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Thyroid hormones are direct modulators of human hair follicle growth and pigmentation

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Abbreviations

ABC = Avidin-biotin complex
ACTH = adrenocorticotrope hormone
AP = Alkaline phosphatase
ATP = adenosine triphosphate
bcl-2 = B-cell lymphoma 2 protein
BM = Basement membrane
BMP = Bone morphogenetic protein
cAMP = Cyclic adenosine monophosphate
cGMP = Cyclic guanosine monophosphate
CK = Cytokeratin
COX1 = cytochrome-c-oxidase subunit 1
CRH = corticotropin releasing hormone
CRH-R = corticotropin releasing hormone receptor
CTS = Connective tissue sheath
CT = Cuticle
CutL 1 = transcription factor
DAPI = 4',6'-diamidin-2'-phenylindol-dihydrochloride
DHT = dihydrotestosterone
DIO = Deiodinase
DIT = diiodinated tyrosine
DNA = Deoxyribonucleic acid
DOPA = 3,4- dihydroxyphenylalanine
DP = Dermal papilla
ECM = Extracellular matrix
eSC = Epithelial stem cell
EPO = erythropoietin
Fig =Figure
FITC = Fluorescein isothiocyanate
FOXN1 = Forehead box N1
HCl = Hydrochloric acid
HF = Hair follicle

HGF = Hepatocyte growth factor

HPU = Hair follicle pigmentary unit

HPT-axis = hypothalamic- pituitary- thyroid axis

Hr = Hairless gene

hr = Hairless protein

HS = Hair shaft

IGF = Insulin-like growth factor

IHC = Immuno- histochemistry

IL-1 = interleukin 1

INFγ = interferon γ

IP3 = Inositol-1,4,5-triphosphate

IR = Immunoreactivity

IRS = Inner root sheath

KGF = Keratinocyte growth factor

LBP = Ligand binding protein

MIT = Monoiodinated tyrosine

MK = Matrix keratinocyte

mSC = Mesenchymal stem cell

NaCl = Sodium chloride

NADPH = Hydrogenated nitrogenamide-adenin-dinucleotide phosphate

NaOH = Sodium hydroxide

NIS = Sodium-iodide symporter

nM = Nano-molar

NT3 = Neurotrophin 3

ORS = Outer root sheath

PAH = Phenylalanine hydroxylase

PBS = Phosphate buffered saline

PCR = Polymerase chain reaction

POD = Programmed organ deletion

POMC = Proopiomelanocortin

PRL = Prolactin

PRL-R = Prolactin receptor

p75NTR = p75 neurotrophin receptor

RNA = Ribonucleic acid

RXR = Retinoid-X-receptor

rT3 = reverse T3

SCF = Stem cell factor = c-kit

SEM = Standard error of the mean

SG = Sebaceous gland

Shh = Sonic hedgehog

SW = Sweat gland

Tab = Table

TBG = thyroid binding globulin

TBS = Tris buffered saline

TdT = Terminal dioxynucleotidyltransferase

TG = thyreoglobulin

TGF-β1 = Transforming growth factor-β 1

TGF-β2 = Transforming growth factor-β 2

TH = thyroid hormones

TNT = Tris buffered saline add triton-x

TPO = thyroid peroxidase

TR = thyroid receptors

TRE = thyroid hormone response element

TRH = thyreotropin releasing hormone

TSA = thyramide signal amplification

TSH = thyreotropin = thyroid stimulating hormone

TUNEL = terminal dUTP nick-end labelling

T3 = triiodothyronine

T4 = tetraiodothyronine (L- thyroxine)

α-MSH = alpha- melanocyte stimulating hormone = melanotropin

6BH4 = L-erythro 5,6,7,8 tetrahydrobiopterin

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1 Introduction

1.1 Background

Since thyroid hormones (TH) provide crucial endocrine controls for human development and metabolism (see below), it is not surprising that TH also affect human skin. TH abnormalities are commonly associated with changes in hair follicle (HF) appearance and function. It has long been observed that prominent hair abnormalities in patients with thyroid dysfunction occur (Freinkel and Freinkel 1972, Messenger 2000, Schell et al. 1991, Ebling 1991). In addition, several *in vivo*-studies have demonstrated that thyroid hormones (TH) exert potent hair-growth modulatory effects e.g. in sheep, rats and mice (Safer et al. 2001, Hale and Ebling 1979, Ferguson 1965). However, these effects are partially conflicting, since application of thyroxine (T4) increased the weight of wool grown in adult sheep (Ferguson 1965), thus indicating a growth promoting effect, while in rats application of thyroxine only shortened anagen but did not stimulate hair growth (Hale and Ebling 1979).

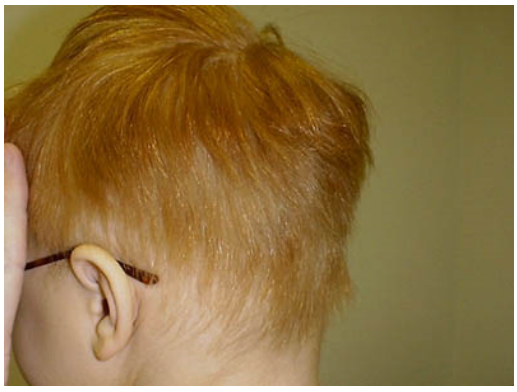


Figure 1.1 Hair loss in thyroid disease

thinning, coarse, dry, lusterless hair, a 7 year old boy was evaluated for growth delay and hair loss. Thinning hair and coarse skin resolved with thyroxine supplementation. Normal growth and development resumed.

From: DermAtlas (www.dermatlas.org)

In humans, diffuse loss of scalp hair follicles, body hair and the lateral eyebrows are clinical signs associated with *hypo*thyroidism, along with the presentation of dry, brittle and dull hair shafts (Freinkel and Freinkel 1972, Schell et al. 1991, Ebling 1991). Confusingly *hyper*thyroid states can also lead to effluvium, despite an apparently increased hair matrix proliferation (Schell et al. 1991). Hyperthyroid HFs produce a thinner hair shaft diameter and brittle, greasy hair (Messenger 2000, Fistarol 2002) and display substantially reduced tensile strength (Stüttgen 1974, Everett et al. 1997). Also, early greying has been claimed to be related to autoimmune thyroid disease, hypothyroidism and non-autoimmune

hyperthyroidism (Kaminski 1997, Lerner 1971), while darkening of gray hair has recently been reported in a few patients after TH administration (Redondo et al. 2007).

The HF is a continuously remodelling and regenerating mini organ, which undergoes phases of massive proliferation (anagen), apoptosis-driven organ involution (catagen), and relative resting (telogen) during hair follicle cycling (Chase 1954, Paus et al. 2000, Schneider et al. 2009). The structure of the HF and its hair shaft is subject to numerous controls, including a very large variety of hormones, growth factors, cytokines and neuropeptides (Paus and Cotsarelis 1999, Stenn and Paus 2001, Dawber 2006, Ohnemus et al. 2006, Schneider et al. 2009). Therefore, it is not surprising that changes in HF structure and hair growth can be important clinical signs of underlying endocrine abnormalities, such as hyperandrogenism, Cushing syndrome, ectopic ACTH production and hyperprolactenemia. Furthermore hormonal treatment can exert substantial hair growth-modulatory affects (e.g. in patients treated with antiandrogens, L-thyroxine, retinoids, or antiestrogens) (Zouboulis et al. 2007, Foitzik et al. 2005, Deplewski & Rosenfield 2000, Paus et al. 2007).

Although the phenomenon of TH-induced hair growth alterations has been appreciated for many decades (Sainton and Simonnet 1931, Rook 1965, Freinkel and Freinkel 1972, Stüttgen 1974), the underlying mechanisms are still very incompletely understood. In particular, it remains to be conclusively documented that TH really exert *direct* effects on human HFs and the yet unclear mechanisms of triiodothyronine (T3) and tetraiodothyronine (T4) affecting human hair shaft formation, HF cycling, HF pigmentation and hair keratinisation. While human scalp HFs do express TH receptors (Billioni et al. 2000, Ahsan et al. 1998), it is not clear whether the clinical hair growth abnormalities seen in patients with thyroid disease reflect direct effects of TH or are the consequence of secondary endocrine abnormalities, e.g. the up-regulation of serum thyroid stimulating hormone (TSH) levels - a defining feature of hypothyroidism (Sawin et al. 1985), or thyroid disease-associated changes in the serum insulin level (Velija-Asimi and Karamehic 2007, Dimitriadis et al. 2006).

BASIC DATA OF HUMAN HAIR FOLLICLES	
Total number	– 5 000 000 (mostly vellus!)
Number of scalp hair follicles	– 100 000 Blondes: + 20% Redheads: – 20%
Average density (scalp) terminal + vellus	1135/cm^2 (newborn)* 615/cm^2 (20–30 years)* 485/cm^2 (30–50 years)* 435/cm^2 (70–80 years)* African-Americans, Asian: lower density Terminal only : ca. 250/cm^2 Bald scalp (45-70 years) : 330/cm^2* Highest density : cheek + forehead !*
Hair embryology	Development progresses at fixed intervals (274–350 µm) in cephalocaudal direction, becoming first visible in eyebrow, upper lip and chin region (9th week) At 16 weeks, hair shafts are formed in these regions Hair follicles are formed in sequential waves of interspersed follicles
Hair cycle distribution (terminal scalp hair)	Anagen: 85–90% Telogen: 10–15% Catagen: <1%
Duration of hair cycle phases (terminal scalp hair)	Anagen: 2–6 years Catagen: 2–3 weeks Telogen: 3 months There are substantial variations in anagen duration: Terminal moustache: 4–14 weeks Terminal arms: 6–12 weeks Terminal legs: 19–26 weeks Vellus: 6–12 weeks Premature anagen induction induced by plucking of telogen hair shafts (depilation) Estrogens prolong anagen Thyroxine promotes growth, corticosteroids retard anagen onset
Number of lifetime cycles	10–20
Physiological hair shedding rate (scalp)	– 100–200/day (substantial interindividual and seasonal variations)
Hair shaft production rate (scalp)	ca 0.35 mm/day, 1 cm/month Hair production is not influenced by cutting/shaving Estrogens reduce hair growth rate Androgens increase hair growth rate and hair diameter in androgen-dependent sites (beard)
Hair shaft diameter and length	Vellus: <0.03 mm; 1–2 mm Terminal: > 0.06 mm; 1 mm Average diameter: Mongoloid hair (circular): 120 µm Caucasian (elliptic): 50–90 µm
Hair shaft structures	Cuticle (outside), cortex, medulla (center) Cuticle maintains hair fiber integrity Cortex contains bulk of hair keratins and keratin-associated proteins Hair fiber strength is largely due to disulfide bonding Medulla consists of loosely connected trichocytes with large intercellular air spaces; provides insulation (animals)
Hair shaft pigmentation	Dark hair: predominance of eumelanin Blond/red hair: pheomelanin predominates Melanosomes of hair cortex larger than those of epidermis
Hair graying (canities)	Generally commences in the third decade of life on the temples, spreading later to crown and occiput; by the age of 50 years, 50% of the population has at least 50% grey hair
Hair patterns	Pubic hair: horizontal (90% of women, 20% of men), acuminate (10% of women, 50% of men) Diffuse chest hair: normally grows only in men, after puberty (until 6th decade) Axillary: appears about 2 years after first pubic hairs, more sparse in Mongoloids than Caucasians; frequently absent in older individuals Trichoglyphics: single, clockwise parietal whirl present in 95% of individuals

Table 1.1 Basic data on human hair

(Paus et al.: Biology of hair and nails. In: Bologna et al. (eds.), Dermatology, 2007)

To address the direct effects of hormones on the human follicle *in vitro*, one can study scalp hair follicles that have been surgically removed from any confounding systemic (neural, vascular) influences and that are organ-cultured under serum-free conditions in the presence of insulin and hydrocortisone (Philpott et al. 1990 & 1996, Foitzik et al. 2005 & 2006, Conrad et al. 2005). Therefore, normal, micro dissected and organ-cultured human scalp HFs are ideally suited to study the human hair growth response to stimulation with TH.

In order to fully appreciate the multiple effects that hormones can exert on hair growth and pigmentation, it is necessary to recapitulate some basic elements of hair follicle biology.

1.2 The human hair follicle

1.2.1 Anatomy

The human HF is an epidermal derived, largely epithelial mini organ, which operates under the control of a highly specialized fibroblast population of mesenchymal origin (i.e. the follicular dermal papilla [DP]). The DP is onion-shaped and contains these densely packed fibroblasts that display inductive and morphogenic properties even in the adult organism (Paus and Cotsarelis 1999, Schneider et al. 2009). The DP is located in the proximal part of the HF and changes its shape and location dependent on the state of hair follicle cycling (see below) (Handjiski et al. 1994). By the number and profound secretory capacities of its fibroblasts the DP determines the size of the hair bulb and, thereby, the diameter of the hair shaft as well as the duration of anagen (Reynolds et al. 1999, Paus 2008, Schneider et al. 2009) (Fig.1.2).

The epithelial part of the HF is composed out of several, concentrically arranged layers of specialized keratinocytes that surround the hair shaft in its centre. The outermost layer of the HF epithelium, the outer root sheath (ORS), is in direct contact and grows *in continuitatem* with the epidermis. The ORS, in turn, is enwrapped by the HF's connective tissue sheath (CTS), which – like the DP - is also derived from the mesoderm and connects the HF and to its surrounding skin compartments (dermis, subcutis) (Paus and Cotsarelis 1999, Schneider et al. 2009).

The HF can be viewed as a multicylindric structure serving as a hair fibre production facility and that guides the directional growth of that hair fibre onto the skin surface. This hair fibre (hair shaft) usually is composed out of a central medulla, around which a cortex and cuticle are arranged. The cuticle is the outermost layer of the hair shaft, is responsible for its shine, and maintains hair shaft integrity (Powell and Rogers 1997). The cortex contains hair keratins and keratin-associated proteins as well as trichocytes aligned to the hair shaft axis and the cuticle (Table 1.1). The medulla consists of loosely connected trichocytes and connecting proteins (Powell and Rogers 1997).

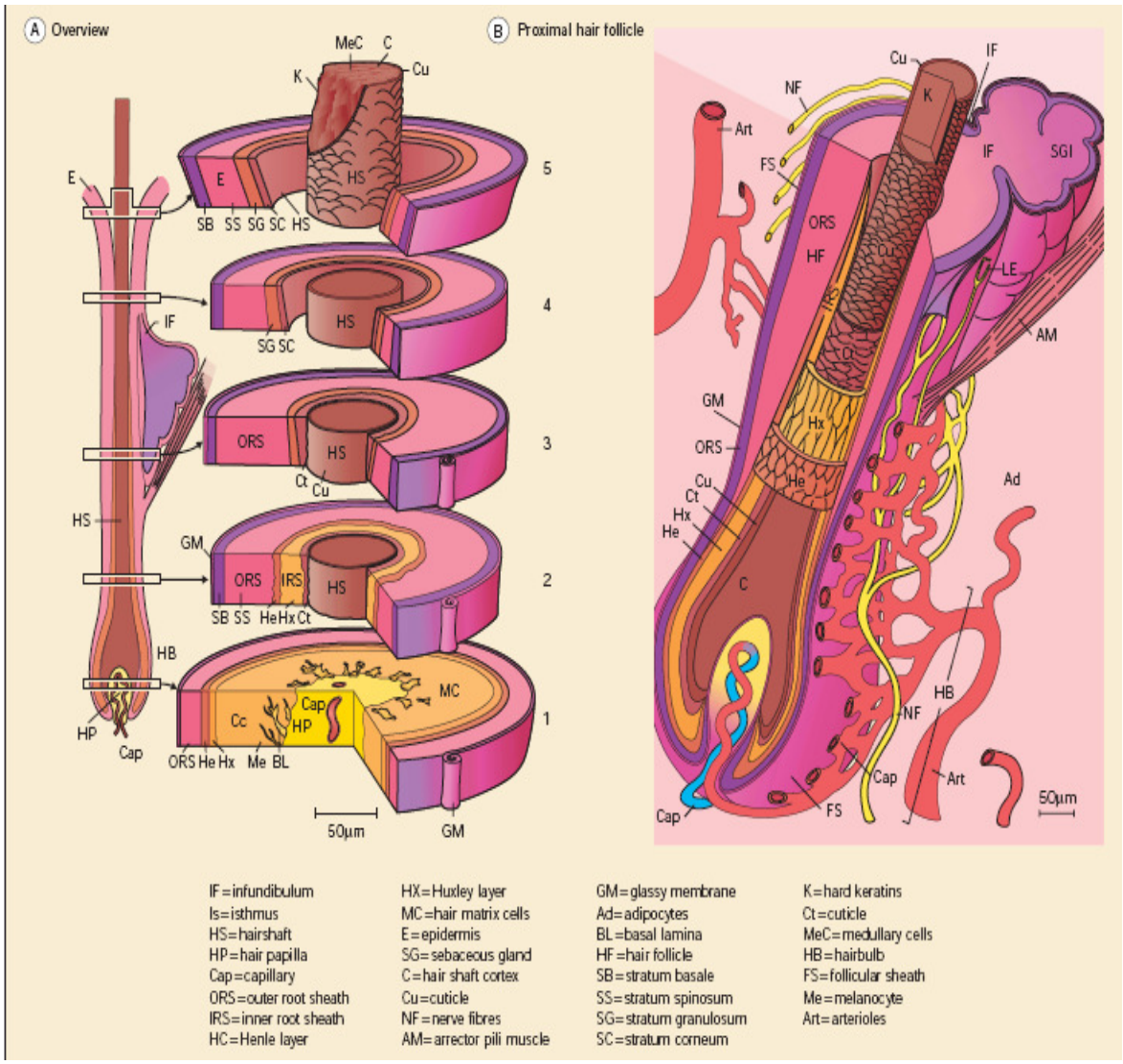


Figure 1.2 The human hair follicle in anagen VI

A) schematic overview, B) proximal HF in skin

(Paus et al.: Biology of hair and nails. In: Bologna et al. (eds.), Dermatology, 2007)

The hair shaft is surrounded by the cuticle of inner root sheath (IRS), which is then covered by Huxley's layer and finally Henle's layer, thus completing the IRS as a guiding structure for the extruded central hair shaft (Fig. 1.2). It moves outwards together with the hair fibre using the companion layer- which might be a part of the outer root sheath (ORS) or an independent cell compartment- as a sheet of drift (Langbein et al. 2002, Stenn and Paus 2001, Gu and Coulombe 2007). The IRS terminates at the level of the sebaceous gland. With the outermost layer of the ORS the eight epithelial layers forming the human HF are completed (Fig. 1.2). Each layer expresses both shared and distinct structural proteins, e.g. keratins (Langbein et al. 2004).

Human terminal HFs can display three main associated structures - the arrector pili muscle, a sebaceous gland and an apocrine gland. These glands empty their specialized products (sebum, apocrine sweat) into the follicular canal via separate ducts that insert into the ORS (infundibulum) (Fig. 1.3). The arrector pili muscle (smooth muscle) allows a variation of the usually oblique angle of hair shafts (Headington 1984). This adrenergically controlled activity of the arrector pili muscle serves thermo-insulatory needs by regulating the amount of air trapped around the associated hair shaft and is involved in reactions to external stressors (Poblet et al. 2002, Paus et al. 2007).

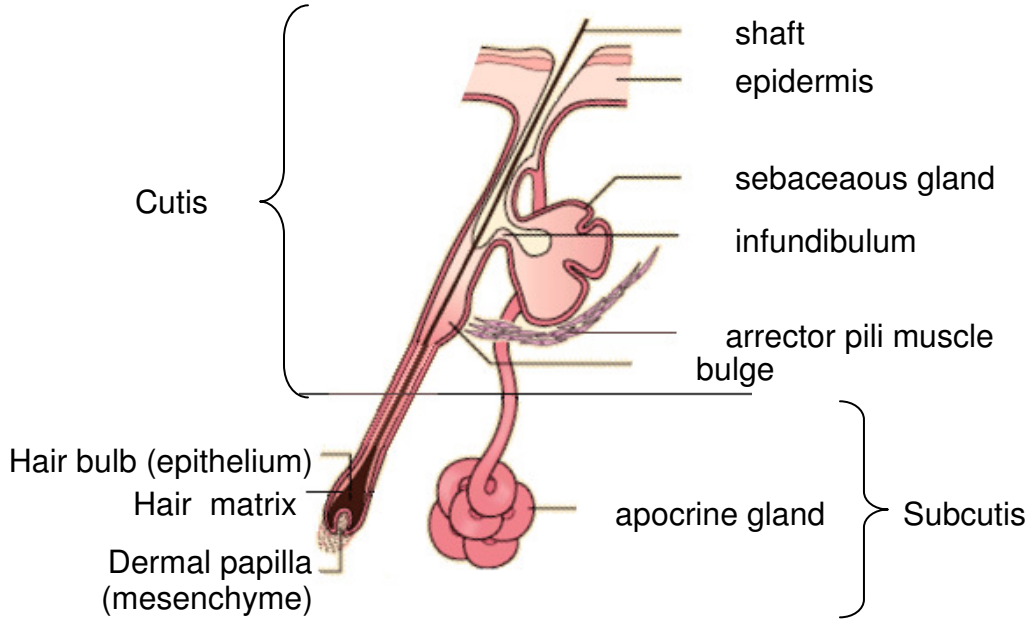


Figure 1.3 The anagen VI HF and its associated structures

(Modified after: Paus et al: Biology of hair and nails. In: Bologna et al. (eds), Dermatology, 2007)

The hair bulb is defined as the proximal part of the HF, containing the dermal papilla, matrix keratinocytes and the surrounding parties of IRS and ORS (Fig. 1.3). In terminal anagen HFs (see below), the hair bulb is situated in the subcutaneous tissue. The anagen hair bulb can be considered the actual “hair shaft factory”, since it contains the very rapidly proliferating hair matrix keratinocytes which when reaching the area above the dermal papilla, differentiate to trichocytes forming the hair shaft (Powell and Rogers 1997, Paus et al. 2007, Cotsarelis and Botchkarev 2008).

Like the HFs of all other mammals, the human HF can be structured into different compartments. At the site of insertion of the arrector pili muscle into the ORS, one finds the bulge region (Fig. 1.3), which is the major seat of follicular epithelial stem cells (Oshima et al. 2001, Ohyama et al. 2006, Cotsarelis 2006). Epithelial stem cells are identified by their very slow-cycling nature and strong expression of keratin 15 and 19 as well as of CD 200 (Oshima et al. 2001, Ohyama et al. 2006, Cotsarelis 2006, Kloepper et al. 2008). These stem cells appear to reside within an immunologically privileged tissue niche (Meyer et al. 2008).
Between the insertion of the arrector pili muscle and the infundibulum of the sebaceous gland the isthmus region, characterized by a particularly dense sensory and autonomous innervation and numerous Merkel cell complexes (Paus et al., 1997), is located (Fig. 1.3). This makes the human HF a highly sensitive tactile organ being able to detect the slightest touch brought about by HF movements. In addition to these sensory and motor functions, the follicular neural plexus might also have important regulatory potential in releasing neurotransmitters, neuropeptides and neutrophins (Paus et al. 1997, Botchkarev et al. 1998, Botchkarev et al. 2000, Botchkarev et al. 2004).

The nutrition of the HF is largely provided by the dermal and subcutaneous vascular plexus that ensheath the entire HF. They form rich, basket-like structures of arterioles, capillaries and venules with numerous shunts situated in the CTS. This vascular plexus inserts into the dermal papilla of terminal hair follicle and ensures access to nutrients, oxygen, regulatory molecules and hormones and allows excretion of toxic metabolites (Sakita et al. 1994, Mecklenburg et al. 2000, Paus et al. 2007).

TRICHOLOGY TERMS	
Hair (follicle) cycle	Autonomous, rhythmic transformation of fully developed hair follicles through phases of regression, growth, and resting; essentially controlled by the follicle itself (by an as yet enigmatic 'hair cycle clock'), yet greatly modulated by numerous systemic/extrafollicular factors
Anagen	Growth stage of the hair follicle cycle
Catagen	Involution of the lower two-thirds of the hair follicle by massive keratinocyte apoptosis
Telogen	'Resting' phase of the hair follicle cycle
Exogen	Phase of active hair shaft shedding (during anagen IV?)
Hair bulb	Lower-most portion of the hair follicle
Hair matrix	Rapidly proliferating keratinocytes that terminally differentiate to produce the hair shaft
Club hair	Fully keratinized proximal tip of hair shaft, formed during late catagen and telogen; brush-like appearance; characteristic for telogen follicles
Vellus hair (follicle)	Very short, non-pigmented, and usually non-medullated; absence of arrector pili muscle; tiny vellus follicles can display extremely large sebaceous glands (face!); undergo full hair cycle, yet much shorter than terminal hair
Terminal hair	Large, usually pigmented and medullated hair
Lanugo hair	Fine hair on the fetal body; shed *in utero* or during the first weeks of life
Vibrissae	Special sensory hair follicles with unique anatomy and biology, found on the upper lips/snout region of rodent skin, but not in humans; largest and most densely innervated hair follicles with special sinusoid blood supply; first hair follicles to develop
Tylotrich hair follicle	Large sensory hair follicles interspersed within truncal skin, most notably in rodents; extra large, long hair shaft, typically associated with double sebaceous gland and an innervated epidermal Merkel cell complex (Pinkus' Haarscheibe); second type of hair follicle to develop
Non-tylotrich pelage hair	Majority of all hair follicles, last to develop
Effluvium	Excessive shedding of hair shafts (=process)
Alopecia	Abnormal hair loss (=result)
Hirsutism	Excessive vellus-to-terminal hair conversion in androgen-dependent areas in women
Miniaturization	Terminal-to-vellus hair conversion (e.g. on the balding scalp during androgenetic alopecia); these miniaturized follicles still display an arrector pili muscle
Arrector pili muscle	Inserts at the level of the bulge; pulls up hair ('goose bumps')
Bulge	Segment of the ORS, located at the level of arrector pili muscle insertion; major seat of epithelial stem cells of the hair follicle
Secondary hair germ	Additional seat of epithelial and also of melanocyte stem cells; located between club hair and DP in telogen hair follicle
Connective tissue sheath (CTS)	Special mesenchymal follicular sheath that is tightly attached to the hair follicle basement membrane and is continuous with the follicular dermal papilla
Follicular dermal papilla (DP)	Onion-shaped, closely packed, specialized fibroblast population with inductive and morphogenic properties ; hair cycle-dependent fibroblast trafficking occurs between CTS and DP; volume of DP determines size of hair bulb and, thus, hair shaft diameter
Inner root sheath (IRS)	Packages and guides the hair shaft; cornifies
Outer root sheath (ORS)	Merges distally into the epidermis and proximally into the hair bulb; does not cornify; provides slippage plane, nutrition, regulatory molecules, and stem cells
Follicle pigmentary unit	Melanin-producing (terminally differentiated?) hair follicle melanocytes located above and around the upper one-third of the DP; transfer eu- or pheomelanosomes to differentiating hair follicle keratinocytes in the precortical matrix; goes largely into apoptosis during each catagen phase, regenerated from melanocyte stem cells in hair germ (and from non-melanogenic ORS melanocytes?) during anagen

Table 1.2 Trichology terms

(Paus et al.: Biology of hair and nails. In: Bologna et al. (eds.), Dermatology, 2007)

Human HFs can be grouped into three major, different types of hair: lanugo hair, vellus hair and terminal hair. **Lanugo hair** is the fine hair produced by fetal skin

HFs, which is shed during the first weeks of life or *in utero*. **Vellus hair** (ca. 4,9 million HF) (Mohn 1958) can be defined as usually non-medullated, non-pigmented, without an arrector pili muscle and very short. **Vellus hair** follows the full hair follicle cycle. **Terminal hair** (about 100,000 HF) is the familiar hair type of the scalp and is characterised by the presence of a medulla, long length and pigmentation (Paus and Cotsarelis 1999, Vogt et al. 2007, Schneider et al. 2009) (Tab. 1.2). In human scalp skin, 2 to 4 terminal and 1 to 2 vellus hair follicles are organized in a so-called “follicular unit” sharing one arrector pili muscular structure (Poblet et al. 2002).

1.2.2 Hair follicle cycling

The human HF undergoes life-long cyclic regression and regeneration dictated by an autonomous molecular oscillator system known as the “hair cycle clock” (Paus and Foitzik 2004), which induces controlled switches in the local signalling milieu that drive the HF through major remodelling events (Paus et al. 2007, Paus and Cotsarelis 1999). However, the exact location (DP or HF epithelium) and the molecular nature of this “hair cycle clock” is still unclear. In addition, a second, extra follicular oscillator system has recently been discovered in the dermis and subcutis of murine skin which impacts on and possible entrains the intra follicular, dominant “hair cycle clock” (Plikus et al., 2008, Plikus and Chuong, 2008). What ever controls this unique cycling mechanism, which is without parallel in mammalian biology, is responsible for driving not only HF remodelling , but also controls the major vascular, pigmentary, neuronal, matrix and connective tissue remodelling events that are tightly coupled to human HF cycling (Stenn and Paus 2001, Paus and Foitzik 2004).

The most proximal portion of the HF, the hair bulb, is also called the ‘cycling portion’ since it displays the most dramatic morphological changes during each hair cycle. The ‘permanent portion’ is formed by the epithelial stem cell-containing bulge region, which also harbours melanocyte stem cells (Nishimura et al. 2002, Aoki et al. 2009), the isthmus, the infundibulum and the follicular ostium with its canal. It shows no dramatic remodelling, although substantial remodelling takes place during each hair cycle even in the ‘permanent portion’, as well (Lindner et al. 1997).

During these cyclic transformations greatly disparate length, the HF passes through eight stages of catagen (catagen I-VIII), i.e. the apoptosis-driven involution phase of HF cycling. These are followed by telogen, the resting phase, and six stages of anagen (anagen I- VI), the growth phase, before the HF re-enters into catagen. The subcategories of the phases (Fig. 1.4) where established and characterized in murine hair follicles (Chase 1954, Müller-Röver et al. 2001), but also apply to the human HF (Kligman 1959, Kloepper et al. 2009).

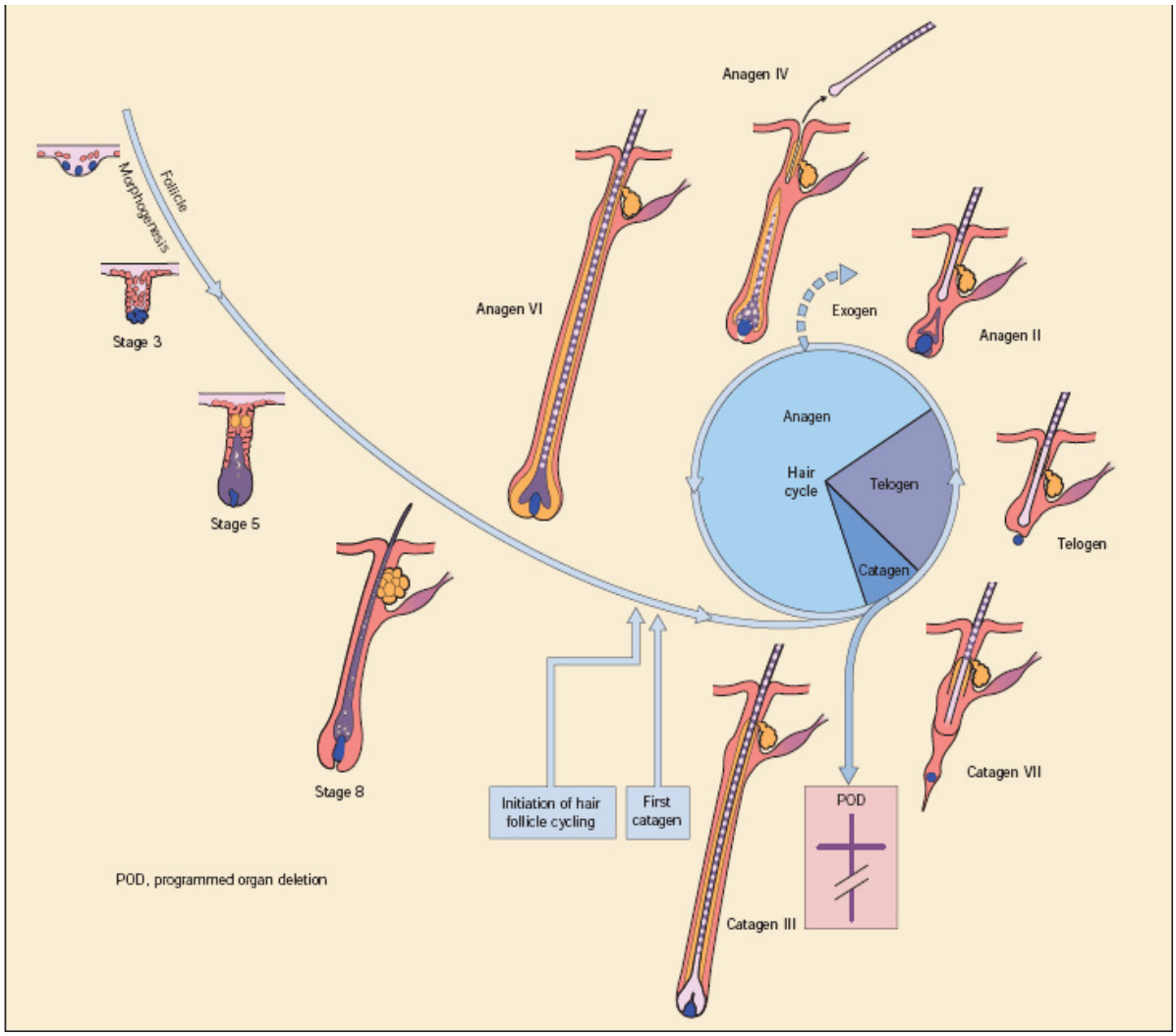


Figure 1.4 HF development and cycling

(Paus et al.: Biology of hair and nails. In: Bologna et al. (eds.), Dermatology, 2007)

Catagen is characterized by apoptosis, terminal differentiation and rapid organ involution. Apoptosis, also known as programmed cell death, occurs primarily in defined apoptotic foci, namely in the hair matrix, the proximal and central ORS and

in melanocytes, but not in the dermal papilla, where high levels of bcl-2 expression appear to suppress apoptosis (Lindner et al. 1997, Müller-Röver et al. 2000). During catagen the HF shrinks and its DP condenses, in part due to the emigration of DP fibroblasts into the proximal CTS (Tobin et al. 2003). The proximal follicle epithelium regresses to a sac-like epithelial strand, above which the club hair forms. The club hair has a characteristic brush-like proximal tip, and is anchored to the secondary hair germ (Fig. 1.4). The club hair is depigmentated at its proximal end, because this portion is generated during late catagen, i.e. at a time when melanogenesis has already ceased (Slominski et al. 1994, Slominski et al. 2005). The process of normal catagen development is highly controlled on several molecular levels. In this control, the zinc finger structure hairless gene (Hr) is one essential coordinator, which guarantees that apoptosis occurs in the right compartments and in the right sequence. In absence of functional Hr-protein (hr) the HF is destroyed during the first time it enters catagen, leading to the characteristic naked skin phenotype of the hairless mutation in mice, rats, or humans (Panteleyev et al. 2000, Paus and Foitzik 2004, Schneider et al. 2009).

In **telogen** biochemical and proliferative activity decline to a minimum compared to the other phases in HF-cycling, even though the HF is less “quiescent” than often assumed during this “resting phase”. In fact, telogen may serve as a crucial ‘brake on anagen’ and therefore have major regulatory importance in hair cycle control (Paus et al. 2000, Stenn and Paus 2001, Paus et al. 1990, Paus and Foitzik 2004, Schneider et al. 2009).

Anagen is marked by massive epithelial cell proliferation in the hair matrix and by an enlargement of the bulb and its DP, not the least due to the immigration of fibroblasts from the CTS into the DP (Tobin et al. 2003, Schneider et al. 2009). Anagen partly recapitulates key morphological aspects HF development, and the HF re-uses some of the same key modulatory factors that drive HF-morphogenesis (e.g. IGF, HGF, KGF; BMP2, TGFβ2, NT3; see table 1.3). The first sign of anagen termination is a retraction of melanocytic dendrites along with a shut-down of melanogenesis (Slominski et al. 1994 & 2005).

modulatory factor (examples)	reference	Effect	hair cycle phase
Noggin	Botchkarev et al., 2001	Signal from DP to epithelial stem cells (ESC)	anagen
STAT3	Sano et al., 2000	Required for anagen onset	telogen
KGF	Rosenquist and Martin, 1996	Signal from DP to epithelium, induction of anagen	anagen
SHH	Wang et al., 2000	epithelial proliferation	anagen
IGF-1	Philpott et al., 1994	epithelial proliferation, anagen maintenance	anagen
CutL 1	Ellis et al., 2001	Differentiation of inner root sheath	anagen/exogen
Cathepsin L	Tobin et al, 2002	Causing exogen	anagen/exogen
BMP	Botchkarev and Paus, 2003	differentiation of HS	anagen
FOXN1	Johns et al., 2005	differentiation of HS	anagen
TGFβ2	Hibino and Nishiyama, 2004	catagen induction	anagen/catagen
NT3	Botchkarev et al., 1998	catagen induction, growth modulator during morphogenesis	anagen/catagen
p75NTR	Botchkarev et al., 2000	catagen induction	anagen/catagen
Prolactin	Foitzik et al., 2006	catagen induction	anagen/catagen
INFγ	Ito et al., 2005	Inhibition of hair elongation, catagen induction (partly via TGFβ2)	anagen/catagen
IL-1	Lindner et al., 1998	catagen induction	anagen/catagen
Hr	Panteleyev et al., 2000	key catagen controller	catagen

Table 1.3 Some factors involved in the control of HF cycling

During cycling a new hair shaft factory has to be constructed in each anagen phase. In the bulge region, as the known epithelial and melanocyte stem cell reservoir (Tiede et al. 2007), bulge epithelial stem cells are mainly responsible for reconstruction of the anagen ORS, whilst the IRS, hair matrix and hair shaft keratinocytes may arise from a distinct stem cell population in the secondary hair germ, which is located between the DP and telogen club hair (see below) and

which persists throughout HF cycling (Paus and Foitzik 2004). However bulge epithelial stem cells can generate all epithelial cell lineages (Cotsarelis 2006).
Hair shaft shedding (termed '**exogen**') is the process of active hair shaft removal from the follicular canal. Exogen is linked to HF cycling, but not dependent upon it (Stenn and Paus 2001, Higgins et al. 2009). Therefore it is not a component of basic hair follicle cycling. While a new hair shaft is built up, the former hair shaft is shed, possibly during anagen IV. Cathepsine L, a lysosomal cysteine protease, appears to be involved in the control of exogen (Tobin et al. 2002, Schneider et al. 2009) (Table 1.3). If HFs malfunction or threaten to degenerate, they can be eradicated permanently by a process that has been coined "programmed organ deletion" (POD). This irreversible immunodestruction of the HF by an auto aggressive inflammatory cell infiltrate may diminish damage which could be caused by a malfunctioning HF, e.g. by protrusion of the hair shaft into the dermis, rather than its extrusion to the skin surface (Eichmüller et al. 1998). .

These signalling interactions result in a special local balance of stimulating and inhibitory factors for each single HF that is responsible for its cyclic transformations. And that markedly differs between defined HF subpopulations in distinct regions of the integument (Paus and Cotsarelis 1999, Schneider et al. 2009). These interregional differences are most obvious in the "paradoxical" effects of androgens, which stimulate e.g. the growth of male beard HFs, but inhibit that of fronto- temporal male and female scalp HFs (Randall 2007, Zouboulis et al. 2007). Recent evidence suggests that estrogens and prolactin also exert important site- and/or gender-specific regulatory effects on human hair follicle growth (Conrad et al. 2005, Langan et al. 2009).
Although HF cycling is essentially independent of functional innervation and blood supply (Maurer et al. 1998, Philpott et al. 1996), substantial remodelling of the HF's vasculature and innervation occurs during each HF cycle and both neural and vascular inputs can modulate HF cycling (Mecklenburg et al. 2000, Paus and Foitzik 2004).

1.2.3 Endocrine controls of human hair follicle cycling

Despite its essentially autonomous growth regulation, the HF is highly sensitive to alterations in systemic endocrine signalling, e.g. by androgens (Randall 2007,

Zouboulis et al. 2007), estrogens (Ohnemus et al., 2006), and prolactin (Foitzik et al. 2009, Langan et al. 2009). . This may serve to coordinate and synchronize the activities of larger cohorts of HFs , e.g. in response to environmental or nutritional changes (Yen 2001).

Though androgens have long been viewed as the main regulators of human hair growth (Randall 2007), the importance of other endocrine controls of human hair growth is being increasingly appreciated (e.g. Ohnemus et al. 2006, Foitzik et al. 2009) (Fig. 1.5).

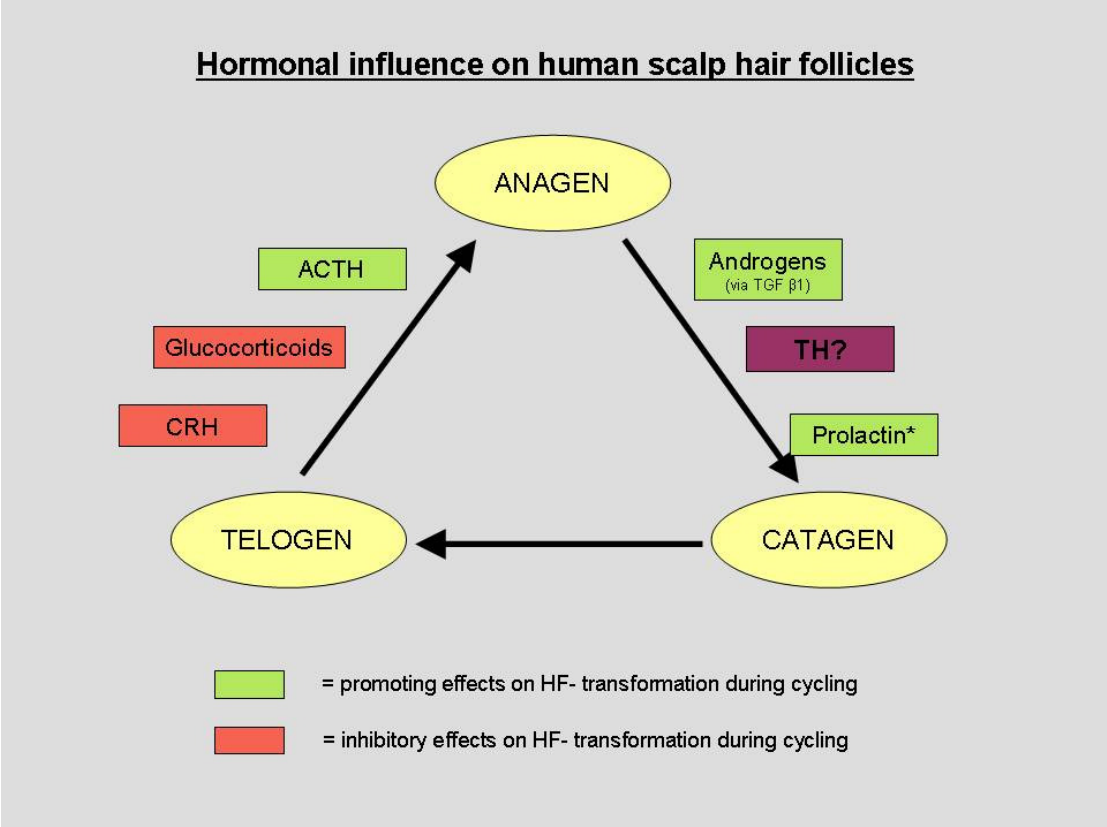


Figure 1.5 Hormonal controls of HF-cycling

Selection of important hormones involved in HF-cycling and homeostasis. *Prolactin: catagen induction in occipital male scalp HFs (Foitzik et al. 2006), but catagen inhibition in female front temporal HFs (Langan et al. 2009).

In addition, the HF and its associated sebaceous gland are becoming increasingly appreciated as astoundingly active producers and metabolizers of a wide range of different hormones, ranging from sex steroids and prolactin, via erythropoietin (Bodó et al. 2007a) and endocannabinoids to all key endocrine mediators of the hypothalamic-pituitary-adrenal axis (HPA: CRH→POMC→ACTH→ cortisol) (Telek et al. 2007, Foitzik et al. 2006).

Centrally released, systemically active mediators of general stress responses are e.g. corticotrophin releasing hormone (CRH), adrenocorticotrope hormone (ACTH), β-endorphin, prolactin (PRL) and catecholamines (Fig. 1.5). CRH is secreted by the hypothalamus to stimulate the HPA axis resulting in secretion of glucocorticoids. These ensure an adaptive response to restore homeostasis of human HF but also suppress immune response (Slominski et al. 2007). As an extra pituitary site of PRL- synthesis (Foitzik et al. 2006) and expressing both PRL and PRL receptors (PRL-R) human anagen VI HFs utilize PRL as an autocrine hair growth modulator with catagen-promoting functions and hair growth-inhibitory effects.

While the central role of androgens in hirsuitism and androgenic alopecia constitutes a central paradigm of clinical trichology, androgens are actually dispensable for fetal HF development and for HF cycling as such. Individuals who lack functional androgen receptors still develop vellus hair and have largely normal scalp hair (Dawber 1997). However, androgens do regulate mostly sexual aspects of hair with “paradoxical” effects, depending on the body site: a comparison of beard growth and balding shows the complex interactions between local signalling milieu (see above) and hormonal influence on the whole organism (Paus et al. 2007). Thus, a rise in systemic androgen levels during puberty is crucial for inducing the vellus-to-terminal transformation of androgen-dependent (i.e. beard, axillary and pubic) HFs. These “paradoxical” effects of androgens probably reflect very different responses of a given hair follicle population to androgen stimulation (e.g. secretion of hair growth promoting IGF-1 versus hair growth-inhibitory TGFβ1 by DP fibroblasts after exposure to androgens (Itami and Inui 2005).

It is on this background of complex, and often seemingly contradictory, endocrine controls of human HF cycling that the current study on the effects of TH on human HF growth, cycling, and pigmentation has to be interpreted.

1.2.4 Hair follicle pigmentation

The pigmentation of human HFs is tightly coupled to the anagen phase of the HF cycle (Slominski et al. 2005). In the anagen hair bulb, the melanocytes essential for hair pigmentation are situated within the hair matrix epithelium, on the basement membrane surrounding the dermal papilla, where they form the HF

pigmentary unit (HPU). These specialized, differentiated HF melanocytes transfer melanin to pre-cortical keratinocytes (Michelet et al. 2009). Two classes of melanins are generated: eumelanin, and phaeomelanin. Eumelanins are “poly-quinones” organized in polymers that are synthesized from tyrosine and DOPA (3,4- dihydroxyphenylalanine). Phaeomelanins are smaller biopolymers than eumelanins and also derive from tyrosine. The relative predominance of eu- versus pheomelanin determines the visible hair colour. Besides by the quality and quantity of its melanins, the resulting hair colour also is influenced by the thickness of the hair shaft (Slominski et al. 2005).

Melanogenesis represents a very complex biochemical cascade of events that is restricted to specialized organelles (melanosomes) and is regulated by a multitude of different factors and co-factors, ranging from transcription factors, via enzymes to hormones, cytokines, neuropeptides and adhesion molecules (Yamaguchi and Hearing 2009, Park et al. 2009). Follicular melanogenesis is initiated by hydroxylation of L-phenylalanine to L-tyrosine by phenylalanine hydroxylase (PAH) (Schallreuter et al. 1998, Slominski et al. 2005). The key enzyme for melanogenesis is tyrosinase, which requires the presence of copper and oxygen for its catalytic activity. Tyrosinase catalyzes the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-dopa). Once L-dopa is formed, melanogenesis can proceed through redox reactions and intra-molecular transformations (Slominski et al. 2005).

The melanoblasts migrate from the neural crest, enter the developing HF and as precursors expressing c-kit they migrate into the stem cell factor (SCF)-supplying hair follicle epithelium (Peters et al. 2002). These melanocytes are maintained by small numbers of melanocyte stem cells located at the HF stem cell niche- the bulge region (Nishimura et al. 2002, Aoki et al. 2009, Sarin and Artandi 2007,). The differentiated, c-kit-positive melanocytes populate the hair bulb (Peters et al., 2002), whose epithelium produces the c-Kit ligand, SCF (Peters et al. 2003). C-kit-negative melanoblasts preferentially target the ORS and the bulge region in the HF. Furthermore, c-kit is required during the hair growth cycle for activation of HF pigmentary unit melanocytes during anagen III-VI (Botchkareva et al. 2001), when active melanin synthesis accompanied by massive tyrosinase activity occurs (Slominski et al. 2005).

The melanocytes of the HF are larger than epidermal melanocytes, develop longer dendrites, but contact fewer keratinocytes than epidermal melanocytes (Scott et al. 2002). As the normally only site of pigment production for the hair shaft the bulb contains highly melanogenic melanocytes, a few less differentiated pigment cells and amelanotic hair bulb melanocytes. Transfer of melanin granules to cortical and medullary keratinocytes in the growing hair shaft has been explained by the extension of filopodia from melanocyte dendrites. This dendrite formation acts as a transporter device for melanosomes towards the keratinocytes (Scott et al. 2002 & 2007, Aspengren et al. 2009). Thus, melanocyte dendricity is an important phenotypic aspect of melanin transfer to keratinocytes. Furthermore melanin synthesis and transfer depend on the availability of melanin precursors and several receptor-dependent and receptor- independent signalling pathways (Tobin 2008, Park et al. 2009).
The produced melanosomes are only minimally digested by keratinocytes, mainly those of the hair cortex, and therefore stain the hair shaft in its full length (Slominski et al. 2005). Melanogenically active melanocytes are restricted to the precortical hair matrix of the anagen hair follicle from where melanosomes are transferred to the hair shaft cortex, and much less to the medulla and cuticle. To ensure renewed hair shaft pigmentation during each new anagen cycle, the activated hair germ stimulates resident, dormant melanocytes to divide in and populate the new anagen hair bulb (Nishimura et al. 2002, Aoki et al. 2009, Slominski et al., 2005).

HF-melanogenesis is highly influenced by several different hormones and cytokines, both stimulatory and inhibitory ones. α-MSH, β-endorphins, histamine, prostaglandins, catecholamines as well as estrogens, androgens and vitamin D enhance the follicular melanogenesis using different second messenger pathways. In contrast, serotonin, melatonin, acetylcholine and dopamine acting via its D2 receptor display an inhibitory effect on melanogenesis. These factors are thought to be essential for the “fine-tuning” of HF pigmentation (Slominski et al. 2004, Park et al. 2009, Tobin 2008). Most recently, even TH have become implicated as a potential regulatory element in hair pigmentation (Redondo et al. 2007), even though it remains unknown whether THs can directly alter human HF pigmentation.

1.3 Thyroid hormones

1.3.1 Thyroid hormones and their metabolism

The thyroid gland contains follicular (epithelial) cells for hormone synthesis. Several follicular cells form a follicle wherein the thyroid hormone can be stored as colloid (Dunn and Dunn 1999). The very complex system of thyroid hormone (TH) synthesis, storage and release is sharply controlled by feedback regulation as well as other regulatory mechanisms to ensure that the healthy thyroid gland is always capable of releasing the necessary amount of TH that is required to maintain systemic/metabolic homeostasis (Molina 2006, Singer 2009).

For TH synthesis, a sodium-iodide symporter (NIS) in the basolateral membrane of thyroid follicular epithelial cells guarantees an energy-dependent iodide uptake. In the apical membrane of these cells lie at least two iodide channels, one of which is called pendrine (Everett et al. 1997, Bizhanova and Kopp 2009), allowing iodide efflux into the follicular lumen. Here the glycoprotein thyroglobulin (TG), containing several tyrosine residues, functions as a matrix for TH-generation and serves as the main part of the colloid at the same time (Dunn and Dunn 1999). The follicular epithelial cells produce TG in a thyrotropin (TSH)-dependent manner and secrete it mainly into the follicular lumen but also in small amounts into the peripheral blood circulation (Fekete and Lechan 2007). Strikingly, TG transcripts have recently even been found in human scalp hair follicles (Bodó et al. 2009b).

In the thyroid follicle lumen, iodide is oxidated to iodine by thyroid peroxidase (TPO), a glycoprotein located at the apical membrane (Kopp 2005). This step relies on the presence of H_2O_2 as a rate-limiting factor. H_2O_2 is regenerated in active thyroid follicular epithelium by thyroid oxidase type 1 and 2 (THOX1, THOX2) (Gérard et al. 2002), contingent on calcium availability and hydrogenated nitrogenamide-adenine-dinucleotide phosphate (NADPH). Then TPO serves as an essential tool for coupling iodine to the tyrosine residues of TG at carbon 3 or 5. The iodination yields monoiodinated (MIT) and diiodinated (DIT) tyrosine residues that are enzymatically united to triiodothyronine = T3 (DIT + MIT) or

tetraiodothyronine = T4 (DIT + DIT) by thyroid peroxidase (Fig.1.6). The generated T3 and T4 can be stored in the colloid for 2-3 months (Nilsson 2001).

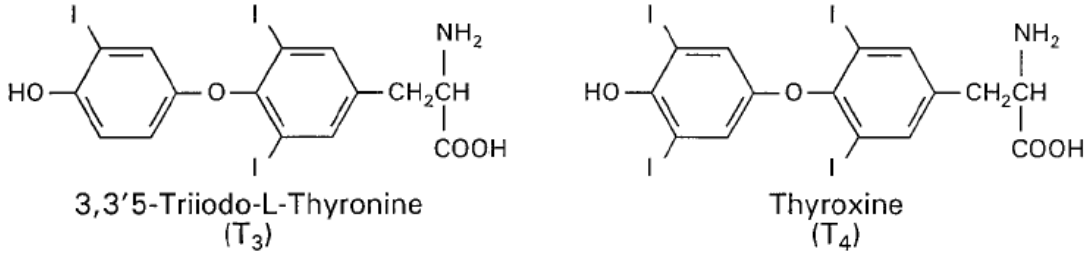


Figure 1.6 Structure of thyroid hormones

These conjugated residues while still bound to TG undergo endocytosis, the formed vesicles fuse with follicular epithelial phagolysosomes in which digestion and cleavage of TG takes place. TG and non conjugated MIT and DIT are reused in hormone synthesis after transportation in the colloid while T3 and T4 are released into the circulation at the basal membrane of the thyroid follicular cell (Nilsson 2001, Molina 2006).

The thyroid gland releases less T3 compared to T4, leading to a higher plasma concentration of T4 (ca. 90nM) compared to T3 (ca. 2nM) (Molina, 2006). T4 serves as a prohormone for T3 and is converted by 5' outer ring deiodination to T3, mainly in the liver but to some extent in all peripheral tissues investigated so far. T4 has a 100 times lower affinity to the thyroid hormone receptor than T3. T4 binds more tightly to binding proteins: both TH are mainly bound to thyroid-binding globulin (TBG) but also to transthyretin and albumin. Only 0.3% of T3 and 0.03% of T4 occur in their free form in human blood. Additionally, T4 has a longer biological half-life of 7 days than T3 (1 day) since only free TH are excreted by the kidneys (Bizhanova and Kopp 2009).

About 80% of T4 are deiodinated in the periphery, while most of the deiodinase activity yields active T3 (33% yield reverse T3 [rT3] with little or no TH activity) (Molina 2006). By further deiodination rT3 and T3 are converted to the biologically inactive T2, although recent studies suggest a role of T2 in rat fat metabolism (Lanni et al. 2005). For deiodination, different deiodinases (DIO) are required, whose expression patterns differ between tissues. DIO 1 is expressed predominantly in liver, kidney and thyroid and catalyzes conversion of T4 to T3/rT3

and T3/ rT3 to T2 and is important for the feedback mechanism. Type 2 DIO has only outer ring deiodination activity and converts T4 to T3. It provides more than 50% of the T3 used in brain, brown adipose tissue, cardiac and skeletal muscle. It is also active in thyroid and pituitary where it is necessary for feedback regulation of TSH secretion. DIO 3 only displays inner ring function thus inactivating T4 to rT3 and T3 to T2. It is mainly expressed in brain, placenta and skin (Köhrle 1999, Safer et al. 2009***).*** Intriguingly, DIO2 has been found to be transcribed by human bulge HF epithelial stem cells *in situ* (Ohyama et al. 2006, Tiede et al. 2009), which suggests that these stem cells can actively deiodinate T4 into T3.

1.3.2 Neuroendocrine controls of thyroid hormone synthesis

TH synthesis and release are tightly regulated by the hypothalamic-pituitary-thyroid axis (HPT-axis) (Larsen et al. 2007, Fekete and Lechan 2007, Singer 2009). At the hierarchical top of this regulatory axis, thyrotropin releasing hormone (TRH), is synthesized in the paraventricular nucleus of the hypothalamus. Then, TRH is released via the median eminence of the hypothalamus. Here nerve endings lead the TRH into the portal capillary plexus which passes the capillary system of the anterior pituitary gland (Fekete and Lechan 2007). TRH binds to G_q-cell membrane receptors (TRH-R) on basophilic cells of the anterior pituitary gland. The resulting activation of phospholipase C and the generation of inositol triphosphate and diacylglycerol raises the intracellular calcium concentration, which serves as the stimulus for exocytosis and release of thyroid -stimulating hormone (TSH) into the systemic circulation (Fekete and Lechan 2007).

TSH binds to a G-protein coupled receptor at the baso-lateral membrane of follicular epithelial cells of the thyroid. Via second messenger pathways, the intracellular cyclic adenosine monophosphate (cAMP) concentration increases whereby TSH provokes a stimulation of all steps involved in thyroid hormone synthesis (e.g. iodine uptake, storage, release, cell proliferation, protection from apoptosis). TSH- and TRH-release underlie negative feedback regulation (Fig. 1.7) by T3 and T4. T4 is converted to T3 mainly peripherally but also in the hypothalamus and anterior pituitary to T3 by type 2 deiodinase. Therefore TSH-release underlies at least a dual inhibition directly via circulating T3 and T4, and

indirectly through inhibition of TRH-release (Shupnik et al. 1989). In addition, dopamine, somatostatine and glucocorticoids at high levels are also potent partial suppressors of TSH-release (Yen 2001, Molina 2006)

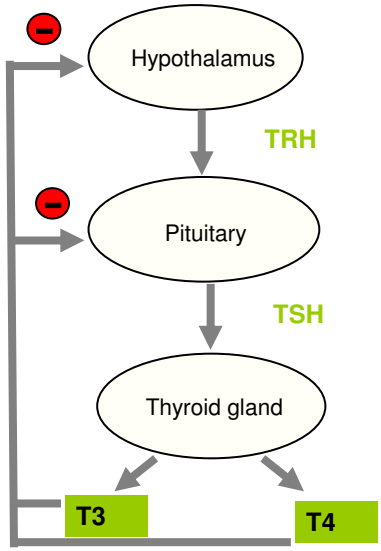


Figure 1.7 Control and feedback regulation of thyroid hormone production along the HPT axis

TRH = thyreotropin releasing hormone, TSH = thyreotropin or thyroid stimulating hormone T3 = triiodothyronine, T4 = thyroxine, ⊖ =inhibition, text written in green = stimulation

In the context of HF biology, this HPT axis (Singer 2009) has recently become particularly relevant, since it was shown that human scalp HFs express TRH, TRH- and TSH- receptors (Gáspár et al. 2009, Bodó et al. 2009b) and that TRH is a strong stimulator of human hair growth in organ culture (Gáspár et al. 2009). Therefore, some up-stream elements of the HPT axis also operate in human skin, thus making the ”thyroid-skin connection” an even more fascinating and clinically relevant research frontier in cutaneous (neuro-)endocrinology (Paus 2010).

1.3.3 Classical and non-classical signalling of thyroid hormones

TH primarily exert nuclear hormone receptor-dependent genomic effects, but also non-genomic TH actions are now recognized (Davis et al. 2008). For their genomic action, TH enter the cell via carrier-dependent, energy-dependent or Na^+-dependent processes. They enter the nucleus and bind to thyroid hormone receptors (TR) of which several isoforms exist (TRα1, TRα2, TRβ1, TRβ2, TRβ3) (Fig. .8). TR are c-erb-A proteins and belong to the nuclear hormones super-family

consisting of thyroid hormone, glucocorticosteroid, retinoic acid, and vitamin D receptors (Aranda and Pascual 2001). TRs bind TH with high affinity and specificity (85% T3, 15% T4), and usually do so in a dimeric form with the retinoid-x-receptor (Moore and Guy 2005). TRs are DNA-binding factors, which, in their unoccupied form, are bound to TH response elements (TRE) in the promoter region of TH target genes where they associated with a couple of co-repressors.

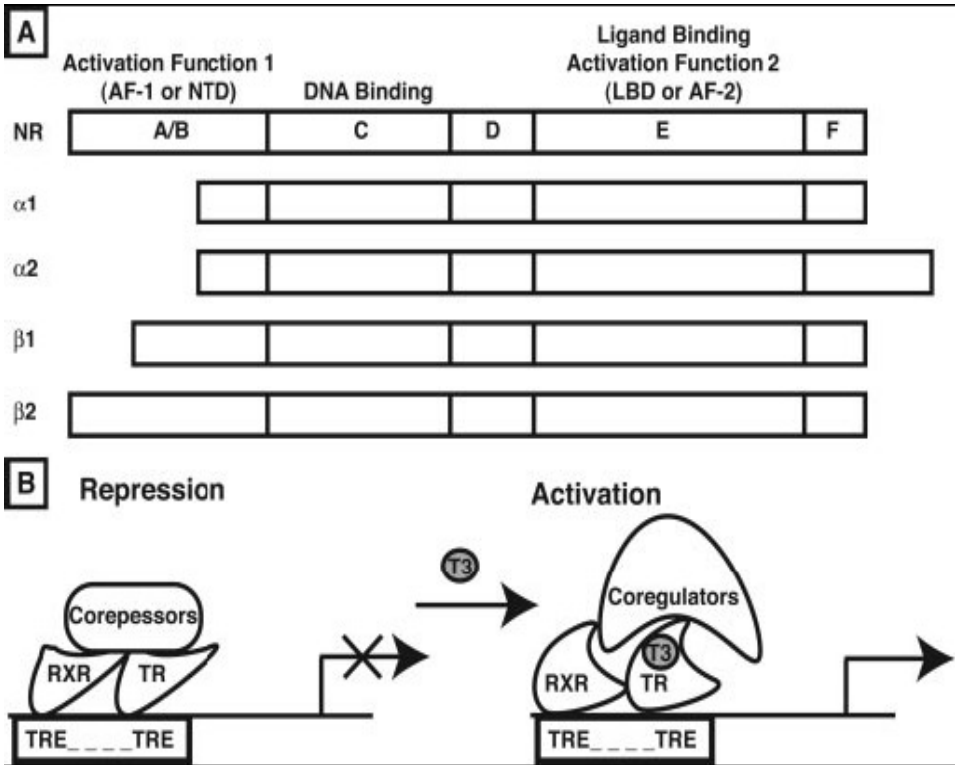


Figure 1.8 Nuclear action of thyroid hormones

(Moore and Guy 2005; A)diagram of the primary structure of the TR subtypes; B) mechanism of TR transcriptional control. TR forms a heterodimer complex with RXR that recognizes specific TREs; AF, activation function; LBD, ligand binding domain; NTD, amino-terminal transactivation domain; T3,triiodothyronine.)

TH binding then leads to co-repressor dissociation and binding of co-activators which results in modulation of the gene transcription (Fig. 1.8).

In contrast to activating TH binding, the TRE can also be negatively regulated (Feng et al. 2000), thus repressing genomic transcription upon ligand binding while the unliganded receptor guarantees a constitutive level of gene transcription.

Activation of the nuclear machinery by T3 has a latency of hours or days (Yen 2001). Nevertheless, a number of TH-effects occur rapidly and are unaffected by

inhibitors of transcription and protein synthesis (Wrutniak-Cabello et al. 2001) e.g. T4 inducing IP3 via cytosolic binding on TRβ and calcium signalling, and TH augmention of the effects of IFNγ via PKC and PKA (Lin et al. 1997). Furthermore cell culture studies indicate a rapid and non-genomic regulation of the Ca2+ATPase enzyme, the Na+ channel via PKC, the K+ channel via PI3-kinase, the Na+/H+ anti-porter via PKC and MAPK by thyroid hormones (Davis et al. 2008). The activation of MAPK suggests a G-protein coupled membrane receptor for thyroid hormones involved, of which the activation via several steps leads to phosphorylation of a serine residue one of the zinc finger structures in TR resulting in increased transcriptional activity (Davis et al. 2008).

In parallel to these fast-acting signalling pathways, T4 was also shown to activate MAPK- kinase (MEK), which phosphorylates tyrosine residues in signal transducers and activators of transcription (STAT1α and STAT3), thus inducing both, the nuclear translocation of both STAT, and further serine phosphorylation by MAPK (Lin et al. 1999). Using these messenger pathways, TH are able to potentiate antiviral or immunomodulatory effects of IFNγ (Lin et al. 1999). Such non-genomic effects of TH have been reported in several tissues, e.g. T4 displaying rapid thermogenic and lipolytic activities or T3 enhancing cardiac output and reducing systemic vascular resistance within 3 minutes via catecholamines, since the action of T3 could be blocked by the β-catecholamine- receptor antagonist esmolol (Schmidt et al. 2002). However, no specific G-protein coupled membrane receptor binding any isoform of TH could be identified so far.

1.3.4 Physiological functions of thyroid hormones

TRs are expressed in all tissues, although the relative expression level of TR isoforms may vary among tissues. In addition, the functional role and relative importance of TH varies greatly between different tissues (Yen 2001). Generally, the TH-induced transcription of Na^+-/K^+-ATPase leads to increased oxygen consumption, but the transcription of uncoupling protein enhances heat generation without adenosine triphosphate (ATP) production (Yen 2001). TH- regulated, protein synthesis and degradation contributes to growth and differentiation.

Furthermore TH are crucial for several developmental processes. One of the most impressive examples is amphibian metamorphosis, which depends on TH (Paris et

al. 2008), while hypothyroidism in neonate humans seems to be associated with a damage of axonal growth of various brain cells (e.g. cerebellum) and a decrease in IQ (Gyamfi et al. 2009). In the fact, the appearance of TH during evolution may have constituted a major developmental landmark, since many of the genes encoding thyroid-associated proteins like TR, TPO, TRH, TSH, NIS and deiodinases all can already be detected in our amphibian ancestors, and have thus been evolutionarily highly conserved. This suggests that the development of TH and their signal transduction systems have played an important role in vertebrate evolution (Paris et al. 2008). .

TH directly affect cellular energy metabolism, namely the key respiratory chain enzyme mitochondrial cytochrome-c-oxidase (Mutvei and Nelson, 1989, Sheehan et al. 2004, Pillar and Seitz 1997). Cytochrome-c-oxidase (also known as complex IV of the mitochondrial respiratory chain) is responsible for the catalyzation of the transfer of four electrons to molecular oxygen. Thereby dioxygen (O_2) is reduced to water. Simultaneously, the enzyme pumps protons across the inner membrane of mitochondria from the matrix to the cytoplasmic side. This process is essential for the oxidative phosphorylation catalyzed by Complex V, the last complex at the inner membrane of mitochondria, resulting in the production of adenosine tri-phosphate (ATP) (Arnold et al. 1998).
Cytochrome-C-oxidase consists of three subunits, the mitochondrial encoded subunits I and II execute the electron transfer function of the holoenzyme while the nuclear encoded subunit III might modulate the proton pumping and/or is involved in the assembly and stability of the mitochondrial encoded subunits (Mutvei and Nelson 1989). Since cytochrome-c-oxidase seems to be a rate limiting enzyme (Sheehan et al. 2004) , it is intriguing to ask whether an effect of T3 and/ or T4 on cytochrome-c-oxidase can be verified in human HFs, since this would likely indicate a major impact of TH on human HF energy metabolism.

TH also display several tissue-specific effects (Table 1.4). For example, in the cardiovascular system, TH have an inotropic and chronotropic effect and decrease the systemic vascular resistance; together, this leads to an increase in cardiac output (Yen 2001). Moreover TH enhance total protein expression in the heart, especially of those proteins that are critical for cardic function such as myosin

heavy chain (Dillmann 1990). Furthermore, in fat, intracellular lipid accumulation and adipocyte proliferation is reduced by TH, and TH increase the number of β-adrenoreceptors on adipocytes, thus promoting catecholamine-mediated lipolysis. TH metabolites can furthermore reduce cholesterol level, and increase lipid metabolism (Moreno 2008). In the liver, TH is involved in triglyceride and cholesterol metabolism and modulates cell proliferation and mitochondrial respiration, while in brain it stimulates the axonal growth of nerve cells. TH are among the essential factors of a normal brain development. (Oppenheimer and Schwartz 1997, Gyamfi et al. 2009). TH is critical for normal bone growth and development. TH increases alkaline phosphatase activity and osteocalcin expression in osteoblasts as well as it affects osteoclasts (Gouveia et al. 2002). TH may act on bone via TH stimulation of growth hormone and insulin-like growth factor I (IGF-I) or by direct effects on target genes (Yen 2001).

Tissue affected	TH- effect	Reference
heart	Increased cardiac output	Yen 2001, Schmidt et al. 2002
	Enhanced myosin heavy chain synthesis	Dillmann 1990
fat	Reduction of cholesterol levels by TH derivatives	Moreno et al. 2008
Vascular system	Lowered systemic vascular resistance	Schmidt et al. 2002
brain	Development	Oppenheimer and Schwartz 1997
	Axonal growth	Gyamfi et al. 2009
bone	Increased turnover	Gouveia et al. 2002
Various tissues	Up regulation of EPO-expression	Ma et al. 2004, Bodó et al. 2007a
	Enhanced expression of COX1 in mitochondria	Sheehan et al. 2004

Table 1.4 Examples for tissue effects of thyroid hormones

Intriguingly, T3 has been reported to up-regulate erythropoietin (EPO) expression in a hepatic epithelial cell line *in vitro* (Ma et al. 2004). EPO is a glycoprotein that essentially works as a cytokine. EPO responds to a fall in partial oxygen pressure

(p O_2) in arterial blood, renal EPO-secretion occurs and enhances erythropoiesis in bone marrow. The putative T3-regulation of EPO maybe relevant in the context of this thesis project, since it was recently identified that human scalp HFs are an important extrarenal source and a non-classical, non-hematopoietic target of EPO (Bodó et al. 2007a, Paus et al. 2009). Hypoxia even up-regulates intrafollicular EPO expression (Bodó et al. 2007a). EPO activates the erythropoietin receptor (EPO-R), which inhibits the pro-apoptotic machinery of EPO-R expressing target cells, induced by changes in gene expression (Jelkmann 2004). EPO suppression of apoptosis is not restricted to erythropoietic cells but is also observed in several other tissues – including human HFs (Bodó et al. 2007a). Thus, EPO may serve as a cell-protectant which may protect cells, for example, against ischemia-induced apoptosis. Moreover, it has been hypothesized that the skin itself is able to directly contribute to the up-regulation of EPO plasma levels in response to hypoxia (Paus et al. 2009)

1.4 Cutaneous effects of thyroid hormone abnormalities

1.4.1 Causes of hypothyroidism and hyperthyroidism

Hypothyroidism can be defined as insufficient TH action with low serum levels of free T4 often along with elevated TSH-levels (Molina 2006). One has to distinguish between primary and secondary hypothyroidism. The primary form results from a disease of the thyroid gland. It might be due to congenital hypothyroidism accompanied by mental retardation and cretinism or to dietary iodine deficiency as well as iatrogenic thyroid gland destruction (e.g. due to radioactive iodine therapy for autoimmune thyroiditis or thyroid carcinoma). Autoimmune thyroid disease, is most prominently represented by Hashimoto thyroiditis (Hashimoto 1912; due to auto antibodies against TPO and/or TG, which can result in hypothyroidism. Typically, the serum TSH-level is high in this case, while T3 can be low or normal in serum (Pearce et al. 2002, Singer 2009)

Secondary hypothyroidism is caused by disorders in the anterior part of the pituitary gland or the hypothalamus leading to an impaired TSH-release (e.g. due to neoplasia in hypothalamus or pituitary gland) and therefore a lack of stimulation

thyroid gland by TSH. TSH, T3 and T4 serum levels, here, are decreased. Some of the patients show TH- resistance, mostly due to a thyroid receptor-β mutation, which is a rare, inherited lower responsiveness of target tissues to TH. Here TSH-, T3- and T4- levels are high, probably as a compensatory mechanism to reach a nearly euthyroid state (Paschke and Ludgate 1997, Fekete and Lechan 2007).

Hyperthyroidism is defined as an excessive functional activity of the thyroid gland with high serum levels of free T3 and free T4. TSH-levels can be low as in the immunogenic form e.g. Grave's disease (Brent 2008) or M. Basodow, defined as immunogenic hyperthyroidism with exophthalmia (von Basedow 1840) or in non-immunogenic forms like toxic goitre. In Grave's disease the TSH-receptor gets stimulated by auto antibodies, a stimulatory immunoglobulin G, yielding excessive production of T3 and T4 whilst TSH-production is repressed via the feedback mechanism (Molina 2006, Brent 2008, Kopp 2005). In toxic goitre, autonomous areas that exist physiologically to fight a lack of exogenous iodide intake are enhanced. A severe iodide deficiency yields goitre and finally results in thyroid autonomy (Singer 2009). Rare causes for hyperthyroidism are sub-acute thyroid inflammation, thyroid carcinoma or excessive exogenous TH-substitution. TSH-secreting adenoma as one of the non-immunogenic forms of secondary hyperthyroidism is characterized by high TSH-serum levels, since the feedback mechanisms outlined above do not work in this case (Larsen et al. 2007, Singer 2009).

1.4.2 Skin and skin appendage abnormalities associated with hypo- and hyperthyroidism

The resulting abnormalities in TH serum levels impact on the function of skin and its appendages, with both similarities and characteristic differences between hypo- and hyperthyroidism. In the skin, hypothyroidism leads to characteristic, clinically detectable changes. Hypothyroid patients typically, though not always, present a pale and cold skin due to cutaneous vasoconstriction, sometimes in association with subcutaneous oedema. The morphological thinning of the epidermis with a thickening of the stratum corneum appears as a coarse, dry and scaly upper skin layer (Table 1.5). In many patients, excessive accumulation of hydrophilic

mucopolysaccharids in the dermis causes a generalized interstitial oedema (myxoedema). This myxoedema can even cause secondary compression complications in the affected region. Furthermore the skin might appear slightly yellow because it suffers from secondary carotenaemia (Fistarol 2002, Stüttgen,1974): TH affects the hepatic transformation of carotene into vitamin A. Therefore, in hypothyroidism there is also a relative hypovitaminosis A (with resulting defects in normal keratinocyte differentiation and epidermal barrier formation), and a higher amount of carotene stays in the circulation. Additionally, hypothyroidism leads to a lessening of eyebrow hair in the lateral third of the eyebrows

By reason of the systemic effects of TH, like enhanced thermo-genesis, heightened cardiac output and raised sweat-production the skin in hyperthyroidism gets warm, soft and moist. Even the thickness of the epidermis is modulated by the T3 serum level, (higher serum-T3 correlates with enhanced epidermal thickness) . Furthermore, at the site of vessels and adnexa haemosiderin deposits can be found in hyperthyroid conditions (Banba et al. 1999). A form of myxoedema that is particularly prominent in the pretibial location can be seen in hyperthyroidism. This myxoedema rather is comparable to the aggregation of mucopolysaccharides in exophthalmia than to myxoedema in hypothyroidism (Fistarol 2002).

Since cytokeratins, which can be grouped into acidic (type I) or basic (type II) keratins, form 30% of the epidermal protein, it is most likely that they are involved in at least some of these changes in human skin due to TH-disorders. Indeed, keratin expression is tightly regulated by various endocrine factors, including THs (Ramot et al. 2009). TH-regulated cytokeratin genes like CK6 and CK14 reportedly display a thyroid hormone responsive element (TRE) in their promoter region via which the transcription of this keratin gene is regulated upon TH binding to TR (Radoja et al. 1997, Freedberg et al. 2001, Blumenberg et al. 1992). As in the rest of the skin epithelium, keratins are also prominently expressed in human HFs, e.g. cytokeratin 6 (CK6, type II) that is typically expressed by proliferating ORS keratinocytes (Langbein and Schweizer 2005), and cytokeratin 14 (CK 14, type I), which is highly expressed by proliferating keratinocytes (Langbein et al. 2004,

Radoja et al. 2004). Therefore, it is conceivable, but remains to be demonstrated, that TH-induced alterations in HF keratin expression could induce in specific changes in HF-morphology and hair shaft structure (Schweizer et al. 2007).

Hypothyroid patients display HFs that are dry, brittle and dull due to a lower sebum production. 50% of the patients suffer from diffuse alopecia which seems to result from a shorter anagen phase and slower growth. Correlating with the myxoedema in the dermis the HFs develop mucine concretions. (Fistarol 2002, Molina 2006). The HFs of hyperthyroid individuals show a thin and fine texture and appear fatty. In hyperthyroidism alopecia also occurs but only in 20-40% of patients. This is explained by a shortening of hair cycling with a shortened anagen- and telogen-phase. There is considered to be a shorter final length of the hair due to the shorter growth phase although proliferation in anagen is enhanced (Schell et al. 1991).Clinically, the most important effect that hair growth-inhibitory or -promoting agents can have is to prolong the duration of anagen, or to induce premature catagen development, since this will lead to enhanced hair growth or telogen effluvium, respectively (Paus and Cotsarelis 1999).

Recently Redondo et al. reported darkening of gray/white hair in some patients after TH administration (Redondo et al. 2007). These observations were made after treatment of myxedema coma and in iatrogenic hyperthyroidism with increased exogenous T3, suggesting an influence of the hormone on HF-pigmentation. Before, “early greying” has been claimed to be related to autoimmune thyroid disease, hypothyroidism and hyperthyroidism (Lerner 1971, Kaminski 1997) indicating hormone- regulated changes in hair pigmentation. These hair effects may reflect a modulatory influence of TH on human HF-cycling (Freinkel and Freinkel 1972, Ebling 1991), since hair follicle melanogenesis is strictly coupled to anagen phase. Alternatively, there might also be a direct, hair cycle-independent effect on TH and HF-melanogenesis. However, it still remains to be conclusively shown that TH affects *human* HF pigmentation at all.

Thyroid state	HF- effects	reference
Hypothyroidism	Dry, brittle and dull hair, mucine concretions	Fistarol 2002
	diffuse hair loss	Molina 2006
	Early greying	Kaminski 1997
Hyperthyroidism	Thin, fine texture and fatty hair, alopecia	Fistarol 2002
	Shortened final length of and growth phase of HF	Schell et al. 1991
	Darkening of grey hair in hyperthyroid state due to treatment with T3	Redondo et al. 2007

Table 1.5 Human HF in hypo- and hyperthyroidism

However, multiple endocrine abnormalities can be associated with thyroid dysfunction [e.g. up regulation of serum TSH as a defining feature of primary hypothyroidism (Sawin et al. 1985, Singer 2009) and thyroid disease-associated changes in the insulin serum level (Velija-Asimi and Karamehic 2007, Dimitriadis et al. 2006)]. Therefore, it is not at all clear whether the recognized effects TH on hair growth and/or pigmentation reflect direct alterations of HF functions by TH, rather than indirect HF responses to secondary endocrine abnormalities. However, it has already been convincingly demonstrated that human scalp HF do express TH-receptors on the gene and protein level (TRβ1) (Ahsan et al. 1998, Billoni et al. 2000). In addition, Billoni et al. have reported that T3 prolongs the "survival" of micro dissected, organ-cultured human scalp HF (Billoni et al. 2000), even though it remained unclear what this claim was based on and exactly was meant by it. In any case, this report encourages one to assume that the detected TRβ1 proteins are functionally active in human HFs.

On this background, we have employed micro dissected, organ-cultured human scalp HFs that are grown under serum-free conditions, supplemented with insulin and hydrocortisone (but in the absence of TH!), as a physiologically relevant preclinical test system for probing the effects of TH on human HF growth, cycling, and pigmentation.

1.5 Aim of the study and specific questions addressed

This study is aimed at elucidating whether TH have any direct effect on isolated, organ-cultured human HFs, i.e. in the absence of systemic endocrine or neural stimuli, by assessing key parameters of human HF growth and pigmentation. Since alterations of human hair are reported in association with thyroid disease, clinically relevant parameters of human hair growth and pigmentation were assessed with top priority. Specifically, the following questions were addressed:

1. Do TH alter any of the key parameters of human HF growth and/or pigmentation (i.e. hair shaft elongation, anagen duration, follicular melanogenesis)? In particular, do THs affect the entry of human anagen VI hair follicles into catagen (Fig. 1.5)?
2. Do TH influence the proliferation or apoptosis of human hair matrix keratinocytes *in situ*?
3. Do TH affect the intrafollicular expression of the key catagen-inducing growth factor, TGFβ2?
4. Are there any differences between T3 and T4 in terms of their modulation of human hair growth and/or pigmentation in organ culture?
5. Do human HFs express deiodinases?
6. Do TH alter the expression of selected cytokeratins in the HF?
7. Since human HFs are an important extrarenal source and a non-classical, non-hematopoietic target of EPO, and since T3 augments EPO expression in a hepatic epithelial cell line: Does T3 up-regulate the HF expression of EPO?
8. Since TH are key regulators of metabolism: Are there any indications that TH might alter human HF mitochondrial metabolism?

1.6 Experimental Design

To address these specific questions, the following experimental design was chosen:

Scalp skin samples were collected from 7 euthyroid female donors undergoing routine face-lift surgery, and anagen VI hair follicles were micro dissected and

organ-cultured under serum-free conditions, in the presence of insulin and hydrocortisone as the only administered exogenous hormones, i.e. under severely "hypothyroid" conditions (Foitzik et al. 2006, Telek et al. 2007, Kloepper et al., 2009). Under these organ culture conditions, even amputated lower HFs, in the absence of bulge HF epithelial stem cells, continue to produce new, pigmented hair shafts at almost the speed of hair shaft production within human scalp *in vivo* for up to 9 days; between day 4 and 9, anagen VI then spontaneously enter into early stages of catagen transformation, until they finally begin to degenerate (Philpott et al. 1996, Kloepper et al. 2009).

During HF organ culture, the effects of selected doses of T3 or T4, which corresponded to the effective T3- concentration with which Billoni et al. (2000) had reported increased "HF survival" as well as physiologic, supra-and infraphysiologic concentration of both TH (Eisenberg et al. 2008), on **hair shaft elongation** were continuously recorded. At the end of experimentation, the following additional hair biology parameters were assessed by quantitative histochemistry or immunohistochemistry :

- **Hair cycle status** (histological assessment, whether the HFs were still in anagen VI or had entered into catagen)
- **HF pigmentation** (measurement of melanin content by Masson-Fontana histochemistry)
- Hair matrix keratinocyte **proliferation** and **apoptosis** *in situ* (measured by Ki67/TUNEL double-immunofluoresence)
- HF protein immunoreactivity for **TGFβ2, EPO, cytochrome-C-oxidase** (COX1), and selected **keratins** (CK6, CK14) *in situ* (measured by immunofluoresence)
- HF transcription of deiodinases that transform T4 into T3 (RT-PCR for D2 and D3)

Where appropriate, qualitative histological assessments were complemented by quantitative histomorphometry or immunohistomorphometry, and the sensitivity of routine immunohistology was enhanced by employing the highly sensitive tyramide signal amplification (TSA) or EnVision method (details, see below).

2 Materials and methods

2.1 Hair follicle isolation and organ culture

2.1.1 Hair follicle sources

The hair follicles used were isolated from skin samples derived from elective plastic surgery (face- lift). These samples have been located mostly at the occipital scalp but also at the fronto-temporal scalp of female patients with an age between 40 and 69 years. All samples were obtained from collaborating plastic surgeons after informed consent , following Helsinki guidelines, and after approval by the institutional Ethics Board of the Medical Faculty of the University of Lübeck (No. 06-109). Since there are intriguing gender-dependent differences in the HF-responsiveness to hormones , e.g. androgens and estrogens (Conrad et al. 2005, Paus et al. 2007, Ohnemus et al. 2006, Langan et al. 2009), it is important to note that all skin samples were standardized according to gender.

To assure that the samples used originated from euthyroid individuals, donor serum was collected before the surgical intervention and later examined for the thyroid status (see Table 2.1). Serum samples were kept at -20°C until further processing. Free T3, free T4, basal TSH and TG levels were assessed at the Endocrinological Laboratory, Dept. of Internal Medicine I, University Hospital Schleswig-Holstein, Campus Lübeck, Lübeck, Germany. Only skin samples of euthyroid donors were used except for the first patient, of whom no serum samples could be obtained, together with the skin samples. One patient with slightly elevated TG, but normal TSH level was also enrolled (Pat. 3). The skin samples were provided from patients undergoing elective face-lift surgery for cosmetic reasons in Bavaria (Dr. Dr. W. Funk, Klinik Dr. Koslowski, Munich, Germany). The scalp skin samples were kept in supplemented William's E medium (Biochrom, Cambridge, UK) at +4°C right after being taken in the operating theatre. However, the time from ischemia until the skin was submerged in William's E medium could not be influenced and controlled. The samples were kept tempered at +4°C and sent directly under sterile conditions in Williams´ E medium to Luebeck by overnight courier for processing within 24h after surgery.

Although a relatively stable temperature during transport can be assumed, minor quality of the samples was excluded by evaluating each sample when arriving. The evaluation included judgement of the medium colour, which can be seen as a reliable quality indicator. A light-red coloured Williams E medium indicates freshness and good tissue conservation while a deep-pink colour can be considered as outdated or unsound. The visual state of the skin sample provided the second quality marker. The samples were observed for disintegration and discolouration as signs for decay. The third quality marker was insufficient hair shaft elongation during the incubation period (HFs that failed to engage in the production of new hair shaft were eliminated from the assays). Only HFs that met these quality-control criteria were included in the subsequent analyses.

Patient no.	**Age**	**TSH (mIU/ml)**	**fT3 (pmol/l)**	**fT4 in (pmol/l)**	**TG (µg/l)**
1	62	Not assessed	Not assessed	Not assessed	Not assessed
2	50	1.60	7.84	14.09	Not assessed
3	40	0.448	4.46	12.15	93.55
4	60	2.89	6.99	17.51	7.84
5	51	2.46	7.22	17.94	3.08
6	49	1.81	6.46	18.89	7.59
7	58	0.761	4.04	17.08	6.80
Reference value*		**0.27- 4.2**	**3.1- 6.8**	**12- 22**	**2- 70**

Table 2.1 Thyroid status of all patients, whose HFs were examined

*Reference values according to the endocrine laboratory, medical clinic I, university of Luebeck, Luebeck, Germany

2.1.2 Micro dissection of human anagen VI hair follicles

HF isolation under sterile conditions followed the standard HF organ culture assay introduced by Philpott et al. (1990), with minor modifications (see below), employing a greatly extended set of experimental read-out parameters an (Bodó et al. 2007a, Foitzik et al. 2006, Ito et al. 2005, Kloepper et al., 2009). During the process of HF isolation, skin samples were kept in Williams´ E medium. Per 100ml William's E 1ml antimycotic/ antibiotic mixture containing 10,000 units of penicillin

(base), 10,000 µg of streptomycin (base), and 25 µg of amphotericin B per ml (Invitrogen, Carlsbad, California) was added.

Skin samples were cut into rectangles of about 1cm length. Under a stereomicroscope (Carl Zeiss MicroImaging Inc., Oberkochen, Germany), epidermis and dermis were mechanically separated from the subcutis after identification of the border between the dermis and the subcutis. The cutting line was placed slightly distal to the border (towards the dermis), so that the reticular structure of the connection between the dermis and the subcutis became clearly visible. With a little pressure on the outer edges of the piece of the sample, macroscopically clearly visible pigmented anagen hair bulbs were then elevated above the subcutis- level and gently plucked out with forceps. Since this is crucial for later HF organ culture and HF vitality, the ORS and CTS of plucked HFs had to stay intact during the whole procedure.

For organ culture, micro dissected and amputated proximal HFs were transferred to fresh culture medium: Williams' E medium (Biochrom, Cambridge, UK), supplemented with 2mmol/L L- glutamine (Invitrogen, Carlsbad, California), 10µg/ ml insulin (Sigma-Aldrich, Taufkirchen, Germany), 10ng/ml hydrocortisone (Sigma-Aldrich, Taufkirchen, Germany), and 1ml penicillin/ streptomycin mixture (10,000 units of penicillin (base) and 10,000 µg of streptomycin (base)/ml; Invitrogen, Carlsbad, California).

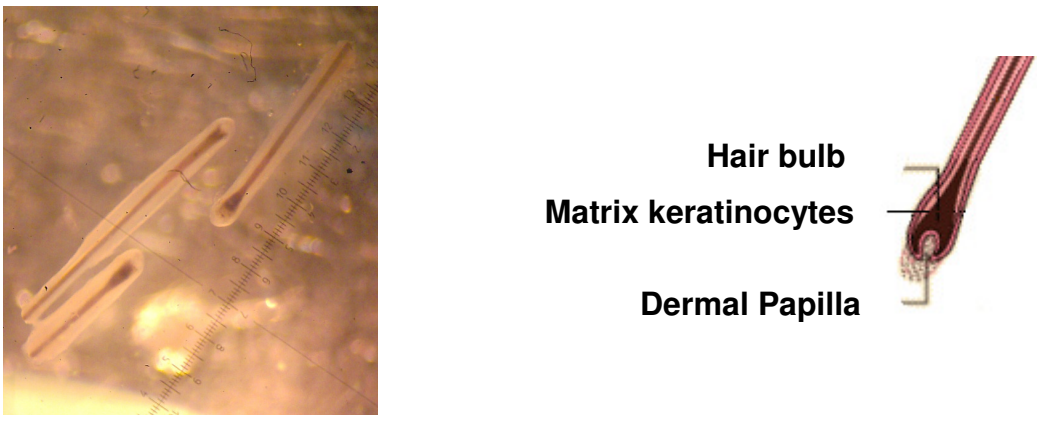


Figure 2.1 Hair follicles in culture well plate and scheme
magnification 20x

For this analysis only pigmented, intact terminal anagen VI hair follicles were used, which were clearly identified under low magnification by morphological criteria, namely the presence of a fully pigmented, characteristically shaped anagen hair bulb (Kloepper et al. 2009) (see Fig. 2.1). These HFs were randomly allocated to 24- multiwell plates (Greiner Bio One, Wemmel, Belgium): 3 hair follicles were cultured in one well (Fig. 2.1), and each well was filled with 500 µl of culture medium. HFs were incubated at 37°C, 95% O_2, 5% CO_2 in a Hera cell incubator (Heraeus, Kendro, Artisan Scientific Corporation, Champaign, IL, United states) for either 5 or 9 days.

2.1.3 T3 and T4-treatment in hair follicle organ culture

The plucked hair follicles were divided into 7 groups to test 3 different concentrations of each thyroid hormone compared to one group as a control. Each group consisted of 18 follicles, which resulted in 6 wells per group on a 24-multi-well plate (only 3 HF were incubated per well). The procedure was repeated 7 times using skin samples of 7 different female patients.

All groups were kept for 24 hours in the culture medium prior to the start of experimentation in order to minimize the effects of stress caused by the micro dissection and plucking process by providing this equilibration period. 1mg of T3 or T4 were dissolved in 1.0 normal natriumhydroxide diluted in culture medium in the concentrations listed below and were added for the first time after the 24 hours equilibration period, and then every second day, during alternating day medium changes. Triiodothyronine (T3) and L- thyroxine (T4) (both: Sigma-Aldrich, Taufkirchen, Germany) were tested at different concentrations and in cultures lasting 5 or 9 as outlined in table 2.2.
We used T3-concentrations of 10^{-12}, 10^{-10} and 10^{-8} mol L^{-1} covering the effective concentration with which Billoni et al. (2000) had reported increased “HF survival”. For T4 ,a physiologic concentration of 10^{-7} mol L^{-1} was applied, as well as infra- (10^{-8} mol L^{-1}) and supra- (10^{-6} mol L^{-1}) physiologic concentrations. As normal ranges of thyroid hormone serum levels for T4 are 5-12 microg/dL (= 6.4 x 10^{-8} mol L^{-1} - 1.5 x 10^{-7} mol L^{-1}) and for T3 are 0.8-1.9 ng/mL(= 1.2 x 10^{-9} mol L^{-1} - 2.9 x 10^{-9} mol L^{-1}) (Eisenberg et al. 2008).

	day 1	day 3	day 5	day 7	day 9
control	Add culture medium (CM)	Medium change	Medium change or freeze	Medium change	freeze
T3 1pM	Add CM with T3 [1pM]	Medium change	Medium change or freeze	Medium change	freeze
T3 100pM	Add CM with T3 [100pM]	Medium change	Medium change or freeze	Medium change	freeze
T3 10nM	Add CM with T3 [10nM]	Medium change	Medium change or freeze	Medium change	freeze
T4 10nM	Add CM with T4 [10nM]	Medium change	Medium change or freeze	Medium change	freeze
T4 100nM	Add CM with T4 [100nM]	Medium change	Medium change or freeze	Medium change	freeze
T4 1µM	Add CM with T4 [1µM]	Medium change	Medium change or freeze	Medium change	freeze

Table *Fejl! Brug fanen Startside til at anvende Abbildungsverzeichnis;Figures på teksten, der skal vises her.Fejl! Brug fanen Startside til at anvende Abbildungsverzeichnis;Figures på teksten, der skal vises her.****2.2 Scheme of HF treatment in organ culture***

At days 5 or 9, groups of 3 HFs cultured together in of one well were removed from the culture medium and placed onto a metal plate for freezing. They were covered with Shandon- Tissue Cryomatrix (Frozen Embedding Compound, Thermo Fisher Scientific Inc., Waltham, MA, United States) and carefully deep-frozen using fluid nitrogen. The hair follicles embedded in Shandon- Tissue were afterwards released from the plate and kept in plastic tubes at -80°C until further processing. For this study, longitudinal frozen sections of 7 µm thickness were produced with a Leica CM 3500 cryostat and devolved on superfrost plus (Menzel GmbH & Co KG, Braunschweig, Germany) slides. To obtain evaluable sections, 5 longitudinal sections through the dermal papilla and the hair shaft were performed on each block of deep frozen hair follicles. Cryoslides were stored at – 80°C until further use.

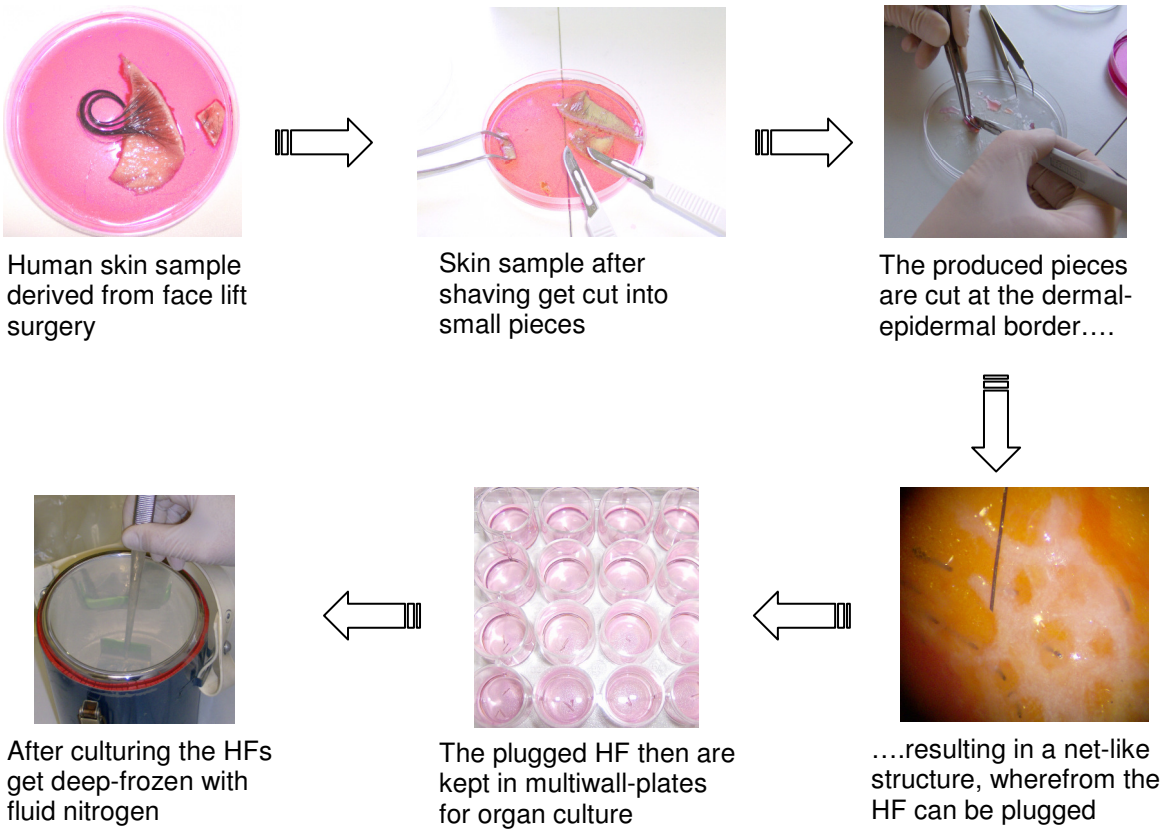


Figure 2.2*Fejl! Brug fanen Startside til at anvende Abbildungsverzeichnis;Figures på teksten, der skal vises her.* ***Illustration of micro dissection of human anagen VI hair follicles, organ culture and deep-freezing***

HF culture and snap-freezing were conducted under highly sterile conditions. Cryosectioning times were kept as short ass possible to diminish the period samples were not kept at -80 °C, but -21 °C.

2.1.4 Measurement of hair shaft elongation

The length of every individual hair follicle was measured using a Zeiss inverted binocular microscope with an eyepiece measuring graticule (Zeiss Stemi 2000-C, Carl Zeiss MicroImaging Inc., Oberkochen, Germany). Therefore, the HFs were paralleled with a measuring scale and their length was assessed from the tip of the DP stalk up to the end of the hair shaft (see Fig. 2.1). Since the eyepiece measuring graticule only provides a virtual unit , the assessed value was expressed as percent of the original HF length. This allows one to compare the hair shaft elongation between groups , donors, and individual HFs. Hair shaft

elongation was measured as described at day 0, 1, 3, 5 (5 day protocol) and at day 0, 1, 3, 5, 7, 9 (9 day protocol).

2.2 Quantitative hair cycle and hair pigmentation histomorphometry

Staging of the hair follicle cycling was carried out according to previously defined, exact morphological criteria (Müller-Rover et al. 2001 Stenn and Paus 2001, Sheehan et al. 2004), using a refined set of objective criteria that was specifically developed for the current thesis project (Kloepper et al. 2009) to determine the percentage of follicles in anagen and early, mid or late catagen. Human anagen hair follicles *in vitro* normally do not enter into the telogen phase in organ culture, because they degenerate before having reached this stage of HF transformation (Philpott et al. 1994). Table 2.3 shows the criteria according to which the HFs were staged, and Figure 2.3 illustrates these criteria.

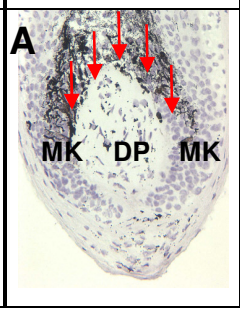
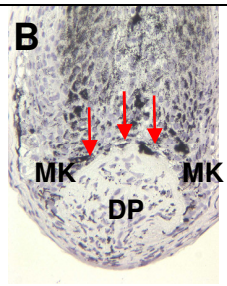
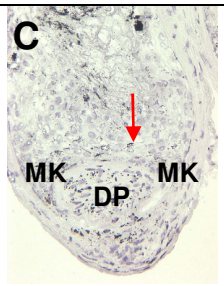
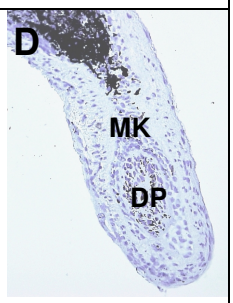
	anagen	**early catagen**	**mid catagen**	**late catagen**
Dermal papilla	onion- shaped	onion- shaped	ball- shaped	ball- shaped
Matrix keratinocytes	nearly surrounding DP	proximal opening	surrounding less than 50% of the DP	touching DP with epithelial stroma
DP-fibroblasts	vertical order	jumbled	dense	Dense
melanocytes	many dendrites	no dendrites	no dendrites, diminished	no dendrites, diminished
morphology				

Table 2.3 Criteria for classification of HF in anagen and different stages of catagen

Figure 2.3 HF in different stages (see Kloepper et al. 2009)

A) anagen HF, B) early catagen HF, C) mid catagen HF, D) late catagen
DP = dermal papilla, MK = matrix keratinocytes, → = indicating melanocytes

The Masson-Fontana histochemistry was used for studying both, hair follicle morphology and for visualizing melanin pigment (Barbosa et al. 1984). The argentaffin melanin is detectable via silver uptake and reduction to its visible metallic state without any reducing agent needed. 7 µm thick cryosections of cultured HFs were air-dried, fixed in ethanol-acetic acid (2:1) and after washing with TBS buffer and distilled water processed with silver nitrate (Merck & Co., Inc., NJ, USA) 10% for 40 minutes. Silver nitrate powder was dissolved in distilled water, 40% NaOH was added droplet- wise until black/ brown precipitate could be seen in the solution. Afterwards the precipitate was soluted by adding ammonium hydroxide. Sodiumthiosulfate (Merck & Co., Inc., NJ, USA) 5% fixed the silver nitrate during 1 minute and counterstaining with Mayer's haematoxylin (Chroma, Muenster, Germany) was performed. The staining was concluded by dehydration of the slides in ethanol of ascending concentrations and finally a xylol- substitute solution (Vogel, Karlsruhe, Germany). Since the human HF has a clearly defined pigmentary unit (see above) and non- pigmented CTS, we used the untreated HF itself as positive and negative control.

2.3 Immunohistochemistry

For all assessments with immonuhistochemistry as positive controls published follicular IR-patterns were reproduced along with every staining carried out as well as negative controls by omitting primary antibody. Only results that were reproducible in at least 3 different patients (yielding to 54 hair follicles per group) were considered and are reported.

To evaluate apoptotic cells in co-localization with a proliferation marker Ki67, a Ki67/TUNEL (terminal dUTP nick-end labelling) double-staining method was used (Bodó et al. 2007a). Cryosections were fixed in paraformaldehyde and ethanol-acetic acid (2:1). After incubation with equilibration buffer for 5 minutes the sections were labelled with a digoxigenin-deoxyUTP (ApopTag Fluorescein, In Situ Apoptosis detection kit; Intergen, Purchase, NY) in the presence of terminal

deoxynucleotidyl transferase (TdT) for 60 minutes at 37°C (ApopTag Fluorescein, In Situ Apoptosis detection kit; Intergen, Purchase, NY) mixed with reaction buffer (3:7). The reaction was stopped by stopping buffer at 37 °C for 10 minutes and was followed by pre- incubation with goat serum 10% and then mouse anti-Ki67 antiserum (1:20 in PBS, over night at 4°C; DAKO, Glostrup, Denmark).

Primary antibody	origin	dilution	Reference tissue (positive control)	Literature
Anti-Ki67	mouse	1:20	murine spleen	(Libiani et al. 2008)
TdT		3:7	4-HC-treated skin	(Bodó et al. 2007b)
Anti-TGF-β2	rabbit	1:50	Human placenta	(Tarrade et al. 2001)
Anti-COX1	mouse	1:40	Human skin	(Li et al. 2007, Bodó et al. 2009b)
Anti-Epo	rabbit	1:50	Human kidney	(Bodó et al. 2007a)
Anti-CK14	mouse	1:200	Human skin	(Olszewska and Sudhoff 2007)
Anti-CK6	mouse	1:10	Human skin	(Demirkesen et al. 1995)

Table 2.4 Primary antibodies used and reference tissues employed as positive controls

2.3.1 Ki67/TUNEL

TUNEL-positive cells were visualized by an anti-digoxigenin FITC-conjugated antibody (ApopTag kit) diluted in blocking solution (56 locking solution : 59 antibody), whereas Ki67 was detected by a rhodamine-labeled goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) 1:200 in PBS with 2% goat normal serum (DAKO, Glostrup, Denmark). Counterstaining was performed with DAPI (Boehringer Mannheim GmbH, Mannheim, Germany) for 1 minute. For all washing steps PBS buffer was used.

Negative controls were performed by omitting TdT and the Ki67 antibody as well as by demonstrating the absence of specific Ki67 or TUNEL immunoreactivity in those cell populations that are expected from the literature to be negative for these markers (Kloepper et al. 2009) . Proliferating matrix/epidermal keratinocytes of normal human skin and frozen sections of human spleen were used as additional positive control tissues for Ki67 expression, while sections of HFs that had been treated with 4-hydroperoxycyclophosphamide -, which strongly up-regulates the

number of TUNEL+ cells in the hair matrix (Bodó et al. 2007b), were used as were used as additional positive control for the TUNEL reaction.

Quantitative immunohistomorphometry was performed in carefully defined, microscopically assessed reference areas as described previously (Foitzik et al. 2006, Bodó et al. 2007a+b): Ki67-, TUNEL-, or DAPI-positive cells were counted in a reference region of the HF matrix defined as all matrix keratinocytes below a line parallel to Auber's line touching the most distal tip of the DP. The percentage of Ki67- positive and TUNEL-positive cells was determined proximal of this line (Fig.2.4). This reference region was chosen instead of the usual region proximal of the Auber's line, because proliferational and apoptotic changes also seemed to affect the matrix keratinocytes distal of the Auber's line (Kloepper et al. 2009).

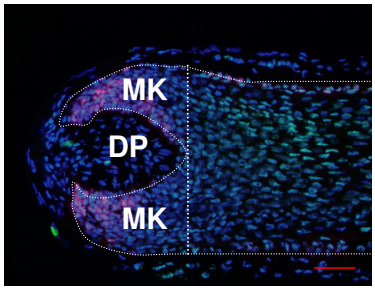


Figure 2.4 reference region for Ki67-/TUNEL staining

All MK counted in area circled by dotted line up to the line at the tip of the DP. MK = matrix keratinocytes, DP = dermal papilla, scale bar = 50 µm

2.3.2 TGFβ2

To investigate transforming growth factor- β2 (TGFβ2) expression, cryosections were acetone-fixed whereupon endogenous peroxidases were blocked with 3% H_2O_2 (Merck & Co., Inc., NJ, USA) for 15 minutes followed by treatment with avidin and biotin (Vector laboratories, Burlingame, CA, USA), each for 15 minutes. After a pre-incubation of 30 minutes with 5% goat normal serum (DAKO, Glostrup, Denmark) in TNT buffer, the slides were incubated first with the primary antibody for TGF- β2 (rabbit anti-human, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) 1:50 in TNT with 2% goat normal serum (DAKO, Glostrup, Denmark), over night at +4 °C. At the second day, the slides were incubated first with biotinylated goat anti-rabbit (Jackson ImmunoResearch) secondary antibodies 1:200 in TNT with 2% goat normal serum (DAKO, Glostrup, Denmark), for 45 min, RT, and then with Streptavidin-conjugated horseradish peroxidise (HRP, 1:100 in TNT) provided

in the TSA kit (Perkin-Elmer, Boston, USA) for another 30 minutes. To complete the amplification, all slides were treated with FITC-tyramide amplification reagent 1:50 in amplification diluent (TSA kit; Perkin-Elmer, Boston, USA) at room temperature. Counterstaining was performed with DAPI (Boehringer Mannheim, Mannheim, Germany) for 1 minute. Negative controls were performed by omitting the primary antibody. For all washing steps TNT buffer was used. As positive control human placenta tissue was used (Tarrade et al. 2001).

2.3.3 COX1

For the detection of COX1 (cytochrome-C-oxidase, Complex IV subunit I) the peroxidase-based avidin-biotin complex method (Vector laboratories, Burlingame, CA, USA) was used, as described (Foitzik et al. 2006, Bodó et al. 2009b). After fixation in acetone at -20°C, quenching of endogenous peroxidases with 3% H_2O_2 (Merck & Co., Inc., NJ, USA), and blocking of non-specific binding sites by pre-incubation with goat normal serum (10% in TBS, DAKO, Glostrup, Denmark) for 20 minutes at RT, cryosections were incubated with monoclonal mouse anti-human Complex IV subunit I antibody (Clone MS404, MitoSciences, Eugene, OR, USA) at a dilution of 1:50 in TBS with 2% goat normal serum (DAKO, Glostrup, Denmark) over night at +4°C.

As secondary antibody, biotinylated goat anti-mouse IgG (PNIM 0816, Beckmann Coulter, Marseille, France) 1:200 in TBS with 2% goat normal serum (DAKO, Glostrup, Denmark) was administered for 45 minutes at RT and then the slides were treated with an avidin-biotin kit peroxidase (Vector laboratories, Burlingame, CA, USA) for 30 minutes followed by AEC substrate- chromogen system (DAKO, Glostrup, Denmark) for 8 minutes. The primary antibodies were replaced by buffer for negative controls. Human skin served as positive control tissue, since it expresses high amounts of COX1 protein in epidermal keratinocytes (Li et al. 2007). Counterstaining was performed with Mayer's hemalaun (Chroma, Muenster, Germany) for 5 minutes. For all washing steps TBS buffer was used.

2.3.4 EPO

Immunostaining for EPO in vehicle/T3/T4-treated follicles was performed using the highly sensitive EnVision® (Dako, Glostrup, Danmark) technique as described before (Bodó et al. 2007a). Acetone- fixed cryosections were, after pre-incubation with goat normal serum (10% in TBS) for 20 minutes, incubated with a rabbit anti-human EPO antiserum (generated by hyper- immunization of rabbits with recombinant human EPO; kindly provided by A. Kromminga, Institute for Immunology, Clinical Pathology, Molecular Medicine, Hamburg, Germany) at a dilution of 1:50 in TBS over night at 4°C. Washing steps were performed with TBS buffer. Slides were then treated with the EnVision labelled polymeric secondary antibody solution (Dako, Glostrup, Denmark) goat anti rabbit/mouse immunoglobulin for 30 minutes at RT, followed by staining with fast red tablets (Sigma-Aldrich, Taufkirchen, Germany) for 5 minutes. Counterstaining was performed with Mayer's haematoxylin (Chroma, Muenster, Germany) for 1 minute. Negative control experiments were performed by omitting primary antibody, while human kidney sections served as positive control tissue, since kidney epithelium is the key source of EPO production (Jelkmann 2004), .

2.3.5 CK14

For the detection of cytokeratin 14 (CK 14) the peroxidase-based avidin-biotin complex method (Vector laboratories, Burlingame, CA, USA) was used. After fixation in acetone at -20°C and blocking of endogenous peroxidases with 3% H_2O_2 cryosections were pre-incubated with goat normal serum (Dako, Glostrup, Denmark) 10% in TBS and incubated with monoclonal mouse anti-human CK 14 antibody (Sigma-Aldrich, Taufkirchen, Germany) 1:200 in TBS with 2% goat normal serum (DAKO, Glostrup, Denmark) over night at 4°C. The staining was performed with biotinylated goat anti-mouse IgG (Beckmann Coulter, Marseille, France) as secondary antibody 1:200 in TBS with 2% goat normal serum (DAKO, Glostrup, Denmark) for 45 min at RT. Washing steps were performed with TBS buffer. Then an avidin-biotin peroxidase kit (Vector laboratories, Burlingame, CA, USA) for 30 minutes followed by AEC substrate-chromogen system (Dako, Glostrup, Denmark) for 5 minutes, both at RT, served as staining substrate.

As negative controls, the primary antibodies were omitted. Human skin (epidermis) was used as a positive control for CK14, since the basal and suprabasal layer of human epidermis is strongly positive for CK14 (Olszewska and Sudhoff 2007). Counterstaining was performed with Mayer's haematoxylin for 1 minute.

2.3.6 CK6

To investigate the expression of human keratin 6 (CK6) (Langbein and Schweizer 2005), acetone-fixed cryosections were pre-treated with non-immune goat serum (Dako, Glostrup, Danmark) 10% in TBS. Cryoslides were first incubated with the primary antibodies against CK6 (mouse anti-human, PROGEN, Heidelberg, Germany) 1:10 in TBS with 2% non-immune goat serum (DAKO, Glostrup, Denmark) over night at 4℃, then with rhodamine-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) 1:200 in TBS with 2% non-immune goat serum (DAKO, Glostrup, Denmark) for 45 minutes at RT. Counterstaining was performed with DAPI (Boehringer Mannheim, Mannheim, Germany) for 1 minute.

Human scalp skin was used as a positive control, since the ORS of human HF strongly expresses CK6 (Demirkesen et al. 1995, Havlickova et al. 2009), while the negativity of CK6-like immunoreactivity in the central hair matrix and the epidermis as well as HF sections without application of primary antibody served as adequate negative controls. Washing steps were performed using TBS buffer.

2.4 Quantitative immunohistomorphometry

For quantitative immunohistochemistry of carefully defined reference areas, densitometric measurement of the intensity of immunoreactivity was performed using the ImageJ/ NIH-image software (National Institute of Health, Bethesda, USA), as previously described (Ito et al. 2005, Foitzik et al. 2006, Bodó et al. 2007a). For light microscopic immunohistochemistry and immunofluorescence, pictures were analysed in RGB colour. Measurements were performed in the ORS at a distance of three microscopic fields in a 200x magnification, distal to the dermal papilla. Only for the assessment of TGF- β2 immunoreactivity, a distance of one microscopic field from the DP was chosen. For each HF, two

measurements were performed, one on each side of the hair shaft, and a mean value was calculated.

Densitometric measurement of melanin staining intensity was also performed with ImageJ/ NIH-image software (National Institute of Health, Bethesda, USA) using 8bits black and white pictures. Previously defined reference areas of interest in the hair matrix (Ito et al. 2005) were used to calculate the average values.

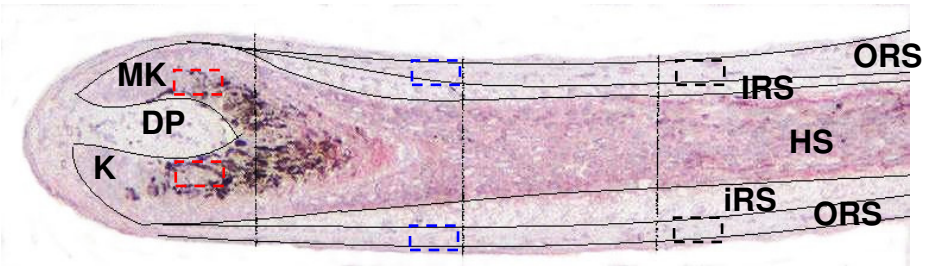


Figure 2.5 reference areas for evaluation of the different stainings

= reference area for densitometric measurement, = reference area for TGFβ2 TSA staining, = reference area for CK6, CK14, COX1 and EPO; MK = matrix keratinocytes, DP = dermal papilla, HS = hair shaft, ORS = outer root sheath, IRS = inner root sheath

2.5 RT-PCR: Deiodinases

Semi quantitative reverse transcriptase (RT)-PCR for D2 and D3 deiodinases

For the semi quantitative PCR analysis of expression of D2 (gene bank accession no. AF093774) and D3 (accession no. NM 001362) total RNA was isolated from micro dissected HFs using the RNA easy kit (Qiagen, Hilden, Germany). 0.5 µg of total RNA was reverse-transcribed with SuperScript First-Strand Synthesis System (Applied Biosystems, Foster City, CA, USA). The quality and quantity of cDNA in all samples were standardized by the amplification of housekeeping gene GAPDH as described previously (Zmijewski et al. 2007).

The PCR conditions for D2 and D3 amplification were as follows: initial denaturation 95°C for 2 minutes followed by 30 cycles of: denaturation at 94°C for 30 seconds; annealing at 60 °C for 30 seconds, elongation 72°C for 30 seconds.

The final elongation step was at 72°C for 5 minutes. The primers used for amplification were described previously (Slominski et al., 2002) and purchased from Intergrated DNA Technology Inc., (Corallville, IA, USA).
For detection of D2, PCR reaction was repeated with a second pair of primers and 0.5 µl of reaction mixture from the first round. PCR products were visualized on 2% agarose gel with ethidium bromide. Data of D2 and D3 expression were normalized to the expression of GAPDH of the same sample. Non-template control (by omitting RNA) and samples not containing reverse transcriptase were used as negative controls, while control RNA from the RNA easy kit served as another positive control.

2.6 Statistical analysis

Statistical analysis was performed using the Mann Whitney U Test for unpaired samples. The p-values were calculated using the statistical analysis software SPSS (SPSS Inc., Chicago, USA). p values of *< 0.5; **≤ 0.01; ***≤ 0.001 were regarded as significantly different.
To evaluate immunohistochemical data, per staining reaction and evaluated reference area, each concentration of T3 and T4 was compared to the vehicle employing the Mann Whitney U Test. The testing was performed per patient and then cumulated. Resulting significant and non-significant outcomes are reported here.
For every parameter examined, 18 HF were cultured per TH- concentration and compared to 18 vehicle-treated control- HF. This procedure was performed with the HF of three different female donors. The resulting data from all three patients were pooled, since HF responses to TH stimulation turned out to congruent between all three patients. This gave a total n of 54 HFs per TH-concentration/control per test/control group.

3 Results

3.1 T4 stimulates human hair matrix keratinocyte proliferation, while both T3 and T4 inhibit apoptosis of these cells

First, we investigated proliferation and apoptosis of matrix keratinocytes by Ki67/TUNEL double-staining and quantitative immunohistomorphometry after T3 and T4-treatment. Whether T3 (1 pM, 100 pM, 10 nM) and/or T4 (10nM, 100nM, 1000 nM) modulate human hair matrix keratinocyte proliferation and apoptosis when added directly to the serum-free medium of micro dissected, organ-cultured normal human scalp HFs was analysed on HFs that were in the stage of maximal growth during HF cycling (anagen VI).

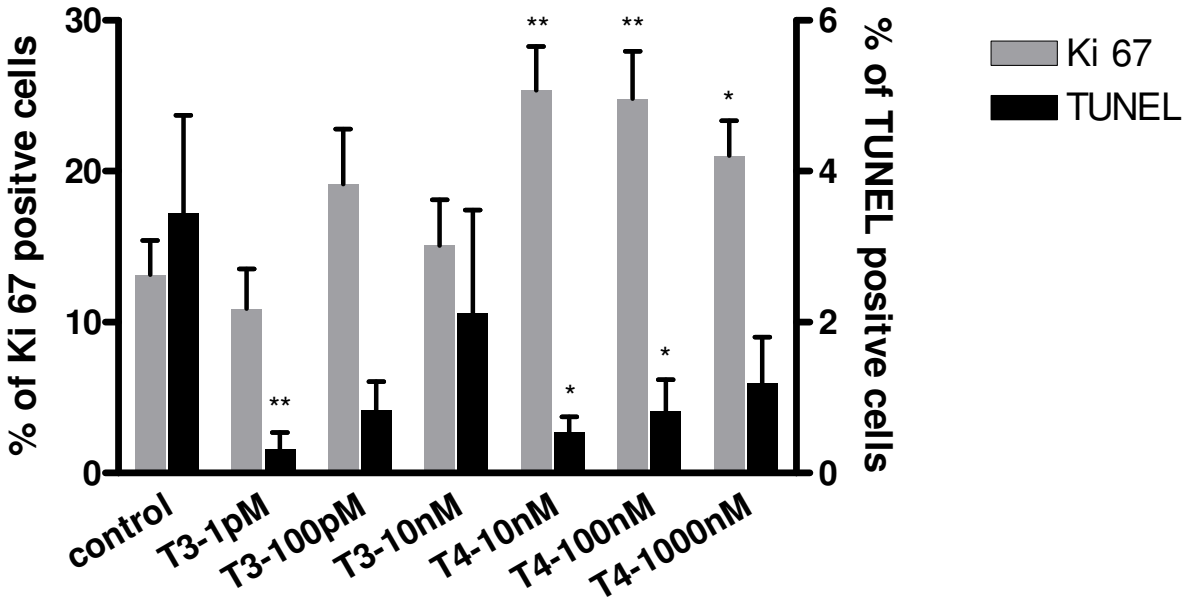


Figure 3.1 T4 stimulates human hair matrix keratinocyte proliferation and both T3 and T4 inhibit hair matrix keratinocyte apoptosis

percentage of positive cells was compared between vehicle and T3/ T4 treated follicles. **P* ≤ 0.05, ***P* ≤ 0.01, significances compared to control; examined on 5 days culture; mean ± SEM.

As shown in Fig. 3.1 and Fig. 3.2, the proliferation of hair matrix keratinocytes was significantly stimulated by T4 seen in the increased amount of Ki67- positive cells,

while the proliferation- modulatory effects of the T3 concentrations tested did not reach the level of significance. However, both T3 and T4 significantly reduced the number of TUNEL-positive matrix keratinocytes displaying a reduced apoptosis in defined reference areas (Fig. 3.1 and 3.2). Thus, both TH exerted slightly differential, but complementary growth-promoting effects on the most rapidly proliferating epithelial cell population of mammalian skin *in situ*.

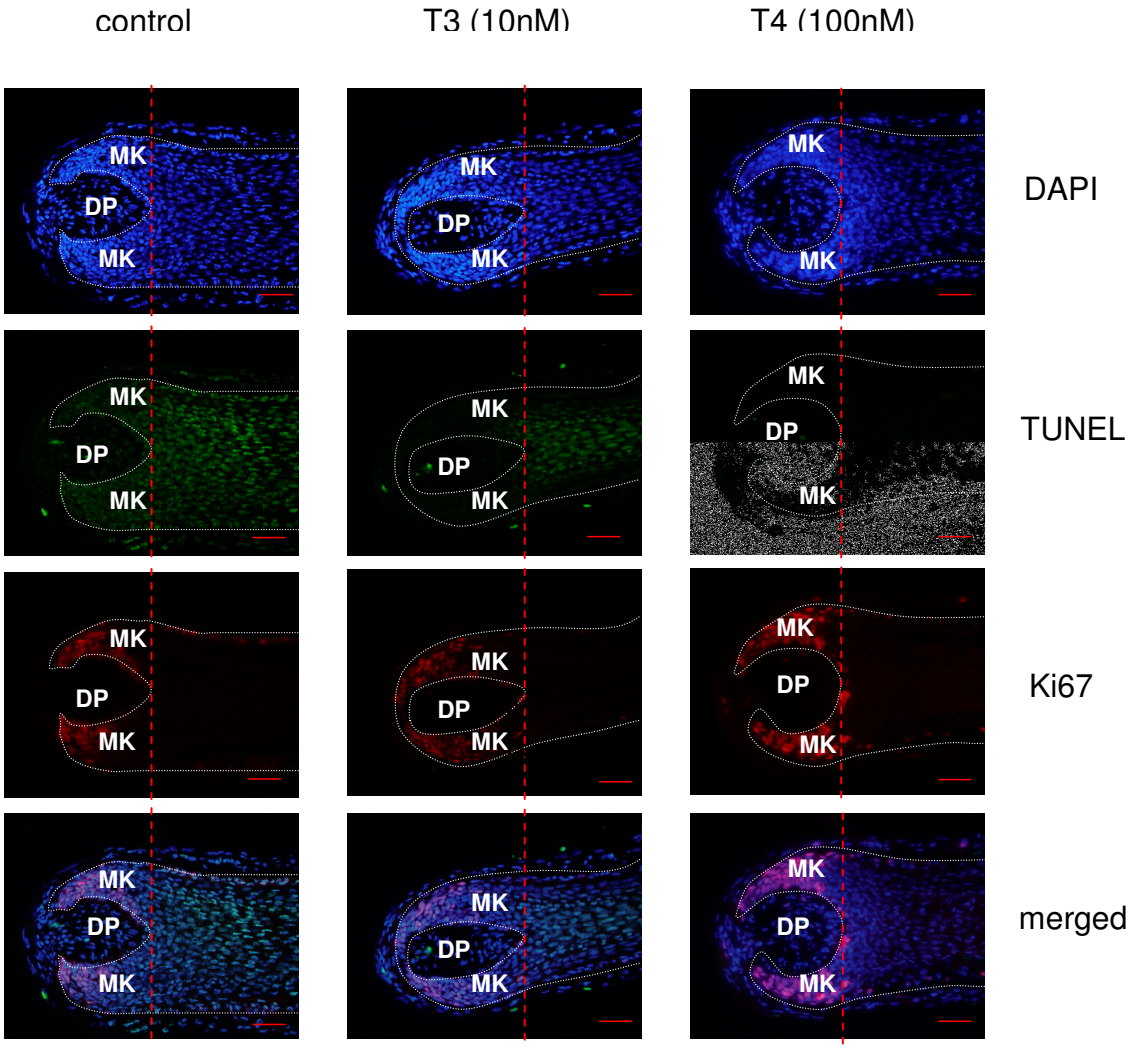


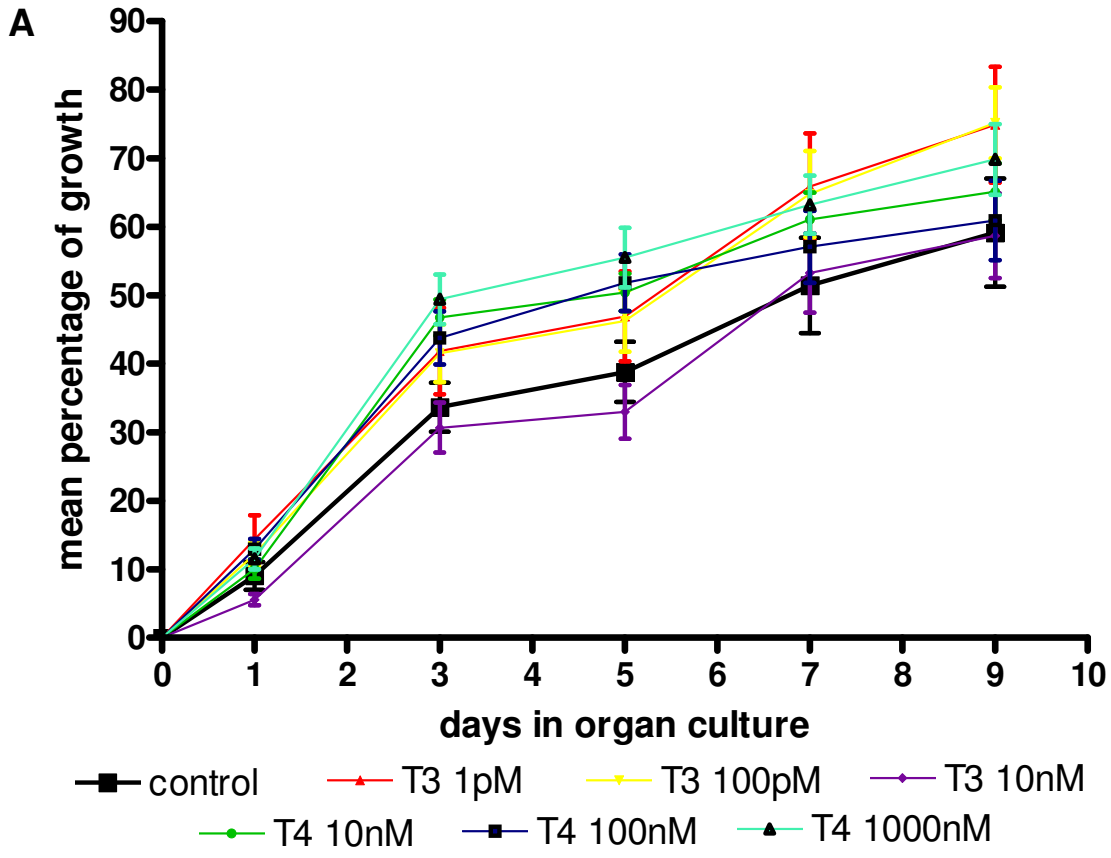
Figure 3.2 Apoptosis and proliferation of TH-treated HFs

Cryosections of cultured HFs treated with vehicle, T3 or T4 were double-immunoabelled for Ki-67(red) and TUNEL (green). After 5 days of culturing; Ki67 and TUNEL positive cells were counted below a line marking the tip of the dermal papilla (indicated in red). DP = Dermal papilla, MK = Matrix keratinocytes.

3.2 T3 and T4 do not significantly alter human hair shaft structure formation *in vitro*

However, during the relatively short HF organ culture period (9 days), these effects on the hair matrix keratinocyte proliferation/apoptosis did not result in marked, histological detectable alterations of the actual hair shaft morphology (Fig. 3.3 C1-C3), and no abnormalities in hair shaft pigmentation became apparent.

Also, hair shaft production over 9 days of serum-free organ culture was not significantly modified by the tested doses of T3 and T4. When measuring the rate of hair shaft elongation *in vitro*, this remained essentially unaltered by THs, compared to vehicle controls, even though two of the tested T3 doses showed a tendency towards slightly enhanced hair shaft formation *in vitro*, compared to vehicle controls. However, no significant, reproducible stimulatory effects on hair shaft production were detected either after T3 or after T4 treatment (Fig. 3.3 A+B) could not be detected ($p>0.05$).



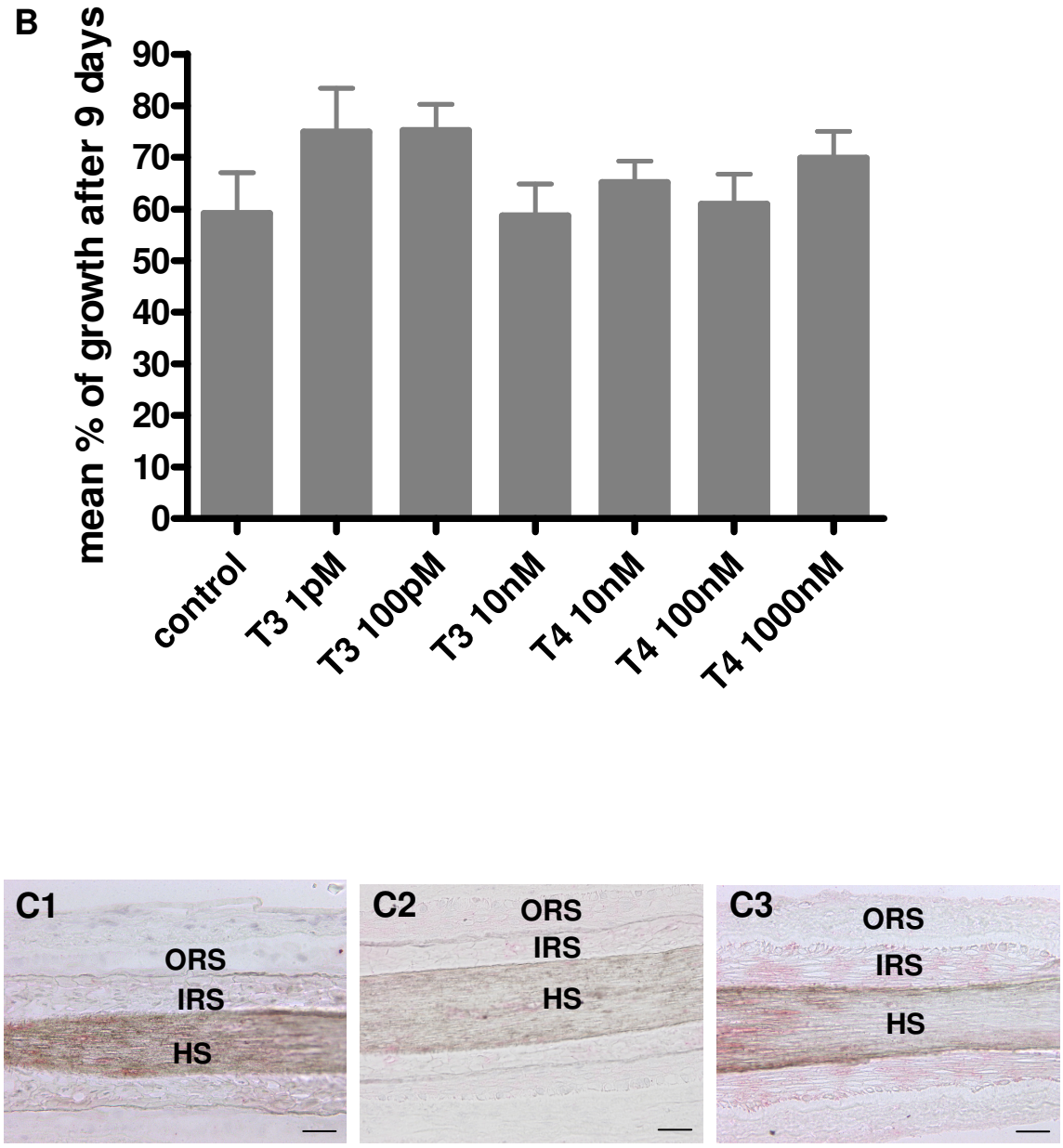


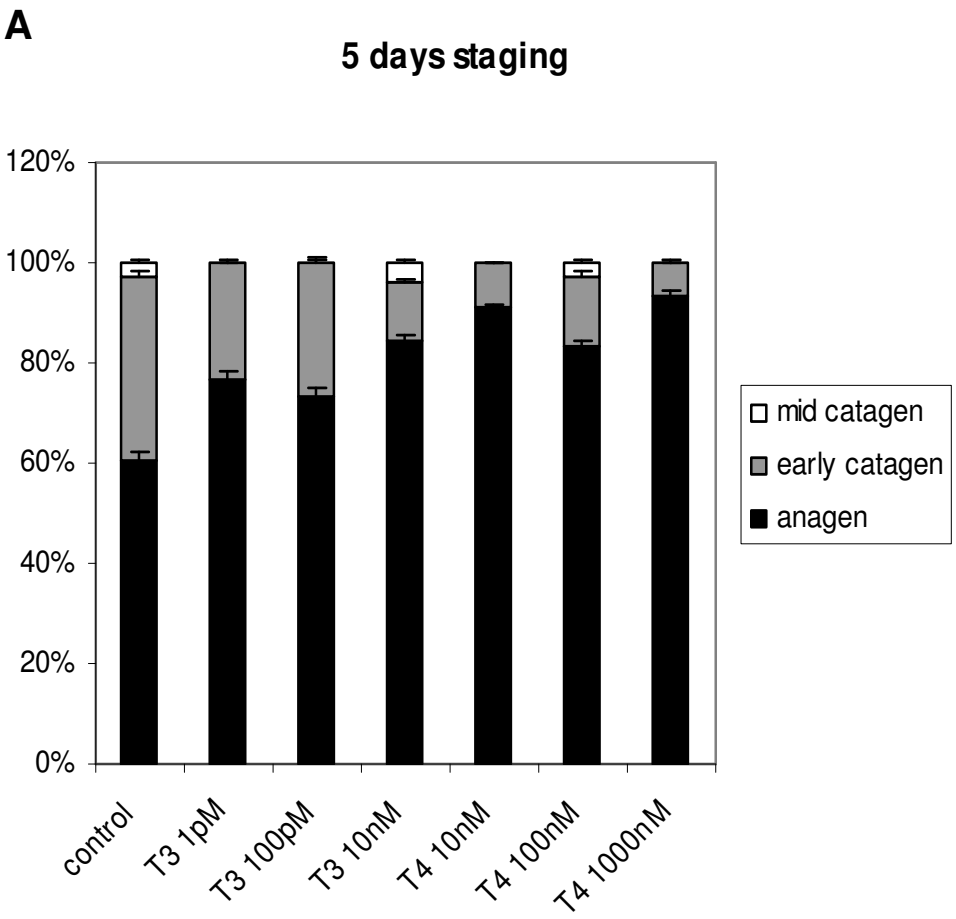
Figure 3.3 Hair shaft elongation and morphology

A) percent of hair shaft elongation measured every second day and compared to day 0, B) percentage of hair shaft elongation after 9 days of HF organ culture, mean ± SEM; all tested concentrations (T3/ T4) not significantly different from the control group ($p>0.05$)., C) hair shaft morphology, C1) control, C2) T3 1pM, C3) T4 1000nM,
scale bars = 50 µm; ORS = outer root sheath, IRS = inner root sheath, HS = hair shaft.

3.3 Both T3 and T4 prolong anagen duration

Clinically, the most important effect that any hair loss-inhibitory or hair growth-promoting agent can have is to prolong the duration of anagen, which is indistinguishable from an inhibition of catagen development. Therefore, we tested by quantitative hair cycle histomorphometry whether TH-treated human HFs showed any evidence of such an effect. Masson-Fontana stained HF sections were employed to assess the hair cycle status, using a set of newly defined , objective criteria that were specifically developed for this thesis project (Kloepper et al. 2009).

Both T3 and T4 increased the percentage of anagen HFs, and decreased the percentage of catagen HFs after 5 (Fig. 3.4 A) or 9 days in organ culture (Fig. 3.4 B). This provides the first objective evidence that THs are potent modulators of human scalp HF cycling, at least *in vitro*.



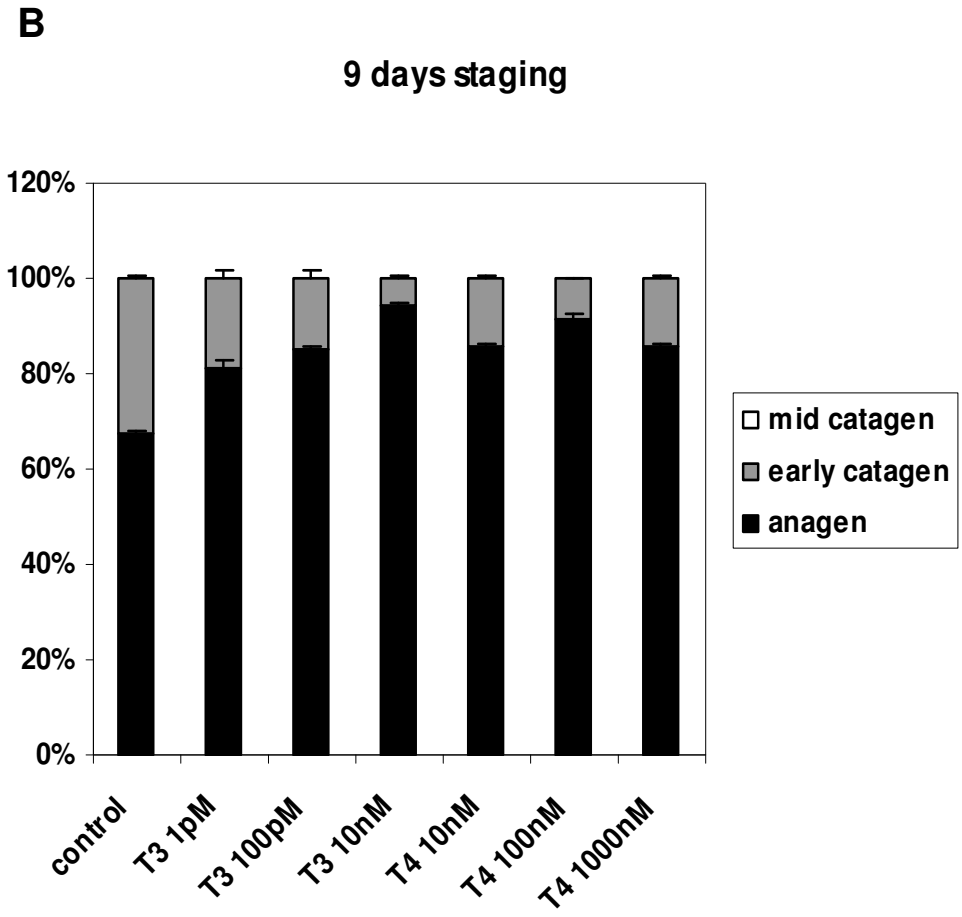


Figure 3.4 Both T3 and T4 prolong anagen duration

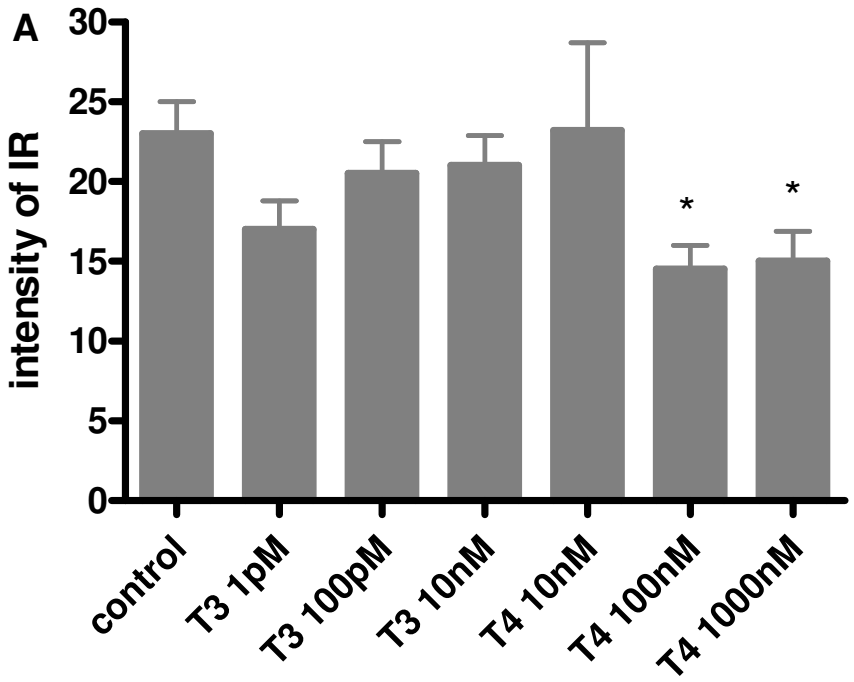
Quantitative histomorphometry shows the percentage of HFs in distinct hair follicle stages (anagen, early catagen, mid catagen); A) Staging of hair follicles cultured for 5 days B) Staging of hair follicles cultured for 9 days; mean in percent ± SEM.

Comparing the hair cycle effects of T3 and T4 over vehicle controls after 5 and 9 days of organ culture did not reveal major differences between both THs. At both end-points, the relative distribution of anagen and catagen HFs displayed a similar pattern. Due to the very limited total number of available human scalp HFs that could be studied, the number of different T3 and T4 doses tested was insufficient to allow any solid conclusions on dose-dependency of the observed hair cycle effects. Nevertheless, the anagen-prolongation by T3 appeared to be in line with a dose-dependent effect. No such trend was seen with T4.

3.4 TGFβ2 immunoreactivity is down-regulated by thyroid hormones

Since, under physiological conditions, TGFβ2 appears to be the most important endogenous stimulus for catagen induction in human scalp hair follicles (Hibino and Nishiyama, 2004, Foitzik et al. 2005, Paus and Foitzik 2004), the hair cycle data generated above were complemented with quantitative immunohistomorphometric assessment of the effect of THs on TGFβ2 immunoreactivity in human HF organ culture. Specifically, we tested whether the anagen prolongation by TH was associated with an altered intrafollicular expression of TGF-β2.

Although substantial inter-individual variations in intrafollicular TGFβ2 IR intensity and patterns were noted between the HFs derived from different patients (data not shown), quantitative immunohistomorphometry revealed that TH treatment results in a discrete, but significant reduction of TGFβ2 IR in the proximal hair bulb epithelium ($p<0.05$) (Fig 3.5 A-C).



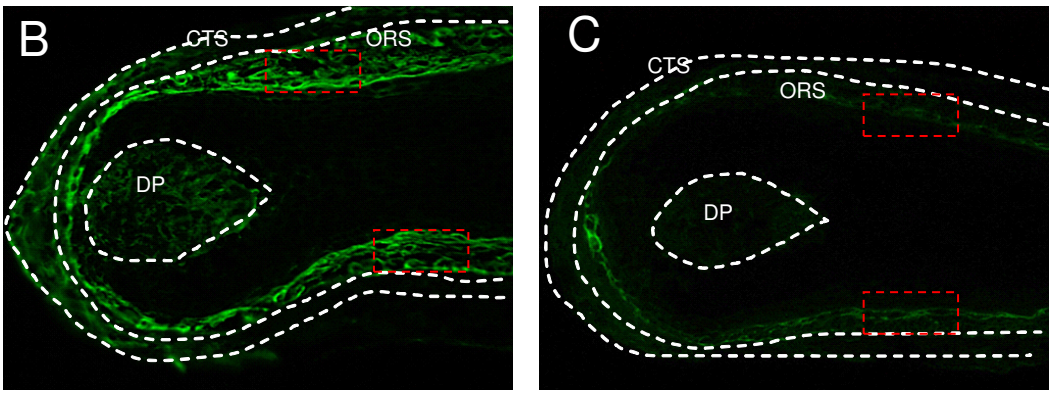


Figure 3.5 Histomorphometry of TGF-β2 immunoreactivity (IR)

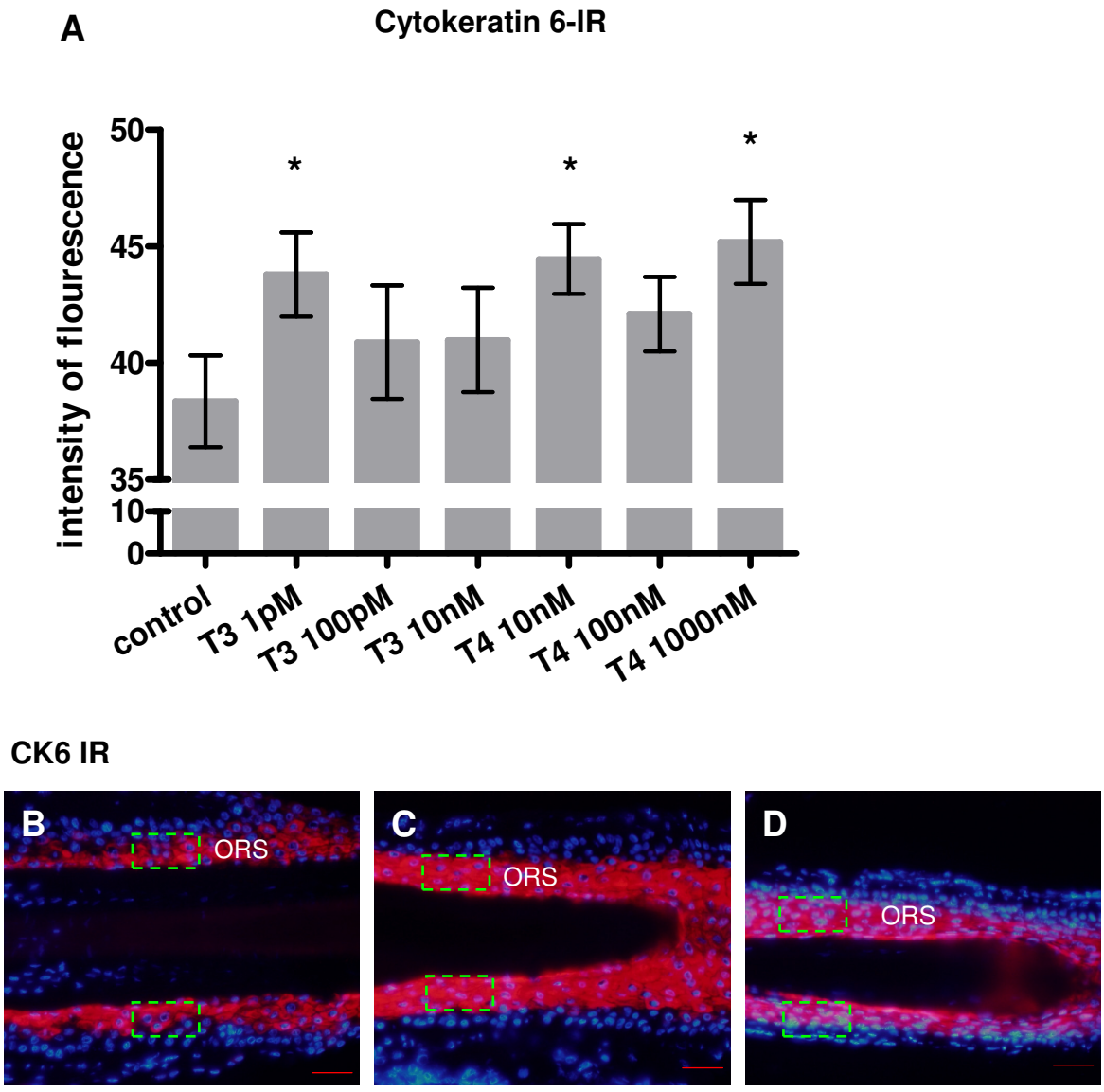
A) quantification of TGFβ2 IR in arbitrary units, using equivalent fields of the distal ORS, p values compared to control, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, mean in percent ± SEM. B) control C) T4; TGFβ2 was evaluated on 9 days culture, ORS = outer root sheath, CTS = connective tissue sheath, DP = dermal papilla, dotted line to facilitate overview (the area between the dotted lines represents the CTS).

This independently confirms a catagen-inhibitory effect of THs, and is in line with the concept that a (direct or indirect) inhibition of the intrafollicular production of TGFβ2, a key catagen-promoting growth factor, may explain at least in part the catagen-inhibitory activity of both T3 and T4.

3.5 T3 and T4 differentially modulate follicular keratin expression

Next, we assessed TH actions on the intra-follicular protein expression for two cytokeratins with recognized TH-responsive elements (TRE), namely human cytokeratin 14 and cytokeratin 6 (Radoja et al. 1997).

Interestingly, CK 6 IR was significantly *increased* after treatment with 1pM T3 and 10nM T4 and 1µM T4 ($p < 0.05$) (Fig. 3.6 A-C, H). Instead, by quantitative immunohistomorphometry, the IR for CK 14 IR was significantly *decreased* in all TH-treated HFs ($p<0.001$) (Fig. 3.6 D-F, G).



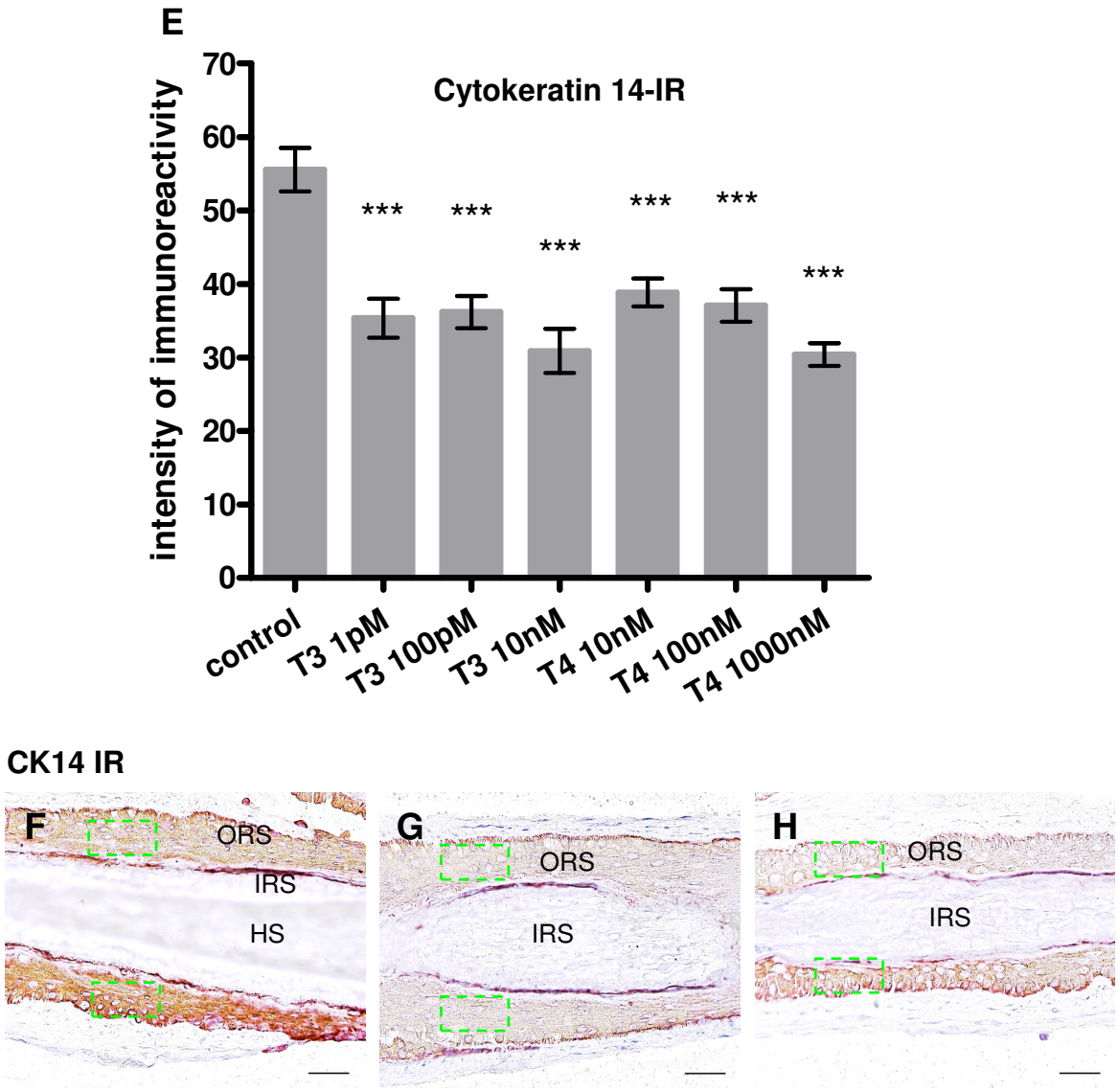


Figure 3.6 Differential effect of TH on human hair keratin protein expression in situ

A) Cytokeratin 6 protein expression detected by IF is significantly altered by both T4 and T3, quantification of CK6-IF,

B-D) Cytokeratin 6 IR in the distal ORS: B) control, C) T3 1pM, D) T4 10nM;

E) T3 and T4 down regulate Cytokeratin 14 IR significantly, quantification of CK14-IR;

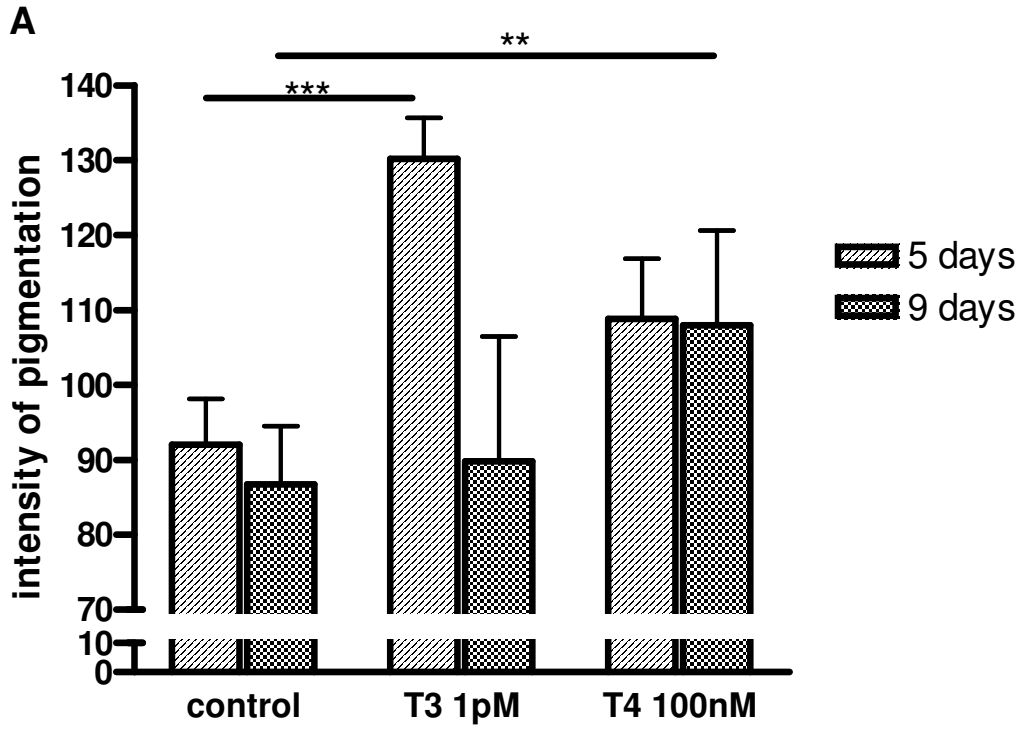
F-H) Cytokeratin 14 IR in the distal ORS: F) control, G) T3 100pM, H) T4 10nM;

cytokeratins evaluated on 5 days culture; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$; mean ± SEM, scale bars = 50 µm; ORS = outer root sheath, IRS = inner root sheath, HS = hair shaft; , = areas measured

3.6 T3 and T4 stimulate hair follicle pigmentation

Next, we tested whether TH modulated intrafolliuclar melanin synthesis. Since follicular melanogenesis is strictly coupled to HF cycling and seizes in catagen (Slominski et al. 2005), it was critical to compare only anagen VI HFs from test and control groups with each other, carefully excluding all catagen HFs from the analysis.

As revealed by quantitative Masson-Fontana histochemistry, both T3 and T4 significantly ($p<0.001$, $p<0.01$) stimulated human HF melanin synthesis, with supra-physiological concentrations of T4 showing the strongest stimulation of HF melanogenesis (Fig. 3.7 A and B1 to D1). Focussing on the measurable effects of TH on HF pigmentation it was noteworthy that, after 5 days of organ culture, T3 showed the strongest HF melanisation-enhancing effect, T3 and T4 were equally pigmentation-enhancing after 9 days. This means that, even though anagen VI HFs already maximally engage in melanogenesis (Slominski et al. 2005), TH can further stimulate intrafollicular pigment production under organ culture conditions.



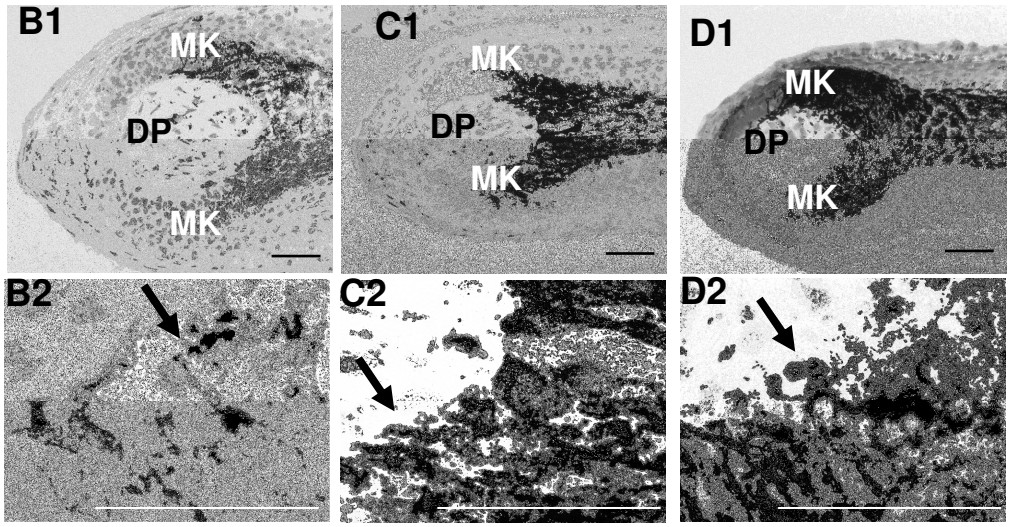


Figure 3.7 T3 and T4 stimulate the hair follicle pigmentation

A) quantification of histochemical staining intensity for melanin (Masson-Fontana histochemistry), $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, mean ± SEM, significances compared to control; B1+2) control, C1+2) T3 1pM, D1+2) T4 100nM; scale bars = 50 µm, magnification in B2,C2 and D2 600x; DP = Dermal papilla, MK = matrix keratinocytes; → = indicating melanocytes.

High power magnification of Masson-Fontana-stained HF sections suggested that TH treatment not only stimulated the total hair bulb melanin content, but also stimulated HF melanocyte dendricity (Fig. 3.7 B2, C2, D2). Unfortunately, the strength of the melanin-associated argentaffine histochemical signals hindered definitive confirmation of this intriguing observation.

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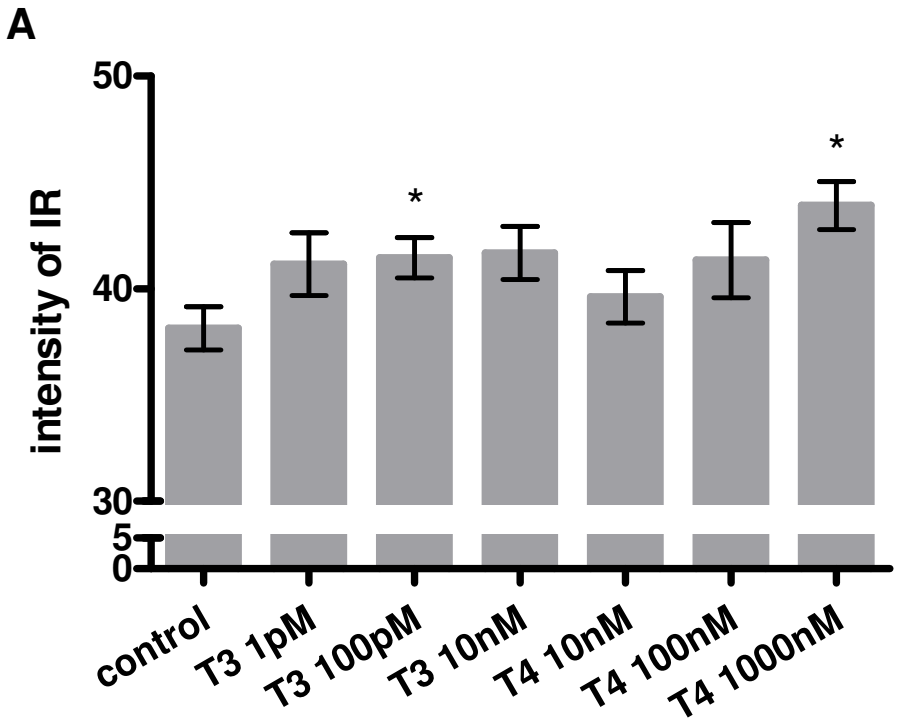
3.7 Thyroid hormones enhance follicular erythropoietin immunoreactivity

Given the reported effect of TH on erythropoietin (EPO) expression in a liver cell line (Ma et al. 2004) and the previous discovery that human HFs are non-hematopoietic targets and extra-renal production sites for erythropoietin, we also examined whether TH can alter the EPO protein expression in human HFs.

As shown in Fig 3.8, both T3 and T4 slightly up-regulated EPO IR in the ORS of human scalp HF after 9 days of organ culture in the presence of these THs. For T3, this up-regulation reached significance at a concentration of 100pM, for T4 at 1µM. Both these concentrations correspond to supraphysiological serum levels (T4: 6.4 x 10^{-8} mol L^{-1} - 1.5 x 10^{-7} mol L^{-1} and T3: 1.2 x 10^{-9} mol L^{-1} - 2.9 x 10^{-9} mol L^{-1};Eisenberg et al. 2008).



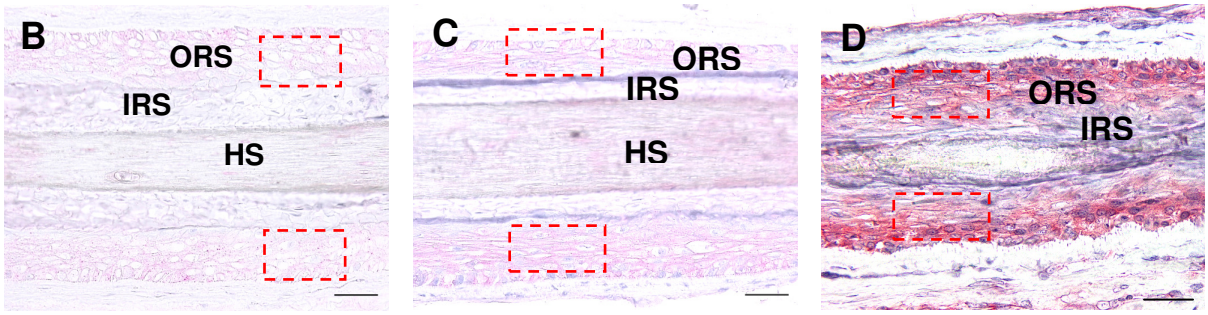


Figure 3.8 Thyroid hormones enhance follicular erythropoietin immunoreactivity (IR) in situ

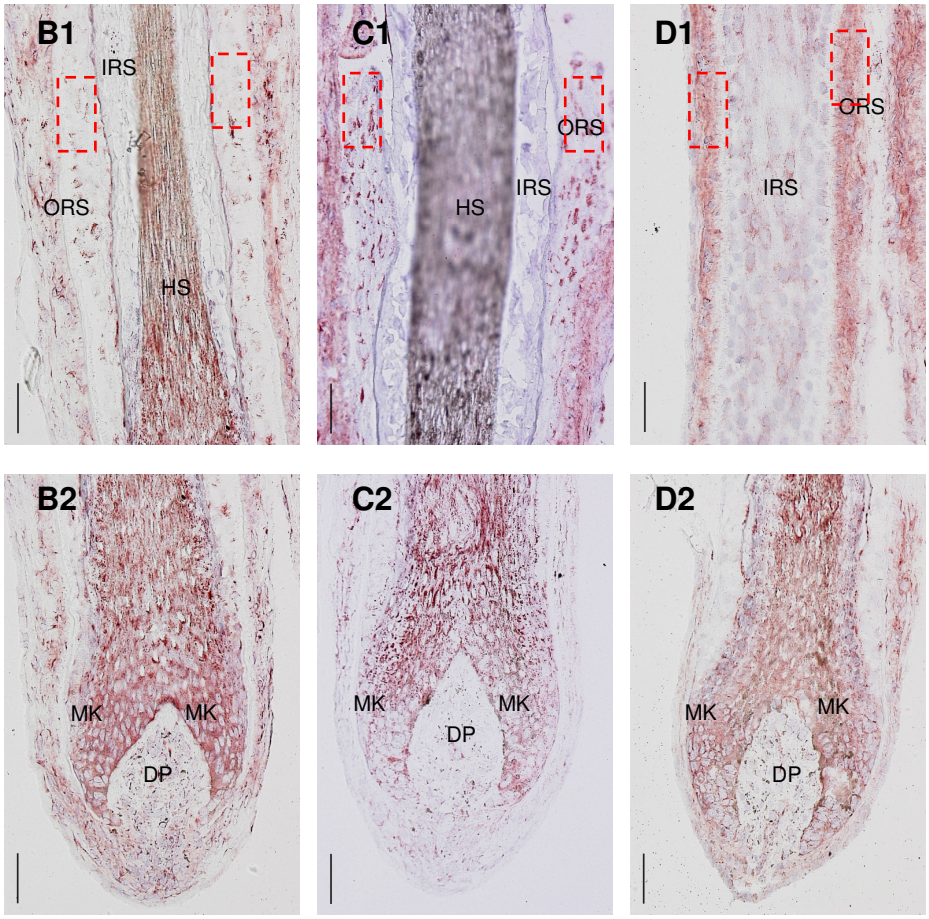
A) quantification of EPO-IR in the ORS, **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001; mean ± SEM, significances compared to control, B) control, C) T3 100pM, D) T4 100nM; 9 days culture; scale bars = 50 µm; ORS = outer root sheath, IRS = inner root sheath, HS = hair shaft; ⬚ = reference areas measured

3.8 Cytochrome-c-oxidase subunit 1 (COX1) immunoreactivity is up regulated by T3 and T4

Furthermore, we investigated whether TH up-regulate protein expression for a key enzyme of mitochondrial energy metabolism, Cytochrome-c-oxidase subunit 1 (COX1)

Here we show by quantitative immunohistomorphometry that T3 and T4 indeed both up-regulate the IR for the key respiratory chain enzyme COX1 in the outer root sheath of human scalp HFs.

COX 1 - IR



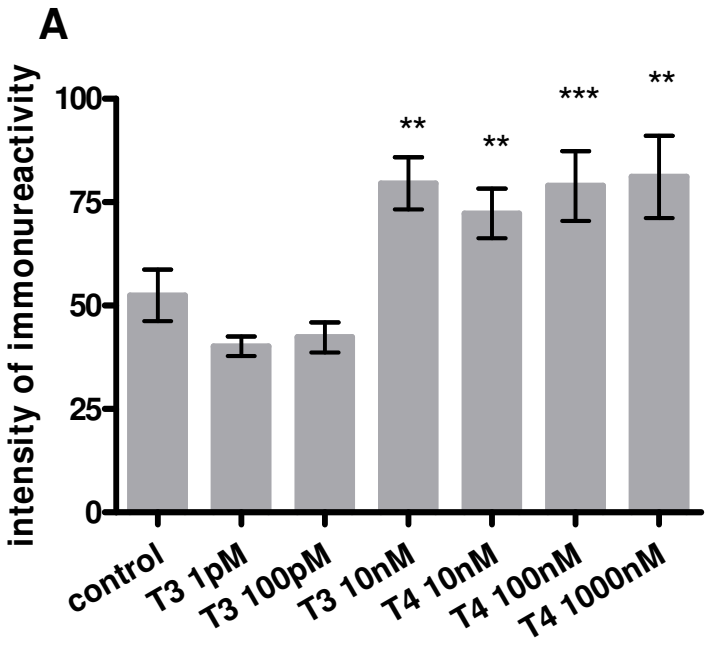


Figure 3.9 Cytochrome-c-oxidase immunoreactivity is stimulated by T3 and T4

A) quantification of COX1- IR, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$; mean ± SEM, significances compared to control; B1+2) control, C1+2) T3 100pM, D1+2) T4 100nM; 9 days culture; scale bars = 50 µm; ORS = outer root sheath, IRS = inner root sheath, HS = hair shaft; [ ] = areas measured.

The IR pattern was strictly spot-like and not homogenously distributed. Since the enzyme is mitochondrial encoded and located only in the respiratory chain of mitochondria (Sheehan et al. 2004), this staining pattern reflects the distribution of COX1 as well as that of mitochondria in human HF. Interestingly, T3 up-regulated COX1 IR only at high concentrations while all administrated T4 concentrations significantly enhanced COX1-IR significantly.

3.9 Deiodinases types 2 (D2) and 3 (D3) are transcribed by human HFs

In the current study, both T4 and T3 exerted (partially differential!) effects on TH-treated human HFs. This already strongly suggested that T4 can indeed be transported into human HF cells in organ culture and is here intracellular deiodinated to T3. In order to further probe this hypothesis, we finally determined by RT-PCR whether deiodinase 2 (D2) (which transforms T4 into T3 by outer ring deiodinisation), and/or deiodinase 3 (D3) (which transforms T4 into inactive, reverse T3) (Köhrle 2000) are transcribed in micro dissected human scalp HFs.

As shown in Fig. 3.10, both deiodinases (D2 and D3) are indeed transcribed in micro dissected human HFs derived from three different individuals at the expected bp length (.D2: 619 bp, D3: 292 bp).

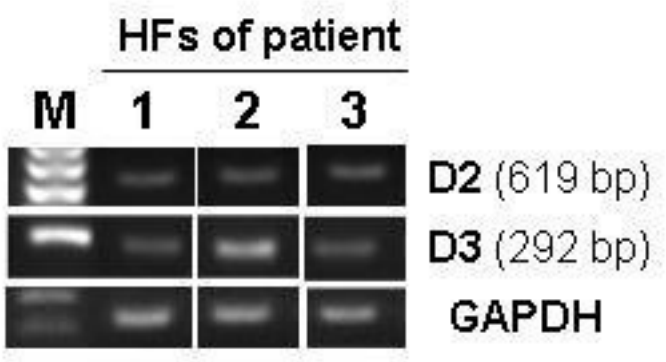


Figure 3.10 Micro dissected human anagen VI hair follicles express deiodinases type 2 (D2) and 3(D3)

Hair follicles from three different female patients (HF 1-3) were micro dissected, mRNA extracted and analysed by semi-quantitative RT-PCR. GAPDH served as housekeeping gene. M: DNA Standard.

4 Discussion

The specific questions that this study has addressed (see Introduction, p. 41), can be answered as follows:

1. TH do alter key parameters of human HF growth and pigmentation *in vitro*, and namely prolong anagen (Fig. 3.4) and stimulate follicular melanogenesis while hair shaft elongation is not significantly altered.
2. Congruently, TH inhibit apoptosis, and T4 also promotes proliferation of human hair matrix keratinocytes *in situ* (Fig. 3.1 & 3.2).
3. In line with their anagen-prolonging effects, TH inhibit the intrafollicular expression of the key catagen-inducing growth factor TGFβ2.
4. There are subtle differences between T3 and T4 in terms of modulation of human hair growth in organ culture, since only T4 but not T3 stimulates hair matrix keratinocytes proliferation. However, pigmentation is enhanced by both T3 and T4, with T4 showing the strongest melanogenic effect.
5. Human HFs do transcribe the deiodinases type 2 and 3.
6. TH alter the expression of cytokeratin 6 and 14 in a differential manner: CK6 is up regulated, while CK14 is down regulated by both T3 and T4.
7. T3 up regulated the expression of EPO by human scalp HFs *in vitro*.
8. TH may alter human HF mitochondrial metabolism, since they enhance the intrafollicular immunoreactivity for COX1, a key respiratory chain enzyme.

Following in the footsteps of Billoni et al (2002), who had already demonstrated TH *receptor* transcript- and protein expression in human HFs (namely, of TRβ2), this study provides the first comprehensive and conclusive evidence that these TH receptors are functional, and shows that human scalp HFs are indeed direct target organs for stimulation by THs. It shows that, in the absence of confounding systemic, neural, or undefined endocrine stimuli, T3 and T4 modulate multiple important parameters of normal HF biology, ranging from HF keratinocyte proliferation, apoptosis and keratin expression via HF cycling to HF pigmentation. Previously, it had already been shown that human HFs are capable of incorporating iodine *in vivo*: treatment of patients with radioactive iodine (I^{131}) that had undergone total thyroidectomy resulted in an accumulation of I^{131} in the scalp hair (Bhargava and Choi 2006).

In the current study, human HFs were obtained from temporal and occipital scalp skin sections of 7 different healthy donors. The donors were euthyroid (including one donor with slightly elevated TG-levels) to exclude pre-experimental changes in the donors' HFs that might have been caused by an altered thyroid status. Unfortunately, from one donor blood values were unavailable, so no investigations could be done regarding his thyroid status. The donor with slightly elevated TG-levels was included in this study, since the levels were just slightly above the normal range, and since all other thyroid investigations were normal (no thyroid disease was documented). The additional examination of anti-thyroid-antibodies like thyroid-peroxidase (TPO) (Kopp 2005) or TSH-receptor antibodies (Fekete and Lechan 2007) would have been optimal, but could not be performed for reasons of insufficient funding. Evaluating these antibodies would have excluded autoimmune thyroid disease. This might have been beneficial in the donor with slightly elevated TG levels, but since TSH-, fT3-, fT4- and TG-levels sensitively indicate thyroid disease, the available data make it highly likely that almost all the investigated HFs were derived from euthyroid donors (Molina 2006, Brent 2008, Kopp 2005). The various quality controls that were performed (see p. 48 - 55), furthermore, ensured that all HFs employed for the current study were of sufficient biological quality.

One major challenge that arises from the current study is to, now, study clinically in hypo- and hyperthyroid patients whether altered TH-serum levels *in vivo* modulate HF growth, cycling, and/or pigmentation and whether these could be restored by adjusting TH-serum levels to the *in vivo*-HF characteristics of the euthyroid state. Clinical investigations should also assess if TH (in hypothyroidism) or TH-inhibitors (in systemic hyperthyroidism) applied topically to the human scalp induce HF alterations *in vivo* that correspond to those observed here in organ culture. Certainly, the current novel findings strongly encourage such clinical follow-up-trials, ideally by the use of modern phototrichogram technology for the accurate *in vivo*-quantification of hair shaft elongation and HF cycling over extended time periods (Gassmueller et al. 2009).

Following the lead of Billoni et al. (Billoni et al. 2000), T3-concentrations of 10^{-12}, 10^{-10} and 10^{-8} mol L^{-1} were used in this study, which cover the effective concentration that these authors had reported to promote "HF survival" of Billoni et al. in addition to slightly infra- and supraphysiological concentrations. For T4, a physiological concentration of 10^{-7} mol L^{-1} was applied as well as infra- (10^{-8} mol L^{-1}) and supra- (10^{-6} mol L^{-1}) physiological concentrations (Eisenberg et al. 2008, Larsen et al. 2007). Thus, a wide range of TH-concentrations was employed to cover the possibilities of both higher and lower hormone uptake in organ culture compared to the *in vivo*-conditions. Systematic studies with an extended dose range would have revealed further insights into the dose-dependency of the effects examined, but could not be performed since this would have required a much larger number of donors and HFs than was available for this study.

Furthermore it needs to be kept in mind that the concentrations referred to as "physiological" are those of free T3 and free T4 measured in human serum, and do not necessarily reflect the actual TH concentration present in peripheral target tissues such as in normal human skin and its appendages *in vivo* (this concentration remains unknown). Since *in vivo* TH reach the HF via its dense vasculature, namely via the well-perfused CTS and the dermal papilla, *in vitro* TH-uptake into the HF occurs only through diffusion and not perfusion. Therefore, one can assume that higher TH-concentrations are necessary in organ culture in order to generate the same intrafollicular T3/T4 levels *in vitro* as the human HF is exposed to *in vivo*. Since the study focussed on exploring whether there are any direct effects of TH on human HFs at all, enzymes that control TH-uptake, such as MTC8 and MTC10 (van der Deure et al. 2010) were not specifically studied. It will be interesting to investigate in follow-up studies where exactly these enzymes are expressed in the human HF, and whether their activity changes during HF cycling and after exposure to substrate (i.e. different concentrations of T3 or T4).

To investigate the effects of TH on human HFs, previously developed standard techniques of quantitative immunohistomorphometry were used, based on highly sensitive and specific immunohistochemistry and immunofluorescence methods (Foitzik et al. 2006, Bodó et al. 2007a+b, Ito et al. 2005). For the assessment of erythropoietin-IR, the highly sensitive EnVision® visualization technique (Bodó et

al. 2007a) was employed, while the even more sensitive TSA-immunostaining method was used to investigate TGF-β2 immunoreactivity in the proximal ORS. Since all immunostainings were performed with adequate negative and positive control (see table 2.4), which yielded the expected results, this attested to the sensitivity and specificity of the observed immunoreactivity patterns.

While the paucity of available human scalp HF precluded systematic dose-response studies, our findings suggest that the predominant *direct* effect of both physiological and supraphysiological T3 and/or T4 concentrations in human hair growth control is that of a hair growth-promoting agent:

- T4 stimulates hair matrix keratinocyte proliferation (Fig, 3.1, Fig.3.2),
- Matrix keratinocyte apoptosis is inhibited by T3 and T4 (Fig, 3.1, Fig.3.2),
- TH prolong anagen duration (i.e. retard spontaneous catagen development) (Fig. 3.4).

This makes the topical application of TH (e.g. of thyroxin in an alcoholic vehicle, just as it is regularly being practised already in clinical dermatology for other steroid hormones, such as glucocorticoids and estrogens) and attractive candidate to be tested as hair growth-stimulatory agent in the human system. The fact that topical TH has already been shown to promote pelage hair growth in mice in vivo (Safer et al. 2001) further encourages one to pursue the current preclinical data in appropriately controlled, double-blind, prospective, randomized clinical trials.

These preclinical data help to explain the previously unclear pathogenesis of the telogen effluvium observed in hypothyroid patients, where a relative TH deficiency had been proposed (but never conclusively demonstrated) to cause premature catagen induction and reduced hair matrix proliferation (Freinkel and Freinkel 1972, Ebling 1991, Schell et al. 1991). The demonstration of the fact that the addition of exogenous TH to human HFs that are cultured in the absence (!) of TH prolongs anagen, stimulates hair matrix keratinocyte proliferation and inhibit their apoptosis provides the first direct evidence for the validity of this old postulate. These findings also correspond well to the reportedly increased hair matrix proliferation in hyperthyroid patients *in vivo* (Schell et al. 1991). The anagen prolongation documented here also may correspond to the (poorly defined and

unclear) parameter of “prolonged HF survival” previously reported by Billoni et al. (2000).

The stimulatory effects of T4 on human hair matrix keratinocyte proliferation stand in striking contrast to that of other steroid hormones. Both 17β-estradiol (E2) and prednisolone (P) enhance apoptosis and inhibit the proliferation of hair matrix keratinocytes *in situ* (in high concentrations) (Bodó et al. 2007b) possibly driving chemotherapy- damaged human HFs into “dystrophic catagen” (Bodó et al. 2009a). Also, topical E2, calcitriols (vitamin D3) and glucocorticoids had all previously been demonstrated to “freeze” murine back skin HFs in telogen and to potently induce premature catagen development in vivo (Reichrath et al. 1994, Paus et al. 1994, Ohnemus et al. 2006). Thus, it deserves to be systematically followed-up, whether topically applied THs may actually be exploited as a stimulator of human hair growth, e.g. in patients suffering from chemotherapy-induced effluvium and alopecia (Bodó et al. 2007b, Safer et al. 2009).

Interestingly, the hair growth promoting effects of TH were not associated with significant stimulatory effects of either T3 or T4 on hair *shaft* growth during the experimental observation period. While this correlates well with the previous results of Billoni et al. (2000), it is conceivable that the relatively short culture period may not have allowed detecting subtle TH effects on human hair shaft formation *in vitro*.

In this context, it needs to be kept in mind that the assay employed here creates a severely “hypothyroid” state *in vitro*, since HF organ culture is performed with serum-free William’s E medium supplemented only with insulin and hydrocortisone (Philpott et al. 1990). Thus, HFs could only have been stimulated by residual quantities of endogenous TH that had already been bound within the HFs (e.g. to intrafollicular TRβ) before HF micro dissection and organ-culture. With every change of medium, these residual TH quantities would have been further reduced/ diluted. Therefore, it is a striking fact that, even in the absence of extra follicular TH, hair shaft production of organ-cultured, amputated human anagen VI HFs *in vitro* progresses at almost the normal speed compared to hair shaft growth seen *in vivo* (Philpott et al. 1994), just as can be seen here in the vehicle-treated control

groups (Fig. 3.3 A+B, Fig. 3.4). This suggests that, despite the profound modulatory effects of TH on several hair biology parameters demonstrated here, extra follicular TH seem dispensable for normal hair *shaft* production under assay conditions and for a limited time period. Naturally, this does not exclude a long-term role for TH in human hair shaft production *in vivo*, especially since hair shaft abnormalities have been clinically reported in both hypo- and hyperthyroid patients (Messenger et al. 2000, Fistarol 2002, Stüttgen 1974). This fact combined with a slight tendency towards enhanced hair shaft production seen in some TH-treated groups raises the possibility that, over weeks and months, TH might well be indispensable for normal hair shaft production of human scalp HFs *in vivo*.

Our study shows that TH do prolong anagen duration significantly *in vitro*, and thus demonstrates one key mechanism for how these steroid hormones may promote hair growth. This finding not only helps to explain the telogen effluvium that can be clinically observed in states of thyroid hormone deficiency (Schell et al. 1991), but also further encourages one to explore topical THs as clinically useful anti-hair loss agents.

Unfortunately, isolated human scalp anagen VI hair bulbs do not run through full cycles in organ-culture and decay shortly after catagen development (Foitzik et al. 2005, Philpott et al. 1990, Philpott et al. 1994, Ito et al. 2005). Therefore, the current organ culture assay cannot clarify another old, as yet unconfirmed hypothesis that hyperthyroidism may also lead to telogen effluvium due to the induction of faster hair follicle cycling (Messenger 2000, Fistarol 2002, Everett et al. 1997, Kaminski 1997). In the current study, high concentrations of T3 or T4 even prolonged anagen duration compared to control (Fig. 3.4). This questions the validity of the old hypothesis of faster HF cycling induced by TH in hyperthyroidism. However, the multiple endocrine changes induced by hyperthyroidism *in vivo* and potential concomitant effects of anti-thyroid auto antibodies (e.g., anti-TSH receptor auto antibodies might stimulate keratinocyte proliferation [Cianfarani et al. 2010]) require caution against overly simplistic transfer of the current *in vitro*- data to the much more complex clinical situation in hyperthyroid patients. Also, the possibility deserves to be considered that the effluvium observed in hyperthyroid patients (Fistarol 2002) might speculatively

reflect TH-induced excessive stimulation of active hair shaft shedding (exogen). Since, unfortunately, there is no assay for studying exogen in organ-cultured human HFs available yet, this intriguing theoretical possibility could not be excluded or verified here.

The current study provides at least one important pointer to the molecular mechanisms by which TH may exert their anagen-prolonging effects: TH down-regulate the intra-follicular protein expression of TGFβ2 (Fig. 3.5), one of the recognized key catagen inducers during human HF cycling, which also inhibits proliferation and stimulates apoptosis of human hair matrix keratinocytes *in situ* and operates as the key terminator of anagen (Hibino and Nishiyama 2004, Itami and Inui 2005, Soma et al. 2002). Despite substantial inter- individual variations, a tendency towards suppression of TGFβ2 could be identified in all HFs investigated here. Although this down- regulatory effect of TH on TGFβ2 may only reflect a small segment of the changes induced by TH in the complex intrafollicular signalling milieu that drive HF transformations from anagen VI towards catagen (see introduction) its inhibition by TH constitute at least one concrete and likely molecular mechanism of TH- action.

The inhibition of intra-follicular TGFβ2 expression by TH is in striking contrast to that of another important, hair growth-modulatory steroid hormone, i.e. alltrans-retinoic acid (tretinoin): It had previously been shown that tretinoin exerts its hair growth-inhibitory, catagen-promoting effects on organ-cultured human HFs at least in part via *up*-regulating TGFβ2 (Foitzik et al., 2005). While alltrans-retinoic acid is another important, hair growth-modulatory steroid hormone (Foitzik et al., 2005), one of its nuclear hormone receptors (RXR) is a known heterodimerization-partner of the thyroid hormone receptor (TR) (Leid et al. 1992). Thus, it is conceivable that RXR and TR ligands interact in the control of HF cycling so as to fine- tune the switch from anagen to catagen by impacting on the intrafollicular TGFβ2 level.

An extended analysis of how TH alter the intra-follicular transcription and protein expression for TGFβ2 is an obvious next step for follow-up studies. Also, it remains to be demonstrated that down-regulation of TGFβ2 is really needed for the anagen- prolonging effects of TH. This could be achieved e.g. by showing that

the latter effects can be abrogated by the co- administration of TGFβ2- neutralizing antibodies (just as it has already been documented for the TGFβ2- dependence of catagen- promoting effects of retinoids [Foitzik et al. 2005]), or by the knock-down of TGFβ2 or TGFβ receptor type II by siRNA technology (e.g. Elbashir et al. 2002) in the presence of TH stimulation.

The current study also revealed for the first time that TH modulate the intrafollicular protein expression for two recognized thyroid hormone-responsive keratins, namely CK6 and CK14 *in situ* (Fig. 3.6). Since the promoter regions of these keratin genes host TREs it is conceivable that TH-TR complexes regulated the transcription of these keratin genes via the classical, TRE-dependent nuclear pathway (Radoja et al. 1997, Freedberg et al. 2001, Blumenberg et al. 1992). In skin, specific keratins can be employed to distinguish between defined keratinocyte subpopulations. For example, cytokeratins 5 and 14 characterize proliferating basal layer keratinocytes, while the cytokeratins 1 and 10 are expressed in terminally differentiating human epidermal keratinocytes (Freedberg et al. 2001). In response to injury (wound healing) or under pathologic conditions (e.g. inflammation-associated hyperproliferation) the keratinocytes can express CK6, CK16, and CK17 (Tomic-Canic et al. 1998, Langbein and Schweizer 2005). Our study showed that TH enhance protein expression of the type II keratin, CK 6, which typically expressed by proliferating ORS keratinocytes under physiological conditions, and decreased expression of the type I keratin, CK 14, which characterizes all proliferating keratinocyte populations in the epidermis and HF (Langbein and Schweizer 2005).

While the current study does not allow drawing definitive conclusions on the functional importance of the regulation of these keratins by TH, it is conceivable that TH modulate ORS keratinocytes functions. The stimulatory effect of TH on CK6 expression may be interpreted to be in line with the concept that this keratin, generally, is thought to be associated with hyperproliferative states of human skin epithelium (Ramot et al. 2009, Langbein and Schweizer 2005). However, the proliferation- *stimulatory* TH effects on hair matrix keratinocytes *in situ* seem to conflict with the simultaneous *down*- regulation of the prototypic keratin of proliferating, basal layer keratinocytes, CK14. However, the expression of both

keratins was measured in the ORS far above (i.e. distal to) the hair matrix, while proliferation was only measured in hair matrix keratinocytes (i.e. in a biologically very different, much less differentiated HF keratinocyte population), these results are not necessarily conflicting: A down- regulatory effect on basal ORS keratinocyte CK14 expression does not exclude a proliferation- stimulatory effect of TH in the hair matrix. Future studies should follow- up the intriguing differential effects of TH on CK6 versus CK14 in all .epithelial skin compartments that express both keratins under physiological or pathological conditions.

Also, it remains to be investigated to which extent TH alter the expression of keratins that are primarily or exclusively expressed in the hair shaft cuticle, cortex or medulla, such as the previously defined expression of hK6irs1 in the IRS (Langbein et al. 2002) and CK15 in the bulge region (Kloepper et al. 2008). This appears important since hair shaft abnormalities have been clinically reported in both hypo- and hyperthyroid patients (Messenger 2000, Fistarol 2002, Everett et al. 1997) it is likely that keratin changes caused by TH also concern the hair shaft. However, the normal speed of hair shaft production and the normal hair shaft morphology in organ- cultured HFs suggests that TH are largely dispensable for normal hair shaft production under assay conditions. Naturally, this does not exclude a long-term role for TH in human hair shaft production *in vivo*, as described above. Since optimized adaption of keratins is crucial for optimal function of human skin and its appendages, TH-mediated changes in keratin-expression could contribute to the environmentally adapted regulation of certain keratins (Ramot et al. 2009). This hypothesis remains to be tested.

The current study also provides the first conclusive evidence that T3 and T4 *directly* stimulate intrafollicular melanin synthesis (Fig. 3.7 A-D). Since intrafollicular melanogenesis is strictly coupled to anagen and HF pigmentation declines sharply during early anagen-catagen transformation of HFs (Slominski et al. 2005), our analysis was restricted to the comparison of anagen VI HFs between test and control groups. The ruled out any confounding influence hair cycle-associated pigmentation changes by TH and excluded the “stimulation” of melanogenesis only reflected the anagen-prolonging effects of TH. Although TH have been proposed for decades to be relevant for hair colour maintenance (e.g.

Lerner 1971), the current results actually provide the first evidence that TH can really stimulate melanogenesis in the mammalian system *in situ*. This correlates nicely with a recent clinical case report that *in vivo*-exposure to high doses of exogenous T4 caused darkening of gray/ white hair (Redondo et al. 2007). While in this report indirect modes of TH action could not be ruled out, our data show that TH directly impacts on human HF pigmentation. Naturally, this suggests that topically applied TH might become a new clinical tool to fight hair greying (Slominski et al. 2005).

The current observation that THs appeared to even stimulate HF melanocyte dendricity (Fig.3.7 B2, C2, D2), even though the employed histochemistry method was insufficiently sensitive to confirm this, suggests that not only melanogenesis as such, but also the transfer of melanosomes from HF pigmentary unit melanocytes to terminally differentiating keratinocytes of the precortical hair matrix is stimulated by TH. If true, this would imply a far-reaching general activation of melanocytes by TH. Therefore, this interesting preliminary observation deserves to be systematically followed up, namely by immunohistochemical analysis of the expression of a key premelanosome marker, gp100 (NKi-beteb), which allows to validate the important hypothesis that THs stimulate melanosome transfer from human HF melanocytes to human HF keratinocytes *in situ* (Singh et al. 2008, Kloepper et al. 2009). While the underlying mechanism(s) and intracellular pathways remain to be dissected, this further illustrates how well-suited organ-cultured human scalp HFs are as a clinically relevant, highly instructive, yet still underexploited discovery tool for the identification of new functions for ancient steroid hormones in cutaneous endocrinology.

As discussed, thyroid diseases can induce distinct hair phenotypes in affected patients (see Introduction, p. 38-39). Since most of the endogenous TH is available as T4 in the serum, while the form considered to be the biologically more “active” one is T3 (Molina 2006), conventional wisdom holds that deiodinases are necessary to convert T4 into T3 within target cells. Since we show here that, not only T3, but also T4 significantly modulates key read-out parameters of human HF biology in organ culture, it is likely that human HFs can convert T4 to T3 – just like all TH-sensitive target tissues (Köhrle 2000). Intriguingly, DIO2 mRNA was already

detected by laser capture micro dissection in the stem cell-rich bulge region of the human HF ORS (Ohyama et al. 2006), while DIO 2 and DIO 3 deiodinase transcripts had previously be found in cultured human skin fibroblasts, melanocytes and keratinocytes (Slominski et al. 2002) and in cultured K15+ human epithelial progenitor cells (Tiede et al. 2009). This corresponds well to the current finding that human HFs transcribe deiodinase genes (Fig. 3.10) and further supports the concept that human HFs have the enzymatic equipment to convert T4 into T3.

The recent finding that human HF epithelial stem cells show an unusually high transcription of deiodinases *in situ* and in cell culture (Ohyama et al. 2006, Tiede et al. 2009) raises the important question whether these crucial epithelial stem cells of human skin are particularly sensitive to TH stimulation, namely by T4, and which stem cell functions are modulated by TH stimulation. Luckily, the most recent establishment of keratin 15 promoter-driven GFP+ human epithelial hair HF progenitor cells (Tiede et al. 2009) offers a powerful new research tool to directly investigate the role of TH in the as yet obscure hormonal controls of HF stem cells (Paus et al. 2008).

The subtle differences observed here between T3 and T4 in the assessed parameters, namely with respect to HF proliferation and HF pigmentation (see e.g. Figs. 3.1, 3.2 and 3.7), raise the possibility that T4 also unfolds separate activities that are independent of its conversion to T3 (Davis et al. 2005). In this context, it is particularly interesting that only T4 stimulated hair matrix keratinocyte proliferation significantly in these experiments, but not T3. This makes it unlikely that the observed T4 effects were mainly due to intrafollicular conversion of T4 to T3 e.g. by DIO2. Instead, this finding might be interpreted as an indication that T4 acts directly on the HF epithelium via cytosolic activation of the IP3- (Davis et al. 2008) or the MAP-kinase (Lin et al. 1999) pathways (see below). However, in the higher concentrations of T3 tested, there was a tendency towards the stimulation of hair matrix proliferation, which might have reached significance if a higher number of HFs could have been tested. Thus, at least some of the observed TH effects likely were mediated by T3, which has a much higher affinity to TRβ2 than T4 (Molina 2006). A deiodinase- inhibitor (iopanoic acid = IOP), which inhibits deiodinases 1 and 2, was shown to suppress the proliferation of primary cultured human

keratinocytes. This effect could be counteracted by application of T3 but not by T4, suggesting that T3 is the main regulator for human epidermal keratinocyte proliferation *in vitro* (Safer et al. 2009). Instead, our data question whether this concept is also valid for human hair matrix keratinocytes *in situ*, and raise the intriguing possibility that this cell population primarily relies on T4 to regulate its proliferation *in vivo*.

Given the many direct TH target genes that display a TRE in their promoter region and the numerous additional negative TRE regulated genes that nevertheless are regulated by TH (Shen et al., 2004) as well as non-classical, receptor-independent TH activities (Davis et al. 2005, Davis et al. 2008) (see table 4.1), multiple additional pathways and molecular targets by which TH may impact on human HF biology must now be considered and systematically explored.

	examples	**reference**
TRE-dependent	Increased turnover	Gouveia et al. 2002
	CK14 and CK6-regulation	Radoja et al. 1997
Negative TRE	TRH- regulation	Hollenberg et al. 1995
	Prohormone-Convertase regultion	Shen et al. 2004
Non- genomic	HIF-1α induction	Moeller et al. 2005
	Increased cardiac output and lowering of vascular resistance	Schmidt et al. 2002

Table 4.1 different pathways of thyroid function

For example, TRE- independent effects of TH even display immunomodulatory impact, since TH may augment IFNγ- effects via PKC and PKA (Lin et al. 1997). Also, since Lin et al. (1997) have published that T4 induces calcium signalling via direct cytosolic binding to TRβ, one reasonable focus of such future studies could be to characterize the differential impact of T4 versus T3 on intracellular calcium signalling in human HF keratinocytes *in vitro*. Non-genomic effects of TH might be important for the fine- tuning of HF defined functions/activities (e.g. hair cycle-dependent hair matrix keratin proliferation/apoptosis and cytokeratin expression).

The role of non-genomic, TRE-independent effects of TH could be probed under physiologically relevant conditions in the human system by stimulating organ-cultured scalp HFs with T4 in the presence of deiodinase inhibitors, or by pretreating HFs with deiodinase siRNA before T4 administration. Any alterations of HF biology read-out parameters that T4 still induces then, would likely not result from its conversion to T3 and may represent non-genomic effects. A comparison with T3 administration with the appropriate inhibitors/ siRNA could clarify, whether also T3 is able to exhibit non-genomic effects.

Since hypothyroidism is frequently associated with macrocytic hypochromic anaemia of moderate severity or hyper-proliferation of immature erythroid progenitors (Larsen et al. 2008), a role for TH on haematopoiesis has long been appreciated, even though the exact underlying mechanisms, including the role of EPO in mediating TH effects on human haematopoiesis, are still debated (Kawa et al. 2009, Gianoukakis et al. 2009). Since it was recently shown that EPO is expressed on the gene and protein level by human HFs and induces differential gene expression changes in the latter (Bodó et al. 2007a), it was particularly intriguing that T3 and T4 increased the intrafollicular protein immunoreactivity for EPO in the central ORS of organ-cultured human scalp HFs (Fig. 3.8). These results are in line with previous reports that TH can stimulate EPO-expression in HepG2 cells, a human hepatoma cell line, and rat kidney (Ma et al. 2004).

In the bone marrow, EPO potently inhibits the apoptosis of erythroid progenitor cells (Jelkmann 2004). In addition, EPO is increasingly understood to operate as a general inhibitor of apoptosis in multiple different systems (Brines and Cerami 2006, Kaushansky 2006, Paus et al. 2009). Therefore, it is conceivable that TH recruit intrafollicular EPO production as yet another player in the regulation of hair matrix apoptosis (e.g. to prolong anagen by suppressing matrix keratinocyte apoptosis). However, in the current study, the up-regulatory effect of TH on EPO expression was only seen in the ORS, not in hair matrix keratinocytes, even though the highly sensitive EnVision® immunostaining technique was used. This makes it less likely that TH recruit EPO to regulate hair matrix apoptosis, and one can currently only speculate whether TH-induced EPO production in the ORS

suppresses apoptosis in human ORS keratinocytes *in situ* functioning e.g. as an endogenous cytoprotectant during HF cycling (Bodó et al. 2007a).

Most recent evidence suggests that EPO can also exert – albeit, greatly divergent - pigmentary effects on human HF in organ culture, as it both up- and down-regulated the melanin content and tyrosinase activity in situ in HFs derived from different donors (Bodó et al. 2009c). Although purely speculative, up regulation of EPO production in the ORS may, therefore, also indirectly affect HF pigmentation, e.g. by modulating the secretion of pigmentation-regulatory signals from the ORS. As TH not only directly enhance hypoxia-induced EPO- formation in the isolated HepG2 cells but also increase hypoxia inducible factor subunit α (HIF-1α), a key upregulator of EPO- production (Ma et al. 2004). Since HIF-1α regulation is reportedly displayed by non- genomic TH- action (Moeller et al. 2005), the exact role of TH as promoters of non-classical EPO production by peripheral tissues certainly is a fascinating new research frontier.

It has long been recognized that TH are key regulators of energy metabolism (Sheehan et al. 2004, Arnold et al. 1998, Pillar and Seitz 1997, Mutvei et al. 1989, Soboll 1993). On the cellular level, this effect appears to be mediated at least in part via up-regulation of cytochrome-c-oxidase (COX1) expression, a key respiratory chain enzyme located at the inner membrane of mitochondria (Sheehan et al. 2004, Arnold et al. 1998, Pillar and Seitz 1997, Scheffler 2008). Here, we show by quantitative immunohistomorphometry that T3 and T4 indeed up-regulate IR for COX1 in the ORS (Fig. 3.9). Functional cytochrome-c-oxidase subunit 1 is required for the stability of complex IV of the respiratory chain (Li et al. 2007). These COX1 data raise the possibility that the proliferation-stimulatory effects of T3/ T4 on human hair matrix keratinocytes (Fig. 3.1, Fig. 3.2) may reflect, at least in part, a TH-induced boostering effect on mitochondrial inner membrane potential, and ultimately the intramitochondrially generated ATP level (Gouveia et al. 2002, Scheffler 2008). Thus, another sensible follow-up experiment would be to compare the ATP levels of TH-treated and control HFs so as to further explore this hypothesis.

The current study nicely illustrates that human HF organ culture (Philpott et al. 1990) offers an excellent, physiologically and clinically relevant test system not only to elucidate this still unclear effects of TH on human hair growth and pigmentation, but also for exploring both classical and non-classical effects of TH on peripheral TH target tissues. As demonstrated here, this simple and highly instructive test system allows one to identify and explore novel functions of TH, thus complementing our – still very incomplete - understanding of the role of steroid hormones in cutaneous endocrinology.

Recently, a novel “microfolliculoid” assay system to further dissect the epithelial and mesenchymal interactions that underlie human HF biology was introduced by Havlickova et al. (2009). This assay offers an attractive research tool for characterizing and experimentally manipulating the signalling exchanges between HF keratinocytes and specialized, inductive HF fibroblasts after the TH stimulation. This assay optimally complements classical human HF organ culture and could facilitate e.g. TR overexpression and knock-down experiments to gain deeper insights into nuclear and non-nuclear effects of TH, using specific antagonists to block the different pathways (Safer et al. 2009). This “microfolliculoid” system could also reveal further insights into the regulation of the HPT-axis equivalent system that may be established in the HF itself: TSH-R was revealed to be expressed in human HF *in situ*. While TSH did not stimulate proliferation, pigmentation or hair growth, extrathyroidal TG- transcription occurred after stimulation with TSH in human HF (Bodó et al. 2009b), indicating that at least this element of the HPT-axis is present in normal human HFs. Additional recent evidence that human scalp HFs express both TRH and TRH-R and that TRH is a strong stimulator of human hair growth in vitro (Gáspár et al. 2009), stand alongside the most recent discovery that TRH stimulates hair matrix proliferation. There, this “microfolliculoid” system should help to further explore whether or not a fully function HPT axis equivalent (Paus 2010) is really established in normal human HFs .

5 Summary

Clinically, insufficient as well as excess levels of thyroid hormones (T3 and T4) can result in modification of hair structure and function and can lead to effluvium or even alopecia. Whether T3 and T4 exert any direct effects on human hair follicles (HFs), and if so, how exactly human HFs respond to T3/T4 stimulation, is still unclear. Therefore, the impact of T3/T4 on human HF *in vitro* was investigated in this study. To this aim, human anagen HFs were micro dissected from skin obtained from euthyroid females (40-69 yrs) undergoing routine facelift surgery, and were treated with T3/T4 or vehicle as control in serum-free organ culture.

Here the study shows that T4 up-regulates the proliferation of hair matrix keratinocytes, whereas their apoptosis is down-regulated by T3 and T4 in organ-cultured normal human scalp HFs. Furthermore, T4 prolongs the duration of anagen, the hair growth phase, *in vitro*. This anagen- prolongation may due to the down-regulation of TGF β_2, the key anagen- inhibitory growth factor. Intrafollicular immunoreactivity for the recognized thyroid hormone-responsive keratins cytokeratin 6 (CK6) and cytokeratin 14 (CK14) is significantly altered by T3 and T4 (CK6 is enhanced, CK14 down regulated). Both T3 and T4 also significantly stimulate intrafollicular melanin synthesis, with supraphysiological concentrations of T4 showing the strongest stimulation of intrafollicular melanogenesis. Since thyroid hormones reportedly can stimulate erythropoietin expression and since previously human scalp HFs were identified as an extrarenal source of erythropoietin, it was also assessed whether thyroid hormones increase intrafollicular erythropoietin immunoreactivity. This is indeed the case. In addition, T3 and T4 also up-regulate the expression of cytochrome-c-oxidase subunit I (COX1), a key respiratory chain enzyme, in the outer root sheath of the HF. Finally, both deiodinase 2 and deiodinase 3 are transcribed in the human HF, thus allowing a intrafollicular transformation of T4 into T3.

Taken together, this study present the first evidence that human HFs are direct targets of thyroid hormones and that T3 and/or T4 modulate multiple, clinically important hair biology parameters, ranging from HF cycling to hair pigmentation.

6 Zusammenfassung

Klinisch können sowohl zu niedrige als auch zu hohe Blutkonzentrationen von Schilddrüsenhormonen (T3 und T4) Veränderungen in der Haarstruktur und der Haarfunktion bewirken und können zu Effluvium oder sogar Alopezie führen. Ob T3 und T4 überhaupt direkte Auswirkungen auf humane Haarfollikel (HF) haben und wenn, wie genau humane HF in diesem Falle auf T3/T4- Stimulation reagieren, ist noch unklar. Daher befasst sich diese Studie mit dem Einfluss von Schilddrüsenhormonen auf humane HF *in vitro*.
Zu diesem Ziel wurden humane anagen HF aus Haut bereitgestellt von euthyroiden Frauen zwischen 40 und 69 Jahren im Rahmen von routinemäßigen Facelift-Operationen isoliert und die HF.wurden mit T3/T4 oder nur Medium als Kontrollgruppe in Serum- freier Organkultur behandelt.

Diese Studie zeigt an mikrodissezierten, normalen humanen Kopfhauthaarfollikeln in Organkultur, das T4 die Proliferation von Haarmatrix- Keratinozyten steigert, während ihre Apoptose durch T3 und T4 verringert wird. Des Weiteren verlängert T4 die Anagen- Phase, die Wachstumsphase des Haares, *in vitro*. Diese Verlängerung des Anagens ist möglicherweise in der Verringerung von TGF β_2, dem Schlüsselfaktor der Anagen- Inhibition, begründet. Die intrafollikuläre Immunreaktivität (IR) für die bekannten Schilddrüsenhormon- reaktiven Keratine Cytokeratin 6 (CK6) und Cytokeratin 14 (CK14) wird durch T3 und T4 signifikant verändert: CK6 wird verstärkt, CK14 vermindert exprimiert. T3 sowie T4 stimulieren außerdem signifikant die intrafollikuläre Melaninsynthese, wobei supraphysiologische Konzentrationen von T4 die stärkste Stimulation der Melaninsynthese zeigen. Da Schilddrüsenhormone Artikeln zufolge die Erythropoietin- Expression stimulieren und vor kurzem humane Kopfhauthaarfollikel als extrarenale Erythropoietinquelle identifiziert wurden, haben wir ebenfalls untersucht, ob Schilddrüsenhormone die intrafollikuläre Erythropoietin IR erhöhen und konnten diese Hypothese bestätigen. Zudem verstärken T3 und T4 die Immunreaktivität der Cytochrome-C-Oxidase subunit I (COX1), einem Schlüsselenzym der mitochondrialen Atmungskette, in der äußeren Wurzelscheide der Haarfollikel. Abschließend konnten wir die Präsenz der Deiodinasen 2 und 3 im menschlichen Haarfollikel feststellen, wodurch neben

dem Abbau der Schilddrüsenhormone auch die Konversion von T4 zu T3 möglich ist.

Zusammengefasst präsentiert diese Studie also den ersten Beweis, das humane HF direkte Zielstrukturen für Schilddrüsenhormone darstellen und demonstriert, das T3 und/oder T4 multiple Parameter der Haarbiologie vom Haarzyklus bis zur Pigmentation beeinflussen.

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7.1 Publications

The results presented in this thesis were published in part in:

van Beek N, Bodó E, Kromminga A, Gáspár E, Meyer K, Zmijewski MA., Slominski A, Wenzel BE., Paus R,
Thyroid hormones directly alter human hair follicle functions: anagen prolongation and stimulation of both hair matrix keratinocyte proliferation and hair pigmentation. ***J Clin Endocrinol Metab*** 93, 4381–4388 (2008) (Impact Factor 5.25)

Abstracts based on data of this thesis:

van Beek N, Bodó E, Kromminga A, Gáspár E, Zmijewski M, Slominski A, Funk W, Wenzel B, Paus R
Evidence that thyroid hormones directly affect human hair follicles: Prolongation of anagen, stimulation of hair matrix proliferation, pigmentation and energy metabolism. *Exp Dermatol* 17, 283-264 (2008).

van Beek N, Bodó E, Kromminga A, Gáspár E, Meyer K, Zmijewski MA., Slominski A, Wenzel BE., Paus R,
Human hair follicles are direct targets for thyroid hormones: involvement in anagen prolongation, hair matrix proliferation, hair pigmentation and metabolism. *J Invest Dermatol* 128, 156-156 (2008).

Additional publications, where the methods learned during the thesis were employed by the candidate, who also contributed original data:

Bodó E, Kromminga A, Bíró T, Borbíró I, Gáspár E, Zmijewski MA, **van Beek N**, Langbein L, Slominski AT, Paus R.

Human Female Hair Follicles Are a Direct, Nonclassical Target for Thyroid-Stimulating Hormone. *J Invest Dermatol* 129, 1126-1139 (2009)

Bodó E, **van Beek N**, Naumann V, Ohnemus U, Brzoska T, Abels C, Paus R.

Modulation of chemotherapy-induced human hair follicle damage by 17-beta estradiol and prednisolone: potential stimulators of normal hair regrowth by "dystrophic catagen" promotion? *J Invest Dermatol* 129, 506-509 (2009)

Ito N, Sugawara K Bodó E, Takigawa M,,**van Beek N,** Ito T, Paus R
Corticotropin-releasing hormone (CRH) stimulates the *in situ* generation of mast cells from precursors in the hair follicle mesenchyme. *J Invest Dermatol* [Epub ahead of print] (2010)

Kloepper JE, Sugawara K, Al-Nuaimi Y, Gáspár E, **van Beek N**, Paus R
Methods in hair research: How to objectively distinguish between anagen and catagen in human hair follicle organ culture. *Exp Dermatol* [Epub ahead of print] (2009).

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7.3 Curriculum Vitae

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October 2007 -February 2009	clinical studies at the University of Luebeck, Luebeck, Germany including elective "Neonatology"
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October 2005 – July 2006	clinical studies at the University of Luebeck, Luebeck, Germany including elective “Clinical Allergology”
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October 2003	beginning of medical studies at the University of Luebeck, Luebeck, Germany

EDUCATION – SCHOOL

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PRACTICAL EXPERIENCE

Since April 2007	working as a student assistant in the Laboratory of Experimental Dermatology, Dept. of Dermatology
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October 2007	internship surgery at the department of plastic surgery, University of Aarhus, Denmark
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