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“The pyogenic cocci, staphylococci and streptococci are constantly found on human skin, most frequently hidden in the depths of the canals of hair follicles. These micro-organisms seize every opportunity to attack the organism, producing such local lesions as acne, pimples, boils and erisipelas [SIC] or even becoming generalized in the blood and tissues as in the septicemias and pyemias. To the skin, therefore, must be assigned a very important function in the prevention of the invasion of micro-organisms, which are found constantly on the surface of the body…”(Metchnikoff 1905)

TABLE OF CONTENTS

FIGURES

TABLES

ABBREVIATIONS

α –MSH	alpha-melanocyte stimulating hormone
ACTH	adrenocorticotropic hormone
ABC	avidin-biotin complex
APM	arrector pili muscle
AMP	antimicrobial peptide
B	bulge
bp	base pair
CCE	cornified cell envelope
CD	cluster of differentiation
CL	companion layer
CTS	connective tissue sheath
DCD	dermcidin
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotide-5'-triphosphate
DP	dermal papilla
E.co.	*Escherichia coli*
EDC	epidermal differentiation complex
EDTA	ethylene-diaminetetraacetate
EP	epidermis
FCS	fetal calf serum
Fig.	figure
FT	follicular trochanter
FPRL	formyl peptide receptor like
GM-CSF	granulocyte macrophage colony-stimulating factor
HBD	human beta-defensin
HF	hair follicle
HIS	hair follicle immune system
HS	hair shaft
IFNγ	interferon-gamma
IgG	immunoglobulin G
IL	interleukin

IR	immunoreactivity
IRS	inner root sheath
LEKTI	Lympho-Epithelial Kazal-Type related Inhibitor
LBP	LPS-binding protein
LPS	lipopolysaccharides
LTA	lipoteichoic acid
mRNA	messenger ribonucleic acid
MHC	major histocompatibility complex
min	minutes
NaCl	sodium chloride
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
ORS	outer root sheath
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Ps.ae.	*Pseudomonas aeruginosa*
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcription
SEM	standard error of the mean
SG	sebaceous gland
S100	soluble at 100% ammonium sulphate at neutral pH
S100A7	psoriasin
SIS	skin immune system
spp.	species
SPINK	Serine Protease Inhibitor Kazal-type
TAE	tris-acetate-EDTA
TBS	tris buffered saline
TIR	Toll-IL-1 receptor
TLR	Toll-like receptor
TNFα	tumour-necrosis-factor-alpha
TSA	tyramide signal amplification
UV	ultraviolet

1 INTRODUCTION

Hair follicles constitute defining features of mammalian species. At the same time the presence of these skin appendages provides major ports of microbial invasion into the mammalian organism, since they interrupt the integrity of the epidermal barrier. Furthermore, the follicular canal itself is very densely colonized by various organisms, whose growth and composition must be kept under tight control in order to guarantee that this component of the skin microflora remains constrained to its physiologically desired activities. It is therefore of pre-eminent importance that the hair follicle epithelium, specifically the distal part of the outer root sheath establishes an effective system of antimicrobial defence and microflora containment as vital part of the skin's innate immune system.

However, in contrast to its cellular protagonists and elements of the acquired hair follicle immune system, the innate hair follicle immune system (iHIS) remains very ill-characterized. Even though a few publications have already provided pointers to important elements of the iHIS in mice and man, for example by demonstrating that the human hair follicle epithelium shows immunoreactivity of the antimicrobial peptides (AMP) human ß-defensin-1 and -2 (Chronnell et al. 2001) and psoriasin (Gläser et al. 2005), the iHIS of human hair follicles remains to be systematically characterized.

The current thesis project attempts to contribute to this endeavour by focussing on two well-characterized AMPs of human skin, psoriasin and RNase7, and on two most recently discovered novel AMPs, hornerin and LEKTI2 (see below). In order to do so, a close collaboration was established between a leading hair research laboratory (Experimental Dermatology Unit, University of Lübeck), where this project was executed under the guidance of Prof. Dr. R. Paus, Prof. Dr. J.-M- Schröder and PD Dr. R. Gläser of the Department of Dermatology, University of Kiel, who had discovered these AMPs in human skin.

In the following, I shall first provide basic background on hair follicle biology and immunology as well as on the biology and functions of AMPs, which is necessary for understanding the experimental design that was chosen to address the following specific questions:

2 SPECIFIC QUESTIONS ADDRESSED

1. Are AMPs like RNase 7, psoriasin, hornerin and LEKTI-2, which are important for the antimicrobial defence of the epidermis, expressed in the human hair follicle?

2. If yes, in which compartments of the human hair follicle are they expressed?

3. Can AMPs in the human hair follicle be stimulated by microbial products like lipopolysaccharides (LPS), lipoteichoic acid (LTA) or protein A?

4. Does the expression pattern change upon stimulation?

5. Does the proinflammatory cytokine interferon-γ (IFNγ) influence the expression of these distinct AMPs in the human hair follicle?

6. Do isolated cells of the human hair follicle express these AMPs on the protein and the mRNA level?

3 LITERATURE

3.1 Overview over hair follicle biology

The key features of mammals include, beside the name giving mammary gland, also the hair coat. This epidermal product has a long evolutionary history. Basically the hair shaft is a further development of the scales of fish and reptiles and is closely related to nails (Paus et al. 1994a). Human hair seems to be rather meaningless in comparison to the protective and warming fur of animals. However, its crucial role in psychosocial and sexual signalling should not be underestimated, as the external signs of well being, social rank and sexual attractiveness are very closely related to the visual appearance especially of the scalp hair. In addition, the hair follicle still is important for UV-light protection, sensory functions and even serves as a stem cell reservoir for keratinocytes and melanocytes (Paus et al. 2008b).

3.1.1 Functional anatomy of the human hair follicle

Over 5 million hair follicles cover the human body, of which about 100 000 are found on the scalp. One can distinguish between three main types of hair follicles: the lanugo, the vellus and the terminal hair follicle. The lanugo hair is very fine and normally shed in utero or during the first weeks of life. Vellus hairs are very short, non-pigmented, normally not medullated and lack the presence of the arrector pili muscle. They are found all over the body surface with the highest density in the cheek and forehead where they in addition can display extremely large sebaceous glands. They undergo full hair cycle, yet much shorter than in terminal hair. Terminal hair is the large usually pigmented and medullated hair which is predominantly found on the scalp. Apart from these differences, all hair follicles share the same functional construction (Paus et al. 2008b; Dawber 1997).

The mature anagen hair follicle resembles an inverted wine glass in whose opening an onion like structure, the dermal papilla, has been placed. This construction is due to the hair follicle’s function as a highly effective factory producing durable, pigmented hair shafts of enormous tensile strength, which must be guided safely to the skin surface without entering into the surrounding dermis. In case the hair shaft gets in contact with the dermis, infections

are the consequence, as it can be seen in ingrown hairs. To ensure the directional growth several multilayered sheaths serve as guiding structures and slippage planes. A hardened cylinder of terminally differentiated keratinocytes, the inner root sheath (IRS), guides and packages the central hair shaft (HS). Using the companion layer (CL) as a slippage plane the IRS moves outwards together with the HS guided by the cells of the outer root sheath (ORS) (Paus et al. 2008b).

The human hair follicle is closely associated with sebaceous glands (forming the pilosebaceous unit) and/or with apocrine glands. It can be divided into the hair root and the lower follicle which constitute the impermanent part of the hair follicle and the permanent part, the isthmus and the infundibulum. One can define these areas by using the guiding structures of the orifice of the sebaceous gland duct and the insertion of the arrector pili muscle (APM) (Whiting 2004) (**Fig. 3.1**).

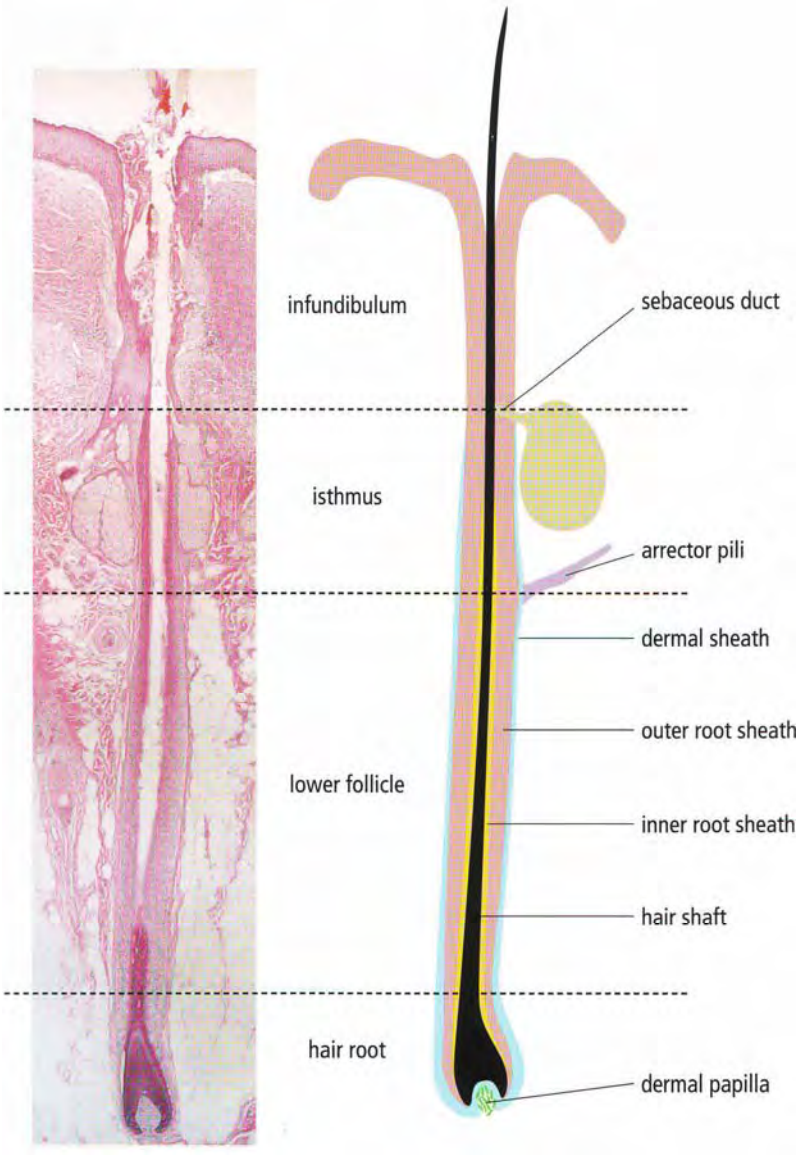

Fig. 3.1 Structure of the human anagen hair follicle in a longitudinal view
On the left a haematoxylin/eosin staining and on the right a schematic drawing (Whiting 2004).

Embedded in the mesenchymal connective tissue sheath (CTS) the epithelial hair follicle compartment consists of at least eight concentric cylinders: ORS (basal layer and suprabasal layers), companion layer, Henle's layer, Huxley`s layer and the cuticle of the IRS, as well as cuticle, cortex and medulla of the hair shaft. Since each of these cylinders represent a distinct lineage of epithelial differentiation, they display characteristic structural proteins (e.g. hair keratins, trichohyalin), adhesion molecules and enzyme activities (Langbein et al. 2001; Langbein et al. 1999) (**Fig. 3.2 & 3.3)**.

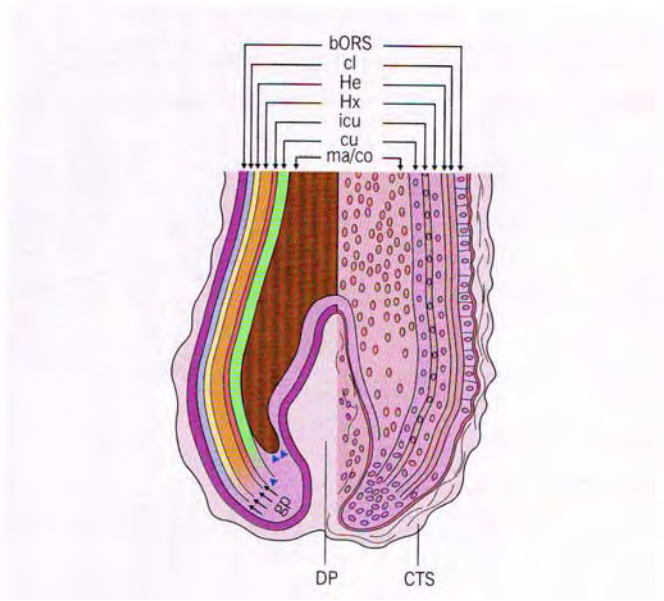

Fig. 3.2 The different layers of the hair follicle epithelium
Cl: companion layer; CTS: connective tissue sheath; cu: hair shaft cuticle; DP: dermal papilla; gp: germinative pool; He: Henle layer; Hx: Huxley layer; IRS: inner root sheath, icu: cuticle of the IRS; ma/co: hair shaft matrix and cortex; ORS: outer root sheath; bORS: basal layer of the ORS. Figure modified from Paus et al. (Paus et al. 2008b).

ORS, IRS and HS originate from three main precursor cell populations descending from epithelial stem cells. These are located in the bulge region of the hair follicle, the area, where the APM inserts into the ORS (Kloepper et al. 2008; Tiede et al. 2007b). In contrast to mice, the bulge region in adult human hair follicles hardly deserves this name as in most cases it is morphologically indistinct from the rest of the ORS. However, in 8% of the hair follicles a protrusion of the ORS, named the follicular trochanter, can be detected, which can serve as a guiding structure to localize this major site of hair follicle stem cells (Tiede et al. 2007a). The epithelial stem cells, from which the ORS is produced, remain within the bulge region in later life, while those that form the IRS and HS are postulated to be deposited in the secondary hair germ (Blanpain et al. 2004; Oshima et al. 2001; Panteleyev et al. 2001).

The hair bulb is the actual place where the hair shaft is produced. Here, one of the most rapidly proliferating cell populations in mammalian tissue, the keratinocytes of the one

layered hair matrix are found. In the precortical hair matrix, above the dermal papilla, these cells initiate their terminal differentiation to trichocytes and receive melanosomes from the melanocytes of the hair pigmentary unit for hair shaft pigmentation. During their further differentiation into hair shaft cuticle, cortex or medulla and their gradual up-wards movement in the follicle, these keratinocytes sequentially express defined sets of keratins.

The volume of the dermal papilla (DP), more precisely the number and the morphogen-secretion profile of the DP fibroblasts, define the size of the hair follicle and the diameter of the hair shaft. There is a remarkable exchange of fibroblasts between the DP and the CTS and a destroyed DP can even be fully reconstituted from the proximal CTS (Jahoda and Reynolds 1996).

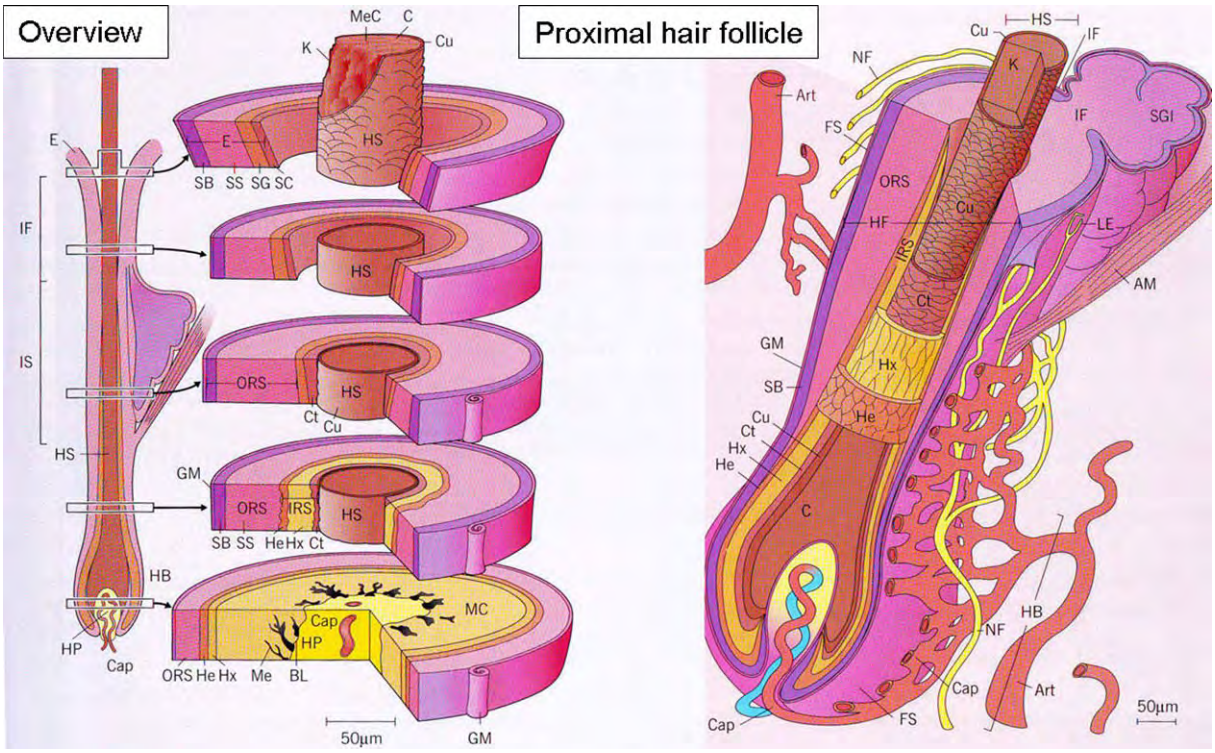

Fig. 3.3 Terminal human hair follicle in anagen VI
Left: Overview illustrating the different layers of the hair follicle in various heights of the hair follicle. Right: Proximal hair follicle. AM: arrector pili muscle; Art: arterioles; BL: basal lamina; C: hair shaft cortex; Cap: capillary; Ct: IRS cuticle; Cu: hair shaft cuticle,; E: epidermis; FS: follicular sheath; GM: glassy membrane; HB: hair bulb; He: Henle layer; HF: hair follicle; HP: hair papilla; HS: hair shaft; Hx: Huxley layer; IF: infundibulum; IRS: inner root sheath; IS: isthmus; K: hard keratins; LE: lanceolate nerve endings; MC: hair matrix cells; Me: melanocyte; MeC: medullary cells; NF: nerve fibres; ORS: outer root sheath; SB: stratum basale; SC: stratum corneum; SG: stratum granulosum; SGl: sebaceous gland; SS: stratum spinosum. Modified from Paus et al.(Paus et al. 2008b).

The hair shaft's main structural component is the cortex: here, densely packed parallelly orientated trichocytes full of longitudinally arranged keratin fibres are cemented together by pertinacious material. This structure gives the hair shaft its high elasticity and resistance to mechanical stress. It is covered by the roof tile-like arranged cells of the cuticle protecting the HS from weathering (Powell and Rogers 1997).

The IRS descends from hair matrix cells that already terminally differentiate before the keratinocytes of the central hair matrix. Thus, the IRS forms a hard cylinder which guides the upwards moving central column of the hair matrix cells differentiating into HS trichocytes. The IRS ends at the level of the insertion of the sebaceous gland duct, since the secretion products of the latter are critical for an orderly IRS dissolution and separation of sheath and shaft (Paus et al. 2008b; Stenn and Paus 2001).

The ORS separates the IRS and its contents from the dermis and continues the epidermal structure in the hair follicle. It comprises of a basal layer attached to the basal membrane (glassy membrane) proliferating and differentiating towards the IRS. In contrast to the epidermis, the ORS keratinocytes do not cornify below the infundibulum (**Fig. 3.3**). The ORS supports and guides the IRS, serves as an energy store (glycogen) and transit route for nutrients and oxygen towards the inner layers of the hair follicle. Most importantly, however, it serves - as mentioned above - as a stem cell reservoir for the hair follicle and for the epidermis (Paus et al. 2008b; Taylor et al. 2000).

Hair follicles grow in an oblique angle towards the epidermal surface. This angle can be varied by the contraction of a small bundle of smooth muscle, collectively called the arrector pili muscle (APM) (**Fig. 3.3**). As the muscle is under adrenergic control, thus, in situations of anxiety or stress, it involuntarily contracts and makes one’s hair “stand up”. This has become rather unimportant in the comparatively nude humans, but is far more impressive in animals. In human scalp skin, there is one APM for each so called follicular unit, a defined group of 2-4 terminal and 1-2 vellus hair follicles (Paus et al. 2008b; Headington 1984).

In addition to this well innervated muscle, the hair follicle itself displays at the level of the bulge and the isthmus a particularly dense and complex sensory and autonomic innervation system and numerous Merkel cell complexes. This enables the hair follicle to serve as a highly sensitive tactile organ, which can sense even the slightest movements of the hair shaft (e.g. evoked by wind). Animals are even equipped with specialized sensory hairs, the so called vibrissae, the largest and most densely innervated hair follicles surrounded by a blood sinus. When the hair is moved, this movement is translated and enhanced by the blood in the sinus and the pressure sensible nerve ends can sense this enhanced signal (Liebich 2004). In addition the hair follicle neural plexus may have important functions by releasing

neurotransmitters, neuropeptides and neurotrophins (Paus et al. 2008b; Peters et al. 2002; Botchkarev et al. 1998a; Botchkarev et al. 1998b).

Besides the complex innervation, the hair follicle also displays a widely ramified perfusion system. Coming from the dermal and the subcutaneous vascular plexus, numerous arterioles, capillaries and venules with plenty of shunts constitute a rich basket-like vasculature. It sheathes the hair follicle weaving through its CTS, and even inserts in the dermal papilla. This ensures the ready access to nutrients, oxygen and hormones to this highly active proliferating organ (Yano et al. 2001; Mecklenburg et al. 2000).
However, the most fascinating part of human hair follicle anatomy is probably that this complex structure does not stay as it is, but constantly undergoes cyclic waves of regressing and rebuilding itself during the whole lifetime.

3.1.2 Hair follicle cycle

Among all mammalian organs the hair follicle is unique in its life-long cyclic activity far out-running the ovarian or endometrial cycle. In this cycle it undergoes three distinct phases changing from growth (anagen) to regression (catagen) and to resting (telogen) (Steen and Paus 2001; Paus and Cotsarelis 1999) (**see Fig. 3.4**). The duration of the anagen phase defines the hair follicle's length and is varying substantially from 2-6 years in terminal scalp hair to only 4-14 weeks in terminal moustache hair or even 6-12 weeks in vellus hair. After a rather short time of 2-3 week of catagen the hair follicle is resting in telogen for approximately 3 months until the new hair starts growing again.

In animals synchronized cycling of the HFs ensures that the fur coat is prepared for seasonal changes in living conditions. In humans this synchronicity can still be seen *in utero* and two postnatal waves in the scalp of the newborn (Paus and Cotsarelis 1999), but later on only asynchronous waves remain and this autonomy and spontaneity of HF cycling ("mosaic pattern") is relatively hard to explain. It might serve the purpose of cleaning the skin of debris and parasites, as well as the shedding of chemicals which are incapsulated within the trichocytes. Furthermore, the HF cycle may regulate the paracrine or even endocrine secretion of hormones and growth factor which are produced within the HF epithelium (Paus et al.

2008a; Ohnemus et al. 2006; Paus and Foitzik 2004; Stenn and Paus 2001; Foitzik et al. 1999).

Anagen is the growing phase of the hair follicle and shares many structural and molecular analogies with HF morphogenesis in the fetus. It is divided in 6 different stages (anagen I-VI), which can be distinguished using defined morphological criteria (Müller-Röver et al. 2001) (**see Fig. 3.4**). In average an anagen scalp hair is growing 0,35mm a day and is not influenced by cutting or shaving.

In catagen (I-VIII) a rapid organ involution driven by controlled apoptosis and terminal differentiation takes place in which the proximal part of the HF regresses entirely (**see Fig. 3.4**). The follicular melanogenesis terminates, the HS production stops and the HS end is sealed off in a so called club. This club hair moves upward until it reaches the permanent, non cycling upper follicle where it remains anchored during telogen. The dermal papilla shows no signs of apoptosis, but nevertheless condenses and also moves upwards connected to the upper follicle only through the epithelial strand, the shrunk proximal HF epithelium (Lindner et al. 1997).

In telogen, the resting phase, the HF lies dormant and no signs neither of apoptosis nor proliferation nor differentiation can be seen (**see Fig. 3.4**) (Alonso and Fuchs 2006; Stenn and Paus 2001).

The only exit from this continuous cycling is the so called programmed organ deletion, in which selected, individual hair follicles are completely eliminated in the consequence of an inflammatory attack on the hair follicle’s stem cells in the bulge region. As this phenomenon occurs in otherwise healthy normal skin, it was postulated that this process is designed for clearing the skin from malfunctioning and undesired hair follicles (**see Fig. 3.4**) (Stenn and Paus 2001; Eichmüller et al. 1998).

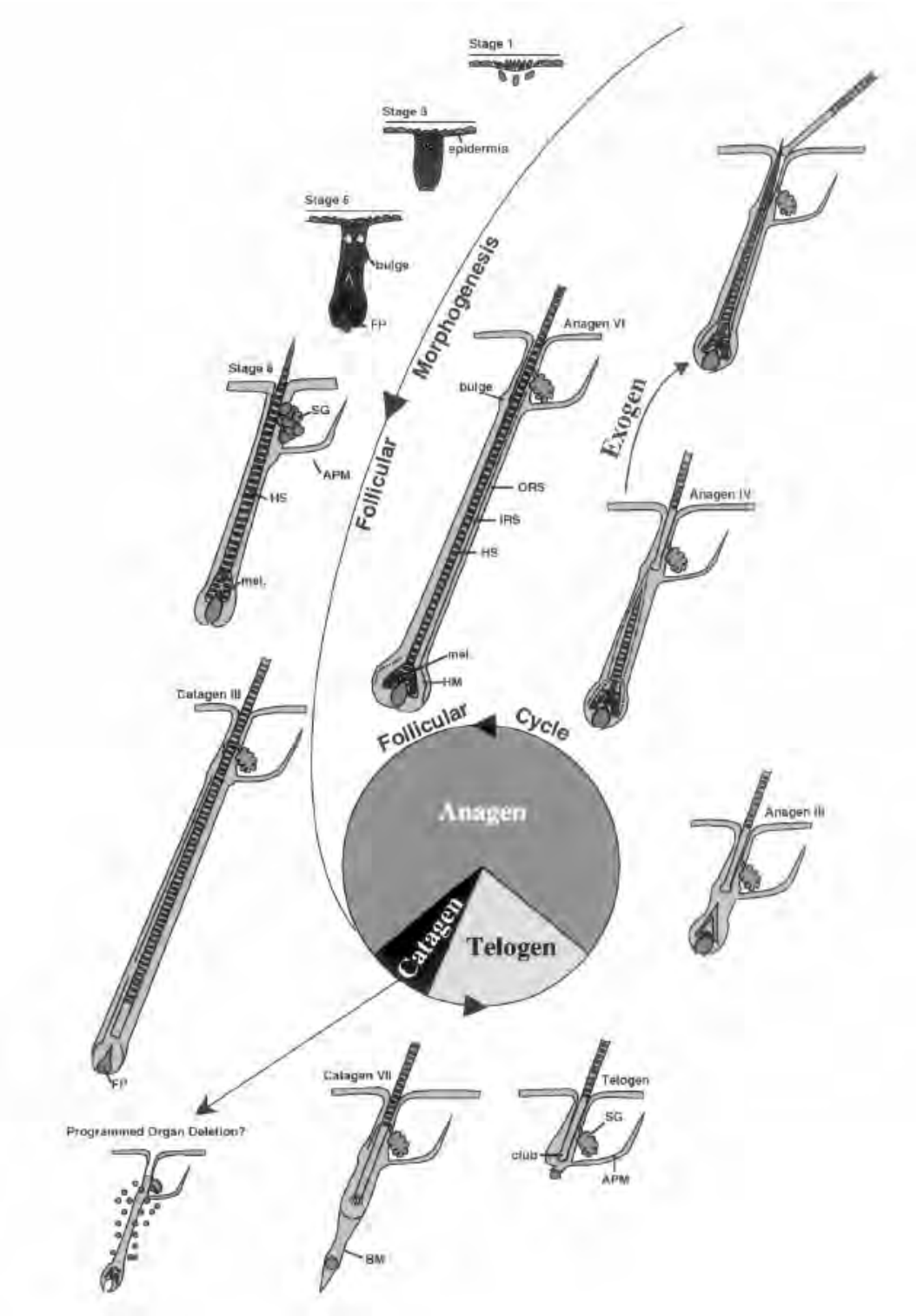

Fig. 3.4 Schematic illustration of the different phases of morphogenesis and the hair cycle
Morphogenesis only occurs once in the lifetime of a hair follicle whereas the cycle repeats many times before it ends. Hair follicle morphogenesis can be classified into nine (0-8) different stages, of which only the most important are shown. It starts from a small epithelial budding in the epidermis right above a mesenchymal condensation and rapidly progresses to the generation of the mature pilosebaceous unit. When this mature hair follicle spontaneously enters catagen, the first hair cycle starts. The cycle is divided into catagen (I-VIII), a regression phase, telogen, the resting phase and anagen (I-VI), the growing phase. The old hair shaft is shed out in exogen. Malfunctioning hair follicles are eliminated and enter the stage of programmed organ deletion (Stenn and Paus 2001).APM: arrector pili muscle; BM: basal membrane; FP: follicular papilla; HM: hair matrix; HS: hair shaft; IRS: inner root sheath; mel.: melanin, ORS: outer root sheath; SG: sebaceous gland

Hair follicle cycling is controlled by the "hair cycle clock", an autonomous molecular oscillator system, which resides in and/or around the hair follicle, that can even continue to operate after transplantation of the hair follicle (Paus and Foitzik 2004; Paus et al. 1999a). Changes in the cycling activity result in unwanted hair loss or gain, making this the most common cause of hair growth disorders seen in clinical practice (Paus 1996).

3.1.3 Hair follicle immunology

The hair follicle is armed with a very efficient immune system. As the risk of infection is highest in the most distal parts of the hair follicle, defence systems are most elaborate there. Therefore, in the distal ORS a dense ring-like net of interacting Langerhans cells and T-cells and a large number of perifollicular mast cells and macrophages can be found (Christoph et al. 2000) (**Fig. 3.5**).

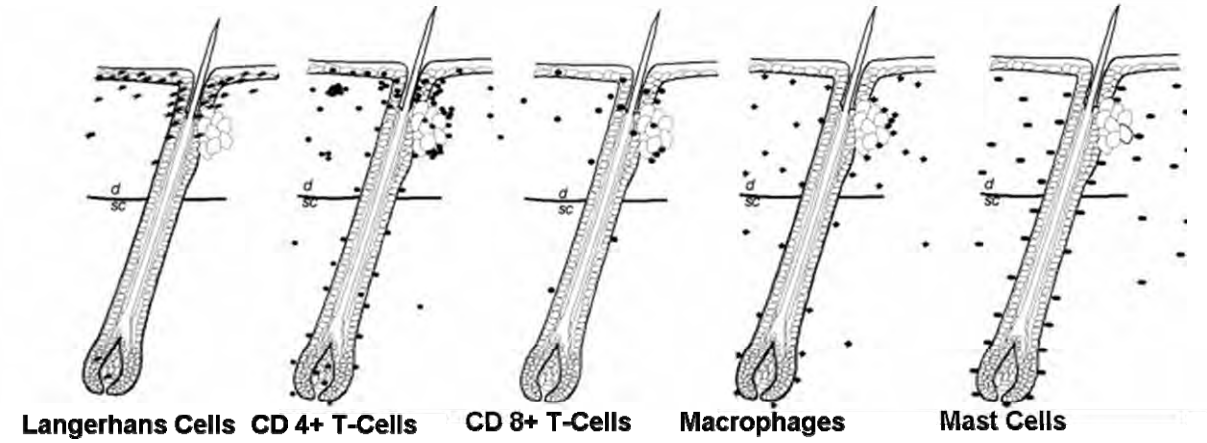

Fig. 3.5 Distribution of immune cells in the normal human pilosebaceous unit in anagen VI
Langerhans cells can be found in the highest density in the distal, infundibular ORS comparable in number and distribution to what is seen in the epidermis. In the isthmus region the number sharply declines and in the proximal part these cells are only found very rarely. CD4+ T-cells are found in highest number in the perifollicular epidermis and in the distal ORS. In the isthmus, the lower hair follicle and the hair bulb this number is markedly lower. CD8+ T-cells can be detected in the epidermis and in the distal ORS, but are almost gone in the isthmus region and below. Macrophages can be seen in the CTS with an increasing number in the proximal hair bulb and in the surrounding dermis. Mast cells were only detected in the CTS, but not in the hair follicle epithelium and in the surrounding dermis. d: dermis; sc: subcutis. Modified from Christoph et al. (Christoph et al. 2000)

Although the hair follicle immune system is an integral part of the skin immune system (see chapter 3.3) it has also distinct peculiarities. First, the distal ORS of human hair follicles harbours an immature, functionally impaired Langerhans cell population. This is supposed to

serve as an intermediary Langerhans cell reservoir between the bone marrow and the epidermis (Gilliam et al. 1998). Second, Langerhans cells and intraepithelial CD4 and CD8 positive lymphocytes are not homogeneously distributed throughout the follicle epithelium but sharply decline in number with increasing distance from the epidermal surface, with very few, if any T cells and Langerhans cells detectable below the level of the bulge (Christoph et al. 2000; Moresi and Horn 1997). Those few Langerhans cells that one can detect by electron microscopy in the anagen hair bulb seem to be immunologically impaired since they do not express MHC class II molecule (Christoph et al. 2000).

One of the most fascinating features in hair follicle immunity is, that the anagen hair bulb and the bulge region establish areas of relative immune privilege (Meyer et al. 2008; Paus et al. 2008b; Paus et al. 2005). Like in other immunologically privileged sites (e.g. the anterior eye chamber or the fetotrophoblast) (Simpson 2006; Niederkorn 2003), this phenomenon is characterized by an absence of MHC class I expression (or very low expression levels), the local generation and secretion of potent immunosuppressive cytokines (e.g. TGFβ1, α-MSH, ACTH, IL 10), impaired antigen presenting functions (via down-regulation of MHC-class II) and the absence of lymphocytes. The immune privilege protects the stem cells in the bulge region and the melanogenesis-associated autoantigens in the hair bulb from immune recognition and destruction. The collapse of the immune privilege therefore ultimately leads to hair loss like in alopecia areata or primary cicatrical alopecia, if the bulge is targeted (Meyer et al. 2008; Gilhar et al. 2007).

Interestingly, hair follicle cycling has a great influence on the immune status of both the hair follicle and the skin, and probably the immune system influences also hair follicle cycling, vice versa (Paus et al. 1999b). For example, it could be shown that the number, location and activation of T-cells, Langerhans cells, mast cells and macrophages varies substantially in murine skin going through different hair cycle waves (Paus et al. 1999b). Mast cells and macrophages can significantly modulate hair growth, especially in mice (Maurer et al. 1997; Maurer et al. 1995). Immunosuppressive substances like glucocorticoids and cyclosporine are among the most potent hair growth modulating substances (Paus et al. 1996; Paus et al. 1994b). In fact almost all of these different hair-cycle modulating cytokines, hormones and neuropeptides have at the same time modulating functions on the immune system (Paus et al. 2008a).

3.2 Microflora of the skin and the hair follicle

Human skin on the one hand acts as a barrier to colonization from the environment, but despite its various defence mechanisms it provides on the other hand also a rather stable microclimate. Some of the inhabitants complete their life cycle on the skin and are considered to be residents whereas others, the transients (visitors) spend only a limited time there (McEwan Jenkinson 1993; Noble 1993).

3.2.1 Resident microflora

The healthy and sterile skin of the fetus becomes rapidly colonized with bacteria soon after the first contact during the birthing process. By the early age of six weeks the microflora qualitatively and quantitatively already resembles the adult skin (Zomorodain et al. 2008; Elias 2007). In average, human skin contains about 10^2 to 10^6 microorganisms/cm^2. These grow in small colonies at the surface and within the uppermost layers of the stratum corneum as well as in the pores of the hair follicles (Schröder and Harder 2006; McEwan Jenkinson 1993). The skin's defence mechanisms restrict but do not prevent this colonization (Lambers et al. 2006). On the contrary, as some of the resident bacteria are also secreting bacteriocins (a kind of antimicrobial peptides in bacteria, see below) and acidic products they are helping to keep the microflora within certain limits and to defend the skin against pathogenic bacteria. In general, the resident flora mostly consists of gram-positive rods including coagulase-negative *staphylococci*, *micrococci* and *peptococcus*, as well as *corynebacterium*, *brevibacterium* and *propionibacterium*. Furthermore the yeast malassezia is present. Among the gram-negative bacteria, only acinetobacter can be commonly found (Tajima et al. 2008; Kozitskaya et al. 2005; Roth and James 1989).

Although the resident flora remains relatively constant, several factors like the pH, humidity and temperature can change the quantitative and qualitative composition of the normal flora (Roth and James 1989). Therefore, as these conditions are different in distinct anatomical sites, the normal flora varies according to the body location. Certain areas like the face, neck and hand are more exposed and others like the axilla or the toe web represent areas of partial occlusion. The normal axillary flora has been described as consisting of large populations dominated of either staphylococcal or coryneform bacteria (Taylor et al. 2003). Toe webs

show also a heavy colonization, which includes gram-negative bacteria and have a higher prevalence of dermatophytes and potentially pathogenic microorganisms. Furthermore, the distribution and density of pilosebaceous units are of significance for the regional variation in the flora as the hair follicle is the site of the largest concentrations of microorganisms in the skin (McBride 1993). It provides a special physicochemical environment with a different pH and oxygen tension than the rest of the skin. Most common organisms found are *Propionibacterium acnes, Propionibacterium granulosum, Staphylococcus epidermidis, Micrococcus spp., Demodex folliculorum* and *Malassezia furfur*, which therefore occur in high population densities on human scalp skin (McBride 1993; Wolff and Plewig 1976).
These species colonize a preferred localization within the hair follicle, depending on their nutritional and oxygen requirements. The lipophilic *malassezia* is found at the hair follicle's orifice, aerobic *staphylococci* and *micrococci* in the infundibulum and anaerobic growing *propionibacteria* are localized around the orifice of the sebaceous glands and more proximal (Wolff and Plewig 1976). The proximal part of the hair follicle is, with the exception of papillomavirus, completely sterile.(Boxman et al. 1997)

3.2.2 Transient microflora

Any organism that inhabits non-cutaneous body sites or is found in the environment can transiently live on the skin. Transient microorganisms are normally unable to multiply and usually die (Brock 2006). Clinically most important are probably the group A streptococci, which rapidly die when placed on normal skin. However, they have been recovered from clinically normal skin, which 10 days later developed impetigo (Roth and James 1989).

3.2.3 Most common bacteria and fungi of skin and hair follicle infections in humans

The most common skin infections presented in general practice include cellulitis, erysipelas, impetigo, folliculitis, furuncles and carbuncles (Stulberg et al. 2002). As can be seen in **table 3.1** these infections are mostly caused by *Staphylococcus* or *Streptococcus spp*. Gram-negative infections are less common and mostly affect only more susceptible patients like

children, patients with diabetes or immunodeficient patients (Brook 2007; Sterry et al. 2006; Kozitskaya et al. 2005).

Group	Bacteria	Diseases (examples)
Gram-positive bacteria		
Staphylococci[a]	*Staphylococcus aureus* *Staphylococcus epidermidis*	folliculitis, furuncle, furunculosis, carbuncle, impetigo, cellulitis, paronychia, toxin-mediated diseases (staphylococcal scalded skin syndrome)
Streptococci[a]	*Streptococcus pyogenes* (group A, β-hemolytic) *Streptococcus viridans*	erysipelas, impetigo (less often), scarlet fever, ecthyma, lymphangitis, necrotizing fasciitis, purpura fulminans
Corynebacteria	*Corynebacterium minutissimum*	erythasma
	Corynebacterium tenuis	trychomycosis axillaris
	Corynebacterium diphteriae	cutaneous diphteria
Borrelia	*Borrelia burdorferi*	Lyme borreliosis including erythema migrans, lymphadenosis cutis benigna, acrodermatitis chronica atrophicans
Treponema	*Treponema pallidum*	syphilis
	Treponema pertenue	yaws
	Treponema carateum	pinta
Mycobacteria	*Mycobacterium leprae*	leprosy
	Mycobacterium tuberculosis	tuberculosis
	Mycobacterium bovis	tuberculosis
	Mycobacterium avium-intracellulare	chronic infections in HIV/AIDS
	Mycobacterium marinum	swimming pool granuloma
	Mycobacterium buruli	buruli ulcer
Actinomycetales	*Actinomyces israelii*	actinomycosis
	Nocardia brasiliensis	nocardiosis
Gram-negative bacteria		gram-negative folliculitis, gram-negative toe web infection, mixed infections, pyoderma in immunosuppressed patients
Enterococcaceae	*Haemophilus ducreyi*	chancroid
	Haemophilus influenzae	facial cellulitis in childhood
	Pseudomonas aeruginosa	nail fold infections, toe web infections, hot top folliculitis, sepsis
	Klebsiella pneumoniae	mixed infections
	Salmonella typhi	typhoid fever
	Escherichia coli	mixed infections
	Yersinia enterocolitica	yersiniosis, erythema nodosum
	Yersinia pestis	plague

Chlamydia	*Chlamydia trachomatis*	lymphogranuloma venereum
Neisseria	*Neisseria gonorrhoeae*	gonorrhea
	Neisseria meningitidis	meningococcal meningitis, pneumonia

Table 3.1 Common cutaneous bacterial infections
[a] Infections of staphylococci and streptococci are referred to as pyodermas. Adapted from Sterry et al. (Sterry et al. 2006)

The most common fungal diseases are caused by dermatophytes. They are often found especially in the toe webs, as these areas are often wet by sweat and hard to be kept dry and therefore offer optimal living conditions for these fungi (**Table 3.2**).

Group	Fungi	Diseases
Trichophyton	*Trichophyton rubrum*	tinea pedis, tinea manuum, tinea corporis, onychomycosis
	Trichophyton mentagrophytes	tinea corporis, tinea faciei, tinea barbae, tinea capitis
	Trichophyton verrucosum	tinea corporis, tinea barbae, tinea capitis (often kerion)
Microsporon	*Microsporon canis*	tinea capitis, tinea corporis
	Microsporon gypseum	tinea capitis, tinea corporis
	Microsporon audouinii	tinea capitis
Candida	*Candida albicans*	cutaneous candidiasis
	Candida krusei	cutaneous candidiasis
	Candida parapsilosis	paronychia
	Candida tropicalis	onychomycosis
	Candida glabrata	part of normal flora, life-threatening disease in immunosuppressed patients

Table 3.2 Common cutaneous fungal infections
Modified from Sterry et al. (Sterry et al. 2006)

3.2.4 Important pathogenicity factors of bacteria

The terms pathogenicity factor and virulence factor are often used in the same sense and are defined as factors that are produced by a pathogen and required for it to cause disease (Pallen and Wren 2007). It has to be noted that defining this term precisely is very difficult as the same factors can be found in pathogenic and non-pathogenic organisms and that the pathogenicity is crucially depending on the host's immune systems.
In the following, certain substances that are important for evasion from the host's defences and cause of disease will be described.

3.2.4.1 Lipopolysaccharides

Lipopolysaccharides (LPS) constitute the main components of the outer membrane of the cell wall of gram-negative bacteria (**Fig. 3.6**).

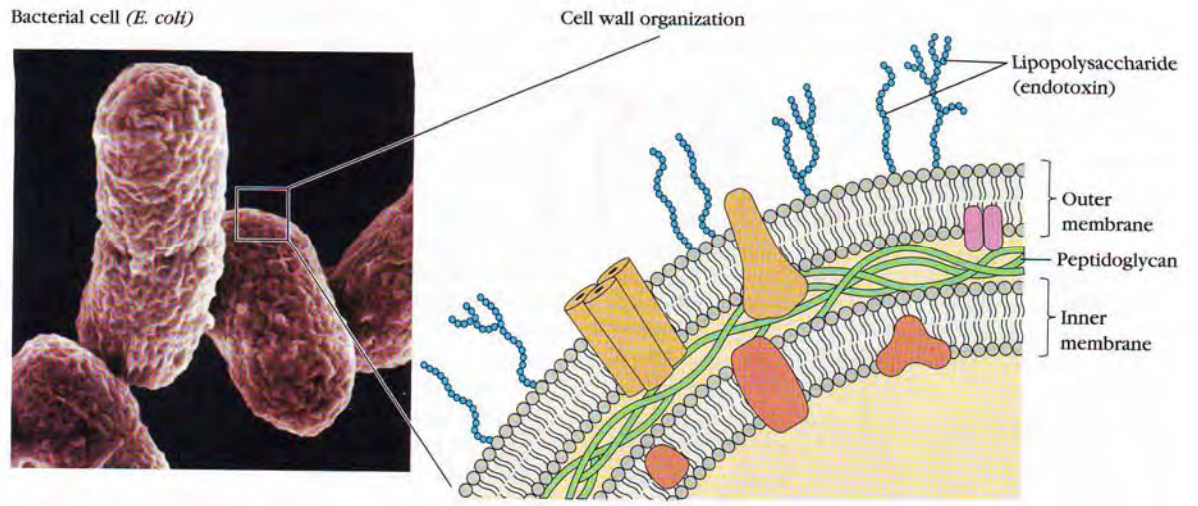

Fig. 3.6 Bacterial cell wall of gram-negative bacteria
The cell wall of gram-negative bacteria like *Escherichia coli* consists of an outer and an inner membrane. Lipopolysaccharides are anchored in the outer membrane (Kuby 2007).

They typically consist of a an inner hydrophobic domain known as Lipid A, a nonrepeating "core" oligosaccharide and an outer distal polysaccharide (or O-antigen) (Beutler and Rietschel 2003; Raetz and Whitfield 2002) (**Fig. 3.7**).

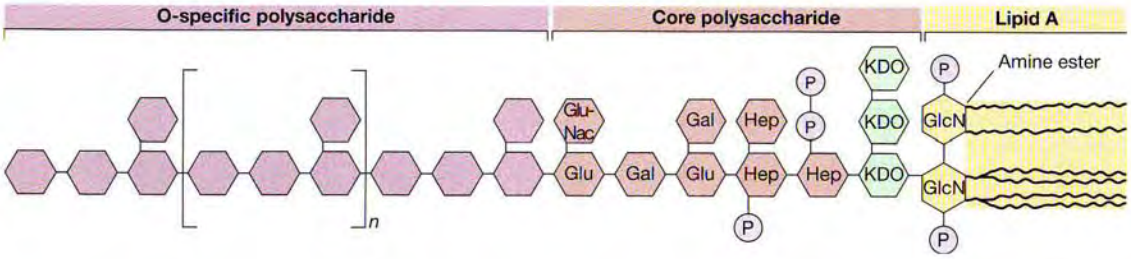

Fig. 3.7 Chemical structure of lipopolysaccharides
They consist of three main parts, an O-specific polysaccharide, a core polysaccharide and lipid A Gal:D-galactose; Glu: D-glucose; GlcN: glucosamine; GluNac: N-acetyl-D-glucosamine; Hep: heptose; KDO: 2-keto-3-deoxyoctonic acid; P: phosphate (Brock 2006).

Lipid A is an unusual glycophospholipid with unique structural features (the fatty acids are connected by amine ester linkage to a disaccharide), that is - apart from differences in structural details - the same in all gram-negative bacteria (Brock 2006; Beutler and Rietschel 2003). Lipoprotein A is the key component acting as an endotoxin in the human/mammalian body which can cause septic shock and death.

Injection of pure LPS into mice or humans is sufficient to mimic most of the features of acute gram-negative infection, including massive production of proinflammatory cytokines such as IL-1, IL-6 and TNF, leading to severe septic shock (Male et al. 2006).
LPS-binding protein (LBP), an acute phase reactant that is present in the blood, binds LPS and transfers it to CD14, a protein that exists soluble in blood and membrane bound on the surface of monocytes and macrophages (Hancock and Scott 2000). The LPS-CD14 complex binds to the Toll like Receptor (TLR) 4, which initiates the start of several pathways all leading towards the production of the proinflammatory cytokines mentioned above.

3.2.4.2 Lipoteichoic acid

Lipoteichoic acid (LTA) is a component of the cell wall of gram-positive bacteria. It consists of a polyalcohol with attached sugars and O-linked alanine and is covalently bound to cell membrane lipids (Brock 2006) (**Fig. 3.8**).

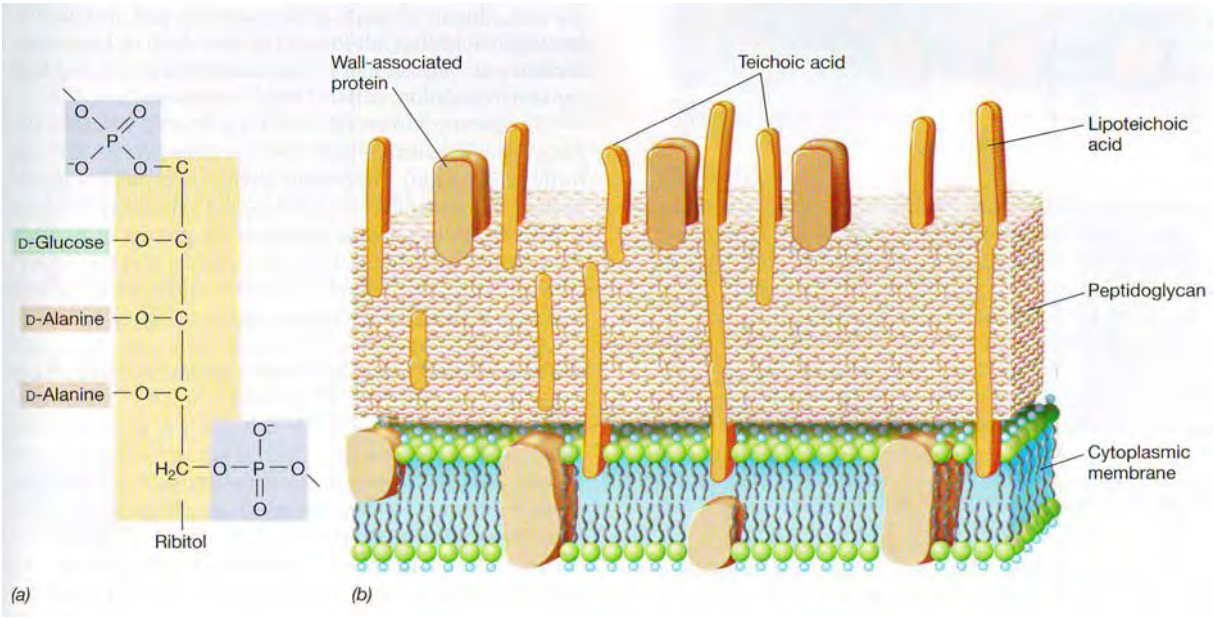

Fig. 3.8 Teichoic acids and the overall structure of the gram-positive cell wall
(a) Structure of the ribitol teichoic acid of *Bacillus subtilis.* The teichoic acid is a polymer of the repeating ribitol units shown here. (b) Diagram of the gram-positive cell wall (Brock 2006).

It was one of the first adhesins identified in gram-positive bacteria (Aly et al. 1997). LTA has been shown to stimulate the production of inflammatory mediators *in vivo*. When injected into animals, LTA and other gram-positive cell wall components elicit many of the characteristic

features of septic shock, including cytokine production, leukocytopenia, circulatory failure, multiple organ dysfunction syndrome and mortality (Hancock and Scott 2000). The recognizing structure in the human body is the toll-like-receptor (TLR) 2.

3.2.4.3 Protein A

Protein A is a 42-kDa protein found in the cell wall of gram-positive bacteria namely *Staphylococcus aureus*, but also exists in a secreted form. It is composed of five homologous domains of 56-61 amino acids, which fold into triple α-helical bundles that are assembled in tandems to produce extended structures in solution. Protein A has the ability to bind the heavy chain constant-region that is shared by many IgG antibodies and therefore making opsonization impossible. Furthermore it binds the V_H3 site of the antibodies' Fab region, and can therefore interact with approximately 30-50% of all circulating B cells in humans. As a "superantigen" it first leads to increased proliferation followed by apoptosis of the B-cells (Kumar et al. 2007; Silverman and Goodyear 2006). Protein A also induces rapid NF-κ-b activation and secretion of proinflammatory cytokines and chemokines such as tumour necrosis factor (TNF)-α and IL-8 in keratinocytes (Ezepchuk et al. 1996) and in human corneal epithelial cells (Kumar et al. 2007).

3.3 Defence mechanisms in the human skin

The skin is the largest organ of the human body with a surface of approximately 1.5-2 m^2. As it is directly interacting with the surrounding environment, one of its most important roles is the protection of the body from potential dangers including UV-radiation, desiccation, overheating, mechanical trauma, infectious agents, toxins and chemical irritants (Sterry et al. 2006; Chuong et al. 2002).

In the following the various lines of defence against potentially pathogenic organisms as shown in **table 3.3**, mainly focussing on the innate immune system will be outlined. Innate immunity - in contrast to adaptive immunity - is ready for immediate activation prior to the attack of pathogens and includes physical, chemical and cellular barriers. Therefore these

mechanisms often eliminate potentially pathogenic organisms long before the adaptive immunity in form of a large population of B- and T-lymphocytes and pathogen specific antibodies is recruited.

Level	Activity
Epidermal surface	microflora and products
	acidic pH
	desiccation
	antimicrobial peptides from the sebum and sweat
	sebaceous and stratum corneum lipids (e.g. free fatty acids)
	protease inhibitors
Epidermis	cornified cell envelope
	continuous renewal and proliferation
	inducible and constitutively expressed antimicrobial peptides
	keratinocyte phagocytic activity
	antigen recognition via toll-like receptors
	cytokine and chemokine production
Dendritic cells of the epidermis and dermis	antigen recognition
	antigen presentation
	cytokine and chemokine production
Mast cells	production of antimicrobial peptides
	recognition of antigen
	proteases
Neutrophils and Macrophages	phagocytosis
	respiratory burst
	production of antimicrobial peptides
Natural killer cells	elimination of virus infected cells
	macrophage activation
	regulator of T- helper cells
B and T-lymphocytes	adaptive immunity

Table 3.3 Multiple levels of cutaneous defence

3.3.1 Physical barrier

First, the skin is covered by the epidermis, a multilayered, cornifying epithelium. It consists of the stratum basale, the stratum spinosum, stratum granulosum, (stratum lucidum) and the stratum corneum. Processing from the stratum basale upwards the cells, produce more and more keratohyalin granula and differentiate finally to the corneocytes, terminally differentiated, anucleated dead keratinocytes, that interlock with each other to vertical columns. During the final stages of terminal differentiation, an insoluble protein structure, the cornified cell envelope (CCE) (**Fig. 3.9**) is assembled beneath the plasma membrane (Candi et

al. 2005; Kalinin et al. 2001). The corneocytes are tightly attached to each other (Madison 2003) glued together with lipid structures (cholesterol, cholesterol esters, ceramides and saturated long chain free fatty acids) organized in characteristic lamellar bilayers filling up the intercellular spaces (Elias 2007; Norlen et al. 1999). As a result corneocytes and the lipid-enriched extracellular matrix form the solid "brick wall" of the stratum corneum that makes invasion for microorganisms extremely difficult if there is no mechanical trauma helping them to overcome this hurdle.

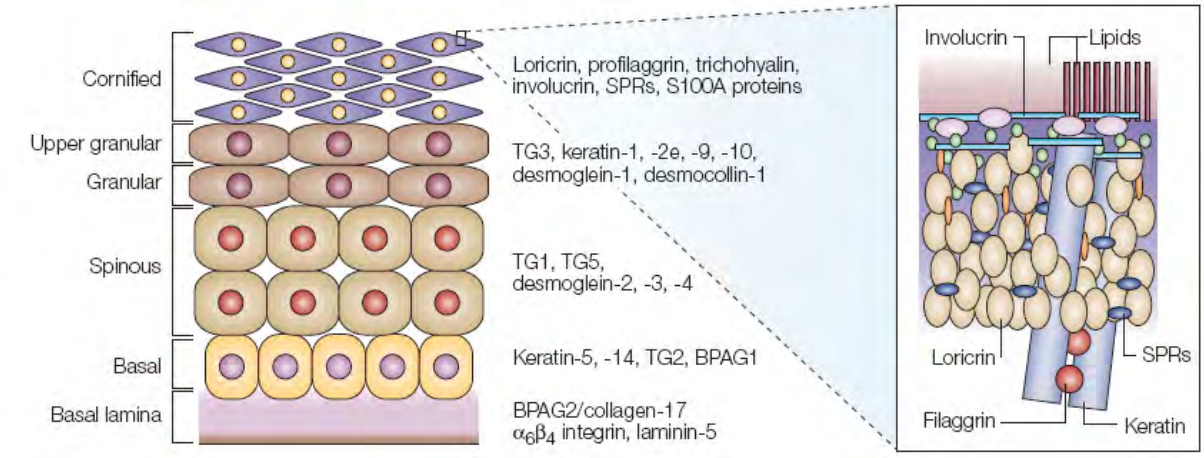

Fig. 3.9 Terminal differentiation in the epidermis
Proteins expressed during skin differentiation are shown (left). At the molecular level, the cornified envelope is formed by proteins that are highly crosslinked by transglutaminases, with specific lipids on the outside, to guarantee specific physical properties. BPAG: bullous pemphigoid antigen; SPR: small proline-rich proteins; TG: transglutaminase (Candi et al. 2005).

Furthermore, the epidermis has a high proliferation rate which leads to continuous renewing of the epithelium and shedding of corneocytes carrying away microorganisms and debris. A normal person sheds 50-60 billion corneocytes per day, adding up to a total annual loss of 4kg (Sterry et al. 2006). The outwards-growth of the hair shaft additionally supports the cleansing of the skin surface of parasites, debris and squames (Paus et al. 2008b).

The hydration status also plays a critical role for microbial colonization. The water content in the stratum corneum drops dramatically towards its surface, creating desiccating conditions, which normally inhibit pathogen colonization (Elias 2007).

3.3.2 Chemical barrier

The skin surface has an acidic pH of about 5 or even below (Lambers et al. 2006) which is partially due to sweat containing lactic acid and the normal microflora, which secretes acidic products that favour their own growth, and partially due to the stratum corneum itself. Whereas normal flora such as micrococci and corynebacteriae grow better at an acidic pH, pathogenic organisms such as staphylococci, streptococci and candida proliferate more avidly at a neutral pH (Elias 2007; Chuong et al. 2002).

Furthermore, the extracellular lipids mentioned before are not only gluing together the corneocytes, but also contain antimicrobial properties. The free fatty acids and sphingosine themselves exhibit potent activity against a variety of bacterial, yeast and viral pathogens (Elias 2007; Bibel et al. 1992) and in addition several antimicrobial peptides like LL-37 and HBD2 are secreted together with these lipids (Braff et al. 2005a; Oren et al. 2003).

In addition the keratinocytes themselves, the sebaceous and the sweat glands and the hair follicle produce various antimicrobial peptides, which prevent the entry of pathogenic microorganisms and which will be discussed in detail later.

3.3.3 Recognition of pathogens

The fast recognition of invading pathogens and immediate action is of eminent importance for a well functioning immune system in an organ facing the environment like the skin. Therefore it is necessary that not only immune cells like macrophages or dendritic cells, but also the keratinocytes themselves can recognize so called pathogen-associated molecular patterns (PAMPs), which are highly conserved among microbes like cell wall components of gram-negative and gram-positive bacteria (Schwarz 2008). This is mediated via pattern recognition receptors, of which the most important ones are probably the Toll-like receptors (TLRs). TLRs are homologues of a receptor (toll) in fruit flies (*Drosophila* spp.) that was initially demonstrated to function in fly development, but later on was identified to also mediate antimicrobial and antifungal immune response (Kuby 2007).

TLRs are membrane spanning proteins that have an extracellular part containing leucin rich repeats which are responsible for ligand binding and an intracellular domain sharing similarities to the Interleukin 1 Receptor called TIR (Toll-IL-1 receptor) domain. This domain has three highly conserved regions called box 1, box 2 and box 3 which serve as binding sites for intracellular proteins participating in the signalling pathways mediated by TLRs (**Fig. 3.10**).

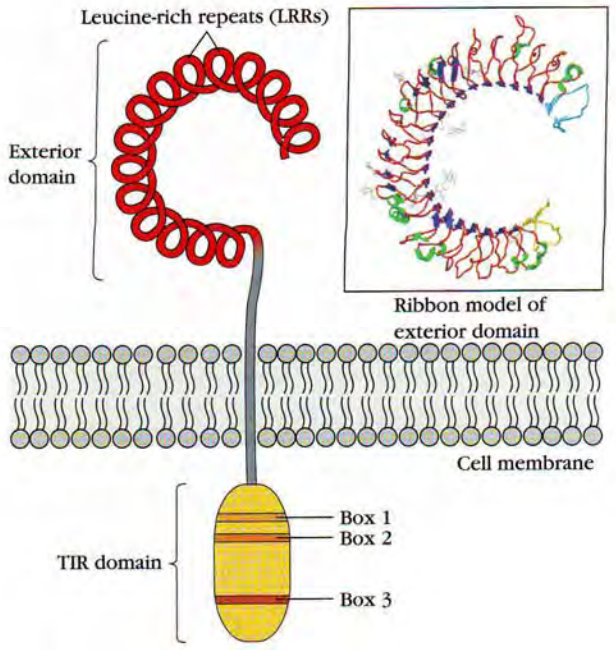

Fig. 3.10 Structure of a Toll-like receptor (TLR)
Toll like receptors have an exterior region that contains many leucin-rich repeats (the ligand binding site), a membrane-spanning domain and an interior domain called the TIR domain (Kuby 2007)

So far, 11 Toll like receptors are known in humans. They can be located in the plasma membrane, but also in intracellular membranes (Kuby 2007). Each TLR recognizes a certain subset of molecules produced by microbes (Beutler et al. 2003; Takeuchi et al. 1999) (**Table 3.4**) often forming dimers by either pairing with itself (like TLR 4) or with another TLR (like TLR 1 and 2 or TLR 2 and 6). These receptors induce via certain transducer molecules (e.g. MyD88) different signalling pathways, like the NF-κB pathway and finally lead to the production of various cytokines, e.g. TNF (Beutler et al. 2003). However, the exact details of the molecular interactions still remain to be discovered.

Expression of TLRs has been detected in various human tissues with varying expression levels. In general, tissues involved in immune response (like the spleen) or exposed to environment (like the skin) were found to have significantly higher TLR expression than, for

instance, the brain or skeletal muscle (Meyer et al. 2007). Human epidermis and primary keratinocytes were reported to express TLR 1, 2, 3, 4, 5, 6, 9 and 10 (Begon et al. 2007; Lebre et al. 2007; Kollisch et al. 2005; Baker et al. 2003; Pivarcsi et al. 2003).

Furthermore, immune cells like dendritic cells, neutrophils and macrophages also express several of the TLRs. Thus, TLRs have an important role as a bridge between innate and adaptive immunity, which is even further emphasized by the fact, that TLR 8 signalling was found to control the function of regulatory T-Cells (Peng et al. 2005).

TLRs	Ligands	Target microbes
TLR1	Triacyl lipopeptides	mycobacteria
TLR2	Peptidoglycans, GPI-linked proteins, Lipoproteins, zymosan	gram-positive bacteria, trypanosomes, mycobacteria, yeasts and other fungi
TLR3	double stranded RNA (dsRNA)	Viruses
TLR4	LPS, F-protein	gram-negative bacteria, respiratory syncytial virus (RSV)
TLR5	Flagellin	bacteria
TLR6	Diacyl lipopeptides, zymosan	mycobacteria, yeasts and other fungi
TLR7	single-stranded RNA (ssRNA)	viruses
TLR8	single-stranded RNA (ssRNA)	viruses
TLR9	CpG unmethylated dinucleotides, herpesvirus	bacterial DNA, some herpes viruses
TLR10,11	Unknown	unknown

Table 3.4 Toll-like receptors and their ligands
TLR 3, 7, 8 and 9 are located in intracellular membranes, the others are expressed on the cell surface (Kuby 2007).

3.3.4 Cellular barrier

The cellular defence system of the skin includes the cellular components of both the innate and the adaptive immune system. All these cells act together as a well-trained team and one cannot definitely answer the question who is the key immune cell in the skin (Schröder et al. 2006). Microbes, trying to invade, first encounter the immunologically active cells of the

epidermis, keratinocytes and to a much lower percentage (2-4% of the total epidermal cell population) Langerhans cells. Keratinocytes are - as mentioned before - equipped with a variety of TLRs and therefore important for the recognition of potential pathogens. Furthermore they are constitutively producing antimicrobial peptides, which are highly effective microbe killing agents (see chapter 3.4). As reaction to recognized pathogens keratinocytes can up-regulate the production of certain AMPs and they produce a variety of cytokines that attract professional phagocyting cells like neutrophils and macrophages (Chuong et al. 2002). Keratinocytes themselves have been reported to be able to phagocyte *Candida albicans* (Csato et al. 1986). However, this is a rather exceptional event that could only be shown for freshly separated cells in a very low percentage *in vitro*. *In vivo*, keratinocytes leave this to the professional phagocytes mentioned before.

Langerhans cells (LCs) are the dendritic cells of the epidermis. They take up foreign antigens as well as self hapten, migrate to the skin – draining lymph node and present the processed antigens to naïve T- Cells leading to T-cell stimulation and differentiation. LCs are characterized by the expression of Langerin (CD207) and MHC II which is necessary for antigen presentation. Moreover, tennis racket-shaped Birbeck granules, which are important for the transmission of HIV1 and its degradation, are typically found in LCs (Loser and Beissert 2007; Schröder et al. 2006). Furthermore LCs were reported to express a specific TLR profile, that enables direct detection of PAMP and differential cytokine production (Flacher et al. 2006).

If microbes manage to pass the epidermal barrier, they face the mast cells, macrophages and dendritic cells in the dermis, as well as neutrophils, B- and T-lymphocytes in the dermis.
Mast cells occur most prominently just below the dermal-epidermal junction and are concentrated around the hair follicle, nerves and blood vessels (Maurer et al. 2003; Weber et al. 2003). Although often regarded as nothing but effector cells in allergic reactions, they actually act as important sentinels of the immune system. They express functional receptors for the detection of bacteria (CD48) and their products (TLR2, 4, 6, 9) but also can be activated by mediators that are produced or up-regulated during bacterial infections like complement components or endothelin-1. Mast cells can have direct antimicrobial activity by releasing cathelicidins (Di Nardo et al. 2003) or chymase (Orinska et al. 2007) and can regulate the toxicity of endogenous mediators (mostly peptides) by releasing proteases (Maurer et al. 2004). Furthermore, by secreting various different cytokines and exosomes,

mast cells are crucial for neutrophil recruitment (Siebenhaar et al. 2007), influence dendritic cell maturation and interact with T-cells (Skokos et al. 2003).

As mentioned before, neutrophils, macrophages and natural killer cells are the professional phagocytes. Neutrophils are the first cells to migrate from the blood to sites of infection (Kuby 2007). They are often found in close proximity to the epidermis whenever wounding (and subsequent microbial invasion) occurs. In fact, neutrophils also seem to be the most common cell type invading inflamed epidermis, far outnumbering lymphocytes (Chuong et al. 2002). Although phagocytosis is their main weapon against invaders, they display several other tools to eliminate pathogens. Via TLRs they are able to directly recognize pathogens. However, binding and phagocytosis increase dramatically, when microbes are opsonized with complement. Furthermore they can produce reactive oxygen species (like O_2^-, HOCL, H_2O_2) and reactive nitrogen species (NO, NO_2) via the so-called respiratory burst. This is used alone or in combination with antimicrobial peptides, in order to kill pathogens.

Macrophages have many similar functions like neutrophils and mostly combat microbes by phagocytosis. In addition, they can also present antigen to T-cells and coordinate other cells via the release of inflammatory mediators and cytokines (Kuby 2007).

Natural killer (NK) cells provide a first line of defence against many different viral infections. They target and kill infected cells, as these are a potential source of large numbers of additional virus particles. They also produce interferon-gamma (IFNγ) and tumour necrosis factor-alpha (TNFα), two important immunoregulatory cytokines, which can stimulate the maturation of dendritic cells. IFNγ is also a powerful mediator of macrophage activation and an important regulator of T- helper cells, linking the NK cells with the adaptive immune system (Kuby 2007).

3.4 Antimicrobial peptides

3.4.1 General overview

Antimicrobial peptides (AMPs) are a widely distributed form of immune defence in nature. They have been isolated from single-celled microorganisms, plants, insects and other

invertebrates, fish, amphibians, birds and mammals including humans (Jenssen et al. 2006). More than 1700 of such peptides have been discovered and the number is constantly increasing. An updated list of AMPs can be found at the ANTIMIC database (http://research.i2r.a-star.edu.sg/Templar/DB/ANTIMIC/) (Brahmachary et al. 2004).

AMPs are mostly small cationic (net charge +2 - +9) and amphiphilic peptides of small molecular weight (consisting of 12 to 100 amino acids) (Hancock and Sahl 2006; Jenssen et al. 2006), but recently, non-cationic antimicrobial peptides, more accurately anionic, [e.g. dermcidin in human sweat (Schittek et al. 2001)] and aromatic peptides as well as peptides derived from oxygen-binding proteins have also been isolated (Vizioli and Salzet 2002). The cationic peptides can be classified into four different structural groups (**Fig. 3.11**): β-sheet peptides stabilized by two or four disulfide bridges (e.g. human α- and β-defensins), α-helical peptides (e.g. LL-37 or margainins), extended structures rich in glycine, proline, tryptophan, arginine and/or histidine (e.g. indolicidin), and loop peptides with one disulfide bridge (e.g. bactenecin) (Hancock and Sahl 2006; Jenssen et al. 2006). They are often synthesized as more or less inactive prepropeptides, which become processed to propeptides and subsequently to several different antimicrobially active derivatives (Radek et al. 2008; Zanetti 2005; Schittek et al. 2001).

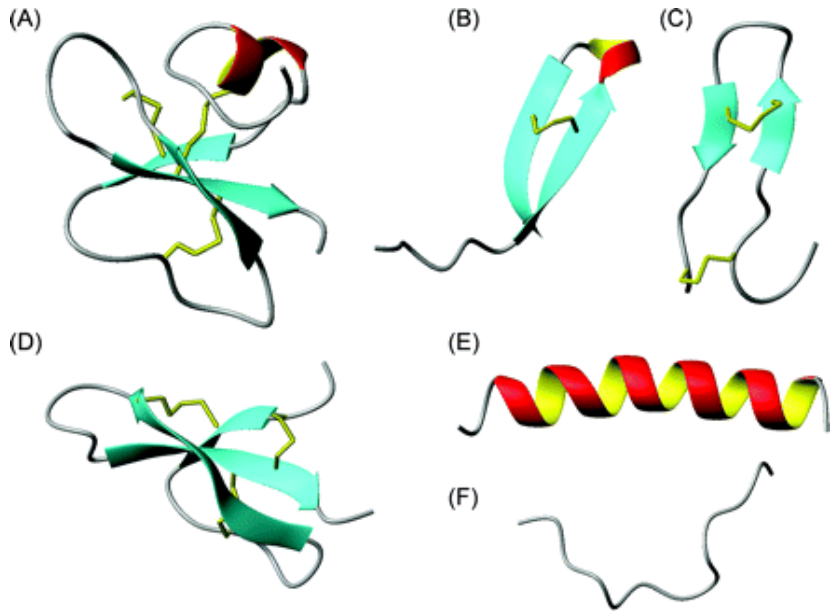

Fig. 3.11 Structural classes of AMPs
Examples of different structural classes of AMPs. A: mixed structure of human β- defensin-2, B: looped thanatin, C: β-sheeted polyphemusin, D: rabbit kidney defensin-1, E: α-helical magainin-2, F: extended indolicidin. Turquois arrows represent β-sheet structures; α-helical structures are depicted in red and yellow. Disulfide bonds are indicated in yellow (Jenssen et al. 2006).

Many AMPs are very similar to neuropeptides regarding their amino acid composition, amphipatic design, cationic charge and size (Brogden et al. 2005). Several neuropeptides even themselves exhibit antibacterial activity. In frogs, for example, it was demonstrated that precursors of AMPs show extensive sequence identities with precursors of opioid peptides (Salzet 2001; Amiche et al. 1994). And in humans, the antimicrobial activity of several neuropeptides like α-MSH, substance P, bradykinin etc. has been proven (Radek et al. 2008; Kowalska et al. 2002; Cutuli et al. 2000).
AMPs produced by bacteria were among the first to be discovered (Mattick and Hirsch 1947). While they do not exactly protect against infection, they contribute to survival of individual bacterial cells by killing other bacteria that might compete for nutrients in the same environment. The bacterial AMPs (also called bacteriocins) are thought to be produced by many or most of the bacteria and are generally extremely potent compared to their eukaryotic counterparts. One of the most extensively studied bacteriocins is, for example, nisin, produced by *Lactococcus lactis*, which has been commonly used as a food preservative for more than 50 years without significant development of resistance (Jenssen, Hamill et al. 2006).

In plants, AMPs also seem to play a fundamental role in defending the organism against bacterial and fungal infections. They can be found in a great number of diverse plant species and were proven to be expressed in various parts of the plant including leaves, flowers, seeds and tubers. Apart from demonstrating these peptides' bactericidal and fungicidal activity *in vitro*, it has been shown that overexpression of some peptides enhance plant tolerance to pathogens and peptide sensitive mutants of pathogens possess significantly decreased virulence towards plant tissues in which these peptides are present (Garcia-Olmedo, Molina et al. 1998; Garcia-Olmedo, Rodriguez-Palenzuela et al. 2001).

Invertebrates lack the presence of adaptive immunity found in vertebrate species. Therefore they have a very effective innate immune system, in which AMPs play a central role. Indeed, for the fruit fly *Drosophila melanogaster* the role of AMPs and the regulation of their expression including the different signalling pathways involved, is well understood (Imler and Bulet 2005) and therefore this animal serves as a model system to study innate immunity. In invertebrates AMPs are found in the hemolymph (plasma and hemocytes), in phagocytic cells and in certain epithelial cells. They can be constitutively expressed, e.g. in the hemocytes of marine arthropods like shrimp oyster and horseshoe crab or inducible upon pathogen recognition like the antifungal peptides in *Drosophila* (Jenssen et al. 2006).

Despite the presence of a functioning adaptive immune system a wide range of peptides has also been isolated in vertebrates. They can either be constitutively expressed or inducible upon stimulation with antigen. Consistent with their role in antimicrobial defence, AMPs in vertebrates are found at sites that routinely encounter pathogens such as the epithelia in the gastrointestinal and urinary tract and the skin, as well as the granules of immune cells (Mookherjee and Hancock 2007; Jenssen et al. 2006).

Amphibian skin glands have been proven to be a rich source of AMPs, with approximately 500 having been described as originating from this source. The α-helical magainins are the prototypic amphibian AMPs with strong membrane-permeabilizing activity towards gram-positive and –negative bacteria, fungi, yeasts and viruses (Zasloff 1987). The mechanisms of action have been extensively studied and therefore these peptides served as a template for the development of the first clinical AMP treatment, although this was finally not successful (Jenssen et al. 2006; Ge et al. 1999). In addition to the skin, AMPs in amphibians are also expressed in the mucosa of the stomach, indicating a role in protection from ingested pathogens.

In mammals AMPs have been isolated from various tissues, but as mentioned before mostly from immune cells, mucosal surfaces and the skin (Fan et al. 2008; Schittek et al. 2008). Furthermore, they can be found in body secretions like sweat and milk (Murakami et al. 2005; Armogida et al. 2004; Schittek et al. 2001). Direct antimicrobial action is not the only, and possibly not even the primary task of mammalian AMPs, as many of them actually suffer losses in their antibacterial properties under serum and tissue conditions (Dürr et al. 2006). Instead, they seem to fulfil important immunomodulatory and other function, which will be explained later. The two most important groups of mammalian AMPs are the cathelicidins and the defensins. They both act as precursor molecules that can release AMPs after proteolytic cleavage.

The cathelicidin family is characterized by a highly conserved cathelin domain, flanked by a signal peptide at the N-terminal and a variable cationic antimicrobial C-terminal (Schauber and Gallo 2008; Dürr et al. 2006). Cathelicidins have been isolated from many mammalian species such as mice, rabbits, sheep, cattle, horses and humans and were recently also found in chicken (Xiao et al. 2006) and fish (Chang et al. 2006). In humans, only a single cathelicidin is found. It is the same situation, e.g. for mice and rats, whereas other species like

cattle and pig have up to seven different cathelicidins (Namjoshi et al. 2008; Braff et al. 2005b).

Defensins contain six cystein residues that form characteristic disulfide bridges (Steinstraesser et al. 2008). According to the alignment of the disulfide bridges and the molecular structure this major AMP family is separated into α-, β- and θ-defensins (Braff et al. 2005b). Distantly related peptides are found in insects and plants. Mammalian defensins exhibit broad spectrum antimicrobial activity against bacteria, fungi and enveloped viruses.
The 6 different α-defensins contain three disulfide bridges in a 1-6, 2-4, 3-5 alignment. Four of them are expressed in human neutrophils, therefore also referred to as human neutrophil peptides (HNP) 1-4 (Harwig et al. 1994). They are stored in the azurophil granules as fully processed, mature peptides. The other two α-defensins (HD 5 and 6) are expressed as propeptides in Paneth cells of the small intestinal crypts and in epithelial cells of the female urogenital tract. The β-defensins contain three disulfide bridges in a 1-5, 2-4 and 3-6 pattern. The four best known β-defensins hBD 1-4 have been identified in various cells types, including epithelial cells of the skin and the lung and peripheral blood mononuclear cells (Fang et al. 2003; Harder et al. 2001; Harder et al. 1997; Bensch et al. 1995).

In recent years more and more proteins that exhibit antimicrobial effects have been discovered in mammals. Often these peptides are known for completely different functions, like the neuropeptides mentioned before, proteinase inhibitors or chemokines (El Karim et al. 2008; Braff et al. 2005b). The antimicrobial functions do not necessarily correlate with the function originally described. Lactoferrin antimicrobial activity for example, does not depend on its antitumour activity (Yang et al. 2004). RNase 3 does not require ribonuclease activity for antibacterial functioning, but for antiviral activity, in which the RNase activity is essential (Domachowske et al. 1998). However, it is important, that studies are critically evaluated as antimicrobial activity assays are often conducted under non-physiological conditions *in vitro* and many of such peptides are inactivated *in vivo* by physiological salt concentrations (Goldman et al. 1997).

3.4.2 Primary modes of action

AMPs represent one of the first lines of defence against invading pathogens. Due to their small size, they can be synthesized more than a 100 times faster than e.g. IgM, if a constant rate of peptide bond formation is assumed. Moreover, small peptides diffuse more rapidly than large proteins and immune cells (Nissen-Meyer and Nes 1997). As primary modes of action the name giving ability to act against bacteria, but also activity against fungi, parasites and viruses will be described.

3.4.2.1 Antimicrobial activity

The best-studied class of cationic AMPs are those with antibacterial activity (Jenssen et al. 2006). Regardless of their actual target of action, all AMPs must interact with the bacterial cytoplasmic membrane. The driving forces behind the antibacterial activity are the essential features of a net positive charge (enhancing interaction with anionic lipids and other bacterial targets), hypdrophobicity (required for membrane insertion) and flexibility (permitting the peptide to change from its solution conformation to its membrane-interacting conformation) (Jenssen et al. 2006; Hancock and Rozek 2002).

The first step of interaction is the electrostatic bonding between the cationic peptide and the negatively charged components present on the outer bacterial envelope, such as phosphate groups within the LPS of gram-negative bacteria, or lipoteichoic acid present on the surface of gram-positive bacteria. Then, in the case of gram-negative bacteria, a process called self-promoted uptake, takes place (Jenssen et al. 2006; Hancock and Scott 2000). In this process the AMPs (possibly prefolded into a membrane-associated structure) interact with the polyanionic surface LPS and competitively displace the divalent cations that bridge and partially neutralize the LPS, resulting in a disruption of the outer membrane (visualized as surface blebbing). Next, the peptides associate with the negatively charged cytoplasmic phospholipid membrane and enter into the interface between the hydrophobic and the hydrophilic portions of the membrane. Afterwards the peptides either form transmembrane pores, which result in membrane permeabilization or they translocate across the membrane and into the cytoplasm without causing major membrane disruption. There are several models proposed how exactly the peptides lead to membrane disruption and with which cellular

components they are interacting finally leading to the bacterium's death. These mechanisms are shown in greater detail in figure **3.12**.

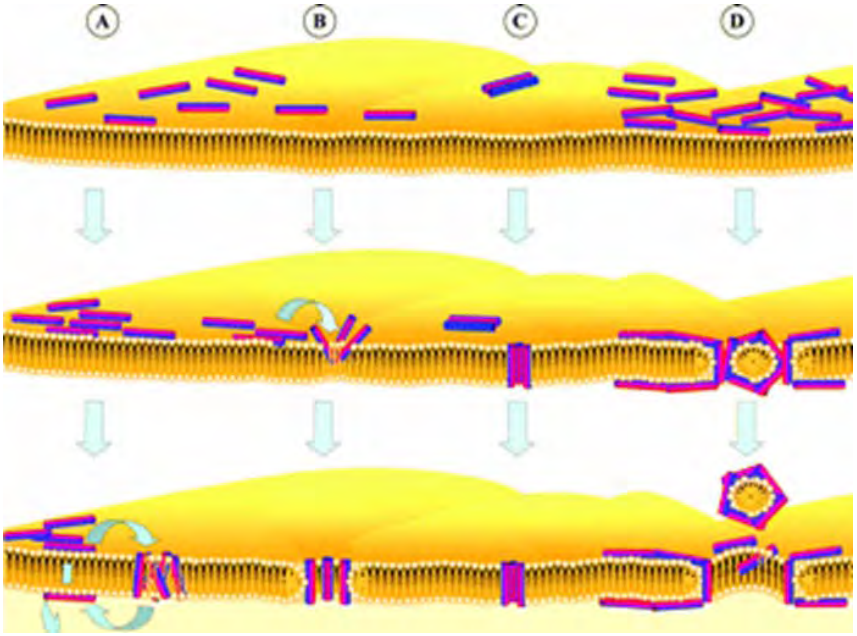

Fig. 3.12 Different ways of antimicrobial action of antimicrobial peptides
Models to explain mechanisms of membrane permeabilization are indicated in A to D. Antimicrobial peptides are shown as blue and red bars. In the "aggregate" model (A) peptides reorient to span the membrane as an aggregate with micelle-like complexes of peptides and lipids, but without adopting any particular orientation. The "toroidal pore" model (B) proposes that peptides insert perpendicular to the plane of the bilayer, with the hydrophilic regions of the peptides associating with the phospholipids head groups while the hydrophobic regions associate with the lipid core. In this process the membrane also curves inwards. In the "barrel stave" model (C) the peptides insert in a perpendicular orientation to the plane of the bilayer forming the "staves" in a "barrel"-shaped cluster with the hydrophilic regions of the peptides facing the lumen of the pore and the hydrophobic regions interacting with the lipid bilayer. The "carpet" model (D) proposes that peptides aggregate parallel to the lipid bilayer, coating local areas in a "carpet"-like fashion. At a given threshold concentration, this is thought to result in a detergent-like activity, causing formation of micelles and membrane pores. Modified from Jenssen et al. (Jenssen et al. 2006) .

The advantage of this rather physical mechanism of action (based on ionic and hydrophobic interactions) lies in the difficulty for the bacteria to create a resistant mutant. Naturally resistants occur, but are very few. They include *Burkholderia cepacia*, whose outer membrane composition does not permit self-promoted uptake, and *Proteus* and *Serratia spp.*, which express proteases that can cleave some cationic peptides (Hancock and Scott 2000).

Inside the membrane AMPs can inhibit the DNA, RNA or protein synthesis, by either inhibiting enzymes that are responsible for protein folding or modifying aminoglycosides. The formation of structural components of the cell wall, e.g. the transglycosilation of lipid II, which is necessary for the synthesis of peptidoglycan, also serves as a target.

3.4.2.2 Anti-endotoxin effects

In addition to the direct antimicrobial effect described above, these peptides can counteract sepsis, by significant reduction of endotoxin-induced inflammatory responses (see chapter 3.4.2) (Mookherjee and Hancock 2007). Due to their high affinity to the polyanionic LPS and to a lesser degree to LTA, they can bind those and therefore prevent the activation of TLR and consequent production of TNFα and IL-6 (Mookherjee and Hancock 2007; Hancock and Scott 2000). It has been shown that, for example, the cathelicidin LL-37 maintains its anti-endotoxin property in the presence of autologous serum even when the peptide was added after stimulation with bacterial endotoxin (Gough et al. 1996). This suggests the presence of other, direct mechanisms, which remain to be elucidated (Mookherjee and Hancock 2007)

3.4.2.3 Antifungal activity

The mode of action against fungi was first described as involving either cell wall lysis or interference with cell wall synthesis (Jenssen et al. 2006; De Lucca and Walsh 1999). But as the number of identified antifungal peptides increases, new modes of action are found. Therefore, the formation of reactive oxygen species and the depletion of mitochondria are subjects of current investigations.

3.4.2.4 Antiparasitic activity

Several AMPs possess an antiprotozoan activity which is basically mediated through permeabilization of the membrane. The porcine cathelicidine PMAP-23 has even been demonstrated to have an antinematodal effect against both the eggs and the worms of *Caenorhabditis elegans* (Park et al. 2004). Via pore formation or direct interaction with the lipid bilayers the cell membrane is disrupted (Jenssen et al. 2006).

3.4.2.5 Antiviral activity

The spectrum of viruses that are affected by AMPs comprise primarily enveloped RNA and DNA viruses with the exception of the non enveloped adenovirus, e.g. feline calicivirus and echovirus 6. The antiviral mode of action can be the blocking of viral entry by interacting with the host cell's heparan sulphate (that serves as an entry point for viruses), with specific cellular receptors (that are important for viral uptake) or with the viral glycoproteins. In addition, AMPs can also block the cell to cell spread of viruses, can damage the viral envelope or stabilize the host cell membrane or even prevent intracellular viral gene/protein expression (Jenssen et al. 2006).

3.4.3 Antimicrobial peptides as key molecules at the interface of innate and adaptive immunity

As in recent years the role of AMPs in immune response is further revealed, it becomes clear that they are much more than just microbicidal substances. Indeed, they have been shown to modulate the immune response in several different ways. Most data on these immunomodulatory activities are available for the probably best studied peptide LL-37, but also for β-defensins or psoriasin. This includes either a direct influence on immune cells, but also indirect via increasing the production of various cytokines.

Immunomodulatory activities include the induction of dendritic cell differentiation (Davidson et al. 2004), conversion of inert self-DNA into a potent trigger of interferon production as the key factor that mediates plasmacytoid dendritic cell activation (Lande et al. 2007), chemotaxis of mast cells (Niyonsaba et al. 2002), influencing the migration of macrophages by modulating their chemokine and chemokine receptor expression (Scott et al. 2002) and increasing the production of various cytokines such as IL-6, IL-8, and GM-CSF (granulocyte macrophage colony-stimulating factor) (Carretero et al. 2008; Gallo 2008; Braff et al. 2005b; Niyonsaba et al. 2005). LL-37 was even shown to be essential for the antimicrobial activity of mast cells (Di Nardo et al. 2003). The authors demonstrated that mast cells from cathelicidin deficient mice had a 50 % reduction in their ability to kill group A *streptococcus*, although these mast cells had the same amount of β-defensin mRNA compared to the mast cells of wild type mice.

β-defensins (HBD2) were shown to be chemotactic for immature dendritic cells and memory T-cells (Yang et al. 1999). HBD2 also promotes histamine release and prostaglandine D2 production in mast cells, suggesting a potential immunotherapeutic role as a vaccine adjuvant to enhance antibody production (Befus et al. 1999).

3.4.4 Additional non-immunological functions of AMPs

Apart from their complex role in immunity, AMPs also seem to be important in various other fields. First, they do have a role in wound healing. Several different AMPs were reported to be up-regulated in wounded tissue (Steinstraesser et al. 2008). The cathelicidin LL-37 for example, was found in increased levels in human skin after sterile incision, which returned to normal as the wound was closed (Dorschner et al. 2001). This was thought to be important for defence reasons. However, as specific antibodies against LL-37 could actually prevent wounds from healing and LL-37 was only found in reduced levels in chronic ulcer epithelium (Heilborn et al. 2003) it became clear, that LL-37 must also have an effect on the wound closure itself. Tokumaru and colleagues could show the influence of LL-37 on keratinocyte migration and postulated a functional link to the epidermal growth factor (Tokumaru et al. 2005). In addition, a most recent study (Carretero et al. 2008) demonstrated the migration of cultured human keratinocytes in an *in vitro* wound model could be confirmed, but with a link to the FPRL (formyl peptide receptor like) -1 receptor. Furthermore LL-37 was able to significantly improve the re-epithelization and granulation tissue formation *in vivo* by adenoviral transfer to excisional wounds in diabetic mice. The human β-defensins (HBD) 1-3 were found to be up-regulated and in a different expression pattern (as the epidermis is destroyed) in burned skin (Kaus et al. 2008; Poindexter 2005). HBD 2 is up-regulated in acute and chronic wounds (Butmarc et al. 2004). HBD 2-4 were also reported to stimulate the migration and proliferation of epidermal keratinocytes (Niyonsaba et al. 2007).
In addition, psoriasin is expressed in high levels in wound exudate and granulation tissue and was demonstrated to be up-regulated in experimental skin barrier disruption (Gläser et al. 2008; Lee and Eckert 2007)

Koczulla et al. demonstrated the influence of LL-37 on angiogenesis in the chorioallantoic membrane assay and a rabbit hind-limb model of ischemia (Koczulla et al. 2003). LL-37 application resulted in neovascularization in both models with direct participation of FPRL-1.

The angiogenic effect could be confirmed by another group in a skinfold chamber model in mice (Steinstraesser et al. 2006).

As mentioned above, a lot of proteins that are well known for other functions are also reported to show antimicrobial activity. This includes neuropeptides like α-MSH, neuropeptide Y or substance P, ribonucleases, proteinase inhibitors and many more, which cannot be described in detail (Rosenberg 2008; Braff et al. 2005b; Kowalska et al. 2002; Cutuli et al. 2000).

In summary, AMPs exhibit a wide variety of functions in the body. However, the many diverse AMPs should not be put into a single conceptual category, as a function seen in one peptide must not necessarily be implied on another (Gallo 2008).

3.4.5 Antimicrobial peptides in the skin

The skin possesses several lines of defence, which include a soluble AMP barrier which is either constitutively expressed or inducible when physical barriers fail to prevent microbial entry (see chapter 3.3). AMPs are expressed in resident cells like keratinocytes and sebocytes, but also from infiltrating cells like granulocytes and macrophages.

The first antimicrobial peptide found in human skin was lysozyme (Ogawa et al. 1971; Klenha and Krs 1967), which was originally described as “bacteriolytic element” in nasal secretion as early as 1922 (Fleming 1922) suggesting that human epithelial glands also secrete antimicrobial compounds (Schröder and Harder 2006). In skin it is located in the cytoplasm of epidermal cells in granular layers and Malpighian cells. Cells in the pilosebaceous unit and hair bulb, as well as parts of eccrine sweat glands were also positive for lysozyme (Papini et al. 1982; Klenha and Krs 1967). It is mainly active against gram-positive bacteria, but also shows activity against gram-negative bacteria like *Escherichia coli* and *Pseudomonas aeruginosa*. However, the contribution of lysozyme to cutaneous defence is still unclear, as lysozyme is expressed exclusively in the cytoplasm and immunoreactive lysozyme is absent in the stratum corneum.

The two most important and most extensively studied AMPs in the skin are the cathelicidins and the β-defensins which were already described before (see chapter 3.4.1). Cathelicidin under physiological conditions is constitutively expressed in neutrophils and sweat glands and is inducible in keratinocytes and mast cells (Schauber and Gallo 2008; Braff et al. 2005b)
HBD1 seems to be constitutively expressed in the epidermis, whereas HBD2 and 3 are inducible under inflammatory conditions. HBD2 is mainly active against gram-negative bacteria whereas HBD3 exhibits broad spectrum antimicrobial activity (Schröder and Harder 2006; Harder et al. 2001).

Although the cathelicidin LL-37 was also reported to be secreted with sweat, dermcidin appears to be the principal sweat AMP that is constitutively and exclusively produced by eccrine sweat glands and secreted into the sweat. This peptide is expressed as a 9.3 kDa precursor, which is proteolytically cleaved, resulting in dermcidin 1 (DCD1), a 47 amino acid containing peptide with broad spectrum antimicrobial activity. DCD1 is effective in low micromolar concentrations unaffected by conditions like low pH and increased salt concentrations which can occur *in vivo* (Rieg et al. 2004; Schittek et al. 2001).

Among the most important AMPs in the skin, there are also RNase 7 and psoriasin, which will be described in detail later (see chapter 3.5.1 and 3.5.2).

The skin is therefore protected by an abundance of different AMPs. They are expressed in different parts of the epidermis which comprises various keratinocyte layers with multiple states of differentiation. Some of these AMPs are constitutively expressed in the uppermost layers of the epidermis, where they are stored in lamellar bodies and then secreted upon final differentiation, so that they are now present at the surface of fully differentiated keratinocytes which form the stratum corneum (Schittek et al. 2008; Elias 2007).

In normal skin with an intact physical barrier these constitutively expressed AMPs control bacterial growth (**Fig. 3.11 A** left). In microwounds, which can occur from physical stress, the physical barrier is disrupted (**Fig. 3.11 A** center) and bacteria and their products can reach living keratinocytes, which in response produce and secret inducible AMPs, without causing inflammation. Only in case of extensive wounding or a massive pathogenic bacterial burden, pro-inflammatory cytokines would be induced and immune cells would be recruited and infiltrate the skin (**Fig. 3.11 B**) (Schröder and Harder 2006).

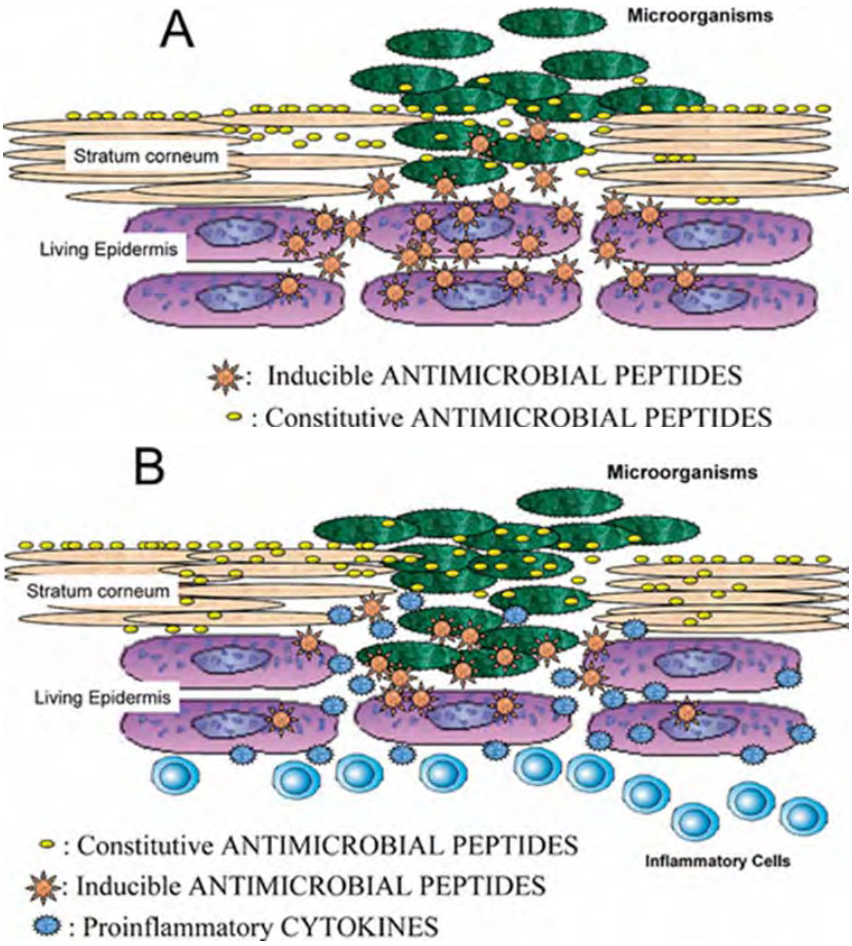

Fig. 3.13 Antimicrobial peptides in the skin
Putative model of a protective function of keratinocyte-derived antimicrobial peptides: In intact skin with an intact physical barrier bacterial growth is controlled by bacteriostatic and bactericidal compounds at the skin surface (A, left). Once the physical barrier is disturbed (A, center), bacteria (or bacterial products) have access to living epidermal keratinocytes, which may now in response secret inducible antimicrobial peptides. When microorganisms induce proinflammatory cytokines and come into contact with keratinocytes and immune cells in deeper areas of the skin, inflammatory cells are recruited and inflammation begins (B) (Schröder and Harder 2006).

A number of studies suggested an association of the activation of the innate immune system and the pathogenesis of inflammatory skin diseases.

Psoriasis is a chronic inflammatory disease which is characterized by a disturbed proliferation and differentiation of keratinocytes accompanied by vascular alterations and epidermal infiltration of activated TH1 lymphocytes and antigen-presenting cells together with a local TH1 cytokine immune response (Büchau and Gallo 2007). Several AMPs like HBD2 and 3, the cathelicidin LL-37, psoriasin, RNase 7 etc. were found to be expressed in increased levels in psoriatic skin. Consistent to that psoriatic skin seldom suffers from bacterial infections (Büchau and Gallo 2007; Harder and Schröder 2005).

Instead, patients suffering from atopic dermatitis often have skin infections with *Staphylococcus aureus*. In acute and chronic lesions of these patients significantly lower levels of antimicrobial peptides like HBD2 and 3 and LL37 are present, which may account for their increased susceptibility to these skin infections (Ong et al. 2002). However, there is no overall lower level of AMPs in atopic dermatitis, as only recently psoriasin protein expression was shown to be up-regulated in the epidermis and its surface of atopic dermatitis patients (Gläser et al. 2008).

There is obviously a very fine line between beneficial high levels of AMPs and disease related high levels of AMPs. It was shown, for example that in rosacea, AMPs are found in very high levels and also in different proteolytically processed forms which might suggest that an exacerbated innate immune system contributes to the pathogenesis of this disease (Schittek et al. 2008; Bevins and Liu 2007; Yamasaki et al. 2007).

3.5 Selected AMPs

In the following four different AMPs found in human epidermis are described in greater detail: RNase 7, psoriasin, hornerin and LEKTI-2. Two of them, RNase 7 and psoriasin, are well known and published and the other two are just newly discovered peptides, whose exact role in immune defence and skin biology still remains to be elucidated.

3.5.1 RNase 7

RNase 7 is a 14.5 kDa highly basic protein which carries four disulfide bridges. It was discovered by analyzing healthy persons' stratum corneum by heparin-affinity purification followed by high performance liquid chromatography (Harder and Schröder 2002). Due to its high sequence similarity and high catalytic activity it was placed in the RNase A superfamily. Among this family there are also for example RNase 2 (eosinophil derived neurotoxin, EDN) and RNase 3 (eosinophil-cationic protein, ECP), which can be found in the granules of eosinophils, RNase 5 (angiogenin), (Dyer and Rosenberg 2006) and RNase 8 (Rudolph et al. 2006), which were all reported to have a role in host defence (**Table 3.5**).

RNase A ribonuclease	Host defence activities
Eosinophil-derived neurotoxin (RNase 2)	chemoatractant for immature human dendritic cells enhances maturation of dendritic cells reduces infectivity for RNA viruses (HRSV, HIV) in tissue culture assays produced in macrophages in response to LPS and TNF-α activates TLR2 and augments TH2 based immune response
Eosinophil cationic protein (RNase 3)	cytotoxic for helminthes, strong bactericidal activity reduces infectivity for HRSV in tissue culture assays
Angiogenin (RNase 5)	moderate bactericidal activity against *Streptococcus pneumoniae*
RNase 8	broad-spectrum moderate bactericidal activity

Table 3.5 Summary of host defence functions for members of the RNase A ribonucleases in humans
Moderate bactericidal activity: low micromolar concentrations in standard overnight incubation assay reduce colony count ten- to 100-fold; strong bactericidal activity: micromolar concentrations reduce colony count 10^4- to 10^7-fold. Table adapted from Rosenberg (Rosenberg 2008).

Besides a high expression level in the skin, RNase 7 mRNA can be detected in several other epithelial tissues including the respiratory tract, the urogenital tract and to a lower extent the gastrointestinal tract (Harder and Schröder 2002).

RNase 7 shows a broad-spectrum antimicrobial activity against several potentially pathogenic gram-negative (*Escherichia coli, Pseudomonas aeruginosa*) and gram-positive (*Staphylococcus aureus, Propionibacterium acnes, Enterococcus faecium*) as well as the yeast *Candida albicans* (Harder and Schröder 2002). Particularly interesting is the high efficacy against a Vancomycin-resistant strain of *Enterococcus faecium* already in nanomolar concentrations. Upon stimulation with bacteria (*Pseudomonas aeruginosa, Stapylococcus aureus and Escherichia coli*) and pro-inflammatory cytokines (TNFα, INFγ and IL1β) the expression level of RNase 7 in cultured primary human keratinocytes could be increased up to 8.5 fold (Harder and Schröder 2002).

Independent from this first report another group published only a few months later the identification of the RNase 7 sequence from the human genome (Zhang et al. 2003). They could also define the unusual lysine-rich nature of the cationic coding sequence and demonstrated in an *in vitro* assay the dose dependent cytotoxicity of RNase 7 against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Furthermore they detected RNase 7 expression additionally in the liver, kidney, skeletal muscle and the heart.

Recently, Huang and colleagues could demonstrate that the lysine residues of RNase 7 are the critical factor for membrane permeabilization and antimicrobial activity and that this is energy independent (Huang et al. 2007). However, the exact mechanism of action remains to be discovered.

3.5.2 Psoriasin (S100A7)

Psoriasin (which is also called S100 A7) is a small protein with a mass of 11 kDa and belongs to the S 100 family. S100 proteins are well known as useful markers in diagnostic immunohistochemistry laboratories dealing with cutaneous tumours, as polyclonal anti S100 antibodies were strongly reactive especially for melanoma. However this multigene family actually consists of more than 20 proteins of low molecular weight and is defined by its property of remaining soluble in 100% saturated ammonium sulphate (McNutt 1998). It is characterized by two calcium binding sites of the helix-loop-helix ("EF-hand type") conformation of which the C-terminal binds calcium with a 100-fold higher affinity than the N-terminal one (Eckert et al. 2004). In response to calcium the S100 proteins undergo a conformational change which enables them to act in the intracellular signal transduction regulating important cell processes like cell proliferation, differentiation and apoptosis (Donato 2001). Fourteen of these proteins have their genes located in the epidermal differentiation complex (EDC) on human chromosome 1q21 (Moubayed et al. 2007; Eckert et al. 2004) and play a role in forming the cornified envelope (**Fig. 3.9**). Beside psoriasin, antimicrobial activity has been reported for several other S100 proteins like the S 100 A8/S100 A9 complex (Miyasaki et al. 1993; Murthy et al. 1993), S100 A12 (Cole et al. 2001) and S100 A15 (Büchau et al. 2007).

Psoriasin was originally described as a protein of unknown function up-regulated in psoriatic skin, using two dimensional protein gel chromatography and comparing psoriatic and normal skin (Madsen et al. 1991). In a further study it could be shown that this elevated expression was not only restricted to psoriasis, but was also found in other inflammatory skin diseases like atopic dermatitis, mycosis fungoides, Darier`s disease and inflammatory lichen sclerosus et atrophicus (Gläser et al. 2008; Algermissen et al. 1996). In addition a chemotactic activity towards CD 4+ T- lymphocytes and neutrophils was reported (Jinquan et al. 1996).

Apart from inflammatory skin conditions elevated expression levels of psoriasin were detected in several tumours like squamous cell carcinoma of the bladder (Celis et al. 1996), the head and neck, the cervix and the lung (Emberley et al. 2004), in adenocarcinoma of the stomach and the breast (Al-Haddad et al. 1999), as well as in various epithelial skin tumours (Moubayed et al. 2007). Some of these expression data are consistent with a role as a chemotactic factor in mediating the inflammatory response, similar to that initially reported in psoriasis (Jinquan et al. 1996). But other data point towards a role of overexpressed psoriasin in tumour invasion and progression (Moubayed et al. 2007; Emberley et al. 2004).

In 2005, psoriasin was shown to be a very potent AMP having its main activity against *Escherichia coli* and a far less activity against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (Gläser et al. 2005). The *Escherichia coli*-cidal activity could be shown *in vitro* and *in vivo*, e.g. very impressively by the fact that inoculation of *Escherichia coli* on the skin of washed fingertips resulted in death of these microbes (**Fig. 3.12).**

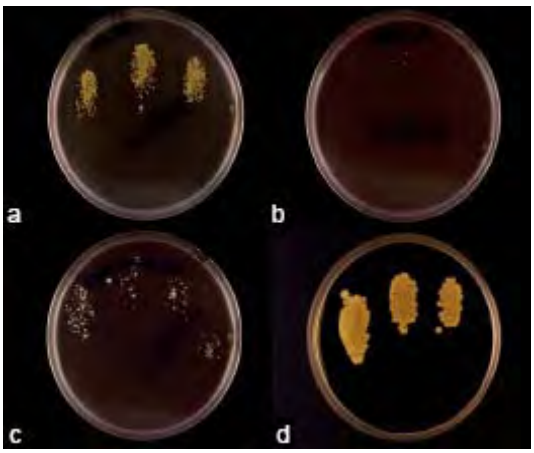

Fig. 3.14 Exposure to human skin kills *Escherichia coli*
Washed fingertips of a healthy volunteer were artificially inoculated for 30 minutes with *Escherichia coli* (a) or *Staphylococcus aureus* (b) and then pressed on an agar-plate Unwashed fingertips served as a control (c) and revealed some bacteria of the commensal flora. (d) shows the outnumbered growth of bacteria on gloved fingertips inoculated with *Escherichia coli* (Gläser et al. 2005).

It was found to be up-regulated in cultured human epidermal keratinocytes after stimulation with bacterial culture supernatants of *Eschericha coli*, IL-1ß, and TNFα, but not by IFNγ. As possible mechanism of action the sequestration of Zn^{2+} was suggested, as the protein showed diminished antimicrobial activity after preincubation in $ZnSO_4$. Immunohistochemistry showed psoriasin in the suprabasal layers of the epidermis, in the sebaceous glands and in the upper part of the hair follicle. In addition, the peptide was found to be secreted on the skin

surface varying substantially between different body locations with highest amounts in presumably highly colonized body parts (Gläser et al. 2005). In another study, it was shown that monomer and covalently cross-linked psoriasin is expressed in high levels in wound exudate and granulation tissue. It was suggested that the keratinocytes surrounding the wound produce and release the peptide into the wound exudates and it could be confirmed that the antimicrobial activity is depending on the zink-binding motif (Lee and Eckert 2007).

Further investigations upon the responsible stimulation mechanisms revealed flagellin to be the principle inducer of psoriasin in epidermal keratinocytes (Abtin et al. 2008). The authors could show that in primary cell culture of human epidermal keratinocytes, psoriasin can be induced by flagellated (wild-type) *Escherichia coli*, but not by a flagellin deficient strain. In addition, the knockdown of TLR5, the known binding site of flagellin resulted in a suppression of *Escherichia coli* induced up-regulation of psoriasin.

3.5.3 Hornerin

Hornerin was first described in humans by Takaishi et al. (Takaishi et al. 2005). Hornerin was immunolocalized in human psoriatic and wounded skin, but not in healthy skin from the trunk. According to these authors, hornerin was mostly found in the peripheral zone of lesional psoriatic skin and in healing human epidermis from day 5 to day 30 and disappeared thereafter. Earlier, the same group had already identified hornerin in mouse skin (Makino et al. 2003; Makino et al. 2001), in which they could localize hornerin IR to the granular and cornified layers of healthy mature murine epidermis, as well as the tongue, the esophagus and the forestomach.

Like psoriasin, hornerin is also a member of the fused S100 gene family (see chapter 3.5.2) and therefore possesses a S100 domain, an EF-hand calcium-binding domain, a spacer sequence and two types of tandem repeats. The S100 family includes other structural proteins expressed late during epidermal differentiation like profilaggrin (Mischke et al. 1996), trychohyalin (Lee et al. 1993) and repetin (Huber et al. 2005; Krieg et al. 1997). Since hornerin was mostly found to be colocalized with profilaggrin in humans (Takaishi et al. 2005) as well as in mice (Makino et al. 2003; Makino et al. 2001) a function in cornification similar to but distinct from filaggrin has been proposed.

The group around Schröder recently reported evidence that derivatives of the full length hornerin, in particular the tandem repeat unit A, exhibit antimicrobial activity at low micromolar concentrations against *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Other derivatives including the tandem repeat unit B- or C-terminus were found to be far less or even not at all antimicrobially active (Wu et al. 2007). In contrary to what was reported before, the same group in addition showed hornerin to be expressed not only in wounded skin but also in normal healthy human skin in varying individual expression levels in various body locations as well as in inflamed skin of patients suffering from atopic dermatitis, psoriasis and verrucae vulgares (Meyer-Hoffert et al. 2007).

3.5.4 LEKTI-2

LEKTI-2 was only recently identified by the group around Schröder upon analysing healthy person's heel stratum corneum (Wu et al. 2008a). It is localized on the SPINK (serine protease inhibitors Kazal type) 9 gene and consists of a typical Kazal domain and an N-terminal signal peptide domain. Apart from the skin SPINK 9 mRNA is highly expressed in thymus and to a lower degree in tonsil, adenoid and bronchial epithelial cells. LEKTI-2 is extremely heterogenous occurring in several N-terminal truncated and in N-terminal extended forms. The recombinant full length protein of 86 amino acids only exerts antifungal activity against *Candida albicans*. However, the mature, naturally occurring peptide of 7 kDa was found to kill *Escherichia coli* (Wu 2005). Antimicrobial assays revealed that of these different N-terminal forms only the N-terminal extended 62- and 63- LEKTI-2 variants exhibit antimicrobial activity. Furthermore, LEKTI 2 specifically inhibits kallikrein 5 (personal communication, Prof. Schröder, Kiel).

The name LEKTI 2 was chosen, because its Kazal domain is highly similar to two domains of the multi- ´Lympho-Epithelial-Kazal-type-Inhibitor` LEKTI. LEKTI is a protease inhibitor protein and is encoded by the gene SPINK5 (Mägert et al. 1999). LEKTI inhibits serinproteases and through this regulates epithelia formation and keratinocyte terminal differentiation. Its defects lead to overdesquamation of corneocytes and skin barrier dysfunction (Komatsu et al. 2002). Mutations affecting LEKTI synthesis result in Netherton syndrome (Trichorrhexis invaginata), a rare autosomal recessive disease, characterized by severe scaling due to defective cornification, hair anomalies (bamboo hair) and immunologic

alterations resembling atopic dermatitis (Schechter et al. 2005). LEKTI is also indirectly involved in the control of innate immunity. It inhibits the kallikreins 5 and 7, which control the activation of the human cathelicidin precursor protein hCAP18 to the antimicrobially active LL-37. Therefore a lack of LEKTI, as shown in SPINK5 deficient mice, results in an increased production of LL-37 and a higher antimicrobial activity of the skin (Yamasaki et al. 2006).
One SPINK protein isolated of the skin of the frog *Phyllomedusa sauvagii* is described to exhibit antimicrobial activity (Gebhard et al. 2004).

3.6 Antimicrobial peptides in the hair follicle

3.6.1 Antimicrobial peptide expression and function in the human hair follicle

Systematic investigations of AMPs in human hair follicles are extremely rare, as most researchers mainly focus on the epidermis and dermis. One of the systematic approaches was conducted by Chronnell et al. in 2001 (Chronnell et al. 2001). Using *in situ* hybridization and immunohistochemistry the authors were analysing the expression pattern of human β-defensin 1 and 2 (HBD 1 and 2) in healthy person's hair follicles as well as in perilesional and intralesional skin of patients suffering from acne vulgaris. Strong HBD 1 and 2 expression was found in the suprabasal layers of the epidermis, in the distal ORS and in the sebaceous gland duct. In acne vulgaris lesions - particularly in pustules - the expression was markedly up-regulated for HBD 2 and to a lesser degree also for HBD 1 compared to the control. This suggests an involvement of β-defensins in the pathogenesis of acne vulgaris, although it might be that the upregulation of these AMPs is a secondary effect due to proinflammatory cytokines.
By other authors an up-regulated expression of HBD2 was also seen in superficial folliculitis (Oono et al. 2003).

Furthermore the same group mentioned before conducted a study on the expression and possible role of adrenomedullin in the hair follicle (Müller et al. 2003). Adrenomedullin was first isolated and described in the adrenal medulla and was found to have a vasodilatatory function (Hinson et al. 2000). But in fact, this peptide was found in a lot of different body

tissues and has a remarkable range of different actions, from regulating cellular growth and differentiation through modulating hormone secretion and finally also antimicrobial effects. Antimicrobial activity is directed against gram-negative as well as gram-positive bacteria (Allaker et al. 1999). In the hair follicle, adrenomedullin protein was strongly expressed in the basal and suprabasal layers of the hair bulb and the proximal ORS. In the distal ORS adrenomedullin immunoreactivity was increasingly suprabasal – a pattern like in the epidermis- especially in proximity to the bulge region where the basal layer was free of immunoreactivity (Müller et al. 2003).

The cathelicidin hCAP18 was reported to be constitutively expressed in human hair follicle epithelium (Braff et al. 2005b).

Lysozyme, the first AMP found in skin is also present in the hair follicle. According to the authors cells in the pilosebaceous unit and hair bulb express lysozyme (Papini et al. 1982).

In the first report of psoriasin as an antimicrobial peptide (Gläser et al. 2005) immunohistochemistry of one single facial hair follicle was shown, with strongest immunoreactivity in the distal ORS.

Bactericidal/permeability increasing protein was originally isolated from leukocytes. It shows cytotoxicity towards gram-negative bacteria and can bind LPS. In human HFs it was reported to have a strong immunoreactivity in the Henle and Huxley layer of the IRS and to be present as RNA transcript in human scalp skin (Takahashi et al. 2004).

α-MSH, which was proven to have antimicrobial activity (Cutuli et al. 2000), could be detected in the ORS of human hair follicle epithelium by RT-PCR and immunohistochemistry (Kono et al. 2001). The authors' interest in this study, however, was mainly directed towards its role in melanogenesis or neuropeptide function.

3.6.2 Antimicrobial peptide expression and function in the hair follicle of other mammalian species

There are even less studies on the expression of AMPs in hair follicles of other mammalian species than humans.

The expression of β-defensin 2, which possesses antimicrobial activity against gram-negative bacteria and *candida*, as well as a bacteriostatic activity against gram-positive bacteria was studied in C 57/BL6 mice (Selleri et al. 2007). In normal skin samples β-defensin 2 showed a weak immunoreactivity in the ORS. Depending on which toll-like-receptor agonists was used for stimulation, the staining pattern changed to be found a) in the IRS and inner layer of the ORS (TLR2), or b) very strong in all sheaths of the hair follicle, the hair shaft and even in the fibroblasts of the dermal papilla (TLR4) or c) in the proximal and middle part of both the IRS and ORS (TLR5).

In the same study as mentioned before (see chapter 3.6.2) bactericidal/permeability increasing protein was in addition to the human hair follicle also shown in the Henle and Huxley layer of rat hair follicles (Takahashi et al. 2004).

4 MATERIAL AND METHODS

4.1 Tissue specimens

Normal human scalp skin from the temporal and occipital region was obtained from 11 female patients aged between 37 and 67 (mean: 57 years +/- 8,49) undergoing routine face-lift surgery after informed consent. All experiments were performed according to the guidelines of the world medical association declaration of Helsinki and with appropriate ethics committee approval (Goodyear et al. 2007; World Medical Association 2007).
The skin was embedded, either immediately or after culture (see chapter 4.2), in Shandon Cryomatrix (Anatomical Pathology, Pittsburgh, PA, USA) and frozen in liquid nitrogen. Samples were cut in 6-7 µm thick cryosections and stored at -80°C until stained. Skin was cut vertically, in order to get length sections of the entire hair follicle.
Per each antigen 3-8 different cryosections, with a sum of 10 hair follicles of at least 5 individuals were examined.

4.2 Full thickness scalp skin organ culture

Full thickness scalp skin organ culture of three different patients was conducted as previously described (Lu et al. 2007). Biopsies of 3-4 mm in diameter were punched out and washed in isolation medium containing William's E medium (Biochrom, Cambridge, UK) with a 1% antibiotic/antimycotic mixture of penicillin G, streptomycin and amphotericin B (100x, Gibco, Karlsruhe, Germany). The skin pieces were then carefully placed in a 6-well plate (4 biopsies/well) containing 4 ml culture medium consisting of William's E medium (Biochrom, Cambridge, UK) enriched with 100 IU/ml penicillin, 10 µg/ml streptomycin (Gibco, Karlsruhe Germany), 10 µg/ml insulin (Sigma-Aldrich, Taufkirchen, Germany), 10 ng/ml hydrocortisone (Sigma-Aldrich) and 2 mmol/l L-glutamine (Invitrogen, Paisley, UK). The biopsies were allowed to float freely with the epidermis facing up and the dermis in the medium (**Fig. 3-1**). They were kept in a CO_2–enriched atmosphere (5 % CO_2 and 95 % air) at a temperature of 37 °C for three days. To minimize artefacts (because of up-regulation of cytokines due to wounding) the skin was left untreated in the culture medium for 48 h. On day 2 the biopsies were incubated for 24 hours with medium and one of the following substances:

(a) 1 µg/ml LPS from *Escherichia coli* O55:B04 (L6529 Sigma-Aldrich),

(b) 1 µg/ml LPS from *Pseudomonas aeruginosa* (Sigma-Aldrich),

(c) 1 µg/ml LPS from both *Escherichia coli* and *Pseudomonas aeruginosa* (Sigma-Aldrich),

(d) 5 µg/ml LTA from *Streptococcus pyogenes* (Sigma-Aldrich),

(e) 5 µg/ml protein A from *Stapylococcus aureus* (Sigma-Aldrich),

(f) 100 IU/ml IFNγ (Prepotech, Rocky Hill, NJ, USA) or

(g) vehicle (PBS).

All substances were kept in a stock solution dissolved in PBS and further diluted with culture medium. On day 3 biopsies were frozen in liquid nitrogen and stored at -80 °C until use.

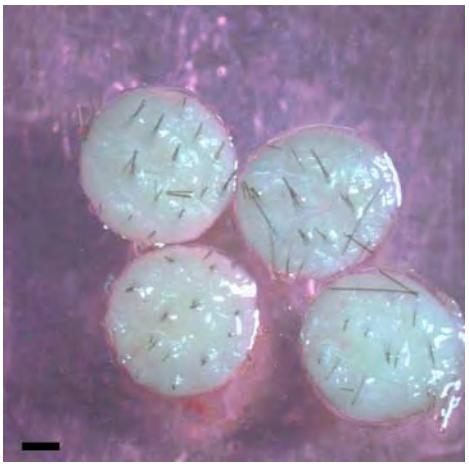

Fig. 4.1 Skin biopsies in culture
3-4 biopsies from human scalp skin are free floating in a well containing 4 ml of supplemented William's E medium. Scale bar represents 1mm.

4.3 Immunohistochemistry

Localization of the immunoreactivity (IR) of the different AMPs was detected using an avidin-biotin-complex (ABC) - alkaline phosphatase method (Vectastain ABC-kit, Vector Laboratories, Burlingame, CA, USA) and additionally the highly sensitive tyramide signal amplification (TSA) (TSA kit; Perkin-Elmer, Boston, USA) method which both have been described before (Kloepper et al. 2008; Telek et al. 2007).

4.3.1 Primary and secondary antibodies

Primary antibodies were all kindly provided from the collaborating group of Prof. Schröder at the Department of Dermatology at the University of Kiel. The specificity of these antibodies was tested by immunodot analysis and Western blot as well as blocking experiments in immunohistochemical stainings. Table **4.1** shows the primary and secondary antibodies as well as the used detection system.

Primary antibody	Origin	Reference	Dilution	Secondary antibody (biotinylated)	Vendor	Secondary detection system
Psoriasin	mouse monoclonal	(Gläser et al. 2005)	1:10000	goat anti-mouse	Immunotech, Beckman Coulter (Fullerton, CA, USA)	ABC-AP
			1:15000			TSA
RNase 7	goat polyclonal	(Köten et al. *submitted*)	1:100	mouse anti-goat	Jackson Immunoresearch (Cambridgeshire, UK)	ABC-AP
			1:400	rabbit anti-goat	Dako Cytomation (Glostrup , Denmark)	TSA
Hornerin	goat polyclonal	(Meyer-Hoffert et al. 2007)	1:200	mouse anti-goat	Jackson Immunoresearch (Cambridgeshire, UK)	ABC-AP
			1:800	rabbit anti-goat	Dako Cytomation (Glostrup , Denmark)	TSA
LEKTI-2	goat polyclonal	personal communication with Prof. Schröder	1:80	mouse anti-goat	Jackson Immunoresearch (Cambridgeshire, UK)	ABC-AP
			1:200	rabbit anti-goat	Dako Cytomation (Glostrup , Denmark)	TSA

Table 4.1 Primary and secondary antibodies and secondary detection systems
ABC-AP: Avidin-Biotin-Complex, alkaline phosphatase, TSA: tyramide signal amplification

4.3.2 Avidin-Biotin-Complex-Alkaline Phosphatase (ABC)

Cryosections (6-7 µm thick) were air dried for 10 minutes (min) and fixed in acetone at -20°C for 10 min. Non-specific binding was blocked for 20 min with 10 % normal serum (originated from the same animal as the secondary antibody) in tris buffered saline (TBS), containing 6.1 g/l tris base and 8.8 g/l sodium chloride (NaCl) at a pH of 7.6. Afterwards, without washing, the slides were immediately incubated with the primary antibody in the appropriate dilution (see chapter 4.3.1) overnight at 4 °C. The following day, the secondary biotinylated antibody (see chapter 4.3.1) (1:200 in TBS) was applied at room temperature for 45 min and followed by an incubation for 30 min at room temperature with the enzyme substrate (ABC reagent). The reaction product was visualized using Fast Red (Sigma-Aldrich) for 6 min. In between each step the slides were washed three times for 5 min in TBS unless stated differently.

Counterstaining was performed with Mayer's haematoxylin (Roth, Karlsruhe, Germany) for 1 min followed by rinsing in tab water for 10 min. Finally, slides were mounted with the aqueous mounting medium Faramount (DakoCytomation, Hamburg, Germany). In the negative control no primary antibody was applied. Apart from that all staining steps were performed as described.

The epidermis in each section served as positive control, as the immunolocalization of these antibodies was already described in the epidermis (Wu et al. 2008a; Meyer-Hoffert et al. 2007; Gläser et al. 2005; Harder and Schröder 2002).

4.3.3 Tyramide signal amplification (TSA)

Sections were air-dried and fixed in acetone at -20 °C as described above. Thereafter endogenous horseradish peroxidase was blocked with 3 % H_2O_2 in phosphate buffered saline (PBS) [containing 1.8 g NaH_2PO_4 x H_2O (sodium dihydrophosphate monohydrate) and 8.0 g NaCl, pH 7.2) for 15 min. Afterwards further blocking steps were conducted using avidin and biotin (both Vector Laboratories, Burlingame, CA, USA) each for 15 min. Preincubation was done with 5 % normal serum (originated from the same animal as the secondary antibody) (DAKO, Glostrup, Denmark) for 30 min followed by an overnight incubation at 4 °C with the primary antibody diluted in TNB (containing 15.76 g/l Tris HCl, 8.77 g/l NaCl and 5 g/l blocking reagent provided in the TSA kit) (see chapter 4.3.1). The following day, a biotinylated secondary antibody (see chapter 4.3.1) was applied for 45 min at room temperature. Next, samples were labelled with streptavidin horseradish peroxidase (TSA kit; Perkin-Elmer, Boston, USA) (1:100 in TNT) for 30 min at room temperature, followed by an application of fluorescein-tyramide (1:50 in amplification diluent provided with the TSA kit) at room temperature for 5 min. Slides were washed thoroughly in between the different steps using TNT buffer (containing 15.76 g/l Tris HCl, 8.77 g/l NaCl and 0.05 % Tween 20, pH 7.5). Sections were counterstained with DAPI (1 µg/ml Boehringer Mannheim, Germany) for 1 min and afterwards mounted with Fluoromount-G (Southern Biotechnologies, Birmingham, USA). As with the other staining method, the negative control was done by omitting the primary antibody and the epidermis served as positive control.

4.3.4 Immunocytochemistry

Cells were grown on a chamber slide (Lab-Tek II Chamber Slide System, Nalge Nunc International Corp., Naperville, USA) and then processed for immunostaining. First, they were washed three times in TBS and afterwards fixed in a mixture of methanol and acetone (7:3) for 5 min at -20 °C. Afterwards the staining protocol for ABC-AP was followed as described before (see chapter 4.3.2).

4.4 Microscopical equipment

For fluorescence as well as for light microscopy the compact all in one type fluorescence microscope biozero BZ8000K from Keyence (Keyence GmbH, Neu-Isenburg, Germany) was used. Photos were taken with the appropriate software, BZ image viewer and BZ analyzer (Keyence). Photos from fresh normal human scalp skin sections were taken with individually adjusted light and contrast condition and in magnifications of 40-630x.
Sections from cultured full-thickness human scalp skin were photographed in a standardized way with set light conditions in a magnification of 40x and 200x (**Fig. 4.2**).

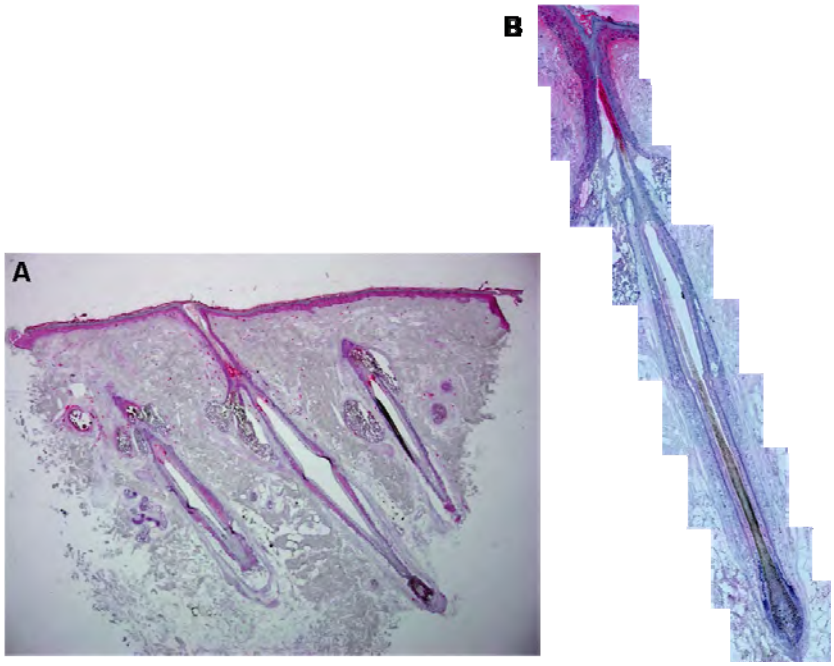

Fig. 4.2 Standardized photos of sections of cultured full thickness human scalp skin cultures
A: 40x magnification of whole section (e.g. RNase 7 staining, ABC-AP method) B: consecutive composite pictures (200x magnification), covering the entire length of the hair follicle (e.g. psoriasin staining, ABC-AP method). The immunopositive cells appear in red; the counterstain with hematoxylin appears blue.

4.5 Quantitative immunhistomorphometry

In a pilot study it was found that differences in the staining intensity occur merely in the infundibular ORS. Therefore only this part was measured by immunohistomorphometry. Incomplete sections, in which the hair follicle could be seen at least up to the orifice of the sebaceous gland where included. In each of two sections of the same hair follicle, 14 previously defined reference regions of interest (in total 28) were measured for relative colour intensity using the NIH Image software (NIH, Bethesda, Maryland) as previously described (**Fig. 4.3**) (Ito et al. 2005; Ito et al. 2004). The mean of these values per each hair follicle was calculated.

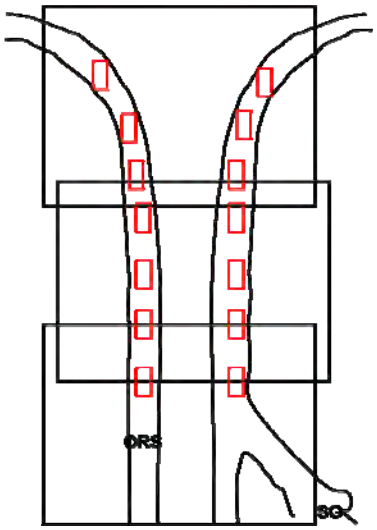

Fig. 4.3 Schematic drawing showing selected reference areas for quantification of immunoreactivity
In each of two sections of the same hair follicle three different pictures up to the orifice of the sebaceous gland were taken with a magnification of 200x. In these, 14 reference areas of interest (28 in total for each hair follicle) were measured for relative colour intensity using the NIH Image programme. ORS: outer root sheath; SG: sebaceous gland

Hair follicles in catagen (**Fig. 4.4**) and telogen were excluded from the evaluation to achieve better comparability.

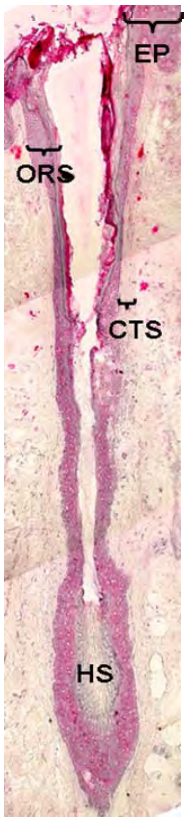

Fig. 4.4 Normal human hair follicle in late catagen/telogen in cultured full-thickness skin biopsies
Section is stained with antibody for RNase 7 which appears in red, ABC-AP method. CTS: connective tissue sheath; EP: epidermis; HS: hair shaft

4.6 Statistical analysis

The mean value for the control group was set as 1 and all values were calculated as multiple of this mean. Data were pooled from at least two different independent experiments. Afterwards they were analyzed using the Mann–Whitney-U test for unpaired samples (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA). P values < 0.05 were regarded as significant.

4.7 Isolation of outer root sheath cells

Cells were isolated using a modified protocol after Detmar et al. (Detmar et al. 1993). Human scalp skin was shaved and gently cut into small pieces following the direction of growth to

preserve the hair follicles. The pieces were incubated with 2.5 units/ml dispase (Roche Diagnostics, Mannheim, Germany) in PBS (Dulbeccco's PBS, PAA Cell Culture Company, Austria) for 16 h at 4 °C. In a second step the skin pieces were exposed to 0.4% collagenase (Serva Electrophoresis GmbH, Heidelberg, Germany) in DMEM (Biochrom, Cambridge, UK) for 1 h at 37 °C. Epidermis, dermis and hair follicles were separated using an operating microscope and microsurgical instruments under sterile conditions. The dermal papilla was cut off. Separated hair follicles were microdissected into three parts (distal, middle, and proximal), washed in PBS and incubated with 0.05 % trypsin/0.02 % EDTA-solution for 15 min at 37 °C. Digestion was stopped using PBS/10 % FCS (fetal calf serum).

Single cell suspensions were cultivated in standard culture flasks supplemented with keratinocyte serum free culture medium (Gibco, Karlsruhe, Germany) in a density of 1.0 x 10^4 per cm^2. Medium was changed every 2 days.

To ensure that the cells are indeed ORS keratinocytes this keratinocyte culture medium was chosen as it prevents the growth of fibroblasts. In addition, cells were parallely stained for keratin 5, 6, 14, 16, and 17, which are specific for ORS keratinocytes (Limat et al. 1991)

4.8 Semi-quantitative RNA detection

4.8.1 RNA isolation

An equivalent number of isolated epidermal keratinocytes and human hair follicle ORS keratinocytes from distal, mid and proximal hair follicle epithelium (10.000 cells/dish), were seeded onto collagen IV/fibronectin (both Sigma-Aldrich, Taufkirchen, Germany) coated 6 cm dishes and grown to confluency. From these total RNA was isolated using RNeasy Mini Kit (Quiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Briefly, cells were lysated by applying RLT buffer and homogenized by passing them 5 times through a 20-gauge needle in order to shear genomic DNA and reduce the viscosity of the lysate. Afterwards, ethanol was added to adjust the binding conditions and the samples were applied to the RNeasy spin column. The RNA binds to the membrane of the column and contaminants were removed with simple wash spins with two different buffers. Finally, the bound RNA was eluted in 50 µl of RNase free water. The amount of isolated RNA was

measured by spectrophotometry at wave lengths of 280 and 260 nm using the BioPhotometer 6131 from eppendorf (Eppendorf, Hamburg, Germany).

4.8.2 Semi-quantitative RT-PCR

Total RNA (100 ng for each sample) was reverse transcribed using the 1st strand cDNA synthesis kit for RT-PCR (AMV) (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's description. The generated cDNA was subjected to PCR analysis in order to amplify psoriasin and RNase 7. This was done using previously reported primers (**Table 4.2**). For comparative analysis the house keeping gene rS26 was amplified as it remains strictly invariant (Vincent et al. 1993). Oligonucleotide primers were commercially synthesized by eurofins MWG GmbH (Heidelberg, Germany), using the following sequences:

Antimicrobial Peptide	Sequence		Reference
psoriasin (S100 A7)	forward	5` AGACGTGATGACAAGATTGAC 3`	Abtin, Eckhardt et al., 2008
	reverse	5` TGTCCTTTTTCTCAAAGACGTC 3`	
RNase 7	forward	5` GGAGTCACAGCACGAAGACCA 3`	Harder and Schröder, 2002
	reverse	5` CATGGCTGAGTTGCATGCTTGA 3`	
rS26	forward	5` GGGAATTCCATATGACAAAGAAAAGAAGGAA 3`	Malygin, Baranovskaya et al., 2003
	reverse	5` GGGAATTCGGATCCTTACATGGGCTTTGGTG 3`	

Table 4.2 Oligonucleotide sequences of used primers

After an initial denaturation at 95 °C for 10 min, there were 40 cycles, each with a denaturation step at 95 °C for 1 min, an annealing step at 56 °C (psoriasin) or 61 °C (RNase 7 and rS26) for 1 min, and an elongation step at 72 °C for 30 s (psoriasin) or 1 min. The cycles were followed by a final elongation at 72 °C for 10 min and held at 4 °C before subjected to gel electrophoresis.

25 µl of the PCR products were run on a 2 % agarose gel containing ethidium bromide (0.5µg/ml) in TAE buffer (containing 4.84 g of tris base, 1.14 ml of 100 % acetic acid and 2 ml of 0.5 M EDTA (PAA Cell Culture Company, Pasching, Austria) per litre with a pH of 8.0). The correct product size was estimated in comparison to the BenchTop 100 bp DNA ladder (Promega, Madison, Wisconsin, USA). The PCR products were visualized and semi-quantified by using the Gel Doc XR system (BioRad, München, Germany) and measuring the respective peak areas with the NIH Image software (NIH, Bethesda, Maryland, USA). The

relative amount of psoriasin and RNase 7 mRNA was corrected by the total mRNA amount as reflected by the expression level of the house keeping gene rS26 (100 %).

5 RESULTS

5.1 Antimicrobial peptides are expressed in normal human scalp hair follicles

Upon systematic immunohistochemical analysis of normal temporal and occipital human scalp skin from female donors, all selected AMPs could be detected in the human hair follicle epithelium.

5.1.1 RNase 7 immunoreactivity

Using two different staining methods, the ABC and the TSA method, immunoreactivity (IR) for RNase 7 was found throughout the entire ORS in all investigated patients (**Fig. 5.1 A**). The IR gradually decreased in intensity processing from distal (**Fig. 5.1 B+C**) to proximal (**Fig. 5.1 D+E**) becoming undetectable in the hair bulb (**Fig. 5.1 F+G**). In addition, several very intensely stained cells located in the dermis around the distal part of the hair follicle were found (**Fig. 5.1 A arrows**). Furthermore a strong IR in the sebaceous glands could be noticed (**Fig. 5.2**). Interestingly these staining patterns could only be obtained, if the used sections were freshly cut, i.e. not older than 48 hours. Older sections gave very faint or no staining at all.

The epidermis as a positive control and the negative control in which the primary antibody was omitted confirmed the specificity of the staining pattern (**Fig. 5.3**)

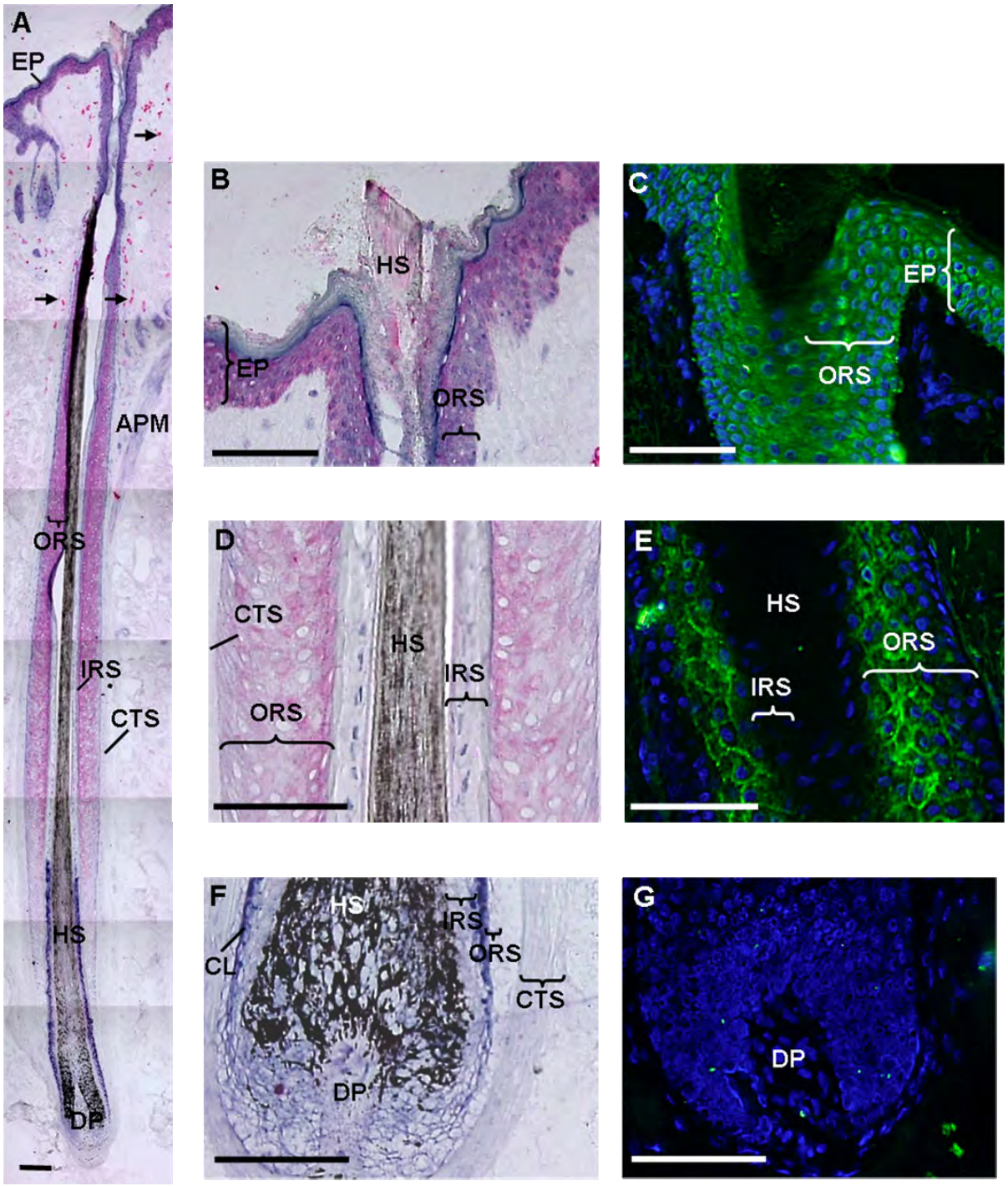

Fig. 5.1 RNase 7 immunoreactivity in normal human anagen hair follicle
RNase 7 IR was seen throughout the entire ORS (A). The intensity of the staining was strongest in the distal part of the hair follicle (B+C), decreased in the middle (D+E) and was absent in the hair bulb (F+G). In addition several intensely stained cells in the dermis around the hair follicle were found (arrows, A). Sections were stained with the ABC-AP method (A, B, D, F) (red: immunopositive cells; blue: counterstain with hematoxylin, black: natural melanin of the hair) and the TSA method (C, E, G) (green: immunopositive cells; blue: cell nuclei). APM: Arrector pili muscle; CL: companion layer; CTS: Connective tissue sheath; DP: Dermal papilla, EP: Epidermis, HS: Hair shaft; IR: immunoreactivity; IRS: Inner root sheath; ORS: Outer root sheath. Bars indicate 50 µm.

Fig. 5.2 RNase 7 immunoreactivity in the sebaceous gland
In the sebaceous gland (SG) strong RNase 7 IR (red) could be noted. The staining method was ABC-AP; the blue counterstain was performed with hematoxylin (blue). Bar indicates 50 µm.

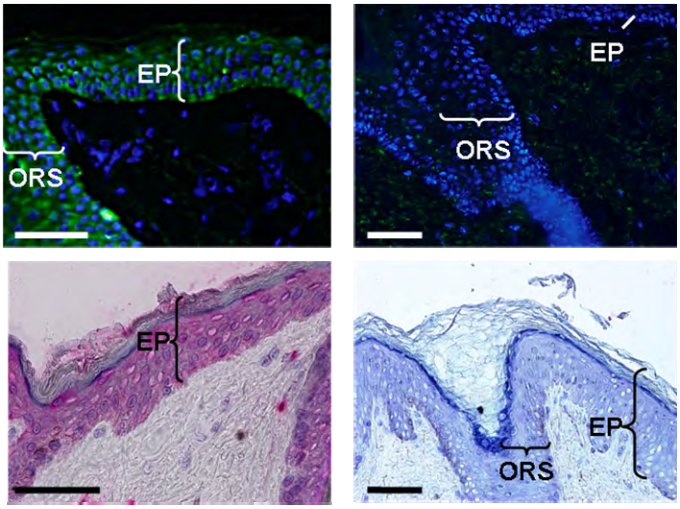

Fig. 5.3 Positive and negative control of Rnase 7 staining
The epidermis served as a positive control (right). In the negative control (left) the primary antibody was omitted. Sections were stained with the ABC-AP method (lower two pictures) (red: immunopositive cells, blue: counterstain with hematoxylin) and the TSA method (upper two pictures) (green: immunopositive cells, blue: cell nuclei). EP: Epidermis; ORS: outer root sheath. Scale bars represent 50 µm.

5.1.2 Psoriasin immunoreactivity

Psoriasin IR was consistently found solely in the uppermost, terminally differentiated layers of the perifollicular epidermis and in the distal part of the ORS, mostly distal of the orifice of the sebaceous gland duct (**Fig. 5.4 A-C**), whereas it almost disappeared in the central ORS (**Fig. 5.4 D+E**) and was absent in the proximal ORS (**Fig. 5.4 F+G**). This was especially apparent in the TSA staining (**Fig. 5.4 C, E, G**). In 50 % of the investigated hair follicles there was also a weak staining in the hair shaft keratin (**Fig. 5.4 B**). Furthermore, weak to moderate psoriasin IR was also seen throughout the sebaceous gland epithelium (**Fig. 5.5**).
The positive and negative control confirmed the specificity of the used antibody (**Fig. 5.6**).

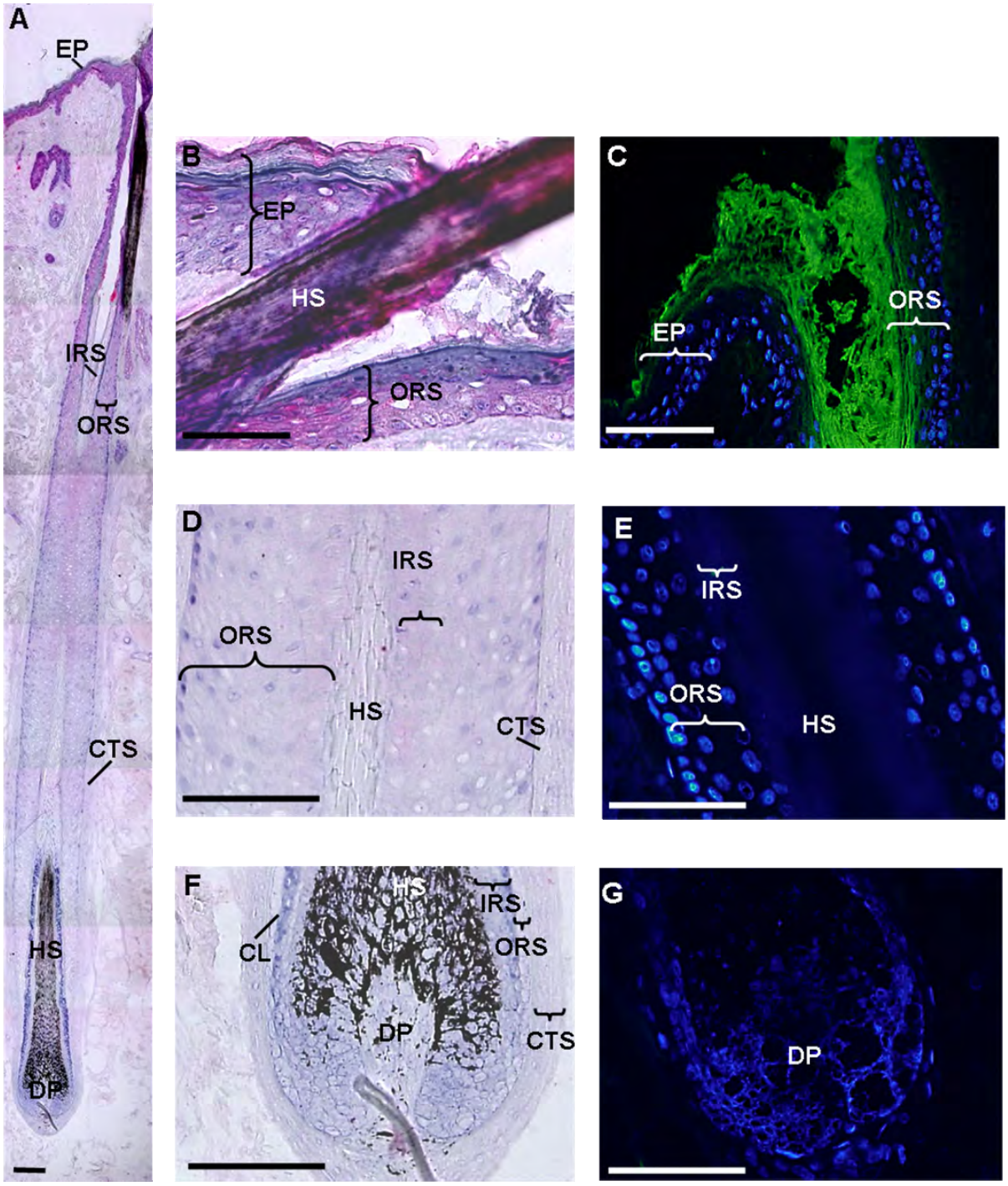

Fig. 5.4 Psoriasin immunoreactivity in normal human anagen hair follicle
Psoriasin IR was detected mostly in the infundibular ORS distal of the orifice of the sebaceous gland duct (A+B), often only in the uppermost layers of the ORS (C). The IR almost disappeared in the middle part (D+E) and was absent in the proximal of the ORS (F+G). Sections were stained with the ABC-AP method (A, B, D, F) (red: immunopositive cells, blue: counterstain with hematoxylin, black: natural melanin of the hair) and the TSA method (C, E, G) (green: immunopositive cells, blue: cell nuclei). APM: Arrector pili muscle; CL: companion layer; CTS: Connective tissue sheath; DP: Dermal papilla; EP: Epidermis; HS: Hair shaft, IR: immunoreactivity; IRS: Inner root sheath; ORS: Outer root sheath. Bars indicate 50 µm.

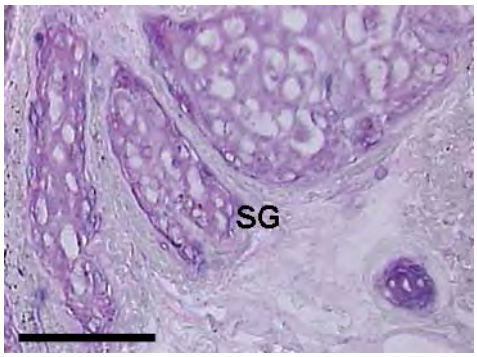

Fig. 5.5 Psoriasin immunoreactivity in the sebaceous gland
The sebaceous gland (SG) revealed rather weak IR for psoriasin (red).The section was stained with the ABC-AP method; the counterstain was performed with hematoxylin (blue). Bar indicates 50 µm.

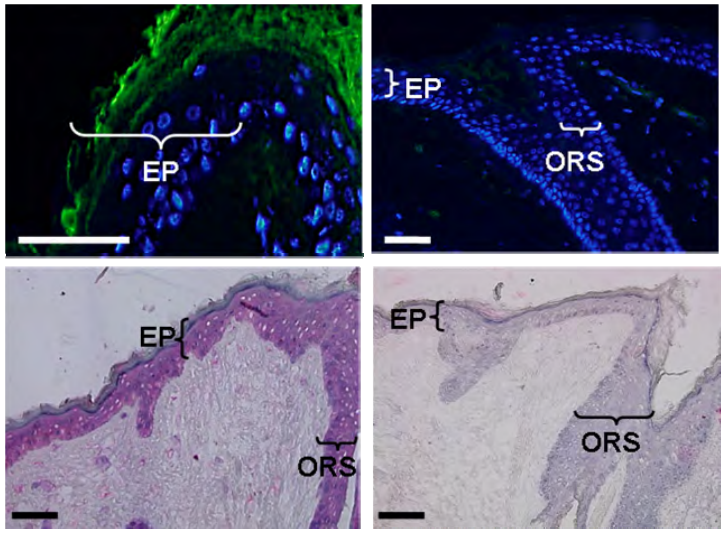

Fig. 5.6 Positive and negative control of psoriasin staining
The epidermis served as a positive control (right). In the negative control the primary antibody was omitted (left). Sections were stained with the ABC-AP method (lower two pictures) and the TSA method (upper two pictures) (green: immunopositive cells, blue: cell nuclei). EP: Epidermis; ORS: outer root sheath. Scale bars represent 50 µm.

5.1.3 Hornerin immunoreactivity

Hornerin IR was found throughout the entire length of normal human scalp hair follicles' ORS (**Fig. 5.7**). The infundibular part showed a very strong suprabasal hornerin staining pattern, which is consistent to what is seen in the epidermis (**Fig. 5.7 B+C**). There, all layers except the basal layer expressed hornerin IR, with strongest expression in the stratum corneum (**Fig. 5.8**). Instead, in central (**Fig. 5.7 D+E**) and proximal parts (**Fig. 5.7 F+G**) the whole ORS epithelium including the basal layer revealed homogeneous, but much weaker IR with a decreasing intensity processing from distal to proximal. The epithelium of the sebaceous gland and duct presented moderate to strong hornerin IR (**Fig. 5.9**). The positive and negative control confirmed the specificity of the staining pattern (**Fig. 5.8**).

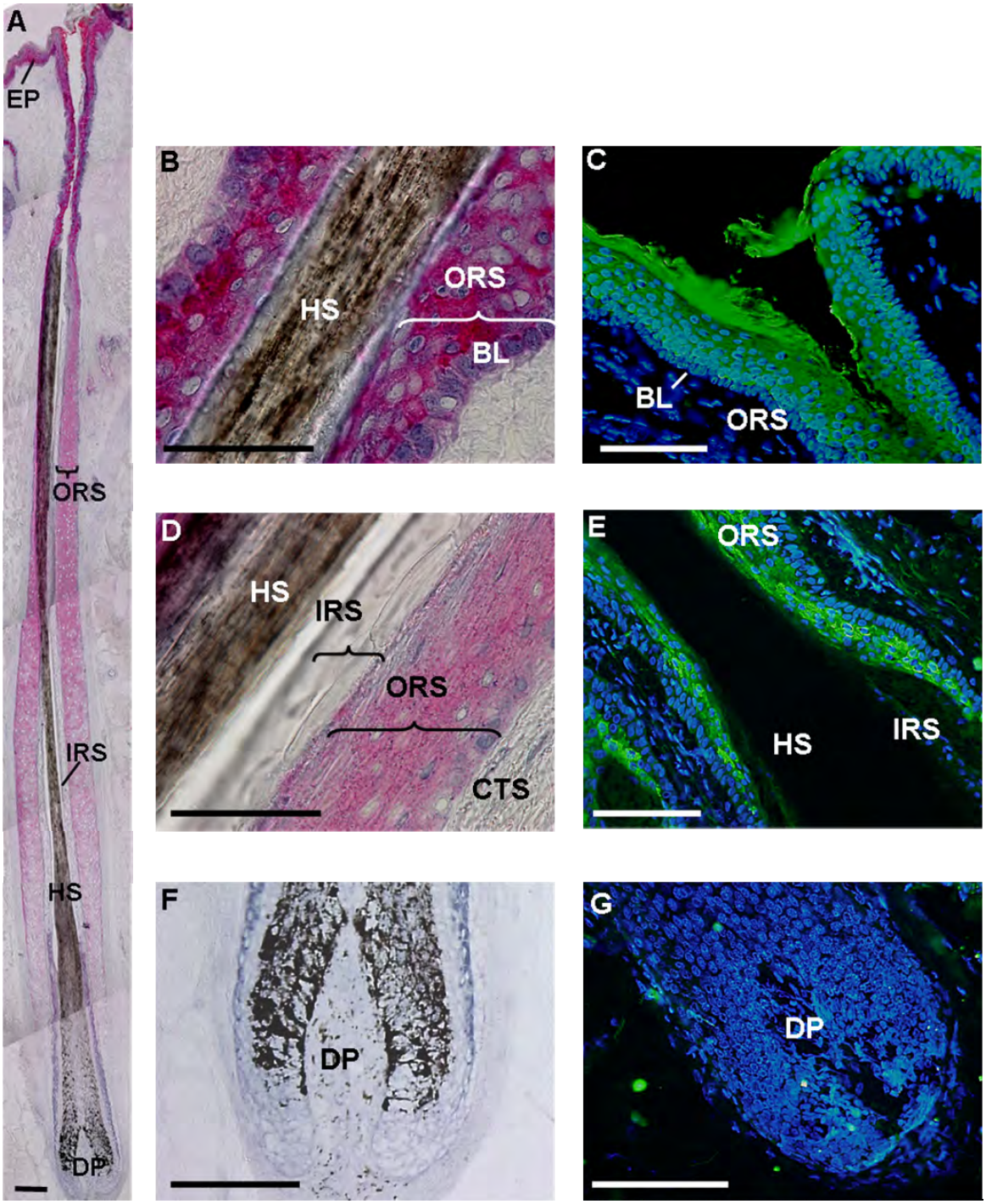

Fig. 5.7 Hornerin immunoreactivity in the normal human anagen hair follicle
Hornerin IR was found throughout the entire length of the hair follicle's ORS (A). In the distal part (B+C) it showed strong suprabasal staining, whereas in the more proximal part (D+E) it was much weaker, but homogeneously distributed. The hair bulb did not express any hornerin IR (F+G). Sections were stained with the ABC-AP method (A, B, D, F) (red: immunopositive cells; blue: counterstain with hematoxylin, black: natural melanin of the hair) and the TSA method (C, E, G) (green: immunopositive cells, blue: cell nuclei). APM: Arrector pili muscle; BL: basal layer; CTS: Connective tissue sheath; DP: Dermal papilla; EP: Epidermis; HS: Hair shaft; IR: immunoreactivity; IRS: Inner root sheath; ORS: Outer root sheath. Bars indicate 50 µm.

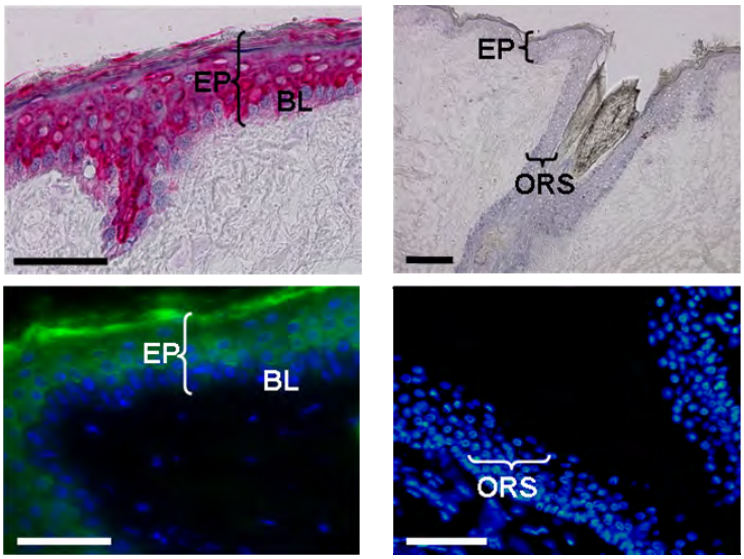

Fig. 5.8 Positive and negative control of hornerin staining
The epidermis served as a positive control (left). In the negative control the primary antibody was omitted (right). Sections were stained with the ABC-AP method (upper two pictures) (red: immunopositive cells; blue: counterstain with hematoxylin) and the TSA method (lower two pictures) (green: immunopositive cells, blue: cell nuclei). BL: basal layer; EP: Epidermis; ORS: outer root sheath. Scale bars represent 50 µm.

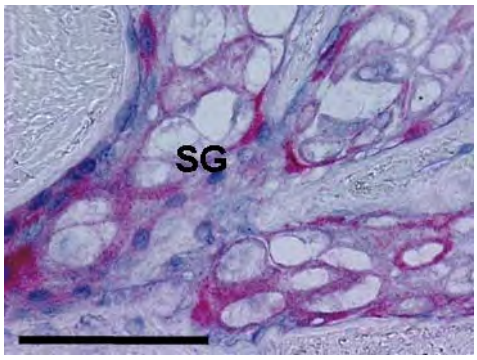

Fig. 5.9 Hornerin IR in the sebaceous gland
The sebaceous gland (SG) epithelium revealed moderate to strong IR for hornerin (red). Section was stained with the ABC-AP method, the counterstain was hematoxylin (blue). Bar indicates 50 µm.

5.1.4 LEKTI-2 immunoreactivity

LEKTI-2 could be found in the entire length of the ORS and in the companion layer of the hair follicle (**Fig. 5.10**). In the distal part of the ORS moderate IR is observed (**Fig. 5.10 B+C**), which is consistent to the staining pattern in the epidermis. Proximal of the orifice of the sebaceous duct the IR for LEKTI-2 in the ORS became very strong, which was especially apparent in the TSA staining (**Fig. 5.10 D+E**), and was attenuated in the very proximal part (**Fig. 5.10 F+G**). In addition, in the most proximal part of the hair follicle a very intense staining of the companion layer could be noticed (**Fig. 5.10 F**).

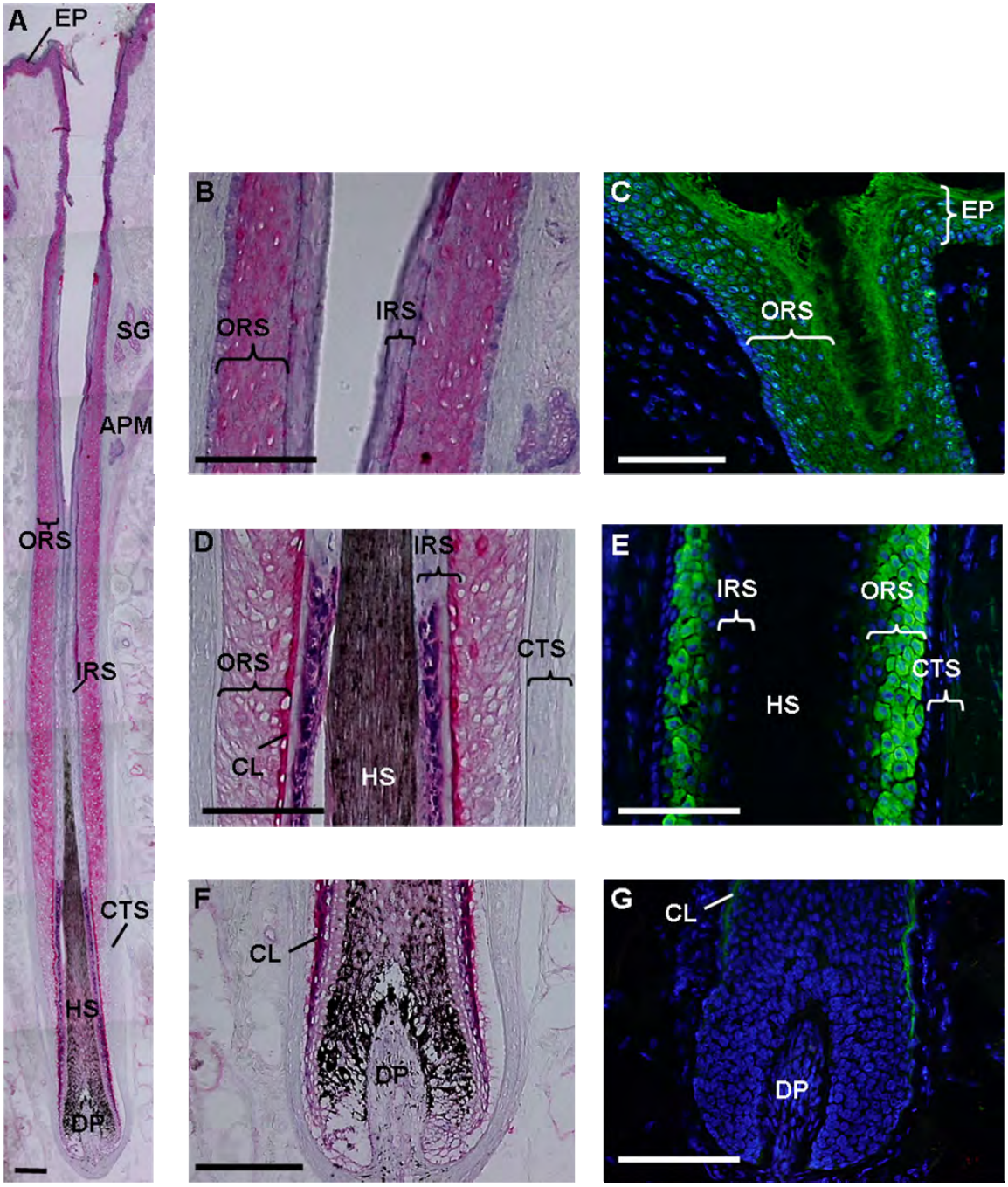

Fig. 5.10 LEKTI-2 immunoreactivity in normal human anagen hair follicle
LEKTI-2 IR was found in the entire ORS and in the companion layer (A). There was moderate IR in the distal part of the ORS (B+C), strong IR in the middle part proximal of the orifice of the sebaceous gland duct (D+E) and weaker IR in the proximal part of the ORS. In addition, a very intense staining of the companion layer could be seen (D, F+G). Sections were stained with the ABC-AP method (A, B, D, F) (red: immunopositive cells; blue: counterstain with hematoxylin, black: natural melanin of the hair) and the TSA method (C, E, G) (green: immunopositive cells, blue: nuclei). APM: Arrector pili muscle; BL: Basal layer; CL: Companion layer; CTS: Connective tissue sheath; DP: Dermal papilla; EP: Epidermis; HS: Hair shaft; IR: immunoreactivity; IRS: Inner root sheath; ORS: Outer root sheath. Bars indicate 50 µm.

The sebaceous gland and duct express strong LEKTI-2 IR as well as the sweat glands (**Fig. 5.11**). The positive and negative control again confirmed the specificity of the antibody (**Fig. 5.12**).

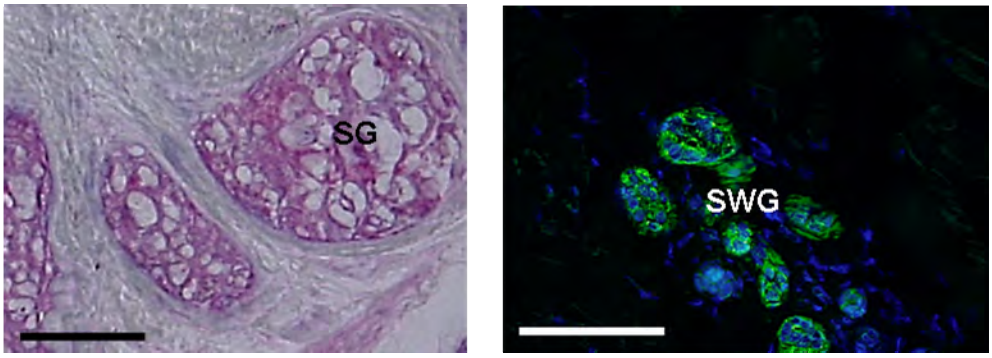

Fig. 5.11 LEKTI-2 immunoreactivity in the sebaceous gland and in the sweat gland
Strong LEKTI-2 IR could be observed in the sebaceous gland (SG) and in the sweat gland (SWG).Sections were stained with the ABC-AP (left) (red: immunopositive cells; blue: counterstain with hematoxylin) and the TSA method (right) (green: immunopositive cells, blue: cell nuclei). Bars indicate 50 µm.

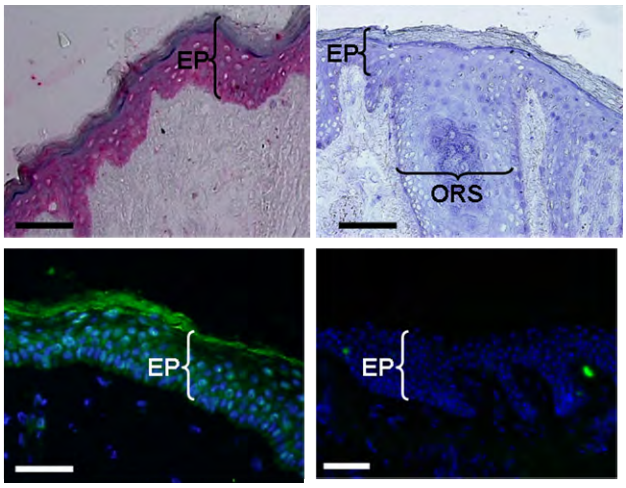

Fig. 5.12 Positive and negative control of LEKTI-2 staining
The epidermis served as a positive control (left). In the negative control the primary antibody was omitted (right). Sections were stained with the ABC-AP method (upper two pictures) (red: immunopositive cells; blue: counterstain with hematoxylin) and the TSA method (lower two pictures) (green: immunopositive cells, blue: cell nuclei). EP: Epidermis; ORS: outer root sheath. Scale bars represent 50 µm.

In summary, all selected AMPs could be detected in normal terminal human hair follicles in anagen VI. An overview of the different staining patterns is given in figure **5.13**.

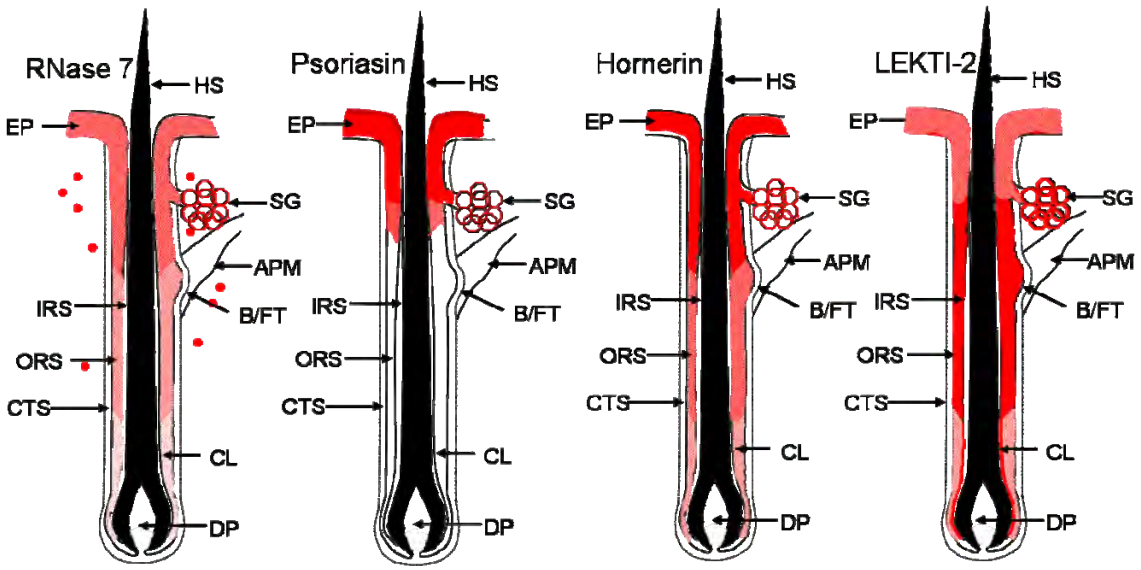

Fig. 5.13 Schematic drawing illustrating the different staining patterns of RNase 7, psoriasin, hornerin and LEKTI-2 in normal human anagen hair follicles
Different grades of red represent the intensity of the immunoreactivity of the distinct antimicrobial peptides. APM: Arrector pili muscle; B/FT: Bulge/ Follicular trochanter; CL: Companion layer; CTS: Connective tissue sheath; DP: Dermal papilla; EP: Epidermis; HS: Hair shaft; IRS: Inner root sheath; ORS: Outer root sheath; SG: Sebaceous gland.

5.2 LPS up-regulated the immunoreactivity of RNase 7 and psoriasin, but not hornerin in the distal outer root sheath

Full-thickness human scalp skin fragments were cultured in serum-free organ culture and treated with LPS from *Escherichia coli* and/or *Pseudomonas aeruginosa* for 24 h. In particular the ORS distal the orifice of the sebaceous gland showed differences in the AMP expression whereas the other parts of the hair follicle did not change in their expression pattern (data not shown). For both, psoriasin (**Fig. 5.14**) and RNase 7 (**Fig. 5.16**), a markedly stronger IR was seen in the LPS treated samples compared to the vehicle treated group.

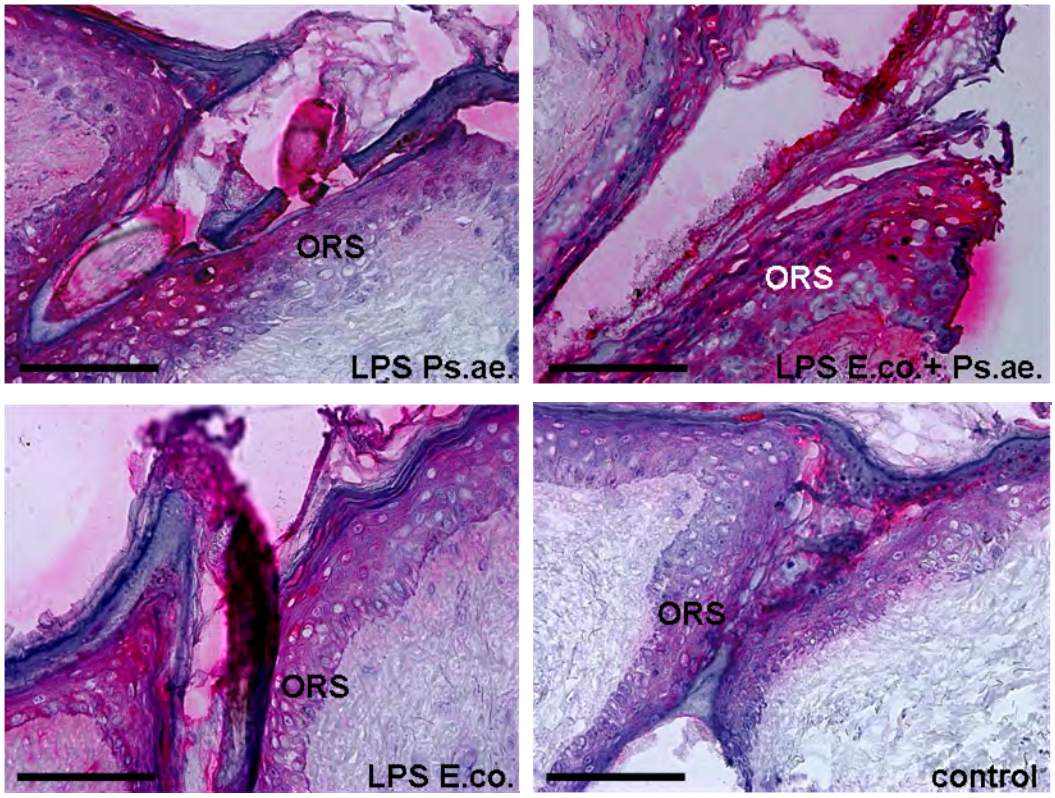

Fig. 5.14 Psoriasin immunoreactivity after 24 hours of LPS treatment in full-thickness human scalp skin organ culture
Representative examples of sections of the LPS-treated and the control group showing the differences in staining intensity. Sections were stained with the ABC-AP method (red: immunopositive cells, blue: counterstain with hematoxylin). E.co.: *Escherichia coli*; LPS: Lipopolysaccharides; ORS: Outer root sheath; Ps. ae.: *Pseudomonas aeruginosa*. Bars represent 50 µm.

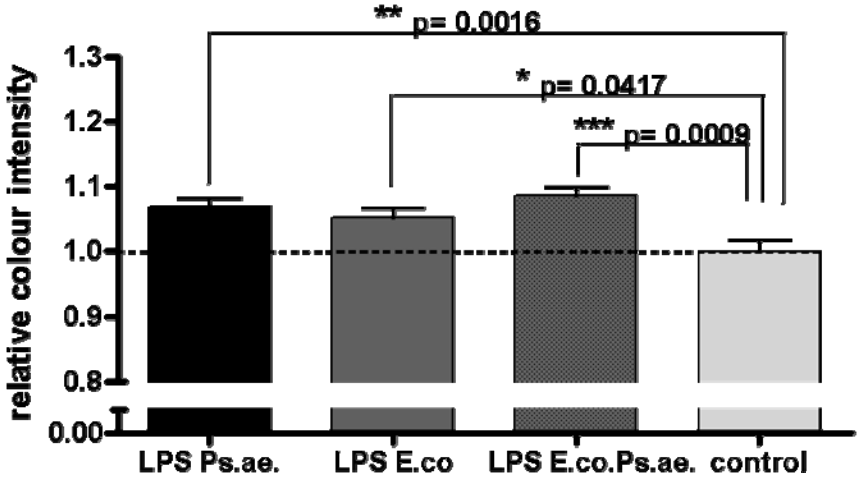

Fig. 5.15 LPS stimulation significantly up-regulated psoriasin immunoreactivity in the distal ORS
Treatment with 1 µg/ml LPS from different species (*Escherichia coli* and/or *Pseudomonas aeruginosa*) significantly up-regulated the relative colour intensity (measured by NIH image) of psoriasin compared to the control group. There was no significant difference between the distinct LPS employed here. The total number of evaluated hair follicles was n=128, LPS Ps.ae.: 35, LPS E.co.: 35, LPS E.co.Ps.ae.: 34, control: 24. Data was pooled from three independent experiments. Statistical analysis was performed using the Mann-Whitney-U test for unpaired samples. Graphs represent mean +/- standard error mean. E.co.: *Escherichia coli*; LPS: Lipopolysaccharides; ORS: Outer root sheath; Ps. ae.: *Pseudomonas aeruginosa.*

By measuring the relative colour intensities with NIH image this optical impression could be quantified. Statistical analysis using the Mann-Whitney-U test revealed a significant up-regulation of psoriasin (**Fig. 5.15**) and RNase 7 IR (**Fig. 5.17**) in the LPS-treated groups compared to the control. For psoriasin IR no significant differences between the distinct LPS employed here could be observed, whereas for RNase 7 IR the combined use of the two distinct LPS led to a significantly lower up-regulation than each LPS alone (**Fig. 5.17**).

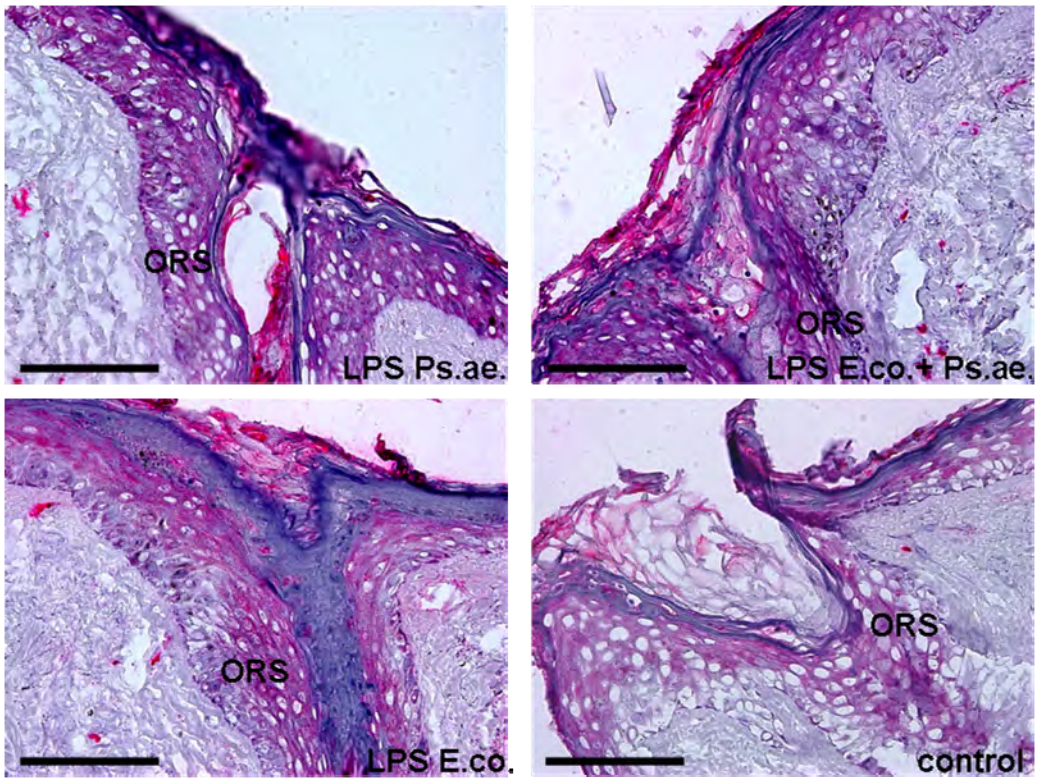

Fig. 5.16 RNase 7 immunoreactivity after 24 hours of LPS-treatment in full-thickness human scalp skin organ culture
Representative examples of sections of the LPS-treated groups and the control showing the differences in staining intensity. Sections were stained with the ABC-AP method (red: immunopositive cells, blue: counterstain with hematoxylin). E.co.: *Escherichia coli*; LPS: Lipopolysaccharides; ORS: outer root sheath; Ps. ae.: *Pseudomonas aeruginosa*. Bars represent 50 µm.

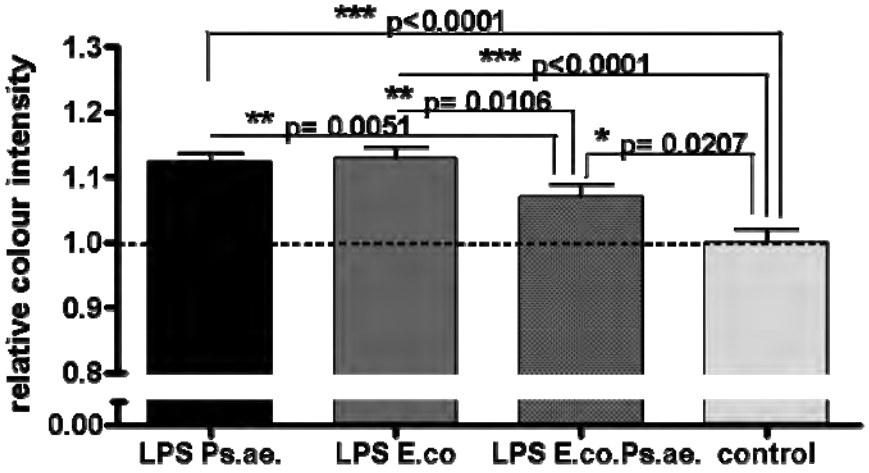

Fig. 5.17 LPS significantly up-regulated RNase 7 immunoreactivity in the distal ORS
Treatment with 1 µg/ml LPS from different species (*Escherichia coli* and/or *Pseudomonas. aeruginosa*) significantly up-regulated the relative colour intensity (measured by NIH image) of RNase 7 compared to the control group. There was also a significant difference between the group treated with LPS of *Ps. aeruginosa* and *E.coli* alone and the group with the combined LPS. The total number of evaluated hair follicles was n=116, LPS Ps.ae.: 40, LPS E.co.: 26, LPS E.co.Ps.ae.: 32, control: 18. Data was pooled from two independent experiments. Statistical analysis was performed using the Mann-Whitney-U test for unpaired samples. Graphs represent mean +/- standard error mean. E.co.: *Escherichia coli*; LPS: Lipopolysaccharides; ORS: Outer root sheath; Ps. ae.: *Pseudomonas aeruginosa.*

Hornerin IR, which was already very strong in control samples, remained unchanged in its intensity regardless the LPS treatment being used (**Fig. 5.18 & 5.19**). However, a slight, but not significant increase for the group treated with LPS from *Pseudomonas aeruginosa* could be noticed.

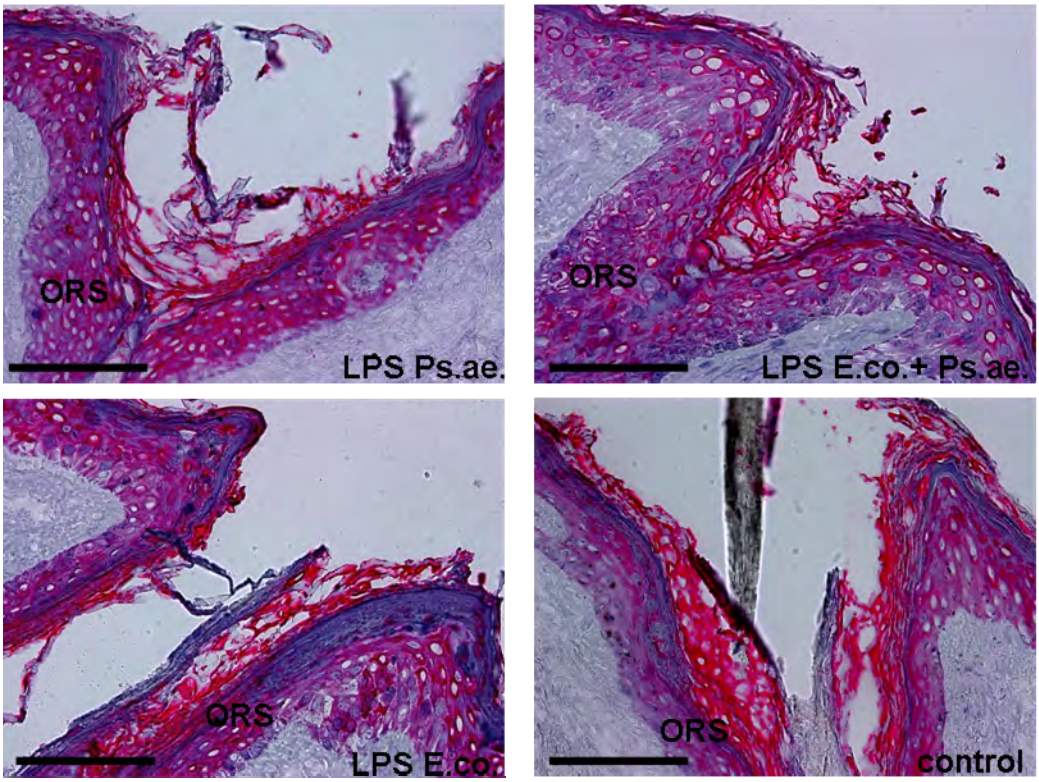

Fig. 5.18 Hornerin immunoreactivity after 24 hours of LPS treatment in full-thickness human scalp skin organ culture
Treatment with 1 µg/ml LPS from different species (*Escherichia coli* and/or *Pseudomonas aeruginosa*) in full thickness human scalp skin organ culture did not change the hornerin immunoreactivity. Sections were stained with the ABC-AP method (red: immunopositive cells, blue: counterstain with hematoxylin). E.co.: *Escherichia coli*; LPS: Lipopolysaccharides; ORS: Outer root sheath; Ps. ae.: *Pseudomonas aeruginosa.* Bars represent 50 µm.

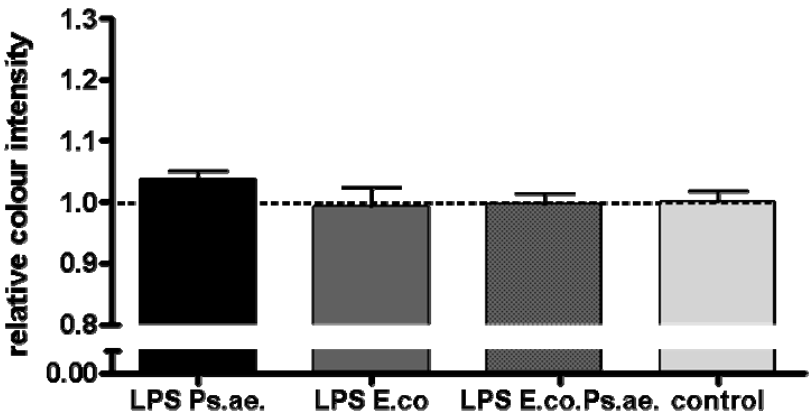

Fig. 5.19 LPS treatment resulted in no significant differences in hornerin immunoreactivity
Treatment with 1 µg/ml LPS from different species (*Escherichia coli* and/or *Pseudomonas aeruginosa*) did not significantly (all p> 0.05) influence the relative colour intensity (measured by NIH image) of hornerin compared to the control group. However, a slight, but not significant increase for the group treated with LPS from *Pseudomonas aeruginosa* could be noticed. There was no difference between the different LPS employed here. The total number of evaluated hair follicles was n=52, LPS Ps.ae.: 12, LPS E.co.: 11, LPS E.co.Ps.ae.: 18, control: 11. Data was pooled from two independent experiments. Statistical analysis was performed using the Mann-Whitney-U test for unpaired samples. Graphs represent mean +/- standard error mean. E.co.: *Escherichia coli*; LPS: Lipopolysaccharides; ORS: Outer root sheath; Ps. ae.: *Pseudomonas aeruginosa.*

5.3 LTA and Protein A differentially regulated AMP immunoreactivity in the human hair follicle epithelium

LTA and protein A treatment in full-thickness skin organ culture both significantly up-regulated psoriasin IR. It deserves to be noticed that the up-regulation after psoriasin IR was significantly higher than the one after protein A treatment (**Fig. 5.20**).

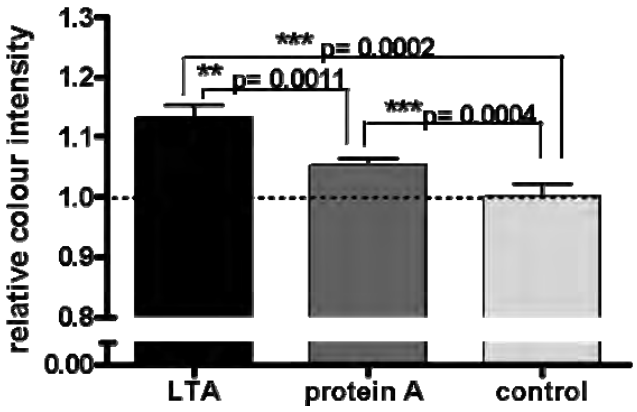

Fig. 5.20 LTA and protein A treatment both up-regulated psoriasin immunoreactivity
Treatment with 5 µg/ml LTA or 5 µg/ml protein A significantly up-regulated psoriasin immunoreactivity in serum free full thickness skin organ culture. The up-regulation after LTA treatment was significantly higher than the one after protein A treatment. The relative colour intensity was measured by NIH image. The total number of evaluated hair follicles was n=44, LTA: 13, protein A: 18, control: 13. Data was pooled from two independent experiments. Statistical analysis was performed using the Mann-Whitney-U test for unpaired samples. Graphs represent mean +/- standard error mean. LTA: lipoteichoic acid.

RNase7 IR was significantly up-regulated only by LTA treatment compared to the control, whereas protein A did not affect RNase 7 IR. In addition, a significant difference between the LTA and protein A treated group could be observed (**Fig. 5.21**).

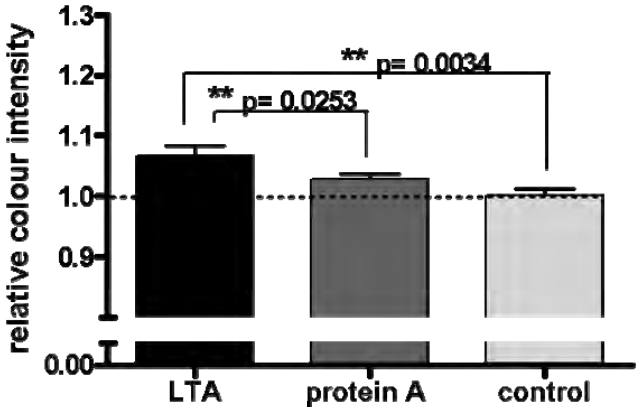

Fig. 5.21 LTA, but not protein A significantly up-regulated RNase 7 immunoreactivity
Treatment with 5 µg/ml LTA, but not with 5 µg/ml protein A significantly up-regulated RNase 7 immunoreactivity in serum free full thickness skin organ culture. There was also a significant difference between the RNase 7 IR after LTA treatment and protein A treatment. The relative colour intensity was measured by NIH image. The total number of evaluated hair follicles was n=55, LTA: 16, protein A: 21, control: 18. Data was pooled from two independent experiments. Statistical analysis was performed using the Mann-Whitney-U test for unpaired samples. Graphs represent mean +/- standard error mean. LTA: lipoteichoic acid.

In contrast to the other two selected AMPs, hornerin IR was significantly down-regulated in the LTA treated group compared to the control. Protein A, however, did not have any influence upon hornerin IR (**Fig. 5.22**).

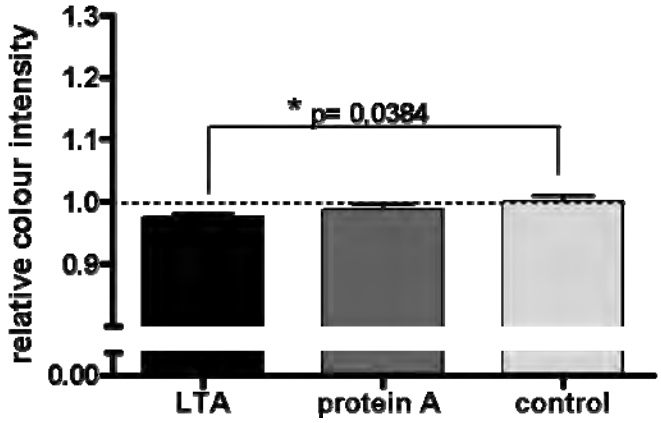

Fig. 5.22 LTA slightly, but significantly down-regulated hornerin immunoreactivity
Treatment with 5 µg/ml LTA, but not with 5 µg/ml protein A significantly down-regulated hornerin immunoreactivity in serum free full thickness skin organ culture. The relative colour intensity was measured by NIH image. The total number of evaluated hair follicles was n=34, LTA: 13, protein A: 12, control: 9. Data represents one experiment. Statistical analysis was performed using the Mann-Whitney-U test for unpaired samples. Graphs represent mean +/- standard error mean. LTA: lipoteichoic acid.

5.4 IFNγ did not stimulate the immunoreactivity of selected AMPs in the human hair follicle epithelium

After 24 hours of treatment in the serum free full thickness skin organ culture, the pro-inflammatory cytokine IFNγ had no influence on the IR neither of psoriasin (**Fig. 5.23**) nor of RNase 7 (**Fig. 5.24**).

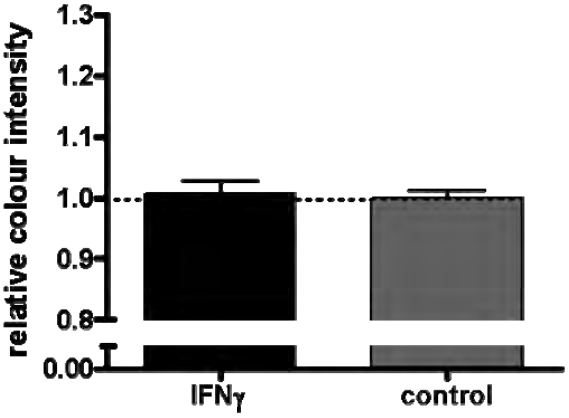

Fig. 5.23 Psoriasin immunoreactivity was not influenced by IFNγ treatment
The relative colour intensity of psoriasin immunoreactivity was not influenced by a treatment of 100 IU/ml IFNγ ($p>0.05$). The total number of evaluated hair follicles was n=8, IFNγ: 5, control: 3. Data represents one experiment. Statistical analysis was performed using the Mann-Whitney-U test for unpaired samples. Graphs represent mean +/- standard error mean. IFNγ: interferon-gamma.

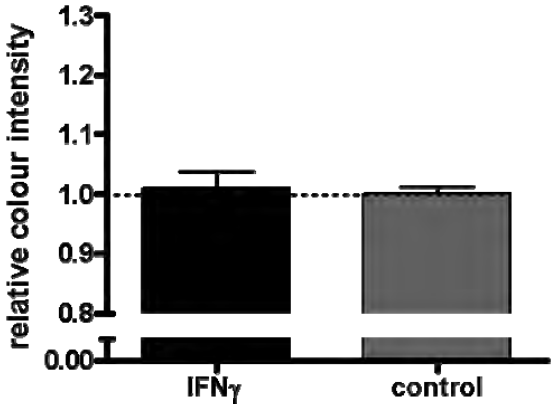

Fig. 5.24 RNase 7 immunoreactivity was not influenced by IFNγ treatment
The relative colour intensity of RNase 7 immunoreactivity was not influenced by a treatment of 100 IU/ml IFNγ ($p>0.05$). The total number of evaluated hair follicles was n=8, IFNγ: 4, control: 4. Data represents one experiment. Statistical analysis was performed using the Mann-Whitney-U test for unpaired samples. Graphs represent mean +/- standard error mean. IFNγ: interferon-gamma.

In contrast, IR for hornerin even showed a significant down-regulation in the distal ORS (**Fig. 5.25**).

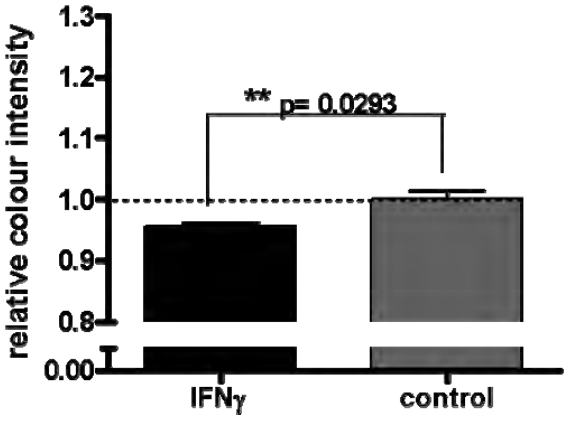

Fig. 5.25 Hornerin immunoreactivity was slightly, but significantly reduced upon IFNγ treatment Treatment with 100 IU/ml IFNγ for 24 h in serum free full thickness skin organ culture significantly decreased the relative colour intensity measured by NIH image. The total number of evaluated hair follicles was n=14, IFNγ: 8, control: 6. Data represents one experiment. Statistical analysis was performed using the Mann-Whitney-U test unpaired samples. Graphs represent mean +/- standard error mean. IR: immunoreactivity; IFNγ: interferon-gamma.

5.5 Antimicrobial peptides in isolated human outer root sheath keratinocytes

5.5.1 RNase 7, but not psoriasin could be immunolabelled in outer root sheath keratinocytes

Primary cells from the distal, middle and proximal ORS all showed intense IR for RNase 7, confirming the findings of the immunohistochemical stainings *in situ* (**Fig. 5.26**). Epidermal keratinocytes served as positive control. In the negative control the primary antibody was omitted.

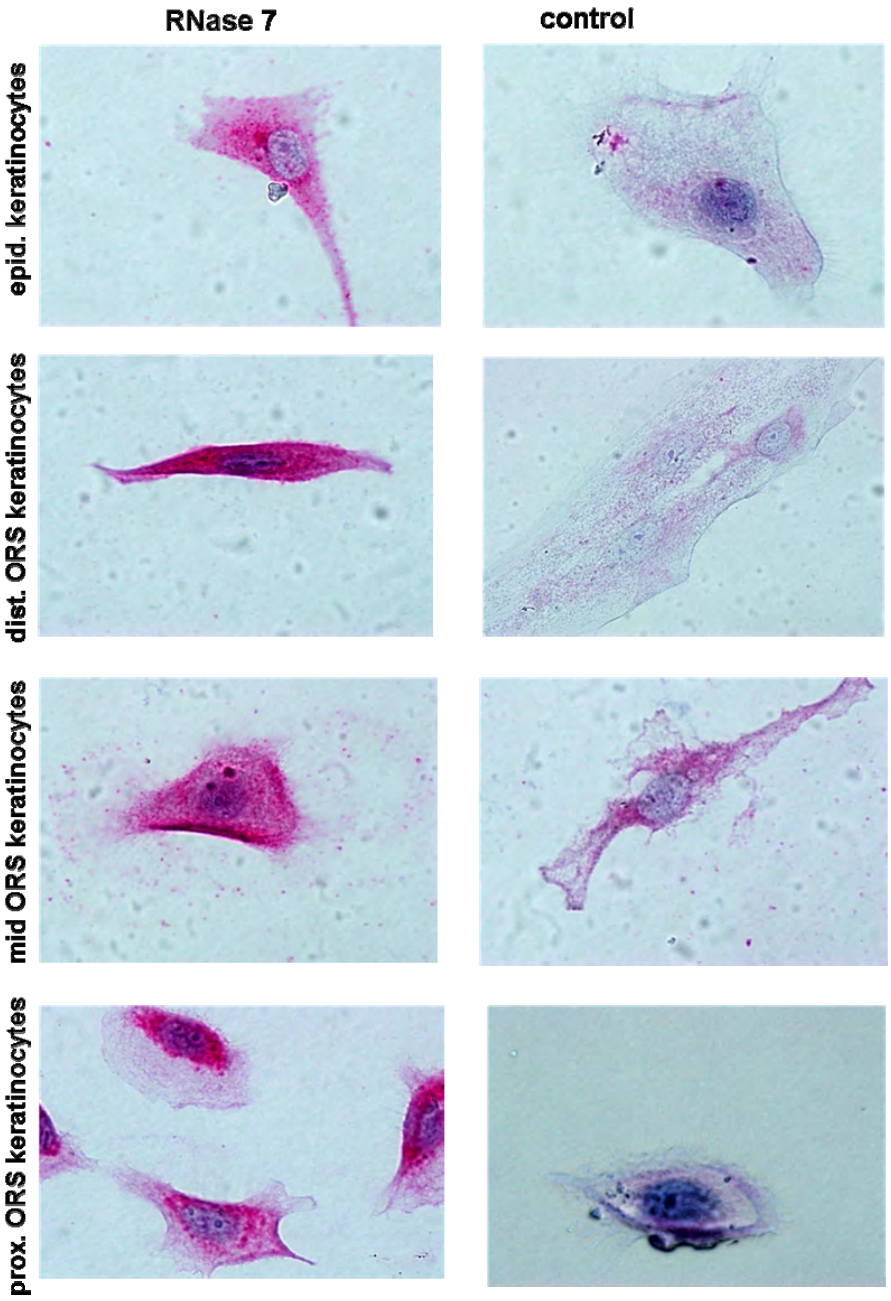

Fig. 5.26 RNase 7 immunoreactivity in isolated epidermal and outer root sheath keratinocytes
RNase 7 immunoreactivity could be detected in isolated ORS keratinocytes of all the different parts of the hair follicle. The epidermal keratinocytes served as a positive control. In the negative controls the primary antibody was omitted. Cells were stained with the ABC-AP method (red: immunopositive cells, blue: counterstain with hematoxylin). dist.: distal; epid.: epidermal; ORS: outer root sheath; prox.:proximal.

However, psoriasin IR was only very weak in the epidermal keratinocytes and not detectable in any of the ORS keratinocytes (**Fig. 5.27**). A background staining was detectable, however clearly distinguishable from the immunopositive stained cells.

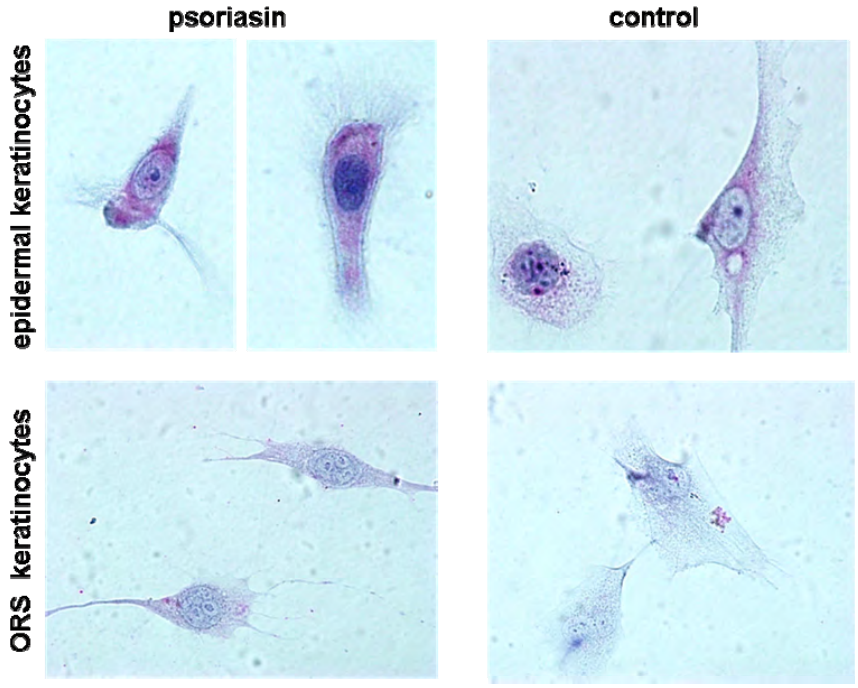

Fig. 5.27 Psoriasin immunoreactivity in epidermal and outer root sheath keratinocytes
Psoriasin immunoreactivity could only be detected very weakly in epidermal keratinocytes, but not in keratinocytes from the outer root sheath (here from the distal ORS). Cells were stained with the ABC-AP method (red: immunopositive cells, blue: counterstain with hematoxylin). ORS: outer root sheath

5.5.2 mRNA of RNase 7 and psoriasin was expressed in isolated outer root sheath keratinocytes

By semi-quantitative RT-PCR both psoriasin and RNase 7 mRNA in the expected size (RNase 7: 235 bp; psoriasin: 127 bp) could be detected in primary cells of ORS keratinocytes of the distal and the middle part, but not of the proximal part of the hair follicle (**Fig. 5.28**).

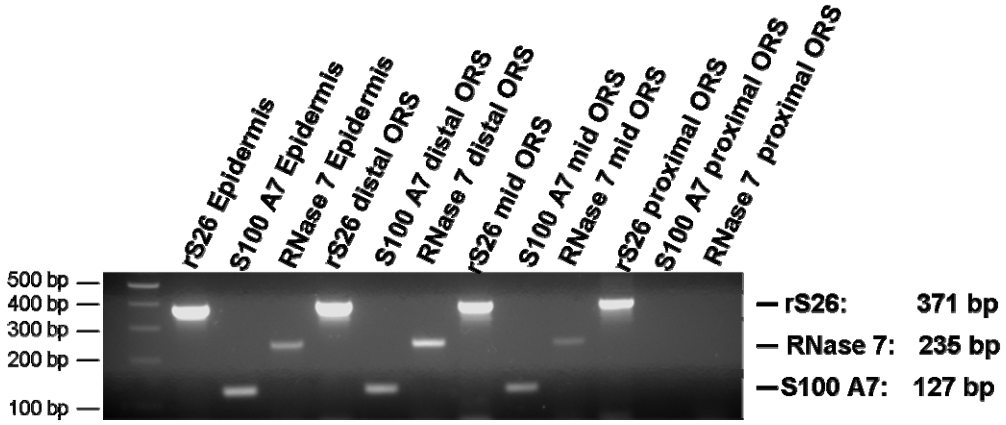

Fig. 5.28 Semi-quantitative analysis of mRNA expression levels of RNase 7 and psoriasin
RNA was isolated from epidermal and ORS keratinocytes, reverse transcribed and subjected to PCR analysis for psoriasin (S100 A7) and RNase 7, as well as for the house-keeping gene rS26. Psoriasin and RNase 7 could be detected in all cells except the ORS keratinocytes of the proximal part of the hair follicle. bp: base pairs; ORS: outer root sheath.

Compared to the house keeping gene rS26 the relative amount of psoriasin mRNA was highest in the keratinocytes from the epidermis (22.3 %), followed by the ones from the distal (17.0 %) and the middle part (13.5%). Instead, the relative amount of RNase 7 mRNA was highest in keratinocytes from the distal part of the ORS (29.2 %), markedly lower in the epidermal keratinocytes (17.2 %) and lowest in the cells isolated from the proximal part of the hair follicle's ORS (4.8 %) (**Fig. 5.29**). Epidermal keratinocytes served as a positive control as mRNA expression of RNase 7 and psoriasin in cultured primary keratinocytes has already been reported (Gläser et al. 2005; Harder and Schröder 2002).

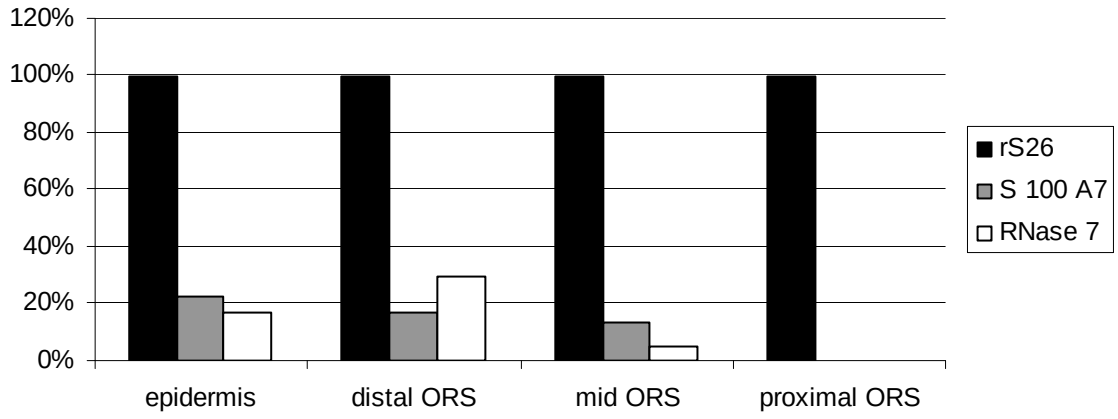

Fig. 5.29 Semi-quantitative analysis of the expression levels of RNase7 and psoriasin
PCR products were analyzed using the NIH Image software (NIH, Bethesda, Maryland, USA) to measure the peak area of each band. The relative mRNA levels of psoriasin (S100 A7) and RNase 7 were corrected by the mRNA amount as reflected by the rS26 level (100 %). Psoriasin mRNA was highest in epidermal keratinocytes and decreased in its amount in the ORS keratinocytes of the proximal and the distal part of the hair follicle. The amount of RNase 7 mRNA had its peak in the ORS keratinocytes followed by the keratinocytes of the epidermis and the mid ORS. In the proximal ORS keratinocytes neither psoriasin nor RNase7 mRNA could be identified. ORS: outer root sheath.

6 DISCUSSION

This study aimed to further investigate selected components of the hair follicle innate immune system, specifically the expression and function of four different AMPs – RNase 7, psoriasin, hornerin and LEKTI-2 – in human scalp hair follicles. Via immunohistochemical analysis it could be demonstrated that all of these peptides are indeed expressed in the ORS of normal human anagen hair follicles and in the sebaceous gland. LEKTI-2 is in addition strongly expressed in the companion layer and in the sweat glands. Furthermore, cultured cells from the distal and middle part of the ORS expressed psoriasin and RNase 7 mRNA.
In full thickness human scalp skin organ culture, microbial products significantly up-regulated the IR of RNase 7 and psoriasin in the distal ORS, whereas hornerin IR stayed at the same level or was even moderately down-regulated. Treatment with the proinflammatory cytokine IFN-γ did not influence the IR of RNase 7 and psoriasin, but resulted in a significant down-regulation of hornerin IR.

In the current study normal human temporal and occipital scalp skin was taken from 11 female patients aged between 37 and 67. To exclude non - biological influences, the donors were supposed to have no pre-treatment and not to be under permanent medication. Per each antigen a representative amount of at least 5 different individuals was examined. This representative amount allowed us to exclude the inter-individual differences that certainly exist to a certain degree. Tissue specimens are mostly from elder woman (mean age: 57 years) but in comparison to tissue specimens from younger persons (37 years) no age related differences could be noticed. Unfortunately, we did not have access to male donors therefore in a strict sense statements about antimicrobial defence in male hair follicles cannot be made.
Complete length sections of hair follicles are rather difficult to obtain, as the cutting plane hardly matches the plane of the hair follicle axis. Thus, if one hair follicle showed the distal part of the hair follicle and the one next to it showed the proximal hair follicle, both were included in the evaluation of AMP expression in normal human scalp hair follicles.

For the tissue collection two critical quality control issues must be considered. Firstly, as the tissue specimens were collected during routine face-lift surgery the exact time from cutting the hairy skin sample until it was put into supplemented William’s E medium could not be exactly controlled. Secondly, scalp specimen from the operating room had to be transported to the laboratory sometimes for several hours. Especially in very warm or cold weather

conditions the supposed temperature of 4 °C might have varied to a certain degree and could not always be maintained. Nevertheless, as soon as the tissue samples entered the laboratory they were immediately processed for culture or cut into pieces and frozen in liquid nitrogen. Furthermore by carefully evaluating the tissue sections, no defects in tissue or reaction quality could be observed.

Cutting the scalp skin and punching out the biopsies implies a wounding stimulus that cannot be avoided. As AMPs do play a role in wound healing and are often found in higher levels in wounded skin, critics might argue that the results merely mirror wounding effects. This problem was circumvented by keeping the biopsies in culture for 48 h before treating them and of course by having a vehicle treated control group.
In the cultured biopsies, sections from hair follicles were taken from all planes in the biopsy including both central and peripheral parts. All hair follicles which were cut up to the level of the insertion of the sebaceous gland duct were evaluated. Although hair follicles which showed morphological signs of catagen did not have a different staining pattern they were excluded from the immunohistomorphometric evaluation in order to have a more homogenous amount of samples.
The counterstain with hematoxylin helped to decide which sections could be included in the immunohistomorphometric evaluation. Cryosections sometimes vary in their thickness, due to temporary warmer conditions on the block during the cutting process. The hematoxylin staining clearly indicated thicker sections, which thus could be excluded. Like this it was assured, that differences in the colour intensity of the respective antibody are in fact correlating to different amounts of antimicrobial proteins.

The data generated in this study suggest that normal human scalp hair follicle epithelium indeed displays a complex antimicrobial defence system which not only comprises human-beta defensins-1 and -2 (Chronnell et al. 2001), but also includes other prototypic AMPs like RNase 7, psoriasin, hornerin and LEKTI-2.

Psoriasin IR has already been shown in facial skin hair follicles to be expressed in the upper layers of the distal part of the hair follicle’s ORS up to the isthmus level proximal of the orifice of the sebaceous gland duct as well as in the surrounding epidermis (Schröder and Harder 2006; Gläser et al. 2005). In this study, on the contrary, it was demonstrated that psoriasin IR in human terminal scalp hair follicles extended less deeply down in the ORS

(**Fig. 5.4**), as suggested by the former study. The observed IR in half of the examined hair shafts (**Fig. 5.4 B**) might be unspecific, as the hair shaft often serves as binding site for all kinds of antibodies (personal communication with Ralf Paus). However, none of the other investigated AMPs showed such a prominent positive staining in the hair shaft and the negative control was always alright. Furthermore, two other proteins from the same family, S100 A8 and S100 A9 were demonstrated to be expressed in the hair shaft medulla by *in situ*-hybridization and immunohistochemistry (Schmidt et al. 2001).

Here, the first immunohistological expression profile for RNase 7, hornerin and LEKTI-2 in human hair follicles is presented.

RNase 7 is expressed over a wide stretch of the hair follicle's ORS (**Fig. 5.1**) and the first evidence of RNase 7 in sebaceous glands is given (**Fig. 5.2**). While RNase 7 shows the highest intrafollicular expression level in the distal ORS, its expression extends to the very proximal parts of the ORS, although it does become weaker. Since the infrainfundibular ORS is thought to represent a "sterile" tissue compartment except for human papilloma virus (Boxman et al. 1997) without any evidence of constitutive bacterial colonization, it is likely that RNase 7 exerts additional, as yet unknown, non-microbiological functions in epithelial cell biology, namely in the human hair follicle epithelium.
In all investigated sections from normal human scalp skin as well as from cultured biopsies numerous very intensely stained cells in the dermis mostly around the distal part of the hair follicle were noticeable. These cells could be mast cells or macrophages as these have been shown to be localized in the dermis around the hair follicle (Christoph et al. 2000). To determine the origin of these cells double stainings of RNase 7 and c-kit (for the detection of mast cells), CD68 (for the detection of macrophages) or MHCII (for the detection of antigen-presenting cells like dendritic cells) would have to be performed.

Hornerin is also expressed over the entire length of the hair follicle's ORS (**Fig. 5.7 A**), with a very intense suprabasal staining in the most distal parts (except the basal layer) (**Fig. 5.7 B+C**) and weaker, but uniformly distributed staining in the proximal parts (**Fig. 5.7 D+E**). In addition, hornerin IR was found in the sebaceous gland (**Fig. 5.9**).
Using immunohistochemistry Takaishi et al. (Takaishi et al. 2005) found hornerin IR in human psoriatic and wounded skin, but not in healthy skin from the trunk. According to these authors, hornerin was mostly found in the peripheral zone of lesional psoriatic skin and in

healing human epidermis from day 5 to day 30 and disappeared thereafter. Hair follicles were not investigated. In contrast, we now clearly show in accordance to recent studies (Meyer-Hoffert et al. 2007) hornerin to be highly expressed throughout the entire epidermis except its basal layer in healthy human scalp skin as well as in the hair follicle epithelium. The negative results of the other studies might be due to technical reasons, as in a very recent study (Wu et al. 2008b) it could be demonstrated that hornerin peptides are extremely fragile and easily degraded and form complex mixtures of peptides and aggregates.
Furthermore, our findings are in accordance with results from mouse skin (Makino et al. 2003; Makino et al. 2001), which have localized hornerin IR to the granular and cornified layers of healthy mature murine epidermis. Similar to RNase 7, hornerin must also have additional functions as it is expressed in parts of the hair follicle where normally no bacteria can be found.

LEKTI-2 IR is very strong throughout most of the ORS, weaker solely in the distal part of the ORS, where the same intensity as in the epidermis can be seen (**Fig. 5.10**). Furthermore the companion layer shows the most intense staining. In addition it is highly expressed in the sebaceous and sweat glands (**Fig. 5.11**). This expression pattern hardly fits to a sole role in antimicrobial defence, as most of the hair follicle's residential flora and potential pathogens are localized in the distal part (McBride 1993; Wolff and Plewig 1976) and one would expect highest amounts of AMPs there. This suggests that the antimicrobial activity of LEKTI-2 is probably only a side function and its main activity is related to other functions in hair follicle biology. LEKTI (from the same family) has a crucial role in the terminal differentiation of keratinocytes and mutations result in severe scaling and hair deformation (Komatsu et al. 2008; Mägert et al. 2002). Therefore, additional functions, for example in cornification for LEKTI-2 are most likely, especially as an inhibitory effect on kallikrein 5 could already be observed (personal communication with Prof. Schröder, Kiel). Functional studies with the recombinant LEKTI-2 protein or with cells overexpressing LEKTI -2 would be a useful tool to investigate further functions. To get a first hint a co-staining with kallikrein 5 and 7 would be possible. Knock-out mice would certainly be the most valuable tool for investigating the sum of biological effects, but before the breeding of such a mouse, in respect to animal welfare, functions have to be further elucidated and localization of LEKTI in the body has to be determined.

The results for psoriasin, RNase 7 and hornerin are perfectly in line with the fact that the hair follicle ostium shows the largest concentration of microorganisms in the skin (Chronnell et al. 2001; McBride 1993). The prominent expression of key AMPs in the distal ORS with its massive microbial burden may, therefore, serve to contain microbial density within defined limits and may further hinder invasion by non-resident, more pathogenic bacteria through this potential port of entry into the organism. RNase7 and hornerin probably also have additional functions in hair follicle biology as both are also expressed in the lower part of the hair follicle epithelium. For LEKTI-2 however, the antimicrobial activity seems to represent only a small field of its potential functions, as its expression is much higher in the mid and proximal hair follicle and amazingly intense in the companion layer. Therefore it was decided not to proceed with LEKTI-2 in the functional studies in full thickness skin organ culture.

In this study it could be demonstrated that RNase 7 and psoriasin are inducible by classical microbial products, but do not appear to be modulated by IFNγ. Also, correlating with emerging evidence from basic AMP research (Abtin et al. 2008; Eberhard et al. 2008; Gläser et al. 2005; Harder and Schröder 2002) our data suggest that distinct microbial products (e.g. LPS versus LTA and protein A) differ in their regulatory effects on intraepithelial AMP expression in normal human scalp hair follicle epithelium *in situ*.

The finding that both psoriasin and RNase 7 in the distal ORS of human scalp hair follicles are up-regulated by prototypic bacterial products represents the first evidence that these intrafollicular AMPs are inducible, like their intraepidermal counterparts. Although psoriasin preferentially kills *Escherichia coli* and shows much less bactericidal activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus epidermidis* (Gläser et al. 2005), it is not only stimulated in the distal human ORS by *Escherichia coli*-LPS, but also to the same extent by *Pseudomonas aeruginosa*-LPS (**Fig. 5.16**). Therefore, similar to the epidermis the potency of the initial stimulus to induce psoriasin does not allow conclusions regarding the efficiency of that peptide against the particular microorganism. Furthermore, our LPS data are in contrast to very recent findings in cultured human epidermal keratinocytes, where LPS did not show any up-regulation of psoriasin on the mRNA level (Abtin et al. 2008). However, this might be due to low copy numbers below the detection level or partially due to very different culture conditions and to the fact that different LPS preparations were used.

Also, psoriasin responds to cell wall-associated molecules of gram-positive bacteria in normal, organ-cultured human hair follicle epithelium (**Fig. 5.21**), even though its primary killing activity appears to be directed against gram-negative bacteria (Gläser et al. 2005).

RNase 7 was demonstrated to be up-regulated in cultured human epidermal keratinocytes after co-culture with heat-inactivated bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Harder and Schröder 2002). In our experiments RNase 7 was up-regulated after treatment with LPS (**Fig 5.17**) and LTA, but not protein A (**Fig. 5.22**). The induction of this peptide is therefore differentially regulated. Not all cell wall components of gram-positive bacteria can actually induce this peptide.

In conclusion, the data obtained suggest, that both psoriasin and RNase 7 are regulated via toll-like receptor TLR 2 and 4, as LPS mostly acts on TLR 4 and lipoteichoic acid on TLR 2 (Kuby 2007). For further elucidating the pathways leading to the induction of these AMPs another experiment in full thickness skin organ culture or cell culture has to be performed with specific blockage of the respective TLR or downstream peptides like MyD88, or with stimulation with endproducts of that pathway like TNFα.

Although IFNγ up-regulated RNase 7 transcript levels in cultured human epidermal keratinocytes (Harder and Schröder 2002), in our study IFNγ did not significantly modulate the protein IR for neither psoriasin nor RNase 7 in hair follicle epithelium (**Fig. 5.23 & 5.24**). On the one hand, this might reflect the very different culture conditions (organ versus cell culture) and the use of different cytokine concentrations in both studies. On the other hand, we cannot exclude that IFNγ altered RNase 7 and psoriasin transcript levels *in situ*, yet that such changes did not result in different AMP protein concentrations *in situ*.

Hornerin on the contrary acts completely different to the other two AMPs in full thickness human scalp skin organ culture. It is not influenced by LPS although the group treated with LPS from *Pseudomonas aeruginosa* appears to have a minimal higher IR compared to the control group (**Fig. 5.19**). This suggests on the one hand, that hornerin possibly reacts on different cell wall components like e.g. flagellin or that the whole bacterium is necessary for stimulation. On the other hand, a more likely explanation is, that hornerin rather belongs to the constitutively expressed AMP in the skin. As it is only expressed in the suprabasal layers of the epidermis and the distal ORS its expression might be more closely linked to the

cornification process and indifferent to stimuli. However, for lipoteichoic acid a slight, but significant down-regulation could be observed (**Fig. 5.22**).

IFNγ also resulted in a down-regulation of hornerin IR (**Fig. 5.25**). As discussed before, that hornerin expression might be closely related to the cornification process, it might be that in inflammatory conditions, hornerin is down-regulated as consequence of a loosening of the cornified envelope.

The mRNA expression of psoriasin in isolated cultured cells of ORS keratinocytes (**Fig. 5.28**) matches perfectly and confirms the immunohistological findings in the human hair follicle *in situ*. Psoriasin mRNA expression is highest in epidermal cells, decreasing in distal and mid ORS keratinocytes and absent in proximal ORS keratinocytes (**Fig. 5.29**), reflecting exactly what is seen in the stained hair follicle. However, immunohistochemical staining of the cells revealed only a faint staining in epidermal keratinocytes, but not in any of ORS keratinocytes (**Fig. 5.27**). In sections from normal human scalp skin psoriasin IR was present only in the upper layers of the epidermis and the hair follicles' ORS. As many other S100 proteins are also solely expressed at a late stage in keratinocyte differentiation, this might suggest that the protein expression of psoriasin is dependent on the degree of differentiation. Therefore it might be that mRNA can already be detected in the cultured cells but the protein will only be expressed in further differentiation. To test this hypothesis, in a further study the staining could be repeated with keratinocytes in which differentiation was induced *in vitro* by increased Ca^{2+} concentration (Yuspa et al. 1989).

Immunohistochemical stainings of primary cells with RNase 7 antibody generally confirm what is seen in immunohistochemical analysis of hair follicles *in situ* (**Fig. 5.26**). However, the keratinocytes from the different parts of the ORS all show the same strong IR, whereas in the sections from human scalp skin a gradual decrease in intensity from distal to proximal could be observed (**Fig. 5.1**). This decreasing expression pattern is partially reflected in the mRNA expression, as the amount of mRNA in distal ORS keratinocytes is markedly higher than in the mid ORS keratinocytes (**Fig. 5.29**).Yet, in proximal ORS keratinocytes no RNase 7 transcripts could be detected, a finding which contradicts the staining patterns. In such a case, there are three standard explanations to be mentioned: First, there is actually no transcript and the seen IR results from passive uptake of the protein, i.e. that RNase 7 was produced from another cell and bound to cell surface proteins. Second, the transcript copy number is so low,

that it is below the detection level in our assay. Yet, if this little amount of RNase 7 transcripts has a long half life, they can still induce substantial protein translation and thereby yield the prominent IR results seen in figure **5.26**. Third, the half life of the RNA transcripts is extremely short, so that RNase 7 mRNA is rapidly degraded even before RNA extraction and stabilization. The fact, that copy numbers in cells of the other parts of the hair follicle's ORS were high enough for detection, makes the second option the most favourable explanation for this discrepancy.
Furthermore it is rather surprising, that the mRNA expression in distal ORS keratinocytes is much higher than in epidermal keratinocytes as the IR *in situ* is more or less the same. As a further step, stimulation of ORS keratinocytes with bacterial agents would be desirable in order to confirm the data generated in the full thickness skin organ culture.

In conclusion, this study suggests that the hair follicle indeed has a complex antimicrobial defence system comparable to the epidermis with differentially regulated AMPs.
As antimicrobial peptides offer themselves as new treatment options in bacterial infections as well as in inflammatory skin diseases, details about the multiple functions and subtle regulation systems have to be further investigated. Regarding the hair follicle it is essential to know, whether the hair follicle's set of AMPs is distinct from the ones in the epidermis or whether these AMPs react differently on certain stimuli in the hair follicle than in the epidermis.

The full thickness skin organ culture employed here offers itself as a highly instructive experimental tool to study AMPs *in situ*. It can complement mere cell cultures as it enables the researcher to study the reactions of the cells in their physiological environment, to compare different compartments like the hair follicle and the epidermis as well as to observe cells in distinct stages of differentiation in close proximity.

The widespread and often imprudent use of broad-spectrum antibiotics in human and veterinary medicine has led to multi-drug-resistant strains of bacteria. This has become an increasing problem worldwide, especially evident within hospitals, where these bacteria are present as nosocomial flora, often leading to endemic infections and avoidable deaths (Backman et al. 2008; Boucher and Corey 2008; Lagamayo 2008).

Therefore health sciences try to explore with increased urgency alternative ways of combating pathogen assaults. AMPs are very attractive candidates in this search for new therapeutics.

New small synthetic peptides based on the wide variety of naturally occurring AMPs have already been designed focussing on enforcing the antimicrobial activity. Currently several AMPs have been produced and four of them already reached phase 3 clinical efficacy trials (Hancock and Sahl 2006; Melo et al. 2006).
However, AMPs are not mere bacteria killing agents. They rather subtly modulate innate as well as adaptive immunity by lowering potentially harmful pro-inflammatory responses as it has been shown in a model of allergic contact dermatitis (Di Nardo et al. 2007). On the one hand, AMPs and AMP-mimetic peptides, therefore, could serve as an entirely new therapeutic approach, not only against bacterial infections, but also against a variety of inflammatory skin disorders like atopic dermatitis (as it has been shown that these often implement defects in the AMP constitution) (Ong et al. 2002). Of special interest regarding the hair follicle is certainly acne vulgaris, where it would be essential to know whether the up-regulation of certain AMPs (Chronnell et al. 2001) is an initial or a secondary event in the pathogenesis of this disease.
On the other hand, however, excessive AMP-mediated signalling may also exert profoundly pro-inflammatory effects. For example, in rosacea an excessive amount of antimicrobial peptides seems to be involved in the pathogenesis of this disease (Bevins and Liu 2007) and also in hidradenitis suppurativa an involvement of overexpressed AMPs is discussed (Kurzen et al. 2008). Thus, to employ AMPs in anti-infection management strategies in clinical reality, may mean to have to walk a fine line between “too much and too little of an essentially good thing”.

Another concern is that, with a more widespread medical use of AMPs, bacterial resistance against these AMPs may be induced – thus up-setting a delicate, long-standing balance between our natural skin microflora and the AMP systems that keep it in check. So far, it appears that the development of resistance against AMPs is unlikely to occur. However, recent observations that staphylococci and streptococci can limit the effectiveness of cationic AMPs for example by reducing the net negative charge of the bacterial cell envelope (Koprivnjak et al. 2008; Kraus and Peschel 2008; Kraus and Peschel 2006) and that the function of cathelicidins is impaired by bacteriostatic antibiotics (Kristian et al. 2007) already flags a warning that resistance against AMPs is a theoretical concern whose importance should not be underestimated.

With progressing knowledge about additional, as yet unknown functions of AMPs and how to stimulate or inhibit single AMPs, would open up a variety of new options of how to treat

different diseases, either in applying AMPs directly or indirectly via stimulation of their synthesis and secretion with specific agents.

Yet, one has to be aware of the risks implied, because AMPs do have multiple functions and are a part of an elaborate system of proteases, AMPs and other factors each at a specific level. Changing one factor (here: AMPs) in this exquisitely balanced anti-infection defence system, always means to change the whole system and risking to eventually imbalance the defence. However, after addressing and resolving these issues and concerns, AMPs have a great potential to be among the most frequently and wide-spread used drugs in the future.

7 SUMMARY

Studies on the antimicrobial defence of human hair follicle epithelium

The hair follicle ostium represents a potential port of microbial entry into the skin and harbours a rich residential microflora, but only rarely shows clinical signs of infection. This suggests the presence of effective local antimicrobial defence systems. So far, research was mostly focussed on the cellular components of the hair follicle's adaptive immune system, although some antimicrobial peptides (AMPs) had already been detected in this region.

Therefore the current study aimed at systematically analysing the immunoreactivity of four selected AMPs in normal human scalp hair follicles *in situ*: RNase 7, psoriasin, hornerin and LEKTI-2. Furthermore the two well characterized AMPs RNase 7 and psoriasin were additionally examined in isolated outer root sheath keratinocytes, via semi-quantitative RT-PCR and immunohistochemistry. In addition, functional studies in full thickness skin organ culture were conducted investigating whether hair follicle AMPs can be stimulated by prototypic bacterial pathogenicity factors [lipopolysaccharides (LPS), lipoteichoic acid (LTA) and protein A] and/ or the pro-inflammatory cytokine interferon-γ (IFNγ).

In this study, all tested AMPs could indeed be detected in the hair follicle epithelium as well as in the sebaceous gland, providing the first immunohistological expression pattern for RNase 7, hornerin and LEKTI-2. RNase 7 immunoreactivity was found throughout the entire outer root sheath (ORS), whereas psoriasin was only observed in the ORS distal of the orifice of the sebaceous duct. Hornerin immunoreactivity was revealed in the entire ORS with a very strong suprabasal staining pattern in the distal part and LEKTI-2 displayed in addition to the strong immunoreactivity in the ORS a very intense staining of the companion layer. Moreover, psoriasin and RNase7 mRNA could be detected in isolated ORS keratinocytes from the distal and the middle part of the hair follicle, but not from the proximal part. Furthermore isolated ORS keratinocytes from all different parts also showed a strong immunoreactivity for RNase 7, but not for psoriasin.

Stimulation of normal human scalp skin biopsies in serum-free organ culture with LPS from different gram-negative microbes significantly up-regulated RNase 7 and psoriasin immunoreactivity in the distal part of the ORS, whereas hornerin remained at about the same

level. LTA and protein A both significantly increased psoriasin IR, while RNase 7 was only up-regulated by LTA, and hornerin was even down-regulated by LTA. IFNγ did not influence neither psoriasin nor RNase 7 immunoreactivity, but significantly down-regulated hornerin immunoreactivity.

To summarize, this study confirms that the epithelium of human hair follicles exhibits an elaborate and partially inducible antimicrobial defence system which includes the peptides RNase 7, psoriasin, hornerin and LEKTI-2. RNase 7 and psoriasin are inducible by bacterial cell wall components, whereas hornerin is constitutively expressed and does not appear to respond prominently to exposure to exemplary bacterial pathogenicity factors.

8 ZUSAMMENFASSUNG

Studien über die antimikrobielle Abwehr des menschlichen Haarfollikelepithels

Die Öffnung des Haarfollikels repräsentiert eine mögliche Eingangspforte für Mikroben und beherbergt zudem eine ausgeprägte Mikroflora, zeigt jedoch relativ selten klinische Zeichen einer Infektion. Dies läßt auf das Vorhandensein von wirksamen lokalen Abwehrsystemen, die auch antimikrobielle Peptide (AMPs) miteinbeziehen, schliessen. Bis zum jetzigen Zeitpunkt standen die zellulären Komponenten des erworbenen Immunsystems des Haarfollikels hauptsächlich im Mittelpunkt der Forschung, obwohl einige AMPs in dieser Region bereits nachgewiesen worden waren.

Deshalb beabsichtigte die aktuelle Studie, die Immunreaktivität von vier ausgewählten AMPs in menschlichen Haarfollikeln der Kopfhaut *in situ* systematisch zu analysieren: RNase 7, psoriasin, hornerin und LEKTI-2. Außerdem wurden zusätzlich die zwei gut charakterisierten AMPs Psoriasin und RNase 7 in isolierten Keratinozyten der äußeren Wurzelscheide mittels semi-quantitativer RT-PCR und Immunhistochemie untersucht. Ferner wurden funktionelle Studien in Vollhautorgankultur durchgeführt, um zu ergründen, inwiefern AMPs des Haarfollikels von prototypischen bakteriellen Pathogenitätsfaktoren [Lipopolysacchariden (LPS), Lipoteichonsäure (LTA) und Protein A] und/oder dem proinflammatorischen Zytokin Interferon-gamma (IFNγ) stimuliert werden können.

In dieser Arbeit konnten in der Tat alle untersuchten AMPs im Haarfollikelepithel und in der Talgdrüse nachgewiesen werden und damit das erste immunhistologische Expressionsmuster im Haarfollikel für Rnase 7, Hornerin und LEKTI-2 erbracht werden. RNase 7 IR wurde über die gesamte Länge der äußeren Wurzelscheide (ORS) gefunden, wohingegen Psoriasin lediglich in den weiter proximalen Anteilen der ORS beobachtet wurde. Hornerin Immunreaktivität präsentierte sich ebenfalls in der ganzen ORS, mit einem äußerst starken suprabasalen Färbemuster im distalen Teil. LEKTI-2 zeigte zusätzlich zu einer starken Immunreaktivität in der ORS auch eine äußerst starke Färbung in der sog. Companion Layer.
Zudem konnte mRNA von Psoriasin and RNase7 in isolierten Keratinozyten der ORS des distalen und mittleren, jedoch nicht des proximalen Anteils des Haarfollikels nachgewiesen werden. Außerdem besaßen isolierte Keratinozyten der ORS aller Anteile eine starke RNase 7 Immunreaktivität, jedoch keine Psoriasin Immunreaktivität.

Die Stimulation von Biopsien aus normaler menschlicher Kopfhaut in serumfreier Organkultur mit LPS von verschiedenen gram-negativen Keimen resultierte in einer signifikanten Hochregulation der Immunreaktivität von RNase 7 und Psoriasin im distalen Anteil der ORS, wohingegen die Immunreaktivität von Hornerin auf dem selben Niveau blieb. LTA und Protein A erhöhten die Immunreaktivität von Psoriasin signifikant, während die von RNase 7 nur durch LTA hochreguliert wurde und die Immunreaktivität des Hornerins von LTA sogar herunterreguliert wurde. IFNγ hatte keinen Einfluss auf die Immunreaktivität von RNase 7 und Psoriasin, bewirkte jedoch eine signifikante Erniedrigung der Immunreaktivivität von Hornerin.

Die Ergebnisse der vorliegenden Arbeit bestätigen somit, dass das Haarfollikelepithel ein komplexes und teilweise induzierbares antimikrobielles Abwehrsystem, das die Peptide RNase 7, Psoriasin und Hornerin und LEKTI-2 einschließt, besitzt. RNase 7 und Psoriasin lassen sich durch bakterielle Zellwandbestandteile induzieren, wohingegen Hornerin konstitutiv exprimiert ist und bei Kontakt mit exemplarisch ausgewählten bakteriellen Pathogenitätsfaktoren weitgehend unverändert bleibt.

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Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschliesslich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 29.10.2008

Katrin Reithmayer