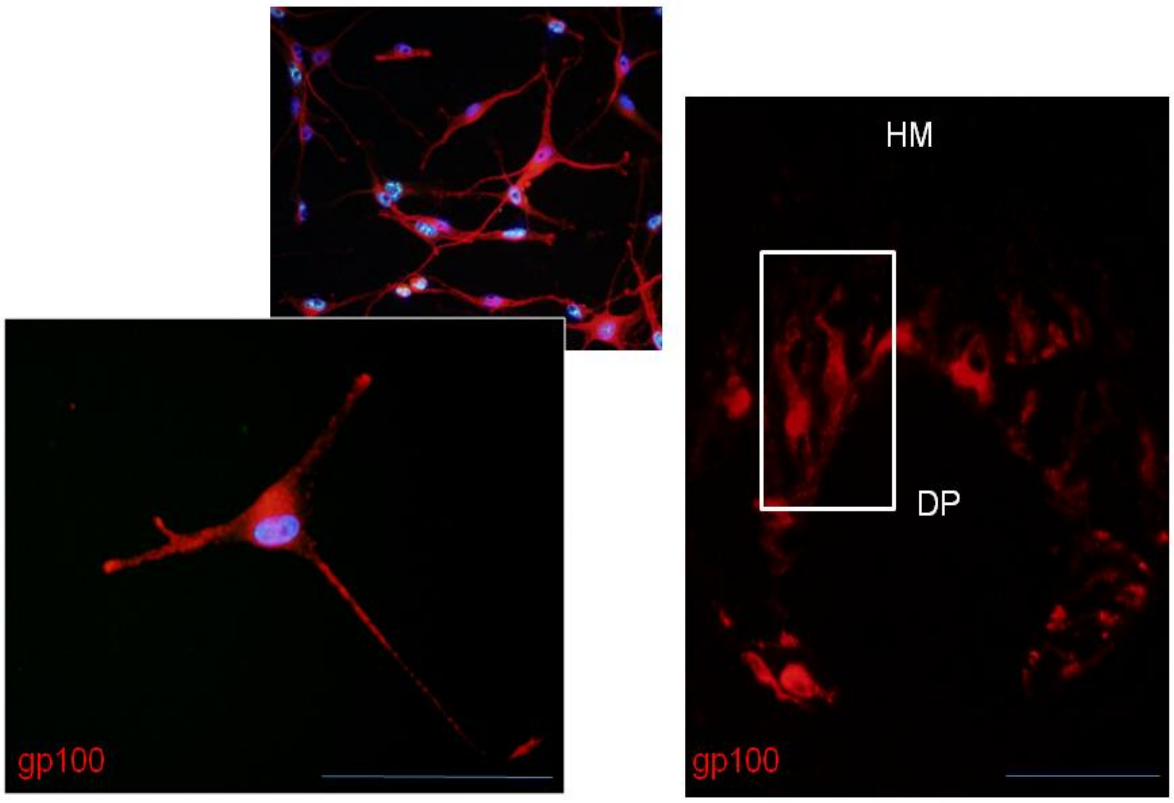

Figure 2. 8: Assessment of dendricity of gp 100+ melanocytes *in vitro* and *in situ*.

The dendricity in gp100+ melanocytes *in vitro* and *in situ* was counted. The number of melanocytes with 2 or more dendrites were expressed as % of the total number of the gp100+ melanocytes. HM: hair matrix; DP: Dermal papilla. Scale bar: 50µm.

2.4.4 Assessment of proliferation of cultered melanocytes

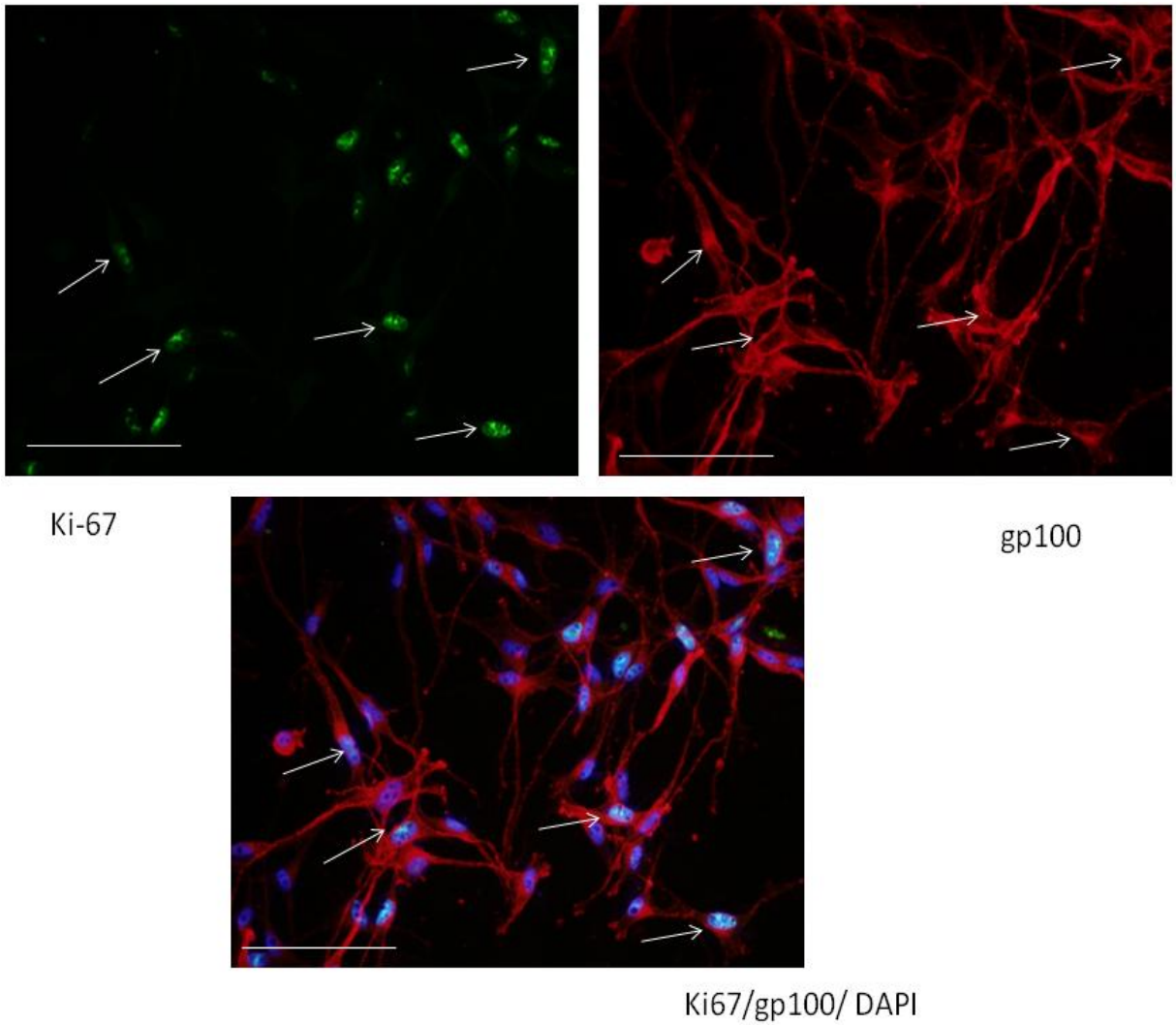

Figure 2. 9: Assessment of proliferation of cultured melanocytes.

Melanocytes with triple positive staining (Ki67/gp100/DAPI) were counted (arrow). The number of melanocytes with triple positive were expressed as % of the total number of the gp100+ melanocytes. Scale bar: 50µm.

2.5 Histomorphometry in human skin

2.5.1 Quantitative melanin histomorphometry

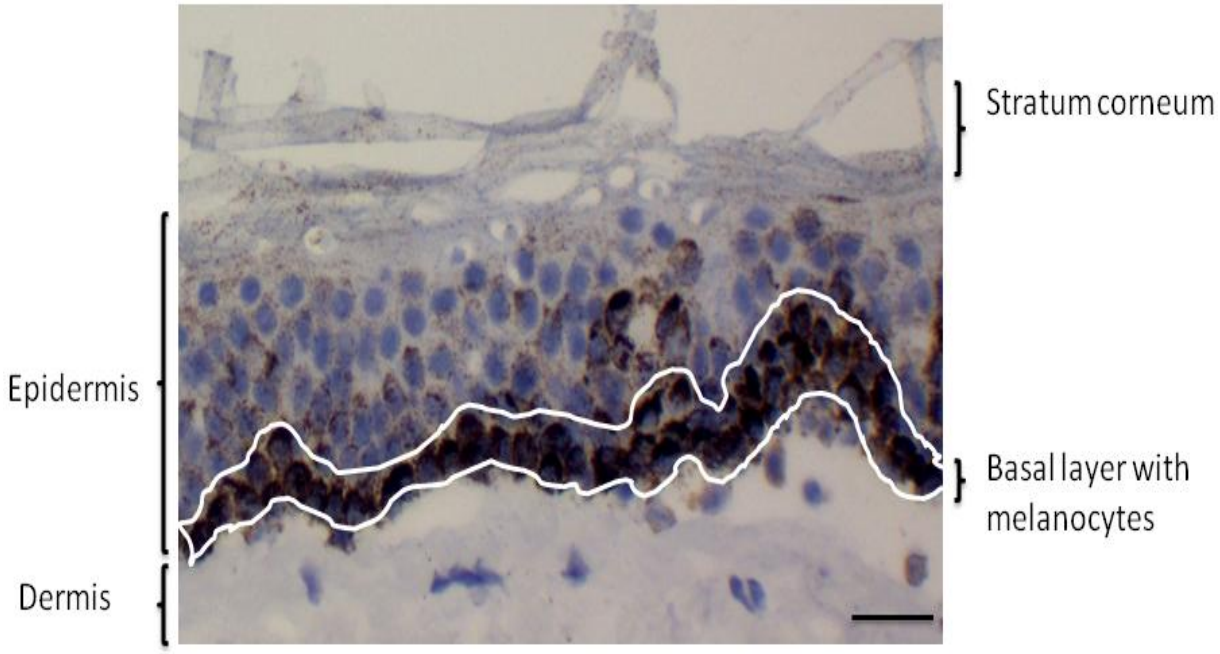

Figure 2. 10: Measurement of the melanin staining intensity in human skin.

The melanin content was measured in human skin. The reference area was selected at the border of the basal layer. All skin sections were in the same format . Scale bar: 50 µm.

2.5.2 Assessment of NKI/beteb (gp100) immunostaining intensity

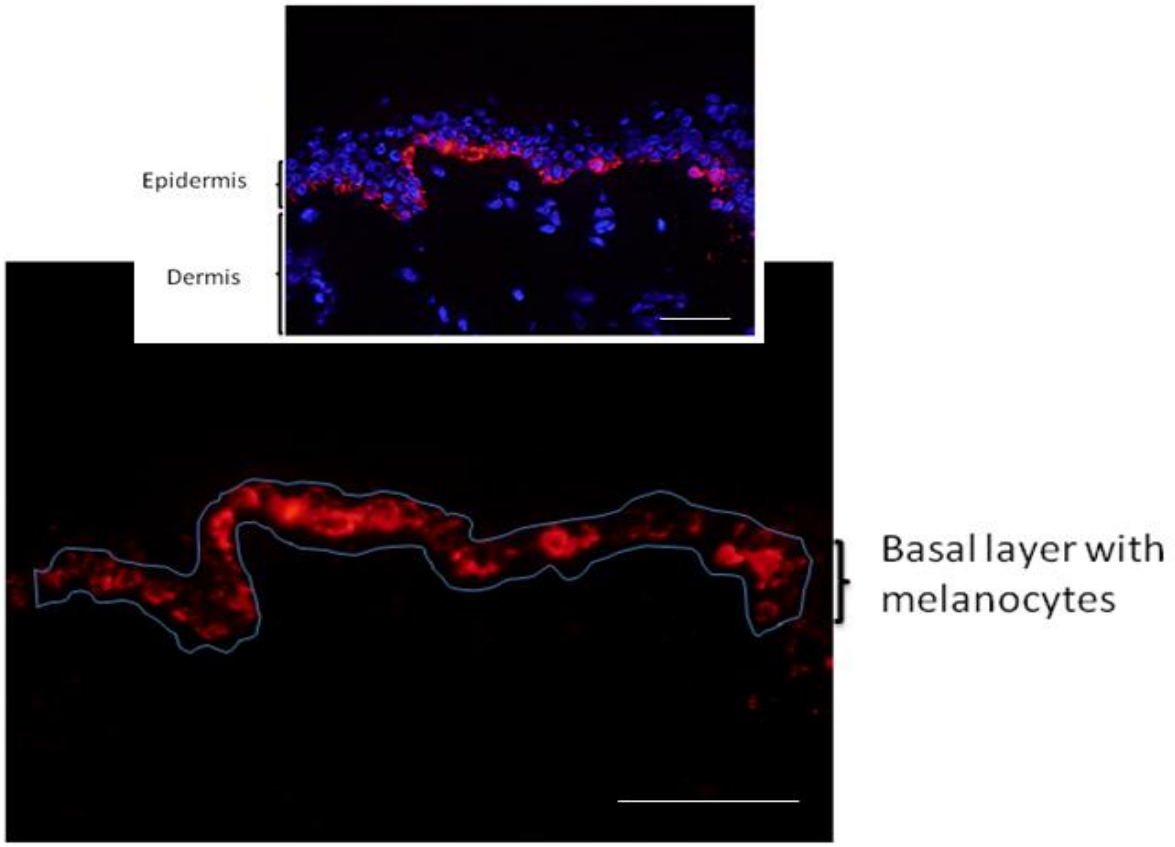

Figure 2. 11: Measurement of the gp100 staining intensity in human skin.

The gp100 immunostaining intensity was measured in human skin. The reference area was selected at the border of the basal layer. All skin sections were in the same format. Scale bar: 50 µm.

2.5.3 Assessment of tyrosinase immunostaning intensity

The measurement of tyrosinase immunnostaning intensity in melanocytes was analogous to gp100 (NKI/beteb).

2.6 Statistical analysis

The statistical analysis software Prism v.3.00 (Graph-Pad Software Chicago, IL, USA) war used for the evaluation of statistical significance. All data were analysed by Mann-Whitney-Test for unpaired samples (expressed as mean ±SEM; p-values *<0.05, **$\leq$0,01, ***$\leq$0,001 were regarded as significant).

3 RESULTS

3.1 TRH stimulates melanin synthesis in organ-cultured normal adult human scalp hair follicles in the absence of a pituitary gland

The first question was whether TRH, which is expressed by human scalp HFs on the gene and protein level (Gáspár et al. 2010), could stimulate the pigmentation of microdissected and organ-cultured adult human scalp HFs *in vitro*.
Only healthy HFs in the active growth stage of the hair cycle (anagen VI), during which the HF engages in maximal melanin production (Slominski et al. 2005; Schneider et al. 2009; Tobin 2011) were used. Masson-Fontana histochemistry staining was employed to visualize the melanin content, which was demarcated as black dots in the hair bulb.

As can be seen in **Figure 3.1**, an elevated melanin content of organ-cultured normal human HFs incubated with TRH, as compared with the vehicle-treated HFs, was qualitatively demonstrated.There are more melanin granules in the proximal hair bulbs, which were incubated with TRH (3nM) after 6 days than in the vehicle treated HFs. Quantitative Masson–Fontana histomorphometry revealed a significantly upregulated amount of histochemically detectable melanin granules in intact anagen hair bulbs compared with vehicle controls, as seen in the graph in **Figure 3.1**.
These qualitative and quantitative Masson-Fontana histochemistry results show that,in the absence of the pituitary gland and serum components, TRH stimulates melanin synthesis in organ-cultured, normal HFs of the human scalp *ex vivo*.

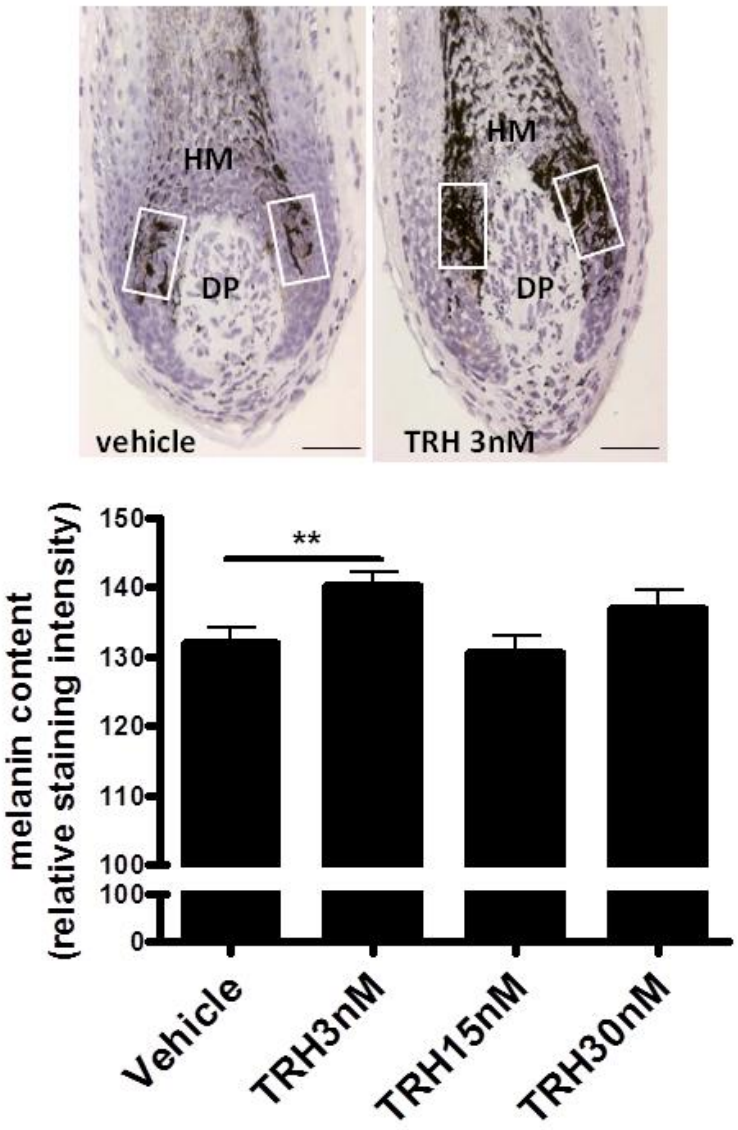

Figure 3. 1: Thyrotropin-releasing hormone (TRH) stimulates melaninsynthesis.

Histochemical staining for melanin in the hair bulb demonstrates elevated pigment production after 6 days in organ-cultured, normal human HFs incubated with TRH, compared with the vehicle-treated control. The increase of the melanin content reached significance with 3 nM TRH. Columns represent mean±SEM; *P<0.05 , **P<0.01, ***P<0.001 versus vehicle (TRH 0 nM), p value was calculated by Mann-Whitney-U-Test for unpaired samples; pooled data from 2 different donors/experiments (n=15–18 HFs per TRH concentration per each experiment),; scale bar=50 µm. DP: Dermal papilla; HM: hair matrix.

3.2 TRH stimulates intrafollicular tyrosinase activity and tyrosinase mRNA expression

Tyrosinase is recognized as the rate-limiting enzyme of melanogenesis (Slominski et al. 2005; Tobin 2011), and its activity reflects the *de novo* synthesis of melanin.
To investigate how TRH-stimulated HF melanin content correlated with tyrosinase enzyme activity and tyrosinase gene expression *in situ*, the tyramide-based tyrosinase assay and quantitative RT-PCR were used (Han et al. 2002).

The photos in **Figure 3.2**, demonstrate qualitatively the elevated tyrosinase activity in organ-cultured, normal human HFs incubated with TRH as compared with to vehicle-treated HFs. Quantitatively, the tyrosinase activity-associated immunofluorescence was significantly upregulated in HFs that had been cultured for 6 days in the presence of 30 nM and 300 nM TRH, as seen in the graph of the **Figure 3.2.**

Moreover, compared with vehicle controls, TRH also stimulated tyrosinase gene transcription *in situ*, as demonstrated by quantitative RT-PCR from mRNA extracts of intact human anagen HFs (**Figure 3.3**). The graph reveals that the tyrosinase mRNA steady-state level was significantly upregulated in HFs that had been cultured for 8 h in the presence of 300 nM TRH, compared with vehicle controls.

These analyses demonstrate the impact of TRH on the key step in the enzymatic control of the melanogenesis by stimulating both the enzyme activity and gene transcription of tyrosinase.

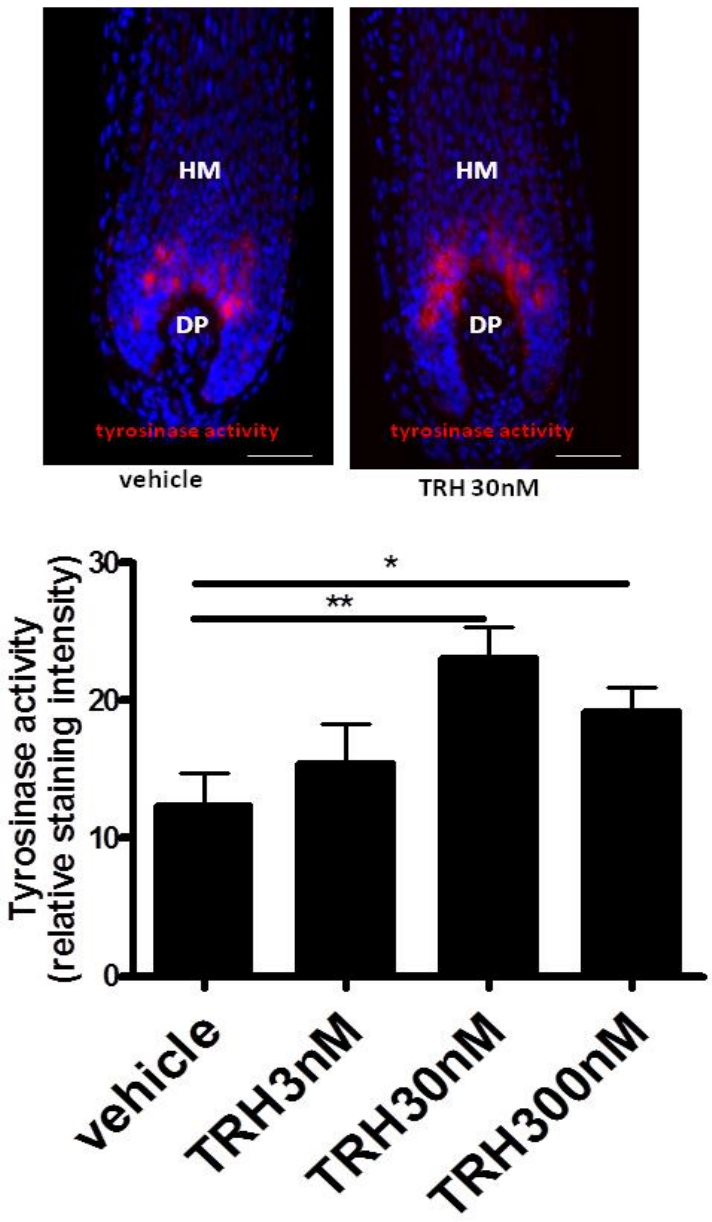

Figure 3. 2: TRH stimulates tyrosinase activity in human scalp hair follicles *in situ*.

Red fluorescence staining represents tyrosinase enzyme activity in the pigmentary unit of TRH-treated HFs. Quantitative analysis of the tyrosinase staining intensity demonstrates a significant increase in activity with 30 nM and 300 nM TRH. Columns represent mean±SEM, *P<0.05 and **P<0.01 versus vehicle (TRH 0 nM); pooled data from 3 different donors/experiments (n=15–18 HFs per TRH concentration per each experiment), scale bar=50 μm. DP: Dermal papilla; HM: hair matrix.

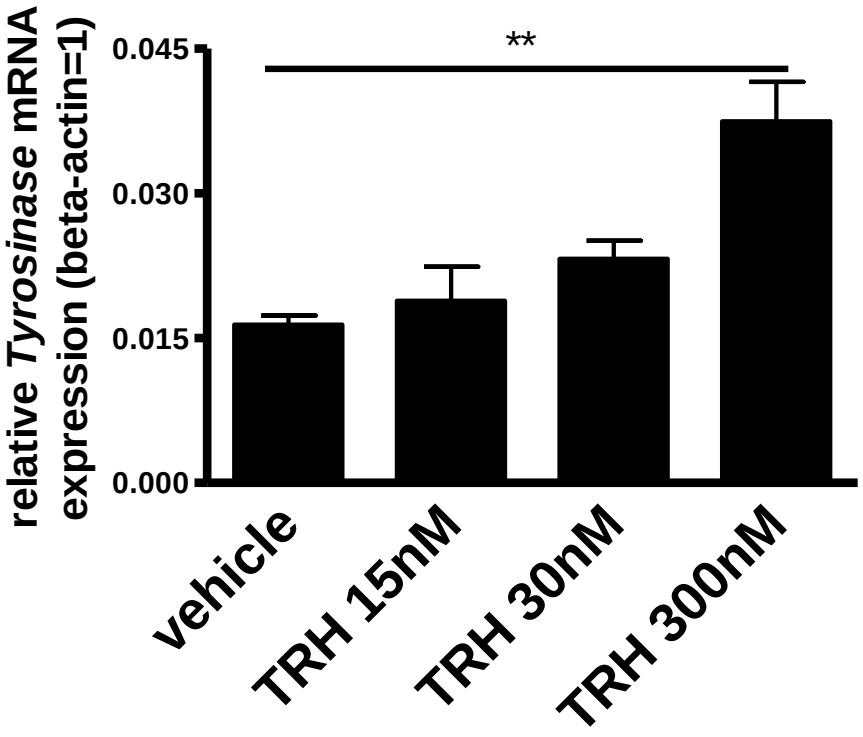

Figure 3. 3: TRH stimulates tyrosinase mRNA expression in human scalp hair follicles *in situ*. Significant increase of tyrosinase mRNA expression was observed in the HFs, which were treated with 300 nM TRH for 8h. Columns represent mean±SEM of three independent experiments (n=15–18 HFs per TRH concentration per each experiment), *P<0.05 and **P<0.01 versus vehicle (TRH 0 nM).

3.3 TRH stimulates melanosome formation and melanocyte dendricity in the human hair follicle pigmentary unit (HFPU)

To corroborate these previous observations by independent, complementary pigment biological parameters, this study investigated next, whether TRH stimulates melanosome formation and melanocyte dendricity in the human HFPU.

For this purpose, the NKI/beteb (gp100) antibody was used to identify the cleaved formed of gp100 present in stages III/IV melanosomes. The gp100 is a structural protein, which critical for the production and maturation of the fibrillar structures within the melanosomes (Singh et al. 2008; Samuelov et al. 2013). The immunodetection of this pigmentation-specific protein provides a very sensitive tool for visualizing both

melanosome transfer from melanocytes to keratinocytes and non-pigmented (amelanotic) melanocytes in the HF (Singh et al. 2008; Kloepper et al. 2010).

Indeed, TRH treated HFs showed significantly higher gp100 immunoreactivity *in situ* compared with controls (**Figure 3.4**.). As assessed by quantitative immunohistomorphometry, TRH significantly increased the total gp100-related immunoreactivity detectable in human anagen hair bulbs, as seen in the graph in **Figure 3.4.**

To induce hair shaft pigmentation, the melanosomes are transferred from melanocytes to neighboring keratinocytes through melanocyte dendrites (Slominski et al. 2005; Kauser et al., 2006; Singh et al. 2008; Tobin 2011; Samuelov et al. 2013). The photos in **Figure 3.5** demonstrate that the melanocytes of TRH treated HFs have more dendrites compared to untreated controls. The effect of TRH on the dendricity of melanocytes was also seen by quantitative analysis, that the number of dendrites per gp100 positive-cell (i.e. more than two dendrites per cell) was significantly increased compared to controls, as seen in the graph in **Figure 3.5**.

In addition, TRH treatment also increased slightly, but not significantly, the total number of human anagen hair bulb melanocytes that were identifiable *in situ* by gp100 immunofluorescence (**Figure 3.6)**.

To conclude, these gp100 data, demonstrates that TRH actually stimulates the melanosome transfer to recipient keratinocytes within the precortical hair matrix of normal human scalp HFs *in situ.*

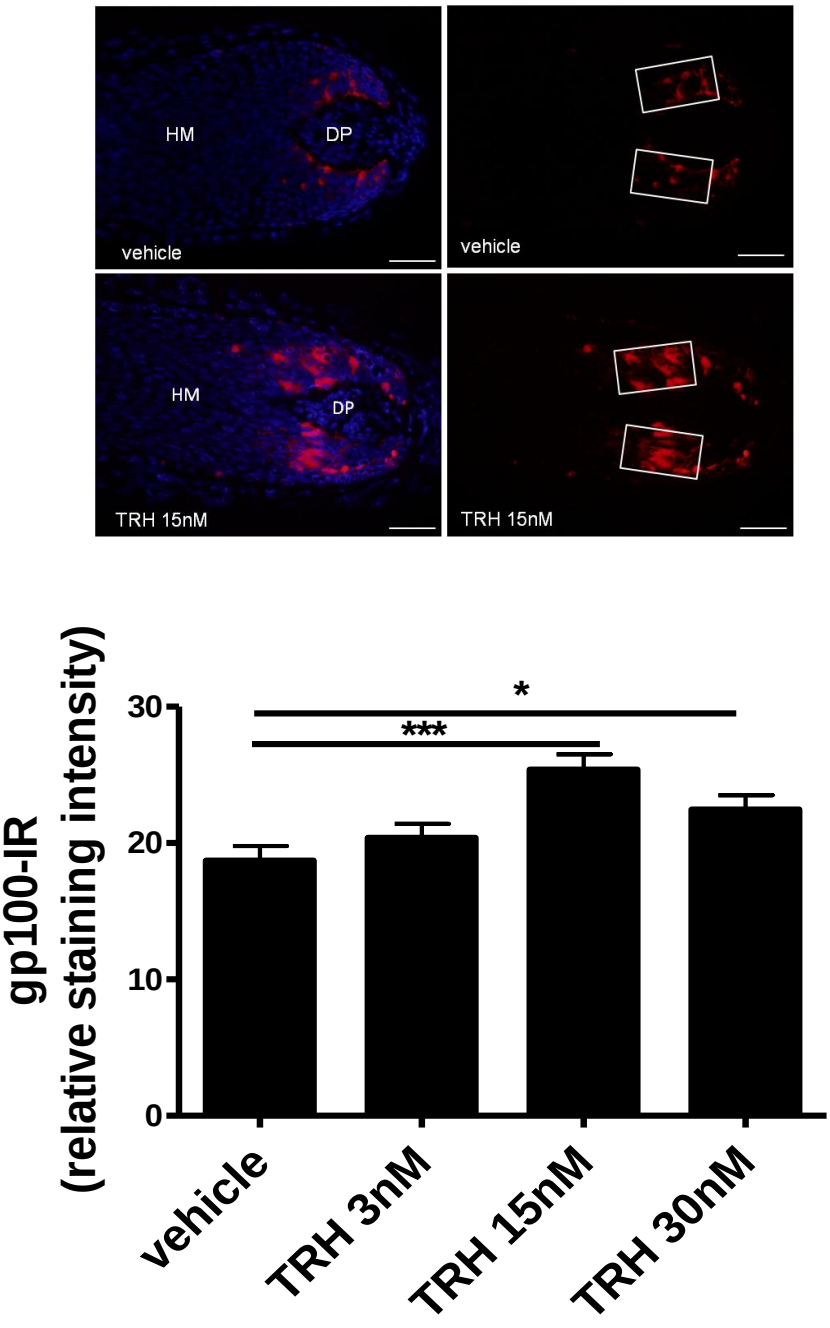

Figure 3. 4: TRH stimulates melanosome formation in human melanocytes *in situ*.

TRH significantly increased the pre-melanosome marker gp100 expression (red) in human anagen hair bulbs. The columns represent mean±SEM of three independent experiments (n=15–18 HFs per TRH concentration per each experiment), *P<0.05 and **P<0.01 versus vehicle (TRH 0 nM); scale bar =50μm. DP, dermal papilla; HM, hair matrix; HPM, hair follicle pigmentary unit; IR, immunoreactivity.

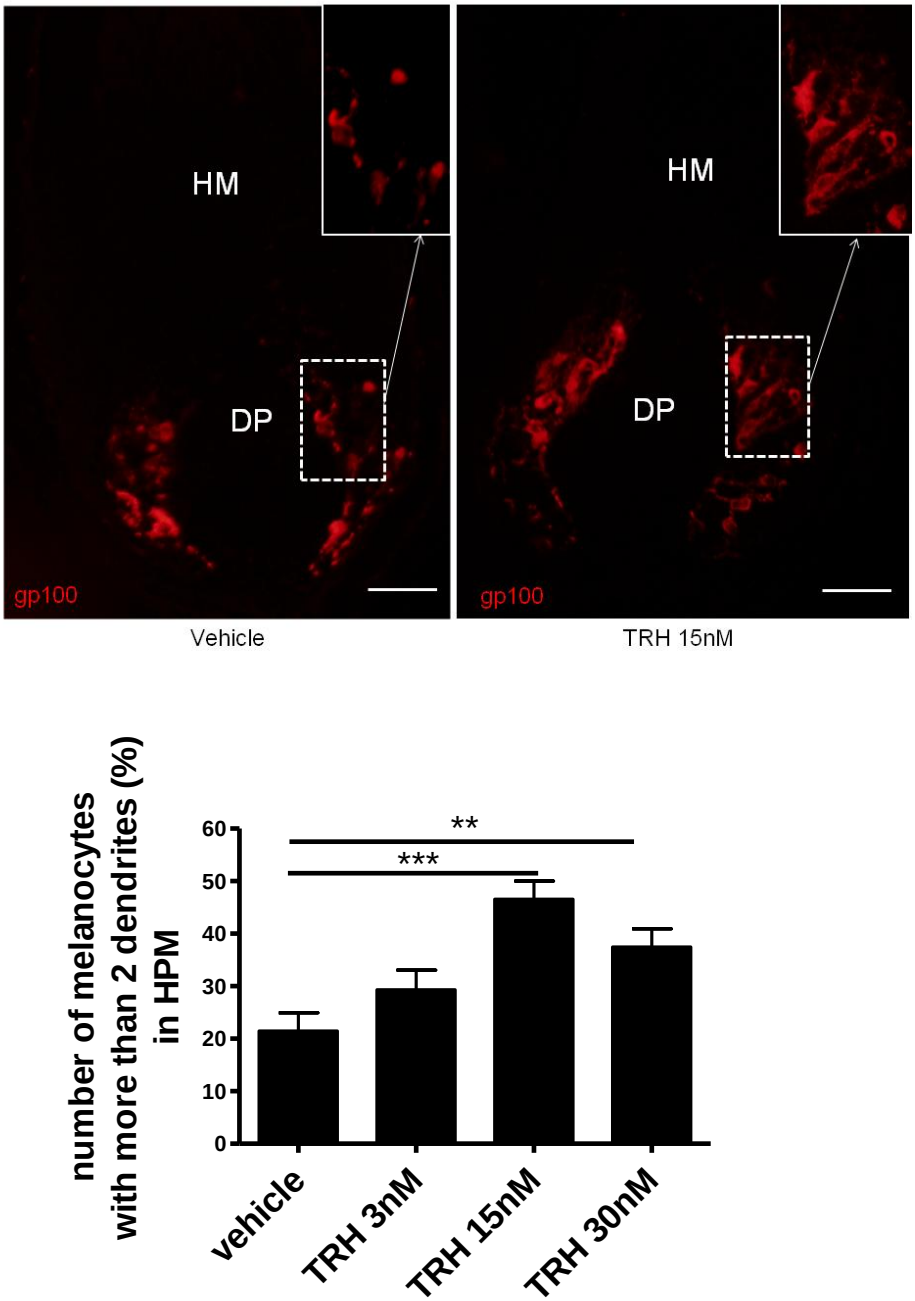

Figure 3. 5: TRH stimulates dendricity in human melanocytes *in situ*.

TRH significantly increased the dendrite formation of human hair follicle melanocytes *in situ*. Columns represent mean±SEM of four independent experiments (n=15–18 HFs per TRH concentration per each experiment), *P<0.05 and **P<0.01 versus vehicle (TRH 0 nM); scale bar = 50μm. DP, dermal papilla; HM, hair matrix; HPM, hair follicle pigmentary unit; IR, immunoreactivity.

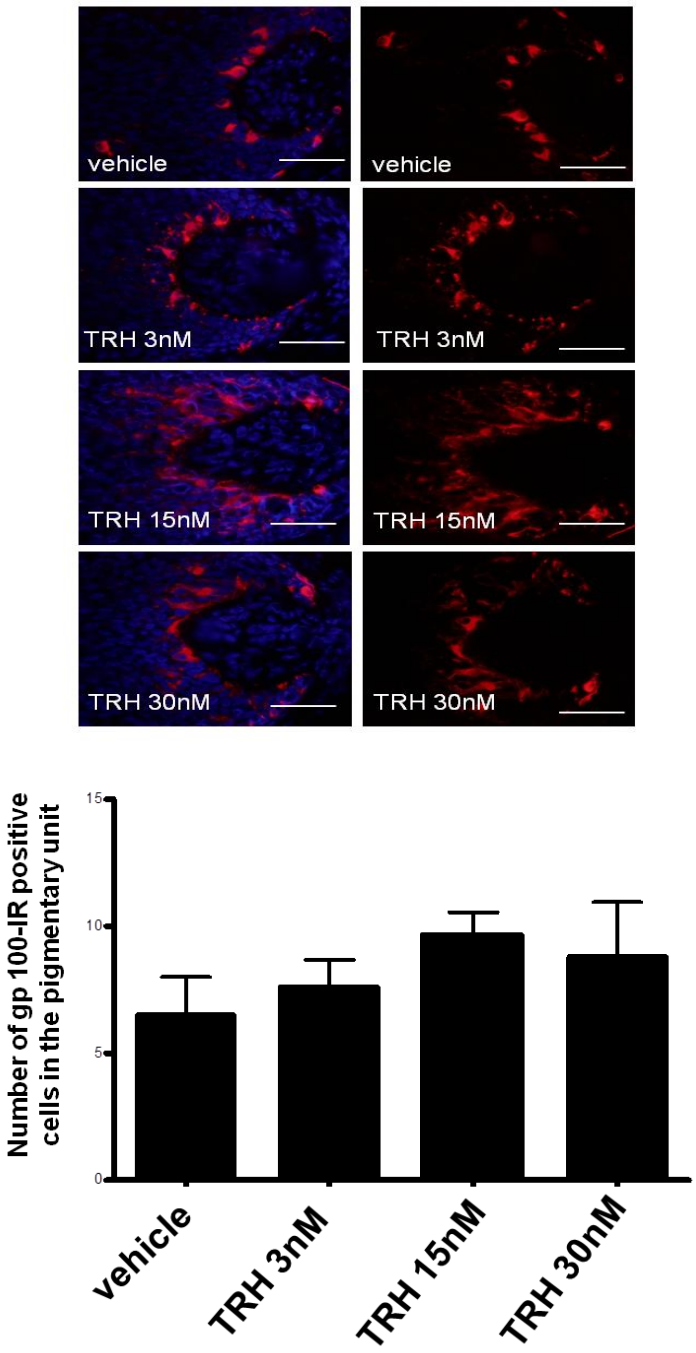

Figure 3. 6: Effect of TRH on the total amount of hair bulb melanocytes.

Thyrotropin-releasing hormone (TRH) increased slightly, but not significantly, the total number of human anagen hair bulb melanocytes that were identifiable *in situ*. Gp100 expression (red) in human anagen hair bulbs. The Columns represent mean±SEM of four independent experiments (n=15–18 HFs per TRH concentration per each experiment), *P<0.05 and **P<0.01 versus vehicle (TRH 0 nM). scale bar = 50μm, DP, dermal papilla; HM, hair matrix; HPM, hair follicle pigmentary unit; IR, immunoreactivity

3.4 TRH stimulates melanogenesis in isolated and cultured human hair follicle-derived melanocytes

The previous data demonstrated the significant pigment-stimulating effect of TRH on HFs *in situ*. The next question is, whether TRH also stimulates pigmentation in primary cultured human HF melanocytes isolated from the outer root sheath of normal adult human scalp HFs; i.e., in the absence of the normal epithelial and mesenchymal signaling environment of HF melanocytes. While these cells normally are amelanotic while they reside within the ORS *in situ* (Slominski et al. 2005; Tobin 2011), they can switch on melanin production after isolation and culture *in vitro* (Kauser et al. 2005).

Figure 3.7 (d,e) show isolated human HF melanocytes in cell culture under the light microscope. It is clearly recognizable that, qualitatively, TRH-treated human HF melanocytes (**Fig. 3.7 e**) have more dendrites compared with control melanocytes (**Fig. 3.7 d**). Nkibeteb (gp100) immunostaining was used to identify melanocytes (**Figure 3.7 b,c)**, and to detect melanocytes with more than two dendrites. Consistent with the *in situ* results, this showed that TRH also significantly stimulated dendricity in isolated, cultured human HF melanocytes, (**Figure 3.7a)**.

Furthermore, to complete the investigation of the effect of TRH on isolated melanocytes, the Nkibeteb (gp100) immunostaining and tyrosinase activity were quantitatively analysed. TRH was again found to stimulate both gp100 expression (**Figure 3.8**), and tyrosinase activity (**Figure 3.9**) in cultured human HF melanocytes. By using the double immunofluorescence of Ki67/gp100, we were able to investigate the proliferation of TRH treated human melanocytes. Intriguingly, even the proliferation of human HF melanocytes was upregulated by TRH *in vitro*, as shown by quantitative Ki67/gp100 double immunofluorescence (**Figure 3.10**).

The results of these investigation show, that TRH directly stimulates melanogenesis in isolated, cultured human HF melanocytes and that inputs from the HF epithelium or the HF mesenchyme are dispensable for this effect.

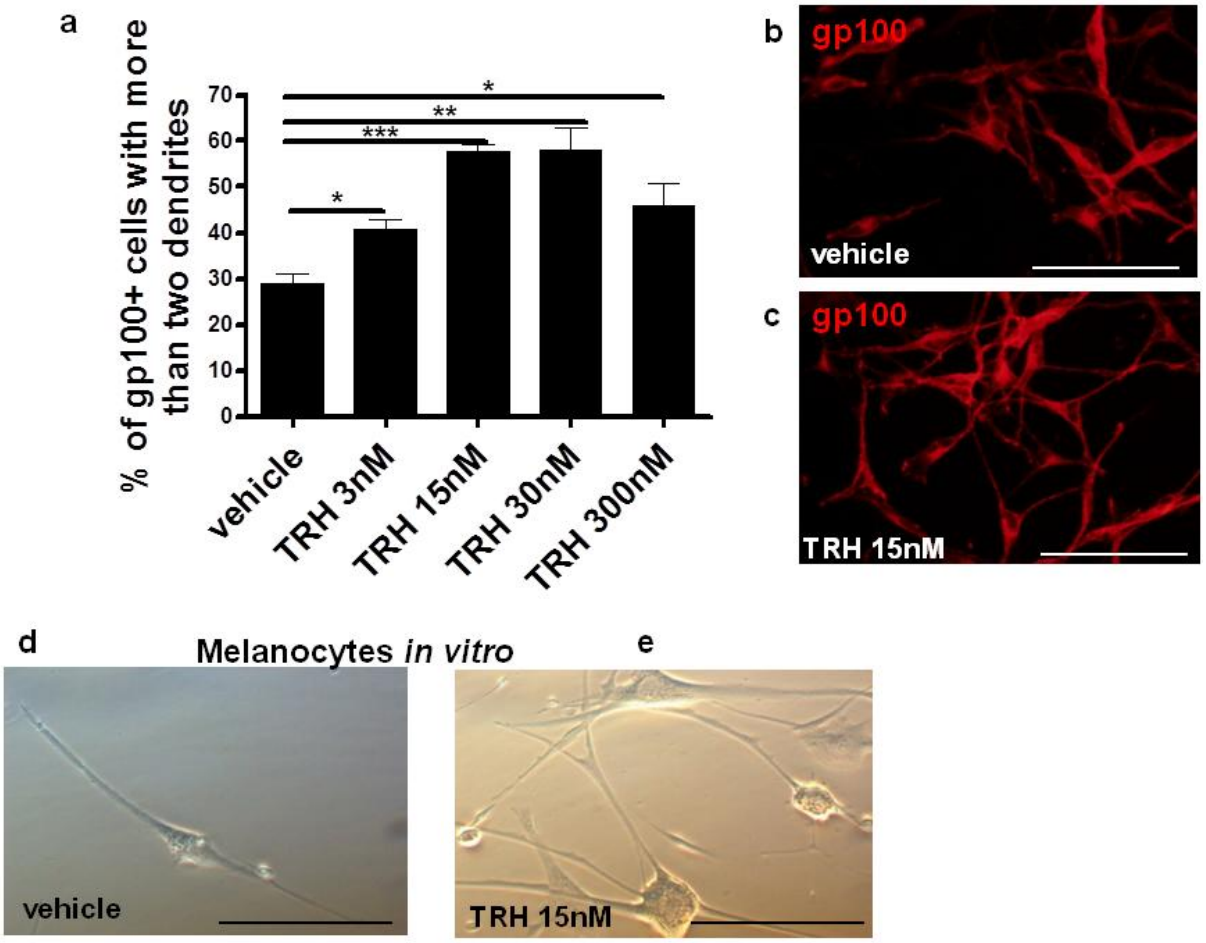

Figure 3. 7: TRH stimulates significantly the dendricity in human melanocytes *in vitro*.

TRH administration *in vitro* significantly stimulated the number of gp100+ melanocytes with more then two dendrites (a). The melanocytes in Nkibeteb (gp100) immunostaining (red) (b,c). Melanocytes in native cells culture (d,e). Columns represent mean±SEM of two independent experiments (n=20-30 melanocytes per TRH concentration per each experiment), *P<0.05, **P<0.01 and ***P<0.001 versus vehicle (TRH 0 nM); scale bar = 50µm.

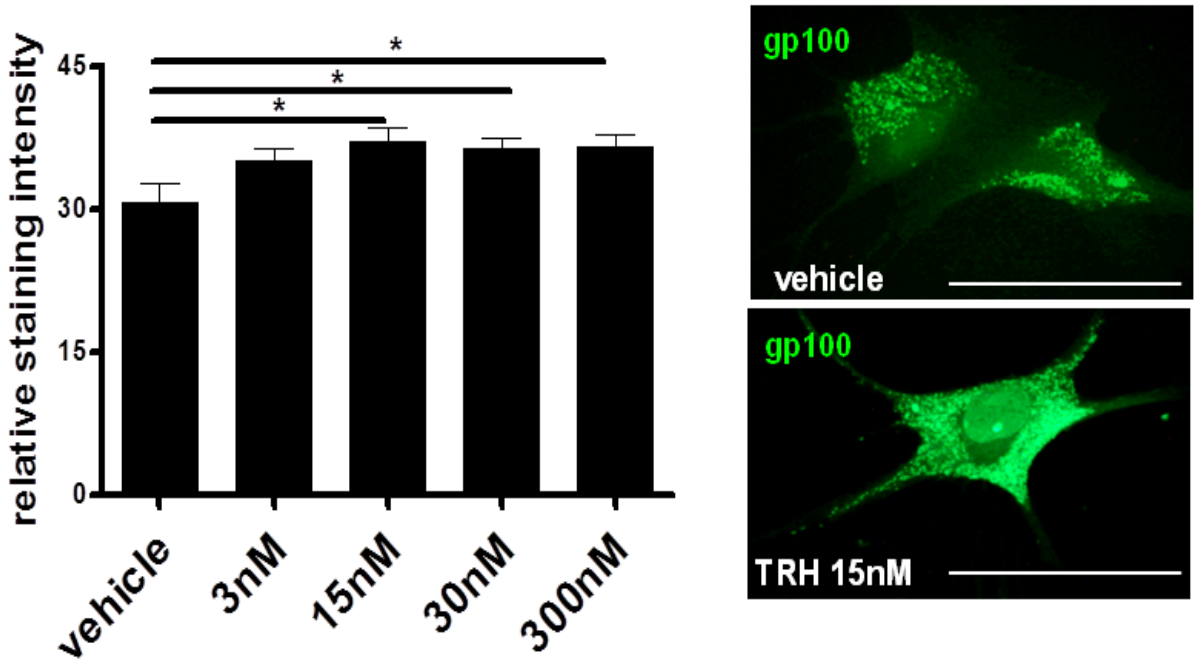

Figure 3. 8: TRH stimulates the gp100 expression of cultured HF melanocytes *in vitro*.
Thyrotropin-releasing hormone administration (15-300 nM) significantly stimulated the gp100 expression (green fluorescence) of cultured HF melanocytes. Columns represent mean±SEM, *P<0.05 versus vehicle (TRH 0 nM); representative results of one experiment (n= 20-23 melanocytes per TRH concentration per experiment), scale bar = 50µm.

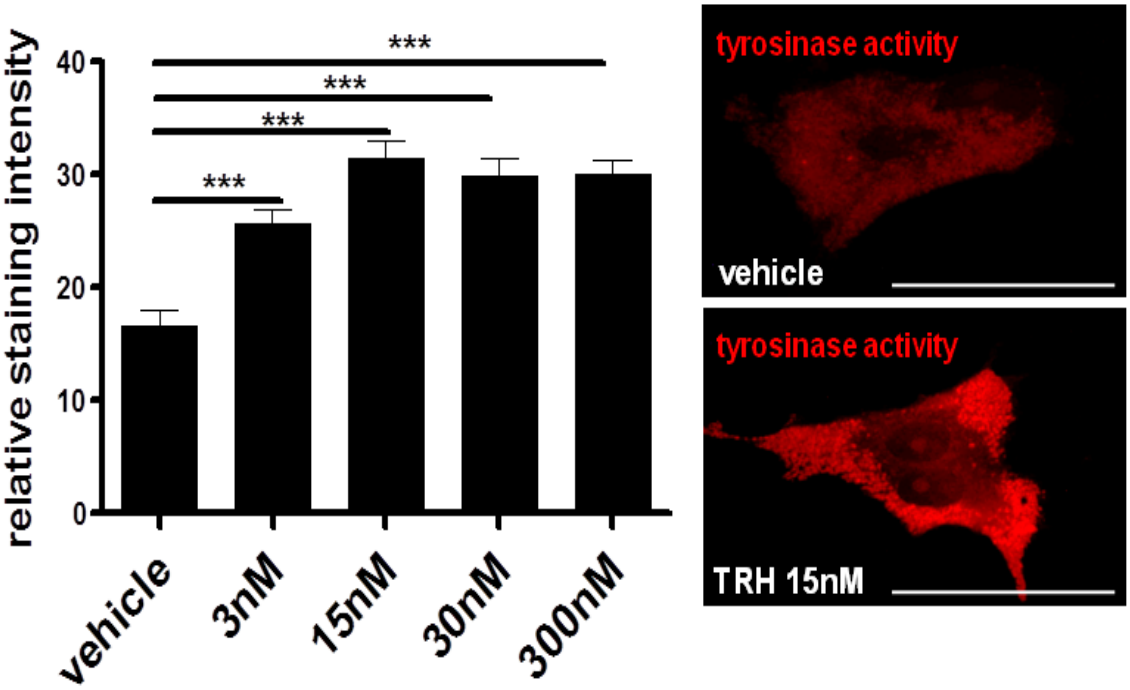

Figure 3. 9: TRH stimulates the tyrosinase activity of cultured HF melanocytes *in vitro*.

Thyrotropin-releasing hormone administration (3-300 nM) significantly stimulated the tyrosinase activity (red fluorescence) of cultured HF melanocytes. The columns represent mean±SEM of one experiment (n= 20-23 melanocytes per TRH concentration per experiment), *P<0.05, **P<0.01 and ***P<0.001 versus vehicle (TRH 0 nM); scale bar = 50μm.

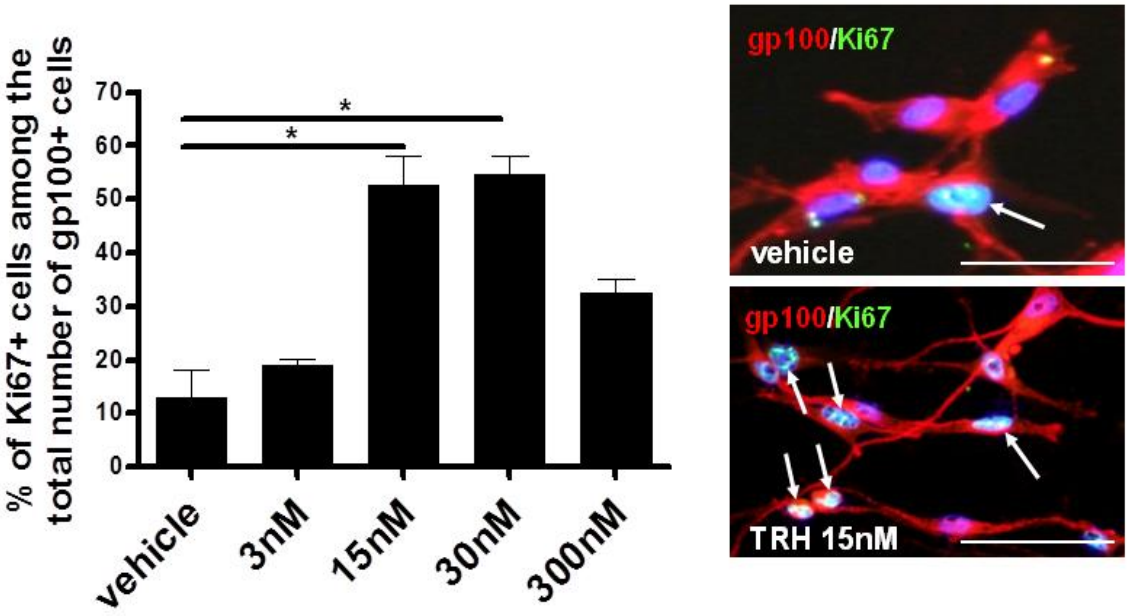

Figure 3. 10: TRH increases proliferation of human HF melaocytes *in vitro*.

Ki67(green)/gp100(red) double immunofluorescence staining shows significantly increased proliferation of cultured HF melanocytes after TRH (15-30nM) treatment. Cell with double immunofluorescence staining (arrows). Columns represent mean±SEM of three independent experiments (n= 80-100 melanocytes per TRH concentration per each experiment), *P<0.05 and **P<0.01 versus vehicle (TRH 0 nM); scale bar = 50µm.

3.5 TRH does not stimulate human epidermal melanogenesis *in situ*

As TRH expression,has been found in both human epidermis (Knuever et al. 2012) and in amphibian skin, where TRH stimulates pigmentation (Jackson and Reichlin 1977; Vaudry et al. 1999), the next question was, whether TRH is an overall pigmentation stimulator in human skin, or whether this effect is specific to the HF.

First, using the Masson-Fontana histochemistry to visualize the epidermal melanin content in the TRH- or vehicle-treated human skin samples, qualitative and quantitative analysis revealed no significant upregulation of histochemically detectable melanin content in the organ cultured human epidermis after TRH stimulation (**Figure 3.11)**.

Analogous to the investigations in HFs and isolated cultured melanocytes described above, further assays (gp100 immunoactivity and tyrosinase activity) were performed with skin samples. In these assays, TRH failed neither significantly upregulated epidermal gp100 immunoreactivity, nor tyrosinase activity *in situ* (**Figure 3.12** and **Figure 3.13**).

Therefore, under physiologically relevant *ex vivo*-conditions, TRH selectively stimulates human HF, but not intraepidermal melanogenesis and melanocyte dendricity.

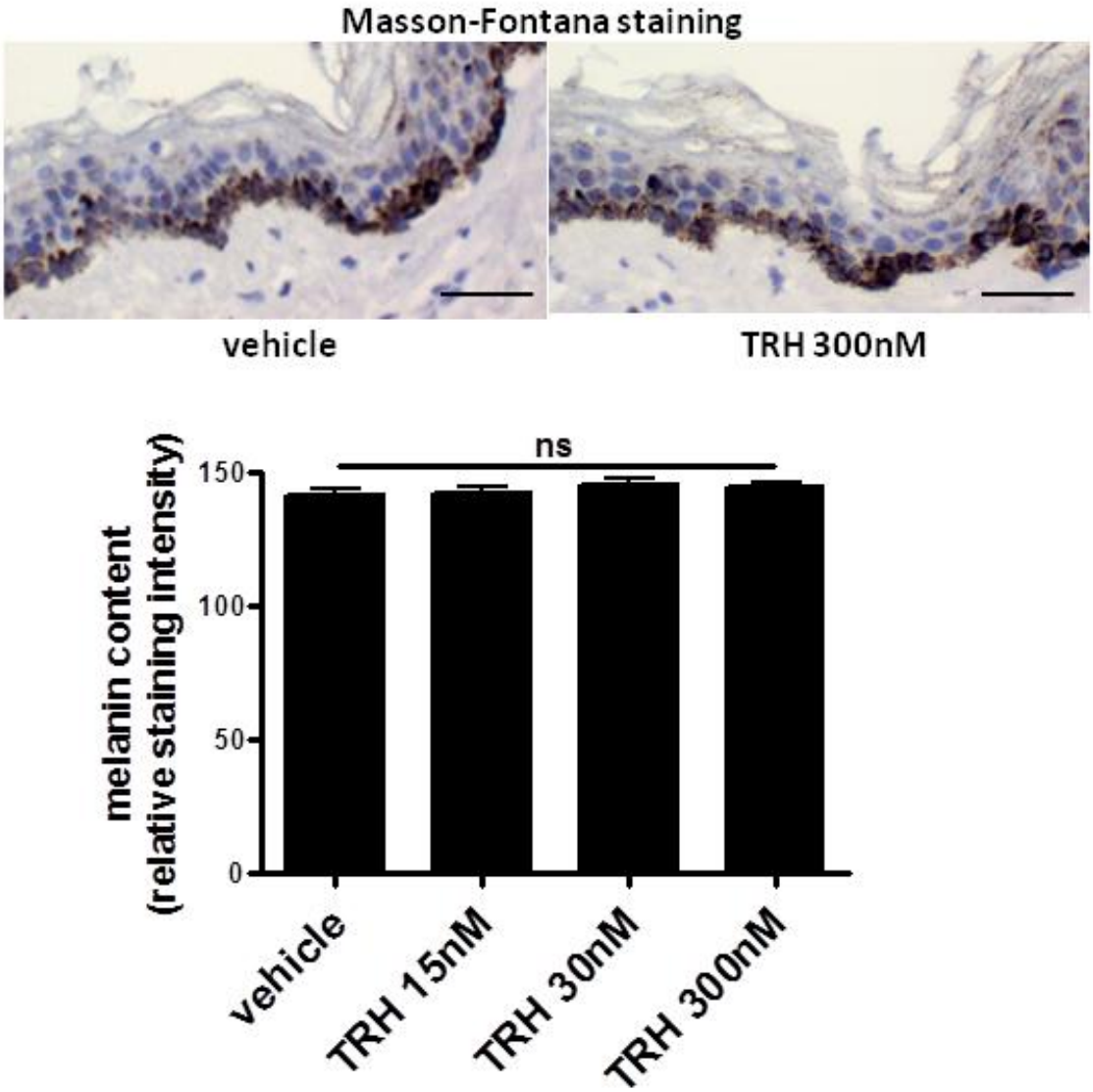

Figure 3. 11: Effect of TRH in melanin formation in human epidermal melanocytes.

Thyrotropin-releasing hormone (TRH) did not stimulate melanin formation in normal human epidermal melanocytes. Columns represent mean±SEM; representative results of one experiment (n= 22-28 skin fragments per TRH concentration per each experiment), NS not significant, scale bar = 50μm

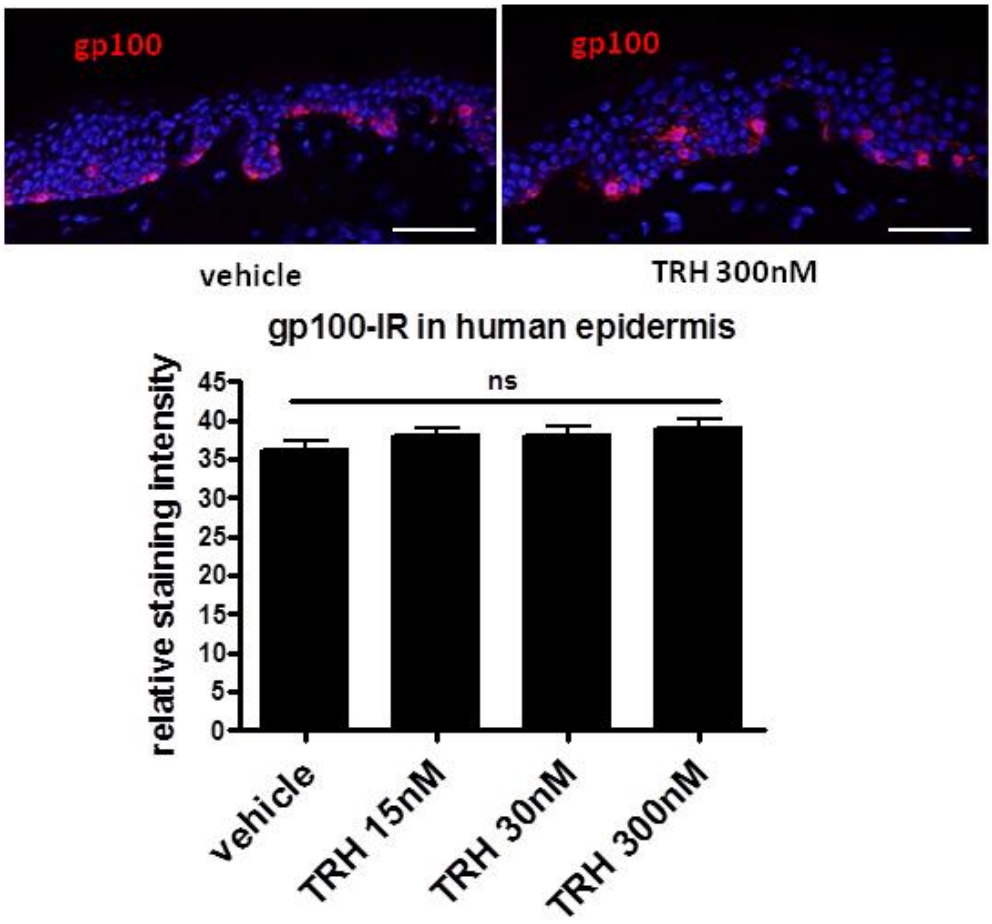

Figure 3. 12: Effect of TRH in gp100 expression in human epidermal melanocytes.

Gp 100 expression (red fluorescence staining) in epidermal melanocytes was not significant increased after TRH treatment. Columns represent mean±SEM; representative results of one experiment (n= 22-28 skin fragments per TRH concentration per each experiment), NS not significant, scale bar = 50µm

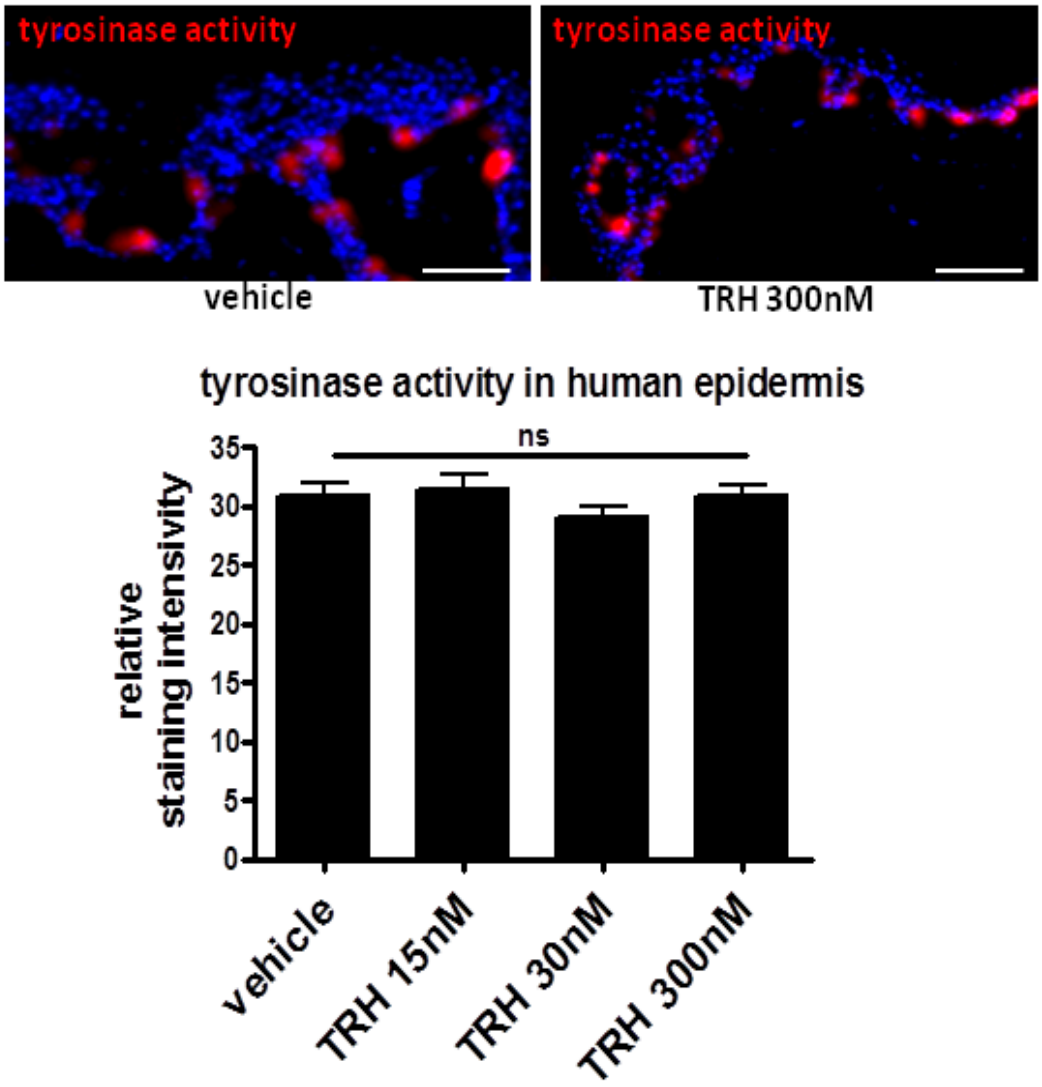

Figure 3. 13: Effect of TRH in tyrosinase activity in human epidermal melanocytes.

Tyrosinase activity (red fluorescence staining) of epidermal melanocytes remained unchanged after 6 days of TRH administration. Columns represent mean±SEM; representative results of one experiment (n= 22-28 skin fragments per TRH concentration per each experiment), NS not significant,
scale bar = 50μm

3.6 The hair follicle pigmentary unit does not seem to express TRH-receptor protein at a detectable level

A major challenge remains to identify the mechanism(s) of action by which TRH may exert its complex pigmentstimulating function on human HF melanocytes *in situ* and *in vitro*. TRH-R mRNA was detected in human HF extracts, although the expression of TRH-R protein *in situ* appeared largely limited to the inner root sheath of the HF (Gáspár et al. 2010). In contrast, isolated human HF melanocytes reportedly do not express TRH-R mRNA *in vitro* (Slominski et al. 2002). This made it difficult to understand how TRH exerts its HF pigmentary effects on the HF pigmentary unit (HFPU).

Therefore, the tyramide signal apflification (TSA) technique was used to define, at a maximal level of sensitivity achievable by immunofluorescence microscopy, the exact anatomical localization of TRH-R protein expression pattern in human scalp HFs (anagen VI), using optimal positive and negative immunoreactivity controls. It is known that the TRH-R is expressed highly in the human anterior pituitary (Sun et al. 2003; Mulla et al. 2009; Gehret and Hinkle 2012), which is used as positive control tissue for this assay.

As seen in **Figure 3.14**, the green fluorescence staining, which represents TRH-R immunoreactivity (TRH-R IR) is visible in thyrotropic cells in the human anterior pituitary (**Figure 3.14a**) and in the IRS of the normal human scalp HF (**Figure 3.14b**). In contrast, in the HFPU and in the ORS, where all HF melanocytes are located (Tobin 2011), specific TRH-R immunoreactivity was absent (**Figure 3.14b).** This independently confirmed earlier (lightmicroscopic) immunohistochemistry data (Gaspar et al. 2010) that the HFPU does not seem to express immuno-detectable levels of TRH-R protein, at least not above the limit of detection of the very sensitive TSA technique used here.

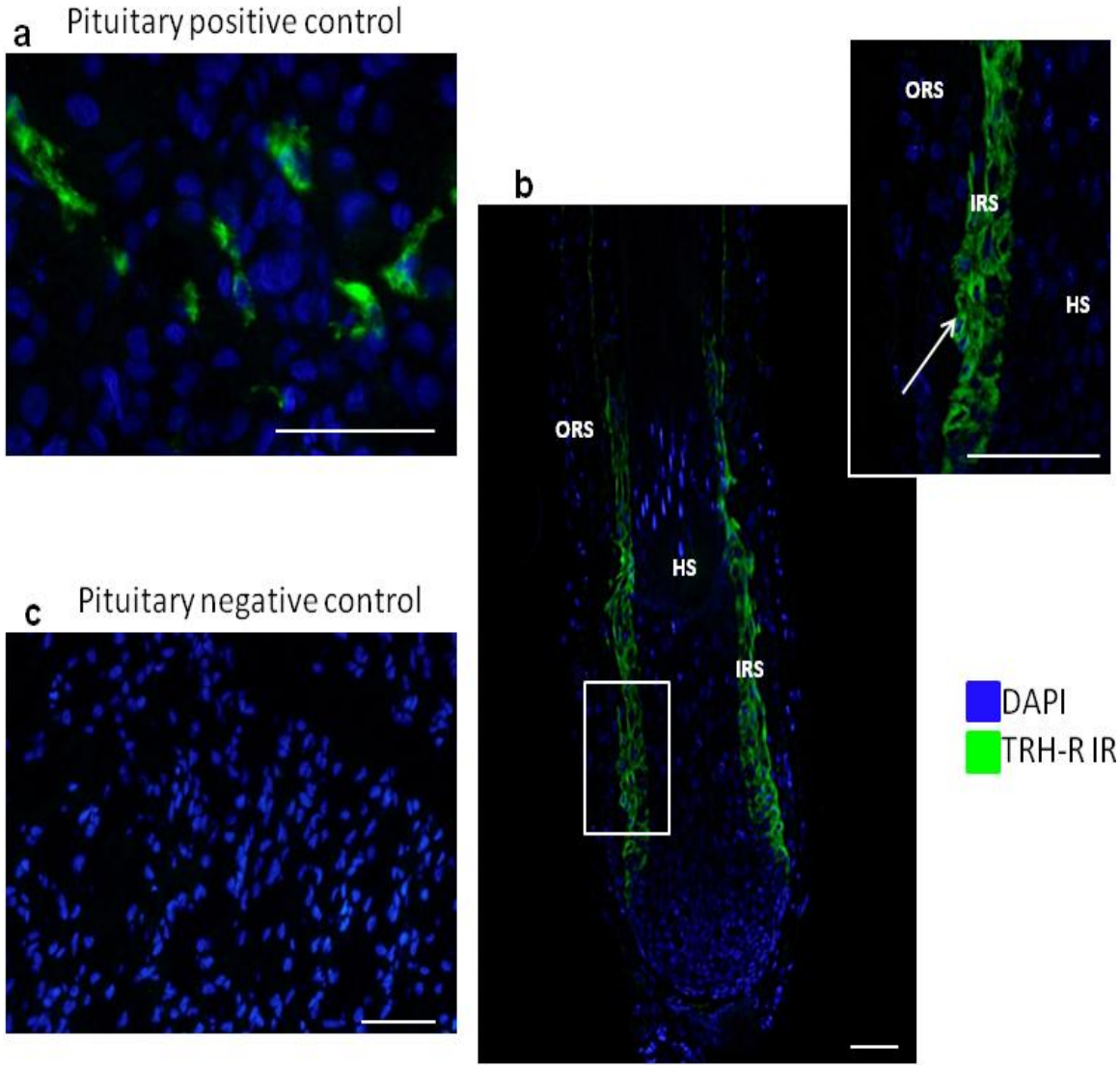

Figure 3. 14: TRH-R expression in human hair follicle.

Green fluorescence staining represents TRH-R immunoreactivity (TRH-R IR) in the inner root sheath (IRS) of the normal human scalp HF and in human pituitary, used as a positive control. Outer root sheath (ORS), hair shaft (HS), scale bar = 50µm.

3.7 Isolated, cultured human HF melanocytes do not express TRH-receptor protein on a detectable level

Slominski et al. have reported that isolated human HF melanocytes do not express TRH-R mRNA *in vitro* (Slominski et al. 2002). Using the TSA technique, it was therefore investigated wether isolated, cultured human HF melanocytes from the human ORS showed no evidence of specific TRH-R immunoreactivity (**Figure 3.15**).

These TRH-R protein expression results suggest that the intrafollicular pigmentary effects of TRH are unlikely to be mediated directly by stimulation of TRH-Rs expressed by melanocytes of the human HFPU.

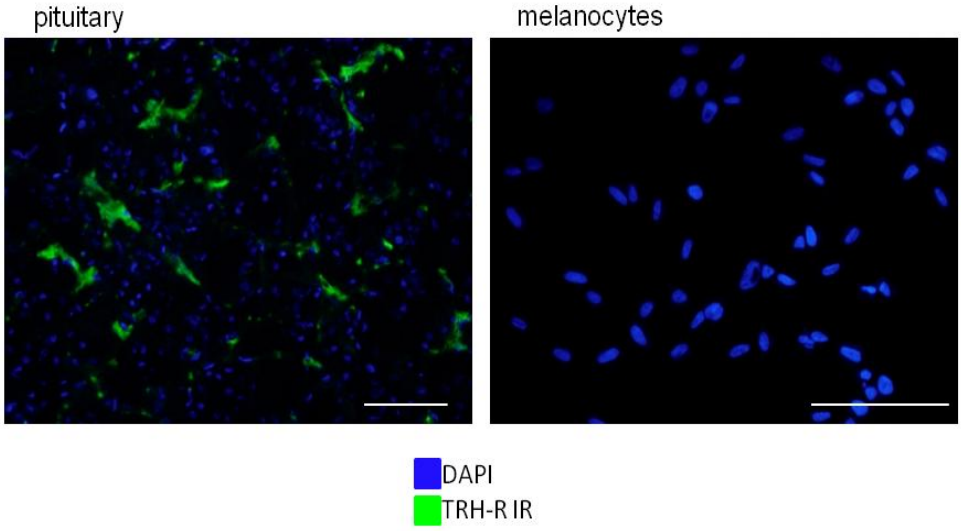

Figure 3. 15: TRH-R immunoreactivity (TRH-R IR) is not shown in cultured human HF melanocytes.

Using human pituitary as positive control, the fluorescence staining (green) of the TRH-R is only seen in human pituitary cells, but not in culterd human HF melanocytes. Scale bar = 50 µm

3.8 POMC-dependent signalling may mediate the pigmentary effects of TRH

The next question was, therefore, whether an indirect mode of action may explain the profound pigmentary effects of TRH. Therefore, this study tested by RT-PCR whether the intrafollicular POMC mRNA steady-state levels in organ-cultured human scalp HFs (Ito et al., 2005) were altered by TRH treatment. POMC is a polypeptide hormone precursor, and its enzymatically cleaved products (namely, ACTH, α-MSH, and ß-endorphin) are key neuroendocrine regulators of melanin production and melanocyte function, notably in human HFs (Tobin and Kauser 2005; Rousseau et al. 2007).

As seen in **Figure 3.16**, after 8 h of incubating organ-cultured HFs with 15 nM TRH, the intrafollicular POMC transcript levels were significantly increased. Interestingly, the tested higher concentration did not exert the same effect.

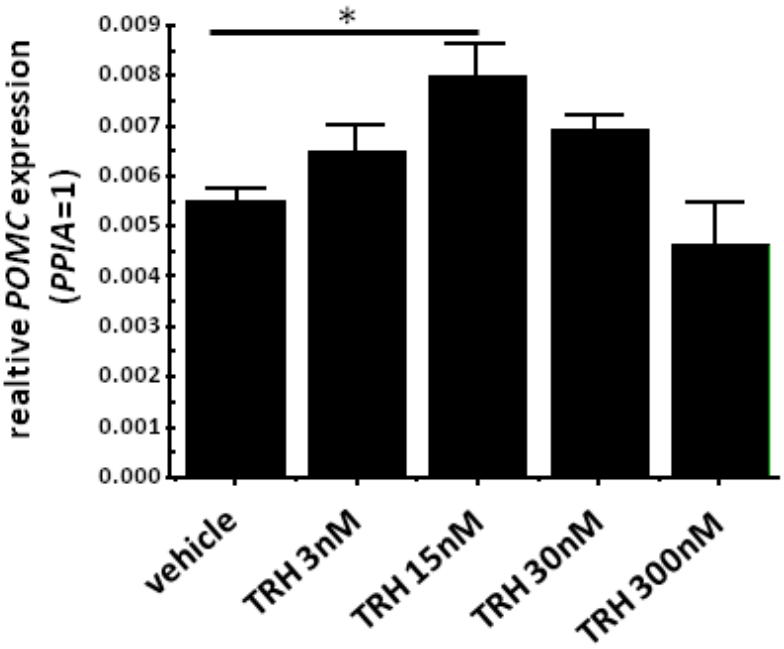

Figure 3. 16: TRH stimulates proopiomelanocortin (POMC) mRNA expression in human scalp HFs *in situ*.

TRH 15 nM significantly upregulated POMC mRNA expression. Columns represent mean±SEM of three independent experiments (n=15–18 HFs per TRH concentration per each experiment), *P<0.05 and **P<0.01 versus vehicle (TRH 0 nM). PPIA: Peptidylprolyl isomerase A (cyclophilin A).

ACTH is one of the cleaved products from the prohormone POMC, and it is known to stimulate human HF melanocytes and intrafollicular melanogenesis by binding to the melanocortin-1-receptor (MC-1R) (Kauser et al. 2005; Rousseau et al. 2007). In light of the above POMC transcription data, the next question was whether ACTH immunoreactivity was actually increased by TRH. To examine this, the intrafollicular immunoreactivity of ACTH was assessed. This protein level analysis showed by

quantitative immunohistomorphometry that TRH significantly upregulated the intrafollicular immunoreactivity for ACTH in the ORS *in situ*, as seen in **Figure 3.17**.

Therefore, it is conceivable that intrafollicularly generated TRH upregulates intrafollicular POMC expression and its processing to melanotropic, POMC-derived peptides, such as ACTH, and thereby stimulates human HF pigmentation indirectly.

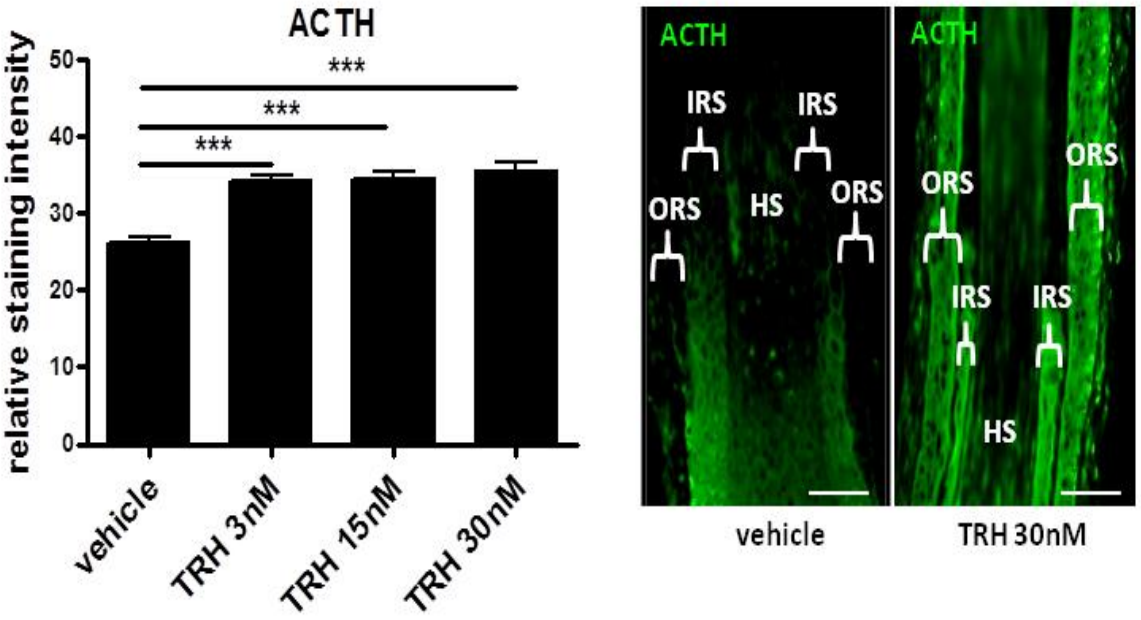

Figure 3. 17: TRH stimulates ACTH immunoreactivity in human scalp HFs *in situ*.
Increased ACTH immunoreactivity (green fluorescence staining) was detectable in the ORS of the TRH (3-30 nM) treated, organ cultured HF. Columns represent mean±SEM, ***P<0.001 versus vehicle (TRH 0 nM).). representative results of one experiment from three independent experiments (n=15–18 HFs per TRH concentration per each experiment). Outer root sheath (ORS); inner root sheath (IRS); hair shaft (HS); scale bar = 50µm.

3.9 TRH may stimulate hair follicle pigmentation via binding to the melanocortin-1-receptor

Both ACTH and α-MSH stimulate human HF melanocytes and intrafollicular melanogenesis by binding to the melanocortin-1 receptor (MC-1R), which is prominently expressed in the human anagen hair bulb (Ito et al. 2005; Slominski et al., 2005; Slominski et al. 2005; Kauser et al. 2006; Tobin 2011). Schiöth et al. found in cultured COS-1 cells that TRH can also use this receptor as an alternative mode of signaling (Schiöth et al. 1999). Moreover, the isolated human HF melanocytes neither expressed TRH-R mRNA *in vitro* (Slominski et al. 2002) nor TRH-R immunoreactivity (**Figure 3.15 above**), yet are well-known to express MC-1R mRNA and protein and to prominently respond to stimulation with ACTH and α-MSH (Abdel-Malek et al., 1999; Kauser et al. 2005; Rousseau et al. 2007). Therefore, it is a very reasonable working hypothessis whether TRH stimulates HF pigmentation via binding to the MC-1R.

To investigate this, the agouti-signaling protein (ASP), a recognized MC-1R antagonist (Lu et al. 1994; Ollmann et al. 1998) was used to inhibit the pigmentary stimulation effect of TRH. The HFs were treated with TRH (3 or 15 nM) in the presence of an excess amount of agouti-signaling protein (ASP; 100 nM).
As seen in **Figure 3.18**, coadministration of TRH with ASP reduced the melanin content, the tyrosinase enzyme activity, and the gp 100 expression *in situ* to the level of vehicle-treated control HFs or even below this level.
This preliminary investigation suggests, that TRH may stimulate HF pigmentation via binding to the melanocortin-1-receptor.

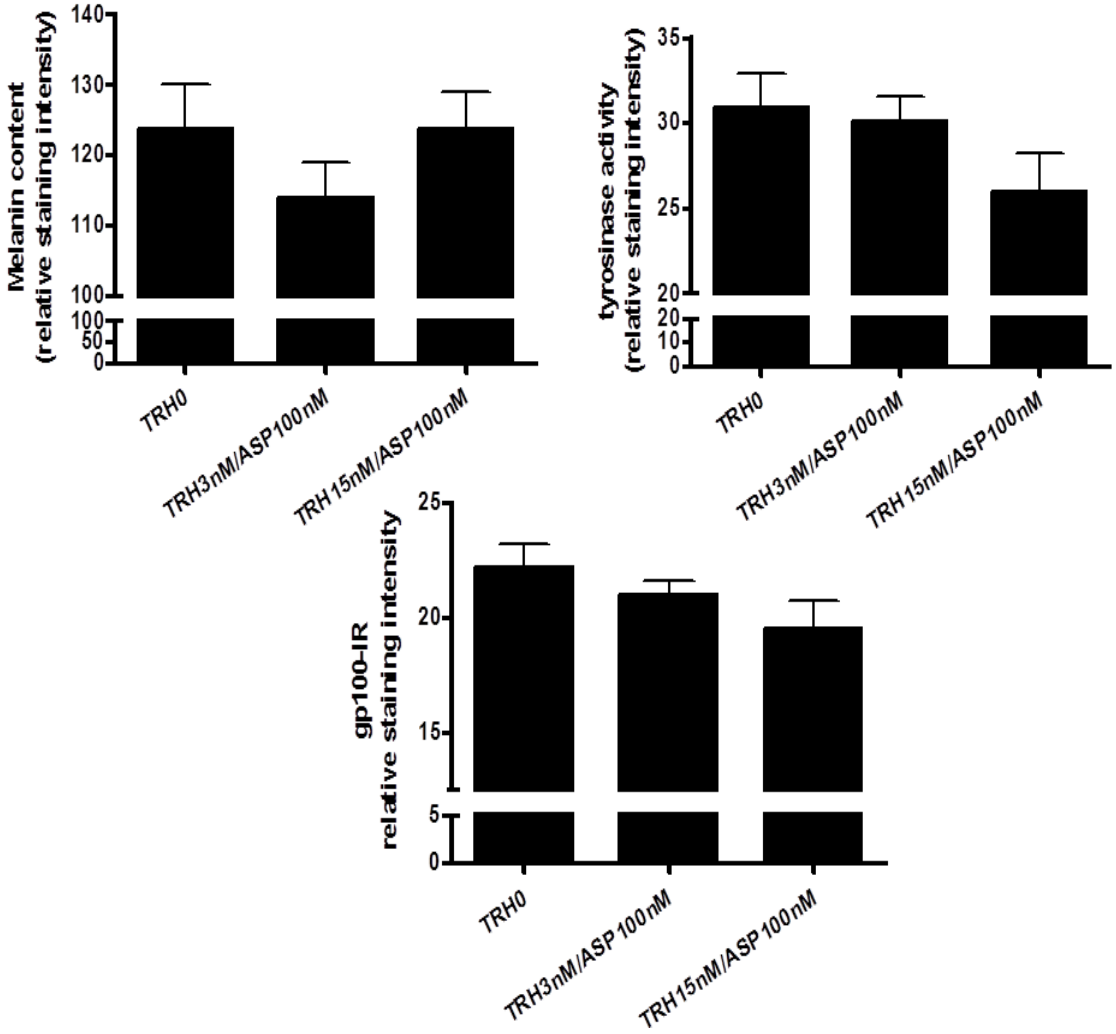

Figure 3. 18: The classical MC1-R competitive antagonist, agouti signalling protein (ASP), partially inhibits the stimulatory effects of TRH on human HFs.

Coadministration of TRH with ASP reduced the melanin content, the tyrosinase enzyme activity and the gp 100 expression *in situ* to the level of vehicle-treated control HFs or even below this level. Columns represent mean±SEM, representative results of one experiment.

3.10 TRH stimulates intrafollicular MITF protein expression

Finally, the impact of TRH on microphthalmia-associated transcription factor (MITF), the ‘‘master regulator of pigmentation’’ (Nishimura et al. 2005; Vachtenheim and Borovansky 2010; Dessinioti et al. 2011) was assessed asking whether TRH upregulated the intrafollicular expression of MITF protein.

As seen in the photos in **Figure 3.19**, the immunoreactivity of MITF in hair bulbs was significantly increased in TRH-treated HFs compared to vehicle controls. In addition, the percentage of MITF-positive cells increased after TRH administration, as seen in in **Figure 3.19**. That TRH upregulate intrafollicular MITF protein expression shows that, whatever its exact mechanism of action may turn out to be, TRH appears to recruit this key pigmentation biology transcription factor into the task.

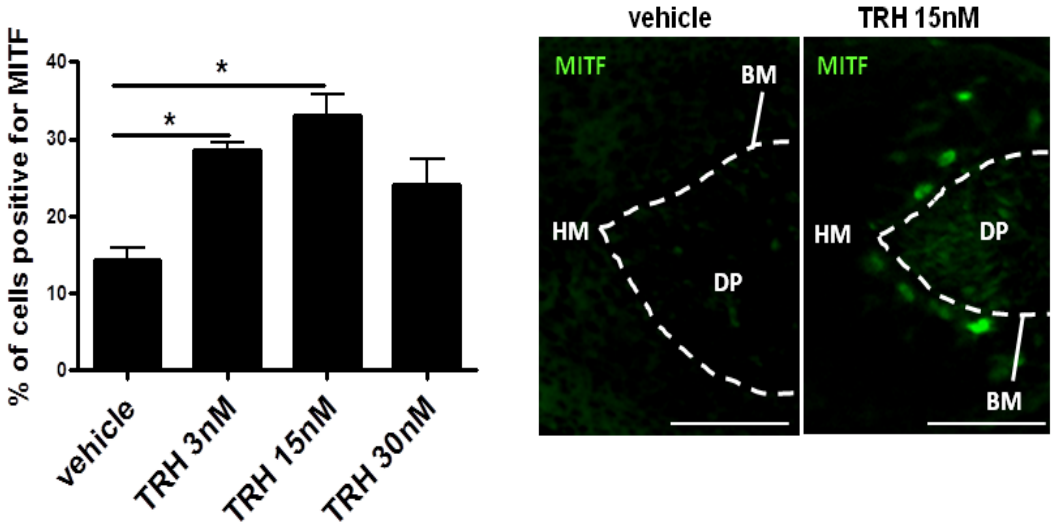

Figure 3. 19: TRH stimulates microphthalmia-associated transcription factor (MITF) immunoreactivity in human scalp HFs *in situ*.

The percentage of MITF-positive cells (green fluorescence staining) increased significantly with TRH administration. Columns represent mean±SEM of three independent experiments (n=15–18 HFs per TRH concentration per each experiment), *P<0.05 and **P<0.01 versus vehicle (TRH 0 nM).). Basal membrane (BM), dermal papilla (DP), hair matrix (HM), scale bar = 50μm.

4 DISCUSSION

It is known that TRH is an important pigmentation stimulator in frogs that helps them to regulate their camouflage (Jackson and Reichlin 1977; Vaudry et al. 1999; Vazquez-Martinez et al. 2003; Jenks et al. 2007; Galas et al. 2009; Jenks et al. 2010). Additionally, TRH has been found to exist in human skin and in human HFs. (Slominski et al. 2002; Gáspár et al. 2010; Knuever et al. 2012). In light of this we decided to investigate if TRH plays a specific role in human skin and hair pigmentation by using highly sensitive immunohistochemical and molecular biological methods.

By employing qualitative and quantitative Masson–Fontana histochemistry, this study presents the first evidence, that TRH exerts direct effects on normal mammalian melanocytes *in situ* and that TRH stimulates significantly melanin production in normal adult human HFs (**Figure 3.1**). These findings not only demonstrate new functions of TRH, which had previously been largely ignored in human skin biology, but also add a potent, selective pigmentation-stimulatory function of TRH to the full range of *non-classical* TRH functions in peripheral human tissues e.g. as a regulator of immune system (Kamath et al. 2009; Kamath 2013), metabolism (Lechan and Fekete 2006; Gertig et al. 2012; Sarvari et al. 2012; Cyr et al. 2013; Gotoh et al. 2013), the autonomic nervous system (Khomane et al. 2011; Thirunarayanan et al. 2012; Volkoff 2012; Zhang and van den Pol 2012), the pancreas (Luo and Jackson 2007), and the heart function (Garcia and Pirola 2005). All of these actions extend well beyond the well-recognized classical thyrotopic function of TRH within the hypothalamic-pituitary-thyroid axis (Joseph-Bravo et al. 2016; Ortiga-Carvalho et al. 2016).

We extended and supported our initial findings by focusing on the rate-limiting enzyme of melanogenesis, tyrosinase (Slominski et al. 2005). Specifically, the *de novo* synthesis of melanin was examined by using *in situ* enzyme histochemistry for tyrosinase activity, and quantitative RT-PCR was used to evaluate tyrosinase gene transcription *in situ*. **Figure 3.2** and **Figure 3.3** demonstrate that TRH can impact the

enzymatic control of melanogenesis by stimulating both tyrosinase gene transcription and enzyme activity.

Interestingly, this finding in a complete human (mini-)organ (Paus et al. 1999, Schneider et al. 2009) stands in apparent contrast to amphibian skin melanophores, which are thought to require pituitary-derived signals for stimulation by TRH, i.e. to respond only indirectly (Vaudry et al. 1999; Vazquez-Martinez et al. 2003; Jenks et al. 2007; Jenks et al. 2010). Instead, we found that not only human HF melanocytes *in situ* ,but also isolated human HFs from the ORS are sensitive to TRH stimulation *in vitro*, even in the absence of pituitary gland-derived melanocortins such as α-MSH. Given that the human hair bulb epithelium expresses TRH on the gene and protein level *in situ* (Gáspár et al. 2010), it is conceivable that also keratinocyte-derived TRH facilitates paracrine effects on melanocytes within the human skin and HF. However, since human HF epithelium is a rich source of melanocortins (Ito et al. 2005, Paus et al. 2014), at least in HF organ culture, theoretically, TRH could also have exerted its pigmentary effects indirectly, i.e. by stimulating the intrafollicular expression of POMC and the enzymatic generation of prigmentation-promoting melanortins from locally produced POMC.

To induce hair shaft pigmentation, mature melanosomes are transferred through melanocyte dendrites within the HFPU to neighboring keratinocytes of the precortical hair matrix (Slominski et al. 2005; Kauser et al. 2006; Singh et al. 2008; Tobin 2011). Therefore, the TRH-induced stimulation of gp100 production (**Figure 3.4**), which plays an essential role in melanosome biogenesis (Singh et al. 2008), enhancement of HF melanocyte dendricity (**Figure 3.5**), and slight increase in total number of hair bulb melanocytes (**Figure 3.6**), together all suggest a considerably more complex regulatory role for TRH in human HF melanocyte biology *in situ*, i.e. one that goes beyond mere stimulation of enzymatic melanogenesis (Slominski et al. 2005). Thus, the gp100 data not only independently confirms the above histochemical and enzymatic pigmentation data, but also show that TRH actually induces an overall stimulates melanin biosynthesis and melanosome transfer to recipient keratinocytes within the precortical hair matrix of normal human scalp HFs.

Whether TRH really is a robust promoter of proliferation of HF melanocyte within the HFPU *ex vivo*, cannot yet be deduced with any certainty from the limited data currently available on this (**Figure 3.6**), which also only showed a very mild effect, and require additional experimental confirmation. If this effect is confirmed, however, this would be both exciting and clinically important in that such a phenomenon would contradict the conventional wisdom that melanocytes of the HFPU represent terminally differentiated cells (Slominski et al. 2005, Tobin 2011) and thus presumably incapable of proliferating. Given that the HFPU even of greying/white HFs in aging human scalp skin still can harbor a few, often even melanogenically active melanocytes (Tobin and Paus 2001, Tobin 2015), this would open up interesting new perspectives for the therapeutic use of TRH in furture anti-greying strategies in order to expand the HFPU melanocyte population by stimulating their intrafollicular proliferation (see also below).

The effects of TRH on human HF pigmentation *in situ*, i.e. where the normal epithelial and mesenchymal signaling environment of HF melanocytes is present, raised the possibility that TRH does not exert any direct effect on melanocytes, but operates by inducing the secretion of pigmentation-stimulatory factors of other resident cells, e.g.keratinocytes. Therefore, this study next investigated the impact of TRH on primary cultured human HF melanocytes isolated from the ORS of normal adult human scalp HFs, which operate in the absence of crucial epithelial and mesenchymal influences (Kauser et al. 2005). Similar to the *in situ* results, TRH also significantly stimulated dendricity (**Figure 3.7**), gp100 expression (**Figure 3.8**), tyrosinase activity (**Figure 3.9**) of melanocytes from human HF. Furthermore, TRH even stimulated the proliferation of isolated human HF melanocytes (**Figure 3.10)** *in vitro*. These observations underscore the hypothesis that TRH can directly stimulate human HF melanocytes and consequently human HF pigmentation and without requiring the presence of other resident cell populations such as hair matrix keratinocytes and/or dermal papilla fibroblasts.

However, an important limiting feature of this *in vitro* experiment is that the HF melanocyte cultures, despite use of the best currently available cell isolation and culture techniques (Tobin et al. 1995, Kauser et al. 2011), are not completely free from contaminating keratinocytes and fibroblasts. Yet, the number of these cells was minimal, and their cellular functions are inhibited by the cell culture procedure employed, namely by the use of a culture medium that strongly favors the survival and function of melanocytes and discriminates against that of keratinocytes and fibroblasts (Tobin et al. 1995). While it is therefore unlikely that our *in vitro* results were influenced by the presence of these cells, this possibility cannot be completely ruled out.

Interestingly, preliminary observations with adult frog skin organ culture in our laboratory, suggested that high doses of TRH can stimulate melanosome dispersion in intracutaneous *Xenopus tropicalis* melanophores *in situ,* in the absence of a pituitary gland (D Pattwell and R Paus, unpublished observation). These frog skin organ-culture observations also suggested that contrary to the conventional knowledge (Jackson and Reichlin 1977; Vaudry et al. 1999), even in amphibian skin TRH can exert direct pituitary gland-independent pigmentation-stimulatory effects. Although these intracutaneous pigmentation stimulatory effects of TRH appear to have been lost in the human epidermal melanocytes *in situ*, we demonstrate here that they have been preserved in the human HFPU. Moreover, this underscores the validity of the concept that epidermal and HF melanocytes represent phenotypically and functionally rather distinct sub-populationsand are under different controls (Slominski et al. 2004; Plonka et al. 2009; Van Raamsdonk et al., 2009; Aoki et al. 2011; Tobin 2011).

Since normal human skin also expresses TRH and the TRH-R (Slominski et al. 2002; Knuever et al. 2012) and since human skin pigmentation is generated via the melanocytes on the basal membrane of the epidermis, we hypothesized that TRH may stimulate human epidermal melanogenesis and tested this in organ-cultured human full-thickness skin biopsies. Unexpectedly, we found that TRH did not significantly upregulate the melanin content (**Figure 3.11**), the gp100 immunoreactivity (**Figure 3.12**), or the tyrosinase activity (**Figure 3.13**) of

intrepidermal melanocytes *in situ*. This demonstrates that TRH selectively stimulates human HF melanogenesis, but not intraepidermal melanogenesis.

After summarizing that TRH selectively stimulates human HF pigmentation, our next major challenge was to find out by which mechanism(s) TRH exerts its complex pigment stimulating function. The first and most obvious idea was that TRH can bind to the TRH-R and thus activate the intracellular signaling pathways recognized for this receptor (see **Fig. 1.13, Fig. 1.14** and (Joseph-Bravo et al. 2016). However, this hypothesis was made unlikely by the immunofluorescence microscopy results reported here, which independently confirmed the TRH-R negativity of both human HFPU melanocytes *in situ* (**Figure 3.14**) and of isolated, cultured human HF melanocytes from the ORS (**Figure 3.15**). This confirms previous immunohistochemical data on intrafollicular TRH-R protein expression from the Paus Lab (Gaspar et al. 2010). If melanogenically active human HF melanocytes express any functional TRH-R protein at all *in situ*, then this was below our experimental detection limit.

Astonishingly, the TRH-R protein was only detectable within the IRS of the HF (**Figure 3.14**), which is thought to play no role HF pigmentation (Gáspár et al. 2010; Tobin 2011). Slominski and colleagues have also reported, that isolated human HF melanocytes do not express TRH-R mRNA *in vitro* (Slominski et al. 2002). All these results suggest that the intrafollicular pigmentary effects of TRH are unlikely to be mediated by the direct stimulation of TRH-Rs expressed by melanocytes within the human HFPU. Instead, it is conceivable that TRH may bind to the TRH-R on IRS-located keratinocytes, which then secrete pigmentation-promoting factors that indirectly impact on HFPU melanocytes within the hair matrix.

Unfortunately, this intriguing hypothesis was impossible to test, as it is currently impossible to isolate and culture IRS-specific HF keratinocytes, which are the first cells in the proximal HF epithelium that terminally differentiate (Schneider et al. 2009). Moreover, any demonstration that IRS keratinocytes secrete factors that exert long-distance effects on the hair matrix would be very striking and a landmark

discovery in hair research, since such secretory effects of the IRS on other compartments of the HF epithelium have never been reported.

However, our HF organ culture data clearly demonstrated that hypothalamus and/or pituitary gland-derived signals are dispensable for the observed pigmentary effects of TRH. Human scalp HFs express an astounding variety of ‘‘hypothalamic’’ and ‘‘pituitary’’ neurohormones (Slominski et al. 2000; Slominski et al. 2002; Kauser et al., 2004; Ito et al. 2005; Kauser et al. 2005; Kauser et al. 2006; Paus 2010, 2011) and TRH upregulated intrafollicular POMC expression and α-MSH and/or ACTH production. In fact, the epithelium of human scalp HFs has an established fully functional peripheral equivalent system of the hypothalamic–pituitary–adrenal axis (Ito et al. 2005), and human HFs are now appreciated to prominently transcribe and translate POMC, from which ACTH and α-MSH are processed (Slominski et al. 2000; Ito et al. 2005; Kauser et al. 2005; Kauser et al. 2006).

The data from the current thesis project render it well-conceivable that the intrafollicularly generated TRH (Gáspár et al. 2010) upregulates intrafollicular POMC expression and its processing to melanotropic peptides, which then stimulates human HF pigmentation indirectly. This consideration was confirmed by using POMC RT-PCR for the amplification of isolated HF-specific mRNA. The detected POMC mRNA steady-state levels were significantly increased within isolated HFs that were previously treated with 15 nM TRH (**Figure 3.16**). Moreover, on the protein level, TRH also significantly increased the intrafollicular immunoreactivity for ACTH *in situ* (**Figure 3.17**), which binds to MC-1R and MC-2R receptors in human HFs (Ito et al. 2005; Kauser et al. 2005) and by that stimulates the intrafollicular melanogenesis.

This mechanistic scenario is supported by clinical evidence: Intravenous administration of TRH to pregnant women, or to a subgroup of patients with Cushing’s disease, reportedly increased serum ACTH levels (Pieters et al., 1982; Pieters et al., 1982). TRH may exploit the intrafollicular production of classical ‘‘pituitary’’ melanotropic hormones to stimulate HF pigmentation indirectly.

However, given the apparently exclusive TRH-R expression in the IRS, one would again have to postulate that terminally differentiated IRS keratinocytes secrete

melanocortins upon TRH stimulation, which then act on the distant HFPU located within the hair matrix of anagen HFs.

As indicated above, the demonstration that the IRS engages in any such paracrine secretory activities would run contrary to current concepts on IRS function (with moulding and anchorage of the hair shaft, but not any secretory activities, currently being thought to represent the main physiological functions of the IRS (Paus and Cotsarelis 1999; Stenn and Paus 2001; Schneider et al. 2009) and would thus be a conceptually very important new development in hair research. However, it has recently been reconsidered whether the ORS also engages in such secretory activities, not only by secreting major hair growth-regulatory growth factors (e.g., TGFß2 and IGF-1), but also in the context of HF energy metabolism (Vidali et al. 2014). Therefore, the hypothesis that IRS keratinocytes may also engage in important (here: pigmentation-related) secretory activities deserves systematic exploration.

Moreover, TRH reportedly can to stimulate non-classical intrafollicular MC1-R-mediated signaling in COS-1 cells *in vitro* (Schiöth et al. 1999) and MC1-R is prominently expressed in the human anagen hair bulb as well as in cultured HF melanocytes (Abdel-Malek et al. 1999; Kadekaro et al. 2003; Ito et al. 2005; Kauser et al. 2005; Slominski et al. 2005; Slominski et al. 2005; Kauser et al. 2006; Kadekaro and Abdel-Malek 2007; Tobin 2011). Therefore, the stimulatory effects of TRH on human HF pigmentation could indeed be TRH-R-independent and could instead (or in addition?) be mediated by binding to the MC-1R.

If TRH actually exerts its complex stimulatory effects on human HF pigmentation via binding to the MC-1R, then competitive inhibition of this receptor would be expected to decrease the pigmentation-stimulating effects of TRH. This hypothesis was tested by administering TRH in the presence of an excess amount of agouti-signaling protein (ASP), the best-established endogenous MC-1R antagonist (Lu et al. 1994; Ollmann et al. 1998; Chai et al., 2003; Patel et al., 2010; Oguro-Okano et al., 2011). The melanin content, the tyrosinase enzyme activity, and the gp100 immunoreactivity *in situ* of the treated HFs with TRH and ASP reduced to the level of the vehicle-

treated HFs or even below this level (**Figure 3.18**). These results underscore that the stimulatory effects of TRH on human HF pigmentation could indeed be mediated, at least in part, by binding to the MC-1R.

In addition, this would explain the – previously unexplained! - ability of HF melanocytes in POMC-knockout mice to still produce eumelanin, despite the absence of POMC products (Slominski et al. 2005). Moreover, the preliminary ASP data reported here in human HF organ culture also question the conventional wisdom that ASP exerts important pigmentary effects only in rodent, but not in human skin (Lu et al. 1994; Le Pape et al., 2008; Ito and Wakamatsu 2011).

That MC-1R is indeed the key receptor in transducing the pigmentary effects of TRH in human HFs *ex vivo* is, therefore, supported by several lines of evidence and argumentation that can be summarized as follows:

(1) The HFPU itself does not express immunohistochemically detectable TRH-Rs;
(2) cultured HF melanocytes do not express detectable TRH-R protein or transcripts, but rather functional MC-1R (Abdel-Malek et al. 1999; Slominski et al. 2002; Kadekaro et al. 2003; Kauser et al. 2005), and yet do respond to TRH stimulation;
(3) TRH can non-classically signal via the human MC1-R (Pieters et al. 1982; Schiöth et al. 1999);
(4) the stimulatory effects of TRH on human hair pigmentation can be partially antagonized by the selective, endogenous, competitive MC-1R antagonist, ASP; and
(5) as mentioned before, the unexpected production of eumelanin in POMC-knockout mice on a C57BL/6 background indicates the existence of an alternative ligand for MC1-R (Slominski et al. 2002) – with intrafollicularly generated TRH being a prime candidate for this hitherto elusive alternative ligand.

However, additional potential mechanisms of action should be considered and explored in future studies. For example, TRH could also stimulate intrafollicular ß-endorphin processing, as this POMC product can also stimulate human HF pigmentation and since human ORS and HFPU melanocytes do express function ß-endorphin receptors (µ-opiod receptors) (Kauser et al. 2004). Even unprocessed POMC itself can stimulate melanin production by human melanocytes *in vitro*

(Rousseau et al. 2007). Since this thesis project has demonstrated, that TRH upregulates intrafollicular POMC transcription, so this additional mechanism of action deserves careful consideration. In theory, even receptor-independent signaling events might contribute to the pigmentary effects of this very small tripeptide neurohormone *in situ* and *in vitro* (to examine and rule out such receptor-independent peptide effects would, of course, be exceptionally challenging, since small peptides can exhibit numerous interactions with other molecules. Yet, the arguments summarized above support of the concept of a predominantly MC1-R-dependent mechanism by which TRH exers its stimulatory effects on the human HFPU.

Microphthalmia-associated transcription factor (MITF) controls more than 25 genes in pigment cells that affect not only melanogenesis, but also melanocyte development, proliferation, survival, and even intrafollicular melanocyte stem cell functions (Nishimura et al. 2005). MITF has consequently been hailed as the master regulator of pigmentation (Vachtenheim and Borovansky 2010), and MITF is activated by MC-1R stimulation (Rouzaud et al. 2006; Chou et al., 2010). Thus, it was an important the final result in the current search for the mechanism(s) by which TRH influences human HF pigmentation, that TRH upregulated the intrafollicular expression of MITF protein (**Figure 3.19**).

Given that many investigators consider the MITF-dependent decline in the number of human HF melanocyte stem cells a chief cause of hair graying (Nishimura et al. 2005; Tobin 2011), while HF melanocyte stem cell survival and activity is MITF-dependent (Nishimura et al. 2005; (Kim et al., 2015; Koludrovic et al., 2015; Mort et al. 2015) it is particularly intriguing to note that TRH can upregulate intrafollicular MITF expression. The proposed key role of MITF in the graying of human hair (Nishimura et al. 2005) and the stimulatory effect of TRH that was observed here on the intrafollicular expression of this master regulator of pigmentation and of HF melanocyte stem cells (Nishimura et al. 2005; Vachtenheim and Borovansky 2010; Tobin 2011), encourage one to explore TRH and/or TRH-mimetic peptides as antigraying agents. The fact that TRH is already regularly used in clinical endocrinology (TRH stimulation test in thyroid medicine should greatly facilitate the

clinical testing of this tripeptide as a new candidate agent for halting or reversing human hair graying.

As TRH is an unusually stable tripeptide, it is conceivable that high intrafollicular TRH doses could clinically be achieved after topical application, e.g., by HF-targeting liposomes (Ciotti and Weiner 2002; Chourasia and Jain 2009) or nanoparticles (Vogt and Blume-Peytavi 2014). This could help to circumvent an undesired rise in systemic TRH levels that may induce excessive thyroid hormone secretion and thus a hyperthyroid state with serious adverse effects.

For this purpose, a randomized, double-blind placebo-controlled study to assess the efficancy of a topical treatment by TRH administered in a HF-targeting vehicle that enriches TRH in the HF and, ideally, retains it here, on the reversal or arrest of graying in healthy volunteers is need, e.g. along the lines of previously published study protocols (Blume-Peytavi et al., 2012).

The current study points to the value of systematically investigating evolutionarily ancient controls of pigmentation that may still be conserved in human skin. In this context, amphibian skin neuroendocrinology (Jackson and Reichlin 1977; Bolaffi and Jackson 1979; Vaudry et al. 1999; Vazquez-Martinez et al. 2003; Jenks et al. 2007; Slominski 2007; Slominski et al., 2008; Galas et al. 2009; Jenks et al. 2010) holds valuable lessons for the mammalian system and may also provide important insights for the management of human pigmentary disorders (Haslam et al., 2013; Haslam et al. 2014). Furthermore, this MD thesis project underscores that human HF organ culture and the highly hormone-sensitive HF melanocyte provide excellent discovery tools for elucidating ancestral, evolutionarily conserved, and clinically relevant non-classical neurohormone functions in human biology (Paus et al. 2014).

Our study also underscores the value of dissecting the physiological controls of human melanocyte biology in organ culture, rather than in non-physiological cell culture systems where melanocytes operate in the absence of crucial epithelial and mesenchymal cues. Human HF organ culture facilitates the study of human melanocyte functions within their natural tissue habitat and under full preservation of

normal keratinocyte–fibroblast–melanocyte interactions (Slominski et al. 2005; Plonka et al. 2009), and so can provide an instructive, accessible, and clinically useful test system for characterizing the neuroendocrine controls of melanogenesis and human melanocyte biology *in situ* (Ito et al. 2005; Kauser et al. 2005; Kauser et al. 2006; Schneider et al. 2009; Paus 2011).

In summary, the current study presents the first evidence that TRH (1) exerts any effects on normal mammalian melanocytes *in situ* and *in vitro*; (2) stimulates melanin production in the appendages of normal adult human skin; (3) selectively stimulates human HF, but not human epidermal melanocytes *in situ*; and (4) that these pigmentation-stimulating effects are independent of the pituitary gland.
Moreover, this MD theis project reveals a novel and apparently HF specific and evolutionarily highly conserved neuroendocrine control system of human pigmentation that may date back at least to the role that these controls already played in amphibian skin biology. This newly identified, physiologically relevant, and melanocyte sub-population-specific control of human pigmentation deserves full exploration not only in melanocyte biology, but also as a potential target to prevent or reverse hair graying (Tobin and Paus 2001; Tobin 2011; Paus et al. 2014).

"Recent developments in human hair follicle pigmentation research since 2011"

When the results of the experiments described in the current dissertation were published (Gaspar et al. 2011), at the time, they represented the first identification of yet another important and novel neuroendocrine regulator of human HF pigmentation since the discovery of beta-endorphin (Kauser et al. 2004; Paus 2011). Since then, five years have passed, but to the best of my knowledge, no other new neuroendocrine regulator of melanogenesis and/or melanocyte function in human skin, namely in human hair follicles, has been convincingly documented in a peer-reviewed original research paper. Therefore, the current data remain at the forefront of pigmentation research despite the fact that they were already generated and published several years ago.

The laboratory in which the experiments had been performed has gone on to explore vasoactive intestinal peptide (VIP) as yet another important, previously unknown neuroendocrine regulator of human hair follicle melanocyte function *in situ* and *ex vivo* and has already presented preliminary evidence in support of this function at international meetings (Bertolini et al., 2016). In addition, the release of VIP from perifollicular nerve endings in human skin may exert local immunoinhibitory functions and serve as yet another neuroendocrinological "guardian of HF immune privilege" (Paus et al., 2005; Gilhar et al. 2012), whose signalling is defective in patients with alopecia areata (Bertolini et al., 2016). Therefore, besides TRH and possibly VIP, there may well be other neurohormones, neuropeptides, and neurotransmitters that also regulate the human hair follicle pigmentary system, but had as yet escaped the attention of professional pigment biologists. This underscores the validity of the claim that human scalp HF and their organ culture provide an excellent discovery tool for the neuroendocrinology of pigmentation (Paus et al. 2014).

In this context, it deserves to be mentioned that completely novel, additional levels of regulation of the human HF pigmentary system have recently been identified. These add to the complexity of how melanocyte functions are regulated in situ by the immediate epithelial environment of melanocytes. For example, it has been shown, again in human scalp HFs *ex vivo*, that the adhesion molecule P-cadherin, which is almost selectively expressed within that part of the HF matrix which contains the HFPU, is another potent, previously unknown regulator of human melanocyte function and melanin production, both in human HFs and in human epidermis *ex vivo* (Samuelov et al. 2013; Samuelov et al. 2015). Therefore, it deserves to be studied whether neuroendocrine regulators of HF pigmentation like ACTH, a-MSH, beta-endorphin and TRH (Paus 2011; Paus et al. 2014) may exert their pigmention-stimulatory effects at least in part also via modulating P-cadherin surface expression and/or the activity of P-cadherin-dependent signaling.

Interestingly, preliminary observations with adult frog skin organ culture in the Paus Lab/Manchester generated after 2011 have suggested that high doses of TRH can stimulate melanosome dispersion in intracutaneous *Xenopus tropicalis* melanophores

in situ, in the absence of a pituitary gland (D Pattwell and R Paus, unpublished observation). These preliminary observations made in organ-cultured, adult frog skin (Meier et al. 2013) raise the possibility that, contrary to conventional wisdom (Jackson and Reichlin 1977; Vaudry et al. 1999), TRH can exert direct, pituitary gland-independent pigmentation-stimulatory effects even in amphibian skin. Although these intracutaneous pigmentation stimulatory effects of TRH appear to have been lost during the evolution of mammalian skin in human epidermal melanocytes *in situ*, the current thesis project demonstrates that they have definitely been preserved in the human HFPU.

Since 2011, the Paus Lab has also shown that TRH stimulates the re-epithelialisation of experimentally wounded, organ-cultured frog and human skin – as a striking example of skin-related TRH effects that have indeed been preserved from lower vertebrates to human primates (Meier et al. 2013; Haslam et al. 2014). This recent research development may also be interesting and relevant from a melanocyte/TRH biology perspective. Recently, it has been reported that direct migration of follicular melanocyte stem cells to the epidermis after wounding or UVB irradiation is dependent on MC1R signaling (Chou et al., 2013). Since paracrine signals released from keratinocytes may play a key role in mediating this (Chou et al. 2013; Ferguson et al., 2015) and since melanocytes may be more important for epidermal repair than is widely appreciated (Paus 2013), it is tempting to ask whether keratinocyte-derived TRH could be one of these neuroendocrine factors. This makes the results from the current thesis project even more interesting and translationally relevant, as they extend their potential importance beyond the field of human HF pigmentation.

In the meantime, the Paus Lab has also shown that TRH operates as an important regulator of mitochondrial energy metabolism in human epidermal and HF keratinocytes *ex vivo* (Knuever et al. 2012); Vidali etal. 2014, Paus et al. 2014). It remains to be clarified whether any of the newly discovered functions of TRH as a regulator of human keratinocyte mitochondrial activity and even biogenesis *ex vivo* (mechanism as yet unknown) also extends to HF melanocyte mitochondria. If this should turn out to be the case, it will be intriguing to evaluate, next, whether or not the complex and selective impact of TRH on the melanocytes of the human HFPU is in any way related to its stimulatory effects on mitochondrial function, not the least

since it is increasingly recognized that mitochondria not only generate ATP in reactive oxygen species, but also exert important regulatory functions, notably within the HF epithelium (Hamanaka et al., 2013; Kloepper et al., 2015).

As to the critical question, whether TRH receptor proteins are really absent from the human HFPU (see above), as is suggested by the current thesis project results (Gaspar et al. 2011), in collaboration with the electronmicrospy service of the Italian Insstitute of Technology, Genova, the Paus Lab has most recently attempted to visualize the intrafollicular TRH-R1 protein expression at the ultrastructual level. Even though this has been complicated by the fact that the originally used, excellent TRH-R1 antibody (Gaspar et al. 2011) is no longer commercially available, first, still very preliminary immuno-transmission electron microscopy results confirm that TRH-R1-associated gold particles are predominantly localized within the IRS of human anagen scalp HFs.

However, these preliminary results have suggested that a few isolated gold grains (above background level) are also seen in other compartments of the HF epithelium, incl. the hair matrix (R Paus, personal communication). Therefore, it is at least conceivable that the human HFPU may indeed express some TRH-R1 molecules at very low-levels (beyond the threshold of immunodetection even by highly sensitive immunofluorescence techniques). Thus, the hypothesis that a direct stimulation of HFPU melanocytes by TRH via its main cognate receptor may also occur *in situ*, after all, is currently being rigorously re-explored by the Paus Lab (University of Manchester).

Finally, peripheral clock gene activity, namely of the core clock components, Clock, BMAL1 and Period1, has most recently surfaced as another important regulatory element of human HF pigmentation, with reduced activity of these clock genes being associated with enhanced pigmentation (Hardman et al. 2015). This is interesting in view of the fact that TRH cells and TRH+ nerve fibers are found in the suprachiasmatic nucleus (SCN), i.e. the central circadian clock (Fliers et al. 1998). Here, TRH may regulate clock activity in the brain, which in turn is thought to regulate circadian fluctuations in TRH serum levels (Philippe and Dibner 2015).

Given that human scalp HF express TRH and TRH receptors (Gáspár et al. 2010) as well as core clock and key clock target genes (Al-Nuaimi et al., 2014), it is reasonable to ask whether the pigmentation-stimulatory effects of TRH in human HFs described in the current dissertation could, at least in part, also be mediated via a modulation of intrafollicular clock and clock target gene activity. First, prelimnary evidence from the Paus Lab suggests that TRH indeed down-regulates the intrafollicular expression of the clock proteins, BMAL1 and Period 1 (Hardman & Paus, University of Manchester, personal communication). Since such an effect of peripheral clock gene expression/activity in human HFs has previously been demonstrated to stimulate HF melanogenesis and to activate human HFPU melanocytes *in situ* (Hardman et al. 2015) peripheral clock activity-mediated effects of TRH may contribute, at least in part, to the overall HF pigmentation phenomena described in the current thesis. This hypothesis is currently under critical examination by the Paus Lab, Manchester.

Therefore, even five years after completion of the experiments reported in the current dissertation, the discovery of the profound and selective stimulatory impact of TRH on human HF pigmentation described here remains as “fresh” and exciting as it was when first published (see *Commentary* by Dosal et al. 2011 (Dosal et al., 2011)) and continues to raise fascinating and translationally relevant, but as yet unanswered questions on the neuroendocrinology of both, human melanocytes and human HFs.

5 SUMMARY

In amphibians, thyrotropin- releasing hormone (TRH) stimulates skin melanophores by inducing secretion of the α-melanocyte-stimulating hormone in the pituitary gland. However, it is unknown whether in humans this tripeptide neurohormone exerts any direct effects on pigment cells, namely, on melanocytes, under physiological conditions. Therefore, this study has investigated whether TRH stimulates pigment production in organ-cultured human hair follicles (HFs), the epithelium of which expresses both TRH and its receptor, and/or in full-thickness human skin *in situ*.

The effect of TRH on hair and skin pigmentation *in situ* was investigated in isolated HFs and human full-thickness skin biopsies, which were organ-cultured in the presence of different TRH concentrations (0, 3, 15, 30, 300 nM). To study the action of TRH on hair pigmentation *in vitro,* the melanocytes from the outer root sheath (ORS) of human anagen VI scalp HFs were isolated, cultured, and then also treated with different TRH concentrations. Quantitave RT-PCR and immunohistochemistry were used to assess TRH dependent changes after application of TRH to the cultures. Finally, the possible mechanisms by which TRH stimulated pigmentation were investigated. From this study it could be concluded that TRH stimulated melanin synthesis, tyrosinase transcription and activity, melanosome formation, melanocyte dendricity, gp100 immunoreactivity, and microphthamia-associated transcription factor expression in human HFs in a pituitary gland independent manner. TRH also stimulated proliferation, gp100 expression, tyrosinase activity, and dendricity of isolated human HF melanocytes. However, intraepidermal melanogenesis was unaffected *in situ*. As TRH upregulated the intrafollicular production of “pituitary”-neurohormes (proopiomelanocortin transcription and ACTH immunoreactivity) and as agouti-signaling protein inhibited TRH-induced HF pigmentation, these pigmentary TRH effects may be mediated in part by locally generated melanocortins and/or by MC-1R signaling. The current MD thesis project introduces TRH as a novel, potent, selective, and evolutionarily highly conserved neuroendocrine factor controlling human pigmentation *in situ*. This physiologically relevant and melanocyte sub-population-specific neuroendocrine control of human pigmentation deserves clinical exploration, e.g., for preventing or reversing hair graying.

6 ZUSAMMENFASSUNG

Thyrotropin Releasing Hormons (TRH), ein kleines Tripeptid-Hormon, bestens bekannt als proximaler Stimulus im Regelkreis der Schilddrüsenhorme.
Amphibien, wie z.B. Frösche, haben die faszinierende, für ihr Überleben vorteilhafte Fähigkeit, sich in zwingenden Situationen mit wechselnden Hautfarben geschickt an die Umgebung anzupassen. Die Forscher haben herausgefunden, dass TRH eine große Rolle in diesen evolutionär ererbten Phänomen spielt. TRH regt die Sekrektion vom α-Melanozyten-stimulierten Hormon (α-MSH) aus der Hypophyse an und dieses induziert in der Haut der Amphibien die Melaninproduktion zur Umweltadaptation.
Folgerichtig wurde die Existenz von TRH und vom TRH-Rezeptor beim Menschen im Haar und in der Haut nachgewiesen. Ob TRH überhaupt eine Rolle bei der Melaninproduktion spielt, war allerdings unbekannt. Daher drängen sich folgende Fragen auf, die zu beantworten und zu belegen wären. 1.) Spielt TRH eine Rolle bei der Pigmentierung menschlicher Haut und Haare unabhängig von Hypophysen? 2.) Wie selektiv ist die pigmentierende Wirkung von TRH? Wirkt TRH nur an Haarfollikeln und/oder auch in der Haut? 3.) Welchen Wirkmechanismus hat TRH? und 4.) Kann TRH eventuell klinisch zum Anti-Graying der Haare benutzt werden?

Mit Erlaubnis der Ethikkommission der Universität zu Lübeck und der von Patienten, sowie der Hilfe von Plastikchirurgen wurde Kopfhaut gesammelt und unserem Labor für Kulturmedien zur Verfügung gestellt. Ausschließlich Materialien, die nicht älter als 24h waren, durften für die Forschung genutzt werden. Im Labor erfolgte die vorsichtige Isolierung der Haarefollikel, die anschließend über Nacht in einem Inkubator im Kulturmedium inkubierten, damit sie sich vom Isolierungsstress erholen konnten. Sorgfältig vorbereitet konnten sie nun mit unterschiedlichen TRH Konzentrationen behandelt werden. Die Auswahl der TRH-Konzentrationen basierten auf den gelungenen Versuchen von Trouslard et al.. Die gleiche Prozedur erfolgte für Hautstücke und für die Melanozyten der äußeren epithelialen Haarwurzelscheide. Für weitere Untersuchungen erfolgte noch die Zerteilung der Haare und Hautstücke in 6µm dicke Cryoschnitte.

Um die möglichen Veränderungen durch die Wirkung von TRH nach der Kultivierung auf der Proteinebene darzustellen, wurden verschiedene immunhistochemische Färbungen verwendet. Mit RT-PCR konnten die Effekte von TRH auf der mRNA Ebene nachgewiesen werden.

Eindeutig zeigte diese Studie, dass TRH die Melaninproduktion, die Transkription und Aktivität vom Schlüsselenzym der Melaninsynthese *Tyrosinase*, die Bildung von Pigmentkörperchen *Melanosomen*, die Dendrizität der Haarmatrixmelanozyten, die glycoprotein 100 *(gp100)* Immunoreaktivität, die Expression des Mikrophthalmie-assoziierten Transkriptionsfaktors *(MITF)* in Haarfollikeln unabhängig von der Hypophyse stimuliert. Gleiches gilt auch für *in vitro,* TRH stimuliert die Proliferation, die gp100 Expression, die Aktivität des Enzyms Tyrosinase und die Dendrizität in isolierten Melanozyten aus Haarfollikeln. Dagegen bleibt die intraepidermale Melanogesesis von TRH unbeeinflusst. TRH steigert die intrafollikuläre Produktion von hypophysären Neurohormonen (Proopiomelanocortin(POMC) und Adrenocorticotropes Hormon (ACTH) Immunaktivität). Und Agouti signaling peptide (ASP) wirkt der pigmentinduzierenden Wirkungen von TRH entgegen.

Erstmalig konnte mit dieser Studie bewiesen werden, dass TRH die Melaninproduktion in menschlichen Haarfollikeln stimuliert. Diese Wirkung von TRH wurde durch den Nachweis der Steigerung der Melaninproduktion und der Aktivität des Schlüsselenzyms der Melaninsynthese Tyrosinase *in situ*, und dessen Transkription in den mit TRH behandelten Haarfollikeln bekräftigt. Anders als bei den Fröschen, wo die pigmentstimuliernde Wirkung von TRH durch die Hypophyse ausgelöst wird, reagieren die Haarfollikel auf TRH auch ohne Einfluss der Hypophyse. Da im menschlichen Haarwurzelepithel TRH im Gen und im Proteinlevel exprimiert, ist es möglich, dass TRH von Keratinozyten parakrine Effekts auf Melanozyten in menschlichen Haarfollikeln ausgeübt hat.
Auch Melanosome, welche in den Melanozyten produziert und an die Keratinozyten weitergegeben werden, um die Haare mit Melanin zu versorgen, wurden untersucht. Dabei wurde die Immunaktivität von gp100, ein Protein, welches für die Produktion von Melanosom eine wichtige Rolle spielt, gemessen. Sowohl die gp100 Immunaktivität, als auch die Dendriten der Melanozyten wurden unter TRH

gesteigert. Der Beweis also dafür, dass TRH seine Wirkung nicht nur bloß auf die Schlüsselenzyme der Melaninsynthesen ausübt, sondern auf alle genannten Prozesse wirkt.

Anschließend wurde die Wirkung von TRH direkt an isolierten Melanozyten untersucht, um den eventuellen Einfluss benachbarter Zellen auszuschließen. Es zeigte hier, dass sowohl die Dendriten der Melanozyten, die gp100 Expression und die Tyrosinase Aktivität aber auch die Proliferation von Melanozyten *in vitro* gesteigert wurde.

Die Untersuchungen in der Haut erbrachten aber keine signifikante Wirkung von TRH auf der epidermalen Pigmentierung. Das könnte bedeuten, während der menschlich-evolutionären Entwicklung ist die Wirkung von TRH auf die epidermalen Melanozyten verloren gegangen. Schlussfolgernd ist anzunehmen, dass die epidermalen und die follikulären Melanozyten unterschiedlichen Populationen und Kontrollmechanismen unterlagen.

Erwiesener Maßen ist somit der Nachweis der pigmentstimulierenden Wirkung von TRH selectiv auf Haarfollikel gelungen. Nun gilt es die pigmentierende Wirkungsmechanismus von TRH zu erforschen. Grundsätzlich kann angenommen aber nicht belegt werden, dass TRH über seinen Rezeptor wirkt, da in der Pigmenteinheit der Haarfollikel und in isolierten Melanozyten kein Rezeptor für TRH nachgewiesen werden konnte. Diese Annahme ist auch deshalb sehr unwahrscheinlich, da anderen Forschunggruppen dieser Nachweis bisher auch noch nicht gelungen ist. Dem gegenüber steht allerdings die Tatsache, dass der TRH-Rezeptor in der inneren Wurzelscheide der Haarfollikel nachgewiesen werden könnte, obwohl diese Schicht nur Keratinnozyten hat und nicht die Pigmentierung der Haare verursacht. Folglich könnte vermutet werden, dass TRH über indirektem Weg, also zuerst auf Keratinozyten wirken, diese daraufhin Signale an die Melanozyten in der pigmentären Einheit weiterleiten und diese zur Melaninproduktion indizieren. Diese Hypothese ist unmöglich nachzuweisen, weil eine Isolierung und Kulturvierung der Keratinozyten aus der inneren Wurzelscheide der Haarfollikeln zurzeit noch nicht möglich ist.

Es ist bekannt, dass die Haarfollikeln verschiedene hypothalamäre und hypophysäre Neurohormone produzieren, wie hier nachgewiesen, eben durch die Expression von POMC und deren Spaltungsprodukt ACTH, diese sich dann an den MC1-R binden

und die Pigmentproduktion dadurch stimulieren. Also könnte es auch sein, dass die pigmenstimulierende Wirkung von TRH indirekt ist und zwar über Produktion von klassischen hypophysären melanotropischen Hormonen, diese dann wiederum die Haarpigmentierung stimulieren.
Weiterhin ist bekannt, dass die Melanozyten in der Pigmenteinheit der Haarfollikel und in den isolierten Melanozyten die MC1R exprimieren und die Forschungsgruppe Schiöth et al. hat nachgewiesen, dass TRH diese MC1R stimulieren kann. Es verleitet uns zu der Annahme, dass TRH die pigmentären Effekte direkt über die Bindung an MC1R ausübt. Dazu wurde Agouti-Signaling-Protein (ASP), welches gegenüber dem MC1R antagonistisch sensitiv wirkt, mit TRH gemischt und die Haarfollikel damit kulturviert. Tatsächlich war die pigmentstimulierende Wirkung von TRH in den Haarfollikeln geblockt und die Wirkungen waren nur auf dem Niveau der nicht behandelten Haarfollikel. Diese Erkenntnis bestärkt die Annahme, dass TRH seine Wirkung über die MC1R ausübt. Um diese tatsächlich zu bestätigen, müssen in Zukunft weitere Untersuchungen durchgeführt werden, zum Beispiel an Melanozyten mit knockout von MC1R. Diese Untersuchungen würden aber den Rahmen dieser Studie übersteigen.
Zusammenfassend konnte bisher gezeigt werden, dass die Wirkung von TRH über MC1R sehr wahrscheinlich ist, weil (1) In der Pigmenteinheit der Haarfollikel konnten keine TRH-Rezeptoren dektiert werden (2) die Melanozyten exprimieren keinen TRH Rezeptor aber MC1R, (3) TRH kann eine Signaltransduktion über MC1R ausüben, (4) die pigmentstimulierende Wirkung von TRH kann teilweise durch das dem MC1R antagonistisch wirkenden Agouti-Signaling-Protein gehemmt werden, (5) TRH stimulates intrafollicular Mikrophthalmie-assoziierten Transkriptionsfaktor (MITF), welches normalerweise über die MC1R aktiviert wird.

Diese Studie zeigt TRH als ein neue, pontente, selective und evolutionär erhaltende neuroendocrine Faktor, welcher die menschliche Pigmentierung stimuliert. Für klinische Anwendung ist es möglich, TRH lokal z.B. als Shampoo zu verwenden, so dass die eventuell entstehende systemische Effekte vermieden werden können.

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8 PUBLICATION

Article

Erzsébet Gáspár, **Kim Thoa Nguyen Thi**, Celine Hardenbicker, Stephan Tiede, Christian Plate, Enikő Bodó, Jana Knuever, Wolfgang Funk, Tamás Biró, and Ralf Paus: Thyrotropin-releasing hormone (TRH) selectively stimulates human hair follicle pigmentation. ***J Invest Dermatol*** **2011; 13:2368-2377.**

Oral Presentation

Kim Thoa Nguyen Thi: Thyrotropin-releasing hormone (TRH) is a novel pigmentary hormone in situ and in vitro. **In 70th Annual Meeting of the Society for Investigative Dermatology (SID), 05.-08. Mai 2010, Atlanta, Georgia, USA.**

Poster

Erzsébet Gáspár, **Kim Thoa Nguyen Thi**, Celine Hardenbicker, Stephan Tiede, Christian Plate, Enikő Bodó, Jana Knuever, Wolfgang Funk, Tamás Biró, and Ralf Paus: Thyrotropin-releasing hormone (TRH) is a novel pigmentary hormone in situ and in vitro. **In 70th Annual Meeting of the Society for Investigative Dermatology (SID), 05.-08. Mai 2010, Atlanta, Georgia, USA.**

Erzsébet Gáspár, **Kim Thoa Nguyen Thi**, Celine Hardenbicker, Stephan Tiede, Christian Plate, Enikő Bodó, Jana Knuever, Wolfgang Funk, Tamás Biró, and Ralf Paus: Thyrotropin-releasing hormone (TRH) ist ein neu pigmentstimulierende Hormon *in situ* and *in vitro*. **4. Lübecker Doktorandentag 2010**

Award

Albert Kligman Travel Fellowship of Society for Investigative Dermatology (SID), 05.-08. Mai 2010, Atlanta, Georgia, USA.

9 ERKLÄRUNG ZUM EIGENANTEIL AN DEN PRÄSENTIERTEN ERGEBNISSEN

In Rahmen des Disserationsprojekts hatte meine Vorgängerin, cand.med. (jetzt: Dr.med.) C. Hardenbicker, herausgefunden, dass Thyrotropin-releasing hormone (TRH) die Melaninproduktion in Haarfolllikel und die Dendrizität der Haarmatrixmelanozyten, steigert.

Um diese neue Entdeckung zu überprüfen, nach zuweisen und zu untermauern habe ich meine Disserationsprojekt gestartet.

1. Mittels Masson-fontana Färbungen habe ich unabhängig von Dr.med.Hardenbicker herausgefunden, dass die Melaninsynthese in den Haarfollikeln, welche in Thyrotropin-releasing hormone (TRH) inkubiert wurden, signifikant höher als in den nicht mit TRH behandelte Haarfollikeln ist.
2. Mittels Nki/Beteb Immunfluoreszenz habe ich neu nachgewiesen, dass TRH sowohl mRNA als auch die Enzymaktivittät des Schlüselemzyms Tyrosinase stimuliert.
3. TRH stimuliert die Dendrizität der Haarmatrixmelanozyten wurde von Dr. med. Hardenbircker in isolierten Haarfollikel beobachet. Diese Effekt habe ich untermauert und bewiesen in isolierten Melanozytenkultur.
4. Mit zahlreichen Experimenten habe ich herausgefunden wie TRH die Pigmentierung der Haarfollikeln stimulieren könnte. Durch diesen Versuchen und deren Ergebniss war es möglich die pigmentbiologische Arbeit zu TRH in der #1 journal des Gebietes „Dermatology“ (J Invest Dermatol) zu veröffentlichen. Das war ein große Erfolg für meine Arbeit.
5. Außerdem habe ich ein Albert Kligman Travel Fellowship zu 70th Annual Meeting of the Society for Investigative Dermatology (SID), 05.-08. Mai 2010, Atlanta, Georgia, USA gewonnen und dufte dort einen Vortrag und eine Posterpresäntation halten.

Nach erfolgreiche Durchführung der Experimenten und Veröffentlichung meiner Ergebnissen widmete ich meine Zeit anschließend Staatexamsvorbereitung. Im Oktober 2012 konnte ich mein Medizinstudium in Regelstudienzeit erfolgreich

abschließen. Unser Glück wurde noch verdoppelt als unsere Tochter kurz danach geboren wurde. Danach war ich lange voll und ganz beschäftigt mit der Vorsorgung unsere Tochter. Nach der Elternzeit begann ich mit der Facharztweiterbildung in Innere Medizin, was mir in den letzen 5 Jahre kaum genug Zeit zur Vervollständigung meiner Dissertation ließ. Nun ist unsere Tochter schon größer und ich habe mehr Berufserfahrung, so dass ich endlich wieder etwas Zeit für die Forschung und den Abschluß meiner Dissertation gefunden habe.

10 DANKSAGUNG

Für meine Doktorarbeit bedanke ich mich recht herzlich bei …

… meinem Doktorvater Herrn **Prof. Dr. Ralf Paus** (Klinik für Dermatologie, Allergologie und Venerologie, Universität zu Lübeck, damals Leiter der Experimentellen Dermatologie der Hautklinik und Professor of Cutaneous Medicine, Dermatology Research Centre, Institute of Inflammation and Repair, University of Manchester) für die Vergabe des Themas, die Konzeption dieses Projektes, die fachliche Betreuung und Supervision, hilfreiche Unterstützung bei der Fertigstelllung dieser Dissertationsschrift, viele wertvolle Ratschläge und seinen Optimismus für das Gelingen dieser anspruchsvollen Arbeit.

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… Herrn **Prof. Tamás Bíro** (Department of Physiology, Research Center for Molecular Medicine, University of Debrecen, Debrecen, Hungary) für die gute Zusammenarbeit und für die Durchführung und Bereitstellung der Ergebnisse der RT-PCR für Tyrosinase und POMC mRNA.

… dem ehemaligen Laborchef des Paus Labors und meinem Mitbetreuer **Dr. Stephan Tiede** für die geduldige Unterstützung, besonders für seine verständnisvolle Betreuung in der späten Phase meiner Arbeit.

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… allen Mitgliedern der **AG Paus** für die familiäre Arbeitsatmosphäre und gute Zusammenarbeit.

… meiner Kollegin **Dr. med. Elisabeth Costello-Boerrigter** für wertvolle Hilfe bei der Englisch-Korrektur dieser Dissertationsschrift (meine Muttersprache is weder Deutsch, noch Englisch, sondern Vietnamesisch…).

… meinen **Eltern, Geschwistern, meinem Mann und meinen Freunden,** die mich in allen Phasen meines Studiums und meiner Promotion eng begleitet haben und die immer für mich da sind!

11 LEBENSLAUF

Persönliche Daten

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Schulbildung

1990 – 2000	Grundschule und Mittelschule in Vietnam
2000 – 2003	Staatliche Regelschule 7 „Ulrich von Hutten“, Erfurt
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2003 – 2006	Oskar Gründler Gymnasium Gebesee
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08/2013 – 12/2015	Assistenzärztin SANA Kliniken Lübeck
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Begonnene Promotion “Thyrotropin releasing hormone (TRH) selectively stimulates human hair follicle pigmentation” (seit Oktober 2009)

Vortrag & Poster in 70th Annual Meeting of the Society for Investigative Dermatology (SID),
05.-08. Mai 2010, Atlanta, Georgia, USA,
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Klinische Erfahrungen

Ärztliche Tätigkeiten

Fach	Klinik	Zeitraum
Medizinische Klinik I Kardiologie	Sana Kliniken Lübeck, Station 11	08/13 – 12/13
Intermediate Care Unit/IMC	Sana Kliniken Lübeck	01/14 – 07/14
Internistische Intensivstation	Sana Kliniken Lübeck	08/14 – 12/15
Medizinische Klinik II Nephrologie	Helios Kliniken Erfurt, Innere Medizin	Seit 12/15

Praktisches Jahr

Fach	Klinik	Zeitraum
Kardiologie	Universitätsklinikum Schleswig-Holstein, Campus Lübeck (UKSH)	4 Wochen
Notaufnahme	Universitätsklinikum Schleswig-Holstein, Campus Lübeck (UKSH)	4 Wochen
Gastroenterologie	Universitätsklinikum Schleswig-Holstein, Campus Lübeck (UKSH)	4 Wochen
Endokrinologie	Universitätsklinikum Schleswig-Holstein, Campus Lübeck (UKSH)	4 Wochen
Gynäkologie und Geburtshilfe	Sana Kliniken Ostholstein, Klinik Eutin	16 Wochen
Chirurgie	Sana Kliniken Ostholstein, Klinik Eutin	16 Wochen
Intensivstation	Sana Kliniken Ostholstein, Klinik Eutin	2 Wochen

Famulaturen

Fach	Klinik	Zeitraum
Thoraxchirurgie	HELIOS Klinikum Erfurt	2 Wochen
Notaufnahme	Staatliches Krankenhaus VinhPhuc/Vietnam	8 Wochen
Internistische Station	Universitätsklinikum Hue/Vietnam	4 Wochen
Polyklinik Kinderchirurgie	Universitätsklinikum Schleswig-Holstein, Campus Lübeck (UKSH)	3 Wochen
Polyklinik Augenheilkunde	Universitätsklinikum Schleswig-Holstein, Campus Lübeck (UKSH)	2 Wochen
Hämatologie	HELIOS Klinikum Erfurt	3 Wochen

Jobs

Blutentnahmedienst – Studentische Hilfskraft – 3 Jahre
Experimentelle Dermatologie – Studentische Hilfskraft – 4 Jahre

Sprachkenntnisse

Vietnamesich	sehr gut, Muttersprache
Deutsch	sehr gut, wie Muttersprache
Englisch	gute Grundkenntnisse
Latein	gute Schulkenntnisse

Sonstige Qualifikationen

ALS-Provider Kurs / European Resuscitation Council (09.05. – 11.05.2014)

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