Aus der Klinik für Dermatologie, Allergologie und Venerologie
der Universität zu Lübeck

Direktor: Prof. Dr. med. D. Zillikens

New pointers to non-classical functions of erythropoietin in human skin and hair follicle biology

Inauguraldissertation
zur
Erlangung der Doktorwürde
der Universität zu Lübeck
- Aus der Medizinischen Fakultät -

vorgelegt von
Friederike Ingeborg Wiersma
aus Eindhoven, Niederlande

Lübeck 2011

1. Berichterstatter: Prof. Dr. med. Ralf Paus

2. Berichterstatter: Priv.-Doz. Dr. med. Matthias Klinger

Tag der mündlichen Prüfung: 30.05.2012
zum Druck genehmigt. Lübeck, den 30.05.2012
- Promotionskommission der Sektion Medizin -

Meinen Eltern

Abbreviations

4-HC	4-hydroperoxycyclophosphamide
ACTH	adrenocorticotropic hormone
AOC2	copper-containing aminase oxidase 2
CIA	chemotherapy-induced alopecia
CLGN	calmegin
CRH	corticotropin-releasing hormone
CRHR	corticotropin-releasing hormone receptor
CTS	connective tissue sheath
DAPI	4’,6-diamidino-2-phenylindole
DP	dermal papilla
EPO	erythropoietin
EPOR	erythropoietin receptor
Exp.	experiment
FITC	fluorescein isothiocyanate
HBA1	haemoglobin alpha 1
HF	hair follicle
HIF	hypoxia-inducible transcription factor
HPA axis	hypothalamic-pituitary-adrenal axis
IL	interleukin
IRS	inner root sheath

KLC3	kinesin light chain 3
mRNA	messenger ribonucleic acid
MR	mineralocorticoid receptor
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ORS	outer root sheath
PBS	phosphate buffered saline
RASL10B	RAS-like family 10, member B
rHuEPO	recombinant human erythropoietin
SCF	stem cell factor
SEM	standard error of the mean
sEPOR	soluble erythropoietin receptor
STAT5	signal transducer and activator of transcription 5
TH	thyroid hormone
TNB	Tris HCl+NaCl+Casein
TNT	Tris-NaCl-Tween buffer
TRH	thyrotropin-releasing hormone
TRHR	thyrotropin-releasing hormone receptor
TSA	tyramide signal amplification
TSH	thyroid-stimulating hormone
TUNEL	terminal dUDP nickendlabelling
qRT PCR	quantitative real-time polymerase chain reaction

Table of contents

List of Figures

List of Tables

1 Introduction

Recent research has uncovered many additional effects of the glycopeptide hormone erythropoietin (EPO) in several tissues that extend well beyond its role in red blood cell differentiation (also called erythropoiesis). One of the recently identified tissues that are influenced by EPO is human skin, whose hair follicles (HFs) were found to produce EPO themselves and to express the EPO receptor (EPOR). Furthermore, it could be shown that EPO reduces the chemotherapy-induced apoptosis in HFs *in vitro* and that it might consequently be an interesting tool for the control of chemotherapy-induced hair loss (Bodo et al., 2007a). The current study is dedicated to following up some of the leads from this study, and to explore additional non-classical functions of EPO-induced signalling in human skin and hair follicle biology.

The following introduction first offers background information on the biology and pigmentation of human skin and HFs, then explains the mechanisms of chemotherapy-induced hair loss. Subsequently, classical and non-classical production sites and functions of EPO are described and currently known connections between EPO and the skin are summarised. Finally, the specific questions addressed by this study are defined and the experimental design chosen to answer these questions is briefly outlined.

1.1 Human skin

The skin is our largest and heaviest organ with an extension of about 1.5-2 m^2 and a weight of 3 kg without fat tissue (Schiebler and Korf, 2007). It consists of three main zones: epidermis (the outer layer), dermis (a fibrous layer that includes blood supply to the skin) and the subcutaneous fat (McKee, 2005; White and Cox, 2006). Additionally, skin comprises hair follicles (HFs), nails and apocrine, eccrine and sebaceous glands as skin appendages (see **Fig. 1 and 3**) (Wolff, 2005).

The epidermis is a multilayered self-renewing sheet of cells composed mainly of keratinocytes (White and Cox, 2006; Moll, 2010; Reifenberger and Ruzicka, 2010). Following the cell division of basal layer keratinocytes, the new keratinocytes undergo terminal differentiation as they migrate towards the skin surface (Wolff,

2005; White and Cox, 2006; Moll, 2010). Other epidermal cells are Langerhans cells for antigen presentation, melanocytes, T-lymphocytes and Merkel cells. The dermis represents a connective tissue sheath of mesenchymal origin consisting of ground substance (polysaccharides and proteins linked to form macromolecules) and two kinds of protein fibre: collagen and elastin (Fitzpatrick et al., 1987; Reifenberger and Ruzicka, 2010).The cell density of the dermis is much lower than the cell density of the epidermis. While most dermal cells are fibroblasts, other cellular constituents are mast cells, macrophages and different leukocyte populations (Ebling et al., 1991; Fritsch, 2009; Reifenberger and Ruzicka, 2010).

Human skin also displays a dense network of sensory and autonomic nerves that influence many functions in skin ranging from vasoconstriction and vasodilatation over cell differentiation and proliferation to wound healing (Roosterman et al., 2006). Several sensory nerve types exist both in the epidermis and in the dermis: for example free epidermal Merkel cells, which react on pressure, and free sensory nerve endings of the dermis. Also, the dermis contains encapsulated endings, e.g. Meissner corpuscles allowing perception of tapping and Vater-Pacini corpuscles which react on vibrations (Roosterman et al., 2006).

The dermis is also equipped with an extensive system of blood vessels organised in two plexuses, a deep and a superficial dermal plexus. These plexuses mainly ensure nourishment and waste removal. Moreover, through opening and closure of many dermal arteriovenous anastomoses situated in the acral parts of the body (fingers, nose, toes, etc.) the dermal blood flow can be regulated. This enables regulation of temperature and blood pressure of the body (Schiebler and Korf, 2007; Moll, 2010). The epidermis does not contain blood vessels but is nourished through diffusion (Moll, 2010). Lymphatic vessels of the skin are also organised in a deep and a superficial plexus. These vessels carry away fluids, proteins, lipids, cells, bacteria and other substances (Banziger-Tobler et al., 2008).

Skin also includes the epidermal-derived skin appendages, i.e. HFs and nails as well as sweat and sebaceous glands (see **Fig. 1**). Two kinds of sweat glands can be distinguished: eccrine and apocrine sweat glands. Eccrine sweat glands can be found almost all over the integument. Through sweat production they contribute to thermoregulation of the body. Apocrine sweat glands, on the other hand, are located only in certain regions such as the axilla, the areola mammae and the

genitoanal region. Apocrine sweat glands act primarily as scent glands (Schiebler and Korf, 2007). Sebaceous glands which produce sebum are usually associated with HFs and form the so-called pilosebaceous unit (see **Fig. 1**) (Paus et al., 2007; Schiebler and Korf, 2007).

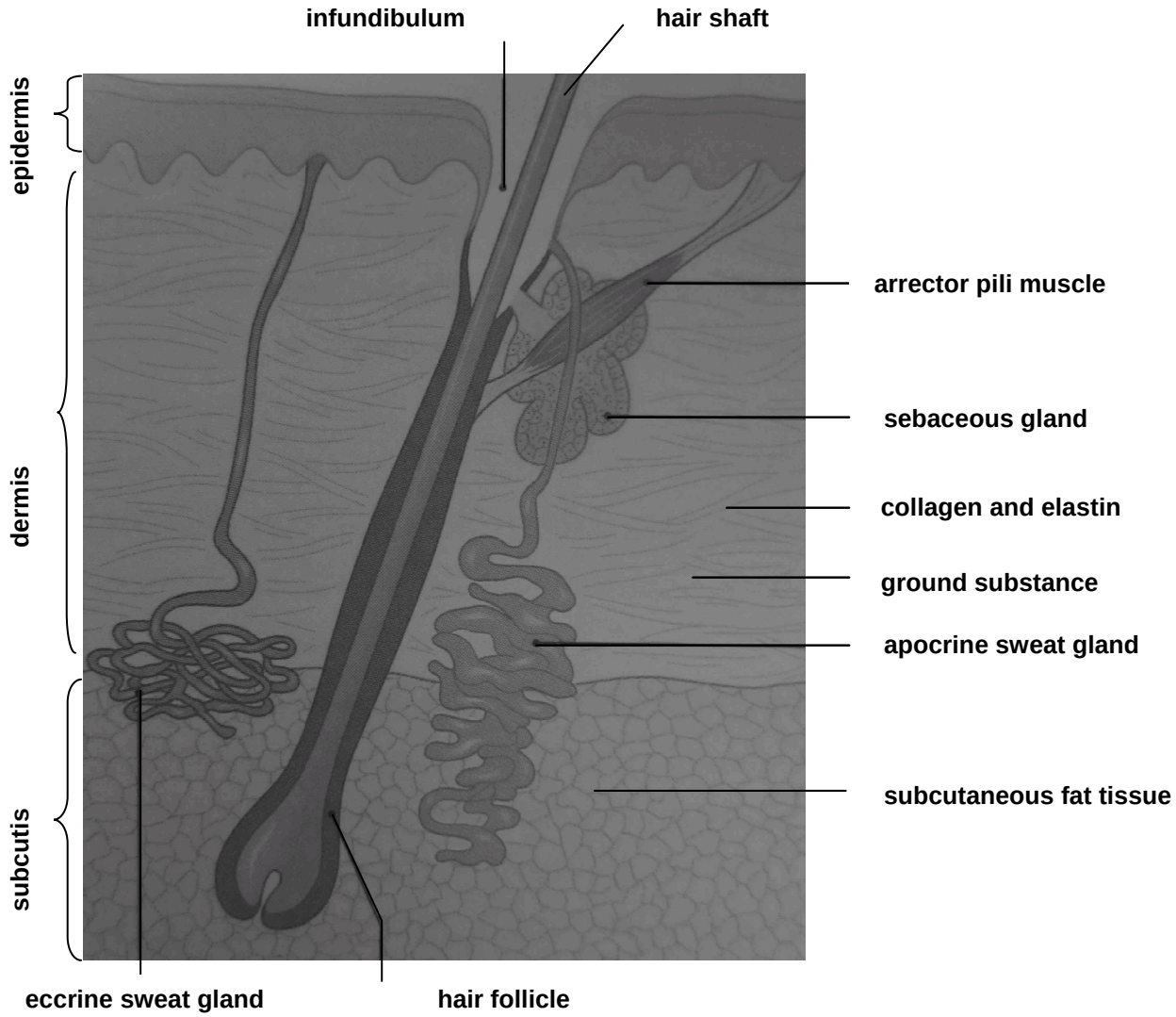


Figure 1. Human skin and its appendages
Skin consists of three main layers, epidermis, dermis and subcutis and the skin appendages, eccrine and apocrine sweat glands, sebaceous glands, hair follicles and nails (nails not shown here). Eccrine sweat glands are not associated with HFs, whereas sebaceous glands and apocrine sweat glands usually secrete their products into the infundibulum of the hair follicle as indicated in this figure. Reprinted by kind permission of the publisher (Zilles and Tillmann, 2010).

Hair and nail share many characteristics: They both belong to the hardest epithelial substances of the mammalian body and serve multiple functions

including psychosocial signalling functions, sensation and protection (Paus et al., 2007). Since HFs were used for several experiments for this thesis project, key characteristics of HF biology will be summarised in a separate chapter (see chapter 1.2).

Due to its localization, the skin serves as a barrier that separates the body's internal homeostasis from the external environment (Slominski and Wortsman, 2000). The physical barrier of the skin, which is mainly localised in the stratum corneum and also in the lower layers of the epidermis, is essential for fending off the uncontrolled loss of water (Moll, 2010). Beyond that, stratum corneum serves as the first barrier to hinder exogenous substances and microbes from penetrating the skin, although to fight the latter many more specialised components of the chemical and immunological skin barrier are involved, like lipids, hydrolytic enzymes, antimicrobial peptides, macrophages and lymphocytes (Madison, 2003; Proksch et al., 2008; Reithmayer et al., 2009). The epidermis is a vital structure, as its destruction over large areas, e.g. through severe burns or toxic epidermal necrolysis, is a life-threatening event (Proksch et al., 2008).
Further important functions of human skin include regulation of temperature, protection against mechanical damages and the detection of sensory stimuli (Fitzpatrick et al., 1987; Moll, 2010; Reifenberger and Ruzicka, 2010). Absorption of ultraviolet light is achieved by skin pigmentation, the process of which is described in more detail in chapter 1.3 (Fitzpatrick et al., 1987; McKee, 2005).

1.1.1 The skin as an endocrine organ

During the last two decades a new perception of skin has emerged that considers skin not only as a target organ of multiple different hormones, but also as a highly active endocrine organ itself that synthesises multiple hormones, neuropeptides and neurotransmitters (Slominski et al., 2008; Chen and Zouboulis, 2009). In fact, the skin may well be the largest peripheral endocrine organ of the human body (compare **Table 1**)(Zouboulis, 2004)!
The skin's best-known endocrine activities are the synthesis of vitamin D by ultraviolet light (Holick, 1994; Slominski and Wortsman, 2000) as well as the synthesis of potent androgens derived from testosterone in puberty leading e.g. to

hair growth and acne (Zouboulis et al., 2007). Also quite well-known are the effects of topic glucocorticoids on skin, as many patients and doctors have observed their desired (mainly reducing inflammation) and undesired effects (such as skin atrophy and pigmentary disorders) when treating various skin diseases (Ference and Last, 2009). However, it has as yet hardly been studied how the exogenous application of glucocorticoids impacts on the intracutaneous production and metabolism of endogenous hormones in human skin.

Another member of the steroid hormone receptor family present in the skin is the mineralocorticoidreceptor (MR). The MR and its ligand aldosterone are mainly involved in renal sodium retention and in regulating blood pressure. However, whether its main ligand aldosterone or glucocorticoids are the main activators of cutaneous MRs, still needs to be clarified (Farman et al., 2010). Nonetheless, recent experiments on mice with overexpression of the MR in basal keratinocytes suggest that the MR is involved in epidermal and HF growth as these so called K5-MR mouse embryos displayed a flat epidermis and the adults showed dystrophic HFs and cysts leading to alopecia (Farman et al., 2010).

The pituitary hormone prolactin – a modulator of lactation and reproduction – is also synthesised in normal human skin and scalp HFs (Foitzik et al., 2006) and exerts multiple effects on the cutaneous system. For example, prolactin modulates hair growth by significantly inhibiting hair shaft elongation and stimulating premature catagen induction (Foitzik et al., 2006; Foitzik et al., 2009). Interestingly, prolactin acts in a gender and site-specific way, as in female front temporal HFs prolactin inhibited catagen induction (Langan et al., 2010a). Remarkably, the two key stimulators of pituitary prolactin secretion, thyrotropin-releasing hormone (TRH) and oestrogen also regulate prolactin and prolactin receptor expression in female human skin and HFs, as *in vitro* experiments have shown (Langan et al., 2010b).

Only recently, increasing evidence has emerged that leptin, the key regulator of food intake, and its receptor are not only present in human epidermis and HFs (Iguchi et al., 2001; Murad et al., 2003; Poeggeler et al., 2010) but may also be an important modulator of hair and skin biology (Poeggeler et al., 2010). There is

evidence that leptin plays a role in wound healing and it has been postulated that leptin may be involved in hair growth and hair cycle control (Poeggeler et al., 2010).

The emerging field of dermato-endocrinology studies the skin and its appendages as both sources and target tissues of multiple different hormones, neuropeptides, and neurotransmitters. This field attempts to clarify the importance of these hormones in skin physiology and skin pathology, how their intracutaneous production and metabolism as well as the expression of their cognate receptors is regulated, and how this amazing intracutaneous endocrine activity might best be manipulated pharmacologically for therapeutic purposes (Slominski and Wortsman, 2000; Ohnemus et al., 2006; Paus et al., 2008; Chen and Zouboulis, 2009; Foitzik et al., 2009; Zouboulis, 2009; Farman et al., 2010; Holick, 2010; Poeggeler et al., 2010).
Furthermore, dermato-endocrinological research may offer a better understanding of endocrine abnormalities that result in skin changes/dermatoses (Smoller and Rongioletti, 2010) and may thus provide better treatment options for these conditions.

1.1.2 Hormone-mediated signalling axes within the skin

The complexity of dermato-endocrinology becomes evident if one considers a few selected, relatively recent discoveries on the existence of intracutaneous, hormone-mediated regulatory axes that appear to coordinate the activities of different hormones.

For example, human skin is not only a source and a target for so-called stress hormones, it also shows the same organisational and signalling structures as the hypothalamic-pituitary-adrenal (HPA) axis. This axis is activated upon psycho-emotional stress resulting in the hypothalamic production and release of corticotropin-releasing hormone (CRH), which stimulates the corticotropin-releasing hormone receptor (CRHR) of the pituitary. CRHR1 signalling mediates the liberation of the proopiomelanocortin (POMC)-derived peptides β-endorphin and ACTH from the anterior pituitary. The latter induces cortisol liberation in the

adrenal gland which enables the body to cope with the stressor (e.g. by mobilizing energy reserves) and suppresses CRH expression through a negative feedback mechanism (Hillhouse and Grammatopoulos, 2006; Slominski et al., 2007).

The skin equivalent of the HPA-axis operates in a similar manner, i.e. certain cutaneous cell types, namely melanocytes and fibroblasts, increase their proopiomelanocortin (POMC) and ACTH production after CRHR stimulation (Slominski et al., 2007). Melanocytes then produce higher levels of cortisol and corticosterone, whereas fibroblasts only produce more corticosterone (Slominski, 2005). Similarly, a peripheral HPA-axis equivalent is present in human HFs itself, too. Ito et al. showed that even isolated HFs, which are disconnected from neural and vascular inputs, secreted substantial levels of cortisol into their culture medium. This is stimulated by CRH and/or ACTH. Moreover, isolated human HFs display a similar negative feedback mechanism of cortisol on CRH expression like the central HPA-axis (Ito et al., 2004a; Ito et al., 2005a). It has been suggested that this recently discovered cutaneous neuroendocrine system may help to maintain local homeostasis (Slominski et al., 2007).

It is not yet clear, whether a similar peripheral axis equivalent also exists for the hypothalamic-pituitary-thyroid (HPT) axis (Paus, 2010). However, there is substantial evidence that members of this axis (thyroid hormones (TH), thyroid-stimulating hormone (TSH) and thyrotropin-releasing hormone (TRH)) and their corresponding receptors are expressed in human skin and HFs. It has been known for a long time that changes of TH levels often affect human skin and HFs, e.g. with the clinical presentation of myxedema, telogen effluvium and hair shaft abnormalities (DeRuiter, 2002) [1]. Recently, it has been documented that thyroid hormones exert multiple complex direct effects on human scalp HFs (van Beek et al., 2008) and that TSH directly impacts on human HF gene expression (Bodo et al., 2009).

[1] DeRuiter (2002) Thyroid Hormone Tutorial: Thyroid Pathology. Endocrine Module (PYPP 5260), Thyroid Section, Auburn University, Spring 2002.
URL: http://www.auburn.edu/~deruija/endp_thyroidpathol.pdf *[date: 19.06.2011]*

The most proximal regulator TRH and its receptor (TRHR) are expressed in the epithelium of human scalp HFs and prolong the hair cycle growth phase (anagen) and promote hair shaft production *in vitro* (Gaspar et al., 2010). The second element of this axis – TSH – was identified a novel modulator of epidermal functions and interestingly its epidermal expression is up-regulated by TRH and down-regulated by THs (Bodo et al., 2010). However, human HFs apparently are **not** able to produce THs themselves, neither constitutively nor after TSH stimulation (Paus, 2010).

This complex endocrine network that modulates human HF and skin functions already consists of many more hormones, but scientists only now begin to understand its various actions. **Table 1** lists some hormones and/or their receptors that are present in human skin and HFs and that might thus be important for skin homeostasis, epidermal and HF cell differentiation, pigmentation and HF cycling.

Only very recently, the glycoprotein hormone erythropoietin (EPO) – best-known for its rescue effects on the developing erythrocyte progenitors – was added to the growing list of hormones that are expressed by human HFs and modulate their biology (Bodo et al., 2007a). As further characterization of the role of EPO in human skin and HFs is the subject of investigation of this thesis, EPO functions, regulation and signalling are described in a separate chapter (chapter 1.5 – 1.5.3).

hormone/-receptor -R	HF	reference	skin	reference
ACTH/-R	+/+	(Ito et al., 2005a)	+/+	(Slominski, 2005)
aldosterone/ mineralocorticoid-R	?/+	(Farman et al., 2010)	?/+	(Kenouch et al., 1994; Farman et al., 2010)
androgen/-R	+/+	(Pelletier and Ren, 2004; Chen and Zouboulis, 2009)	+/+	(Pelletier and Ren, 2004; Slominski, 2005; Zouboulis et al., 2009)
cortisol/-R	+/+	(Ito et al., 2005a)	+/+	(Slominski et al., 2007)
CRH/-R	+/+	(Slominski et al., 1999; Kono et al., 2001)	+/+	(Slominski et al., 1999; Slominski et al., 2007)
EPO/-R	+/+	(Bodo et al., 2007a; LeBaron et al., 2007)	+/+	(Bodo et al., 2007a; LeBaron et al., 2007)
oestrogen/-R	+/+	(Pelletier and Ren, 2004; Zouboulis et al., 2007)	+/+	(Sominski, 2005; Zouboulis et al., 2007)
growth hormone/-R	?/+	(Simard et al., 1996)	?/+	(Slominski et al., 2000a)
leptin/-R	+/+	(Iguchi et al., 2001; Poeggeler et al., 2010)	+/+	(Murad et al., 2003; Poeggeler et al., 2010)
luteinizing hormone/-R	+/+	(Pabon et al., 1996)	?/+	(Pabon et al., 1996)
melatonin/-R	+/+	(Kobayashi et al., 2005; Fischer et al., 2008)	+/+	(Fischer et al., 2008)
prolactin/-R	+/+	(Foitzik et al., 2006; Foitzik et al., 2009)	+/+	(Foitzik et al., 2006; Foitzik et al., 2009)
TH/-R	-/+	(Billoni et al., 2000; van Beek et al., 2008)	-/+	(Slominski and Wortsman, 2000)
TRH/-R	+/+	(Gaspar et al., 2010)	+/?	(Ellerhorst et al., 2004)
TSH/-R	?/+	(Bodo et al., 2009)	+/+	(Slominski et al., 2002; Bodo et al., 2010; Cianfarani et al., 2010)

Table 1 A selection of hormones and their receptors expressed in human skin and hair follicles
(+ = expression, - = no expression, ? = expression as yet unknown)

1.2 Biology of human hair follicles

Since human HFs are used as the study model in this present thesis project, key features of their biology deserve to be briefly summarised:

The hair follicle (HF) is a characteristic feature of mammals (Paus and Foitzik, 2004; Schneider et al., 2009). Humans carry about 5 million HFs, 100 000 thereof on the scalp (Paus et al., 2007). There are three main types of HFs: fetal lanugo hair, the very thin and short, non-pigmented vellus hair that cover most parts of the body and thirdly the large, pigmented, clearly visible hair of the scalp, the eyebrows, the pubic hair and the beard hair, which form the group of terminal hair (Paus et al., 2007). Unlike in other mammals, in humans the function of HFs is not so much thermo-isolation, but rather communication and attractiveness. This importance is reflected in the affliction that most people undergo when confronted with hair loss (Paus et al., 2007). In most cases, hair growth disorders (alopecia, androgen-dependent balding) are the result of changes in the hair cycle, i.e. the growth stage (anagen) is shortened (Paus and Foitzik, 2004). Normally, anagen duration is 2-6 years, regression (catagen) lasts 2-3 weeks and “resting” (telogen) 3 months (Paus et al., 2007).

The HF is a complex miniorgan of the skin that consists of mesenchymal parts – the dermal papilla (DP) and the connective tissue sheath (CTS) that surrounds the entire HF – and of epithelial parts arranged in at least 8 concentric layers that form the infundibulum, isthmus, bulge and hair bulb (compare **Fig. 2 and 3**) (Paus et al., 2007; Schneider et al., 2009). The anagen HF can be divided into two parts: The lower-most portion called hair bulb which undergoes dramatic morphological changes during each hair cycle and is therefore called the “cycling portion“ of the HF. The upper “permanent portion“ does not visibly cycle (however, also the “permanent portion“ undergoes remodelling during each hair cycle) and consists of the bulge region, isthmus, infundibulum and follicular ostium and canal (see **Fig. 2**) (Paus et al., 2007; Schneider et al., 2009).

The infundibulum is the most distal part of the HF which describes the part of the HF that reaches from the sebaceous gland duct to the epidermal surface. The next lower part of the HF called isthmus is located between the sebaceous gland duct

and the bulge region. The lower isthmus contains the bulge region – the area where the arrector pili muscle inserts (see **Fig. 2**) (Schneider et al., 2009). It is the major seat of epithelial and melanocytic HF stem cells (Paus et al., 2007).

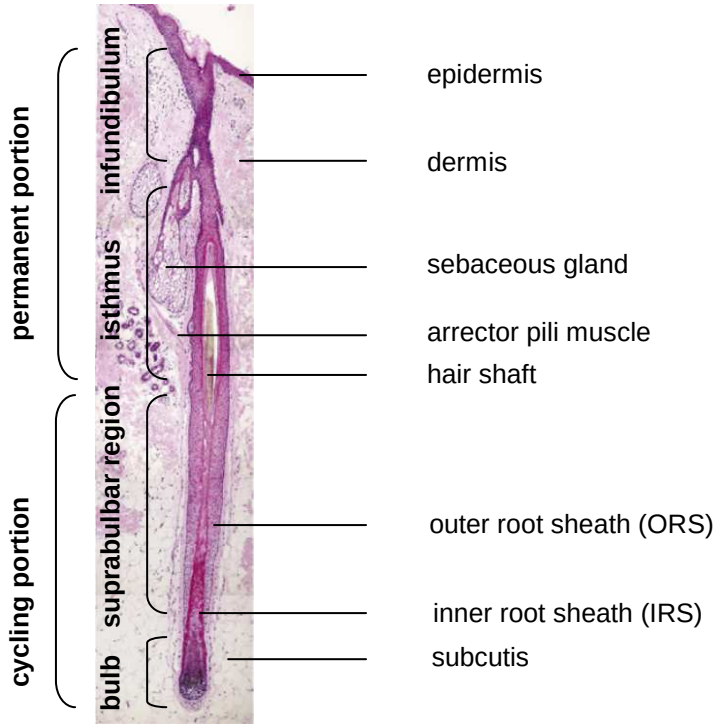


Figure 2. Longitudinal section through a human scalp hair follicle (anagen VI)
Reprinted by kind permission of Dr. Katja Meyer and the publisher (Schneider et al., 2009).

The hair bulb is the most proximal part of the HF and the anagen hair bulb is the place where the actual hair shaft is produced. It contains a very rapidly proliferating cell population called hair matrix keratinocytes. In the precortical hair matrix, just above the DP, these rather undifferentiated cells differentiate into trichocytes and finally form the hair shaft (Paus et al., 2007). Here, also the pigmentation of the hair shaft takes place, as hair matrix keratinocytes receive melanosomes from the melanocytes of the hair follicle pigmentary unit (cf. below) (Paus et al., 2007).

The hair shaft consists of three layers of terminally differentiated keratinocytes: medulla, cortex and cuticle. The medulla in the middle is surrounded by the main structural component that achieves the remarkable elasticity and resistance of the hair shaft, the cortex. The outer-most layer of the hair shaft is the hair cuticle,

whose cells are arranged like roof tiles protecting the hair shaft from weathering (see **Fig. 3**) (Powell and Rogers, 1997; Paus et al., 2007). The hair shaft is situated in the hair canal, i.e. the tubular connection between the epidermis and the most distal part of the IRS (Schneider et al., 2009).

The DP is an onion-shaped mesenchymal structure of specialised fibroblasts located in the proximal HF (see **Fig. 3**) (Benninghoff and Drenckhahn, 2002; Schneider et al., 2009). The volume of the DP determines the size of the HF and the diameter of its hair shaft. The DP is also thought to be an important control device for HF cycling, in which the DP undergoes dramatic changes of shape and location (compare below) (Paus and Cotsarelis, 1999; Paus et al., 2007). During the hair cycle, there is also a cycle-dependent extensive trafficking of fibroblasts between the DP and the CTS; a destroyed DP can even be fully rebuild by the fibroblasts of the CTS (Jahoda and Reynolds, 1996).

Every HF has a dense blood perfusion system that consists of arterioles, capillaries and venules coming from the dermal and subcutaneous vascular plexuses (Paus et al., 2007). The perifollicular blood vessels weave through the HFs' CTS and even insert into the DP of terminal HFs (Mecklenburg et al., 2000; Yano et al., 2001). This enables the supply of all key regions of the HF with nutrients, oxygen and hormones as well as the removal of toxic by-products, e.g. from pigmentary pathways (Paus et al., 2007). Like the lower portion of the HF, the vascular system undergoes substantial changes, too, and in mice, active hair growth in anagen is even coupled to new formation of blood vessels (= angiogenesis) (Mecklenburg et al., 2000).

HFs not only display a dense vascular system, but also a very dense innervation system especially of the bulge and isthmus region. This accounts for the enormous tactile sensitivity of HFs that even register slight hair shaft movements, e.g. caused by wind or insects.
Moreover, the follicular neural plexus releases neurotransmitters, neuropeptides and neurotrophins and may thus fulfil important trophic and regulatory functions (Botchkarev et al., 1997; Paus et al., 1997; Botchkarev et al., 1998; Peters et al., 2001; Botchkarev et al., 2004; Botchkarev et al., 2006).

However, interestingly neither intact HF innervations nor blood supply are *absolutely* necessary for the continuation of hair growth and cycling as even microdissected organ-cultured human scalp HFs in anagen can transform to the early catagen stages (Philpott et al., 1996; Maurer et al., 1998).

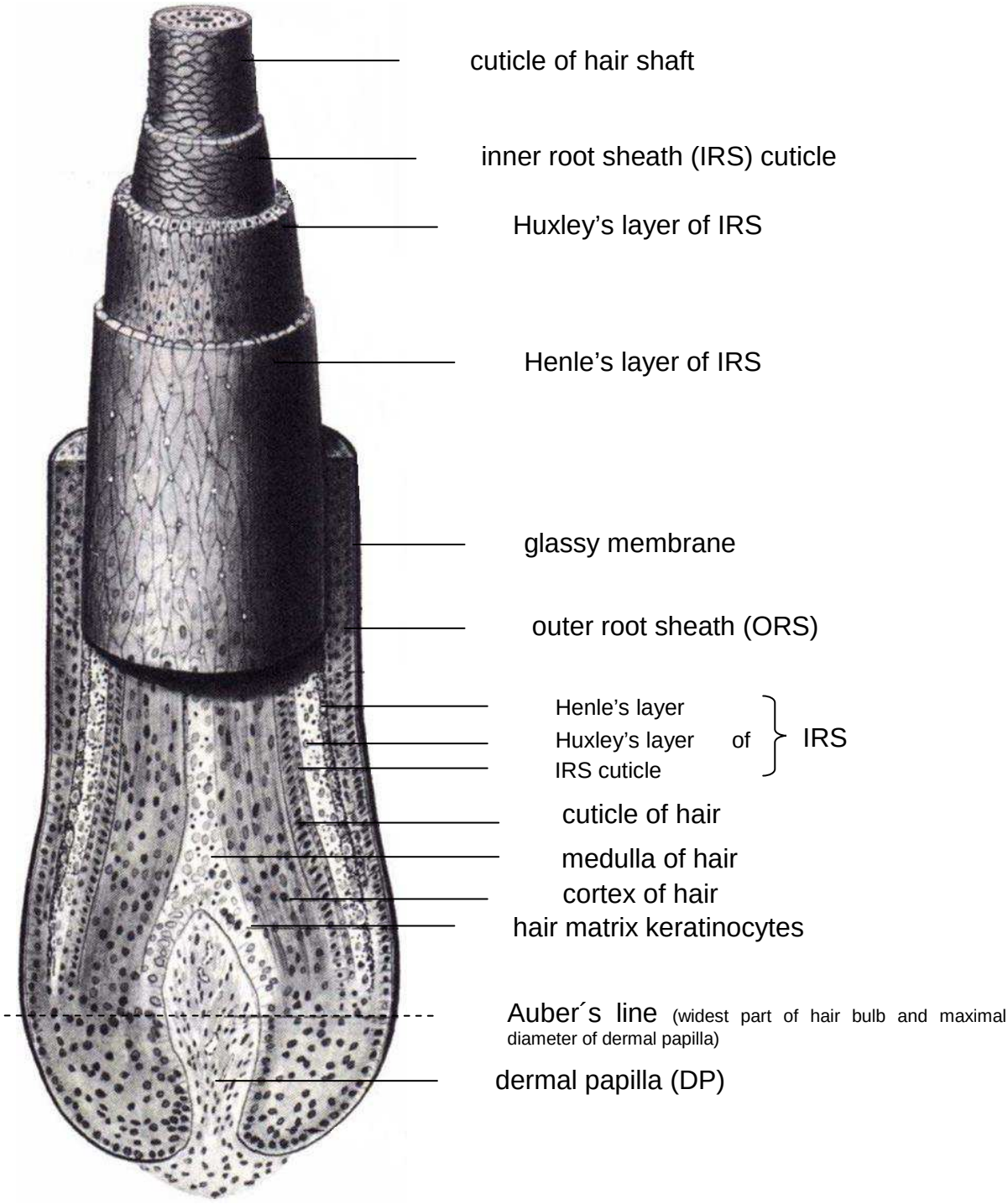


Figure 3. Schematic representation of a human HF
Reprinted by kind permission of the publisher (Benninghoff and Drenckhahn, 2002).

1.2.1 Hair follicle cycle

The HF is unique in the mammalian body as it is the only organ that has lifelong cyclic activity. Other cycling organs such as the ovary or the endometrium have a much shorter span of cycling. In its cycle, the HF undergoes cyclic transformations from stages of growth (anagen), regression (catagen) and relative quiescence (telogen) (Stenn and Paus, 2001; Paus and Foitzik, 2004). The length of the HF's product, the hair shaft, is determined by the duration of anagen, which lasts 2–6 years in terminal scalp hair. Catagen has a duration of only 2–3 weeks and telogen of 3 months. Approximately 85-90% of the terminal scalp HFs are found to be in the growth phase, whereas only 10-15% rest in telogen and less than 1% are in catagen. In average, a human scalp hair grows 0.35 mm per day and is not influenced by shaving or cutting (Paus et al., 2007).

The synchronised HF cycle of some mammals (e.g. rodents) allow adaption to seasonal changes through rhythmic seasonal shedding of hair shafts. The first two waves of HFs in the developing human are still synchronised, but after birth each HF follows its own cycling rhythm (Stenn and Paus, 2001). The purposes for asynchronised HF cycling are not as evident but may include:

- generation of different hair shaft lengths in different regions (e.g. short hair shaft of eyebrows and eyelashes)
- cleaning of the body surface from debris and even parasites
- means of excretion of toxic substances (e.g. heavy metals)
- protection from improper formation of the follicle
- protection against malignant degeneration (as the HF is one of the fastest growing tissues of the organism) (Stenn and Paus, 2001; Paus and Foitzik, 2004)

Anagen, the growth phase, shares many similarities with fetal HF morphogenesis (compare **Fig. 4**). It can be divided into 6 phases (anagen I – VI) and is marked by massive proliferation and differentiation of keratinocytes in the hair matrix. In anagen, the lower (cycling) portion of the HF is completely regenerated and hair shaft growth and pigmentation occur (see **Fig. 4**) (Stenn and Paus, 2001; Paus et al., 2007).

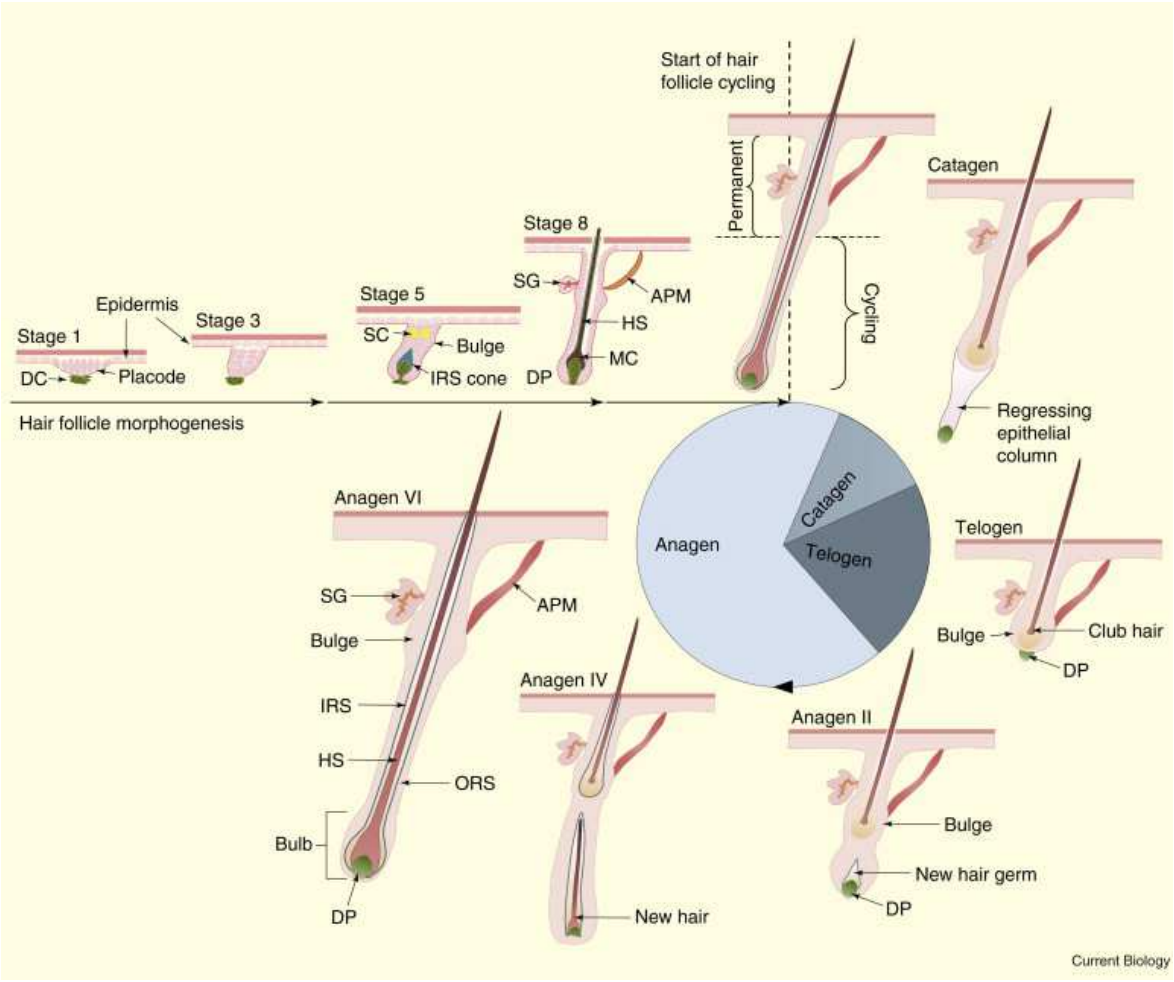


Figure 4. Key stages of follicular morphogenesis and the follicular cycle
After completion of HF formation (= morphogenesis), which occurs but once in lifetime *in utero*, the HF enters its first cycle. From then on, the HF undergoes lifelong cyclic regression and regeneration, which occurs in defined phases: catagen (regression), telogen (relative quiescence) and anagen (growth phase). As indicated, the HF can be divided into a permanent upper and a cycling lower portion, which undergoes dramatic visible changes during the cycle. Reprinted by kind permission of the publisher (Schneider et al., 2009).

Catagen is marked by the involution of the lower part of the HF as a consequence of keratinocyte apoptosis. As this includes the actual hair shaft factory (= anagen bulb), hair shaft production ends. Further characteristics are the termination of melanogenesis (the melanin-producing HF melanocytes largely undergo apoptosis, too), the non-apoptotic (!) condensation of the DP and the formation of a club hair which moves upward until it reaches the upper permanent part of the HF where it remains anchored during telogen (see **Fig. 4**) (Alonso and Fuchs, 2006; Paus et al., 2007; Schneider et al., 2009).

After completion of this involution process, the HF enters the resting phase called telogen where the HF lies dormant. However, this phase might be much more important for the hair cycle as the term “resting phase“ implies (Stenn and Paus, 2001; Alonso and Fuchs, 2006).

1.3 Skin and hair follicle pigmentation

As the current thesis project puts emphasis on investigating potential effects of EPO on pigmentation, essentials of cutaneous pigment biology will be summarised briefly.

Skin and HF pigmentation is mainly achieved by melanin, a biological pigment derived from the amino acid tyrosine (see **Fig. 5**). Melanin is produced in specialised organelles of melanocytes – the lysosome-like structures called melanosomes – and is then transferred to adjacent keratinocytes (Slominski et al., 2004; Lin and Fisher, 2007; Coelho et al., 2009). Two different types of melanin can be distinguished: black-brown eumelanins and yellow-reddish pheomelanins. Eumelanin and pheomelanin are polymers derived from tyrosine in a complex process called melanogenesis (see **Fig. 5**) (Tobin, 2008, 2011). These two types of melanin are both present in hair shafts and skin, where their quality and quantity determine the colour of hair and skin. Interestingly, both types of melanin can be released by the same melanocyte (Slominski et al., 2004).

Melanogenesis is a multi-step process that requires several enzymes and cofactors (Tobin, 2008; Hirobe, 2011). The key enzyme and thus the rate-limiting enzyme is tyrosinase, a copper-dependent enzyme which catalyses the hydroxylation of L-tyrosine to L-dopa. The following chemical reactions of melanogenesis, i.e. oxido-reduction reactions and intramolecular transformation, can occur spontaneously after L-dopa is formed (compare **Fig. 5**) (Lerner and Fitzpatrick, 1950; Slominski et al., 2004; Slominski et al., 2005; Yamaguchi and Hearing, 2009).

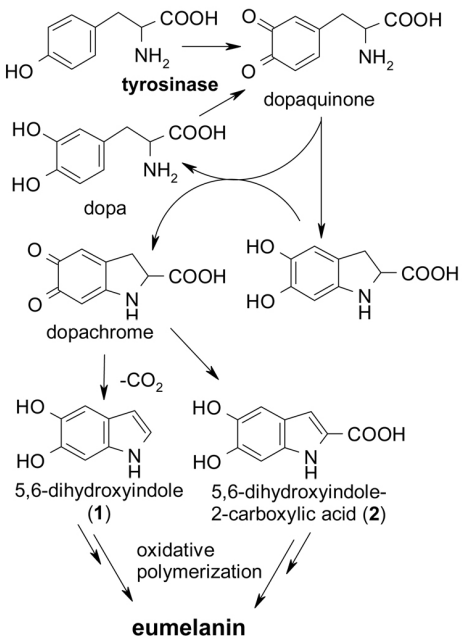


Figure 5. The tyrosinase-catalysed oxidation of tyrosine
Through several reactions, the brown-black pigment called eumelanin is created. Reprinted by kind permission of the publisher (d'Ischia et al., 2009).

The melanocytes then transfer the synthesised melanin-containing melanosomes first to the tip of their dendrites and then to keratinocytes in the epidermis or cortical and medullary keratinocytes of the growing hair shaft, presumably by the same means (Slominski et al., 2004; Ando et al., 2009). The process of this transfer is not yet completely understood and several hypothesis have been proposed including exocytosis, cytophagocytosis, fusion and membrane vesicle transport (Yamaguchi and Hearing, 2009). However, ultrastructural observations have suggested that dynein and kinesin are important for the microtubule-associated transport of melanosomes in human melanocyte dendrites (Hara et al., 2000; Park et al, 2009).

The structural and functional unit of one melanocyte and approximately 36 epidermal keratinocytes is called epidermal melanin unit (see **Fig. 6**). A corresponding follicular melanin unit (also called HF pigmentary unit) consists of one melanocyte and only approximately 5 hair matrix keratinocytes (Fitzpatrick and Breathnach, 1963; Tobin et al., 1999; Tobin, 2008; Miot et al., 2009; Tobin, 2011). There are more differences between epidermal and follicular melanin units than the higher epidermal melanocyte-keratinocyte ratio: Histologically, follicular melanocytes are larger, more dendritic and produce larger melanosomes.

Furthermore, follicular melanocyte activity is tightly coupled to the hair growth cycle as this activity only occurs in anagen (Slominski and Paus, 1993; Paus et al., 2007; Tobin, 2008, 2011). In contrast, epidermal melanocytes seem to produce melanin continuously. In general, melanin granules in hair shafts tend to remain intact leading to an evenly stained hair shaft in its full length, unlike in the epidermis where melanin granules are almost completely digested by keratinocytes (Oliveira and Junior, 2003; Tobin, 2008).

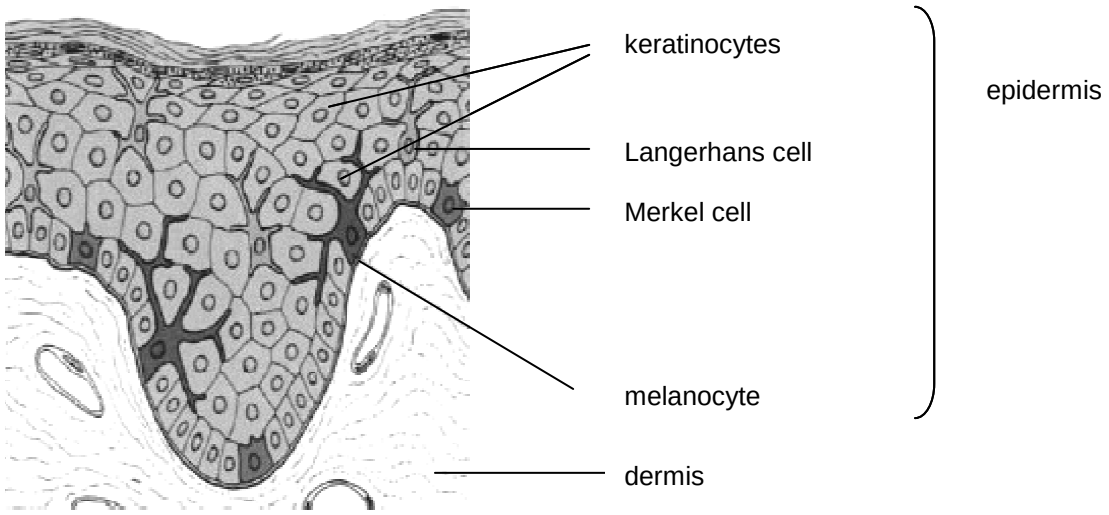


Figure 6. Epidermal melanin unit
The melanocytes are situated in the basal layer of the epidermis (here shown in dark grey) and one melanocyte supplies approximately 36 keratinocytes (here shown in light grey) with the melanin-containing melanosomes. From the tips of their dendrites, the melanocytes transfer the melanosomes to adjacent keratinocytes. Reprinted by kind permission of the publisher (Miot et al., 2009).

The development of follicular and epidermal melanin units commences in embryonic and fetal development stages (Slominski et al., 2005).

C-kit expressing melanoblasts migrate from the neural crest and populate the basal layer of the epidermis and then enter the developing stem cell factor (SCF) supplying HF epithelium. Differentiated c-kit positive melanocytes populate the HF bulb that produces SCF whereas c-kit negative melanoblasts move into the ORS and bulge region in developed HFs (Peters et al., 2002; Oliveira and Junior, 2003; Slominski et al., 2005). However, anagen HFs do not only host melanotic dopa-positive melanocytes in the basal layer of the infundibulum and the upper DP, but also amelanotic dopa-negative melanocytes in some parts of the ORS, in the

periphery of the bulb and in the most proximal matrix (Oliveira and Junior, 2003; Tobin, 2008, 2011).

The exclusively cyclic presence of melanogenically active HF melanocytes during the anagen phase of HF cycling (as opposed to the constant presence of amelanotic melanocytes in the outer root sheath and melanocyte stem cells in the HF bulge) has provoked several theories. Currently, the overall view is that immature melanocytes are recruited into the reforming anagen hair bulb from a melanocyte stem cell reservoir in the upper HF. The immature melanocytes then begin to differentiate by producing the enzymatic and organelle equipment necessary for melanogenesis. The maturing melanocytes also develop dendrites, a transporter device to transfer melanosomes towards keratinocytes. In catagen, HF melanocytes undergo apoptosis and are no longer detectable (Oliveira and Junior, 2003; Paus et al., 2007; Tobin, 2008).

As skin is the main barrier to the external environment, it relies on melanocytes to provide protection against the harmful effects of solar radiation (Slominski et al., 2004; Lin and Fisher, 2007). However, how exactly pigmentation protects the skin is not known yet. It is presumed though that melanin shields the nucleus (Coelho et al., 2009). The reduced protection in individuals with fair skin is thought to account for their much higher risk of sunburn and development of cutaneous malignancies (squamous cell and basal cell carcinomas and melanoma) (McKee, 2005). Melanin may also serve as an antioxidant defence for skin and HFs (Slominski et al., 2004). Beside protection, skin and hair colour contribute also highly to our appearance and to inter-individual communication (Tobin, 2008). Interestingly, melanocytes are now recognised to fulfil many more functions than just melanin production. There is increasing evidence that melanocytes play an important role in the innate immune system of the skin. They produce many immunologically active proteins and seem to be directly involved in the immunological barrier of the skin (Plonka et al., 2009). Furthermore, melanocytes may be regulators of epidermal functions through their produced melanosomes (Plonka et al., 2009).

There is a complex regulation of melanogenesis by hormones, cytokines, neurotransmitters, growth factors and nutrients (Slominski et al., 2004; Slominski et al., 2005). Among others, the following substances have been reported to stimulate melanogenesis in skin and/or hair follicles: α-MSH (melanocyte-stimulating hormone), ACTH (adrenocorticotropic hormone), β-endorphin, prostaglandins, histamine, SCF, oestrogens, androgens (Hearing, 1999; Slominski et al., 2000b; Slominski et al., 2004), CRH (Kauser et al., 2006) and thyroid hormones (van Beek et al., 2008). Several inducers of melanocyte dendricity have been identified, e.g. α melanocyte-stimulating hormone, ACTH, β-endorphin and ultraviolet light *in vivo* (Hunt et al., 1994; Romero-Graillet et al., 1997; Slominski et al., 2004).
Whether or not the glycopeptide hormone EPO is part of the complex hormonal regulation of human skin and hair pigmentation, had never been investigated so far.

1.4 Chemotherapy-induced alopecia

Since we shall investigate in this thesis the effects of EPO on human skin and HF not only under physiological conditions, but also in response to chemotherapy, the problem of chemotherapy-induced alopecia (CIA) needs to be briefly introduced.

In the chemotherapeutic treatment of cancer, hair loss (alopecia) is still one of the most frequent and distressing problems (Kiebert et al., 1990; Hesketh et al., 2004; Bodo et al., 2007b).
Unfortunately, most protective agents are only able to reduce alopecia in one specific chemotherapeutic agent at best (Wang et al., 2006). Also, the few agents tested in humans (e.g. minoxidil and AS101) could not stop CIA, only shorten the duration and reduce the severity of CIA (Wang et al., 2006). The molecular pathways that lead to apoptosis induced by chemotherapy in HFs include the transcription factor p53 (Botchkarev et al., 2000; Bodo et al., 2007b) and the Fas signalling pathway (Sharov et al., 2004).
One agent often used in experimental designs to examine chemotherapeutic effects is the cyclophosphamide-derivative 4-hydroperoxycyclophosphamide (4-HC), which spontaneously converts to 4-hydroxy-cyclophosphamide. The latter is

also the product of hepatic cytochrome P450 reactions of cyclophosphamide itself, and then breaks down to the reactive intermediates phosphoramide mustard and acrolein and thus forming interstrand DNA cross-links, the primary action by which cyclophosphamide and 4-HC induce apoptosis (Takamizawa et al., 1975; Colvin, 1999; Murata et al., 2004; Bodo et al., 2007b).

In vitro studies of organ-cultured microdissected human HFs have shown a significant reduction of 4-HC-induced intrafollicular apoptosis when EPO was co-administered (Bodo et al., 2007a). After this study, two important questions have remained open: First, through which anti-apoptotic pathway does EPO reduce CIA? As several different molecules ranging from STAT5 to NF-κB over PKB/Akt (compare **Fig. 10)** are involved in EPO signalling, further studies need to concentrate on the underlying mechanisms. However, in this project, we concentrated on the second open question. It was our aim to answer whether EPO is capable of reducing chemotherapy-induced apoptosis also in human HFs *in situ* and thus coming one step closer to a possible pharmacological use of EPO as a protective agent against CIA in patients receiving chemotherapy.

1.5 Erythropoietin

Bodo et al. have previously shown that the erythropoiesis key regulatory hormone EPO and its receptor are among the important endocrine elements expressed by human HFs (Bodo et al., 2007a; Paus et al., 2009). As this thesis examines EPO functions in human skin and HFs in more detail, some essentials of EPO biology will be summarised in the following chapter.

1.5.1 Structure and classical functions of erythropoietin

EPO is a glycoprotein hormone which is mainly produced in the cortex of the kidneys by peritubular fibroblasts (Jelkmann, 2007b). It is a hormone that serves as an essential viability and growth factor for erythrocyte progenitors (Jelkmann, 2004). By binding to its specific receptor on erythroid progenitor cells, EPO primarily rescues these cells from apoptosis (Fisher, 2003; Jelkmann, 2011). EPO amounts are usually indicated in International Units (IU). One EPO IU is defined by exerting the same erythropoiesis-stimulating effect as 5 µmol of cobaltous chloride (Jelkmann, 2009). Basal EPO plasma concentration ranges from 6 to 32 IU/l and

in patients with anaemia, EPO plasma levels can be elevated up to 10,000 IU/l or more (Jelkmann, 2011).

About a century ago, Carnot and Deflandre first postulated that a humoral factor regulates the production of erythrocytes (Carnot and Deflandre, 1906; Fisher, 2003). In 1977, the hormone was purified for the first time (Miyake et al., 1977) and a few years later the gene for EPO could be cloned (Jacobs et al., 1985; Lin et al., 1985). Since the 1950s it has been known that EPO is produced in the kidneys (Jacobson et al., 1957) in peritubular cells (Koury et al., 1988). After the first clinical trials with recombinant human EPO (rHuEPO) in 1987 (Eschbach et al., 1987) it has been used for treating renal anaemia on a widescale since 1989 (Fisher, 2003).

Structurally, EPO is a highly glycosylated protein with a total molecular mass of 30.4 kDa. The 165 amino acids that form the peptide part of the molecule are completed by 4 carbohydrate chains that make up 40% of the total molecule (see **Fig. 7**) (Jelkmann, 2004). This glycosylation is essential for the survival of EPO, as especially the three N-linked carbohydrate chains stabilise the protein whereas the fourth O-linked chain seems to be much less important (Delorme et al., 1992; Jelkmann, 2004). However, the carbohydrate portion is not required for receptor-binding (Jelkmann, 2004).

EPO folds – like other members of class I cytokines – into 4 alpha helical bundles, (Jelkmann, 2004) as Cheetham et al. have verified by nuclear magnetic resonance spectroscopy in 1998 (Cheetham et al., 1998). The four alpha helices are arranged in an up-up and down-down topology, here lettered A-D from the N-terminus (**Fig. 7**). The antiparallel helices A and D are connected by a disulfide bond between cysteine 7 and 161 and a second disulfide bond between cysteine 29 and 33 links helix A and B as indicated by arrows (Cheetham et al., 1998; Banks et al., 2009).

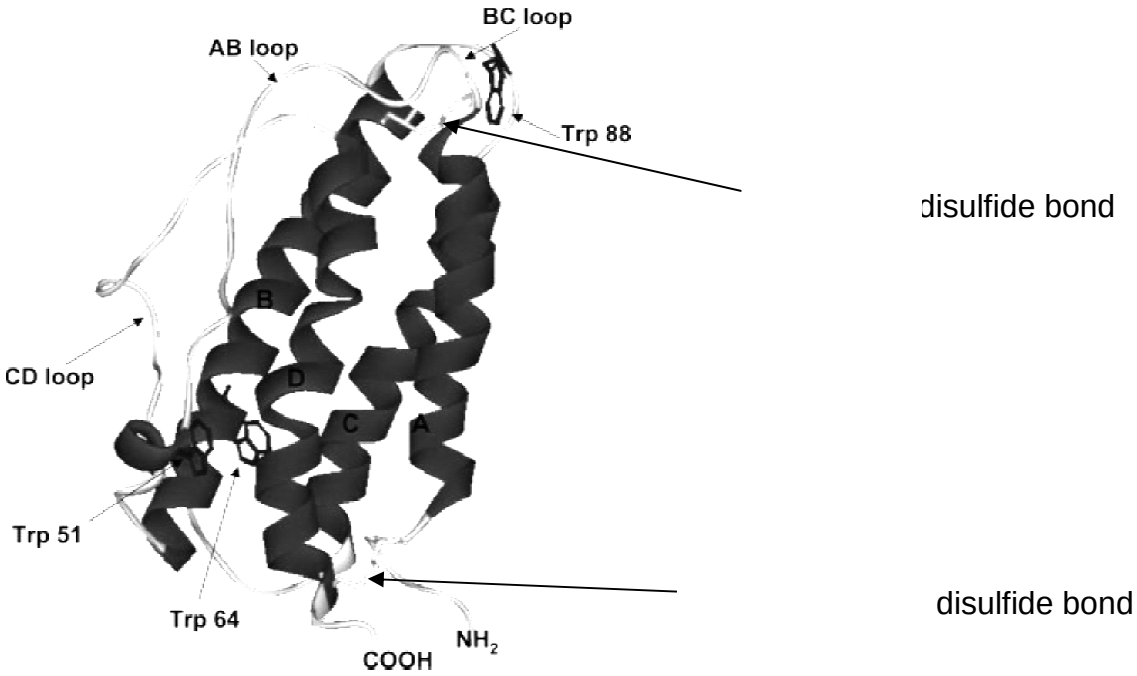


Figure 7. Kinetic folding mechanism of erythropoietin
EPO's four alpha helices lettered A-D and the connecting disulfide bonds. Reprinted by kind permission of the publisher (Banks et al., 2009).

Three of the helices (A, C and D) interact with the haematopoietic EPOR whereas helix B is not in contact with the two binding sites of the haematopoietic EPOR. However, helix B seems to be responsible for EPO's tissue protective effects. The observation that small helix B derived peptides are tissue protective but not haematopoietic is in line with this hypothesis (Brines et al., 2008). In chapter 1.5.3, these non-classical tissue protective functions of EPO will be dealt with in more detail.

As mentioned before, the kidneys are the main production sites of EPO in adults. EPO gene expression is induced under hypoxic conditions via hypoxia-inducible factors (HIF) (see **Fig. 8**). When in a state of severe hypoxia, EPO levels of individuals with intact renal functions can be increased up to 1000-fold (Jelkmann, 1992, 2004; Stockmann and Fandrey, 2006). HIFs are a family of transcription factors which have been first discovered as regulators of EPO gene expression, but later it became clear that they control all kinds of intracellular hypoxic responses throughout the body (Smith et al., 2008). They are part of the regulation of hundreds of genes involved in angiogenesis, glycolysis, cell growth, apoptosis and vasomotor regulation (Semenza, 2004; Smith et al., 2008). Of the three known

subtypes of the HIF-α unit (HIF-1α , -2α, -3α) that all dimerise with the HIF-β subunit (also known as aryl hydrocarbon receptor nuclear translocator [ARNT]), HIF-1α and HIF-2α seem to share functional characteristics (Wiesener et al., 1998) whereas HIF-3α is rather involved in negative regulation of hypoxia (Chen et al., 2009). While first HIF-1α was detected, it is now known that HIF-2α is the primary transcription factor in EPO gene expression (Jelkmann, 2011).
Both HIF-1α and HIF-1β are continuously produced, but under normoxic conditions, the hydroxylation of the proline residues immediately leads to the degradation of HIF-1α in the ubiquitin proteasome pathway. **Fig. 8** shows that in contrast to normoxia, hypoxia prevents the degradation of the HIF-1α subunit which then enters the nucleus, dimerises with HIF-1β to form the HIF heterodimer that then exerts its effects on gene regulation, the most important regulatory element being the hypoxia response element (HRE), to which HIF can bind (Jelkmann, 2004; Chen et al., 2009; Semenza, 2009). For a more detailed description, see **Fig. 8**.

Other regulators of EPO production are GATA-2 and NF-κB, which inhibit EPO gene expression (Imagawa et al., 1997) especially in inflammatory diseases (Jelkmann, 2004, 2007a). In normoxia, GATA-2 suppresses the EPO promoter, whereas in hypoxia GATA-2 levels decrease (Jelkmann, 2011).

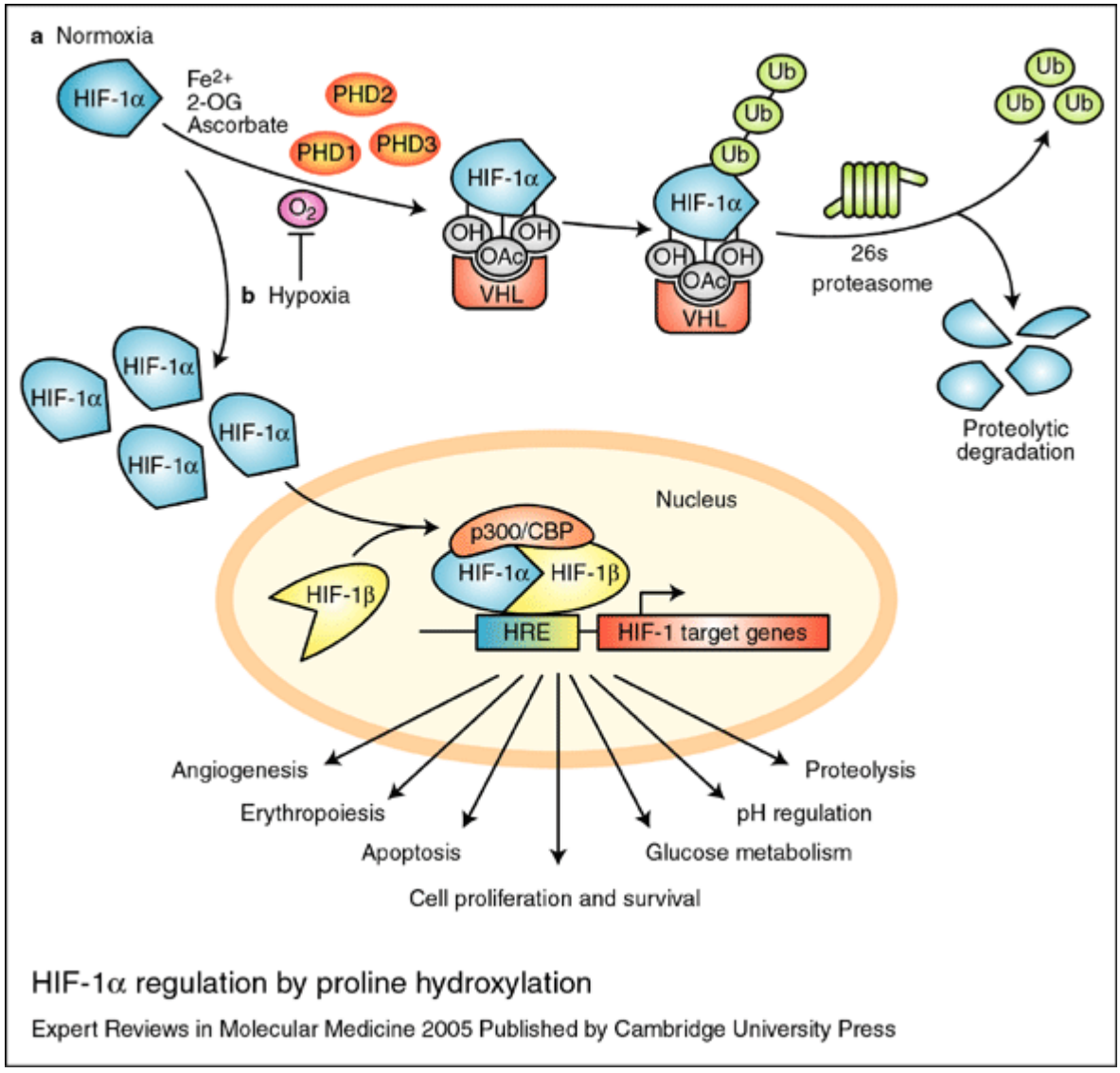


Figure 8. HIF-1α regulation by proline hydroxylation

a) Normoxia: HIF-1α is continuously produced, but in states of normoxia, it is destroyed rapidly in proteasomes. In the presence of O_2, HIF-1α is hydroxylated at proline residues by oxygen- and iron-dependent prolyl-hydroxylase domain (PHD) containing enzymes. This process increases the affinity for the von-Hippel-Lindau tumour suppressor protein (VHL) which binds to the hydroxylated HIF-1α leading to its polyubiquination (Ub). This complex is then immediately degraded by proteasomes.

b) Hypoxia: Only under hypoxic conditions, HIF-1α escapes the proteolytic degradation but survives instead and enters the nucleus where it dimerises with HIF-1β. This newly emerged heterodimer recruits the transcriptional coactivator p300/CBP, binds to the hypoxia response element (HRE) and activates for example the EPO gene.

Reprinted by kind permission of the publisher (Carroll and Ashcroft, 2005).

1.5.2 EPO signalling

The homodimeric EPO receptor (EPOR) is a member of the cytokine class I receptor family, to which also (among others) the receptors for IL-2 to IL-7, growth hormone and prolactin belong (D'Andrea and Zon, 1990; Youssoufian et al., 1993). The EPOR protein has a predicted molecular mass of approximately 60 kDa, although the EPOR of erythroid cells can possess different sizes (Jelkmann, 2011). Glycosylation, ubiquination and other modifications by proteins have been postulated to account for the varying molecular mass of EPO (Jelkmann et al., 2008). EPOR, like other members of the cytokine receptor superfamily, consists of a single transmembrane spanning region, a cytoplasmic region and an extracellular region (see **Fig. 9**).

The latter contains a WSXWS motif, which is essential for ligand binding, internalization and signal transduction (Jelkmann et al., 2008).

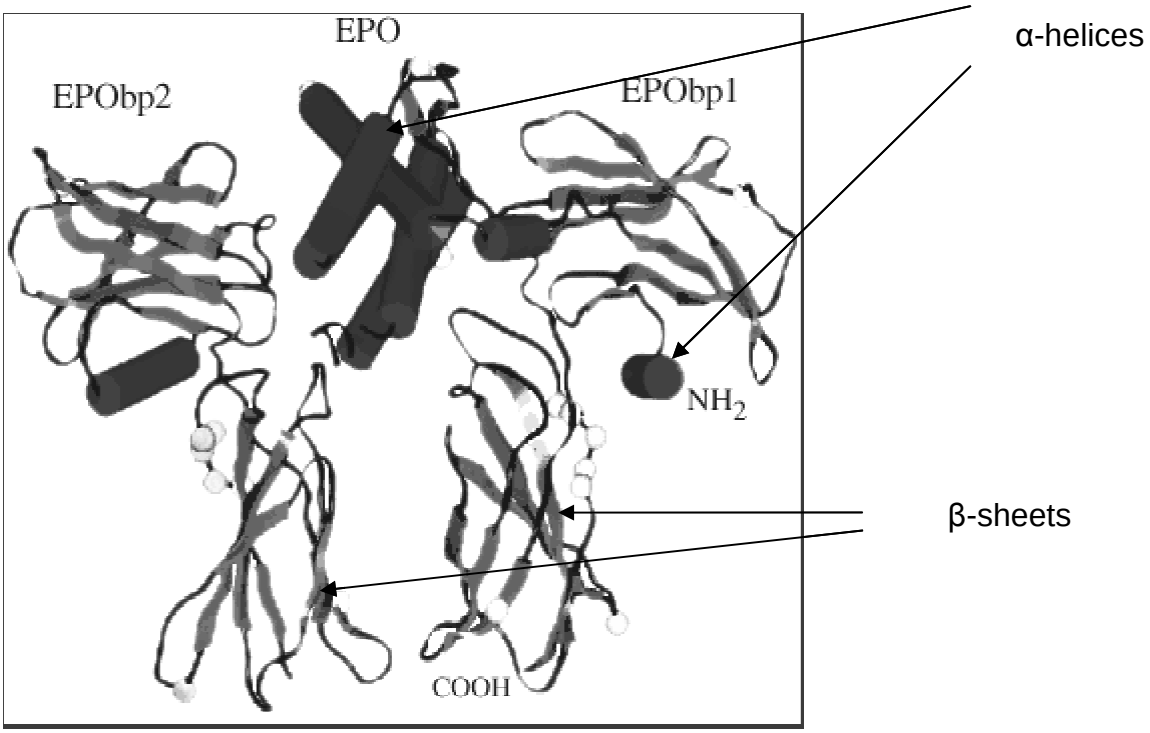


Figure 9. Chrystal structure of EPO and two extracellular EPOR domains
Two soluble extracellular EPO receptor domains (EPObp1 and 2) dimerise upon EPO binding. α-helices are shown as cylinders and β-sheets as ribbons. Reprinted by kind permission of the publisher (Syed et al., 1998).

EPO binding to the EPOR is followed by conformal changes of the receptor, phosphorylation of an associated Janus kinase 2 (JAK2) tyrosine kinase, phosphorylation of the EPOR and thus binding and phosphorylation of several signalling molecules (Tauchi et al., 1995; Remy et al., 1999; Jelkmann, 2004; Rossert and Eckardt, 2005). In **Figure 10**, the most important pathways by which EPO can reduce apoptosis are shown.

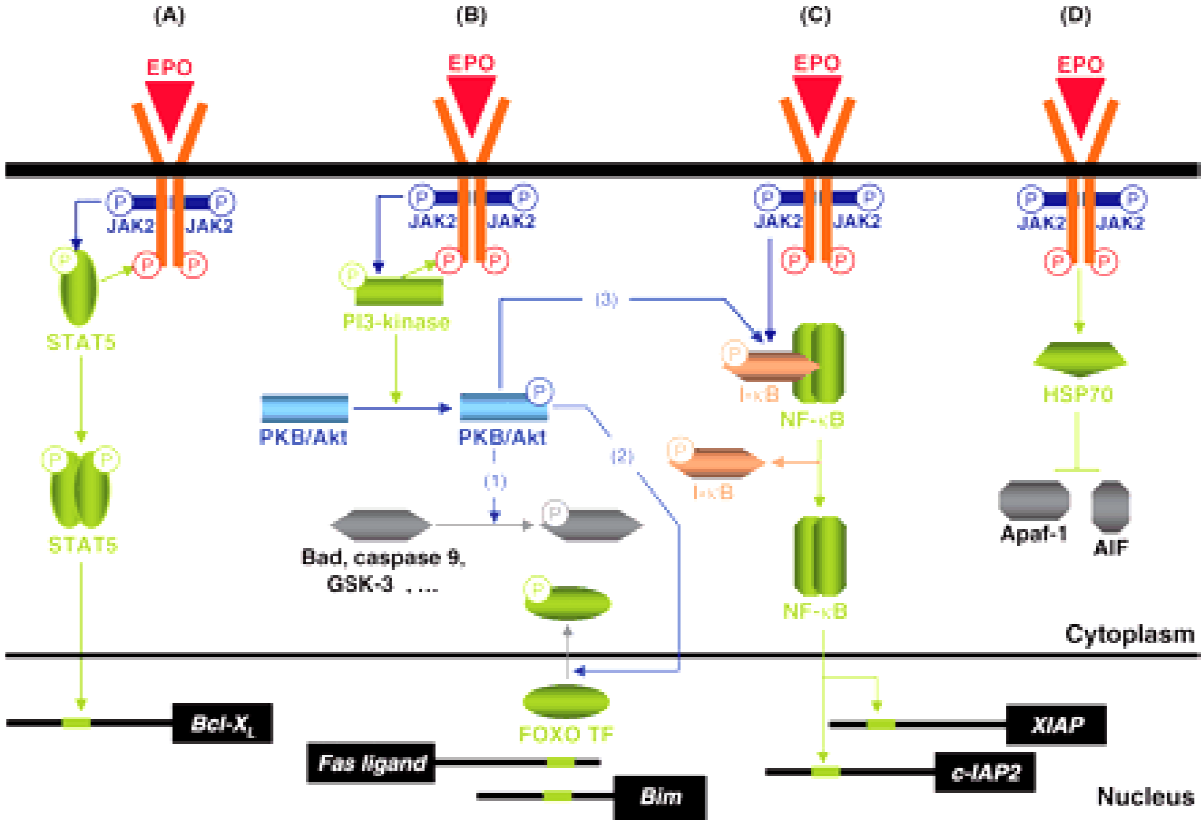


Figure 10. Schematic representation of anti-apoptotic pathways of EPO
(A) Homodimerisation of STAT5, which activates e.g. the anti-apoptotic Bcl-X_L. **(B)** Activation of the phosphatidyl-inositol-3-kinase (PI3-kinase), which leads to the inactivation of pro-apoptotic molecules, such as Bad or caspase 9. **(C)** Activation of NF-κB, which enhances the transcriptional activity of anti-apoptotic genes, such as x-linked inhibitor of apoptosis protein (XIAP) and cellular inhibitor of apoptosis protein 2 (c-IAP2). **(D)** Activation of heat shock protein (HSP) 70, which inhibits pro-apoptotic molecules. Reprinted by kind permission of the publisher (Rossert and Eckardt, 2005).

1.5.3 Non-classical production sites and functions of EPO

Apart from its classical production site in peritubular kidney cells and its classical function as chief regulator of erythropoiesis, EPO is now recognised as a tissue-protective factor that operates in a wide range of other tissues than the bone marrow (Arcasoy, 2008; Jelkmann et al., 2008; Arcasoy, 2010).

For example, EPO mRNA has also been detected in brain, liver, spleen, lung and testis (Fandrey and Bunn, 1993; Arcasoy, 2008) and EPOR mRNA is expressed in brain, heart, blood vessels, kidneys, liver, testis, retina and skeletal muscle (Digicaylioglu et al., 1995; Juul et al., 1998; Jelkmann et al., 2008). In 2007, Bodo et al. could show that also human scalp hair follicles express EPO on the mRNA and protein level *in situ* and express transcripts for the EPOR (Bodo et al., 2007a). All these observations have led to major research efforts on the role of EPO signalling in those tissues both in developing and in adult systems. It could be shown that in the developing embryonic murine heart, the lack of EPO and EPOR is accompanied by ventricular hypoplasia (Wu et al., 1999). In adults, EPO was found to also have apoptosis-inhibitory effects in several other tissues and to oppose the actions of pro-inflammatory cytokines (Brines and Cerami, 2008).

To give some examples, it has been reported that the infusion of EPO can protect neurons against ischaemic injury (Sakanaka et al., 1998; Ehrenreich et al., 2002; Siren et al., 2009). Similar protective findings of EPO have been described when applying EPO after a blunt trauma of the head (Brines et al., 2000) or the spinal cord (Gorio et al., 2002; Fang et al., 2009). Rodents treated with EPO after transient or permanent coronary artery occlusion show reduced myocardial damage (Moon et al., 2003; Parsa et al., 2003; Prunier et al., 2009). There are even reports that EPO may be successful in the neuroprotection in schizophrenia (Ehrenreich et al., 2004; Ehrenreich et al., 2008).

Apart from these protective effects there is a discussion whether EPO promotes tumour growth (Jelkmann, 2007b; Tovari et al., 2008) as EPO and EPOR are highly expressed in various solid tumours (Westenfelder and Baranowski, 2000; Acs et al., 2001; Arcasoy et al., 2002; Kumar et al., 2006).

However, doubts have recently arisen about the reliability of the previous reports on EPOR expression in both tumours and normal extra-haematopoietic tissues.

Sinclair et al. claim that most of the findings are not trustworthy as they are based on either the non-specific EPOR antibody or on other methodical inadequacies (Sinclair et al., 2010). In their own experiments on several non-haematopoietic tissues such as endothelial, cardiac, neuronal and renal cells, the group detected either no or only very low EPOR protein expression levels (Sinclair et al., 2010). Similarly, members of the same group have claimed that all the tumour cell lines examined expressed EPOR mRNA only at low levels. Partly, these tumour cell lines expressed also EPOR protein at low levels and only in one case EPOR was detected on the cell surface. Furthermore, they reported that EPOR activation was *not* possible in any of the examined cell lines (Swift et al., 2010). However, these results remain to be confirmed by other groups.

1.6 Erythropoietin and the skin

As demonstrated in the preceding chapter, during the last years EPO has been appreciated to be involved in a lot more processes and tissues than previously thought. Moreover, there are several reports of EPO/EPOR signalling in human and rodent skin, e.g. melanocytes have been shown to express both EPO protein and the EPOR. Dermal papilla cells of HFs and epidermal cells as well as cutaneous mast cells have been reported to express the EPOR (Kumar et al., 2005; Isogai et al., 2006; LeBaron et al., 2007; Mirmohammadsadegh et al., 2010).

In 2007, LeBaron et al. reported that in rats, EPO treatment generates strong activation of the STAT5 pathway in dermal papilla cells of HFs and a weaker activation in epidermal cells. Thus the team provided first evidence for the existence of functional EPOR in the cutaneous system of rats by classifying the two cell types as EPO-target cells.

Encouraged by these observations, Bodo et al. continued to investigate this EPO-skin connection and published the intriguing finding that human skin produces EPO and expresses the EPOR (Bodo et al., 2007a). In particular, this group from the laboratory where this thesis project was executed examined EPO protein expression *in situ* in human scalp skin using the highly sensitive EnVision technique. This technique revealed EPO protein expression in HF keratinocytes of

the central ORS and in blood vessels of human scalp skin. In contrast, epidermal and follicular melanocytes did not seem to express EPO protein and most intensive staining was seen in ORS keratinocytes (Bodo et al., 2007a). Moreover, the group revealed by ELIZA assay the HF's capacity to secrete substantial amounts of EPO itself. The microdissected HFs secreted a total of 2.4 mU/ml EPO protein into the medium after 48 hours of organ culture under serum-free conditions. Using quantitative real-time reverse transcriptase PCR, the previous results could be confirmed, as both EPO and EPOR transcripts were detected in freshly microdissected hair bulbs of anagen human scalp HFs (Bodo et al., 2007a).

Furthermore, Bodo et al. published first evidence of a possible cutaneous EPO regulation system. Similar to the kidney, EPO was up-regulated in hypoxia-treated HFs and since HFs also transcribe HIF-1α, they might also detect hypoxia through rising amounts of HIF-1α protein. Although in the experiments performed by Bodo et al., EPO did not significantly modulate two key parameters in HF research, namely hair shaft elongation and proliferation/apoptosis of matrix keratinocytes *in vitro*, EPO *was* able to inhibit apoptosis in organ-cultured human scalp HFs treated with the cyclophosphamide-derivative 4-HC (Bodo et al., 2007a). This apoptosis-reducing effect makes EPO interesting for a potential use in chemotherapy-associated hair loss (Bodo et al., 2007a).

First gene expression profiling by microarray analysis showed that several genes respond to EPO treatment. For this experiment, the HFs of two female patients were cultured with either vehicle or 100 IU/ml EPO for 6 hours. After RNA isolation, a human whole genome oligo microarray (44K) was performed as a commercial service by Miltenyi Biotec GmbH (Cologne, Germany). This service is based on Agilent Microarray technology and covers more than 40,000 well-characterised human genes. The group implemented two selection strategies: First only those "differentially expressed" candidate genes were selected that showed a significant ($p<0.01$) equidirectional up- or down-regulation in both examined patients (minimal transcription change > 1.5-fold) (see **Fig. 11)**. The second, less stringent, analysis selected those genes that were substantially modulated **in only one** of the two patients (transcription changed > 5-fold, $p<0.01$) as shown in **Table 2**.

Abbreviation	Sequence Description	Fold Change
LOC126536	Homo sapiens hypothetical protein LOC126536	–27.1
CTSZ	Homo sapiens cathepsin Z	–25.0
FABP1	Homo sapiens fatty acid binding protein 1	–23.5
PRDM14	Homo sapiens PR domain containing 14	–20.6
APOB48R	Homo sapiens apolipoprotein B48 receptor	–19.8
TPH2	Homo sapiens tryptophan hydroxylase 2	–18.6
CHIT1	Homo sapiens chitinase 1	–13.3
PLCB2	Homo sapiens phospholipase C, beta 2	–11.4
RPS4Y1	Homo sapiens ribosomal protein S4, Y-linked	–9.9
MIA2	Homo sapiens melanoma inhibitory activity 2	–9.9
UCP2	Homo sapiens uncoupling protein 2	–9.4
RBMY1E	Homo sapiens RNA binding motif protein, Y-linked, family 1, member E	–9.4
CDW52	Homo sapiens CDW52 antigen	–9.2
KCNV2	Homo sapiens potassium channel, subfamily V, member 2	–8.7
ZNF409	Homo sapiens mRNA for KIAA1056 protein	–8.4
FBXL18	Homo sapiens hypothetical protein FLJ11467	–8.3
RPS4Y2	Homo sapiens ribosomal protein S4, Y-linked 2	–8.0
L3MBTL	Homo sapiens mRNA for KIAA0681 protein	–7.9
LRRC21	Homo sapiens leucine rich repeat containing 21	–7.4
SLC24A1	Homo sapiens mRNA for KIAA0702	–7.3
GAGED3	Homo sapiens G antigen, family D, 3	–7.2
CCL19	Homo sapiens chemokine (C-C motif) ligand 19	–7.1
DNAI2	Homo sapiens mRNA for intermediate dynein chain	–7.0
DDI1	Homo sapiens DNA-damage inducible protein 1	–6.9
ADAMTS10	Homo sapiens a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 10	–6.8
TM7SF4	Homo sapiens DC-specific transmembrane protein	–6.8
HK3	Homo sapiens hexokinase 3 (white cell), nuclear gene encoding mitochondrial protein	–6.6
CART1	Homo sapiens cartilage paired-class homeoprotein 1	–6.6
PDE6C	Homo sapiens phosphodiesterase 6C, cGMP-specific, cone, alpha prime	–6.4
AOC2	Homo sapiens amine oxidase, copper containing 2, transcript variant 1	–6.3
IL20	Homo sapiens interleukin 20	–6.1
NM_001012763	Homo sapiens gonadotropin-releasing hormone receptor, transcript variant 2	–6.0
TDGF1	Homo sapiens teratocarcinoma-derived growth factor 1	–6.0
LCP1	Homo sapiens lymphocyte cytosolic protein 1	–5.6
KCNH6	Homo sapiens cDNA FLJ33650 fis, highly similar to Rattus norvegicus potassium channel	–5.4
PRDM7	Homo sapiens PR domain containing 7	–5.4
FPR1	Homo sapiens formyl peptide receptor	–5.3
M27126	Human lymphocyte antigen	–5.2
PDLIM2	Homo sapiens PDZ and LIM domain 2	–5.2
EDN3	Homo sapiens endothelin 3, transcript variant 2	+5.4
LOC440040	PREDICTED: Homo sapiens similar to Metabotropic glutamate receptor 5 precursor	+6.2
PTPRB	Homo sapiens protein tyrosine phosphatase, receptor type	+6.6
ABCA13	Homo sapiens ATP binding cassette gene, sub-family A, member 13	+7.6

Table 2 List of genes substantially altered after EPO administration in only one of the patients

HFs of two patients were incubated for 6 h with either EPO (100 IU/ml) or vehicle; after RNA isolation a microarray was performed (>5-fold, $p<0.01$).

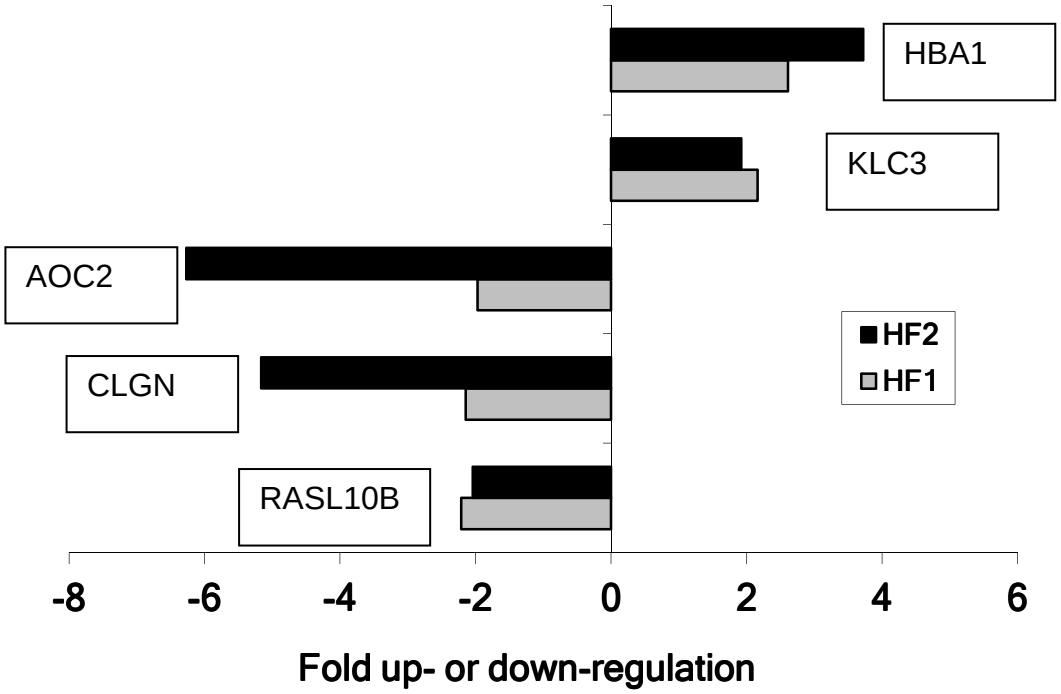


Figure 11. Possible EPO-target genes detected via microarray
HFs of two donors (HF1 and HF2) were incubated for 6 h with either vehicle or 100 IU/ml EPO. The microarray analysis revealed 5 genes significantly altered upon EPO treatment compared to RNA extracted from vehicle-treated HFs in both patients (>1.5-fold, $p<0.01$): haemoglobin alpha 1 (HBA1), kinesin light chain 3 (KLC3), copper-containing aminase oxidase (AOC2), calmegin (CLGN) and RAS-like family 10, member B (RASL10B).

Bodo et al. proposed that the EPO-induced down-regulation of RAS10B, a small GTPase with tumour suppressor potential (Zou et al., 2006), may be associated with EPO's anti-apoptotic properties (Bodo et al., 2007a). Furthermore this group detected some gene expression changes of genes that are involved in neuroendocrinology of the skin, for example tryptophan hydroxylase 2 (TPH2), the rate-limiting step in the production of the neurotransmitter serotonin.

Recently though, another group performed similar experiments on both human and mouse HFs (Kang et al., 2010). Their results are partially consistent with the previous study, but also conflict in some respects with the findings by Bodo et al. described above. In particular, Kang et al. confirmed that EPO protein expression can be mainly found in the ORS of HFs. However, Kang et al. reported that neither EPOR mRNA nor EPOR protein were expressed in cultured human ORS keratinocytes. Since cultured human papilla cells though were shown to express a functional (i.e. responding to EPO by phosphorylation) EPOR by both RT-PCR

and immunoblot analysis, the group postulated that EPO originating from ORS cells may operate in a paracrine fashion on DP cells (Kang et al., 2010).

However, the data the Korean group reported on hair shaft elongation and proliferation of matrix keratinocytes in human organ-cultured HFs are contradictory to those of our laboratory: Kang et al. presented that EPO significantly enhanced hair shaft elongation and increased Ki-67 positive matrix keratinocytes around the DP whereas Bodo et al. had detected no such effect in the corresponding experiments. As gender-dependent hormone signalling differences are known for example for oestrogens, androgens and prolactin (Ohnemus et al., 2006; Langan et al., 2010a), a possible explanation for this discrepancy could be that Bodo et al. used HFs from female patients, whereas the Korean group used HFs from male donors (Kang et al., 2010).

In 2006, Isogai et al. presented for the first time that human cutaneous mast cells express the EPOR in their secretory granules. In immunohistochemically examined skin samples from patients with different dermatological diseases, mast cells were positively stained by a commercially available anti-soluble EPOR antibody, but did not react with an antibody against the C-terminal peptide of the EPOR (Isogai et al., 2006). Further confirmation that the EPOR on mast cells may not represent the complete receptor molecule was obtained by immunoblot analysis that revealed two protein bands at 20 and 43 kDa. This reflects neither the reported size of the whole EPOR molecule (60 kDa) nor the reported size of the soluble form of the EPOR (34 kDa). Therefore, the authors speculated that the EPOR in the secretory granules of mast cells may be bound to an unknown substance (Isogai et al., 2006).

There is evidence that EPO promotes wound healing in the subcutaneous tissue of rats *in vivo*: when applying a special wound healing model consisting of fibrin Z-chambers accelerated fibrin-induced wound healing could be observed when recombinant EPO was administered (Haroon et al., 2003). Correspondingly, endogenous EPO blocking by using soluble EPOR and anti-EPO monoclonal antibodies resulted in delayed wound healing (Haroon et al., 2003; Brines et al., 2008). Pyroglutamate helix-B-surface peptide (pHBSP), a non-erythropoietic,

tissue-protective peptide that has been derived from the tertiary structure of EPO promoted wound healing of punch biopsy wounds in rats (Brines et al., 2008).
Also, in mice with experimental burn injuries, reepithelialisation was quicker in rHuEPO-treated mice and a similar improvement of wound healing was found in genetically diabetic mice after rHuEPO application (Galeano et al., 2004; Galeano et al., 2006). In these experiments an increased angiogenesis could be observed indicating that this may be the main factor for the wound healing improvements.
Clinical trials in systemic sclerosis patients with severe skin ulcers showed great improvements ranging from significant reduction to complete resolution of the ulcers after treatment with rHuEPO (Ferri et al., 2007).

Boutin et al. recently presented new evidence that murine skin might serve as a critical mediator of systemic responses to hypoxia and that EPO might not only be the key regulator of erythropoiesis but also a regulator of other forms of systemic response to hypoxia. In particular, they assume that the dermal response to hypoxia is important for the acute and chronic adaption to hypoxia with only the chronic response leading to an increased plasma EPO level (see **Fig. 12**) (Boutin et al., 2008). They reported that epidermal deletion of HIF-1α inhibits renal EPO synthesis in response to hypoxia and that conversely mice with an epidermal deletion of von-Hippel-Lindau tumour suppressor protein, a negative regulator of HIF, have increased EPO synthesis and polycytaemia. Also the same group described that the dermal administration of nitric oxide (NO) donors increases EPO levels (Boutin et al., 2008).

However, elements of this work have raised controversy, in particular the finding of Boutin et al. that exposure of murine skin to hypoxia *in vivo* does not increase EPO expression (Paus et al., 2009). Also parts of the experimental design (Semenza, 2008) and some of the conclusions drawn and concepts deduced from their findings were criticised (Paus et al., 2009). Based on the observation that human skin and its HFs express EPO protein and that (likely HIF-1α-mediated) oxygen-sensing may be present in human HFs, Paus et al. propose a different scenario that also includes skin as a major structure in oxygen sensing: namely that skin itself might enhance systemic EPO plasma levels in states of hypoxia by up-regulation of intracutaneous EPO production (see **Fig. 12**) (Paus et al., 2009).

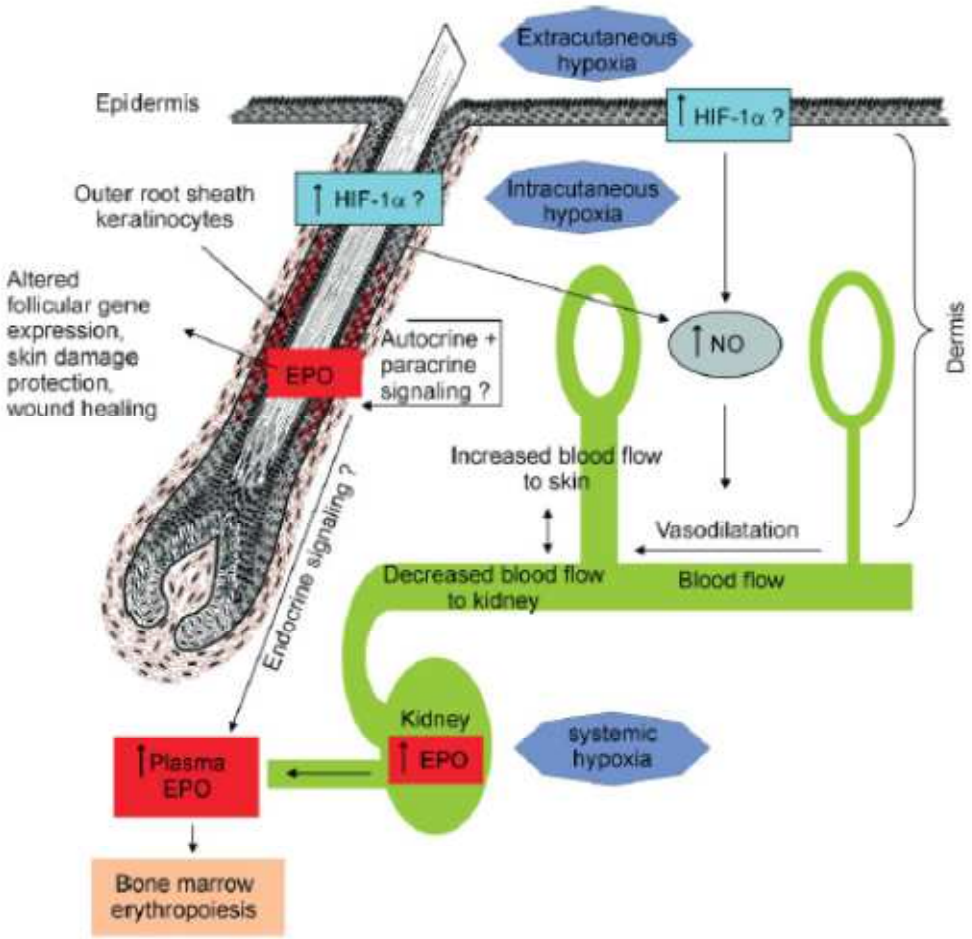


Figure 12. Dermal and follicular response to extra- and intracutaneous hypoxia – hypothetical scenario
Boutin et al. have suggested that in mice renal EPO production is controlled through oxygen-sensing in the skin. In (more chronic) hypoxia, blood flow to the skin is increased by HIF-1α-mediated nitric oxide (NO) production. This is accompanied by decreased blood flow to the kidney which leads to a rise of plasma EPO levels (Boutin et al., 2008). Paus et al. have advanced the hypothesis that ORS keratinocytes of anagen HFs may contribute to systemic plasma EPO levels as they are an extrarenal source of EPO (Bodo et al., 2007a). Reprinted by kind permission of the publisher (Paus et al., 2009).

These authors postulate that the local cutaneous EPO production – with skin being the largest mammalian organ – might actually account for significant systemic EPO levels. If future experiments confirm this hypothesis, the field would be opened for clinical investigations along the line whether stimulation of intracutaneous EPO production may suffice to counteract declining renal EPO production (Paus et al., 2009).

All currently available pointers for EPO in skin biology and pathology are summarised in **Table 3**.

cell type	**EPO/EPOR expression and/or effect**	**reference**
human melanocytes	EPO and EPOR expression	(Kumar et al., 2005; Mirmohammadsadegh et al., 2010)
human melanoma cells	EPO and EPOR expression activates the phosphoinositide 3-kinase/Akt pathway	(Kumar et al., 2005; Mirmohammadsadegh et al., 2010)
DP cells of HFs of rats	strong activation of the STAT5 pathway	(LeBaron et al., 2007)
human DP cells	(functional) EPOR expression	(Kang et al., 2010)
epidermal cells of rats	weak activation of the STAT5 pathway	(LeBaron et al., 2007)
cutaneous human mast cells	EPOR expression in secretory granules; probably not the complete EPOR molecule	(Isogai et al., 2006)
human ORS keratinocytes	EPO expression	(Bodo et al., 2007a; Kang et al., 2010)
part of skin	**EPO effect**	**reference**
human scalp HFs	inhibition of apoptosis induced by 4-HC	(Bodo et al., 2007a)
human scalp HFs	enhanced hair shaft elongation and increased Ki-67 positive matrix keratinocytes in male HFs; *in contrast, Bodo et al. had not found these effects in female human scalp HFs!*	(Bodo et al., 2007a; Kang et al., 2010)
rodent skin	accelerated wound healing associated with increased angionesis	(Haroon et al., 2003; Galeano et al., 2004; Galeano et al., 2006; Brines et al., 2008)
human skin of patients with systemic sclerosis	significant improvement of skin ulcers	(Ferri et al., 2007)
murine skin as a major structure of oxygen sensing	EPO as a regulator of many forms of systemic response to hypoxia	(Boutin et al., 2008)
	hypothesis: skin itself might enhance systemic EPO plasma levels in states of hypoxia by up-regulation of intracutaneous EPO production	(Paus et al., 2009)

Table 3 Summary of currently available pointers to a role of EPO in skin biology and pathology

1.7 Major open questions in cutaneous EPO research

Given that skin is a major endocrine organ, it is important to also evaluate the full dimensions of physiological, pathological and pharmacological EPO functions in human skin biology. The previously published EPO experiments on human and animal skin or its isolated cell populations have offered first evidence of not only the mere expression of EPO/EPOR expression in skin, but also of the functionality of this signalling pathway in human skin.
However, as EPO/EPOR signalling within the cutaneous system has only recently been discovered, many questions in the field remain wide-open.

First of all, further localization of the exact cell types that express EPO/EPOR is required to verify or to complete our current, still very limited understanding of EPO biology in human skin. Secondly, for further confirmation of the functionality of cutaneous EPOR-mediated signalling, assessment of gene changes as well as other changes of cell biology induced by EPO treatment would be helpful.
As a next step, further experiments on human HFs obtained from both sexes are necessary to clarify the conflicting results obtained by Bodo et al. and Kang et al. (see previous chapter). In this regard, also the issue whether there actually is a gender difference in EPOR expression levels and/or pattern, as speculated by Kang et al., needs to be examined systematically. Alternatively, other and/or additional influencing factors must be investigated. As EPO signalling can induce several different signalling cascades (see **Fig. 10**), a detailed determination of the pathways following EPOR activation in human skin and HFs is needed.

Several authors have claimed the presence of EPO and EPOR in cultured melanocytes (Kumar et al., 2005; Mirmohammadsadegh et al., 2010). However, it remains to be assessed whether the main function of these cells – i.e. to produce pigment – is altered by EPO/EPOR signalling. This is especially important as epidermal and follicular pigmentation is intensively regulated by multiple hormones and other factors (Slominski et al., 2004), of which EPO might be one.

Similarly, the significance of EPOR present in human cutaneous mast cells has not yet been investigated and requires further experiments. Next, first hints that EPO treatment reduces chemotherapy-induced alopecia (CIA) deserve to be followed up in further detail (Bodo et al., 2007a). It needs to be shown that this effect can not only be triggered in microdissected HFs but also in full-thickness skin. After that, this anti-apoptotic signalling cascade would have to be evaluated in an *in vivo* system, and the same applies to all other previous findings on cutaneous EPO effects.

Finally, future experiments will have to clarify the impact of cutaneous EPO production on systemic EPO levels (Paus et al., 2009).
Out of this wide range of open questions, we chose to concentrate on further evaluation of EPO-induced gene expression changes in the human HF and to examine whether apoptosis/proliferation is also altered in full-thickness skin. Besides, we wanted to evaluate changes due to EPO treatment in mast cell and melanocyte biology *in situ*. Since the experimental part of this work had already been finalised when Kang et al. published their conflicting EPO data, we did not re-examine EPO effects on human hair shaft growth.

1.8 Specific questions defined

On this basis, the following questions were addressed:

1. Can the EPO responsive genes that have been detected by microarray technique also be confirmed on the mRNA level by qRT PCR and/or on protein level by immunohistochemistry?
2. Does EPO influence HF pigmentation? If so, are there changes in the number, the dendricity and/or in the tyrosinase activity of human HF melanocytes?
3. As it has been reported that mast cells express the EPOR (Isogai et al., 2006), does EPO alter human skin mast cell numbers or degranulation in the CTS of HFs?
4. Is the apoptosis reduction of EPO in chemotherapy-treated HFs also seen in unmanipulated human HFs within full-thickness skin *in situ*?

5. Is any apoptosis-reducing effect of EPO also seen in the epidermis and dermis of organ-cultured full-thickness human skin?
6. Does EPO alter cell proliferation in human full-thickness skin *in situ*?

1.9 Experimental design

To address these specific questions, we have chosen the following design that allows to dissect the direct effects of EPO on human HFs and full-thickness skin *in vitro,* i.e. in the absence of confounding vascular, neural, endocrine, renal and any other systemic influences. Uninflamed human scalp skin was randomly collected from a total of 8 healthy male and female donors undergoing elective plastic surgery. Full-thickness scalp skin biopsies or its microdissected HFs were divided into different treatment groups, organ-cultured under serum-free conditions and then examined through histochemical and immunohistochemical stainings, as previously described (Philpott et al., 1990; Bodo et al., 2007a; Lu et al., 2007). We also employed the cyclophosphamide-derivative 4-HC (Bodo et al., 2007b) to study whether EPO modulates apoptosis of chemotherapy-damaged human HFs within organ-cultured full-thickness human skin. After photodocumentation, hair and skin biology parameters were assessed by staining patterns and qualitative and/or quantitative (immuno)histomorphometry.

Additionally, HFs from one additional patient were used to follow up the previous microarray gene expression leads by qRT PCR in order to confirm the robustness of gene expression changes induced by EPO treatment.

2 Materials and methods

2.1 Human skin and HFs: tissue source, preparation and organ-culturing

Following the Helsinki Guidelines, after informed consent and ethics approval (No. 06-109) human frontotemporal and occipital scalp skin for HF preparation was obtained from a total of 8 healthy female and male patients undergoing elective plastic surgery with collaborating surgeons (mainly Dr. Dr. W. Funk, Klinik Dr. Koslowski, Munich). The age range of the donors was 54 to 68 years, with an average of 60.6 years. The skin samples were transported on ice in falcon tubes containing William's E medium supplemented with 250 IU/ml penicillin, 250 mg/ml Streptomycin and 12.5 mg/ml amphotericin B (Gibco, Paisley, Scotland).

The following day, HFs were isolated in isolating medium consisting of William's E (Biochrom, Cambridge, UK) supplemented with a 1% antibiotic/antimycotic mixture (100x, Gibco, Karlsruhe, Germany) containing penicillin G, streptomycin and amphotericin B. After shaving, the skin sample was cut in pieces of approximately 5-10 mm^2. Then the piece of skin was cut with a scalpel blade just above the subcutis at the dermo-subcutaneous fat interface. Under a binocular dissecting microscope, the HF bulbs were removed from the subcutaneous fat tissue. This was achieved by using a blunt forceps to press down gently and stabilise the fat tissue so that it was easier to grip the HF and pull it out with a watchmakers' forceps.

The HFs of five of the patients were organ-cultured in five independent experiments (also referred to as assay) as described in the next chapter. The HFs obtained from one additional patient were used for qRT PCR analysis (see chapter 2.2).

The scalp skin obtained from 3 other patients was used for skin organ culture as described in chapter 2.1.2.

2.1.1 Human hair follicle organ culture

The intact hair follicles were maintained in 24-well plates (see **Fig. 13**). Each well contained serum-free William's E medium (Biochrome, Cambridge, UK) supplemented with 2 mmol/l L-Glutamin (Invitrogen, Paisley, UK), 10 ng/ml hydrocortisone (Sigma-Aldich, Taufkirch, Germany), 10 µg/ml insulin (Sigma-Aldich), and 1% antibiotic / antimycotic mixture (Gibco, Karlsruhe, Germany) (Philpott et al., 1990; Bodo et al., 2007a). The well plates were kept at 37 °C in an atmosphere of 5% CO_2 and 95% air.

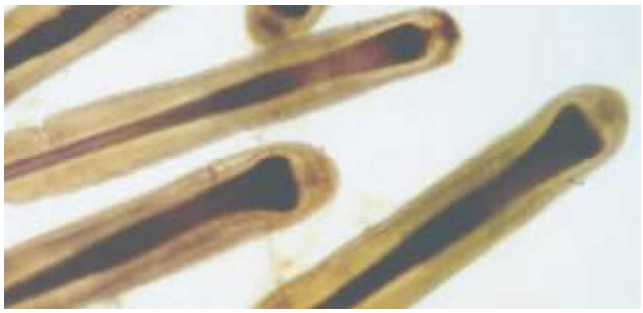


Figure 13. Hair follicles in culture
Reprinted by kind permission of the publisher (Tobin, 2008).

For a total of five independent experiments, HFs of five different donors were separated randomly in two groups and treated with either vehicle or 100 IU/ml EPO (Roche/ Boehringer Mannheim, Mannheim, Germany) for five days (or for 9 days in one assay). EPO was first added on day 1 (one day after HF isolation) and the medium was renewed every second day. After 5 days (one culture after 9 days) follicles were covered with embedding kit (Shandon cryochrome^{TM}, Pittsburg, PA, USA), frozen in liquid nitrogen and stored at -80 °C until cryosectioning and staining. Later, the frozen samples were cut into cryosections of 6 µm on a Leica cryostat (Leica, CM 3050s).

2.1.2 Human skin organ culture

Human skin samples were obtained from a total of 3 donors (age range 51-64) and processed similar to the HFs (see chapter 2.1.1). The skin biopsies of the first patient were used for a preliminary study to determine the necessary incubation period of 4-HC. It turned out that the 48 day incubation period, that had successfully induced apoptosis in organ-cultured HFs before (Bodo et al., 2007b),

was too short to reliably induce apoptosis in all skin compartments. Therefore we modified our protocol and used an incubation period of 7 days for the actual experiments.

Skin biopsies were prepared with single-use punches of 2 and 4 mm and were left floating in serum-free, supplemented William's E medium (Biochrome, Cambridge, UK) (see **Figure 14**). The skin biopsies were randomly divided into four groups: vehicle (only supplemented William's E medium), EPO
(+ 100 IU/ml EPO), 4-HC (+ 10 µmol/l 4-HC) and 4-HC + EPO (+ 100 IU/ml EPO, + 10 µmol/l 4-HC). 4-HC was obtained from Niomec (Bielefeld, Germany) and because of its short half-life time it was prepared immediately before use and kept on ice. Skin biopsies were first incubated overnight and the next day (day 1) EPO was added. On day 2, medium was exchanged and the test substances were added. EPO was hereby given some preincubation time and added one hour prior to 4-HC. After that, vehicle and test substances were exchanged every two days. After 9 days the skin biopsies were frozen in liquid nitrogen as described above, then stored at -80 °C and cut into cryosections of 7 µm.

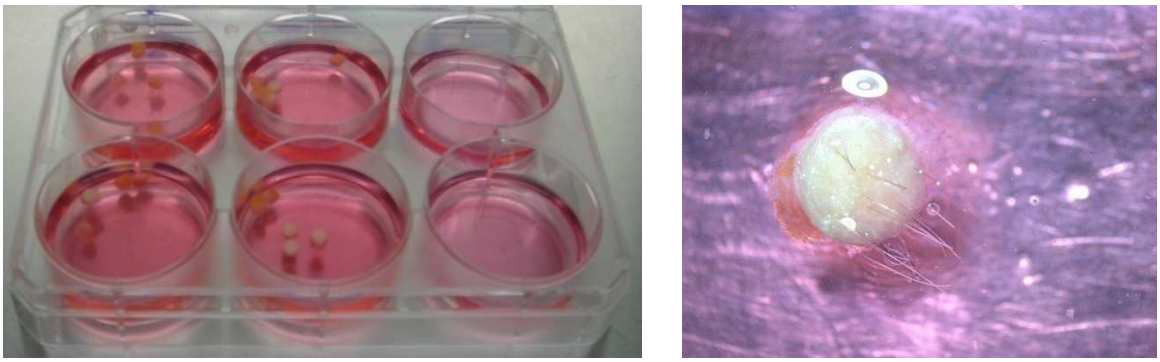


Figure 14. Full-thickness human scalp skin punch biopsies
The punch biopsies (with preserved HFs) were organ-cultured in 6-well plates for 9 days.

2.2 Quantitative real-time PCR (qRT PCR)

For quantitative real-time polymerase chain reaction (qRT PCR), cultured HFs of one patient were treated for 6 hours with either vehicle or 100 IU/ml EPO. The following RNA extraction and qRT PCR was kindly performed by Prof. Támas Bíró (Department of Physiology, University of Debrecen, Debrecen, Hungary). RNA extraction was achieved using TRIzol (Invitrogen, Paisley, UK) according to standard protocols. The well-established TaqMan® Gene Expression Assays (Applied Biosystems, Darmstadt, Germany) were used (Heid et al., 1996; Wong and Medrano, 2005). There are more than 1,000,000 pre-designed assays commercially available to detect a wide range of genes in several species. Every assay consists of two specific primers and a matching specific TaqMan® probe that allows the quantification of mRNA amounts. The TaqMan® probe is 5´end labelled by a FAM™ reporter dye (6-carboxyfluorescein) and 3´end labelled by a non-fluorescent quencher dye. The underlying principle is that in an intact probe, the short distance of the quencher dye to the reporter dye inhibits (quenches) the fluorescence of the reporter dye via Förster-type energy transfer. The sequence of the probe is designed in such a way that the probe specifically anneals within the DNA region to be amplified, i.e. between the forward and reverse primer site. During each PCR cycle, the AmpliTaq Gold® DNA polymerase cleaves the reporter dye from the annealed probe by its 5´ to 3´ nucleolytic activity. Consequently, the separation of the quencher dye from the reporter dye results in the loss of fluorescence inhibition and the fluorescence signal of the reporter dye can be measured. The fluorescence signal is detected by a special fluorescence detection system and is directly proportional to the accumulation of PCR products (Heid et al., 1996).[2]

Then the PCR cycle in which the fluorescence signal exceeds a defined level called threshold is determined (the so-called threshold cycle C_T in ABI PRISM® literature (Applied Biosystems, Darmstadt, Germany)) (Wong and Medrano, 2005). To enable better comparison, the fluorescence intensity is normalised to the

[2] TaqMan® Universal PCR Master Mix, Protocol, Applied Biosystems, ©2002, 2010.
URL: http://www3.appliedbiosystems.com/cms/groups/mcb_support//documents/generaldocuments/cms_042996.pdf *[date: 19.06.2011]*

fluorescence emission intensity of a passive reference dye.[3] For this normalisation process, usually, housekeeping genes that have a stable expression are used (Wong and Medrano, 2005). For our experiment, beta-actin and GAPDH were used as endogenous controls ("housekeeping genes").

Five different genes were examined: three genes that had been selected according to the criteria listed under 1.5 (**Fig. 11)**, namely haemoglobin alpha 1 (Schechter, 2008; Tsiftsoglou et al., 2009), kinesin light chain 3 (Hara et al., 2000) and calmegin (Watanabe et al., 1995; Yoshinaga et al., 1999; Yamagata et al., 2002). Additionally GATA-1 and GATA-2 who are known to play an important role in EPO signalling (Jelkmann, 2007a) were chosen (compare chapter 1.5.1). The primer sequences for the genes to be examined are not published by the company. Instead, Assay ID numbers are used to identify the primers. The Assay ID numbers used for our experiment are summarised in **Table 4.** qRT PCR was carried out with components of the TaqMan® Universal Master Mix (Applied Biosystems, Darmstadt, Germany) according to standard protocols. After the preparatory step which takes place at 55 °C for 2 minutes and 95 °C for 10 minutes, 60 cycles followed with every cycle consisting of 15 seconds of denaturation at 95 °C and 1 minute of annealing and amplification at 60 °C. The fluorescence signal emitted from the cleaved reporter dye was detected by the ABI PRISM® 7000 Sequence Detection System.

Gene symbol	Gene name	Assay ID
ACTB	beta actin	Hs99999903_m1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
HBA1	haemoglobin, alpha 1	Hs00361191_g1
KLC3	kinesin light chain 3	Hs00377103_m1
CLGN	calmegin	Hs00189073_m1
GATA-1	GATA binding protein 1	Hs00231112_m1
GATA-2	GATA binding protein 2	Hs00231119_m1

Table 4 Assay ID numbers for TaqMan® gene expression assays used

[3] Application note, Real time PCR: Understanding C_T, Applied Biosystems.
URL: http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_053906.pdf *[date: 19.06.2011]*

2.3 Immunohistochemistry and histochemistry

2.3.1 Ki-67 / TUNEL double-immunofluorescence

To evaluate the amount of apoptotic and proliferating cells in untreated and treated human skin biopsies (with vehicle, EPO, 4-HC or EPO + 4-HC), the Ki-67 / TUNEL (terminal dUDP nickendlabelling) double-staining method was used (Bodo et al., 2007a; Bodo et al., 2007b; Kloepper et al., 2010).

7 µm thick cryostat sections were fixed in a 1% paraformaldehyd-PBS (phosphate buffered saline) solution (10 minutes, room temperature) and in ethanol-acetic acid (2:1, 5 minutes, -20 °C) and washed in between with PBS (2 times for 5 minutes, room temperature). Then the slides were covered with digoxygenin-deoxyUTP in the presence of terminal desoxynucleotidyl transferase (TdT) for 60 minutes at 37 °C (ApopTag Fluorescein In Situ Apoptosis Detection Kit, Intergen, Purchase USA). After preincubation with normal goat serum (10% in PBS, 20 minutes, room temperature) the skin biopsies were incubated with the primary antibody mouse anti-Ki-67 antigen (Clone MIB-1; DAKO, Glostrup, Denmark) diluted 1:20 in PBS with 2% normal goat serum (overnight, 4 °C). Always washing with PBS between the following steps, the apoptotic and proliferating cells were made visible by applying the secondary antibodies. Therefore, first the biopsies were incubated (30 minutes, room temperature) with a FITC-conjugated anti-digoxygenin-antibody (ApopTag kit) and second with a rhodamine-labelled goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, USA)(1:200 in PBS, 2% normal goat serum). Counterstaining was achieved with 4',6-diamidino-2-phenylindole (DAPI) (Boehringer / Mannheim, Mannheim, Germany) to visualise the nuclei. Finally the slides were mounted with Fluoromount-G (Southern Biotechnology, Birmingham, USA). As positive control, murine spleen was used and for negative control, the terminal desoxynucleotidyl transferase (TdT) and Ki-67 antibody were omitted (Bodo et al., 2007a).

2.3.2 Masson-Fontana histochemistry

In order to evaluate whether EPO regulates pigmentation, we performed Masson-Fontana histochemistry which detects melanin (Barbosa et al., 1984; Bodo et al., 2007b) on human HF sections.

Therefore the slides were air-dried for 10 minutes and fixed in ethanol-acetic acid (10 minutes, -20 °C). Using several washing steps in between (with PBS), the slides were then processed in silver nitrate (10 %, with sodium hydroxide solution and ammonia) (56 °C, dark). Then the specimens were put in sodium thiosulfate (5%, 1 minute). Counterstaining was achieved with haematoxylin, dehydration with ethanol/xylol and mounting with synthetic resin mounting (Eukitt, O. Kindler GmbH & Co, Freiburg, Germany).

2.3.3 Tyramide-based tyrosinase activity *in situ* assay

In 2002, Han et al. described a new method for measuring the activity of tyrosinase, the key enzyme of melanogenesis and the rate-limiting enzyme for HF melanin production (Slominski and Paus, 1993; Slominski et al., 2005), the so-called tyramide-based tyrosinase activity *in situ* assay (Han et al., 2002). This assay was employed in the current study.

Using several washing steps with PBS or 0.1% NP-40/PBS in between, the slides were fixated in methanol/acetone (1:1, -20 °C), and treated with H_2O_2 (3% in PBS, 10 min) to quench endogenous peroxidase activity. To reduce background staining, samples were blocked with 5% bovine serum albumin (fraction V, Boehringer Mannheim, Mannheim, Germany). The actual reaction of this assay is that the tyrosinase enzyme reacts with the added biotinyl tyramide (1:50, 15 minutes; TSA kit, Perkin Elmer, Boston, USA). The biotinylated products of this reaction were visualised by a fluorescent-conjugated streptavidin complex (5% streptavidin-Cy3, 60 minutes, 1:600; Sigma, St. Louis, MO, USA). DAPI (Boehringer Mannheim, Mannheim, Germany) was used for counterstaining the nuclei.

2.3.4 Tyramide signal amplification (TSA) for calmegin detection

Since by both microarray assay and qRT PCR analysis, transcription of the calmegin gene was found to be down-regulated in human HFs after EPO treatment, it was our objective to determine whether this was also the case at protein level and, furthermore, where in the human HFs calmegin protein is expressed.

To clarify this question, we used the highly sensitive TSA staining method (TSA kit, Perkin Elmer, Boston, USA) on untreated skin and cultured human HFs (Ito et al., 2004b; Bodo et al., 2005; Lu et al., 2009).

Using several washing steps with Tris-NaCl-Tween (TNT) buffer (0.1 mol/l Tris-HCl (pH 7.5), 0.15 mol/l NaCl and 0.05% Tween 20) in between, after fixation in acetone (10 minutes, -20 °C) endogenous peroxidase activity was quenched with H_2O_2 (3% in PBS, 15 minutes, room temperature).Then the slides were pretreated with avidin and biotin (both 15 minutes; Vector Laboratories, Burlingame, CA, USA). After 30 minutes preincubation with normal rabbit serum (5% in TNB; Jackson ImmunoResearch, West Grove, PA, USA) the samples were incubated by the primary antibody calmegin (sc-49899, Santa Cruz Biotechnology, Santa Cruz, California, USA) at a dilution of 1:1000 in TNB (Tris-buffered saline with 0.5% casein) supplemented with 2% normal rabbit serum (overnight, 4 °C). The next day, a secondary biotinylated rabbit anti-goat antibody (E0466, DAKO, Glostrup, Denmark) was applied to the slides at a dilution of 1:200 in TNB supplemented with 2% normal rabbit serum (45 minutes, room temperature). Next, the streptavidin conjugated horseradish peroxidase (TSA kit, Perkin Elmer, Boston, USA) diluted 1:100 in TNB was applied. Amplification of this reaction was achieved by applying FITC-tyramide amplification reagent (1:50 in amplification diluents (provided in TSA kit)). Counterstaining was performed with DAPI (Boehringer Mannheim, Mannheim, Germany) and mounting with Fluoromount (Southern Biotechnologies, Birmingham, USA). Murine testis sections were used as positive control (Yoshinaga et al., 1999), and for negative control the primary antibody was omitted.

2.3.5 Immunofluorescence staining for NKI/beteb

Since especially for the assessment of the melanocyte-dendrite ratio it was often quite difficult to distinguish properly between melanocytes and keratinocytes, we decided to perform an additional, highly specific melanocyte staining, namely NKI/beteb (Adema et al., 1993). The antigen recognised by the NKI/beteb antibody is the “silver protein” (gp100), which is important for the anchoring of melanin polymers to the melanosome matrix and is thus recognised as the key premelanosomal marker (Vancoillie et al., 1999; Singh et al., 2008). NKI/beteb stains not only the melanocytes in the epidermis and in the hair bulb but also the “dormant”, amelanotic HF melanocytes in the ORS as well as other relatively undifferentiated HF populations (Oliveira and Junior, 2003; Singh et al., 2008).

The technique used was an immunofluorescence staining. Several washing steps in between were done with PBS. After 10 minutes air drying and fixation in 4% paraformaldehyde (30 minutes, room temperature), the cryosections were preincubated with normal goat serum (5% in PBS, 60 minutes). Then incubation with the primary antibody mouse-anti NKI/beteb (Monosan, MON7006-1, Uden, The Netherlands) diluted 1:20 in 0.3% Tritonx, and PBS supplemented with 2% normal goat serum followed. The next day, the secondary antibody, a rhodamine-labelled goat anti-mouse antibody (A-11030, Alexa Flour), was applied in a dilution of 1:200 in 0.3% TritonX, PBS and 2% normal goat serum for 90 minutes. Counterstaining was achieved with DAPI (Boehringer Mannheim, Mannheim, Germany) and mounting with Fluoromount (Southern Biotechnologies, Birmingham, USA).

For negative control, the primary antibody was omitted. The detection of HF melanocytes with their characteristic dendritic morphology and location served as an optimal positive control (Singh et al., 2008).

2.3.6 Chloroacetate esterase reaction (Leder esterase histochemistry)

The chloroacetate esterase reaction is a routine histochemical staining to detect mature mast cells (Leder, 1964, 1970; Wong et al., 1982). However, it is not specific for mast cells, also neutrophils and monocytes are stained (Leder, 1979), but in the CTS of HFs one expects the positive cells to be mast cells as neutrophil granulocytes and monocytes are usually absent in this mesenchymal skin compartment.

Cryosections of cultured human HFs were incubated in distilled water for 5 minutes, and then treated with incubating medium for 40 minutes. The latter consists of 10 mg napthol-ASD chloroacetate, 1 ml of N,N-dimethylformamide, 35 ml Sorenson's working buffer and 0.2 ml of nitrosylated pararosaniline (0.1 ml of 4% sodiumnitrite in aqua plus 0.1 ml of pararosaniline solution). After washing in running tap water, counterstaining was achieved with light green (1 minute) that mainly stains collagen. Another washing step followed, then the slides were dried (5 minutes), cleared in xylol substitute (Merck, Darmstadt, Germany) and mounted with synthetic resin (Eukitt, O. Kindler GmbH & Co, Freiburg, Germany).
This staining protocol was obtained from the website "stainfile" and newly introduced into the laboratory[4].

2.4 Histomorphometry

2.4.1 Quantitative analysis with densitometry

For both melanin staining (Masson-Fontana) and immunofluorescence staining for calmegin, the intensity of the staining was quantified using the NIH/Image software (Bethesda, Maryland, USA), as previously described (see **Fig. 15**) (Ito et al., 2004b; van Beek et al., 2008). For pigmentation intensity, the pictures of the Masson-Fontana stained HFs were transformed into black-and-white 8 bit pictures and previously defined areas of interest were measured (Ito et al., 2005b). The fluorescence pictures were measured in the colour mode. The photos of all the

[4] StainsFile Leder Esterase for mast cells. URL: http://stainsfile.info/StainsFile/stain/cell/leder.htm *[date: 19.06.2011]*

samples of one experiment were taken with the same exposure time and the same magnification. In every sample, defined areas were measured leading to a mean value.

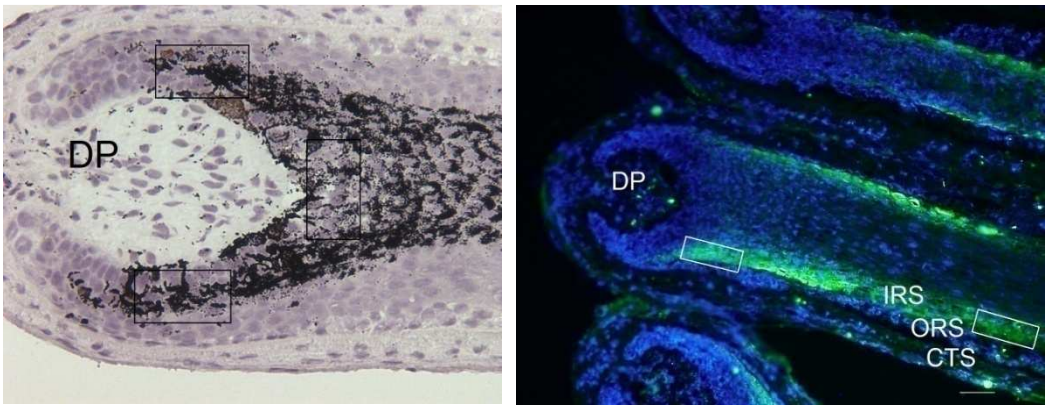
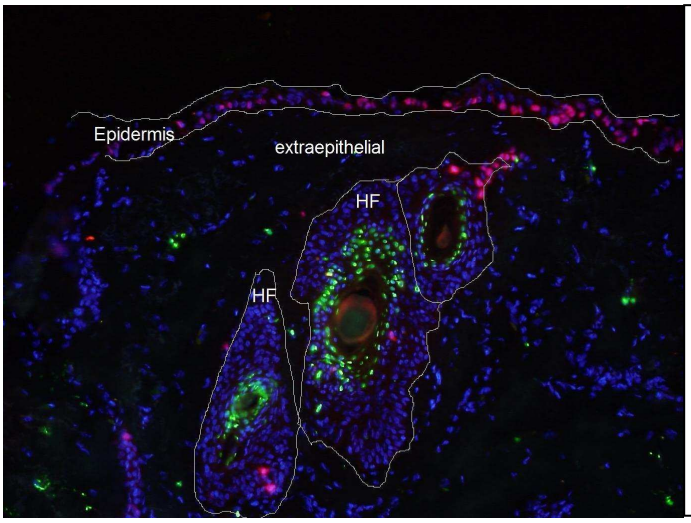


Figure 15. Assessed reference areas for Masson-Fontana histomorphometry (left) and calmegin immunohistomorphometry (right)
DP: dermal papilla, IRS: inner root sheath, ORS: outer root sheath, CTS: connective tissue sheath.

2.4.2 Assessment of apoptotic / proliferating cells

By employing previously described quantitative immunomorphometrical techniques, the number of apoptotic (TUNEL) and proliferating (Ki-67) cells could be analysed in treated and untreated Ki-67 / TUNEL stained human skin biopsies (Foitzik et al., 2006; Bodo et al., 2007a; Bodo et al., 2007b).



TUNEL cells (green) and Ki-67 cells (red) were assessed in three different skin compartments in relation to the total cell amount:

- **A)** basal layer of epidermis
- **B)** ORS and IRS of HFs
- **C)** extraepithelial (=dermis)

Figure 16. Reference regions for Ki-67 / TUNEL immunohistomorphometry

All apoptotic and proliferating cells were counted in three different compartments of the skin, namely in the basal layer of the epidermis, in the ORS and IRS of HFs and in dermal extraepithelial parts (extraepidermal, extrafollicular) as indicated in **Fig. 16**. Since the edges of the full-thickness skin punch biopsies were often not intact due to the punching process, in every sample the outermost parts were not included in the analysis. The numbers of Ki-67 and TUNEL positive cells are indicated as percentage to the total number of DAPI positive cells in these compartments (mainly keratinocytes and melanocytes or in the extraepithelial parts fibroblasts and mast cells).

2.4.3 Assessment of mast cell degranulation

Using a 200 fold magnification, Leder esterase-stained mast cells in the CTS of human HFs were counted using three categories: not degranulated, moderately degranulated (less than 50% of the granules released from the cell) and extensively degranulated (more than 50% of the granules released), as described previously (Siebenhaar et al., 2007a).

2.4.4 Assessment of number and dendricity of melanocytes

First, the number of melanocytes and the number of dendrites per melanocyte were counted in Masson-Fontana stained EPO- or vehicle-treated human HFs (magnification 600x). For confirmation, the same was repeated in NKI/beteb stained HFs.

2.4.5 Microscopy

For microscopical investigation of the specimens, the all-in-one type microscope Keyence Biozero 8000 was used for both fluorescence and light microscopy (Keyence, Osaka, Japan). This portable fluorescence microscope allows to produce easily high-quality images without requiring a darkroom and facilitates digital image analysis.

2.5 Statistical analysis

For statistical analysis of the HFs, the data of the EPO-treated group was compared with those of the vehicle-treated group. For human skin biopsies, the EPO-, 4-HC-, and 4-HC + EPO-treated groups were compared to the vehicle group. Additionally, the 4-HC + EPO-treated group was compared to the 4-HC-treated groups. In all experiments, the mean and the standard error of the mean (SEM) were calculated. To evaluate statistical relevance, p-values were assessed by using the Mann-Whitney-U-test for unpaired samples when comparing two groups. When more than two groups were compared, the Kruskal-Wallis-test was applied (SPSS Inc., Chicago, USA). Significance level α was set 0.05 and not adjusted.

Statistical analysis for the degranulation of mast cells was performed with the chi-square test (χ^2-test) as described previously (Maurer et al., 2004; Siebenhaar et al., 2007b).

The $\Delta\Delta C_T$ method was used to show the relative gene expression changes in EPO-treated HFs compared to the expression in vehicle-treated HFs as assessed by qRT PCR (Livak and Schmittgen, 2001).

3 Results

3.1 Selected microarray results were confirmed by qRT PCR analysis

Out of the previously published microarray data (compare **Fig. 11 and Table 2**) from the laboratory where this thesis project was executed (Bodo et al., 2007a) we selected the three most interesting EPO-target candidate genes: haemoglobin alpha 1, kinesin light chain 3 and calmegin. For these genes, the previous microarray results were confirmed by qRT PCR in an independent third patient so as to simultaneously confirm the microarray results by qRT PCR and to check how reproducible these gene expression results are between HFs from different individuals. Haemoglobin alpha 1 and calmegin were chosen as we wished to further confirm the new finding of their expression in human HFs (Bodo et al., 2007a). We also opted for kinesin light chain 3, a molecule that connects EPO to one of the HF's key properties – i.e. pigmentation – through involvement of kinesin in melanosome transport (Hara et al., 2000). Additionally, GATA-1 and -2 were chosen because of their known involvement in EPO biology (Jelkmann, 2007a).

Using the TaqMan® Gene Expression Analysis technology for qRT PCR, mRNA levels for these target genes could be measured involving the $\Delta\Delta C_T$ method as described earlier (Livak and Schmittgen, 2001). The relative expression for all the target genes was calculated by this method and is shown in the chart below (**Fig. 17**). As shown in **Fig. 17**, qRT PCR performed on HF RNA which was extracted after 6 hours of EPO or vehicle treatment confirmed that haemoglobin alpha 1 (HBA1) and kinesin light chain 3 (KLC3) were up-regulated in EPO-treated HFs. In contrast, calmegin (CLGN) was massively down-regulated (it could not even be measured anymore) after EPO treatment. Additionally, here we show that the zinc finger containing transcription factor GATA-2 is expressed in human HFs and that EPO increases its transcription. However, GATA-1, which is an important transcription factor during erythropoiesis (Jelkmann, 2007a), is not expressed at all in human HFs.

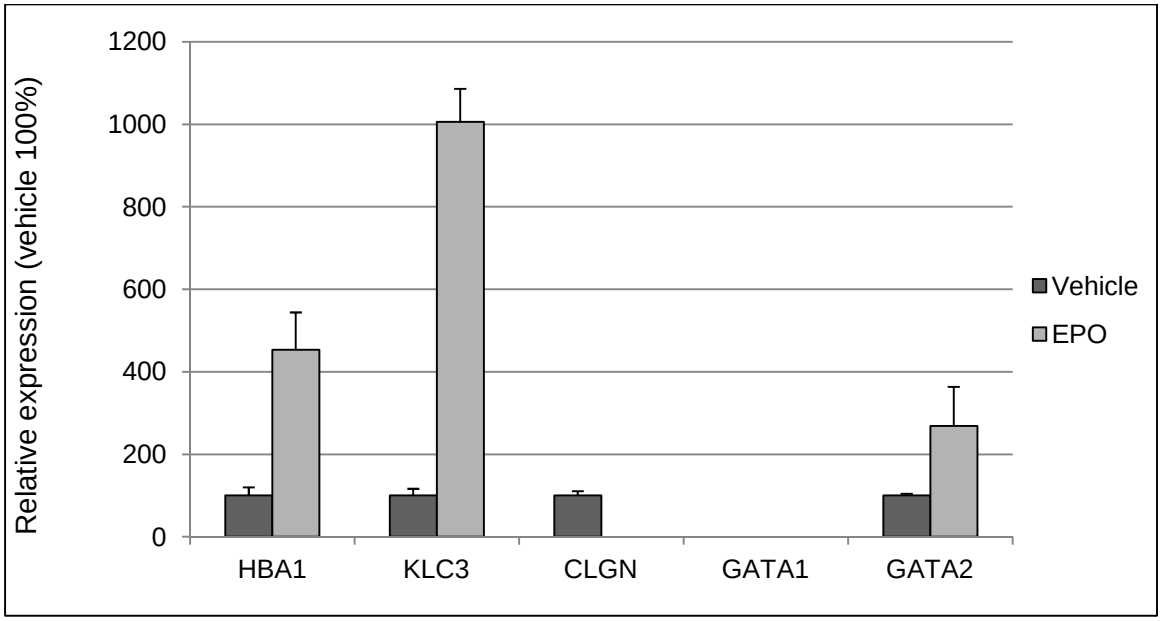


Figure 17. Relative gene expression changes of 5 selected genes in HFs
HFs were organ-cultured with 100 mU/ml EPO or vehicle for 6 h (N = 20 HFs per group). Gene expression levels of haemoglobin alpha 1 (HBA1), kinesin light chain 3 (KLC3), calmegin (CLGN), GATA-1 and GATA-2 of HFs of both groups were compared to each other via qRT PCR. Using the $\Delta\Delta C_T$ method, the relative gene expression changes in EPO-treated HFs are shown here compared to the expression in vehicle-treated HFs which is set 100%; Error bars: SEM.

3.2 Calmegin is expressed in the IRS of human HFs

Next, we attempted to follow up the protein *in situ*-expression for one of these qRT PCR-confirmed genes that are differentially regulated by EPO in human HFs. For this, calmegin was selected because it was our aim to verify our novel finding of calmegin expression in human scalp HFs. Moreover, calmegin might be a new interesting target gene of EPO as its gene expression is substantially altered under EPO treatment (compare **Fig. 11 and 17**).

First, to examine whether human skin expresses calmegin protein at all, which had previously been detected only in endoplasmatic reticulum of male germ cells (Yoshinaga et al., 1999; van Lith et al., 2007), we immunostained untreated full-thickness scalp skin sections of eight different patients via the sensitive TSA method. As a positive control, we used murine testis (the employed antibody recognises both murine and human calmegin [Santa Cruz datasheet nr. sc-49899]), where calmegin is expressed during the second half of

spermatocytogenesis and the beginning of spermiogenesis (i.e. from the pachytene stage of primary meiosis to the early maturation stage of the spermatids) (Yoshinaga et al., 1999). These processes take place in the seminiferous tubules of the testis, with the germ cells differentiating from the outside to the inside of the lumen, where the fully developed sperm can be found (van Lith et al., 2007). Consequently, as the early stages of spermatocytogenesis and the late stages of spermiogenesis do not show calmegin expression, one would expect that the innermost and the outermost cells of the seminiferous tubules are not stained with the calmegin antibody. This is exactly the case, as can be seen in **Fig. 18**, while the negative control showed no specific immunoreactivity above some minor background staining (not shown).

Having thus convincing positive and negative control evidence for a working calmegin immunofluorescence protocol, we went on to analyse calmegin protein expression in human skin sections. This revealed prominent specific immunoreactivity in the IRS of human scalp HFs in anagen VI (**Fig. 19 and 20**). It could not be determined with certainty whether or not the occasional, much less prominent extrafollicular immunoreactivity observed outside the HF (see **Fig. 19, right side**), which was interpreted as a tissue handling-related artefact, was truly specific.

To the best of our knowledge, this is the first time that the – supposedly testis-specific (Watanabe et al., 1994; van Lith et al., 2007; Muro and Okabe, 2010) chaperone molecule calmegin has been identified on both the gene and protein level in mammalian skin, namely in the IRS of its HFs.

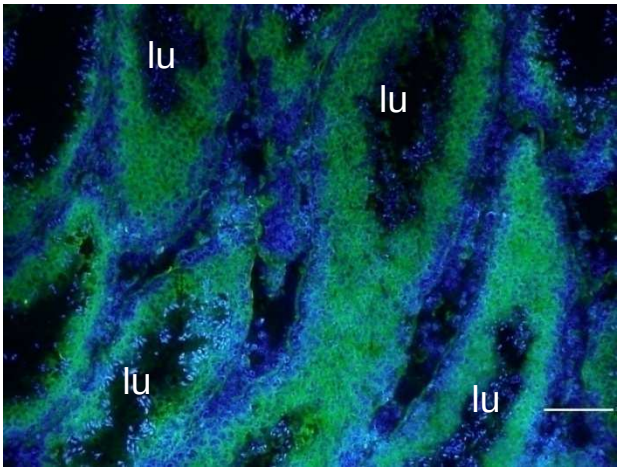


Figure 18. Murine testis as a positive control for calmegin immunoreactivity
TSA immunohistochemistry; blue: DAPI-counterstaining; green: calmegin; scale bar = 100 µm; lu: lumen of the seminiferous tubule with maturing sperm.

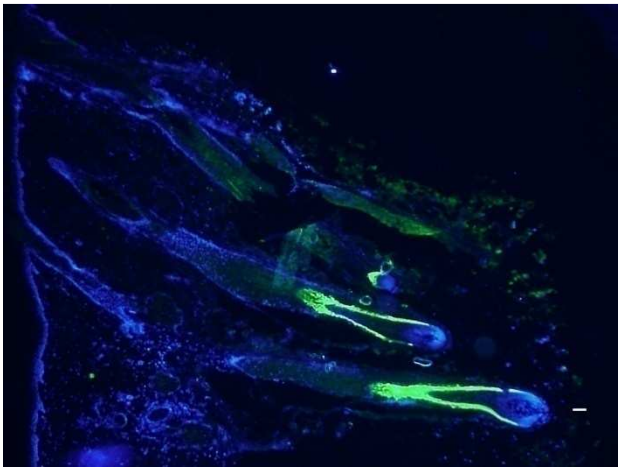
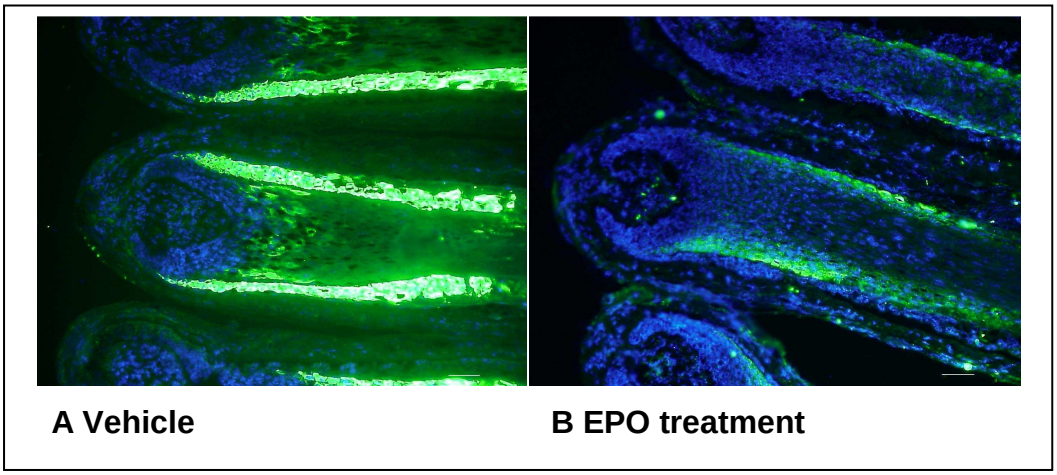


Figure 19. Calmegin protein-related immunoreactivity in the IRS of human HFs
Untreated full-thickness skin; TSA immunohistochemistry; blue: DAPI-counterstaining; green: calmegin; scale bar=100 µm; magnification 20x.

3.2.1 Calmegin intensity decreased after EPO treatment

After having confirmed the presence of calmegin in HFs, we wanted to examine whether we can verify the decrease of calmegin on the protein level after EPO treatment, as microarray and qRT PCR results had shown on the mRNA level. To achieve this, we used the TSA staining method. In three different experiments we used 14-22 EPO- and vehicle-treated organ-cultured anagen HFs. Measuring the intensity of calmegin staining in the IRS of the HF revealed a significant decrease of calmegin expression after EPO treatment (compare **Fig. 20**).



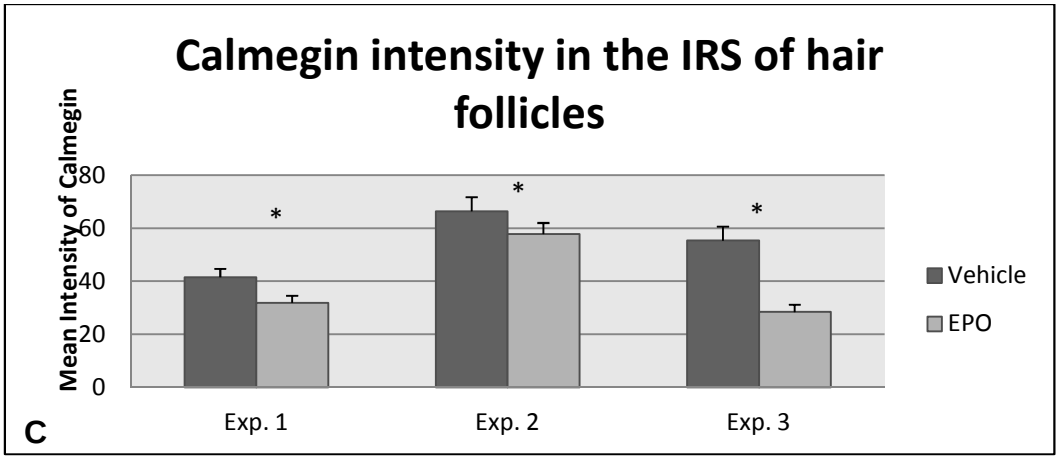


Figure 20. EPO reduced follicular calmegin immunoreactivity
TSA immunohistochemistry of **A)** vehicle- and **B)** EPO-treated HFs (100 mU/ml, 5 days). **C)** Quantification of calmegin intensity; N = 14-22 HFs per group; *p<0.05 EPO compared to vehicle; error bars: SEM; scale bars = 50 µm.

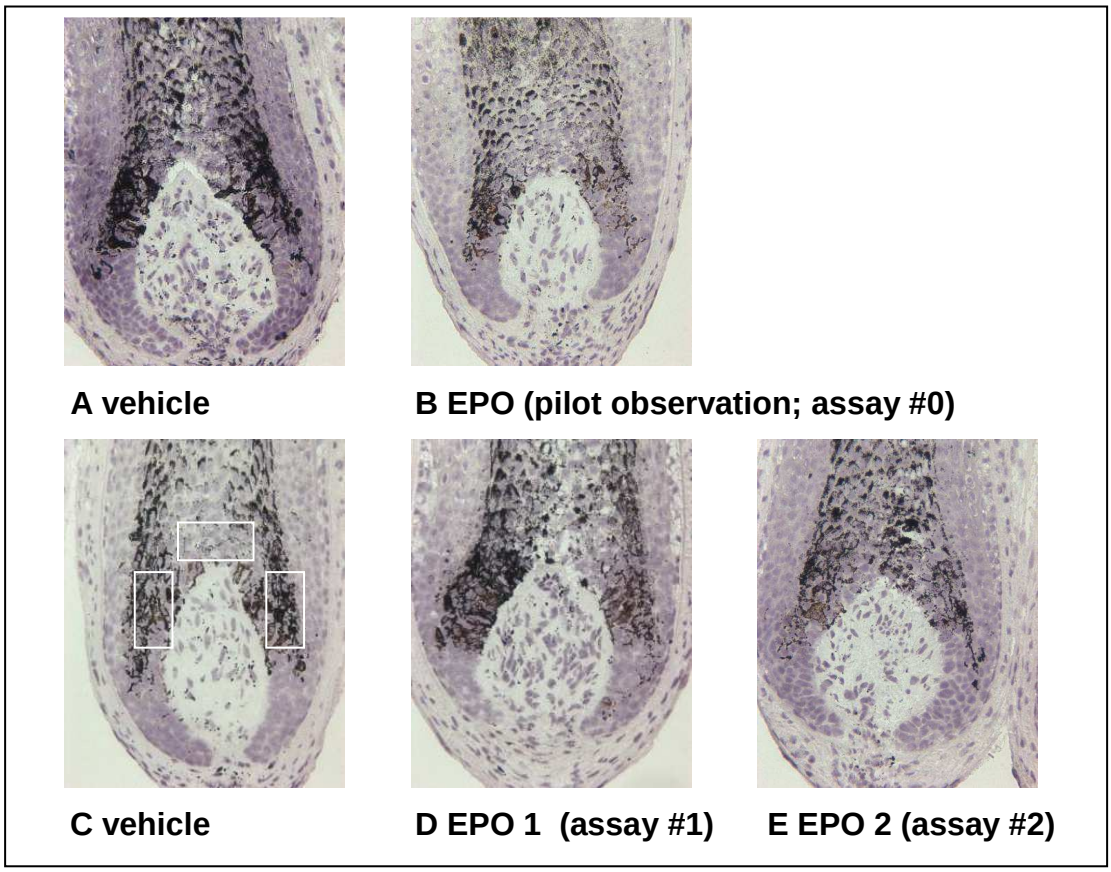
3.3 EPO is a new pigmentation-modulatory hormone in the HFs of some, but not all human individuals

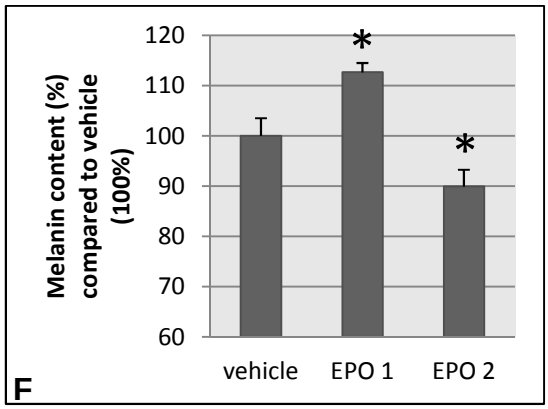
While studying the effects of EPO on microdissected human scalp hair, we noticed that in one male donor the melanin content in EPO-treated HFs (5 days, 100 IU/ml EPO) was significantly lower than in the control group (assay #0) as assessed by quantitative Masson-Fontana histochemistry. In contrast, previous HF microarray results from our laboratory (see chapter 1.6 and **Fig. 11**) had shown that kinesin light chain 3, a molecule that has been implicated as an important regulator of pigmentation (Vancoillie et al., 2000), was up-regulated after EPO administration (Bodo et al., 2007a).

Therefore, we decided to look more closely at EPO-influenced alterations in HF pigmentation using the Philpott system of organ-cultured, microdissected and amputated anagen hair bulbs, which has proved to be an excellent tool for investigating recognised and novel regulators of human HF pigmentation (Kauser et al., 2004; Ito et al., 2005a; Kauser et al., 2005; van Beek et al., 2008). By quantitative Masson-Fontana histochemistry, we found that most HF assays showed a response to EPO stimulation (in total 4 out of 5). Unexpectedly, however, there were huge inter-individual differences in the response to EPO-stimulation (see **Fig. 21** and **Table 5**).

A significantly reduced melanin content of proximal anagen hair bulbs was seen in the HFs of two patients (assay #0 and #2, shown as EPO 2), while a significant increase of the histochemically detected HF melanin content was seen in the HFs of two other donors (assay #1 and #3, shown as EPO 1). One patient did not respond at all (assay #4) (compare **Fig. 21**).

Unfortunately, due to the painfully limited number of human scalp skin samples that routinely are available for study, these different effects could not be examined in a larger cohort of donors. Therefore, no sufficiently reliable statements can be made on the relative distribution of responders versus non-responders and of individuals whose HF pigmentation is stimulated versus those whose melanin production is inhibited by EPO – other than that there is massive inter-individual variation in the response to the HF pigmentary unit to stimulation by EPO under assay conditions. Analysis of gender- , location- or age-dependent differences between the studied HFs also failed to reveal a consistent pattern within the very small cohort of donors that was available for study (see **Table 5**).





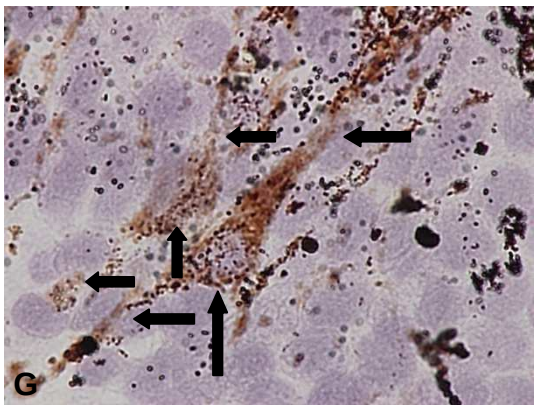


Figure 21. Melanin content after EPO treatment was both in- and decreased
Masson-Fontana histochemistry; **A)** and **B)** The pilot observation (assay #0, see also **Table 5**) showed strong decrease of melanin pigment after EPO treatment. **C-E)** Repeat experiments showed both increase (assay #1 and #3, shown as EPO 1) **D)** and decrease (assay #0 and #2, shown as EPO 2) **E)** of melanin content after EPO treatment (100 mU/ml). White squares indicate measure region. **F)** Quantification of these melanin content changes; for better comparison, the pigmentation intensity of test HFs is shown compared to vehicle = 100%. Data from assay #1 and #3 and from assay #0 and #2 were pooled. N = 29-31 HFs per group; * $p<0.05$; error bars: SEM. **G)** High power magnification (1200x) of melanocytes ↑ with dendrites ←.

Assay number	Origin of skin region	Gender of skin donor	Age of skin donor	Length of HF culture	EPO effect on melanin content
#0	frontotemporal	male	59 years	5 days	decreased
#1	frontotemporal	female	62 years	9 days	increased
#2	occipital	female	60 years	5 days	decreased
#3	frontotemporal	male	68 years	5 days	increased
#4	frontotemporal	male	54 years	5 days	unchanged

Table 5 Properties of hair follicles used for experiments on pigmentation
Assay #0 = pilot observation. Assay #1 and #3 are pooled to EPO 1 and assay #0 and #2 are pooled to EPO 2.

Contradictory effects of EPO treatment were also shown with respect to tyrosinase activity *in situ*, which could be measured in the HFs of only two donors (since HF sections from the other three donors had been fully consumed for the additional analyses reported below): As shown in **Fig. 22**, EPO treatment induced higher tyrosinase activity *in situ* in the HFs of one of the donors which had also shown increased histochemically detected melanin content. In another donor, who had failed to respond to EPO stimulation by Masson-Fontana-detectable changes in HF pigmentation (assay #4), there was also no effect on the tyrosinase activity-related immunoreactivity by this enzyme histochemistry method (not shown).

Next, we counted the number of melanocytes and the amount of dendrites of each demarcated melanocyte (which reflects melanosome transfer activity (Singh et al., 2008)) in both Masson-Fontana-stained sections and NKI/beteb-immunostained slides, as the latter technique is the most sensitive currently available standard method for detecting even immature, amelanotic HF melanocytes (Kauser et al., 2004, 2005; Singh et al., 2008; Tobin, 2008).
At 600x magnification, the melanocytes and their dendrites were counted in the precortical matrix above Auber's line. However, as shown in **Fig. 23** EPO administration neither significantly altered the amount of melanocytes nor the number of dendrites per melanocyte.

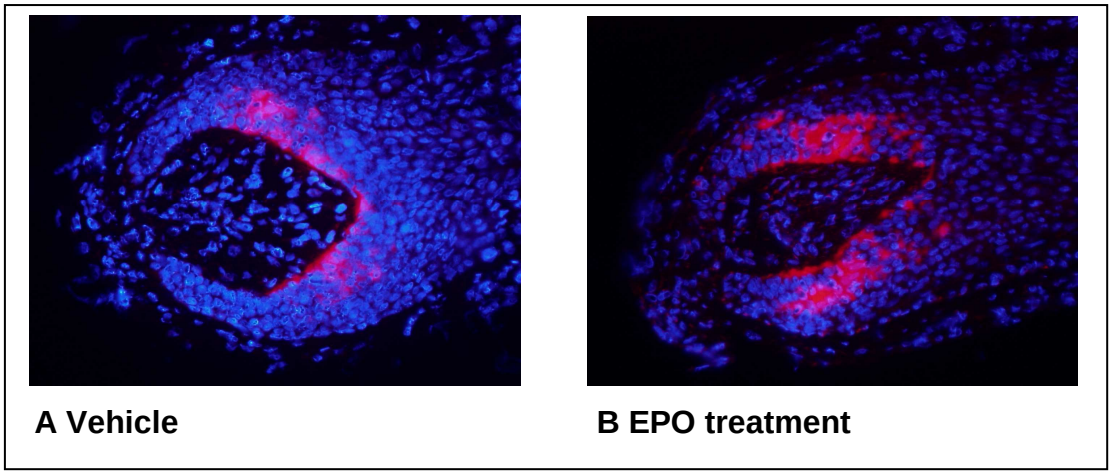
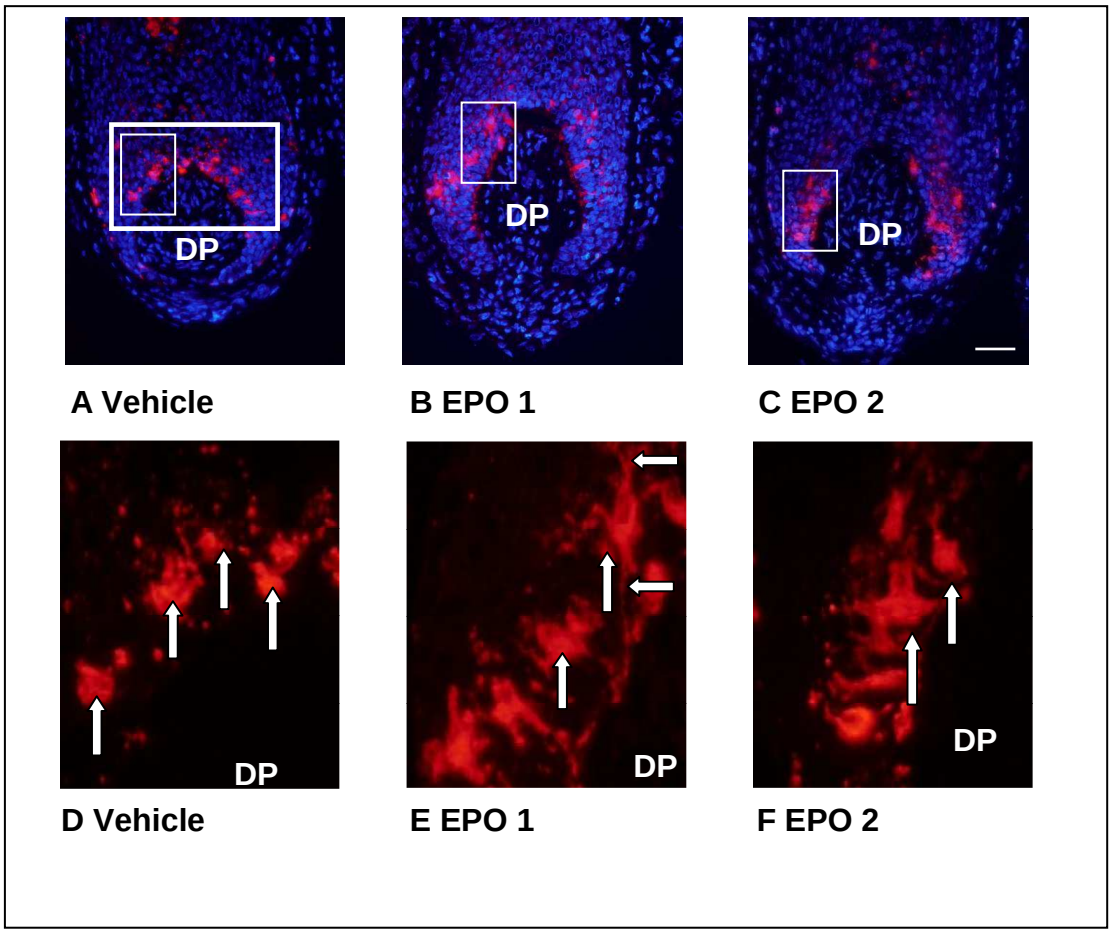


Figure 22. EPO treatment induced higher tyrosinase activity *in situ* in the HFs of one of the donors in which also histochemically higher melanin content was detected
Tyramide-based tyrosinase activity in situ assay of a **A)** vehicle-treated HF and **B)** EPO-treated HF (100 mU/ml). N = 6-9 HFs per group.



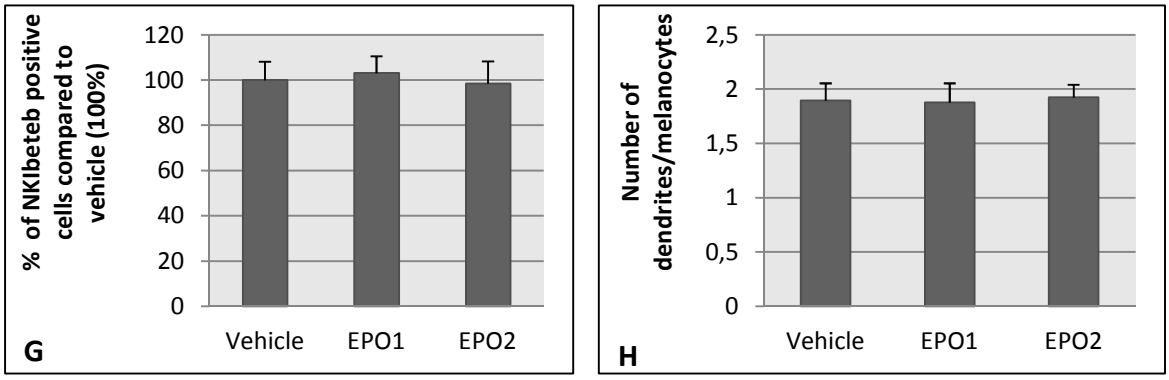


Figure 23. Melanocyte number and number of dendrites per melanocyte did not alter after EPO treatment

A)-F) Representative immunofluorescence NKI/beteb stained sections showed that melanocyte number and melanocyte dendricity neither changed in the two donors who responded to EPO with higher melanin content (shown as EPO 1) nor in the two donors who responded with lower melanin content (shown as EPO 2).

D)-F) Melanocytes of the small rectangle above are shown without counterstaining; ⇧ melanocytes; ⇦ melanocyte dendrites; DP dermal papilla; scale bar 50 µm.

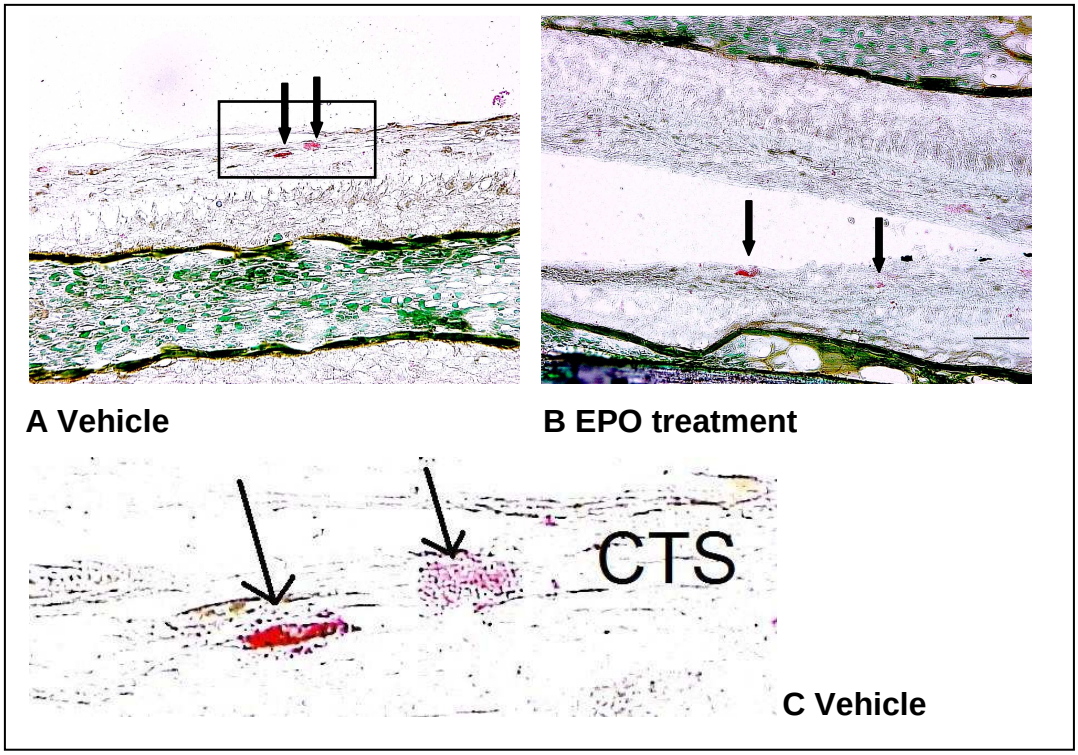
G)-H) Only NKI/beteb quantitative results are shown here, as results in Masson-Fontana stained slides were the same. Melanocytes and their dendrites were counted in the labelled region (large square in **A)**). Data from assay #1 and #3 were pooled to EPO 1 and data from assay #0 and #2 were pooled to EPO 2. N = 14-23 HFs per group; $p>0.05$; values in % and adjusted to one vehicle value; error bars: SEM.

3.4 EPO did not change the number and degranulation status of CTS mast cells

Since EPO has been proposed to play a role in mast cell biology (see chapter 1.6), we also examined the effect of EPO on human skin mast cells *in situ*. Mast cells can be easily visualised histochemically. Furthermore, they can be manipulated pharmacologically within their natural mesenchymal tissue habitat in the connective tissue sheath (CTS) of microdissected, organ-cultured human scalp HFs (Peters et al., 2007; Ito et al., 2010) (see **Fig. 24**).

In the 133 HFs analysed after 5 days of organ culture a total number of 576 mast cells was detected in the vehicle group while 577 mast cells were histochemically demarcated in the EPO-treated group. Therefore, EPO had no effect on the total number of mature, Leder esterase-demarcated human CTS mast cells *in situ.* Next, it was analysed whether EPO treatment altered the histochemically assessable state of mast cell activation, as evidenced by their granulation/degranulation status. As shown in **Fig. 24**, EPO treatment did not

induce any significant decrease or increase in the degranulation of human CTS mast cells *in situ*.



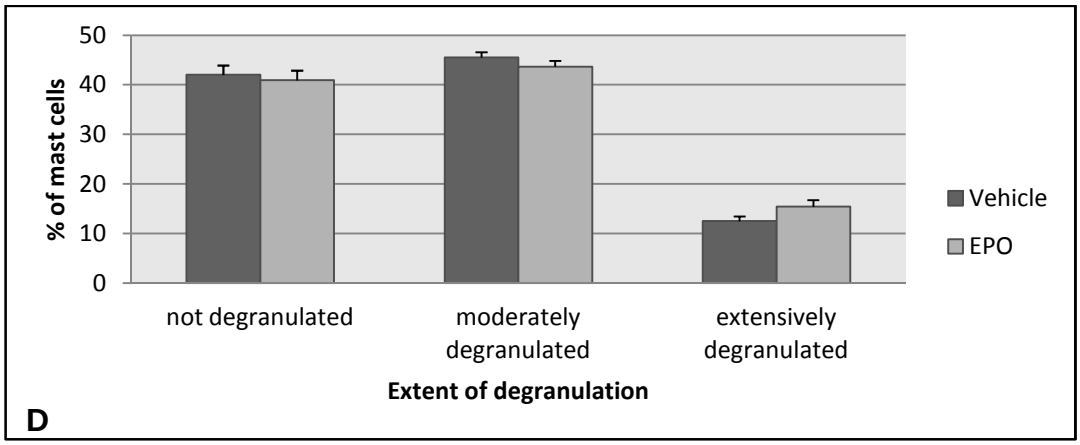


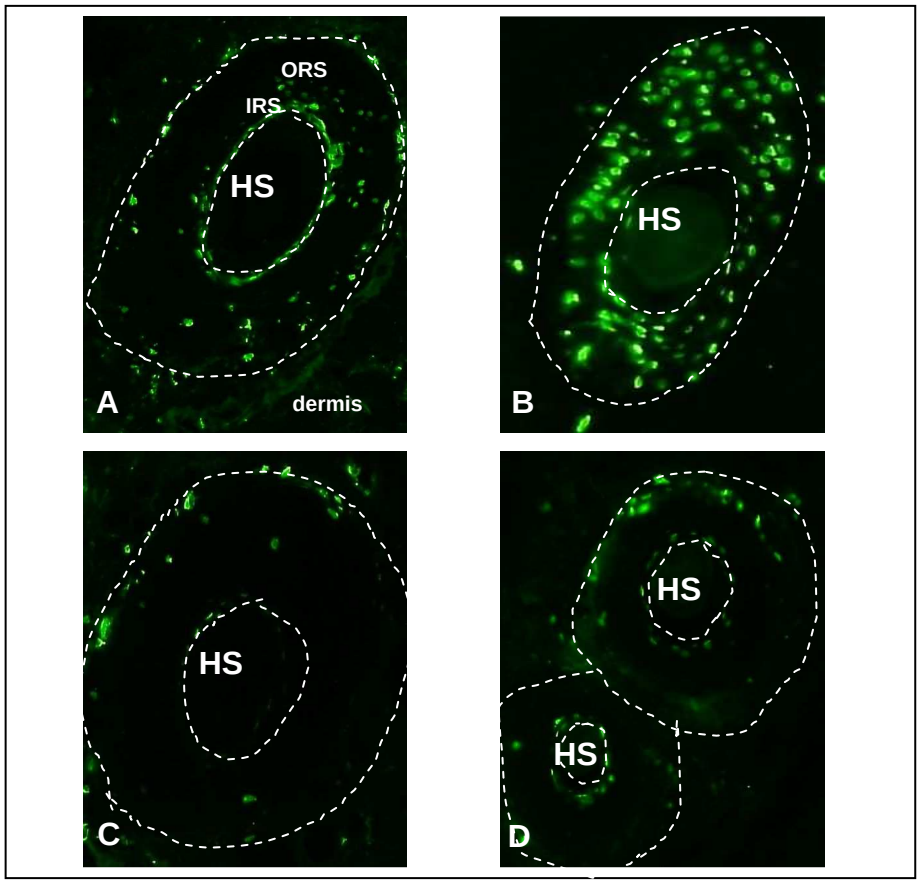
Figure 24. EPO treatment did not alter mast cell degranulation in the CTS of cultured HFs
A-C) Follicular mast cells ↓ in a **A)** vehicle-treated section and in a **B)** EPO-treated section. **C)** high power magnification of a non-degranulated and degranulated mast cell of the labeled region in **A)**. **D)** Quantification of mast cell degranulation; data were pooled from 3 independent experiments. N = 64-69 HFs per group, $p>0.05$; mean in % of total mast cells; error bars: SEM; p values calculated using the χ^2-test.

3.5 EPO reduced apoptosis in chemotherapy-treated full-thickness skin

Next, we wished to clarify whether the apoptosis-inhibitory effects of EPO that had been observed after short-term EPO treatment of microdissected, amputated, and 4-HC-treated human anagen hair bulbs (Bodo et al., 2007a) also extended to full-length human HF growing within their natural tissue environment. The preliminary investigation had shown that a 48 hour incubation period of 4-HC only provokes a very subtle increase of apoptotic cells in the organ-cultured punch biopsies of human full-thickness scalp skin. Also here, EPO pre- and co-treatment resulted in a lower apoptosis rate. However, the number of apoptotic (TUNEL) cells was too low to reliably study a possible EPO-induced inhibition of apoptosis (Exp. 0, data not shown). For the next two experiments (Exp.1 and 2), we used a longer 4-HC treatment period of 7 days which induced clearer effects.

As expected, after 4-HC treatment, the number of apoptotic (TUNEL-positive) cells was significantly higher than in the vehicle-treated group in all examined skin compartments, i.e. in the IRS and ORS of HFs (**Fig. 25**), in the basal layer of the epidermis (**Fig. 26, A-E**) and in extraepithelial parts of the dermis (**Fig. 26, A-D, F**). Furthermore, in all compartments EPO pre- and co-administration significantly inhibited 4-HC-induced apoptosis (**Fig. 25 and 26**). Surprisingly, in the first experiment (Exp.1) EPO alone significantly induced apoptosis in the basal layer of the epidermis (**Fig. 26, E**).

The skin we used for Exp. 1 unfortunately displayed only few HFs and could therefore not be used to examine 4-HC and EPO effects in the HF compartment.



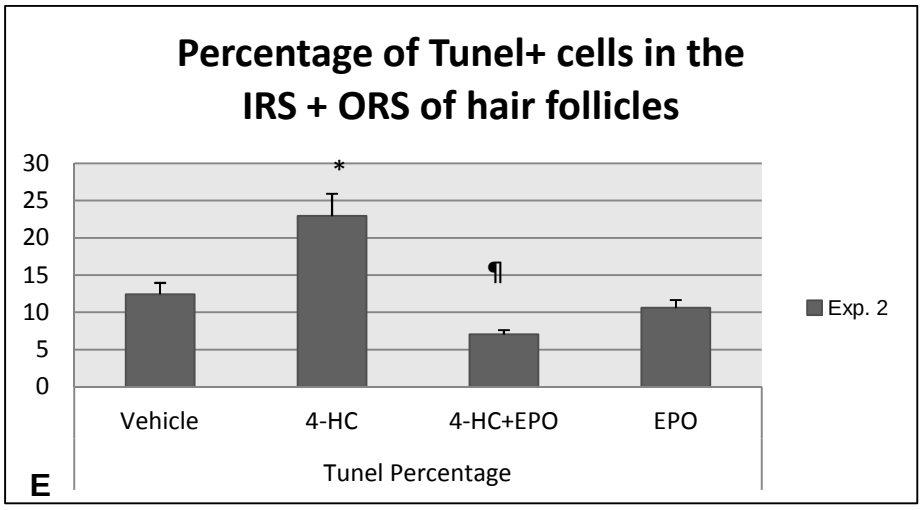


Figure 25. Apoptotic (TUNEL) cells in the IRS and ORS of HFs in human full-thickness scalp skin

Sections of punch biopsies of human full-thickness scalp skin that were organ-cultured for 9 days and treated for 7 days with **A)** vehicle, **B)** 4-HC (10 µmol/l), **C)** 4-HC + EPO and **D)** EPO (100 IU/ml) were double-stained with Ki-67 / TUNEL immunofluorescence staining and counterstained with DAPI. For better illustration, only TUNEL+ cells (green) are shown here. **E)** Percentage of apoptotic (TUNEL+) cells in relation to all DAPI + cells in the IRS and ORS of HFs. * $p<0.05$ compared to vehicle; ¶ $p<0.05$ compared to 4-HC; error bars: SEM; N = 31-76 HFs per group. Dotted lines in A-D illustrate the contours of HFs and the hair shaft (HS); IRS: inner root sheath; ORS: outer root sheath.

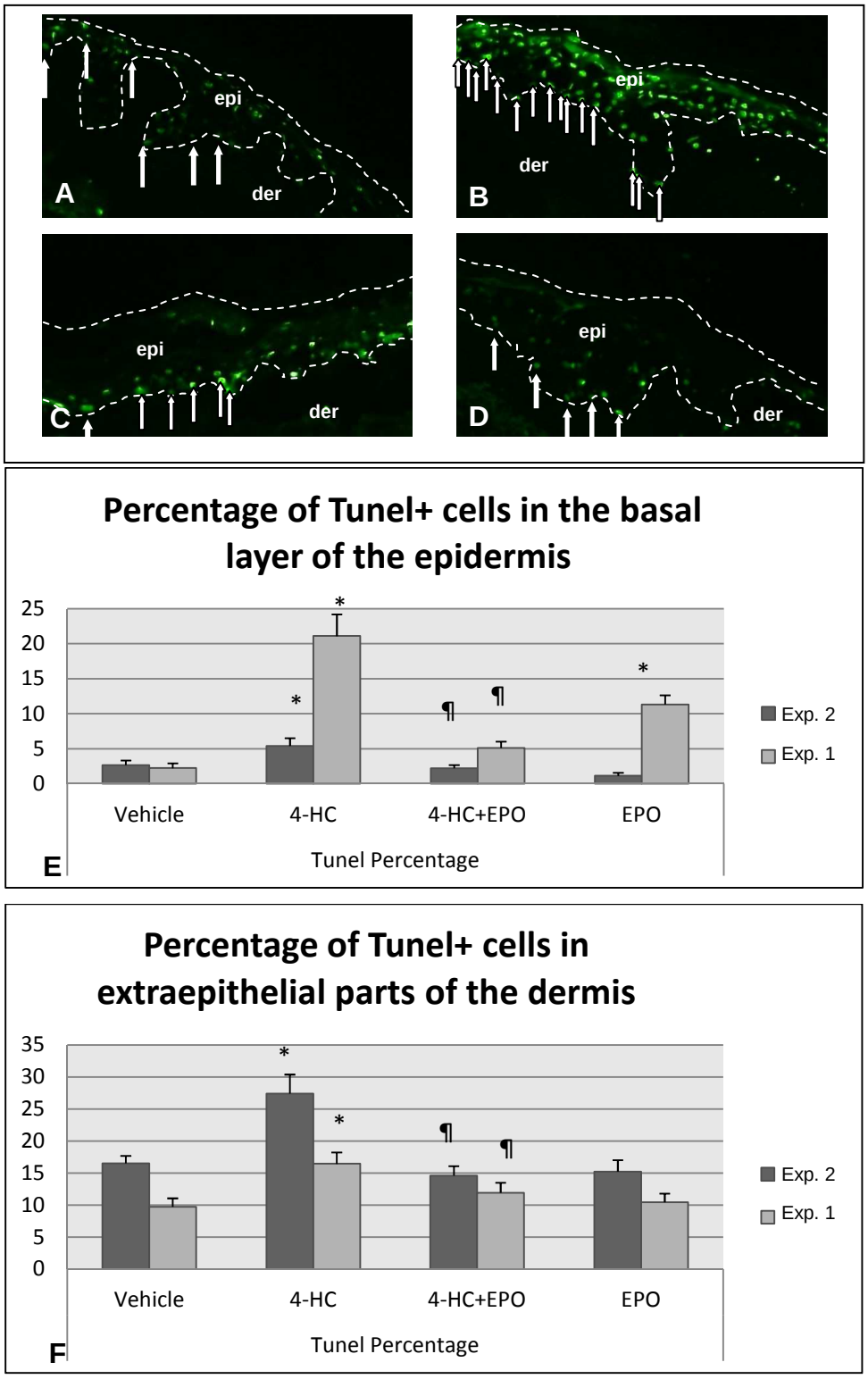


Figure 26. Apoptotic cells in the basal layer of the epidermis and the extraepithelial part of the dermis in human full-thickness scalp skin

Sections of punch biopsies of human full-thickness scalp skin that were organ-cultured for 9 days and treated for 7 days with with **A)** vehicle, **B)** 4-HC (10 µmol/l), **C)** 4-HC + EPO and **D)** EPO (100 IU/ml) were double-stained with Ki-67 / TUNEL and counter-stained with DAPI. For better illustration, only TUNEL cells (green) are shown here. **E)** Percentage of apoptotic (TUNEL+) cells in relation to all DAPI + cells in the basal layer of the epidermis and **F)** in the extraepithelial parts of the dermis. * $p<0.05$ compared to vehicle; ¶ $p<0.05$

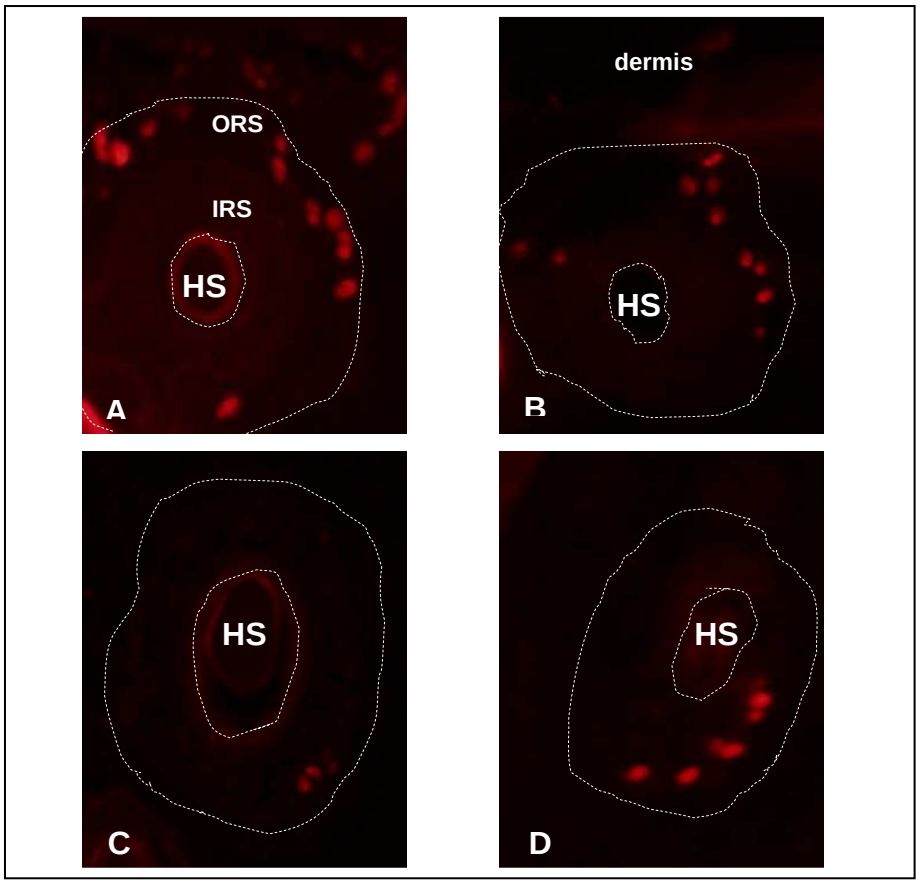
compared to 4-HC; error bars: SEM. Punch biopsies were received from two donors and cultured in two independent experiments (Exp. 1 and 2); N = 25-43 epidermal and dermal visual fields per group; dotted lines illustrate the contours of the epidermis, only TUNEL cells in the basal layer of the epidermis were counted as indicated by ⇧ ; epi: epidermis; der: dermis.

In conclusion, in line with its recognised anti-apoptotic properties, EPO tends to counteract chemotherapy-induced enhanced apoptosis in various compartments of human skin *in situ*, namely in the IRS and ORS of HFs, in the basal layer of the epidermis and in extraepithelial parts of the dermis.

3.6 EPO reduced proliferation in all skin compartments

Finally, we checked whether and how EPO alters proliferation in various human skin compartments in the presence and absence of chemotherapy, using the cyclophosphamide-derivative 4-HC as a recognised, potent inhibitor of human HF keratinocyte proliferation *in situ* (Bodo et al., 2007b).

Not only the proportion of apoptotic cells, but also the proportion of proliferating (Ki-67) cells was altered under EPO and/or 4-HC administration. In both experiments (Exp. 1 and 2) EPO displayed the tendency to lower (!) the number of proliferating cells compared to the vehicle-treated group in all compartments of the skin (see **Fig. 27, A, D, E and Fig. 28, A, D-F**). Similar to EPO administration, 4-HC treatment resulted in a lower proliferation rate in all compartments, too (see **Fig. 27, A, B, E and Fig. 28, A, B, D, F**). 4-HC/EPO co-treatment provoked a compartment-depending response: in the IRS and ORS of HFs and in the basal layer of the epidermis, which can be referred to as epithelial parts of skin, the number of proliferating (Ki-67) cells was lowered (see **Fig. 27 and 28, A, C, E)** but in extraepithelial parts (= dermis) 4-HC and EPO together increased the number of Ki-67 cells (compare **Fig. 28, A, C, F**).



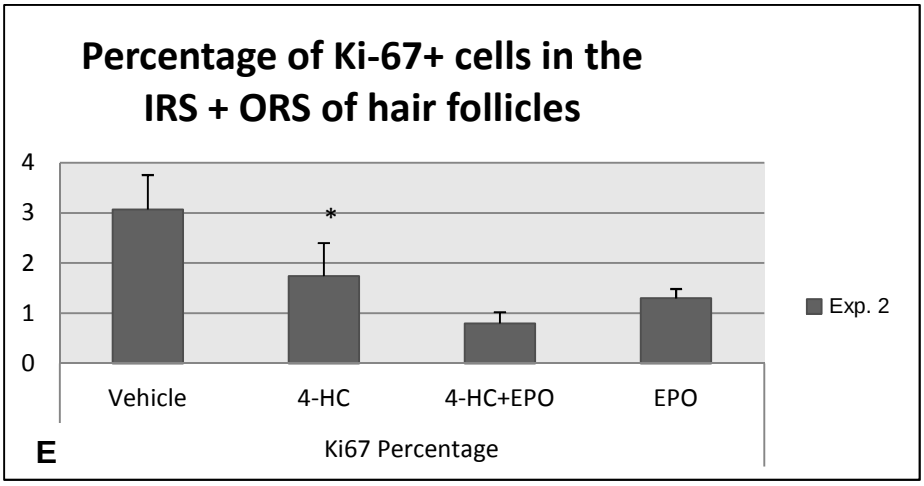


Figure 27. Proliferating (Ki-67+) cells in the IRS and ORS of HFs within skin
Sections of punch biopsies of human full-thickness scalp skin that were organ-cultured for 9 days and treated for 7 days with with **A)** vehicle, **B)** 4-HC (10 µmol/l), **C)** 4-HC + EPO and **D)** EPO (100 IU/ml) were double-stained with Ki-67 / TUNEL and counter-stained with DAPI. For better illustration, only Ki-67+ cells (red) are shown here. **E)** Percentage of proliferating (Ki-67) cells in relation to all DAPI + cells in the IRS and ORS of HFs.
* $p<0.05$ compared to vehicle; error bars: SEM; N = 31-76 HFs per group. Dotted lines illustrate the contours of the HFs and hair shafts (HS); IRS: inner root sheath; ORS: outer root sheath.

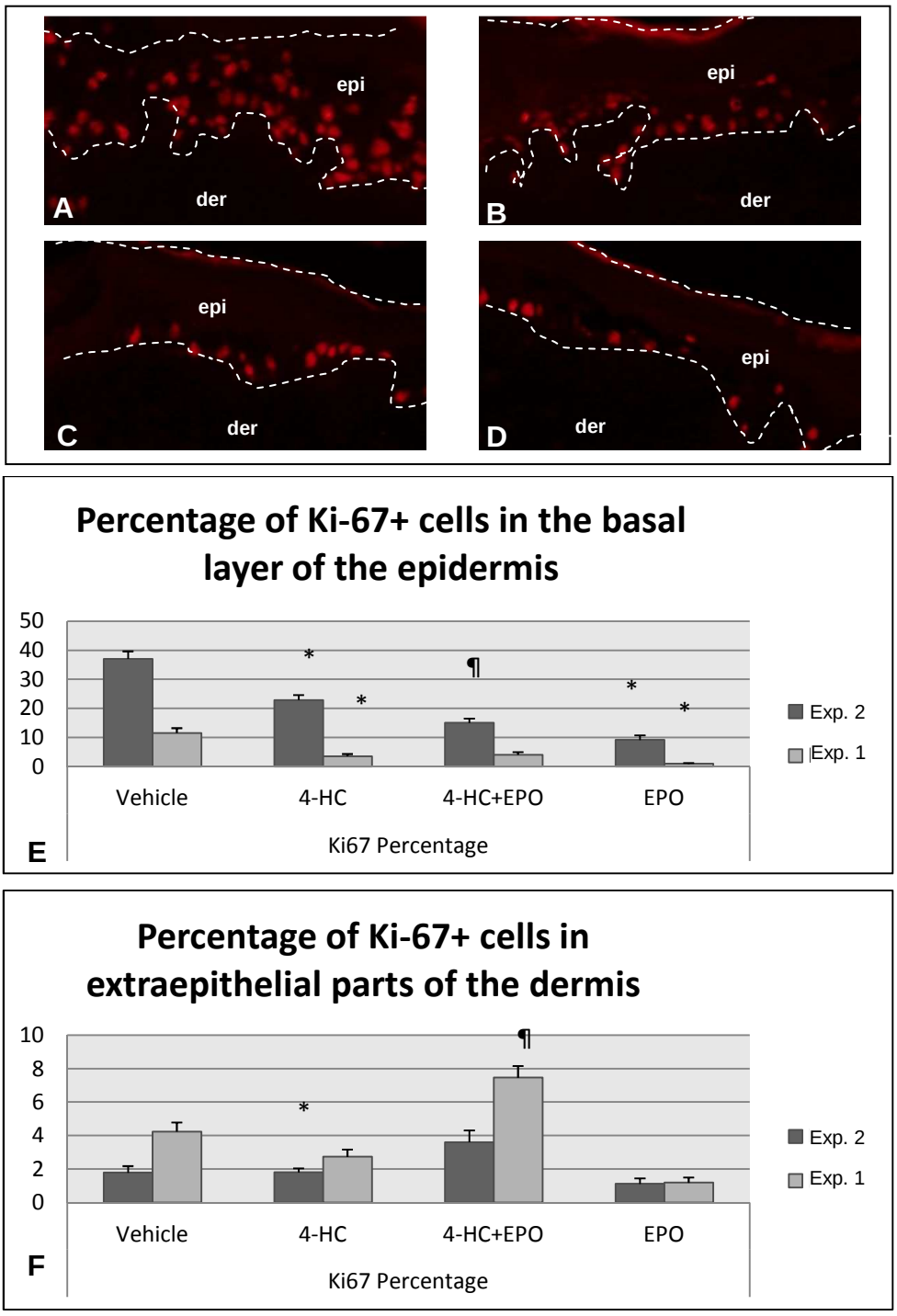


Figure 28. Proliferating cells in the basal layer of the epidermis and the extraepithelial part of the dermis in human scalp skin

Sections of punch biopsies of human full-thickness scalp skin that were organ-cultured for 9 days and treated for 7 days with with **A)** vehicle, **B)** 4-HC (10 µmol/l), **C)** 4-HC + EPO and **D)** EPO (100 IU/ml) were double-stained with Ki-67 / TUNEL and counter-stained with DAPI. For better illustration, only Ki-67+ cells (red) are shown here. **E)** Percentage of proliferating cells in the basal layer of the epidermis and **F)** in extraepithelial parts of the dermis; * $p<0.05$ compared to vehicle; ¶ $p<0.05$ compared to 4-HC; error bars: SEM. Punch biopsies were received from two donors and cultured in two independent experiments (Exp. 1 and 2); N = 25-43 epidermal and dermal visual fields per group. Dotted lines illustrate the contours of the epidermis; epi: epidermis; der: dermis.

4 Discussion

This thesis project provides new (although mostly preliminary) indications that EPO is indeed involved in several aspects of skin physiology in which it had not previously been implicated. EPO significantly changes the expression level of several genes transcribed in human skin and/or hair follicles, and joins the growing list of pigmentation-modulatory factors. Also, it acts in an anti-apoptotic manner in chemotherapy-treated organ-cultured human skin.
The questions specified in chapter 1.8 can be answered as follows:

1. Haemoglobin alpha 1, kinesin light chain 3 and calmegin were selected for qRT PCR detection of EPO-induced changes of mRNA levels. Using this technique, the previously reported gene changes can be confirmed in the HFs of a third patient. For calmegin, EPO-induced changes are even confirmed on protein level *in situ* by immunohistochemistry.

2. EPO is a new pigmentation-modulatory hormone. However, the pigmentary response of HFs derived from different individuals to EPO varied dramatically: Among those individuals that responded to EPO, some showed increased and others decreased pigmentation (melanin content), which seemed to correlate with tyrosinase activity. However, the number and dendricity of human HF melanocytes were unaffected by EPO treatment.

3. Human skin mast cells in the CTS of HFs do not respond to EPO treatment, as assessed by counting the number and degranulation status of mast cells.

4. EPO reduces massive apoptosis provoked by chemotherapy in unmanipulated, full-length human HFs within full-thickness skin *in situ*.

5. Similarly, EPO also reduces apoptosis in other examined compartments of the skin, i.e. in the basal layer of the epidermis and in the skin mesenchyme (dermis).

6. EPO alters cell proliferation in normal human full-thickness skin *in situ*. In all examined human skin compartments, EPO lowered the proliferation rates.

Out of the 5 candidate genes that were either significantly up- or down-regulated in both examined patients as had been previously detected by microarray (**Fig. 11**) (Bodo et al., 2007a), we tested the three most promising ones by qRT PCR in a third patient. This confirmed an up-regulation of haemoglobin alpha and kinesin light chain 3 transcription and a down-regulation of calmegin expression and supports the novel concept that haemoglobin alpha, kinesin light chain 3 and calmegin are novel (direct or indirect) target genes of EPO regulation. Naturally, our experimental design (organ culture) does not allow us to distinguish direct from indirect EPO-responsive genes.

Calmegin was also visualised on the protein level by immunohistochemistry. Unfortunately, however, the antibodies and immunostaining protocols used for immunohistochemical detection of kinesin light chain and haemoglobin alpha failed to elicit specific and reproducible immunoreactivity patterns that were clearly above the background. Therefore, those proteins could not be localised in human HFs or skin.

Although their functional importance still remains to be systematically explored (for instance by gene knock-down experiments using gene-specific siRNA so as to check whether gene silencing affects e.g. hair growth or pigmentation), some of the genes differentially expressed after EPO administration may indeed be important, novel EPO-target genes in hair follicle biology. For example, kinesin molecules are expressed in human skin cells (namely in human melanocytes, keratinocytes, fibroblasts and neuroblastoma cells) (Vancoillie et al, 2000). They are very likely to play an important role in microtubule-based melanosome transport in human melanocytes and are thought to be involved in skin pigmentation (Vancoillie et al., 2000; Park et al, 2009). The hair pigmentary effects of EPO observed in the current thesis project, therefore, may well reflect EPO-induced changes in the microtubule-based melanosome transport within the human HF pigmentary unit.

It has previously been reported that the beta globin chain is expressed in single hair (Liu et al., 1990), and Bodo et al. have presented the novel finding that the alpha globin chain is also transcribed in human HFs (Bodo et al., 2007a). Our qRT PCR results (chapter 3.1) confirm the expression of alpha globin chain mRNA in human HFs and its up-regulation upon EPO treatment. However, our attempts to demonstrate the presence of alpha globin chain *protein* in human HFs have so far been unsuccessful. Apart from its most obvious role of oxygen transport in erythrocytes, several other roles of haemoglobin have been proposed. The most interesting function in the current context is haemoglobin's possible involvement in heat transduction through its oxygenation-deoxygenation cycle (Giardina et al., 1995). This phenomenon has been investigated at least for some animals and also for the human fetus (Clementi et al., 1994; Giardina et al., 1995). Since skin forms an important barrier to protect the body from heat loss, it is tempting to speculate that intracutaneous haemoglobin (or parts of these molecules) may also be involved in heat transduction processes.

EPO gene expression is controlled by several transcription factors, e.g. hypoxia-inducible transcription factors (HIFs) and – less important – GATA transcriptions factors. GATA-2 that was up-regulated in qRT PCR, appears to inhibit EPO gene expression (Liu et al., 1990; Tabata et al., 2001; Jelkmann, 2007a, 2011). Therefore, this may be a mechanism of negative endocrine feedback regulation of EPO in human HFs. Also, there is evidence that GATA-2, a transcription factor expressed in haematopoietic cells (Weiss et al., 1994), is expressed in mast cells and has an important regulatory function during mast cell development (Harigae et al., 1998). Naturally, qRT PCR results on RNA extracts from entire HFs do not reveal the exact HF cell type that expresses GATA-2, but it is conceivable that the observed EPO-induced changes in GATA-2 transcription may reflect expression changes in the abundant mast cells of the HF´s CTS (Ito et al., 2010).

By qRT PCR and immunohistochemistry, we present the first evidence here that the calnexin-homologous chaperone calmegin is expressed in the inner root sheath of human hair follicles. Furthermore, in agreement with previous microarray results (Bodo et al., 2007a) and with our present qRT PCR data, calmegin protein

is down-regulated in EPO-treated organ-cultured anagen HFs. The endoplasmic reticulum-located calmegin is generally claimed to be a testis-specific Ca^{2+}-binding protein that is important for male germ cell development (Watanabe et al., 1994). However, as we show here, calmegin is *not* exclusively expressed during distinct stages of spermatogenesis, but is also found in at least one extratesticular expression site: human skin. This raises the possibility that there could be additional extratesticular expression sites of calmegin and strongly suggests that the physiological functions of this chaperone protein extend well beyond spermatogenesis.

During spermatogenesis several sequences of complex processes involving mitotic, meiotic and postmeiotic actions take place until the highly specialised sperm are formed (Yoshinaga et al., 1999; Phillips et al., 2010). To regulate these complex processes, the testis needs a stage-specific well-coordinated programme of gene expression (Eddy, 2002; Phillips et al., 2010). One of these genes is the calmegin-gene, which is only active from the pachytene stage of primary meiosis until the early maturation phase of spermiogenesis.
As a chaperone, calmegin is responsible for the proper folding of certain proteins (probably sperm surface proteins that mediate the interactions between sperm and egg). Although displaying morphologically normal spermatogenesis, calmegin null mutant mice are infertile due to failure of the sperm to bind to the zona pellucida of the egg (Ikawa et al., 1997; Yamagata et al., 2002).

Our current data suggest that calmegin may also exert functions in HF biology. As we show, calmegin expression in human HFs is down-regulated on gene and protein level when EPO is added to the organ-culture medium. Whether this calmegin response to EPO treatment is limited to the HF epithelium remains to be dissected. As most *hormonally regulated* proteins in HF biology have, sooner or later, turned out to play defined physiological functions, it is unlikely that follicular calmegin expression in the IRS is a mere epiphenomenon. Theoretically it is conceivable that calmegin exerts some role in protein binding in the HF, too, by analogy with its role in the testis. The fact that we could identify calmegin in the human HF IRS, which is a hardened inner cylinder of terminally differentiated keratinocytes guiding and packaging the central hair shaft (Paus et al., 2007;

Schneider et al., 2009), invites the hypothesis that one or several proteins necessary for the formation of the IRS are folded by the chaperone calmegin.

Among the few published studies and experiments on EPO concerning the testis, nothing has as yet been reported about an impact of EPO on spermatogenesis. Rather, EPO may rescue the seminiferous tubules in testicular torsion of rats (Koseoglu et al., 2009) and stimulate testosterone production in men (Foresta et al., 1994). Given that most human scalp HFs are exquisitely testosterone-sensitive (Paus et al., 2007) and that human pilosebaceous units both synthesise and metabolise androgens in substantial quantities (Chen and Zouboulis, 2009), it is also possible that EPO impacts on intrafollicular androgen levels. Clearly, the determination of the exact intrafollicular functions of both calmegin and EPO, and of how EPO regulates intrafollicular calmegin constitutes an intriguing and so far entirely unexplored novel aspect of human hair biology.

Here we also show for the first time that EPO is a novel pigmentation-modulatory hormone in human HFs, thus adding a new non-haematopoietic function to its growing list. In our experiments, the HFs of four out of the five examined individuals responded to EPO treatment, but in different ways. Two individuals showed increased HF pigmentation and two decreased HF pigmentation. Why different individuals respond so very differently to stimulation with the same hormone (EPO) remains a mystery, though. The discrepant pigmentary response can neither be explained by sex or age of the donor, nor by differences in the skin region HFs were derived from, nor by the duration of EPO treatment (compare **Table 5**).

In theory, this may reflect inter-individual differences in the expression levels of peripheral tissue EPOR, but unfortunately a reliable antigen-specific EPOR antibody is still not available, so that this possibility could not be checked on the protein level. Comparative qRT PCR analyses for differences between EPOR transcript steady-state levels may provide first indications as to whether or not this hypothetical explanation for the large observed differences in the pigmentary EPO response is plausible. However, even if there are differences in the relative intrafollicular expression levels of EPOR, this would not really offer a satisfactory

explanation for a diametrically opposed target organ response to stimulation with the same hormone. (This is somewhat reminiscent of the diametrically opposed target organ response of human scalp and beard HFs to stimulation with the same androgen, dihydrotestosterone (DHT) (Stenn and Paus, 2001; Paus et al., 2007)).

Furthermore, inter-individual differences in the intracellular signal transduction pathway(s) activated after EPOR stimulation could also account for the observed discrepancies in the pigmentary EPO response of human scalp HFs. Substantial differences in post-receptor signalling are a reasonable possibility, since EPO signalling involves several pathways (see **Fig. 10**), and their preferential employment may, in *peripheral* human tissues, differ between distinct individuals. However, dissection of such hypothetical signal transduction differences was clearly beyond the scope of the current thesis project.

We demonstrate that HF melanocytes *in situ* respond to EPO by regulating their melanin content and their tyrosinase activity while melanocyte number and dendricity did not change after EPO treatment. Whether the pigmentary effects of EPO within the human HF pigmentary unit reflect a direct action of EPO on human HF melanocytes or an indirect one mediated by hair matrix keratinocytes is not clear yet. While some other hormones and factors that influence human skin and HF pigmentation have also been found to enhance melanocyte dendricity, such as endothelin 1 (Hara et al., 1995; Park et al., 2009), prostaglandin 2α (Scott et al., 2004; Park et al., 2009), histamine (Yoshida et al., 2000; Park et al., 2009) and possibly also thyroid hormones (van Beek et al., 2008), EPO apparently does not promote human melanocyte dendrite formation *in situ*.

Melanosome transport from melanocytes to keratinocytes via the dendrites' tip is mainly controlled by two classes of microtubule-associated motor proteins: kinesin and dynein (Park et al., 2009). Interestingly, the previous microarray (Bodo et al., 2007a) and our confirmatory qRT PCR results have revealed that kinesin light chain 3 is up-regulated in HFs after EPO treatment. Therefore, it would be interesting to investigate in future studies, namely with the help of instructive melanocyte-keratinocyte co-culture systems that allow the study and manipulation

of melanosome transfer, whether or not EPO really modulates the kinesin-dependent melanosome transfer.

An interesting association between ocular and coat pigmentation and susceptibility to oxygen-induced retinopathy in rats has recently been reported (van Wijngaarden et al., 2007). Apparently, mice with dark coat and eye colour have a much higher risk of suffering from oxygen-induced retinopathy after cyclic hyperoxic exposure than albino rats with red eyes. Whether this correlation is causal or coincidental is not known yet. However, it appears that the expression of EPO (and vascular endothelial growth factor as pro-angiogenic factors) during the period of hyperoxic exposure correlates with the risk of developing oxygen-induced retinopathy: Those rat strains with high EPO expression levels during hyperoxic exposure are less likely to suffer from retinopathy (van Wijngaarden et al., 2007). This shows that also in rats quite differing EPO expression levels can be observed maybe even in association with the phenotype.

With the chosen assay design, EPO treatment led neither to an alteration in the number of mast cells nor to any decrease or increase of the degranulation level of mast cells in the HF´s CTS. In 2005, it has been reported that the receptor for erythropoietin is present on cutaneous mast cells (Isogai et al., 2006). However, probably this group only detected the soluble form of the EPOR (sEPOR), since only the antibody against sEPOR (and not the anti-cEPOR antibody) reacted with the granules of mast cells.

Soluble receptors have been described for several members of the type I transmembrane cytokine superfamily (Harris and Winkelmann, 1996; Lee et al., 2010). They correspond to the extracellular domain of the complete receptor and are rather secreted by the cell than expressed on the cell surface (Harris and Winkelmann, 1996; Khankin et al., 2010). The sEPOR binds EPO and thus prevents EPO from binding to the EPOR (Baynes et al., 1993; Soliz et al., 2007). Not much is known about the functions of sEPORs but they are thought to be regulators of their ligand concentration (Morishita et al., 1996). A recent publication (Soliz et al., 2007) adds support to this theory: they claim that the down-regulation of sEPOR (and consequently maintenance of high EPO-levels) in states of chronic

hypoxia is necessary for an adequate ventilatory acclimatization to hypoxia. Therefore, it is possible that the sEPOR on mast cells is fulfilling regulatory functions of EPO levels rather than being involved in signal transduction. However, further experiments would be needed to examine this intriguing possibility of mast cells contributing to the regulation of cutaneous EPO levels.

On the other hand, it is also quite possible that the supposed "EPOR" detection on mast cells by Isogai et al. was just a false-positive result. After all, the group itself was puzzled by the inappropriate size of the detected "EPOR" (43 kDa), which was too small for the whole EPOR molecule but too big for the soluble form, which reportedly has a size of 34 kDa (Isogai et al., 2006). Isogai et al. explain that the EPOR they discovered on mast cells may have been the soluble form of the EPOR bound to an unknown substance and therefore bigger in size than expected (Isogai et al., 2006). This theory has not yet been confirmed by independent investigators, and another possible explanation might be that the purported EPOR might in fact have represented a different cross-reacting antigen. Consequently, further research is required to determine which form of the EPOR – if any at all – is present on mast cells. Regardless of whether the reported structure on mast cells is the regulatory soluble EPOR or no EPOR at all, since we did not see any changes in mast cell number or degranulation status, we did not follow this up any further.

As previously reported for human scalp HFs (Bodo et al., 2007a), pre- and co-treatment with EPO in full-thickness skin reduces chemotherapy-induced apoptosis. As a chemotherapeutic agent the cyclophosphamide-derivative 4-HC was used. Cells in all examined skin compartments showed increased apoptosis rates after 4-HC treatment compared to vehicle treatment. As found in the previously performed HF experiments (Bodo et al., 2007a), EPO co-treatment did significantly down-regulate chemotherapy-induced apoptosis in all skin compartments. These findings are in line with many other reports that found anti-apoptotic effects of EPO in lots of different tissues (see chapter 1.5.3) (Arcasoy, 2008; Jelkmann et al., 2008; Arcasoy, 2010). There are several pathways by which EPO can reduce apoptosis involving STAT5, PKB/Akt, caspase 9, Bcl-X_L and Fas ligand (compare **Fig.10**). At this moment, we can only speculate about

which anti-apoptotic signalling cascades are involved. However, since Fas as a key p53 target has been identified as a crucial molecular player in the HF response to cyclophosphamide (Sharov et al., 2004), one plausible pathway by which EPO inhibits apoptosis in human skin may be the prevention of Fas-gene activation by EPO (compare **Fig. 10**).

These observations support the concept of clinically exploring EPO not only as a possible agent against chemotherapy-induced alopecia (CIA), which is still one of the most disturbing side-effects of chemotherapy (Kiebert et al., 1990; Hesketh et al., 2004; Bodo et al., 2007b), but also against other forms of chemotherapy-induced general skin damage (e.g. epidermal atrophy, acral erythema, nail changes (Chu and DeVita, 2010; Kamil et al., 2010)). However, the crux of the matter remains how to selectively inhibit apoptosis in “good” cells, namely rapidly proliferating skin and mucous membrane epithelium, without simultaneously protecting malignant cells from chemotherapy-induced apoptosis (Bodo et al., 2007b). For the skin and its HFs, direct topical EPO application is conceivable, e.g. via appropriate liposomal vehicles. However, it is questionable whether this would lead to sufficient EPO concentrations in the skin and whether the enormous costs associated with the external application of (very expensive!) synthetic EPO could justify the potential benefits. Moreover, this might also lead to systemic EPO elevation and thus could induce serious EPO-related undesired effects (e.g. hypertension and cardiovascular events (Jelkmann, 2008)). Alternatively however, one could attempt to up-regulate the endogenous, intracutaneous EPO production in human skin by topically applied substances that chemically "imitate" a state of hypoxia, thus up-regulating HIF-1α activity, and/or that directly stimulate intracutaneous EPO gene expression.

In addition to undesired apoptosis-protective effects of such a strategy on tumour cells, one has to bear in mind that the induction of apoptosis (both in the HFs and the skin) is a protective response of the body to eliminate severely damaged or transformed cells. Thus, if EPO is applied to or is up-regulated within the skin, such damaged cells (e.g. cells that have accumulated major chemotherapy- or ultraviolet-light–induced DNA damage) might be hindered from undergoing apoptosis. However, as the HF is a continuously renewing miniorgan (see chapter

1.2) one might argue that, in this special case, the potential risk that comes along with an EPO "rescue" of damaged cells may not be as harmful as in other organs and tissues.

Two investigations with divergent results of EPO-mediated changes concerning proliferation in human HFs have so far been reported. While the group in whose laboratory the current thesis project was executed did not find significant proliferation differences in the hair matrix between EPO- and vehicle-treated HFs (Bodo et al., 2007b), a Korean group recently reported an increased number of Ki-67 positive matrix keratinocytes around the dermal papilla in HFs that had been treated with EPO (Kang et al., 2010). However, the two experimental designs were not identical. Both groups used supplemented William's E medium, although Bodo et al. also added 10 µg/ml insulin and a slightly different antibiotic mixture (penicillin G, streptomycin and amphotericin B versus streptomycin alone). While Bodo et al. used only female HFs, Kang et al. used male ones. Also, Bodo et al. used the higher EPO concentration of 100 IU/ml (versus 10 IU/ml) and a longer incubation period (9 days versus 6 days).

After organ-culturing human skin (rather than isolated, microdissected HFs) with either vehicle or EPO, as Kang et al. (2010), we too detected changes in proliferation rate, yet opposite to what those authors had reported: EPO itself (and, of course, 4-HC) significantly reduced proliferation in all examined skin compartments. Instead, the combination of 4-HC with EPO seemed to lower proliferation rates in epithelial compartments, while the opposite trend was seen in the dermis.

Thus, remarkably, EPO application in organ-cultured human full-thickness skin was accompanied by reduced (!) proliferation rates in all human skin compartments *in situ,* whereas in several other tissues EPO reportedly increased proliferation. Apart from proliferation-promoting effects on cells of the erythroid lineage, *in vitro*, EPO also promotes proliferation in endothelial cells (Anagnostou et al., 1990; Ribatti et al., 1999; Jelkmann, 2007b), in skeletal muscle satellite cells (Ogilvie et al., 2000), in human bone marrow mesenchymal cells (Zeng et al., 2008), in astrocytes (Sugawa et al., 2002) and in breast cancer cells (Fu et al., 2009).

At present we have no convincing explanation for the observation why EPO alone reduced proliferation, but exerted differential proliferation-modulatory effects in distinct skin compartments when combined with the cytostatic metabolite 4-HC. Of course, a much larger number of skin organ culture assays (with skin derived from distinct human individuals) than could be examined in the current study is needed, before one can conclude that this is a reproducible proliferation response pattern. Such repeat experiments are also called-for since large differences were observed in the number of proliferating cells in all groups (for example, in the basal layer of the epidermis (37% Ki-67+ cells in Exp. 2 versus 12% in Exp. 1).
If the differential response pattern summarised above is eventually confirmed, one may speculate that the addition of 4-HC triggers distinct damage response patterns in different skin cell populations (in some of which entry into the cell cycle could be part of this damage response, while in others the proliferation-inhibitory properties of 4-HC may predominate).

These rather substantial differences in response patterns observed in organ-cultured skin or HFs derived from different patients seen in the current study are also notable in the pigmentary experiments. This suggests that HFs and entire organ-cultured territories of human skin depend on numerous biological variables (e.g. differences in the production of other hormones and cytokines, in the gene expression profile, in receptor polymorphisms, inflammatory signalling milieu, in stem cell survival; differences in the general health state and medication of skin donors; and in skin and HF traumatisation during surgery, transport, microdissection, and organ culture set-up). In other words, among other things different degrees of skin wounding in the operating theatre and during skin punching preparation or HF microdissection in the laboratory have to be taken into account when assessing apoptosis and proliferation. In particular, the borders of the punch biopsies showed massively increased apoptosis (most probably due to skin wounding): therefore in all sections the outermost microscopic fields were not included in the analysis in order to minimise the wounding influence.
Taken together, this huge spectrum of variables makes it impossible to standardise when using tissue from different individuals, whose skin is harvested and processed at different time points and by different physicians/researchers.

Thus, for practical purposes, it is probably most appropriate to consider each assay of organ-cultured HFs or human skin biopsies (especially those derived from different skin regions or even different individuals) as a microcosmos of its own, in which large variations in the response to a defined test agent may well be the rule rather than the exception.

As well as these general considerations, the ongoing debate about homodimeric versus heterodimeric EPORs and associated post-receptor signalling variations may have to be taken into account. Non-haematopoietic tissues have been claimed to require the heteromeric EPOR (Brines et al., 2004; Brines and Cerami, 2008) and not the homodimeric EPOR, which is expressed by erythrocytic progenitor cells. This heterocomplex consists of one EPOR molecule and CD131, the common β receptor chain that is also present in the granulocyte-macrophage colony stimulating factor and in the IL-3 and IL-5 receptor (Jubinsky et al., 1997; Brines et al., 2004). This heteromeric EPOR complex is believed to exhibit a lower affinity for EPO than the homodimeric receptor. Consequently, it is claimed that the "tissue protective" non-haematopoietic EPOR does not respond to the low concentrations of EPO present in the circulation but rather to the much higher, locally produced EPO levels (Brines and Cerami, 2008; Erbayraktar et al., 2009).

EPO signalling is even more complex than is already indicated by the many possible signalling pathways that can be activated after EPOR stimulation shown in **Fig. 10**. For example, changes in the available form of SCF lead to changes in EPOR activation. In particular, stroma cells that only express a membrane-restricted isoform of SCF induce a more sustained and elevated tyrosine phosphorylation of both c-kit and the EPOR (Kapur et al., 1998)! This leads to greater proliferation of erythrocytic progenitor cells compared to stroma cells expressing the soluble SCF (Kapur et al., 1998). Thus, SCF/c-kit signalling might provide a means of regulating EPOR activation (Kapur et al., 1998). This may also be relevant in the current context, as the human HF itself is a prominent source of SCF (Ito et al., 2010). Thus, inter-individual differences in intrafollicular and intracutaneous SCF production may contribute to the observed large variations in the response to EPO.

This study shows for the first time that the apoptosis-reducing effect of EPO administration also applies to the *full-length* HFs located in organ-cultured, full-thickness skin punch biopsies. As CIA is still a frequent and very disturbing side effect of chemotherapy (Hesketh et al., 2004), it is of immense clinical importance to develop satisfactory new pharmacological approaches to reduce or prevent hair loss in chemotherapy patients. Yet there is still a long way until a possible pharmacological use of EPO against hair loss in patients who receive chemotherapy. The process will have to include clinical investigations, e.g. as clinical trials evaluating topical EPO application as a tool to prevent CIA. Many patients who receive chemotherapy also suffer from tumour anaemia that is often treated with systemic EPO doses. Therefore it would be a good starting point to investigate systematically the prevalence and extension of CIA in this patient cohort compared to a cohort of tumour patients that do not receive EPO treatment.

In summary, this study shows that EPO changes intrafollicular gene expression and is a new player in the regulation of human HF pigmentation. Also, EPO significantly limits chemotherapy-induced apoptosis in all compartments of human skin while reducing intracutaneous proliferation.
Taken together, these findings provide further evidence for the functionality and importance of EPOR-mediated signalling in normal human skin and reveal novel pointers to potential, previously unknown non-haematopoietic functions of EPO in the human system.

5 Summary

Erythropoietin (EPO) – long appreciated as the principal regulator of red blood cell production – is now also recognised as an important tissue protective factor in many other tissues that express EPO and/or the EPO receptor (EPOR), e.g. brain, heart, liver and lung. Most recently, our group discovered that even human hair follicles not only express the EPOR but also generate remarkable amounts of EPO themselves. Intriguingly, EPO here significantly reduced apoptosis induced by the cyclophosphamide derivative 4-hydroperoxycyclophosphamide (4-HC), a chemotherapeutic agent. Since the skin is a major endocrine organ, it is of great importance to further explore the physiological function of EPO signalling in human skin and its appendages.

For this purpose, we decided to obtain additional evidence of the functionality of EPOR expressed in human hair follicles by comparing the gene expression levels of selected genes that had previously been identified as candidate EPO-responsive genes, by quantitative real-time polymerase chain reaction (qRT PCR) in the presence and absence of EPO stimulation. Since other groups had reported that the EPOR is expressed in melanocytes and in mast cells, we also specifically investigated EPO's influence on the pigmentary system and on hair follicle-associated mast cells. Finally, we examined whether EPO also exerts anti-apoptotic and/or proliferation-modulatory functions in organ-cultured human full-thickness skin exposed to chemotherapy, using 4-HC.

To answer these questions, human hair follicles were microdissected and full-thickness skin was punch-biopsied from skin obtained from healthy persons undergoing plastic surgery. Subsequently, hair follicles or skin biopsies were organ-cultured in a serum-free medium with either vehicle or EPO. Additionally, two further groups of skin biopsies were treated with 4-HC and 4-HC + EPO. After processing, the different (immuno)histologically stained specimens were compared to each other.

The following results were obtained:

1. EPO modulated the mRNA steady-state levels of haemoglobin alpha 1, kinesin light chain 3 and calmegin, as measured by qRT PCR. For calmegin, EPO-induced changes could even be confirmed on protein level *in situ* by immunohistochemistry.
2. We were able to show for the first time that EPO is a novel pigmentation-modulatory hormone. However, the pigmentary response to EPO of hair follicles derived from different human individuals varied dramatically: Among those individuals that did respond to EPO, some showed increased and others decreased pigmentation (melanin content). This response correlated with changes in the tyrosinase activity in melanocytes. The number and dendricity of follicular melanocytes were unaffected by EPO treatment.
3. Hair follicle-associated mast cells did not respond to EPO treatment, as assessed by counting the number and degranulation status of mast cells.
4. EPO reduced massive apoptosis provoked by chemotherapy in unmanipulated human hair follicles within normal human skin.
5. Similarly, EPO also reduced apoptosis in the other compartments (epidermis and dermis) of human skin.
6. In all examined human skin compartments, EPO lowered proliferation rates.

These findings provide further evidence for the functionality and importance of EPOR-mediated signalling in normal human skin and reveal previously unknown non-haematopoietic functions of EPO in the human system.

6 Zusammenfassung

Neben der seit langen bekannten Funktion als Hauptregulator bei der Produktion roter Blutzellen wird Erythropoietin (EPO) jetzt auch als wichtiger gewebeschützender Faktor in vielen anderen Geweben, die EPO und/oder den EPO Rezeptor (EPOR) exprimieren, wie z.B. Gehirn, Herz, Leber und Lunge, angesehen. Erst kürzlich entdeckte unsere Forschungsgruppe, dass auch menschliche Haarfollikel nicht nur den EPOR exprimieren, sondern selbst bemerkenswerte Mengen EPO produzieren. Faszinierenderweise reduzierte EPO signifikant die Apoptoserate in Haarfollikeln, die mit dem Cyclophosphamidabkömmling 4-Hydroperoxycyclophosphamid (4-HC), einem Chemotherapeutikum, behandelt worden waren. Da die Haut ein wichtiges endokrines Organ ist, ist es äußerst wichtig, die physiologischen Funktionen von EPO-Signalwegen in der menschlichen Haut und ihren Hautanhangsgebilden weiter zu erforschen.

In der vorliegenden Arbeit wollten wir weitere Beweise für die Funktionalität des in menschlichen Haarfollikeln exprimierten EPOR erbringen. Dazu verglichen wir die Genexpressionsniveaus ausgewählter Gene, die zuvor schon als EPO-responsive Kandidatengene identifiziert worden waren, mit und ohne EPO-Stimulation mittels quantitativer Echtzeit-Polymerase-Kettenreaktion (qRT PCR). Da andere Arbeitsgruppen beschrieben hatten, dass der EPOR auf Melanozyten und Mastzellen exprimiert wird, erforschten wir auch gezielt den Einfluss von EPO auf das Pigmentierungssystem und auf Haarfollikel-assoziierte Mastzellen. Schließlich untersuchten wir, ob EPO auch antiapoptotische und/oder proliferations-modulierende Wirkung auf menschliche organkultivierte Vollhaut hat, die Chemotherapie in Form von 4-HC ausgesetzt war.

Zur Beantwortung dieser Fragen wurden aus der Haut gesunder Personen, die sich einer Schönheitsoperation unterzogen hatten, Haarfollikel mikrodisseziert sowie Vollhautbiopsien gewonnen. Anschließend wurden die Haarfollikel oder Hautbiopsien in einem serumfreien Medium entweder mit Trägersubstanz oder mit EPO organkultiviert. Zusätzlich wurden zwei weitere Gruppen von Hautbiopsien

mit 4-HC und 4-HC + EPO behandelt. Nach ihrer Verarbeitung verglichen wir die Proben, die mit verschiedenen (immun)histochemischen Färbungen behandelt wurden, miteinander.

Dabei erhielten wir folgende Ergebnisse:

1. EPO modulierte die Steady-State-Niveaus von Hämoglobin alpha 1, Kinesin light chain 3 und Calmegin mRNA, wie mit Hilfe von qRT PCR gemessen wurde. Für Calmegin konnten die EPO-induzierten Änderungen sogar auf Proteinniveau *in situ* mittels Immunhistochemie bestätigt werden.
2. Wir konnten erstmals zeigen, dass EPO ein neues pigmentierungs-modulierendes Hormon ist. Jedoch unterschieden sich die Pigmentierungs-reaktionen von Haarfollikeln verschiedener menschlicher Individuen auf EPO beachtlich. Einige der Individuen, die auf EPO reagierten, zeigten eine erhöhte, andere hingegen eine verminderte Pigmentierung (Melaningehalt). Diese Reaktion korrelierte mit den Änderungen der Tyrosinaseaktivität der Melanozyten. Die Anzahl und Dendrizität follikulärer Melanozyten wurde von der Behandlung mit EPO nicht beeinflusst.
3. Die Haarfollikel-assoziierten Mastzellen reagierten nicht auf Behandlung mit EPO, wie die Bestimmung der Anzahl und des Degranulationszustandes der Mastzellen belegte.
4. EPO reduzierte die durch Chemotherapie hervorgerufene, massive Apoptoserate unmanipulierter Haarfollikel in der menschlichen Haut.
5. Ebenso verringerte EPO auch die Apoptoserate in den anderen Kompartimenten (Epidermis und Dermis) der menschlichen Haut.
6. In allen untersuchten Hautkompartimenten verminderte EPO die Proliferationsrate.

Diese Ergebnisse stellen weitere Belege zur Funktionalität und Bedeutung von EPOR-vermittelten Signalwegen in normaler menschlicher Haut dar und enthüllen zuvor unbekannte nicht-hämatopoietische Funktionen von EPO beim Menschen.

7 References

Acs G, Acs P, Beckwith SM, Pitts RL, Clements E, Wong K, Verma A (2001)
Erythropoietin and erythropoietin receptor expression in human cancer. Cancer Res 61: 3561-5

Adema GJ, de Boer AJ, van 't Hullenaar R, Denijn M, Ruiter DJ, Vogel AM, Figdor CG (1993)
Melanocyte lineage-specific antigens recognized by monoclonal antibodies NKI-beteb, HMB-50, and HMB-45 are encoded by a single cDNA. Am J Pathol 143: 1579-85

Alonso L, Fuchs E (2006)
The hair cycle. J Cell Sci 119: 391-3

Anagnostou A, Lee ES, Kessimian N, Levinson R, Steiner M (1990)
Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. Proc Natl Acad Sci U S A 87: 5978-82

Ando H, Ichihashi M, Hearing VJ (2009)
Role of the ubiquitin proteasome system in regulating skin pigmentation. Int J Mol Sci 10: 4428-34

Arcasoy MO (2008)
The non-haematopoietic biological effects of erythropoietin. Br J Haematol 141: 14-31

Arcasoy MO (2010)
Non-erythroid effects of erythropoietin. Haematologica 95: 1803-5

Arcasoy MO, Amin K, Karayal AF, Chou SC, Raleigh JA, Varia MA, Haroon ZA (2002)
Functional significance of erythropoietin receptor expression in breast cancer. Lab Invest 82: 911-8

Banks DD, Scavezze JL, Siska CC (2009)
Kinetic folding mechanism of erythropoietin. Biophys J 96: 4221-30

Banziger-Tobler NE, Halin C, Kajiya K, Detmar M (2008)
Growth hormone promotes lymphangiogenesis. Am J Pathol 173: 586-97

Barbosa AJ, Castro LP, Margarida A, Nogueira MF (1984)
A simple and economical modification of the Masson-Fontana method for staining melanin granules and enterochromaffin cells. Stain Technol 59: 193-6

Baynes RD, Reddy GK, Shih YJ, Skikne BS, Cook JD (1993)
Serum form of the erythropoietin receptor identified by a sequence-specific peptide antibody. Blood 82: 2088-95

Benninghoff A, Drenckhahn D (2002)
Anatomie. 16 edn, vol 2. Elsevier GmbH Urban & Fischer Verlag, München

Billoni N, Buan B, Gautier B, Gaillard O, Mahe YF, Bernard BA (2000)
Thyroid hormone receptor beta1 is expressed in the human hair follicle. Br J Dermatol 142: 645-52

Bodo E, Biro T, Telek A, Czifra G, Griger Z, Toth BI, Mescalchin A, Ito T, Bettermann A, Kovacs L, Paus R (2005)
A hot new twist to hair biology: involvement of vanilloid receptor-1 (VR1/TRPV1) signaling in human hair growth control. Am J Pathol 166: 985-98

Bodo E, Kany B, Gaspar E, Knuver J, Kromminga A, Ramot Y, Biro T, Tiede S, van Beek N, Poeggeler B, Meyer KC, Wenzel BE, Paus R (2010)
Thyroid-stimulating hormone, a novel, locally produced modulator of human epidermal functions, is regulated by thyrotropin-releasing hormone and thyroid hormones. Endocrinology 151: 1633-42

Bodo E, Kromminga A, Biro T, Borbiro I, Gaspar E, Zmijewski MA, van Beek N, Langbein L, Slominski AT, Paus R (2009)
Human female hair follicles are a direct, nonclassical target for thyroid-stimulating hormone. J Invest Dermatol 129: 1126-39

Bodo E, Kromminga A, Funk W, Laugsch M, Duske U, Jelkmann W, Paus R (2007a)
Human hair follicles are an extrarenal source and a nonhematopoietic target of erythropoietin. Faseb J 21: 3346-54

Bodo E, Tobin DJ, Kamenisch Y, Biro T, Berneburg M, Funk W, Paus R (2007b)
Dissecting the impact of chemotherapy on the human hair follicle: a pragmatic in vitro assay for studying the pathogenesis and potential management of hair follicle dystrophy. Am J Pathol 171: 1153-67

Botchkarev VA, Botchkarev NV, Albers KM, van der Veen C, Lewin GR, Paus R (1998)
Neurotrophin-3 involvement in the regulation of hair follicle morphogenesis. J Invest Dermatol 111: 279-85

Botchkarev VA, Botchkareva NV, Peters EM, Paus R (2004)
Epithelial growth control by neurotrophins: leads and lessons from the hair follicle. Prog Brain Res 146: 493-513

Botchkarev VA, Eichmuller S, Johansson O, Paus R (1997)
Hair cycle-dependent plasticity of skin and hair follicle innervation in normal murine skin. J Comp Neurol 386: 379-95

Botchkarev VA, Komarova EA, Siebenhaar F, Botchkareva NV, Komarov PG, Maurer M, Gilchrest BA, Gudkov AV (2000)
p53 is essential for chemotherapy-induced hair loss. Cancer Res 60: 5002-6

Botchkarev VA, Yaar M, Peters EM, Raychaudhuri SP, Botchkareva NV, Marconi A, Raychaudhuri SK, Paus R, Pincelli C (2006)
Neurotrophins in skin biology and pathology. J Invest Dermatol 126: 1719-27

Boutin AT, Weidemann A, Fu Z, Mesropian L, Gradin K, Jamora C, Wiesener M, Eckardt KU, Koch CJ, Ellies LG, Haddad G, Haase VH, Simon MC, Poellinger L, Powell FL, Johnson RS (2008)
Epidermal sensing of oxygen is essential for systemic hypoxic response. Cell 133: 223-34

Brines M, Cerami A (2008)
Erythropoietin-mediated tissue protection: reducing collateral damage from the primary injury response. J Intern Med 264: 405-32

Brines M, Grasso G, Fiordaliso F, Sfacteria A, Ghezzi P, Fratelli M, Latini R, Xie QW, Smart J, Su-Rick CJ, Pobre E, Diaz D, Gomez D, Hand C, Coleman T, Cerami A (2004)
Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. Proc Natl Acad Sci U S A 101: 14907-12

Brines M, Patel NS, Villa P, Brines C, Mennini T, De Paola M, Erbayraktar Z, Erbayraktar S, Sepodes B, Thiemermann C, Ghezzi P, Yamin M, Hand CC, Xie QW, Coleman T, Cerami A (2008)
Nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin. Proc Natl Acad Sci U S A 105: 10925-30

Brines ML, Ghezzi P, Keenan S, Agnello D, de Lanerolle NC, Cerami C, Itri LM, Cerami A (2000)
Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. Proc Natl Acad Sci U S A 97: 10526-31

Carnot P, Deflandre C (1906)
Sur l'activité hemopoietique de serum au cours de la regenation du sang. CR Acad Sci 1906 143: 384-6

Carroll VA, Ashcroft M (2005)
Targeting the molecular basis for tumour hypoxia. Expert Rev Mol Med 7: 1-16

Cheetham JC, Smith DM, Aoki KH, Stevenson JL, Hoeffel TJ, Syed RS, Egrie J, Harvey TS (1998)
NMR structure of human erythropoietin and a comparison with its receptor bound conformation. Nat Struct Biol 5: 861-6

Chen L, Endler A, Shibasaki F (2009)
Hypoxia and angiogenesis: regulation of hypoxia-inducible factors via novel binding factors. Exp Mol Med 41: 849-57

Chen WC, Zouboulis CC (2009)
Hormones and the pilosebaceous unit. Dermatoendocrinol 1: 81-6

Chu E, DeVita VT (2010)
Physicians' Cancer Chemotherapy Drug Manual. 10 edn. Jones and Bartlett Publishers International, London

Cianfarani F, Baldini E, Cavalli A, Marchioni E, Lembo L, Teson M, Persechino S, Zambruno G, Ulisse S, Odorisio T, D'Armiento M (2010)
TSH receptor and thyroid-specific gene expression in human skin. J Invest Dermatol 130: 93-101

Clementi ME, Condo SG, Castagnola M, Giardina B (1994)
Hemoglobin function under extreme life conditions. Eur J Biochem 223: 309-17

Coelho SG, Choi W, Brenner M, Miyamura Y, Yamaguchi Y, Wolber R, Smuda C, Batzer J, Kolbe L, Ito S, Wakamatsu K, Zmudzka BZ, Beer JZ, Miller SA, Hearing VJ (2009)
Short- and long-term effects of UV radiation on the pigmentation of human skin. J Investig Dermatol Symp Proc 14: 32-5

Colvin OM (1999)
An overview of cyclophosphamide development and clinical applications. Curr Pharm Des 5: 555-60

D'Andrea AD, Zon LI (1990)
Erythropoietin receptor. Subunit structure and activation. J Clin Invest 86: 681-7

Delorme E, Lorenzini T, Giffin J, Martin F, Jacobsen F, Boone T, Elliott S (1992)
Role of glycosylation on the secretion and biological activity of erythropoietin. Biochemistry 31: 9871-6

Digicaylioglu M, Bichet S, Marti HH, Wenger RH, Rivas LA, Bauer C, Gassmann M (1995)
Localization of specific erythropoietin binding sites in defined areas of the mouse brain. Proc Natl Acad Sci U S A 92: 3717-20

d'Ischia M, Napolitano A, Pezzella A, Meredith P, Sarna T (2009)
Chemical and structural diversity in eumelanins: unexplored bio-optoelectronic materials. Angew Chem Int Ed Engl 48: 3914-21

Ebling F, Eady R, Leigh I (1991)
Anatomy and Organization of Human Skin. In: Champion R, Burton J, Ebling F (eds) Textbook of Dermatology, 5th edn. Blackwell Scientific Publications, London, pp 49-124

Eddy EM (2002)
Male germ cell gene expression. Recent Prog Horm Res 57: 103-28

Ehrenreich H, Bartels C, Sargin D, Stawicki S, Krampe H (2008)
Recombinant human erythropoietin in the treatment of human brain disease: focus on cognition. J Ren Nutr 18: 146-53

Ehrenreich H, Degner D, Meller J, Brines M, Behe M, Hasselblatt M, Woldt H, Falkai P, Knerlich F, Jacob S, von Ahsen N, Maier W, Bruck W, Ruther E, Cerami A, Becker W, Siren AL (2004)
Erythropoietin: a candidate compound for neuroprotection in schizophrenia. Mol Psychiatry 9: 42-54

Ehrenreich H, Hasselblatt M, Dembowski C, Cepek L, Lewczuk P, Stiefel M, Rustenbeck HH, Breiter N, Jacob S, Knerlich F, Bohn M, Poser W, Ruther E, Kochen M, Gefeller O, Gleiter C, Wessel TC, De Ryck M, Itri L, Prange H, Cerami A, Brines M, Siren AL (2002)
Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med 8: 495-505

Ellerhorst JA, Naderi AA, Johnson MK, Pelletier P, Prieto VG, Diwan AH, Johnson MM, Gunn DC, Yekell S, Grimm EA (2004)
Expression of thyrotropin-releasing hormone by human melanoma and nevi. Clin Cancer Res 10: 5531-6

Erbayraktar Z, Erbayraktar S, Yilmaz O, Cerami A, Coleman T, Brines M (2009)
Nonerythropoietic tissue protective compounds are highly effective facilitators of wound healing. Mol Med 15: 235-41

Eschbach JW, Egrie JC, Downing MR, Browne JK, Adamson JW (1987)
Correction of the anemia of end-stage renal disease with recombinant human erythropoietin. Results of a combined phase I and II clinical trial. N Engl J Med 316: 73-8

Fandrey J, Bunn HF (1993)
In vivo and in vitro regulation of erythropoietin mRNA: measurement by competitive polymerase chain reaction. Blood 81: 617-23

Fang XQ, Fang M, Fan SW, Gu CL (2009)
Protection of erythropoietin on experimental spinal cord injury by reducing the expression of thrombospondin-1 and transforming growth factor-beta. Chin Med J (Engl) 122: 1631-5

Farman N, Maubec E, Poeggeler B, Klatte JE, Jaisser F, Paus R (2010)
The mineralocorticoid receptor as a novel player in skin biology: beyond the renal horizon? Exp Dermatol 19: 100-7

Ference JD, Last AR (2009)
Choosing topical corticosteroids. Am Fam Physician 79: 135-40

Ferri C, Giuggioli D, Sebastiani M, Colaci M (2007)
Treatment of severe scleroderma skin ulcers with recombinant human erythropoietin. Clin Exp Dermatol 32: 287-90

Fischer TW, Slominski A, Zmijewski MA, Reiter RJ, Paus R (2008)
Melatonin as a major skin protectant: from free radical scavenging to DNA damage repair. Exp Dermatol 17: 713-30

Fisher JW (2003)
Erythropoietin: physiology and pharmacology update. Exp Biol Med (Maywood) 228: 1-14

Fitzpatrick T, Bernhard J, Soter N (1987)
Correlation of Pathophysiology of the Skin. In: Fitzpatrick T, Eisen A, Wolff K, Freedberg I, Austen F (eds) Dermatology in General Medicine, 3rd edn. Mc Graw-Hill, New York, pp p69-73

Fitzpatrick TB, Breathnach AS (1963)
[the Epidermal Melanin Unit System.]. Dermatol Wochenschr 147: 481-9

Foitzik K, Krause K, Conrad F, Nakamura M, Funk W, Paus R (2006)
Human scalp hair follicles are both a target and a source of prolactin, which serves as an autocrine and/or paracrine promoter of apoptosis-driven hair follicle regression. Am J Pathol 168: 748-56

Foitzik K, Langan EA, Paus R (2009)
Prolactin and the skin: a dermatological perspective on an ancient pleiotropic peptide hormone. J Invest Dermatol 129: 1071-87

Foresta C, Mioni R, Bordon P, Miotto D, Montini G, Varotto A (1994)
Erythropoietin stimulates testosterone production in man. J Clin Endocrinol Metab 78: 753-6

Fritsch P (2009)
Dermatologie und Venerologie für das Studium. 1 edn. Springer Medizin Verlag, Heidelberg, pp 6-76

Fu P, Jiang X, Arcasoy MO (2009)
Constitutively active erythropoietin receptor expression in breast cancer cells promotes cellular proliferation and migration through a MAP-kinase dependent pathway. Biochem Biophys Res Commun

Galeano M, Altavilla D, Bitto A, Minutoli L, Calo M, Lo Cascio P, Polito F, Giugliano G, Squadrito G, Mioni C, Giuliani D, Venuti FS, Squadrito F (2006)
Recombinant human erythropoietin improves angiogenesis and wound healing in experimental burn wounds. Crit Care Med 34: 1139-46

Galeano M, Altavilla D, Cucinotta D, Russo GT, Calo M, Bitto A, Marini H, Marini R, Adamo EB, Seminara P, Minutoli L, Torre V, Squadrito F (2004)
Recombinant human erythropoietin stimulates angiogenesis and wound healing in the genetically diabetic mouse. Diabetes 53: 2509-17

Gaspar E, Hardenbicker C, Bodo E, Wenzel B, Ramot Y, Funk W, Kromminga A, Paus R (2010)
Thyrotropin releasing hormone (TRH): a new player in human hair-growth control. Faseb J 24: 393-403

Giardina B, Messana I, Scatena R, Castagnola M (1995)
The multiple functions of hemoglobin. Crit Rev Biochem Mol Biol 30: 165-96

Gorio A, Gokmen N, Erbayraktar S, Yilmaz O, Madaschi L, Cichetti C, Di Giulio AM, Vardar E, Cerami A, Brines M (2002)
Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma. Proc Natl Acad Sci U S A 99: 9450-5

Han R, Baden HP, Brissette JL, Weiner L (2002)
Redefining the skin's pigmentary system with a novel tyrosinase assay. Pigment Cell Res 15: 290-7

Hara M, Yaar M, Byers HR, Goukassian D, Fine RE, Gonsalves J, Gilchrest BA (2000)
Kinesin participates in melanosomal movement along melanocyte dendrites. J Invest Dermatol 114: 438-43

Hara M, Yaar M, Gilchrest BA (1995)
Endothelin-1 of keratinocyte origin is a mediator of melanocyte dendricity. J Invest Dermatol 105: 744-8

Harigae H, Takahashi S, Suwabe N, Ohtsu H, Gu L, Yang Z, Tsai FY, Kitamura Y, Engel JD, Yamamoto M (1998)
Differential roles of GATA-1 and GATA-2 in growth and differentiation of mast cells. Genes Cells 3: 39-50

Haroon ZA, Amin K, Jiang X, Arcasoy MO (2003)
A novel role for erythropoietin during fibrin-induced wound-healing response. Am J Pathol 163: 993-1000

Harris KW, Winkelmann JC (1996)
Enzyme-linked immunosorbent assay detects a potential soluble form of the erythropoietin receptor in human plasma. Am J Hematol 52: 8-13

Hearing VJ (1999)
Biochemical control of melanogenesis and melanosomal organization. J Investig Dermatol Symp Proc 4: 24-8

Heid CA, Stevens J, Livak KJ, Williams PM (1996)
Real time quantitative PCR. Genome Res 6: 986-94

Hesketh PJ, Batchelor D, Golant M, Lyman GH, Rhodes N, Yardley D (2004)
Chemotherapy-induced alopecia: psychosocial impact and therapeutic approaches. Support Care Cancer 12: 543-9

Hillhouse EW, Grammatopoulos DK (2006)
The molecular mechanisms underlying the regulation of the biological activity of corticotropin-releasing hormone receptors: implications for physiology and pathophysiology. Endocr Rev 27: 260-86

Hirobe T (2011)
How are proliferation and differentiation of melanocytes regulated? Pigment Cell Melanoma Res 24: 462-78

Holick MF (1994)
McCollum Award Lecture, 1994: vitamin D--new horizons for the 21st century. Am J Clin Nutr 60: 619-30

Holick MF (2010)
Vitamin D: extraskeletal health. Endocrinol Metab Clin North Am 39: 381-400, table of contents

Hunt G, Donatien PD, Lunec J, Todd C, Kyne S, Thody AJ (1994)
Cultured human melanocytes respond to MSH peptides and ACTH. Pigment Cell Res 7: 217-21

Iguchi M, Aiba S, Yoshino Y, Tagami H (2001)
Human follicular papilla cells carry out nonadipose tissue production of leptin. J Invest Dermatol 117: 1349-56

Ikawa M, Wada I, Kominami K, Watanabe D, Toshimori K, Nishimune Y, Okabe M (1997)
The putative chaperone calmegin is required for sperm fertility. Nature 387: 607-11

Imagawa S, Yamamoto M, Miura Y (1997)
Negative regulation of the erythropoietin gene expression by the GATA transcription factors. Blood 89: 1430-9

Isogai R, Takahashi M, Aisu K, Horiuti Y, Aragane Y, Kawada A, Tezuka T (2006)
The receptor for erythropoietin is present on cutaneous mast cells. Arch Dermatol Res 297: 389-94

Ito N, Ito T, Betterman A, Paus R (2004a)
The human hair bulb is a source and target of CRH. J Invest Dermatol 122: 235-7

Ito N, Ito T, Kromminga A, Bettermann A, Takigawa M, Kees F, Straub RH, Paus R (2005a)
Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol. Faseb J 19: 1332-4

Ito N, Sugawara K, Bodo E, Takigawa M, van Beek N, Ito T, Paus R (2010)
Corticotropin-releasing hormone stimulates the in situ generation of mast cells from precursors in the human hair follicle mesenchyme. J Invest Dermatol 130: 995-1004

Ito T, Ito N, Bettermann A, Tokura Y, Takigawa M, Paus R (2004b)
Collapse and restoration of MHC class-I-dependent immune privilege: exploiting the human hair follicle as a model. Am J Pathol 164: 623-34

Ito T, Ito N, Saathoff M, Bettermann A, Takigawa M, Paus R (2005b)
Interferon-gamma is a potent inducer of catagen-like changes in cultured human anagen hair follicles. Br J Dermatol 152: 623-31

Jacobs K, Shoemaker C, Rudersdorf R, Neill SD, Kaufman RJ, Mufson A, Seehra J, Jones SS, Hewick R, Fritsch EF, et al. (1985)
Isolation and characterization of genomic and cDNA clones of human erythropoietin. Nature 313: 806-10

Jacobson LO, Goldwasser E, Fried W, Plzak LF (1957)
Studies on erythropoiesis. VII. The role of the kidney in the production of erythropoietin. Trans Assoc Am Physicians 70: 305-17

Jahoda CA, Reynolds AJ (1996)
Dermal-epidermal interactions. Adult follicle-derived cell populations and hair growth. Dermatol Clin 14: 573-83

Jelkmann W (1992)
Erythropoietin: structure, control of production, and function. Physiol Rev 72: 449-89

Jelkmann W (2004)
Molecular biology of erythropoietin. Intern Med 43: 649-59

Jelkmann W (2007a)
Control of erythropoietin gene expression and its use in medicine. Methods Enzymol 435: 179-97

Jelkmann W (2007b)
Erythropoietin after a century of research: younger than ever. Eur J Haematol 78: 183-205

Jelkmann W (2008)
Developments in the therapeutic use of erythropoiesis stimulating agents. Br J Haematol 141: 287-97

Jelkmann W (2009)
Efficacy of recombinant erythropoietins: is there unity of international units? Nephrol Dial Transplant 24: 1366-8

Jelkmann W, Bohlius J, Hallek M, Sytkowski AJ (2008)
The erythropoietin receptor in normal and cancer tissues. Crit Rev Oncol Hematol 67: 39-61

Jelkmann WE (2011)
Regulation of erythropoietin production. J Physiol 589: 1251-8

Jubinsky PT, Krijanovski OI, Nathan DG, Tavernier J, Sieff CA (1997)
The beta chain of the interleukin-3 receptor functionally associates with the erythropoietin receptor. Blood 90: 1867-73

Juul SE, Yachnis AT, Christensen RD (1998)
Tissue distribution of erythropoietin and erythropoietin receptor in the developing human fetus. Early Hum Dev 52: 235-49

Kamil N, Kamil S, Ahmed SP, Ashraf R, Khurram M, Ali MO (2010)
Toxic effects of multiple anticancer drugs on skin. Pak J Pharm Sci 23: 7-14

Kang BM, Shin SH, Kwack MH, Shin H, Oh JW, Kim J, Moon C, Kim JC, Kim MK, Sung YK (2010)
Erythropoietin promotes hair shaft growth in cultured human hair follicles and modulates hair growth in mice. J Dermatol Sci 59: 86-90

Kapur R, Majumdar M, Xiao X, McAndrews-Hill M, Schindler K, Williams DA (1998)
Signaling through the interaction of membrane-restricted stem cell factor and c-kit receptor tyrosine kinase: genetic evidence for a differential role in erythropoiesis. Blood 91: 879-89

Kauser S, Slominski A, Wei ET, Tobin DJ (2006)
Modulation of the human hair follicle pigmentary unit by corticotropin-releasing hormone and urocortin peptides. Faseb J 20: 882-95

Kauser S, Thody AJ, Schallreuter KU, Gummer CL, Tobin DJ (2004)
beta-Endorphin as a regulator of human hair follicle melanocyte biology. J Invest Dermatol 123: 184-95

Kauser S, Thody AJ, Schallreuter KU, Gummer CL, Tobin DJ (2005)
A fully functional proopiomelanocortin/melanocortin-1 receptor system regulates the differentiation of human scalp hair follicle melanocytes. Endocrinology 146: 532-43

Kenouch S, Lombes M, Delahaye F, Eugene E, Bonvalet JP, Farman N (1994)
Human skin as target for aldosterone: coexpression of mineralocorticoid receptors and 11 beta-hydroxysteroid dehydrogenase. J Clin Endocrinol Metab 79: 1334-41

Khankin EV, Mutter WP, Tamez H, Yuan HT, Karumanchi SA, Thadhani R (2010)
Soluble erythropoietin receptor contributes to erythropoietin resistance in end- stage renal disease. PLoS One 5: e9246

Kiebert GM, Hanneke J, de Haes CJ, Kievit J, van de Velde CJ (1990)
Effect of peri-operative chemotherapy on the quality of life of patients with early breast cancer. Eur J Cancer 26: 1038-42

Kloepper JE, Sugawara K, Al-Nuaimi Y, Gaspar E, van Beek N, Paus R (2010)
Methods in hair research: how to objectively distinguish between anagen and catagen in human hair follicle organ culture. Exp Dermatol 19: 305-12

Kobayashi H, Kromminga A, Dunlop TW, Tychsen B, Conrad F, Suzuki N, Memezawa A, Bettermann A, Aiba S, Carlberg C, Paus R (2005)
A role of melatonin in neuroectodermal-mesodermal interactions: the hair follicle synthesizes melatonin and expresses functional melatonin receptors. Faseb J 19: 1710-2

Kono M, Nagata H, Umemura S, Kawana S, Osamura RY (2001)
In situ expression of corticotropin-releasing hormone (CRH) and proopiomelanocortin (POMC) genes in human skin. Faseb J 15: 2297-9

Koseoglu B, Yilmaz E, Ceylan K, Uzun E, Bayram I, Hizli F (2009)
The protective effect of erythropoietin infusion on testicular torsion/detorsion: an experimental study. Int Urol Nephrol

Koury ST, Bondurant MC, Koury MJ (1988)
Localization of erythropoietin synthesizing cells in murine kidneys by in situ hybridization. Blood 71: 524-7

Kumar SM, Acs G, Fang D, Herlyn M, Elder DE, Xu X (2005)
Functional erythropoietin autocrine loop in melanoma. Am J Pathol 166: 823-30

Kumar SM, Yu H, Fong D, Acs G, Xu X (2006)
Erythropoietin activates the phosphoinositide 3-kinase/Akt pathway in human melanoma cells. Melanoma Res 16: 275-83

Langan EA, Ramot Y, Goffin V, Griffiths CE, Foitzik K, Paus R (2010a)
Mind the (gender) gap: does prolactin exert gender and/or site-specific effects on the human hair follicle? J Invest Dermatol 130: 886-91

Langan EA, Ramot Y, Hanning A, Poeggeler B, Biro T, Gaspar E, Funk W, Griffiths CE, Paus R (2010b)
Thyrotropin-releasing hormone and oestrogen differentially regulate prolactin and prolactin receptor expression in female human skin and hair follicles in vitro. Br J Dermatol 162: 1127-31

LeBaron MJ, Ahonen TJ, Nevalainen MT, Rui H (2007)
In vivo response-based identification of direct hormone target cell populations using high-density tissue arrays. Endocrinology 148: 989-1008

Leder LD (1964)
[on the Selective Enzyme-Cytochemical Demonstration of Neutrophilic Myeloid Cells and Tissue Mast Cells in Paraffin Sections.]. Klin Wochenschr 42: 553

Leder LD (1970)
Diagnostic experiences with the naphthol AS-D chloroacetate esterase reaction. Blut 21: 1-8

Leder LD (1979)
The chloroacetate esterase reaction. A useful means of histological diagnosis of hematological disorders from paraffin sections of skin. Am J Dermatopathol 1: 39-42

Lee JH, Wang, LC, Yu HH, Lin YT, Yang YH, Chiang BL (2010)
Type I IL-1 receptor (IL-1RI) as potential new therapeutic target for bronchial asthma. Mediators Inflamm 2010: 567351

Lerner AB, Fitzpatrick TB (1950)
Biochemistry of melanin formation. Physiol Rev 30: 91-126

Lin FK, Suggs S, Lin CH, Browne JK, Smalling R, Egrie JC, Chen KK, Fox GM, Martin F, Stabinsky Z, et al. (1985)
Cloning and expression of the human erythropoietin gene. Proc Natl Acad Sci U S A 82: 7580-4

Lin JY, Fisher DE (2007)
Melanocyte biology and skin pigmentation. Nature 445: 843-50

Liu TC, Chang JG, Lin CP, Lee LS, Lin SF, Liu HW, Chen TP (1990)
Detection of beta-globin gene from single hairs. Gaoxiong Yi Xue Ke Xue Za Zhi 6: 181-5

Livak KJ, Schmittgen TD (2001)
Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-8

Lu Z, Fischer TW, Hasse S, Sugawara K, Kamenisch Y, Krengel S, Funk W, Berneburg M, Paus R (2009)
Profiling the response of human hair follicles to ultraviolet radiation. J Invest Dermatol 129: 1790-804

Lu Z, Hasse S, Bodo E, Rose C, Funk W, Paus R (2007)
Towards the development of a simplified long-term organ culture method for human scalp skin and its appendages under serum-free conditions. Exp Dermatol 16: 37-44

Madison KC (2003)
Barrier function of the skin: "la raison d'etre" of the epidermis. J Invest Dermatol 121: 231-41

Maurer M, Peters EM, Botchkarev VA, Paus R (1998)
Intact hair follicle innervation is not essential for anagen induction and development. Arch Dermatol Res 290: 574-8

Maurer M, Wedemeyer J, Metz M, Piliponsky AM, Weller K, Chatterjea D, Clouthier DE, Yanagisawa MM, Tsai M, Galli SJ (2004)
Mast cells promote homeostasis by limiting endothelin-1-induced toxicity. Nature 432: 512-6

McKee P (2005)
The structure and function of the skin. In: McKee P.H. CE, Granter S.R. (ed) Pathology of the Skin with clinical correlations, 3rd edn. Elsevier Mosby, Philadelphia, Pa, pp 1-36

Mecklenburg L, Tobin DJ, Muller-Rover S, Handjiski B, Wendt G, Peters EM, Pohl S, Moll I, Paus R (2000)
Active hair growth (anagen) is associated with angiogenesis. J Invest Dermatol 114: 909-16

Miot LD, Miot HA, Silva MG, Marques ME (2009)
Physiopathology of melasma. An Bras Dermatol 84: 623-35

Mirmohammadsadegh A, Marini A, Gustrau A, Delia D, Nambiar S, Hassan M, Hengge UR (2010)
Role of erythropoietin receptor expression in malignant melanoma. J Invest Dermatol 130: 201-10

Miyake T, Kung CK, Goldwasser E (1977)
Purification of human erythropoietin. J Biol Chem 252: 5558-64

Moll I (2010)
Duale Reihe: Dermatologie. 7 edn. Georg Thieme Verlag, Stuttgart, pp 3-15

Moon C, Krawczyk M, Ahn D, Ahmet I, Paik D, Lakatta EG, Talan MI (2003)
Erythropoietin reduces myocardial infarction and left ventricular functional decline after coronary artery ligation in rats. Proc Natl Acad Sci U S A 100: 11612-7

Morishita E, Narita H, Nishida M, Kawashima N, Yamagishi K, Masuda S, Nagao M, Hatta H, Sasaki R (1996)
Anti-erythropoietin receptor monoclonal antibody: epitope mapping, quantification of the soluble receptor, and detection of the solubilized transmembrane receptor and the receptor-expressing cells. Blood 88: 465-71

Murad A, Nath AK, Cha ST, Demir E, Flores-Riveros J, Sierra-Honigmann MR (2003)
Leptin is an autocrine/paracrine regulator of wound healing. Faseb J 17: 1895-7

Murata M, Suzuki T, Midorikawa K, Oikawa S, Kawanishi S (2004)
Oxidative DNA damage induced by a hydroperoxide derivative of cyclophosphamide. Free Radic Biol Med 37: 793-802

Muro Y, Okabe M (2010)
Mechanisms of Fertilization--A View from the Perspective of Gene Manipulated Mice. J Androl

Ogilvie M, Yu X, Nicolas-Metral V, Pulido SM, Liu C, Ruegg UT, Noguchi CT (2000)
Erythropoietin stimulates proliferation and interferes with differentiation of myoblasts. J Biol Chem 275: 39754-61

Ohnemus U, Uenalan M, Inzunza J, Gustafsson JA, Paus R (2006)
The hair follicle as an estrogen target and source. Endocr Rev 27: 677-706

Oliveira IO, Junior HLA (2003)
Current knowledge on the biology of melanocytes in the human hair follicle. An bras Dermatol 78: 331-43

Pabon JE, Bird JS, Li X, Huang ZH, Lei ZM, Sanfilippo JS, Yussman MA, Rao CV (1996)
Human skin contains luteinizing hormone/chorionic gonadotropin receptors. J Clin Endocrinol Metab 81: 2738-41

Park HY, Kosmadaki M, Yaar M, Gilchrest BA (2009)
Cellular mechanisms regulating human melanogenesis. Cell Mol Life Sci 66: 1493-506

Parsa CJ, Matsumoto A, Kim J, Riel RU, Pascal LS, Walton GB, Thompson RB, Petrofski JA, Annex BH, Stamler JS, Koch WJ (2003)
A novel protective effect of erythropoietin in the infarcted heart. J Clin Invest 112: 999-1007

Paus R (2010)
Exploring the "thyroid-skin connection": concepts, questions, and clinical relevance. J Invest Dermatol 130: 7-10

Paus R, Arck P, Tiede S (2008)
(Neuro-)endocrinology of epithelial hair follicle stem cells. Mol Cell Endocrinol 288: 38-51

Paus R, Bodo E, Kromminga A, Jelkmann W (2009)
Erythropoietin and the skin: a role for epidermal oxygen sensing? Bioessays 31: 344-8

Paus R, Cotsarelis G (1999)
The biology of hair follicles. N Engl J Med 341: 491-7

Paus R, Foitzik K (2004)
In search of the "hair cycle clock": a guided tour. Differentiation 72: 489-511

Paus R, Peker S, Sundberg JP (2007)
Biology of Hair and Nails. In: Bolognia J.L. JJL, Rapini R.P. (ed) Dermatology, 2nd edition edn. Mosby, London, pp 1007-32

Paus R, Peters EM, Eichmuller S, Botchkarev VA (1997)
Neural mechanisms of hair growth control. J Investig Dermatol Symp Proc 2: 61-8

Pelletier G, Ren L (2004)
Localization of sex steroid receptors in human skin. Histol Histopathol 19: 629-36

Peters EM, Botchkarev VA, Botchkareva NV, Tobin DJ, Paus R (2001)
Hair-cycle-associated remodeling of the peptidergic innervation of murine skin, and hair growth modulation by neuropeptides. J Invest Dermatol 116: 236-45

Peters EM, Liotiri S, Bodo E, Hagen E, Biro T, Arck PC, Paus R (2007)
Probing the effects of stress mediators on the human hair follicle: substance P holds central position. Am J Pathol 171: 1872-86

Peters EM, Tobin DJ, Botchkareva N, Maurer M, Paus R (2002)
Migration of melanoblasts into the developing murine hair follicle is accompanied by transient c-Kit expression. J Histochem Cytochem 50: 751-66

Phillips BT, Gassei K, Orwig KE (2010)
Spermatogonial stem cell regulation and spermatogenesis. Philos Trans R Soc Lond B Biol Sci 365: 1663-78

Philpott MP, Green MR, Kealey T (1990)
Human hair growth in vitro. J Cell Sci 97 (Pt 3): 463-71

Philpott MP, Sanders DA, Kealey T (1996)
Whole hair follicle culture. Dermatol Clin 14: 595-607

Plonka PM, Passeron T, Brenner M, Tobin DJ, Shibahara S, Thomas A, Slominski A, Kadekaro AL, Hershkovitz D, Peters E, Nordlund JJ, Abdel-Malek Z, Takeda K, Paus R, Ortonne JP, Hearing VJ, Schallreuter KU (2009)
What are melanocytes really doing all day long...? Exp Dermatol 18: 799-819

Poeggeler B, Schulz C, Pappolla MA, Bodo E, Tiede S, Lehnert H, Paus R (2010)
Leptin and the skin: a new frontier. Exp Dermatol 19: 12-8

Powell BC, Rogers GE (1997)
The role of keratin proteins and their genes in the growth, structure and properties of hair. Exs 78: 59-148

Proksch E, Brandner JM, Jensen JM (2008)
The skin: an indispensable barrier. Exp Dermatol 17: 1063-72

Prunier F, Pottier P, Clairand R, Mercier A, Hajjar RJ, Planchon B, Furber A (2009)
Chronic erythropoietin treatment decreases post-infarct myocardial damage in rats without venous thrombogenic effect. Cardiology 112: 129-34

Reifenberger J, Ruzicka T (2010)
Haut und Hautanhangsorgane. In: Zilles K, Tillmann B (eds) Anatomie, 1st edn. Springer Medizin Verlag, Heidelberg, pp 587-98

Reithmayer K, Meyer KC, Kleditzsch P, Tiede S, Uppalapati SK, Glaser R, Harder J, Schroder JM, Paus R (2009)
Human hair follicle epithelium has an antimicrobial defence system that includes the inducible antimicrobial peptide psoriasin (S100A7) and RNase 7. Br J Dermatol 161: 78-89

Remy I, Wilson IA, Michnick SW (1999)
Erythropoietin receptor activation by a ligand-induced conformation change. Science 283: 990-3

Ribatti D, Presta M, Vacca A, Ria R, Giuliani R, Dell'Era P, Nico B, Roncali L, Dammacco F (1999)
Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. Blood 93: 2627-36

Romero-Graillet C, Aberdam E, Clement M, Ortonne JP, Ballotti R (1997)
Nitric oxide produced by ultraviolet-irradiated keratinocytes stimulates melanogenesis. J Clin Invest 99: 635-42

Roosterman D, Goerge T, Schneider SW, Bunnett NW, Steinhoff M (2006)
Neuronal control of skin function: the skin as a neuroimmunoendocrine organ. Physiol Rev 86: 1309-79

Rossert J, Eckardt KU (2005)
Erythropoietin receptors: their role beyond erythropoiesis. Nephrol Dial Transplant 20: 1025-8

Sakanaka M, Wen TC, Matsuda S, Masuda S, Morishita E, Nagao M, Sasaki R (1998)
In vivo evidence that erythropoietin protects neurons from ischemic damage. Proc Natl Acad Sci U S A 95: 4635-40

Schechter AN (2008)
Hemoglobin research and the origins of molecular medicine. Blood 112: 3927-38

Schiebler T, Korf H-W (2007)
Anatomie. 10 edn. Steinkopff Verlag, pp 213-26

Schneider MR, Schmidt-Ullrich R, Paus R (2009)
The hair follicle as a dynamic miniorgan. Curr Biol 19: R132-42

Scott G, Leopardi S, Printup S, Malhi N, Seiberg M, Lapoint R (2004)
Proteinase-activated receptor-2 stimulates prostaglandin production in keratinocytes: analysis of prostaglandin receptors on human melanocytes and effects of PGE2 and PGF2alpha on melanocyte dendricity. J Invest Dermatol 122: 1214-24

Semenza GL (2004)
Hydroxylation of HIF-1: oxygen sensing at the molecular level. Physiology (Bethesda) 19: 176-82

Semenza GL (2008)
O2 sensing: only skin deep? Cell 133: 206-8

Semenza GL (2009)
Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology (Bethesda) 24: 97-106

Sharov AA, Siebenhaar F, Sharova TY, Botchkareva NV, Gilchrest BA, Botchkarev VA (2004)
Fas signaling is involved in the control of hair follicle response to chemotherapy. Cancer Res 64: 6266-70

Siebenhaar F, Sharov AA, Peters EM, Sharova TY, Syska W, Mardaryev AN, Freyschmidt-Paul P, Sundberg JP, Maurer M, Botchkarev VA (2007a)
Substance P as an immunomodulatory neuropeptide in a mouse model for autoimmune hair loss (alopecia areata). J Invest Dermatol 127: 1489-97

Siebenhaar F, Syska W, Weller K, Magerl M, Zuberbier T, Metz M, Maurer M (2007b)
Control of Pseudomonas aeruginosa skin infections in mice is mast cell-dependent. Am J Pathol 170: 1910-6

Simard M, Manthos H, Giaid A, Lefebvre Y, Goodyer CG (1996)
Ontogeny of growth hormone receptors in human tissues: an immunohistochemical study. J Clin Endocrinol Metab 81: 3097-102

Sinclair AM, Coxon A, McCaffery I, Kaufman S, Paweletz K, Liu L, Busse L, Swift S, Elliott S, Begley CG (2010)
Functional erythropoietin receptor is undetectable in endothelial, cardiac, neuronal, and renal cells. Blood 115: 4264-72

Singh SK, Nizard C, Kurfurst R, Bonte F, Schnebert S, Tobin DJ (2008)
The silver locus product (Silv/gp100/Pmel17) as a new tool for the analysis of melanosome transfer in human melanocyte-keratinocyte co-culture. Exp Dermatol 17: 418-26

Siren AL, Fasshauer T, Bartels C, Ehrenreich H (2009)
Therapeutic potential of erythropoietin and its structural or functional variants in the nervous system. Neurotherapeutics 6: 108-27

Slominski A (2005)
Neuroendocrine system of the skin. Dermatology 211: 199-208

Slominski A, Malarkey WB, Wortsman J, Asa SL, Carlson A (2000a)
Human skin expresses growth hormone but not the prolactin gene. J Lab Clin Med 136: 476-81

Slominski A, Paus R (1993)
Melanogenesis is coupled to murine anagen: toward new concepts for the role of melanocytes and the regulation of melanogenesis in hair growth. J Invest Dermatol 101: 90S-7S

Slominski A, Tobin DJ, Shibahara S, Wortsman J (2004)
Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol Rev 84: 1155-228

Slominski A, Wortsman J (2000)
Neuroendocrinology of the skin. Endocr Rev 21: 457-87

Slominski A, Wortsman J, Kohn L, Ain KB, Venkataraman GM, Pisarchik A, Chung JH, Giuliani C, Thornton M, Slugocki G, Tobin DJ (2002)
Expression of hypothalamic-pituitary-thyroid axis related genes in the human skin. J Invest Dermatol 119: 1449-55

Slominski A, Wortsman J, Luger T, Paus R, Solomon S (2000b)
Corticotropin releasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. Physiol Rev 80: 979-1020

Slominski A, Wortsman J, Paus R, Elias PM, Tobin DJ, Feingold KR (2008)
Skin as an endocrine organ: implications for its function. Drug Discov Today Dis Mech 5: 137-44

Slominski A, Wortsman J, Plonka PM, Schallreuter KU, Paus R, Tobin DJ (2005)
Hair follicle pigmentation. J Invest Dermatol 124: 13-21

Slominski A, Wortsman J, Tuckey RC, Paus R (2007)
Differential expression of HPA axis homolog in the skin. Mol Cell Endocrinol 265-266: 143-9

Slominski AT, Botchkarev V, Choudhry M, Fazal N, Fechner K, Furkert J, Krause E, Roloff B, Sayeed M, Wei E, Zbytek B, Zipper J, Wortsman J, Paus R (1999)
Cutaneous expression of CRH and CRH-R. Is there a "skin stress response system?" Ann N Y Acad Sci 885: 287-311

Smith TG, Robbins PA, Ratcliffe PJ (2008)
The human side of hypoxia-inducible factor. Br J Haematol 141: 325-34

Smoller B, Rongioletti F (2010)
Clinical and Pathological Aspects of Skin Diseases in Endocrine, Metabolic, Nutritional and Deposition Disease. In: Smoller B, Rongioletti F (eds). Springer, Berlin

Soliz J, Gassmann M, Joseph V (2007)
Soluble erythropoietin receptor is present in the mouse brain and is required for the ventilatory acclimatization to hypoxia. J Physiol 583: 329-36

Stenn KS, Paus R (2001)
Controls of hair follicle cycling. Physiol Rev 81: 449-94

Stockmann C, Fandrey J (2006)
Hypoxia-induced erythropoietin production: a paradigm for oxygen-regulated gene expression. Clin Exp Pharmacol Physiol 33: 968-79

Sugawa M, Sakurai Y, Ishikawa-Ieda Y, Suzuki H, Asou H (2002)
Effects of erythropoietin on glial cell development; oligodendrocyte maturation and astrocyte proliferation. Neurosci Res 44: 391-403

Swift S, Ellison AR, Kassner P, McCaffery I, Rossi J, Sinclair AM, Begley CG, Elliott S (2010)
Absence of functional EpoR expression in human tumor cell lines. Blood 115: 4254-63

Syed RS, Reid SW, Li C, Cheetham JC, Aoki KH, Liu B, Zhan H, Osslund TD, Chirino AJ, Zhang J, Finer-Moore J, Elliott S, Sitney K, Katz BA, Matthews DJ, Wendoloski JJ, Egrie J, Stroud RM (1998)
Efficiency of signalling through cytokine receptors depends critically on receptor orientation. Nature 395: 511-6

Tabata M, Tarumoto T, Ohmine K, Furukawa Y, Hatake K, Ozawa K, Hasegawa Y, Mukai H, Yamamoto M, Imagawa S (2001)
Stimulation of GATA-2 as a mechanism of hydrogen peroxide suppression in hypoxia-induced erythropoietin gene expression. J Cell Physiol 186: 260-7

Takamizawa A, Matsumoto S, Iwata T, Tochino Y, Katagiri K, Yamaguchi K (1975)
Studies on cyclophosphamide metabolites and their related compounds. 2. Preparation of an active species of cyclophosphamide and related compounds. J Med Chem 18: 376-83

Tauchi T, Feng GS, Shen R, Hoatlin M, Bagby GC, Jr., Kabat D, Lu L, Broxmeyer HE (1995)
Involvement of SH2-containing phosphotyrosine phosphatase Syp in erythropoietin receptor signal transduction pathways. J Biol Chem 270: 5631-5

Tobin DJ (2008)
Human hair pigmentation--biological aspects. Int J Cosmet Sci 30: 233-57

Tobin DJ (2011)
The cell biology of human hair follicle pigmentation. Pigment Cell Melanoma Res 24: 75-88

Tobin DJ, Slominski A, Botchkarev V, Paus R (1999)
The fate of hair follicle melanocytes during the hair growth cycle. J Investig Dermatol Symp Proc 4: 323-32

Tovari J, Pirker R, Timar J, Ostoros G, Kovacs G, Dome B (2008)
Erythropoietin in cancer: an update. Curr Mol Med 8: 481-91

Tsiftsoglou AS, Vizirianakis IS, Strouboulis J (2009)
Erythropoiesis: model systems, molecular regulators, and developmental programs. IUBMB Life 61: 800-30

van Beek N, Bodo E, Kromminga A, Gaspar E, Meyer K, Zmijewski MA, Slominski A, Wenzel BE, Paus R (2008)
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: 4381-8

van Lith M, Karala AR, Bown D, Gatehouse JA, Ruddock LW, Saunders PT, Benham AM (2007)
A developmentally regulated chaperone complex for the endoplasmic reticulum of male haploid germ cells. Mol Biol Cell 18: 2795-804

van Wijngaarden P, Brereton HM, Coster DJ, Williams KA (2007)
Genetic influences on susceptibility to oxygen-induced retinopathy. Invest Ophthalmol Vis Sci 48: 1761-6

Vancoillie G, Lambert J, Mulder A, Koerten HK, Mommaas AM, Van Oostveldt P, Naeyaert JM (2000)
Kinesin and kinectin can associate with the melanosomal surface and form a link with microtubules in normal human melanocytes. J Invest Dermatol 114: 421-9

Vancoillie G, Lambert J, Nayaert JM (1999)
Melanocyte biology and its implications for the clinician. Eur J Dermatol 9: 241-51

Wang J, Lu Z, Au JL (2006)
Protection against chemotherapy-induced alopecia. Pharm Res 23: 2505-14

Watanabe D, Okabe M, Hamajima N, Morita T, Nishina Y, Nishimune Y (1995)
Characterization of the testis-specific gene 'calmegin' promoter sequence and its activity defined by transgenic mouse experiments. FEBS Lett 368: 509-12

Watanabe D, Yamada K, Nishina Y, Tajima Y, Koshimizu U, Nagata A, Nishimune Y (1994)
Molecular cloning of a novel Ca(2+)-binding protein (calmegin) specifically expressed during male meiotic germ cell development. J Biol Chem 269: 7744-9

Weiss MJ, Keller G, Orkin SH (1994)
Novel insights into erythroid development revealed through in vitro differentiation of GATA-1 embryonic stem cells. Genes Dev 8: 1184-97

Westenfelder C, Baranowski RL (2000)
Erythropoietin stimulates proliferation of human renal carcinoma cells. Kidney Int 58: 647-57

White G, Cox N (2006)
Fundamental Dermatology and Terminology. Diseases of the Skin a Color Atlas and Text, 2nd edn. Elsevier Mosby, Philadelphia, Pa, pp 1-10

Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, Wood SM, Gatter KC, Harris AL, Pugh CW, Ratcliffe PJ, Maxwell PH (1998)
Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. Blood 92: 2260-8

Wolff H (2005)
Einführung. In: Braun-Falco O, Plewig G, Wolff H, Burgdorf W, Landthaler M (eds) Dermatologie und Venerologie, 5 edn. Springer Medizin Verlag, Heidelberg, pp 3-21

Wong E, Morgan EW, MacDonald DM (1982)
The chloroacetate esterase reaction for mast cells in dermatopathology: a comparison with metachromatic staining methods. Acta Derm Venereol 62: 431-4

Wong ML, Medrano JF (2005)
Real-time PCR for mRNA quantitation. Biotechniques 39: 75-85

Wu H, Lee SH, Gao J, Liu X, Iruela-Arispe ML (1999)
Inactivation of erythropoietin leads to defects in cardiac morphogenesis. Development 126: 3597-605

Yamagata K, Nakanishi T, Ikawa M, Yamaguchi R, Moss SB, Okabe M (2002)
Sperm from the calmegin-deficient mouse have normal abilities for binding and fusion to the egg plasma membrane. Dev Biol 250: 348-57

Yamaguchi Y, Hearing VJ (2009)
Physiological factors that regulate skin pigmentation. Biofactors 35: 193-9

Yano K, Brown LF, Detmar M (2001)
Control of hair growth and follicle size by VEGF-mediated angiogenesis. J Clin Invest 107: 409-17

Yoshida M, Takahashi Y, Inoue S (2000)
Histamine induces melanogenesis and morphologic changes by protein kinase A activation via H2 receptors in human normal melanocytes. J Invest Dermatol 114: 334-42

Yoshinaga K, Tanii I, Toshimori K (1999)
Molecular chaperone calmegin localization to the endoplasmic reticulum of meiotic and post-meiotic germ cells in the mouse testis. Arch Histol Cytol 62: 283-93

Youssoufian H, Longmore G, Neumann D, Yoshimura A, Lodish HF (1993)
Structure, function, and activation of the erythropoietin receptor. Blood 81: 2223-36

Zeng QB, Cheng FJ, Zhang WG, Tang JM, Chen L, Liu QH, Gao QP, Wang JN (2008)
Erythropoietin promotes proliferation of human bone marrow mesenchymal stem cells in vitro. Zhongguo Shi Yan Xue Ye Xue Za Zhi 16: 1392-7

Zilles K, Tillmann B (2010)
Anatomie. 1st edn. Springer Medizin Verlag, Heidelberg

Zou H, Hu L, Li J, Zhan S, Cao K (2006)
Cloning and characterization of a novel small monomeric GTPase, RasL10B, with tumor suppressor potential. Biotechnol Lett 28: 1901-8

Zouboulis CC (2004)
The human skin as a hormone target and an endocrine gland. Hormones (Athens) 3: 9-26

Zouboulis CC (2009)
The skin as an endocrine organ. Dermatoendocrinol 1: 250-2

Zouboulis CC, Chen WC, Thornton MJ, Qin K, Rosenfield R (2007)
Sexual hormones in human skin. Horm Metab Res 39: 85-95

8 Danksagung

An dieser Stelle möchte ich allen danken, die zum Gelingen dieser Arbeit beigetragen haben.

Insbesondere bedanke ich mich bei meinem Doktorvater Prof. Dr. Ralf Paus für die Vergabe des Themas und für die Unterstützung und Hilfestellung beim Erstellen dieser Arbeit und bei meiner engagierten Betreuerin Dr. Enikö Bodó, die mir in allen Phasen der Dissertation immer zur Seite stand. Außerdem danke ich dem Klinikdirektor Prof. Dr. Zillikens.

Für das tolle Arbeitsklima, die Unterstützung in allen Bereichen und die schönen Momente innerhalb und außerhalb des Labors danke ich allen weiteren Mitgliedern der Arbeitsgruppe Paus. Sie alle haben den experimentellen Teil der Dissertation zu einem unvergesslichen Erlebnis gemacht: Astrid Becker, Nina van Beek, Nadine Dörwald, Nancy Ernst, Dr. Tobias Fischer, Lütfiye Hastedt, Dr. Elisabeth Gáspár, Annika Hanning, Céline Hardenbicker, Benedikt Kany, Patrick Kleditzsch, Dr. Jennifer Klöpper, Jana Knuever, Dorothee Langan, Dr. Katja Meyer, Viktoria Naumann, Christian Plate, Dr. Burkhard Pöggeler, Dr. Yuval Ramot, Dr. Katrin Reithmayer, Gabriele Scheel, Anne Spiegelberger, Dr. Koji Sugawara, Dr. Stefan Tiede und Antje Winter-Keil.

Für ihre immerwährende Unterstützung beim Studium und der Doktorarbeit sowie in allen Lebenslagen danke ich meinen lieben Eltern Hannelore und Jacob und meinen lieben Geschwistern Maarten, Caroline, Simon und Margarete.

Für die sprachlichen Korrekturen bin ich meinem Bruder Maarten, meiner Mutter und Julian sehr dankbar.

Außerdem danke ich meinen Freunden Anna, Anne, Cordula, Omidreza, Sonja und Shahpour, die mich während des gesamten Studiums, vor allem aber auch beim Erstellen dieser Arbeit immer wieder unterstützt haben – sowohl moralisch als auch mit Rat und Tat.

9 Lebenslauf

An dieser Stelle erscheint in der gedruckten Version der Lebenslauf.

10 Publications

Parts of this thesis were published in:

Articles:

Bodo E, **Wiersma F**, Funk W, Kromminga A, Jelkmann W, Paus R **(2010)**
Does erythropoietin modulate human hair follicle melanocyte activities in situ?
Exp Dermatol. 19 (1): 65-67, 2010 Jan

Abstract (ADF Tagung 2008):

Wiersma F, Bodo E, Kromminga A, Funk W, Jelkmann W, Paus R **(2008)**
Searching for new pointers to non-classical functions of erythropoietin (EPO) in human skin and hair biology
Exp. Dermatol. 17, 242- 290, 2008