A new model of blood vessel remodeling:
Hair follicles modulate the perifollicular vasculature by synthesizing key regulators of blood vessel homeostasis

THESIS
submitted in partial fulfilment of the requirements for the degree
PHILOSOPHICAL DOCTOR
- Ph.D. -
in the field of pathology
at the School of Veterinary Medicine Hannover

by
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Hannover, Germany 2002
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Oral examination: 03.06.2002
... Ein Künstler tut wohl, sein Kunstwerk nicht öffentlich sehen zu lassen, bis er es vollendet hat, weil nicht leicht jemand raten noch Beistand tun kann; ist es hingegen vollendet, so hat er alsdenn den Tadel oder das Lob zu überlegen und zu beherzigen, solches mit seiner Erfahrung zu vereinigen und sich dadurch zu einem neuen Werke auszubilden und vorzubereiten. In wissenschaftlichen Dingen hingegen ist es schon nützlich, jede einzelne Erfahrung, ja Vermutung öffentlich mitzuteilen, ja es ist höchst rätlich, ein wissenschaftliches Gebäude nicht eher aufzuführen, bis der Plan dazu und die Materialien allgemein bekannt, beurteilt und ausgewählt sind.

J.W.v.G.

MEINER FRAU

ANJA

IN LIEBE UND DANKBARKEIT

GEWIDMET
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abbreviations:

aa  amino acid
Ang-1  Angiopoietin-1
Ang-2  Angiopoietin-2
ANOVA  analysis of variance
DAPI  4´,6-diamidine-2´-phenylindole-dihydrochloride
GAPDH  glyceraldeyde-3-phosphate dehydrogenase
H&E  hematoxylin & eosin
HF  hair follicle
mRNA  messenger RNA
MVD  Microvessel density (microvessels per microscopic field)
n.s.  no statistically significant difference
p.d.  post depilation (day after anagen induction by depilation)
p.p.  post partum
PBDG  porphobilinogen deaminase
PDGF  Platelet-derived growth factor
PEC  Proliferating endothelial cell
PECAM-1  Platelet /endothelial cell adhesion molecule –1 (CD31)
REGWQ  Ryan-Einot-Gabriel-Welsch range
SD  standard deviation
SEM  standard error of the mean
TBS  tris-buffered saline
TEM  Transmission electron microscopy
TGF  transforming growth factor
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tbody>
<tr>
<td>TNP-470</td>
<td>O-chloroacetylcarbamoyl-fumagillol</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxyribonucleotidyl transferase Nick End Labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor (vascular permeability factor)</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>Vascular endothelial growth factor receptor-1 (fms-like tyrosine kinase, Flt-1)</td>
</tr>
<tr>
<td>VEGFR-2</td>
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1. INTRODUCTION

Hair follicles are characteristic cutaneous appendages in mammals. They serve for protection of the skin, thermal isolation, dispersion of sweat gland products, camouflage, and inter- and intraspecies communication (PAUS and COTSARELIS 1999, CHUONG et al. 2002). In man, hair has a great psychosocial importance and patients with hair loss (i.e. alopecia) or excessive hair growth (i.e. hirsutism) suffer tremendously (PAUS and COTSARELIS 1999). This applies especially to patients who suffer from hair loss due to anti-cancer chemotherapy (PAUS et al. 1994a). Despite the high demand for treatment of hair growth disorders, there is currently no effective treatment available, since still too little is known about the physiology and pathophysiology of hair growth (STENN and PAUS 2001).

1.1 Physiology of hair growth

Hair follicles vary greatly between species and even within species depending on their localization (PAUS and COTSARELIS 1999). However, they all have the same basic structure and they all undergo three principal phases of follicular activity (Figure 1.1): during anagen, a population of rapidly proliferating hair matrix cells generates the hair shaft which penetrates the epidermis to form the hair coat of mammals. The duration of the anagen phase is determined genetically. Then, proliferation of the matrix cells eventually ceases and the proximal portion of the hair follicle regresses by epithelial cell apoptosis within a relatively short period of time (catagen), to be followed by a phase of relative quiescence (telogen) (PAUS and COTSARELIS 1999).
Figure 1.1 (page 12): Schematic representation of development and cycling of hair follicles (from PAUS and COTSARELIS 1999): Schematic drawing of selected stages of the morphogenesis and the three phases of follicular cycling (anagen, catagen, telogen). The roman numerals indicate morphologic substages of anagen and catagen. The pie chart shows the proportion of time the hair follicle spends in each stage. Hair follicle morphogenesis begins with a condensation of dermal fibroblasts and a subsequent condensation of epidermal keratinocytes above them (stage 1). Keratinocytes proliferate and produce the hair germ which finally encloses the condensed dermal fibroblasts that now represent the dermal papilla. Keratinocytes above the dermal papilla differentiate and build the inner root sheath and the hair shaft. Keratinocytes in the middle of the outer root sheath represent the bulge, the presumed seat of follicular stem cells (stage 6). Above the bulge, the sebaceous gland and the arrector pili muscle develop (stage 8). After catagen-induction, the proximal portion of the hair follicle regresses by apoptosis and forms the involuting epithelial column (catagen VII). Telogen hair follicles reveal club hairs and lack the involuted proximal portion (telogen). During anagen –development, keratinocytes proliferate and enclose the dermal papilla again (anagen III). The old club hair is eventually lost while a new hair shaft is formed by the hair matrix (anagen VI).

1.2 The blood supply to the hair follicle

In principle, normal development and cycling of hair follicles depends on the interaction of epithelial and mesenchymal cells (STENN and PAUS 2001). Based on these interactions, hair follicles can develop and grow without a blood supply, as has been shown by different organ culture techniques (e.g. HARDY 1952, PHILPOTT 1999). However, since keratinocytes of the hair follicle matrix are among the most metabolically active cells within the whole mammalian organism (STENN and PAUS 2001), a blood supply is indispensable for long term maintenance of hair growth, bringing nutrients and oxygen as well as various hormones to the hair follicle (DURWARD and RUDALL 1958). The intensive circulation in anagen skin suggests, that it is associated with the high metabolic activity of hair follicle matrix cells (DURWARD and RUDALL 1949, MONTAGNA and ELLIS 1958, HASHIMOTO et al. 1990, SAKITA et al. 1994), and indeed fenestrated capillaries, indicating the need for a rapid exchange of molecules between the vascular system and the tissue, have been demonstrated around the hair bulbs (MCLEOD 1970, BRAVERMAN and KEHYEN 1981, SAKITA et al. 1994).

Thus, an insufficient blood supply of hair follicles could be one factor involved in hair follicle pathology. There are indeed some, but very few evidences in the literature
indicating that a correlation between decreased cutaneous vascularization and decreased hair growth exists. Firstly, the anastomoses of both the dermal and the hypodermal blood vessel plexus in the skin decrease progressively with age, so that ultimately some areas of the skin are poorly supplied with blood, possibly explaining decreased follicular activity in aged individuals (HOEPKE 1927, CHIALE 1927, ELLIS 1958). Secondly, in diseases that are characterized by a disturbed vascularization of the skin (e.g. vasculitis), at least in animals, atrophy of the hair follicles is a common feature, suggesting that not fulfilling its nutritional needs leads to follicular atrophy and absence of hair growth (YAGER and WILCOCK 1994). Thirdly, minoxidil, used to promote hair growth in androgenetic alopecia, is supposed to have an effect on the vasculature. It upregulates the expression of VEGF on human dermal papilla cells (LACHGAR et al. 1998), induces fenestration of perifollicular capillaries (SAKITA et al. 1999), and stimulates cutaneous blood flow (WESTER et al. 1984). Supporting perifollicular vascularization could therefore be exploited as a therapeutic approach against alopecic diseases. In contrast, limiting or reducing the blood supply to hair follicles could represent an interesting approach to treat excessive hair growth.

It was once shown that induction of angiogenesis, i.e. the growth of new capillaries from pre-existing ones, in rabbit ear-skin can stimulate telogen hair follicles to enter anagen (HOECKEL et al. 1984). However, since the angiogenic substance (a purified macrophage-derived angiotropin) used in these experiments was hardly defined, it cannot be excluded that other factors were in fact responsible for the observed anagen-induction. Experiments with rotated skin grafts and transposed skin flaps in rats have indeed shown that the start of follicular activity precedes its vascularization, suggesting that vascularization is not the initial mechanism of anagen-development (EBLING and JOHNSON 1959).

The observations mentioned above are only vague evidences suggesting a relationship between cutaneous vascularization and hair growth. Therefore, it is interesting to explore whether the cutaneous vascular system develops in accordance to hair follicle morphogenesis and whether it changes in accordance to the hair growth cycle in the adult organism.
**Figure 1.2:** Schematic representation of the microvascular organization in human skin (from BRAVERMAN 1989). e = epidermis; a = arteriole; v = venule; f = subcutaneous fat. The cutaneous vessels are organized in two horizontal plexus. The upper one is located in the superficial dermis, the lower one is located at the dermal-subcutaneous junction. Ascending and descending vessels connect these plexus and give rise to small capillaries that surround the adnexal structures.

**Figure 1.3:** Demonstration of blood vessels by endogenous alkaline phosphatase activity on a frozen sagittal section through the bulb of an anagen hair follicle in the human scalp (from MONTAGNA and ELLIS 1958). An extensive capillary tuft within the dermal papilla (large arrow) is visible. There are crosscuts of small capillaries also detectable in the connective tissue sheath (small arrows).
Figure 1.4: Scanning electron micrograph of vascular corrosion casts of rat dorsal skin in anagen (from SAKITA et al. 1994). (a) Section tangential to a hair follicle. A basket-like capillary network around the hair follicle (H) is seen. The subcutaneous artery (A) and vein (V) are travelling parallel to the skin surface near the buttom of the hair follicle. Some arterial branches (a) from the subcutaneous artery ascend along the hair follicle and supply the lateral side of the hair follicle. The capillaries surrounding the upper part of the hair follicle are connected with those located below the epidermis (e). The venous tributaries (v), collecting both the capillaries located below the epidermis and those of the hair follicle, descend along the hair follicle and finally drain into the subcutaneous vein. (b) Section showing the buttom of the capillary network around the hair follicle. The buttom of the hair follicle (H) is supplied by the arterial branches from the subcutaneous artery (A) and drain into the veins continuous with the subcutaneous vein (V).
1.2.1 Development and anatomy of the perifollicular microcirculatory system

The cutaneous blood supply and especially the coarse anatomy of perifollicular blood vessels has already been described in the late 19th century (TOMSA 1873, SPALTEHOLZ 1893). However, descriptions of the anatomy of the cutaneous blood supply are difficult to compare, as they depend on the location and thickness of the skin investigated, the type and number of adnexal structures, its specific relation to subcutaneous bones and muscles, and the technique used to identify the blood vessels (MONTAGNA and PARAKKAL 1974).

The assembly of the dermal microvasculature in human skin begins during embryonal development (JOHNSON and HOLBROOK 1989). Later on, these simple capillary-like vessels mature and become organized into two well defined layers which are parallel to the epidermis and are connected with each other by multiple smaller vessels (JOHNSON and HOLBROOK 1989). It can be debated whether there are distinct vascular plexus in the skin, since the apparently separate plexus actually are interconnecting vessels of different sizes at all levels of the dermis and with various spatial relationships (MONTAGNA and PARAKKAL 1974). In principal however, the cutaneous vascular organization simply is addressed to as two major plexus of blood vessels (BRAVERMAN 1997): a lower horizontal plexus at the dermal-subcutaneous interface, formed by perforating vessels from the underlying muscles and subcutaneous fat, and an upper horizontal plexus in the dermis (BRAVERMAN 1997) (Figure 1.2). Other plexus have been defined, but are variably developed in different species (DURWARD and RUDALL 1958, AMAKIRI 1976). The lower plexus contains the biggest vessels of the skin and consists of a wide-meshed vascular network, whereas the upper plexus shows a fine-meshed network of small caliber vessels. Multiple, fairly prominent vertical vessels connect both plexus. They also give rise to interconnecting vessels that surround the adnexal structures. A dense capillary network is located around the sebaceous glands, around the pilary canal at isthmus level, and around the lower third of the follicular outer root sheath (DURWARD and RUDALL 1958, MONTAGNA and ELLIS 1958). The latter consists
of parallel, longitudinally oriented vessels that are interconnected by an irregular basket-like network of small vessels which may cover the follicle completely (UNNA 1908, MONTAGNA and PARAKKAL 1974, SAKITA et al. 1994) (Figure 1.4). Capillaries can also be found within the follicular dermal papilla, although there are marked variations. Human terminal hair follicles have a rich network of capillaries within the dermal papilla (Figure 1.3) (MONTAGNA and ELLIS 1958), as do large hair follicles e.g. in rat, sheep, ox, guinea pig, and rabbit (DURWARD and RUDALL 1958). In small hair follicles of the rat, however, as well as in all pelage hair follicles of the mouse, the dermal papilla is devoid of capillaries (HARDY 1952). Hence the size of the capillary tuft within the dermal papilla might directly be related to the size of the hair follicle and thus might be determined by the diffusion limit of oxygen (RYDER 1956). Differences in the follicular blood supply between species, and even between different types of hair follicles within one individual, are not only restricted to the dermal papilla. In fact, the blood supply to the whole follicle appears to vary according to its size and the type of fibre that is produced (DURWARD and RUDALL 1958). Large hair follicles of rats, for example, have a pronounced basketwork of closely-meshed capillaries around them, whereas smaller awl follicles and zigzag follicles have a less dense and irregular oriented network (DURWARD and RUDALL 1958) (Figure 1.5).

Opposed to the extensive literature on the human cutaneous vasculature, surprisingly little is known about the cutaneous vascular system in mice. After birth, a rich capillary network develops in the hypodermis above the panniculus carnosus and encircles the follicles (HARDY 1952). Since no photodocumentation of the blood vessel development in murine skin is available, one aim of this study was to investigate this aspect thoroughly with regard to its association to hair follicle morphogenesis (PAUS et al. 1999)
1.2.2 Variations of the perifollicular vasculature according to the hair growth cycle

Differences in the appearance of the perifollicular microcirculatory system have not only been noticed between different types of hair follicles, but also between growing and resting follicles (MERKEL 1919, DURWARD and RUDALL 1949, HARDY 1952, DURWARD and RUDALL 1958, FORBES 1967, MONTAGNA and PARAKKAL 1974). For example, dye-injection studies in rat and rabbit skin have revealed dramatic changes in the appearance of cutaneous blood vessels during synchronized switches of large groups of hair follicles from the resting (telogen) to the growing stage of the hair cycle (anagen) and vice versa (DURWARD and RUDALL 1958) (Figure 1.6). Anagen hair follicles are surrounded by a much more extensive capillary system than are telogen follicles (SAKITA et al. 1994). During catagen-development, vessels in the proximal perifollicular region of rat hair follicles appear reduced and contracted (DURWARD and RUDALL 1958). Thus a reduction of perifollicular blood vessels during catagen–development can be presumed, and indeed a rather diffuse alkaline phosphatase activity was demonstrated in capillaries within the dermal papilla of human catagen hair follicles, which has been interpreted as a sign of blood vessel degeneration (ELLIS and MORETTI 1959). Differences in the appearance of perifollicular blood vessels are not only restricted to pelage hair follicles, but have also been demonstrated in sinus hair follicles of the cat (GODYNICKI et al. 1997).

The observations mentioned above demonstrate vast morphological differences in the perifollicular vasculature between growing and resting hair follicles. However, these observations do not clarify whether the morphological changes in the cutaneous vasculature are due to differences in tissue perfusion or whether they are based upon active remodeling processes in the perifollicular vessels itself, including angiogenesis and vascular regression. Therefore, another aim of this study was to clarify, whether angiogenesis and subsequent regression of perifollicular blood vessels are features of the hair growth cycle.
Figure 1.5: India ink injection into the vascular system of rat skin (from DURWARD and RUDALL 1958). Note the variations in blood supply to different hair follicle types. Whereas large hair follicles are closely surrounded by blood vessels, the multiple small hair follicles have a less tight contact to blood vessels.
Figure 1.6: **India ink injection into the vascular system of rat skin** (from DURWARD and RUDALL 1958). The picture shows the subsequent transition of hair follicles from telogen (left) to anagen (right). Note the higher density of blood vessels surrounding anagen hair follicles.
1.3 Vascular remodeling

The capacity of vessels to remodel persists throughout life and is particularly evident in the reproductive cycle of the endometrium and during pregnancy (COWAN and LANGILLE 1996). Cutaneous vessels also have a remarkable capacity for adaptation (MONTAGNA and PARAKKAL 1974). In the adult skin, however, angiogenesis is still thought to be restricted to pathological conditions (FOLKMAN and SHING 1992, FOLKMAN 1995), such as wound healing (e.g. ARNOLD and WEST 1991, TONNESEN et al. 2000), hyperproliferative inflammatory skin diseases like psoriasis (DETMAR et al. 1994), and in association with a wide range of tumors (e.g. FOLKMAN 1987, 1990, 1996, CARMELIET and JAIN 2000).

1.3.1 Angiogenesis

Angiogenesis is the growth of new capillaries from pre-existing vessels (CARMELIET 2000). Angiogenic sprouting is one important, but not the only mechanism of blood vessel formation in the adult organism (AUGUSTIN 2001). Vessels may also arise by bridging or splitting of pre-existing vessels, i.e. intussusceptive angiogenesis (Figure 1.7), or simply by growth in length and width, i.e. remodeling angiogenesis (RISAU 1997, CARMELIET 2000, AUGUSTIN 2001). These forms can occur separately or in combination. The intussusceptive and remodeling form of angiogenesis most likely expands the pre-existing vascular network and, in the adult organism, occurs most notably during the cyclic changes of the endometrial vasculature (ROGERS et al. 1998, PATAN et al. 2001).

Angiogenesis is a multi-step process. It involves vasodilation, loosening of interendothelial cell contacts, relieve of periendothelial cell support, degradation of the vascular basement membrane, proliferation of endothelial cells, migration of endothelial cells towards the angiogenic stimulus, formation of endothelial sprouts and organization of new vascular channels (CARMELIET 2000). These processes are regulated by a multitude of growth factors and other signaling mechanisms
and their occurrence may vary according to the tissue and type of angiogenesis (CARMELIET 2000, HAAS et al. 2000).

Figure 1.7: Schematic representation of different forms of angiogenesis (modified from CARMELIET 2000). Sprouting, bridging or intussusception of pre-existing vessels leads to generation of new “daughter” vessels.

1.3.2 Maturation and regression of blood vessels

Opposed to pathological, i.e. unidirectional angiogenesis, under physiological conditions vessels will finally either mature or regress (NICOSIA and OTTINETTI 1990, BENJAMIN 2000).

Maturation of newly formed blood vessels requires recruitment of extracellular matrix and perivascular cells. These events are mediated by the interaction of a variety of cytokines, chemokines and growth factors (OETTGEN 2001). Endothelial cell–derived PDGF-BB (platelet–derived growth factor-BB) and pericyte–derived TGF-β1 (transforming growth factor-β1) have been shown to be of major importance for the recruitment of pericytes, which stabilize the immature neovasculature and thus limit the plasticity window that allows remodeling and pruning of vessels (ANTONELLI-ORLIDGE et al. 1989, LINDAHL et al. 1997, BENJAMIN et al. 1998). Pericyte recruitment, however, is probably not the only mechanism of vessel stabilization (EBERHARD et al. 2000).

Lack of blood vessel maturation may finally lead to blood vessel regression which is typically associated with tissue involution/ regression, as it is seen during corpus luteum regression (AUGUSTIN et al. 1995). It has especially been studied in the
ovary (AUGUSTIN et al. 1995, MODLICH et al. 1996) and in various tumor models (BENJAMIN and KESHEI 1997, SGONC et al. 1998), and it is accompanied by apoptosis or degeneration of endothelial cells. After dissociation of tight junctions, these endothelial cells detach from their underlying basement membrane, protrude into the vessel lumen and finally may constrict and occlude small vessels (MODLICH et al. 1996, HONMA and HAMASAKI 1998). Thus endothelial cell survival is a crucial step in determining the fate of newly formed blood vessels, and a multitude of factors has been shown to inhibit or promote endothelial cell survival (Figure 1.8, Table 1.1) (CARMELIET 2000).

1.8: Schematic representation of vasculogenesis/angiogenesis, stabilization, maturation and eventual regression or sprouting of vessels (modified from CARMELIET 2000). (A) VEGF from the perfused tissue mediates (via VEGFR) assembly of endothelial cells (EC). (B) Endothelial cells recruit pericytes (PC) by synthesizing PDGF-BB (acting via PDGFR-β). Pericytes produce angiopoietin-1 (Ang1) and stabilize endothelial cell contacts and EC – PC interactions. (C) TGF-β1 mediates recruitment of smooth muscle cells (SMC) and synthesis of extracellular matrix (ECM) (via TGF-βR). (D) Angiopoietin-2 (Ang2) may destabilize the vessel (via Tie2) by loosening EC - PC interactions and mediating degradation of ECM. This leads either into angiogenesis in the presence of angiogenic stimuli (E) or into regression of the vessel, when survival factors are absent (F).
1.3.3 Regulation of vascular remodeling

Vascular homeostasis in the skin, often described as vascular quiescence (DETMAR 1996), must strictly be controlled in a spatiotemporal manner (LIEKENS et al. 2001). This requires the orchestration of a variety of cellular and molecular mechanisms dictated by the perfused tissue (HANAHAN 1997, DETMAR 1996, FOLKMAN 1997, YANCOPOULOS et al. 2000). There is a row of evidences that, within the skin, the epidermis and not the dermis regulates the homeostasis of cutaneous vascularization (MALHOTRA et al. 1989, DETMAR et al. 1995, DETMAR 1996, DETMAR et al. 1998). Consequently, it would be conceivable that the hair follicle meets its vastly changing metabolic demands by regulating its own critical blood supply. This, however, has not yet been shown experimentally. There is only one report supporting this theory by demonstrating an angiogenic potential of anagen rat hair bulbs in a rabbit corneal pocket angiogenesis assay (STENN et al. 1988).

Multiple endogenous stimulators and inhibitors of angiogenesis and of blood vessel remodeling have been identified during the past decade (FOLKMAN 1997, CONWAY et al. 2001). Many of these factors are pleiotropic, i.e. they mediate different functions in a variety of tissues. The two most important families of vascular growth factors are largely specific for endothelial cells. These are the vascular endothelial growth factor -family (most importantly VEGF-A), and the angiopoietin –family (Figure 1.9) (LIEKENS et al. 2001, CONWAY et al. 2001).

1.4 Vascular endothelial growth factor

The vascular endothelial growth factor (VEGF, vascular permeability factor) -family consists of 5 different glycoproteins (VEGF-A, VEGF-B, VEGF-C, VEGF-D, PIGF) that are closely related to TGF-β2 and PDGF-BB (MCDONALD and HENDRICKSON 1993; ACHEN and STACKER 1998). VEGF-A (hereafter called VEGF) was the first being described and is the best studied member of the VEGF family (LEUNG et al. 1989). It is a central regulator of vasculogenesis and angiogenesis (FERRARA 1999, CARMELEIT et al. 1996). It promotes endothelial cell proliferation and migration
(ACHEN and STACKER 1998, NEUFELD et al. 1999), increases vascular permeability (SENGER et al. 1983), and it is a survival factor for newly-formed blood vessels (ALON et al. 1995, BENJAMIN and KESHET 1997), not only in the very initial phases of vasculogenesis, but also in later stages of angiogenesis and vascular maintenance (CARMELIET et al. 1996, FERRARA et al. 1996, GALE and YANCOPOULOS 1999). VEGF appears to be vital in a dose-dependent manner, since even the loss of a single VEGF-allele results in embryonic lethality (CARMELIET et al. 1996, FERRARA et al. 1996).

When investigating the effects of VEGF on the vasculature, it is important to note that VEGF occurs in different isoforms that derive from the alternative splicing of the primary transcript, involving exons 6 and 7 of the VEGF gene (ROBINSON and STRINGER 2001). These exons encode for heparan-binding regions that influence VEGF binding to the extracellular matrix, solubility of each isoform, and their mitogenic potential. Alternative splicing of VEGF mRNA may also have an important influence on the regulation of VEGF activity (GRUNDSTEIN et al. 2000, ROUT et al. 2000, ROBINSON and STRINGER 2001). Whereas a high amount of VEGF164 is sequestered to the extracellular matrix or the cell membranes, the isoform VEGF120 is freely diffusible (FERRARA et al. 1992, PARK et al. 1993). Thus VEGF proteins may become available to endothelial cells either by diffusion of readily synthesized short isoforms, or by cleavage from sequestered longer isoforms (PARK et al. 1993).

All VEGF isoforms, irrespective of their different affinities, act on endothelial cells via two specific tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1), that are predominantly expressed on endothelial cells (Figure 1.9) (DEVRIES et al. 1992, MILLAUER et al. 1993, NEUFELD et al. 1999). There are further VEGF receptors (e.g. neuropilin-1) that are isoform-specific and probably function as co-receptors, but their exact function in mediating VEGF-signals is yet unknown (SOKER et al. 1998). VEGFR-1 and VEGFR-2 exert different biological effects. VEGF binding to VEGFR-2 induces endothelial cell proliferation and also is involved in their protection from apoptosis (SHALABY et al. 1995). In contrast, VEGFR-1 binding does not induce proliferation, but stimulates endothelial cell migration and tube formation (FONG et al. 1995, SEETHARAM et al. 1995, BARLEON et al. 1996).
VEGFR-1 might also be involved in down-regulating VEGF activity to ensure that the right numbers of endothelial cells are generated (GALE and YANCOPOULOS 1999). Hence the temporal and spatial expression of VEGF-receptors on endothelial cells plays an important role in the regulation of endothelial cell behaviour.

VEGF protein has been demonstrated in human epidermal keratinocytes (BROWN et al. 1992, VIAC et al. 1997) and in keratinocytes of the human hair follicle (GOLDMAN et al. 1995, WENINGER et al. 1996, KOZLOWSKA et al. 1998). VEGF mRNA and protein have also been reported to occur in human dermal papilla fibroblasts (LACHGAR et al. 1996, KOZLOWSKA et al. 1998), although its synthesis by mesenchymal cells is still controversial (SENGER and VAN DE WATER 2000). For example, no fluorescence could be demonstrated in any mesenchymal cell of transgenic mice, expressing the green fluorescent protein (GFP) under a portion of the human VEGF-promoter (KISHIMOTO et al. 2000), and no VEGF mRNA had been detectable in mesenchymal cells by in situ –hybridization (YANO et al. 2001).

Figure 1.9: Schematic representation of two families of vascular growth factors and their receptors (modified from YANCOPOULOS et al. 2000). (a) VEGF-family; (b) angiopoietin-family; (+) and (-) indicate whether the particular factor activates or blocks the receptor; (?) indicates that a potential interaction has not yet been confirmed experimentally. The major regulators of blood vessel homeostasis are highlighted in red.
1.5 Angiopoietins

The angiopoietins (Angiopoietin-1, Angiopoietin-2) and their common receptor (Tie-2) are the second family of growth factors being specific for endothelial cells. They mostly regulate endothelial cell- and blood vessel- homeostasis (MUSTONEN and ALITALO 1995). Like VEGF, angiopoietin-1 (Ang-1) is also involved in early blood vessel development, but it is supposed to play a later role in angiogenesis (SURI et al. 1996). It primarily mediates blood vessel maturation and stabilization (SURI et al. 1998, HAYES et al. 1999), which includes complex interactions between the endothelial cells and the surrounding matrix (INGBER and FOLKMAN 1989), and it induces formation of leakage-resistant vessels (THURSTON et al. 1999, THURSTON et al. 2000). Ang-1 exerts its effect via binding to a tyrosine kinase receptor (Tie-2) ([Figure 1.9]) (DUMONT et al. 1992, PARTANEN et al. 1992). This receptor is involved in both angiogenesis and vascular maintenance (SATO et al. 1995, WONG et al. 1997). A second tyrosine kinase receptor for angiopoietins (Tie-1) has been described. However, although the absence of this receptor leads to embryonic death due to defects in vasculogenesis, until now its ligands, its signaling pathways and its specific functions are unknown (DAVIS and YANCOPOULOS 1999, MARRON et al. 2000).

The second member of the angiopoietin–family, angiopoietin-2 (Ang-2), shows about 60% homology to Ang-1. It binds to the same receptor but does not stimulate its phosphorylation and thus is an endogenous antagonist of Ang-1 (MAISONPIERRE et al. 1997). Ang-2 is predominantly expressed at sites of vascular remodeling. Its effect is thought to be coordinated with VEGF: Ang-2 blocks the Ang-1 mediated stabilization of vessels, thus promoting vessel sprouting in the presence of VEGF. In the absence of VEGF, this destabilization of vessels leads to vascular regression ([Figure 1.10]) (MAISONPIERRE et al. 1997).

Ang-1, Ang-2, and Tie-2 mRNA are expressed at low levels in normal human and murine skin (WONG et al. 1997, SURI et al. 1998, BROWN et al. 2000), but their association with hair follicles has not been determined so far.
Figure 1.10: Schematic representation of the roles of VEGF and angiopoietins during vessel remodeling (modified from YANCOPOULOS et al. 2000). Vasculogenesis (A) and Angiogenesis (B) are stimulated by VEGF. Angiopoietin-1 (Ang-1) mediates stabilization and maturation of vessels (C). Angiopoietin-2 (Ang-2) blocks Ang-1 effects and thus destabilizes vessels (D). Unstable vessels regress in the absence of VEGF (E) or undergo angiogenesis in the presence of VEGF (F).
1.6 The C57BL/6-mouse model for hair research

In mice, hair growth is synchronized and occurs as a wave which sweeps posteriorly and dorsally from the throat region so that all follicles in a particular region are in the same hair cycle stage (DRY 1926, CHASE et al. 1953). This synchronization enables one to investigate a large amount of hair follicles within the same phase of the hair growth cycle. Furthermore, a temporally highly defined hair growth cycle (Figure 1.11) can be initiated in mice by simply applying a wax and rosin mixture to the back skin of telogen mice, and peeling it off after hardening, thus stripping all telogen hair shafts (PAUS et al. 1990). Since in mice cutaneous melanocytes are restricted to the hair follicles and produce pigment in anagen only, the hair cycle stage can in principle be determined by the skin colour of animals (Figure 1.11, 2.2). Compared to rats, the growth wave of mice shows a much higher degree of synchronicity, even when it is induced by depilation (PAUS, unpubl. observation). Finally, the vast amount of transgenic mice allows to dissect the function of various factors that are involved in blood vessel homeostasis. These unique model systems do not occur in any other species.

Therefore, the hair growth cycle of mice represents a suitable model for investigating the relationship between hair follicles and the perifollicular vascular system.
Figure 1.11: Time scale for the hair growth cycle in female C57BL/6 mice (from MÜLLER-RÖVER et al. 2001). Note the changes of skin pigmentation and skin thickness after depilation.
### Activators and Inhibitors of Angiogenesis

<table>
<thead>
<tr>
<th>Activators</th>
<th>Function</th>
<th>Inhibitors</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF, VEGF-C, PIGF, and domologues</td>
<td>Stimulate angiogenesis (suppress angiogenesis in some context), permeability; VEGF-C: stimulates lymphangiogenesis; PIGF: role in pathologic angiogenesis</td>
<td>VEGFR-1, soluble</td>
<td>Sink for VEGF, VEGF-B, PIGF (VEGFR-1) and for VEGFα (NP-1)</td>
</tr>
<tr>
<td>VEGF receptors (VEGFR)</td>
<td>VEGFR-2: angiogenic signaling receptor; VEGFR-3: (lymph)angiogenic signaling receptor; neuropilin-1 (NP-1): binds specifically VEGFαα, coreceptor of VEGFR-2</td>
<td>Angiopoietin-2</td>
<td>Antagonist of Ang1: induces vessel regression in the absence of angiogenic signals</td>
</tr>
<tr>
<td>Angiopoietin-1 (Ang1) and Tie-2 receptor</td>
<td>Ang1: stabilizes vessels by tightening endothelial-smooth muscle interactions; inhibits permeability; Ang2: destabilizes vessels before sprouting</td>
<td>Thrombospondin-1 (TSP-1)</td>
<td>Extracellular matrix protein: type I repeats inhibit endothelial migration, growth, adhesion, survival; related TSP-2 also inhibits angiogenesis</td>
</tr>
<tr>
<td>PDGF-BB and receptors</td>
<td>Recruit smooth muscle cells</td>
<td>Meth-1, Meth-2</td>
<td>Inhibitors containing metalloprotease, thrombospondin and disintegrin domains</td>
</tr>
<tr>
<td>TGF-β1, endoglin, TGF-β receptors</td>
<td>Stabilize vessels by stimulating extracellular matrix production</td>
<td>Angiostatin and related plasminogen kringles</td>
<td>Proteolytic fragments of plasminogen; inhibit endothelial migration and survival</td>
</tr>
<tr>
<td>FGF, HGF, MCP-1</td>
<td>Stimulate angiogenesis (FGF, HGF) and arteriogenesis (FGF, MCP-1)</td>
<td>Endostatin</td>
<td>Fragment of type XVIII collagen; inhibits endothelial survival and migration</td>
</tr>
<tr>
<td>Integrins αvβ3, αvβ5</td>
<td>Receptors for matrix macromolecules and proteinases (MMP2)</td>
<td>Vasostatin, calreticulin</td>
<td>Calreticulin and N-terminal fragment (vasostatin) inhibit endothelial growth</td>
</tr>
<tr>
<td>VE-cadherin, PECAM (CD31)</td>
<td>Endothelial junctional molecules; essential for endothelial survival effect</td>
<td>Platelet factor-4</td>
<td>Heparin-binding CXC chemokine inhibits binding of bFGF and VEGF</td>
</tr>
<tr>
<td>Ephrins</td>
<td>Regulate arterial/venous specification</td>
<td>Tissue inhibitors of MMP (TIMPs), MMP-inhibitors; PEX</td>
<td>Suppress pathologic angiogenesis; PEX: proteolytic fragment of MMP2, blocks binding of MMP2 to αvβ3</td>
</tr>
<tr>
<td>Plasminogen activators, matrix metalloproteinases</td>
<td>Proteinases involved in cellular migration and matrix remodeling: liberate bFGF and VEGF from the matrix; activate TGF-β1; generate angiostatin</td>
<td>Interferon (IFN) α, β, γ, IP-10, IL-4, IL-12, IL-18</td>
<td>Cytokines and chemokines, inhibiting endothelial migration; IFNα downregulates bFGF</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor-1</td>
<td>Stabilizes nascent vessels by preventing matrix dissolution</td>
<td>Prothrombin kringle-2, anti-thrombin III fragment</td>
<td>Fragments of the hemostatic factors suppress endothelial growth</td>
</tr>
<tr>
<td>Nitric oxide synthase, Cyclooxygenase-2</td>
<td>Nitric oxide and prostaglandins stimulate angiogenesis and vasodilation</td>
<td></td>
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<tr>
<td>Other activators</td>
<td>Chemokines (also present in or affecting non-endothelial cells) play a pleiotropic role in angiogenesis</td>
<td></td>
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**Table 1.1: Overview on activators and inhibitors of angiogenesis** (modified from CONWAY et al. 2001)
1.7 Aims of the study

The purpose of this study was to investigate the potential for a new therapeutical approach to manipulate hair growth disorders by influencing the blood supply to the hair follicle. Since hardly anything is known about the relationship between hair follicle growth and cutaneous vascularization, we aimed to investigate whether hair follicles can actively influence their blood supply and whether hair growth depends on this influence. We decided to use the C57BL/6-mouse model for hair research because morphogenesis and cycling of hair follicles can precisely be determined and investigated in this model.

Firstly, since data on the perifollicular vasculature in mice is sparse, this study aimed at dissecting the development and the anatomy of the perifollicular vascular system in murine skin. Furthermore, we wanted to investigate the factors that are involved in its guidance and in the relationship between blood vessels and hair follicle morphogenesis. Secondly, we wanted to investigate whether there is actual blood vessel growth (angiogenesis) associated with anagen-development in the skin and if so, whether this angiogenesis is necessary for normal anagen-development. Thirdly, we wanted to examine whether there is vascular regression associated with hair follicle involution, as would be presumed if anagen-associated angiogenesis occurs. If so, we wanted to dissect the cellular events in order to compare them to other model systems of physiologic blood vessel regression. Fourthly, we wanted to explore the molecular controls that are involved in regulating angiogenesis and regression of the perifollicular vasculature and whether they derive from the hair follicle itself or from other cutaneous cells.

Finally, this study should investigate whether the cyclic reconstruction of the perifollicular vascular system, if it occurs at all, represents a suitable model for physiological blood vessel remodeling. Although angiogenesis has intensively been investigated during the last decades (FOLKMAN 1995, CARMELIET and JAIN 2000), our understanding of the cellular and molecular mechanisms of vascular remodeling still is very limited (YANCOPOULOS et al. 2000). This is mainly due to a lack of suitable in vivo models in which functional studies can be performed (AUGUSTIN...
The early embryonic lethality of mice with targeted mutations of genes that are critical for blood vessel morphogenesis and maintenance limits detailed mechanistic experiments (KORFF et al. 2001). Therefore, model systems of physiological angiogenesis that, beside vascular growth, encompass eventual maturation or regression of vessels are essential research tools for investigating the finely-tuned balance of angiogenesis stimulators and inhibitors (YANCOPOULOS et al. 2000, AUGUSTIN 2001).

1.7.1 Questions addressed by the study

I. Development of the cutaneous microvasculature and its association with hair follicle morphogenesis

1) Does a distinct perifollicular vascular network exist in mouse skin?

2) How and when does it develop?

3) Do hair follicles express VEGF and angiopoietins, the two major families of vascular-specific growth factors, during development of the perifollicular vasculature?

II. Remodeling of the cutaneous microvasculature in association with hair follicle cycling

1) Do morphological changes of the cutaneous vascular system occur in association with hair follicle cycling in mouse skin?

2) Does the cutaneous microvessel density vary throughout the hair growth cycle in mouse skin?

3) Does angiogenesis, i.e. the growth of new capillaries, occur during synchronized anagen-development in murine skin?

4) Is this anagen-associated angiogenesis necessary for normal anagen-development of hair follicles?
5) Does vascular regression occur during synchronized transformation of hair follicles from anagen to catagen to telogen in murine skin?

6) What are the cellular events that are associated with the vascular regression in mouse skin?

III. Vascular growth factors in hair follicle cycling

1) Where is the VEGF protein localized in mouse skin throughout the hair growth cycle?

2) Is the VEGF mRNA alternatively spliced in mouse skin?

3) Does expression of these splice variants change in accordance to hair follicle activity?

4) How do mRNA levels of different VEGF isoforms and their receptors change quantitatively throughout the hair growth cycle in mouse skin?

5) Which vessels express VEGFR-1 protein during the hair growth cycle?

6) Does neutralization of VEGF alter the cutaneous vascular system and/or hair follicle cycling?

7) Do mice, that lack a functional VEGF protein in keratin 5-expressing cells, exhibit abnormalities in hair follicle morphogenesis and the first spontaneous catagen-development?

8) Does treatment with recombinant murine VEGF influence catagen-development of hair follicles?

9) Do hair follicles express Angiopoietin-1 and Angiopoietin-2 protein during the hair growth cycle?

10) Are mRNAs of Angiopoietin-1, Angiopoietin-2 and their receptor differently expressed in murine skin throughout the hair growth cycle?
2. MATERIAL AND METHODS

2.1 Animals and tissue collection

2.1.1 C57BL/6 mice

Female C57BL/6 mice were purchased from the animal facility of the University Hospital Eppendorf and were housed in community cages under standardized conditions (12 h light/dark-cycle, water and mouse chow ad libitum).

Hair follicle morphogenesis was investigated in these mice during embryo-fetal and early postnatal development, according to published comprehensive guidelines (PAUS et al. 1999).

2.1.2 VEGF<sup>f/f</sup> K5-Cre<sup>+</sup> mice

Skin tissue from mice in which the VEGF gene was inactivated in keratin 5 – expressing keratinocytes, i.e. in cells of the interfollicular epidermis and the follicular outer root sheath, was obtained from Prof. Dr. E. Tschachler, Department of Dermatology, University of Vienna Medical School, Vienna, Austria. Two loxP sites, i.e. a sequence motif that is recognized by the enzyme Cre recombinase, had been inserted by homologous recombination in either site of exon 3 of the VEGF gene, finally resulting in homozygous VEGF<sup>f/f</sup> – mice. These animals had been intercrossed with mice that express Cre under the keratin 5 – promoter (K5-Cre<sup>+</sup> -mice), which led to homozygous floxed mice that expressed the K5-Cre – transgene. In these double-transgenic mice, Cre binds to loxP and excises exon 3 of the VEGF gene, resulting in animals that lack functional VEGF mRNA in keratin 5 –expressing cells (Figure 2.1). These animals were 10-20% smaller than the non-transgenic littermates, and showed retarded wound healing (ROSSITER et al. 2002). Skin tissue from these mice and from non-transgenic controls was embedded as described in 2.1.5 and was analysed with regard to hair follicle morphogenesis and the cutaneous microvessel density.
Figure 2.1: Conditional gene ablation by the Cre-loxP system (modified from SAUER 1998). The target gene (here exon 3 of the VEGF gene) is modified by homologous recombination in embryonal stem (ES) cells so that it is flanked by two directly repeated loxP sites (for simplicity, only one allele is shown). Mice are generated from these ES cells by standard procedures. The loxP–modified (floxed) mouse is mated with a Cre-transgenic mouse that expresses the enzyme Cre-recombinase under control of a tissue-specific promoter (here keratin 5–promoter). This generates a double–transgenic mouse in which the loxP–modified gene is deleted in those cells in which the Cre-transgene is expressed (here keratin 5–expressing cells). Other cells retain the target gene.
2.1.1 Anagen-induction by depilation

Anagen was induced as previously described by depilation in the back skin of 6- to 9-week old mice with all back skin hair follicles in telogen (PAUS et al. 1990). Briefly, a mixture of bees wax (Aldrich Chemical Company, Milwaukee, WI, USA) and rosin gum (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was heated and applied onto the back skin of mice. After hardening, the mixture was peeled off, removing all telogen hair shafts from their follicles. This procedure has been shown to induce a very synchronous anagen – development in the back skin of the animals, which can be followed macroscopically by simple determination of the skin color, as is demonstrated in Figure 2.2 (PAUS et al. 1990). Back skin was harvested from 3 animals at days 0, 3, 5, 8, 12, 17, 19, 25, 29 and 34 after depilation. Experiments were conducted according to federal guidelines and all procedures were approved by the local authorities (BAGS Hamburg, G12/00, G22/00).

2.1.2 Angiogenesis inhibition in vivo

To investigate the effect of angiogenesis inhibition on the hair growth cycle, mice were treated with the synthetic fumagillin derivative TNP-470 (provided by Schering AG, Berlin, Germany). This compound specifically inhibits angiogenesis and thus is able to delay angiogenesis – dependent processes such as cutaneous wound healing in vivo (CASTRONOVO and BELOTTI 1996, KLEIN et al. 1999). It has already been used in clinical trials (CASTRONOVO and BELOTTI 1996), and no toxic effects on keratinocytes are known (CASTRONOVO, personal communication). TNP-470 was administered intraperitoneally once a day beginning with the day of depilation (day 0 p.d.) until animals were sacrificed for skin embedding. A dosage of 6 mg TNP-470 per kg body weight, dissolved in peanut oil with 0.24% ethanol was used. A group of eight mice was treated with TNP-470, and a control group of eight mice was treated with the vehicle only. Firstly, five animals of each group were analysed after 6 days of treatment. Secondly, three animals of each group were analysed after 8 days of treatment. Skin color was assessed macroscopically and the percentage of hair follicles in each hair cycle stage of anagen–development was assessed
Material and Methods

microscopically, after embedding and processing of back skin as described (2.1.3). Endogenous alkaline phosphatase activity was used as a marker of the dermal papilla to determine the exact hair cycle stage of individual hair follicles (HANDJISKI et al. 1994). The hair cycle stage of at least 100 hair follicles per mouse was assessed and recorded. The percentages of hair follicles in each hair cycle stage were compared between the two groups using the Wilcoxon test for comparison of two independent samples.

2.1.3 Treatment with a VEGF neutralizing antibody

To investigate the role of VEGF during anagen–development in the depilation–induced hair growth cycle, mice were treated with a VEGF–neutralizing polyclonal antibody, raised in goats that were immunized with a recombinant mouse VEGF (R&D Systems GmbH, Wiesbaden, Germany). 100 µg of the affinity–purified antibody were reconstituted in 1000 µl sterile PBS.

In a first set of experiments, six mice were depilated as described in 2.1.1. Three mice were treated daily with the VEGF–neutralizing antibody (20µg/mouse/d) subcutaneously from the day of depilation (d 0) until day 8 after depilation (d 8). The other three animals served as controls and were treated with PBS only. Eight days after depilation, animals were sacrificed and back skin was taken and embedded as described in 2.1.4. Frozen sections were stained for endogenous alkaline phosphatase activity (HANDJISKI et al. 1994) and used to record the hair follicle stage of individual hair follicles in back skin (MÜLLER-RÖVER et al. 2001). The hair cycle stage of at least 100 hair follicles in back skin was evaluated and recorded for statistical analysis. Sections stained for PECAM-1 served for evaluation of microvessel density as described in 2.4.2. The diameters of hair follicle bulbs were measured at Auber´s line of the dermal papilla in H&E–stained sections.

In a second set of experiments, seven mice were depilated. Four mice were treated with the VEGF–neutralizing antibody (25 µg/mouse) intraperitoneally on days 8, 11 and 14 after anagen–induction by depilation. Three mice served as PBS–treated controls. The animals were sacrificed on day 16 of the depilation–induced hair cycle,
i.e. just before the hair follicles spontaneously enter catagen–development. The skin samples were processes and evaluated as described above.

### 2.1.4 Treatment with recombinant mouse VEGF

To further investigate the role VEGF plays for vascular remodeling during the hair growth cycle, mice were treated with a recombinant murine VEGF\(_{164}\) protein (R&D Systems GmbH, Wiesbaden, Germany). Six animals were depilated, and recombinant murine VEGF (300 ng/mouse) dissolved in PBS were injected subcutaneously every day between day 14 and 18 after depilation into three mice. The other animals received injections of PBS alone. Skin was taken on day 19 p.d. and was analysed as described above.

![Figure 2.2: Photodocumentation of the depilation-induced hair growth cycle in C57BL/6 mice.](image)

Mice are depilated when all hair follicles in back skin are in telogen. Note the pink skin color of telogen mice. After anagen-induction, the skin color becomes grey, due to the melanogenesis that is associated with anagen-development (black arrows). At day 17 after depilation, catagen – development is initiated spontaneously, and the wave of catagen-development proceeds from the cranial to the caudal region of the back skin. Catagen-induction is associated with a stop in melanogenesis, thus it is visible macroscopically by the brighter skin color (white arrows).
Material and Methods

1. Anaesthetize animals by i.p. injection of ketamine/xylazine.
2. Shave back skin with electric clippers. Sacrifice animal by cervical dislocation.
3. Using sharp scissors, cut a hole into the skin at the base of the tail.
4. Insert the closed scissors and push them forward until they reach the neck area.
5. Open scissors and pull them back, separating the skin from the underlying connective tissue.

6. Freeze the outermost slice in liquid N₂. (Note: Use a cryotub or similar for optimal freezing.)
7. Using a very sharp scalpel blade, cut the skin into pieces of approx. 5 mm width along the median axis.
8. Place the skin on a cardboard that is covered with aluminum foil, carefully remove the skin.
9. Cut off the back skin along the shaved margin.

10. Use the innermost slices for histology.
11. Carefully cut the cardboard and aluminum foil between the two slices.
12. Use the left slice for fixation in formalin and paraffin-embedding, and continue with the right slice as indicated in 14-21.

13. Fixation (24-48 h, at +4°C) in 4% neutr. buffered formaldehyde.

15. Spread embedding medium (frozen specimen embedding resin) onto the skin.
16. Carefully grasp the upper end of the skin slice with forceps and pull it backwards onto the lower end of the slice as indicated in the cartoon.
17. Freeze the folded skin specimen on the aluminum foil in isopentane, cooled by liquid N₂.

18. Dip the form into liquid N₂, then remove the frozen block from the form and store it at -80°C.
19. Place the frozen skin specimen as indicated in the cartoon in a tablet form that is filled with embedding resin.
20. Remove the frozen skin specimen from the aluminum foil.
2.1.5 Embedding of skin

Mice were anaesthetized with ketamin/xylazin (Ketanest, Park-Davis, Freiburg, Germany/ Rompun, Bayer, Leverkusen, Germany) and the back skin was shaved using electric clippers. Then, animals were sacrificed by cervical dislocation, and the back skin was dissected at the level of the subcutis, just below the subcutaneous muscle layer (Figure 2.3). Tissue from the back skin of mice was either natively frozen, embedded in frozen specimen embedding resin (Shandon, Pittsburgh, PA) or was fixed in 4% buffered formalin overnight, embedded in paraffin and sectioned (5 µm) onto adhesive microscope slides (SuperFrostPlus, Menzel-Gläser, Germany), as described in Figure 2.3 (PAUS et al. 1999). Representative tissue samples of some animals were fixed in Karnovsky’s fixative, post-fixed in 2% osmium tetroxide and uranyl acetate, and embedded in resin as previously described (TOBIN et al. 1991).

2.2 Immunohistology

2.2.1 PECAM-1 (CD31)

Endothelial cells were demonstrated in frozen sections by staining for CD31 (PECAM-1). PECAM-1, an endothelial cell adhesion molecule, is a widely used panendothelial cell marker, and facilitates the assessment of the vascular status of a tissue (NEWMAN 1997, EBERHARD et al. 2000). Frozen sections were fixed in
acetone for 10 min, dried and then washed in Tris-buffered saline (TBS, pH 7.4). After pre-incubation with 10% normal goat serum in TBS, slides were incubated with a monoclonal rat antibody raised against the murine homologue of CD31 (Pharmingen, San Diego, CA, USA). This antibody was used in a dilution of 1:2000 in TBS, and 2% normal goat serum were added. After over-night incubation, slides were washed and treated with a polyclonal goat antibody, directed against rat IgG (Dianova GmbH, Hamburg, Germany) in a concentration of 1:200 in TBS. 2% normal goat serum and 4% normal mouse serum were added.

For light microscopical detection, a biotinylated antibody was used. This was followed by the ABC-detection system. The latter was either conjugated to alkaline phosphatase or to peroxidase (Vector, Burlingame, CA, USA). According to the employed enzyme, Vectastain AP substrate kit I or DAB (Vector, Burlingame, CA, USA) were used as substrates. Slides were counterstained with hematoxylin, dehydrated and permanently mounted.

For immunofluorescent detection, a fluorescein isothiocyanate (FITC)- or Cy3-conjugated secondary antibody was used. Slides were then stained with DAPI, a cell nuclei marker, and mounted in Fluormount (Southern Biotechnology Associates, Birmingham, AL, USA). Sections without the primary antibody and sections with an antibody to an irrelevant antigen served as negative controls.

### 2.2.2 Ki-67 / PECAM-1 double staining

Sections were treated as described above to stain for PECAM-1. After incubation with the secondary antibody, conjugated to FITC, sections were pre-treated again with 10% normal goat serum in TBS and were finally incubated with a polyclonal rabbit antibody, raised against the murine Ki-67 protein (Dianova GmbH, Hamburg, Germany). This antibody was diluted 1:100 in TBS and 2% normal goat serum were added. After over-night incubation, sections were treated with a Cy3-conjugated goat antibody, raised against rabbit IgG (Dianova GmbH, Hamburg, Germany), diluted 1:200 in TBS with 2% normal goat serum and 4% normal mouse serum. Double immunoreactive cells were identified as cells that revealed a red intranuclear staining
(Ki-67) which is surrounded by a green cytoplasmic staining (CD31), resulting in a small yellow-appearing zone around the nucleus, where both colors superimpose. Sections without either primary antibody served as negative controls.

### 2.2.3 Vascular endothelial growth factor (VEGF)

For detection of VEGF-immunoreactivity, paraffin sections were deparaffinized in xylene, rehydrated in graded alcohols and washed in 0.05 M TBS (pH 7.4). They were then incubated for 15 min in 3% H$_2$O$_2$ to quench endogenous peroxidase activity and subsequently incubated with normal rabbit serum (10% in TBS) to block unspecific binding of the secondary antibody. Sections were incubated over-night with an affinity-purified goat polyclonal antibody raised against a peptide mapping at the amino terminus of the mature form of murine VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) in a dilution of 1:150 in TBS. After washing in TBS, slides were incubated with a biotin-conjugated rabbit anti-goat IgG antibody (Dianova GmbH, Hamburg, Germany) diluted 1:300 in TBS for 30 min at room temperature. After thorough washing, slides were incubated with a PAP (goat) complex (DAKO, Hamburg, Germany), washed, and then incubated with a peroxidase-conjugated avidin-biotin-complex (Vector, Burlingame, CA) for 30 min at room temperature each. Labeling with peroxidase was developed using a 3`3-diaminobenzidine substrate kit (Vector, Burlingame, CA), counterstained with methylene green (DAKO, Hamburg, Germany), dehydrated and mounted. For negative controls, the primary antibody was incubated with a five-fold excess of the blocking peptide (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight.

### 2.2.4 Angiopoietin-1 and Angiopoietin-2

For the detection of Ang-1 and Ang-2 –immunoreactivity in paraffin sections of mouse skin samples, a similar protocol was used as has been described for VEGF (2.2.3). As primary antibody, an affinity-purified goat polyclonal antibody raised 1) against a peptide mapping at the amino terminus of human angiopoietin-1 or 2)
against a peptide mapping at the carboxy terminus of human angiopoietin-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used in a dilution of 1:100 and 1:200, respectively. Both antibodies were known to react with the equivalent protein of mouse origin.

2.2.5 VEGFR-1 (Flt-1) / PECAM-1 – double staining

Double-staining for VEGFR-1 and PECAM-1 was performed on frozen sections as follows: Endogenous peroxidase was quenched in 3% H₂O₂ for 15 min. Endogenous avidin and biotin were blocked using an avidin/ biotin blocking kit (Vector, Burlingame, CA) according to the manufacturer’s protocol. All steps were interspersed by washing in TBS. Sections were pretreated with normal goat serum and incubated with a rat anti PECAM-1 antibody as described above. After washing, sections were pretreated with TNB buffer (NEN Life Science Products, Boston, MA) for 20 min at room temperature and incubated over-night with a rabbit affinity-purified polyclonal antibody raised against a peptide mapping at the carboxy terminus of Flt-1 of human origin (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000 in TNB buffer. Sections were washed in TNT (NEN Life Science Products, Boston, MA) and incubated with a biotin-labeled anti rabbit-IgG antibody raised in goat (Dianova, Hamburg, Germany), diluted 1:200 in TNB. After washing in TNT, sections were incubated with a streptavidin-horseradish peroxidase complex (NEN Life Science Products, Boston, MA), diluted 1:100 in TNB buffer and with a tyramide-fluorescein complex (NEN Life Science Products, Boston, MA), diluted 1:50 in amplification diluent (NEN Life Science Products, Boston, MA). Sections were washed in TNT and TBS and were treated with a goat antibody against rat IgG, labeled with Cy3 (Dianova, Hamburg, Germany). Sections were washed in TBS again and mounted in Fluoromount (Southern Biotechnology Associates, Birmingham, AL).
2.3 In situ apoptosis detection

Apoptotic endothelial cells were labeled using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Intergen Company, Oxford, UK) as described previously (LINDNER et al. 1997), followed by CD31-immunofluorescence staining as described above.

2.4 Morphometry

All experiments were conducted using a well known inbred strain of mice that had extensively been investigated previously with regard to hair follicle morphogenesis and cycling. These previous experiments had shown that depilation of telogen skin induces a simultaneous, very homogenous anagen–development of all hair follicles (PAUS et al. 1990). Furthermore, the hair cycle stage of each hair follicle can easily be assessed morphologically, which facilitates the investigation of different microscopic fields with hair follicles of exactly the same stage of the hair growth cycle. Therefore, very little variations between individual animals of the same time point were observed, and relatively small numbers of animals appeared to be sufficient in order to detect statistical differences of various parameters between different groups.

2.4.1 Assessment of mean vessel diameter and mean vessel length

Employing CD31-stained mouse skin sections and a digital imaging system (Openlab, Imprivision, Coventry, U.K.), the diameter of 150 transversally cut vessels and the length of 150 longitudinally cut vessels was measured in sections of telogen skin (day 0 p. d.) and anagen VI skin (day 12 p.d.). The obtained data was logarithmically transformed, and the log-normal distributed data was statistically analysed using the two-sided student’s t-test for independent samples.
2.4.2 Assessment of microvessel density

Sections stained for CD31 with the immunoperoxidase technique were investigated at 400x magnification. 90 microscopic fields were evaluated for microvessel density (MVD), i.e. the number of microvessels per microscopic field (VARTANIAN and WEIDNER 1994, HUDLICKA et al. 1998). The microscopic fields were positioned distal to the subcutaneous muscle layer, so as to evaluate major parts of the subcutaneous and dermal compartment of mouse skin. Because single CD31-immunoreactive cells cannot be classified with certainty as either myelomonocytic cells (ALBELDA et al. 1991) or endothelial cells, e.g. of small, obliquely cut capillaries, we counted CD31-immunoreactive cell aggregates in a restricted manner. Only those cell aggregates that showed formation of a central lumen or that could clearly be identified as endothelial cells, i.e. which formed longitudinal, occasionally branching structures, were counted as one unit each.

The mesenchymal skin compartment, where all blood vessels are located, changes with the hair growth cycle, as the thickness of all skin compartments and the size of the hair follicles fluctuates significantly during the murine hair cycle (CHASE et al. 1953, HANSEN et al. 1984). To avoid data distortion by these changes, we evaluated the effect of a correction formula, previously used to study the hair cycle-dependent amount of mast cells in murine skin (PAUS et al. 1994a). However, this compartment correction did not reveal any changes in the hair cycle-dependent MVD compared to the original data (data not shown).

Due to the above mentioned changes in skin thickness during the entire hair growth cycle in mice, we later aimed to establish another technique that fully considers this phenomenon. This new technique was used to more accurately evaluate changes in the microvessel density during anagen to telogen development of hair follicles and was also used to evaluate differences in cutaneous microvessel density between differently treated animals (2.1.2 - 2.1.4). Sections immunostained for CD31 were used again, but the number of immunoreactive crosscuts of vessels was evaluated, using a frame with a width of 370 µm. The sections were evaluated at 250x magnification and immunoreactive vascular structures were counted in the whole
dermal and hypodermal tissue from the dermoepidermal junction to the subcutaneous muscle layer. 60 microscopic fields with hair follicles of the same hair cycle stage were evaluated and the average number of microvessels per reference area was calculated.

For evaluation of changes in the microvessel density throughout anagen- and catagen–development of hair follicles, i.e. for comparison of multiple groups, statistical analysis was performed using analysis of variance (ANOVA) to check for equality of means. The means of the different hair cycle stages were then assessed for statistically significant differences using the Scheffé-test and the Ryan-Einot-Gabriel-Welsch range (REGWQ).

For comparison of the microvessel density between two groups of animals, normal distribution of values was verified by normal probability plot, and differences between both groups were evaluated using the two-sided student’s t-test for unpaired samples.

2.4.3 Assessment of the number of endothelial cell nuclei within a reference area

Sections stained for CD31 and counterstained with 1 µg/ml DAPI (4´,6-diamidine-2´-phenylindole dihydrochloride) (Roche Diagnostics GmbH, Mannheim, Germany) were used to count endothelial cell nuclei in the interfollicular dermis and subcutis within a field, limited by the dermoepidermal junction, the upper margin of the subcutaneous muscle layer and the connective tissue sheath of two neighboring, longitudinally cut hair follicles. Endothelial cell nuclei were counted at 250x magnification in 60 interfollicular areas.

Firstly, two groups (telogen and anagen VI) were examined and their means were compared statistically after logarithmic transformation, employing the two-sided student’s t-test for equality of means of two independent groups.

Secondly, a more detailed analysis of the number of endothelial cells per reference area was performed in skin samples of the anagen to telogen transformation. In each
animal, 30 interfollicular areas were evaluated with regard to the number of endothelial cell nuclei. Statistical analysis was performed using analysis of variance (ANOVA) to check for equality of means. The means of different hair cycle stages were then assessed for statistically significant differences using the Scheffé-test and the Ryan-Einot-Gabriel-Welsch range (REGWQ).

2.4.4 Assessment of proliferating endothelial cells
Sections stained for CD31 and Ki-67 were investigated at 400x magnification. In each animal, 60 microscopic fields with hair follicles of the same hair cycle stage were evaluated for the number of double-immunoreactive cells. The microscopic fields were positioned distal to the subcutaneous muscle layer. Statistical analysis was performed using analysis of variance (ANOVA) to check for equality of means. Means of the different hair cycle stages were then assessed for statistically significant differences using the Scheffé-test and the Ryan-Einot-Gabriel-Welsch range (REGWQ).

2.5 Semi-thin sections and transmission electron microscopy
Semi-thin sections (0.5-1.0 µm) were cut from Karnovsky’s fixative-fixed, resin-embedded tissue samples, stained with toluidine blue/borax and examined by oil-immersion light microscopy. Ultra-thin sections were stained with uranyl acetate and lead citrate and then examined using a Jeol 1200X electron microscope (TOBIN et al. 1991).

2.6 Semiquantitative reverse transcription-PCR (RT-PCR) for VEGF-mRNA:
Total RNA was isolated from 30 mg of back skin from three different mice of selected hair cycle stages (days after anagen–induction by depilation), using a single-step
guanidine thiocyanate-phenol-chlorophorm method (Quiagen, Hilden, Germany), according to the manufacturer’s protocol. 3 µg of total RNA were used for reverse transcription, employing the 1st strand cDNA synthesis kit and random primers (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer’s protocol. Samples without reverse transcriptase served as negative controls.

The following sets of oligonucleotide primers (Invitrogen, Karlsruhe, Germany) were used to amplify specific cDNA: \( \beta\)-actin: 5´ - GAA AAC GCA GCT CAG TAA CAG TCC G -3´ and 5´ - TAA AAC GCA GCT CAG TAA CAG TCC G -3´; VEGF: 5´ - GCA CCC TGG CTT TAC TGC TGT ACC -3´ and 5´ - CCG AAA CCC TGA GGA GGC TCC -3´ (GORDEN et al. 1997). The primers for VEGF were localized in exon 1 and exon 8 of the VEGF gene (Figure 2.4) and allowed amplification of all VEGF mRNA splice variants that are known to derive from alternative splicing (Figure 2.5).

PCR reactions were performed in a thermal cycler (Whatman Biometra, Göttingen, Germany) with Taq polymerase, employing the PCR core kit (Quiagen, Hilden, Germany). The following cycles were used: \( \beta\)-actin: 94°C, 3 min (1x); 94°C, 1 min; 60°C, 45 sec; 72°C, 45 sec (30x); 72°C, 5 min (1x); VEGF: 94°C, 3 min (1x); 94°C, 1 min; 60°C, 1 min; 72°C, 1 min (45x); 72°C, 5 min (1x). For semiquantitative PCR-analysis, the cDNA concentration of each sample was adjusted according to the level of \( \beta\)-actin mRNA. \( \beta\)-actin mRNA was used as internal standard, after we had compared its levels with the levels of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA in our samples. Since no difference in the expression of mRNA levels of both “housekeeping” genes was detected, it was assumed that \( \beta\)-actin is a reasonable internal standard for semiquantitative PCR analysis of mouse skin (data not shown).

PCR products were electrophoresed in a 2.0% high resolution agarose/ ethidium bromide gel and photographed under UV light. Total RNA from murine lung tissue was processed as described above and used as a positive control (BURCHARDT et al. 1999). The mean intensity of each band was measured using 1D Image Analysis Software (Kodak, Rochester, NY, USA). Intensities of the VEGF bands were adjusted according to the intensity of the \( \beta\)-actin band in each sample. The mean intensity
(measured in arbitrary units) was calculated from three individual samples per hair cycle stage. Since conventional RT-PCR is at best semiquantitative, we did not compare the means for statistical differences.

### Material and Methods

1. caagcgccgca agagagcggg ctgcctcgca gtccgagccg gagagaggga gcgcgacgcg
2. ggcgcggccag gacgcctctc gaaaccatg
3. gtcacagtta gacccgaggt gacacaggt gtcggtgtctg cgtatgagct
4. ttcaccggctc cagctggtcg caggctgcct ggtggtgtctg cgtatctgtg
5. tgcacagtta gacccgaggt gacacaggt gtcggtgtctg cgtatgagct

### Figure 2.4: Complete nucleotide sequence of mouse VEGF mRNA

**Legend:**
- **blue:** coding sequence
- **red:** binding sites of forward and reverse primers for PCR
- **black:** start and stop codon

**Figure 2.5: Schematic representation of splice variants of mouse VEGF mRNA.**
2.7 Quantitative real-time (TaqMan) RT-PCR

Quantitative real-time RT-PCR based on the TaqMan technique (Figure 2.7) was performed in order to accurately quantify the mRNA levels of the two most important VEGF isoforms and both VEGF receptors. Total RNA was isolated from selected animals as described above, but 5 µg of total RNA were used for oligo dT-primed reverse transcription. cDNAs were diluted 1:50 and 10µl were used per PCR reaction. PCR reactions for each data point were performed in triplicates.

Primers and fluorogenic TaqMan probes for mouse VEGF\textsubscript{164}, VEGF\textsubscript{120}, VEGFR-1, VEGFR-2, Ang-1, Ang-2, and Tie-2 were designed by GenExpress (Berlin, Germany) (Table 2.1). Primers and custom-synthesized TaqMan probes, labeled with FAM (6-carboxyl-fluorescein) as reporter dye and TAMRA (6-carboxy-tetramethyl-thodamine) as quencher dye were from TIB MOLBIOL (Berlin, Germany).

PCR was carried out in an ABI PRISM 7700 Sequence Detector (Applied Biosystems) in 96 well optical plates, heat sealed with an optical cover sheet (Abgene, Hamburg, Germany) in a total volume of 25 µl, using the Universal Mastermix (Applied Biosystems) according to the manufacturer’s protocol (Figure 2.6). Annealing temperature and primer concentrations were optimized for each primer/probe combination. All reactions were run as two step PCRs as follows: 2 min at 52°C, 10 min at 95°C (1x); 15 sec at 95°C and 1 min at 60°C (40x). Probe concentration was always 100 nM. Primer concentrations were 300 nM for VEGFR-2, and 900 nM for the other primer pairs. The target sequences were cloned and quantified at GenExpress (Berlin, Germany) and serial dilutions were used to generate standard curves for quantification. The standard dilutions were included in every PCR experiment. VEGF\textsubscript{120} primer combinations did not show any signal with sequences from VEGF\textsubscript{164}. Results of the experiments mentioned above were used to calculate intra-assay variation and the total variation of the assay.

Assays were performed as triplicates for each sample. The mean of each triplicate was calculated and the three values per time point were compared by analysis of variance and subsequent Scheffé-test and REGWQ range.
2.8 Statistical analysis

To evaluate for statistical significance, the mean, the standard deviation and the 95% confidence interval of each group were calculated. Normal distribution was determined by normal probability plot. Where necessary, data was logarythmically transformed. P-values were calculated with statistical tests, as indicated for each parameter. In general, differences were calculated for a significance niveau of 5%. All statistical analyses were performed on statistical software (SPSS, Chicago, IL).

Figure 2.6: Schematic representation of the principle of quantitative TaqMan RT-PCR (from BUSTIN 2001). Amplification is performed in closed, optical tubes of a 96 –well microplate that is placed in a combined thermal cycler/ detector, the ABI PRISM 7700. A laser is directed to each of the 96 sample wells, and the fluorescence emission data for each sample are collected once every few seconds as the PCR products are being generated. The starting copy number is determined by monitoring when PCR product is first detected – the higher the starting copy number of the target, the sooner a significant increase in fluorescence is observed. The data are fed to a computer, which analyses the results, eliminating the need for post-PCR processing.
Figure 2.7: Schematic representation of the TaqMan assay (from BUSTIN 2001). Three parameters affect performance of the 5′-nuclease assay: the quenching of the intact probe, its hybridisation efficiency and the efficiency of cleavage by the polymerase. (A) The RT step synthesises a cDNA copy of the mRNA. (B) After denaturation, primers and probe anneal. The proximity of the two dyes quenches the signal from the fluorescent dye at the 5′ end of the probe. (C) Polymerisation proceeds at the same temperature as the annealing step. (D) The polymerase displaces and hydrolys the labelled probe. (E) The fluorescent dye is released from its proximity to the quencher, and fluorescence is detected. This signal is directly proportional to the number of molecules present at the end of the previous or beginning of the current cycle.
<table>
<thead>
<tr>
<th>gen (accession no.)</th>
<th>primer/probe</th>
<th>sequence 5’-3’</th>
<th>matching nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse VEGF&lt;sub&gt;164&lt;/sub&gt; (M95200)</td>
<td>primer, forward</td>
<td>CCGATCAAACCTCACTACAA</td>
<td>405 – 423, located in exon 4</td>
</tr>
<tr>
<td>mouse VEGF&lt;sub&gt;120&lt;/sub&gt; (M95200)</td>
<td>primer, forward</td>
<td>CCGATCAAACCTCACTACAA</td>
<td>405 – 423, located in exon 4</td>
</tr>
<tr>
<td>mouse VEGFR-1, Flt-1 (D88689)</td>
<td>primer, forward</td>
<td>AGGCTTTCAGATTCATACTAG</td>
<td>3895 – 3914</td>
</tr>
<tr>
<td>mouse VEGFR-2, Flk-1 (X70842)</td>
<td>primer, forward</td>
<td>AGGAGAGATTGTCTTCTTAG</td>
<td>4370 – 4389</td>
</tr>
<tr>
<td>mouse angiopoietin-1 (NM 009640)</td>
<td>primer, forward</td>
<td>CAACCAGGCGCAGA</td>
<td>366 - 380</td>
</tr>
<tr>
<td>Mouse angiopoietin-2 (NM 007426)</td>
<td>primer, forward</td>
<td>AGGTCCAGAACGGGCTAGG</td>
<td>314 - 331</td>
</tr>
<tr>
<td>mouse tyrosine kinase receptor Tie-2 (X71426)</td>
<td>primer, forward</td>
<td>CCGGTGTTTCCATGTTTCTGCTT</td>
<td>3258 - 3278</td>
</tr>
</tbody>
</table>

Table 2.1: Primer and probe sequences used for quantitative (TaqMan) RT-PCR analysis.
3. RESULTS

This study provides evidence for a close interrelation between hair follicle morphogenesis/cycling and the cutaneous vascularization in mice. As opposed to telogen skin, a dense capillary network surrounds hair follicles in later stages of morphogenesis and anagen-development. This is associated with vascular remodeling, comprising angiogenesis and vascular regression, which occur spatially and temporally restricted according to the activity of hair follicles. The latter are shown to be the predominant source of key regulatory factors of vascular homeostasis (VEGF and angiopoietins), suggesting that hair follicles modulate the observed vascular reorganization, and thus regulate their blood supply according to their metabolic demand. Finally, it is shown that hair growth depends on this interaction between the hair follicle and its blood supply, although the regulation of the vascular remodeling is seemingly complex and cannot be easily manipulated pharmacologically.

Since the anatomy of the skin varies tremendously between different species, and even within one species depending on the region of the body, it is of major importance to clarify the nomenclature that is used in this study. This nomenclature is represented in Figure 3.0. According to this scheme, the skin consists of an avascular epidermis that is separated from the underlying dermis by a basement membrane zone. As opposed to humans, in murine skin the dermis cannot be separated in a papillary and a reticular part, but is generally referred to as the dermal connective tissue. Without any clearly definable separation, the dermis, that consists of a dense network of fibroblasts and collagen fibers, turns into a connective tissue of much looser consistency with multiple interspersed fat cells. This layer is often named the subcutis, implicating that it is clearly separable from the cutis. This is, however, not the case, especially since the multiple adnexal structures of mouse skin reach deep into this compartment. Therefore, we refer to the loose connective tissue with multiple fat cells as the hypodermis, which is demarcated in the deepness by the subcutaneous muscle layer (panniculus carnosus).
3.1 Development of the cutaneous vasculature in association with hair follicle morphogenesis

The aim of this study was to investigate remodeling processes in the vascular system that surrounds hair follicles in mouse skin. There is, however, hardly any data available about the cutaneous blood supply in mice. Therefore, we firstly investigated its development and anatomy, and layed emphasis on its association with hair follicle morphogenesis.
3.1.1 Development of the cutaneous vascular system comprises development of a distinct perifollicular vascular network

Hair follicle morphogenesis begins at approximately day 14.5 of embryo-fetal development with the formation of an epithelial hair germ and a mesenchymal condensation of dermal fibroblasts that later become the dermal papilla (PAUS et al. 1999). At day 16.5 of embryo-fetal development, the dermis and the hypodermis can already be separated microscopically, since the former exhibits a denser tissue architecture. At this time of development, there is a single blood vessel plexus detectable at the dermal–hypodermal interface (Figure 3.1.1 A,B). Hair follicles grow into the dermal tissue and at stage 3 of morphogenesis (for a detailed description of the stages of hair follicle morphogenesis see PAUS et al. 1999), they reach the above mentioned blood vessel plexus (Figure 3.1.1 C). At this early stage of hair follicle development (approximately day 18.5 of embryo-fetal development) a close association between hair bulbs and the surrounding vasculature is already detectable, since the latter begins to surround the proximal portion of hair follicles (Figure 3.1.1 C). In neonatal mice, another blood vessel plexus has developed which is located just beneath the epidermis (Figure 3.1.1 D). Many hair follicles have reached morphogenesis stage 5 and 6 by that time and their hair bulbs are located in the hypodermal tissue surrounded by a multitude of blood vessels that all derive from the lower plexus (Figure 3.1.1 D). With subsequent development of hair follicles, the hypodermis increases tremendously in width. The blood vessels of the lower plexus spread throughout the hypodermis and become longitudinally orientated to the hair follicles. They finally come into close proximity to the outer root sheath of hair follicles and especially surround the proximal half of them (Figure 3.1.1 E-H). In contrast to the well vascularized hypodermis, the dermal tissue appears to be only sparsely vascularized during early postnatal development (Figure 3.1.1 E,F).
3.1.2 Outer root sheath keratinocytes are a major source of blood vessel–specific growth factors during cutaneous development

The above mentioned observations suggest a close relationship between hair follicle morphogenesis and blood vessel development in mouse skin. It was therefore interesting to investigate, whether hair follicles synthesize and secrete growth factors that govern blood vessel development.

It is well known, that vasculogenesis and angiogenesis crucially depend on two families of blood vessel–specific growth factors: VEGF is the most important growth factor during vascular development. Mice that lack even one of the two VEGF allels or one of its two main receptors die during early embryonal development, due to severe defects in the cardiovascular system (FERRARA et al. 1996, CARMELIET et al. 1996, FONG et al. 1995, SHALABY et al. 1995). Angiopoietins are equally crucial for vascular development, since animals without angiopoietin-1 or without the angiopoietin–receptor (Tie-2), also die due to sever malformations of the vasculature (DUMONT et al. 1994, SURI et al. 1996).

Therefore, we aimed to investigate by immunohistology, whether vascular specific growth factors are detectable in mouse hair follicles, to which compartment of the hair follicle the immunoreactivity is localized, and when it is detectable during hair follicle development.

3.1.2.1 VEGF-immunoreactivity is prominent in outer root sheath keratinocytes of hair follicles

In prenatal and neonatal skin, VEGF–immunoreactivity can be detected in the interfollicular epidermis. This immunoreactivity is restricted to the suprabasal epidermal cell layers (Figure 3.1.2 C,D). Hair follicle plugs that derive from the basal epidermal layer do not exhibit VEGF–immunoreactivity (Figure 3.1.2 C). However, when hair follicles have reached stage 5 of morphogenesis and thus keratinocytes have begun to differentiate into an inner and an outer root sheath, the latter becomes strongly VEGF–immunoreactive. With subsequent elongation of the hair follicle, VEGF–immunoreactivity remains in the outer root sheath, whereas matrix
keratinocytes, the proliferating compartment of the hair follicle, and keratinocytes of the inner root sheath are not immunoreactive (Figure 3.1.2 D). Many mesenchymal cells of the dermis show marked VEGF–immunoreactivity during very early postnatal development of the skin (Figure 3.1.2 C,D). This strong immunoreactivity, however, is lost during the first postnatal days and is replaced by a moderate VEGF–immunoreactivity in only few cells of the dermal mesenchyme (Figure 3.1.2 E).

According to the dermal fibroblasts, cells of the dermal papilla are slightly immunoreactive during early cutaneous development and thus during early stages of hair follicle morphogenesis (Figure 3.1.2 C,D). This immunoreactivity, however, is lost or reduced to a very faint immunostaining when the hair follicles mature (Figure 3.1.2 E). The walls of large vessels above the subcutaneous muscel layer exhibit marked VEGF–immunoreactivity throughout cutaneous development.

### 3.1.2.2 Angiopoietin-1–immunoreactivity is prominent in outer root sheath keratinocytes of hair follicles

Ang-1–immunoreactivity is detectable in the suprabasal layers of the interfollicular epidermis during early cutaneous development (Figure 3.1.3 A,B). Consequently, hair follicle plugs, that derive from the basal cell layer of the epidermis, do not exhibit Ang-1–immunoreactivity (Figure 3.1.3 A,B). Once hair follicles have reached stage 5 of morphogenesis, and a distinction between the inner and the outer root sheath can be made, the outer root sheath is markedly immunoreactive (Figure 3.1.3 B). Immunoreactivity is retained in this compartment throughout hair follicle morphogenesis (Figure 3.1.3 D,E). Approximately 4 days after birth, the suprabasal immunoreactivity of the interfollicular epidermis is lost. Instead, some basal cells with dendritic morphology become Ang-1–immunoreactive (Figure 3.1.3 C). With regard to morphology and distribution of these cells, they likely represent γ/δ T-cell receptor (TCR)-positive dendritic epithelial lymphocytes, that have been shown to populate the epidermis early during postnatal cutaneous development (PAUS et al. 1998). This observation is of interest, since generation of angiopoietins has not been described for epithelial dendritic lymphocytes so far. However, it does not appear to
be of any relevance for the present study and is thus explored in a separate investigation. Immunoreactivity for Ang-1 changes markedly in the mesenchymal compartment of the skin during cutaneous development. In prenatal skin, only very faint immunoreactivity can be found in few cells of the dermis and hypodermis (Figure 3.1.3 A). In perinatal skin, a strong immunoreactivity is detectable in many cells of the dermal mesenchyme (Figure 3.1.3 B). This, however, is lost at approximately 4 days after birth and is replaced by a strong immunoreactivity of only few cells within the dermis and hypodermis (Figure 3.1.3 D,E,F). These cells likely represent macrophages and pericytes, which have already been demonstrated to synthesize Ang-1 (OTANI et al. 1999, MAISONPIERRE et al. 1997). Furthermore, Ang-1–immunoreactivity can be detected in the walls of large vessels in the subcutaneous connective tissue (Figure 3.1.3 B).

3.1.2.3 Angiopoietin-2–immunoreactivity is not detectable in keratinocytes of the hair follicle

Immunoreactivity for Ang-2 in prenatal skin is restricted to a faint staining of the suprabasal epidermal layers (Figure 3.1.4 B). No staining is detectable in hair follicle keratinocytes even after they have reached stage 5 of morphogenesis (Figure 3.1.4 B,G), and no staining is detectable in the dermal mesenchyme during early postnatal stages of cutaneous development (Figure 3.1.4 C,D). Immunoreactivity, however, is rather strong in some dendritic cells within the basal epidermal layer (Figure 3.1.4 B-G) and in some cells of the dermal and hypodermal connective tissue (Figure 3.1.4 F). These cells are likely to represent macrophages and pericytes that have already been shown to express Ang-2 (MAISONIERRE et al. 1997, OTANI et al. 1999).

3.2.1.4 Co-localization of Angiopoietin-1- and VEGF-immunoreactivity in the hair follicle epithelium suggests that they govern the development of the cutaneous vasculature

Summarizing the above described results of VEGF, Ang-1 and Ang-2–immunoreactivity, it is evident that immunoreactivity for VEGF and Ang-1 are
predominantly co-localized to the same tissue compartments and are temporally associated with one another. In prenatal skin, both growth factors are localized to the suprabasal epidermis. In perinatal skin, this is accompanied by strong immunoreactivity in the dermal connective tissue. Between 1 and 4 days after birth, however, immunoreactivity in the dermal compartment is lost. The major source of both growth factors in postnatal skin are the outer root sheath keratinocytes of hair follicles. Furthermore, blood vessels of the lower horizontal plexus are immunoreactive for both VEGF and Ang-1. In contrast, Ang-2-immunoreactivity is not detectable in any hair follicle compartment. Immunoreactivity is restricted to cells of the dermal and hypodermal mesenchyme, which likely represent macrophages and pericytes (Figure 3.1.5).
Figure 3.1.1: Development of the cutaneous vascular system in mice. PECAM-1–immunostaining of frozen sections from different stages of hair follicle development. Endothelial cells are labeled red. Counterstained with hematoxylin. d=dermis; hd=hypodermis; pc=panniculus carnosus (subcutaneous muscle layer); white arrow =upper horizontal vascular plexus; black arrow =lower horizontal vascular plexus. Borders between epidermis, dermis and hypodermis are marked by the dotted lines in E-H. Note the increase in hypodermal thickness during cutaneous development and the dispersion of the blood vessels throughout it. (A,B) E16.5; HF morphogenesis stage 2 (A) and stage 2-3 (B); (C) E18.5; HF morphogenesis stage 3; (D,E) day 1 p.p.; HF morphogenesis stage 6; (F) day 4 p.p.; HF morphogenesis stage 7; (G) day 8 p.p.; HF morphogenesis stage 8; (H) day 12 p.p.; mature anagen-like HFs. Original magnification: 250x (A-D); 100x (E-H).
Figure 3.1.2: VEGF–immunostaining of paraffin sections from different stages of hair follicle development. VEGF protein is labeled brown. Counterstained with methylen green. (A) positive control: mouse lung tissue. Note VEGF-immunoreactivity in bronchial (arrows) and alveolar (arrowhead) epithelium. (B) negative control: mouse skin, day 1 p.p.; compare to D. (C) mouse skin day 18.5 of embryonal development. HF morphogenesis stage 2; hp=hair plug; dp=dermal papilla. (D) day 1 p.p.; HF morphogenesis stage 5; ors=outer root sheath; d=dermis. Note immunoreactivity in the wall of large subcutaneous vessels (arrows). (E) day 6 p.p.; e=epidermis; pc=panniculus carnosus (subcutaneous muscle layer); the center shows a tylotrichic hair follicle; note the suprabasal pattern of VEGF–immunoreactivity in the interfollicular epidermis (e), the strong immunoreactivity in the outer root sheath of all hair follicles (ors), and the very faint immunoreactivity of the dermal papilla (dp).
Figure 3.1.3: Angiopoietin-1 immunostaining of paraffin sections from different stages of hair follicle development. Ang-1 protein is labeled red. Counterstained with hematoxylin. (A) day 18.5 of embryonal development; hair follicles in stage 1 (arrowhead) and stage 2 (arrow) of morphogenesis. d=dermis; dp=dermal papilla. (B) day 1 p.p.; HF morphogenesis stage 5; e=epidermis. Note strong immunoreactivity in the outer root sheath of hair follicles (arrow) and in the wall of subcutaneous vessels (arrowheads). (C) day 4 p.p.; note immunoreactivity of dendritic cells in the basal epidermis (arrowheads), possibly representing γδ TCR-positive intraepithelial dendritic lymphocytes, and in the dermal connective tissue, possibly representing macrophages and pericytes. (D) day 8 p.p.; pc=panniculus carnosus (subcutaneous muscle layer); note strong immunoreactivity in the outer root sheath (arrow). (E) day 12 p.p.; note strong immunoreactivity in the outer root sheath of hair follicles (arrow) and in many cells of the dermal and hypodermal mesenchyme. (F) day 12 p.p.; note immunoreactivity of pericytes (vessel marked by the arrow). (G) positive control: mouse ovary; strong immunoreactivity in the corpus luteum (cl), lack in immunoreactivity of the follicular granulosa cells (f).
Figure 3.1.4: Angiopoietin-2–immunostaining of paraffin sections from different stages of hair follicle development. Ang-2 protein is labeled brown (A-E) or red (F,G). Counterstained with methylen green or hematoxylin. (A) positive control: mouse ovary. Note Ang-2–immunoreactivity in corpus luteum (cl) and in blood vessels of theca interna, and lack of immunoreactivity in the granulosa cells of follicles (f). (B) mouse skin day 18.5 of embryonal development. HF morphogenesis stage 3; hp=hair plug; dp=dermal papilla; pc=panniculus carnosus (subcutaneous muscle layer). (C) day 1 p.p.; HF morphogenesis stage 5. Note very faint immunoreactivity in the suprabasal epidermis (e) and lack of immunoreactivity in the follicular outer root sheath. Ang-2–immunoreactivity is restricted to some cells within the dermis and hypodermis. (D,E) day 4 p.p.; note immunoreactivity of cells in the dermis and hypodermis, and within the basal epidermal layer (possibly representing γδ -TCR -positive intraepithelial dendritic lymphocytes, arrowheads). (F,G) day 8 p.p.; immunoreactivity is still present in basal epidermal cells (F, arrowheads); and in cells of the hypodermis with close proximity to hair follicles, possibly representing macrophages and pericytes (G, arrowheads).
Figure 3.1.5: Schematic representation of immunoreactivity patterns for VEGF, Angiopoietin-1 and Angiopoietin-2 during hair follicle morphogenesis. d=dermis; e=epidermis; hd=hypodermis; j=dermo-hypodermal junction; lhp=lower horizontal blood vessel plexus; ors=outer root sheath; pbv=perifollicular blood vessels; pc=panniculus carnosus (subcutaneous muscle layer); uhp=upper horizontal blood vessel plexus.
3.2 Remodeling of the cutaneous microvasculature in association with hair follicle cycling

3.2.1 There are strong evidences of vascular remodeling during the hair growth cycle

The above mentioned observations of blood vessel development in mouse skin demonstrate the existence of a distinct perivascular circulatory system in anagen mouse skin and suggest a close functional relationship between these perifollicular blood vessels and the hair follicles themselves. Since hair follicle keratinocytes possess the capability to generate key regulatory growth factors for vascular homeostasis (VEGF, Ang-1), it seems possible, that they modulate their blood supply in accordance to their metabolic demand. This would encompass an active growth of perifollicular vessels, i.e. angiogenesis, during synchronized anagen–development of hair follicles and a subsequent regression of vessels after catagen has been induced and once the demand of the hair follicles of an extensive blood supply is reduced.

In order to verify this hypothesis, we firstly aimed to investigate whether active processes of vascular remodeling, i.e. angiogenesis and vascular regression, are associated with hair follicle cycling.

3.2.1.1 The cutaneous microvasculature undergoes substantial morphological rearrangements throughout the murine hair cycle

Although hair cycle–associated morphological rearrangements of the cutaneous microvasculature have roughly been described decades ago in rats and rabbits (DURWARD and RUDALL 1949, DURWARD and RUDALL 1958) (Figure 1.6), it is not known, whether these differences also occur in mouse skin and whether they are merely based on passive changes in vessel perfusion or represent true vascular remodeling. Therefore, we have carefully investigated the morphology of dermal and hypodermal blood vessels throughout the murine hair growth cycle in H&E-stained sections from paraffin-embedded tissue, and in frozen sections, immunostained for
PECAM-1, an endothelial cell adhesion molecule. All sections were examined with regard to blood vessel and endothelial cell morphology.

A striking difference in the arrangement of cutaneous blood vessels in mouse back skin was evident when telogen and anagen VI skin were compared. Both revealed an intensive circulatory system within the hypodermis. In telogen skin, a dense capillary network was visible between the subcutaneous muscle layer (*panniculus carnosus*) and the proximal end of the hair follicles. The dermis, in contrast, revealed less blood vessels, which occasionally followed the hair follicles longitudinally. An upper horizontal plexus of blood vessels, that ran parallel to the epidermis, was visible at the isthmus levels of hair follicles (*Figure 3.2.1 A*). As opposed to telogen, anagen VI skin showed an intensive circulation around the hair follicle bulbs. A multitude of large vessels was detectable that ran longitudinal to the hair follicles. They gave rise to multiple connecting capillaries which surrounded the hair follicles (*Figure 3.2.1 B*).

Although the thickness of the skin varies tremendously between telogen and anagen VI skin, an equal or even higher density of blood vessels was evident in anagen skin compared to telogen skin. Furthermore, vessels in unmanipulated telogen mouse skin were much smaller in diameter and length, and more tortuous than vessels in anagen VI skin (*Figure 3.2.2 A-D*). In *telogen* skin, small crosscuts of vessels were detectable by PECAM-1–immunostaining in the hypodermal and dermal mesenchyme. Endothelial cell nuclei were of flat, elongated shape (*Figure 3.2.2 E*). In contrast, vessels in *mid-anagen* skin had a straight course, revealed many branches and often showed a distinct central lumen. Their nuclei were ovoid in shape and appeared to be larger than those in telogen skin (*Figure 3.2.2 G*). In *anagen VI* skin, many peribulbar vessels were easily recognized, exhibiting a wide lumen and endothelial cell nuclei with an elongated, flattened phenotype (*Figure 3.2.2 I*). During *early catagen* -development, peribulbar vessels were still easily recognizable (*Figure 3.2.2 K*), but were contracted and reduced (*Figure 3.2.2 H*). When the hair follicles had further regressed, the peribulbar vessels remained as small structures with substantially smaller lumens, and condensed or irregularly shaped endothelial cell nuclei (*Figure 3.2.2 L*). Some perifollicular blood vessels even exhibited partial occlusion by microemboli (*Figure 3.2.2 M*).
Toluidin blue-stained semi-thin sections from epon-embedded tissue were examined with regard to endothelial cell morphology in perifollicular vessels. Endothelial cells with chromatin separation, indicative of mitosis, were only found between day 3 and 12 after anagen-induction by depilation (p.d.), i.e. during anagen II to anagen VI (Figure 3.2.3). The number of mitotic endothelial cells (counted in 2-5 blocks, derived from 2-3 animals per time point) peaked on day 8 p.d. (no statistical analysis was performed due to the limited data). These cells were absent in telogen and catagen skin (data not shown).

In both catagen and telogen skin, endothelial cells with chromatin condensation and emargination, indicative of apoptosis, were found (Figure 3.2.4), while such endothelial cells were absent in early and late anagen VI skin (data not shown). Perifollicular blood vessels with endothelial cells, exhibiting these features of apoptosis, were found most prominently in the subcutis, next to the regressing hair bulb and less frequently in the upper dermis in close proximity to the follicular isthmus and the sebaceous gland. However, these endothelial cells were only detectable infrequently, and degenerative changes of endothelial cells, i.e. nuclear indentations/ infoldings, appeared to occur much more frequently than characteristic hallmarks of apoptosis (Figure 3.2.4 A). Furthermore, endothelial cell detachment from the vessel wall was observed in several cutaneous and subcutaneous perifollicular vessels (Figure 3.2.4 B,D). Detached cells were found floating in the vessel lumen of both catagen and telogen skin (Figure 3.2.4 D).

### 3.2.1.2 Diameter and length of subcutaneous vessels are larger in anagen compared to telogen skin

To verify the occurrence of morphological differences in the cutaneous vasculature between telogen and anagen skin, we measured the diameters and lengths of cutaneous blood vessels in the hypodermis of anagen and telogen skin. Vessels had a significantly larger median diameter (p < 0.001) and measurable length (p < 0.001) in anagen, compared to telogen skin (Figure 3.2.5 A,B).
3.2.1.3 The microvessel density in murine skin increases during synchronized anagen–development and decreases after all hair follicles have entered telogen again

To further evaluate changes in the cutaneous vascular pattern, the microvessel density (MVD), i.e. the number of microvessels per microscopic field (VARTANIAN and WEIDNER 1994, HUDLICKÁ et al. 1998), was assessed during the entire depilation-induced murine hair growth cycle. PECAM-1-immunoreactive cell aggregates that showed distinctive formation of a central lumen or that formed longitudinal, occasionally branching structures with the obvious appearance of blood vessels were counted. Analysis of variance clearly indicated a significant (p<0.001) difference between the means of all groups. The Ryan-Einot-Gabriel-Welsch range (REGWQ-test) identified three different ranges of mean values (Figure 3.2.6). Telogen skin of day 0 and day 34 after depilation and anagen III skin were within the same range of MVD. Anagen –stages IV to VI and catagen stage VI represented another range of MVD. The microvessel density of telogen skin 25 days after depilation represented a separate range, statistically different from the others. Scheffé-test for multiple comparisons of means revealed a highly significant increase in MVD between anagen III and anagen IV (p<0.001) and a highly significant decline of MVD between day 25 and day 34 p.d. (p<0.001) (Figure 3.2.6). The constant and statistically significant increase in MVD between telogen skin (day 0 p.d.) and anagen VI skin (d 12 p.d.) suggested anagen–associated vascular growth. However, MVD did not decline during catagen–development of the hair follicles and was actually highest on day 25 after depilation, which might be related to the fact that the whole dermal and hypodermal compartment decrease in thickness (CHASE et al. 1853, HANSEN et al. 1984) and thus might actually increase the density of microvessels. Thereafter, it significantly decreased while the hair follicles remained in the telogen stage of the hair growth cycle, suggesting that new blood vessels that had been built during anagen, had regressed again (Figure 3.2.6).
Figure 3.2.1: Arrangement of the vascular system in telogen and anagen VI mouse skin. Blood vessels were stained red by immunofluorescent detection of PECAM-1 in 100µm-thick frozen sections of back skin. The cutaneous architecture was demonstrated by DAPI-staining of all cell nuclei (grey-blue). Both images were superimposed. (A) telogen skin. (B) anagen VI skin. A lower (1) and an upper (2) horizontal plexus of blood vessels is evident in both telogen and anagen VI skin. Hair follicles are indicated by asterisks. e = epidermis; d = dermis; hd = hypodermis; pc = panniculus carnosus (subcutaneous muscle layer). The arrows mark small capillaries that cross-connect larger ascending blood vessels in anagen skin and surround hair follicles. Note the tremendous change in skin thickness which is evident by comparing (A) and (B), that are of the same magnification. Original magnification: 100x.
Figure 3.2.2 (page 73): Hair cycle-dependent rearrangements of the cutaneous microvasculature. (A – D) Histology of mouse back skin, immunohistochemically stained for CD31, an endothelial cell–specific marker, and schematic drawing of the CD31–immunoreactive cell aggregates. Note the differences in the arrangement of CD31–immunoreactive cutaneous vessels between telogen skin (A, B) and anagen VI skin (C, D). E = epidermis; pc = panniculus carnosus (subcutaneous muscle layer). Scale bars represent 200 µm. (E – H) Histology of mouse back skin, immunohistochemically stained for CD31. There are hair cycle–dependent changes in the diameter, length and arrangement of cutaneous vessels and in the morphology of endothelial cell nuclei. In telogen skin (E) cutaneous vessels are small in diameter and length. Capillaries show flattened endothelial cell nuclei (insert). Dermal vessels exhibit larger diameters and lengths in anagen IV skin (F). They show maximal diameters and lengths in anagen VI skin, where vessels exhibit many branches (G) and endothelial cell nuclei have an oval shape (insert). During hair follicle regression (catagen, H) cutaneous vessels retain their elongated course, but become condensed as the surrounding subcutaneous tissue diminishes in size. These hair cycle–dependent changes in the cutaneous mesenchyme become very obvious by comparing (E) to (H), which are of the same magnification. Scale bars represent 80 µm. (J – M) Haematoxylin & eosin–stained paraffin sections of mouse back skin in different phases of the hair growth cycle. Anagen VI skin (J) exhibits straight peribulbar vessels with considerable lumen and flattened, elongated endothelial cells (arrows). DP = dermal papilla. In early catagen skin (K) peribulbar vessels are still easily recognizable but exhibit a less flattened, elongated endothelial cell nuclear morphology (arrows). ES = epithelial strand. In advanced catagen skin (L) the proximal portion of the hair follicle has moved upwards and tortuous and narrow vessels remain in the subcutaneous mesenchyme (insert). They exhibit reduced diameters and condensed endothelial cell nuclei (arrows). In telogen skin (M) peribulbar vessels show small diameters, are short and tortuous. Endothelial cell nuclei are mostly elongated and flattened. Some vessels exhibit endothelial cell nuclei condensation (arrowhead) and obstruction by cell emboli (arrow). Scale bars represent 17 µm (J – L) and 8 µm (M).
Figure 3.2.3: Mitotic endothelial cells in anagen skin. Toluidin blue-stained semithin section of mouse back skin in early anagen. Mitotic endothelial cell with separated chromatin (black arrow, insert). Compare with the non-mitotic endothelial cells (white arrows). ORS = follicular outer root sheath. F = fat. C= collagen fibrills.
Figure 3.2.4: Endothelial cell degeneration in catagen skin. Toluidine blue–stained semithin section of mouse back skin during catagen-development. (A) subcutaneous arteriole with apoptotic (arrows) and degenerating endothelial cells (EC). Note the condensation of the apoptotic nuclei and infoldings of the nuclear envelope of the degenerating cells. PC = pericyte; catagen skin (d 19 p.d.). (B) degenerating/detaching EC (arrow) in a dermal venule. EC = morphologically unaltered EC. WBC = white blood cell; early catagen (d 17 p.d.). (C) apoptotic EC (arrow) in a perifollicular vessel; EC = morphologically unaltered EC; HS = hair shaft; catagen (d 20 p.d.). (D) degenerating EC (open arrow) in a subcutaneous vessel. Note the floating degenerating cell (arrow) in the lumen of the vessel; EC = morphologically unaltered EC; telogen (d 25 p.d.).
Figure 3.2.5: Differences in the mean vessel diameter (A) and the mean vessel length (B) between telogen and anagen VI mouse skin. Each parameter was assessed in 150 hypodermal vessels in three different animals per group; shown are mean values ± 95% confidence interval; *** = p < 0.001; two-sided student’s t-test for unpaired samples.
Figure 3.2.6: Cutaneous microvessel density (MVD) varies according to the hair growth cycle. MVD was assessed at selected time points of the depilation-induced murine hair cycle by immunohistochemical staining of CD31. The evident, although insignificant increase in MVD between catagen VI (d 12) and telogen (d 25) is probably due to quantitative changes of the dermal and hypodermal tissue; for each point of time, microvessels were counted in 60 microscopic fields in three different mice; shown are means ± 95% confidence interval; n.s. = no statistically significant difference; *** = p<0.001. Analysis of variance and Scheffé-test. The red lines indicate the three ranges identified by the REGWQ-test.
3.2.2 Evidences of angiogenesis occur during anagen-development of hair follicles

3.2.2.1 Anagen skin encompasses more endothelial cell nuclei than telogen skin

The changes in vessel morphology during anagen–development and the increase in microvessel density during this hair cycle phase suggest that growth of new capillaries from pre-existing ones, i.e. angiogenesis, is associated with anagen. To verify this hypothesis, the number of endothelial cell nuclei was counted in a reference area, so as to explore whether it is indeed higher in anagen compared to telogen skin, as would be expected if vascular growth was occurring. The interfollicular region was chosen as reference area, because the space between two hair follicles remains relatively unaltered during the entire hair cycle. Indeed, a significantly lower (p=0.009) number of endothelial cell nuclei could be detected in telogen skin (d 0 p.d.) compared to anagen VI skin (d 12 p.d.) (Figure 3.2.7).

3.2.2.2 Large numbers of Ki-67 antigen–immunoreactive (proliferating) endothelial cells can be detected only during anagen

To further confirm that growth of microvessels occurs during synchronized anagen–development, as is suggested by an increase in the endothelial cell number, frozen sections of mouse skin were double-stained for PECAM-1 and the Ki-67 antigen, a nuclear antigen that is tightly associated with cell proliferation (GERDES et al. 1983, GERDES et al. 1997, ENDL and GERDES 2000). Double-immunoreactive cells were referred to as proliferating endothelial cells (PEC) and were counted per microscopic field. PECs could be detected only very rarely in telogen and early anagen skin, but were found very frequently in anagen IV to anagen VI skin (Figure 3.2.8 A-D). Analysis of variance and REGWQ-test revealed a significant (p<0.001) difference in the mean number of PECs between the investigated time points of the depilation–induced hair cycle. Scheffé-test showed a significant increase in the number of PECs per reference area between anagen III and anagen IV (p=0.029) and anagen IV and
anagen V (p<0.001), and a significant decline between anagen V and anagen VI (p<0.001) and anagen VI and catagen II (p<0.01). This suggests that cessation of endothelial cell-proliferation is an early marker of hair follicle regression. PEC values of telogen, anagen III, catagen II and catagen VI were within the same range as identified by the REGWQ-test and not statistically different from one another as indicated by Scheffé-test (Figure 3.2.8 E). PECs were predominantly observed in the perifollicular vasculature, associated with branching cutaneous vessels (Figure 3.2.8 C,D). Rarely PECs were detectable in larger vessels of the lower and of the upper horizontal plexus (Figure 3.2.8 B).

3.2.2.3 Proliferating endothelial cells are detectable only in anagen skin by transmission electron microscopy

To verify the occurrence of mitotic endothelial cells during anagen–development, transmission electron microscopy was performed. Small capillary-type vessels of the hypodermis with close proximity to the proximal portion of the hair follicle were chosen for analysis. Many of these vessels exhibited activated endothelial cells, highly suggestive of endothelial cell mitosis. These morphological features were best demonstrated in mid-anagen skin (d 8 p.d.) (Figure 3.2.9).

3.2.2.4 The angiogenesis-inhibitor TNP-470 retards anagen-development

After it had been demonstrated that synchronized anagen–development of hair follicles in murine skin is indeed associated with angiogenesis of perifollicular microvessels, we aimed to investigate its functional relevance for hair growth. C57BL/6-mice were depilated to induce anagen and were treated intraperitoneally with TNP-470, a synthetic fumagillin-derivative, used as a reference inhibitor of angiogenesis (CASTRONOVO and BELOTTI 1996). A slight difference in the color of back skin between the TNP-470 -treated mice and controls, which is indicative of profound differences in the speed of anagen development (PAUS et al. 1990, PAUS et al. 1994a) could already be observed at day 6 of treatment. While the skin of
control mice was slightly grey in the caudal parts of the back, reflecting massive anagen-associated follicular melanization (SLOMINSKI and PAUS 1993), TNP-470 -treated animals retained a pink skin colour. This difference in skin colour became even more obvious at day 8 p.d., when the skin of TNP-470 -treated animals was slightly grey, reflecting the onset of follicular melanogenesis, while the skin of control mice was almost completely black, indicating the presence of far-advanced stages of anagen development (Figure 3.2.10).

These macroscopic observations demonstrated a significant retardation in anagen-development by TNP-470, which was confirmed histologically and by quantitative histomorphometry. 6 days after anagen induction by depilation, control mice exclusively showed hair follicles in anagen III (mainly anagen IIIc), whereas mice treated with TNP-470 had follicles in different stages of early anagen development, i.e. anagen I to anagen III (Figure 3.2.11) (for a detailed description of the stages of hair follicle cycling in mice see MUELLER-ROEVER et al. 2001).

Statistical analysis was performed with the Wilcoxon test for two independent samples. A significant difference of the percentage of hair follicles in stages anagen II and anagen III (p=0.019) was evident between both groups, and thus suggested retardation in the anagen development after TNP-470 administration (Figure 3.2.12 A).

Analysis of the microvessel density in frozen skin samples demonstrated that treatment with TNP-470 had indeed influenced the cutaneous vascular system: a significantly (p=0.021) lower microvessel density was evident in the skin of TNP-470–treated animals, compared to controls (Figure 3.2.11).

Animals that had been treated with TNP-470 for 8 days still exhibited hair follicles in anagen stages I and II, and the most advanced anagen stage of hair follicles was stage V. In contrast, control mice had hair follicles in stages III to VI of anagen -development. Again, statistically significant differences in the percentage of hair follicles in anagen III (p=0.034) and anagen V (p=0.032) could be demonstrated between both groups (Figure 3.2.12 B).
Figure 3.2.7: The number of endothelial cell nuclei per interfollicular area is lower in telogen than in anagen VI skin. The number of endothelial cell nuclei was assessed in 60 interfollicular areas in three individual animals per group; shown are mean values ± 95% confidence interval; ** = p<0.01. two-sided student’s t-test for unpaired samples.
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Figure 3.2.8: Proliferating endothelial cells occur in the cutaneous vasculature during anagen–development only. Double-immunofluorescent staining for CD31 (green) and the Ki-67–antigen (red); note the lack of Ki-67–immunoreactive follicular keratinocytes in telogen skin (A). Double-immunofluorescent cells exhibit a red intranuclear staining and a green cytoplasmatic staining and were interpreted as proliferating endothelial cells (PEC). No PECs could be detected in telogen or very early anagen skin (A). In anagen IV skin (day 5 p.d.), some PECs become detectable, mostly in small ascending vessels, very rarely in vessels of the lower horizontal plexus (B, arrow). Many PECs are detectable in anagen V skin (day 8 p.d.) (C) and anagen VI skin (day 12 p.d.) (D). PECs are mainly detected in longitudinal, branching perifollicular vessels (C, D, arrowheads). Scale bars = 80µm (A-C), and 50 µm (D). (E) The number of PECs per microscopic field changes during the murine hair growth cycle. PECs were counted in 75 microscopic fields in three different animals per hair cycle stage, using 400x magnification. Mean ± 95% confidence interval. *p<0.05; ***p<0.001; n.s.=no statistically significant difference. Analysis of variance and Scheffé-test. The red lines indicate different ranges identified by the REGWQ-test.
Figure 3.2.9: Mitotic endothelial cell in anagen skin. Transmission electron microscopy of an activated endothelial cell in anagen skin (day 8 p.d.), highly suggestive for endothelial cell mitosis. C = collagen fibrils; EC = endothelial cell; L = capillary lumen; MEC = mitotic endothelial cell; RBC = red blood cell.
Figure 3.2.10: Inhibition of angiogenesis retards depilation–induced anagen–development. C57BL/6 mice after treatment with the fumagillin-derivative TNP-470 (6 mg/kg/d) for 8 days (A) compared to vehicle–treated controls (B). All mice were depilated and treated daily intraperitoneally. The difference in anagen–development can be assessed macroscopically by the skin color: during the depilation–induced hair cycle of the C57BL/6 mouse, the skin color changes from pale (telogen) via slight grey (early anagen) to dark grey-black (anagen VI). Whereas controls show a dark grey skin color (B), TNP-470–treated animals retain the pale to slightly grey skin color of early anagen (A).
Figure 3.2.11: Histology of anagen–retardation by angiogenesis–inhibition. Shown is the skin of mice, either treated with the vehicle alone (A) or with TNP-470 (B). 6 days after depilation. Whereas control animals predominantly reveal hair follicles in anagen IIIc, TNP-470–treated animals show hair follicles of earlier anagen –stages, i.e. anagen II. Note the difference in skin thickness that is associated with the different anagen –stages. (C) TNP-470–treated mice exhibit a significantly lower cutaneous microvessel density. Mean ± 95% confidence interval. Two-sided student’s t-test for unpaired samples.
Figure 3.2.12: Histomorphometry of anagen–retardation by angiogenesis–inhibition. Hair follicles (%) in different stages of anagen–development in back skin of mice, either treated with TNP-470 (6 mg/kg/d) or with the vehicle alone (control). (A) Analysis after 6 days of treatment. While 100% of hair follicles in back skin of control mice are in anagen III, TNP-470–treated animals still reveal a significant percentage of hair follicles in earlier anagen stages I and II; seven mice were investigated in each group and the hair cycle stage of at least 60 hair follicles was assessed per mouse; mean ± SEM; * = p<0.05. Wilcoxon test. (B) Analysis after 8 days of treatment; three mice were investigated in each group and the hair cycle stage of at least 60 hair follicles per mouse was assessed; mean ± SEM; * = p<0.05. Wilcoxon test.
3.2.3 Evidences of vascular regression occur throughout the anagen to telogen–transformation of hair follicles

3.2.3.1 The number of endothelial cells decreases before the decline of microvessel density during catagen to telogen transformation of hair follicles

Since our assessment of the microvessel density during the murine hair growth cycle had revealed a slight but significant increase between anagen VI and telogen skin (Figure 3.2.6), i.e. when the hair follicles undergo involution, we speculated that this increase might in fact be related to the hair cycle-dependent decline in skin thickness (CHASE et al. 1953, HANSEN et al. 1984). Therefore, we thoroughly investigated the microvessel density and the number of endothelial cells during the anagen to catagen to telogen transformation again. This time, we modified the technique how to assess the microvessel density, in order to take into account the changes in the thickness of various skin compartments. Furthermore, we counted all PECAM-1–immunoreactive structures that clearly were no monocytic cells.

A statistically significant difference (p<0.001) between the means of all groups was found by analysis of variance and the REGWQ-test. Again, a slight, although statistically not significant, increase in the MVD was obvious between anagen VI and catagen VI skin. This time, however, no increase in MVD could be demonstrated between catagen VI and telogen skin. Indeed, Scheffé-test showed a significant decline (p=0.028) of the MVD between catagen VI and telogen skin (d 25 p.d.). This significant decline continued between 25 and 34 days after depilation (p<0.001) (Figure 3.2.12). It is important to note that these vascular changes occur during a time period when all hair follicles remain morphologically unaltered in telogen and when the skin thickness also remains constant.

We also studied the number of endothelial cells within a reference area again. Significant differences were detected between the means of all groups using analysis of variance and a subsequent Scheffé-test and the REGWQ range. The number of endothelial cell nuclei, reflecting the number of endothelial cells in the interfollicular dermis and hypodermis, declined significantly (p<0.001) between anagen VI and
telogen skin, confirming our previous data (3.2.2.1). Precisely, a significant decline was evident between catagen II and catagen VII (p=0.01) and between catagen VII and telogen skin (p<0.001). The number of endothelial cell nuclei per reference area reached its minimum already on day 25 p.d., i.e. when all hair follicles in murine back skin have just traversed from catagen into telogen. Thereafter, the number of endothelial cell nuclei was constant for the remainder of telogen (Figure 3.2.12).

Thus the number of endothelial cell nuclei declined earlier than the density of cutaneous microvessels, suggesting loss of endothelial cells as a major mechanism in catagen –associated vascular regression.

3.2.3.2 Nucleosomal fragmentation in endothelial cells is detectable during catagen to telogen transformation of hair follicles

In order to verify the role that endothelial cell apoptosis plays in the reduction of the number of endothelial cells, an immunofluorescence double-staining technique for apoptosis and endothelial cells was performed. Double-immunofluorescent cells were indeed detectable in catagen skin. Interestingly, several of these double-immunofluorescent cells occurred in clusters rather than as isolated cells (Figure 3.2.13). No double-immunofluorescent cells were found in anagen skin (data not shown). However, the number of double-immunofluorescent cells in catagen skin was too low and variable among individual mice that it could not be quantitated reliably. This strongly suggests that other mechanisms beyond endothelial cell apoptosis account for the substantial decline in endothelial cell number and microvessel density that is associated with the spontaneous, synchronized catagen to telogen transformation of murine pelage hair follicles.

3.2.3.3 Apoptotic and degenerating endothelial cells are detectable during catagagen to telogen transformation of hair follicles

Transmission electron microscopy (TEM) was performed to investigate the cellular events that occur when endothelial cells and finally cutaneous microvessels
decrease in number, i.e. during the catagen to telogen transformation. TEM revealed the presence of both apoptotic and degenerative changes in endothelial cells during the catagen to telogen transformation. Using the skin microvasculature-classification scheme of BRAVERMAN and KEH-YEN (1981), endothelial cells in several types of vessels were affected. These included endothelial cells in collapsed extra-papillary, ascending (i.e. arterial type) capillary loops (approx. 6 µm across, Figure 3.2.14 G), extra-papillary descending (i.e. venous type) capillary loops (approx 10 µm across, Figure 3.2.14 B), post-capillary venules (approx. 25 µm across, Figure 3.2.14 D), and arterioles (up to 75 µm across, Figure 3.2.14 F). Endothelial cells were observed to detach from their associated basement membrane and either exhibited classic features of apoptosis, i.e. condensation of nuclear chromatin and cytoplasm, fragmentation of the nucleus, and budding of the whole cell (WYLLIE et al. 1980), or degenerative changes morphologically distinct from apoptosis (WALKER et al. 1988). Apoptotic endothelial cells exhibited typical nuclear chromatin condensation/emargination (Figure 3.2.14 A) and were occasionally observed to shed into the vessel lumen (Figure 3.2.14 F). Degenerating endothelial cells in catagen and telogen skin exhibited intranuclear pockets, pinching of the nuclear envelope, blebbing of the cell membrane and cytoplasmic swelling (Figure 3.2.14 B). Deletion of endothelial cells appeared to occur by both apoptosis and degenerative change alongside each other in the same vessel (Figure 3.2.14 F). In addition, isolated pyknotic cells were observed in regions close to the proximal hair follicle that may have been previously vascularized, as they were sometimes associated with basement membrane material (data not shown). Apoptotic or degenerating endothelial cells detached and appeared to be shed into the vessel lumen, occasionally associated with subsequent vessel constriction and collapse (Figure 3.2.14 C - G).
Figure 3.2.12: Decline in microvessel density (dotted line, left y-axis) and number of endothelial cell nuclei (continuous line, right y-axis) during the anagen VI to telogen transformation of hair follicles. The x-axis shows different time points of the hair growth cycle. The corresponding hair cycle stage is depicted in the schematic drawing below. Shown are mean values ± 95% confidence interval. *=p<0.05; **=p<0.001; n.s.=no statistically significant difference. Analysis of variance and Scheffé-test.
**Figure 3.2.13:** Apoptotic endothelial cells in catagen skin. Double immunofluorescence for CD31 (red) and apoptosis (green); counterstained with DAPI (blue). Clusters of apoptotic endothelial cells (arrows) in a dermal vessel close to an obliquely cut hair follicle (HF) and its sebaceous gland (SG). The insert shows a phase contrast image of this section. Telogen skin (d 25 p.d.). Original magnification: 250x.

**Figure 3.2.14 (page 93):** Apoptotic and degenerating endothelial cells in catagen skin.
Transmission electron microscopy of murine back skin. **(A)** Capillary endothelial cell (EC) with typical nuclear condensation, suggestive of apoptosis. Despite this nuclear change, the cell is not yet rounded up; early catagen (d 17 p.d.). L = vascular lumen. **(B)** Degenerating extra-papillary descending capillary in the deep dermis; note the significant cytoplasmic blebbing (black arrows), pinch–like indentation/ infoldings of the nuclear envelope (white arrows) and increased nuclear heterochromatism; advanced catagen (d 19 p.d.). **(C)** Detachment of degenerating endothelial cells (EC) from their basal lamina. Note that part of this venule is devoid of endothelial cell coverage (arrow); late catagen (d 20 p.d.). **(D)** Detachment of endothelial cell (EC) from the basal lamina in a post-capillary venule; note that this endothelial cell retraction has left wide spaces (arrows) on both sides of the vessel; the affected endothelial cells also exhibit cytoplasmic bebbing; a cell of unknown origin, possibly a monocyte (M) is present within the lumen; late catagen (d 20 p.d.). **(E)** Vascular occlusion by an embolus of unknown origin, possible endothelial cell (*); note the advanced state of degeneration of that cell occluding this capillary; advanced catagen (d 19 p.d.). **(F)** Collapse and occlusion of a dermal arteriole (encircled by the dotted line); note the presence of an apoptotic cell (arrowhead) and fragments of a degenerated cell of unknown origin (arrow); little luminal space remains in this vessel; telogen (d 25 p.d.). **(G)** Total collapse of a small capillary in the subcutis; a degenerating endothelial cell completely fills the capillary space; note the advanced nuclear change indicated by heterochromatin condensation and infoldings/ indentations of the nuclear profile; note also the disorganized collagen layer (arrows); catagen (d 18 p.d.).
3.3 Hair follicles express vascular growth factors in dependence of their state of activity

The investigations mentioned above had demonstrated a temporally restricted vascular growth and regression during the hair growth cycle in mice. These events are associated with proliferation and apoptosis/ degeneration of endothelial cells and they are strictly correlated with the hair follicle activity. In accordance to our observations during hair follicle morphogenesis, namely that the hair follicle epithelium is a major source of vascular–specific growth factors, we speculated that hair follicles can modulate the perifollicular vasculature by synthesizing key regulators of vascular homeostasis.

To further clarify this issue, the expression patterns of the three major regulators of blood vessel growth and regression, i.e. VEGF, Angiopoietin-1 and Angiopoietin-2, and of their receptors were analysed on protein and mRNA–levels during the depilation –induced hair growth cycle in mouse skin.

3.3.1 Hair cycle –dependent expression of vascular endothelial growth factor (VEGF)

3.3.1.1 VEGF immunoreactivity is predominantly localized to the follicular epithelium throughout the hair growth cycle

Firstly, we examined the protein expression of VEGF by immunohistochemistry. The antibody employed was one, raised against a peptide mapping to the amino-terminus of the mouse VEGF protein. Therefore, it recognized several isoforms of the murine VEGF protein (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Strong cytoplasmic immunoreactivity was detectable in all layers of the interfollicular epidermis and in few cells within the dermal mesenchyme throughout the entire hair growth cycle (Figure 3.3.1). Telogen: During telogen, VEGF immunoreactivity was strong in the follicle epithelium along the hair canal, in the entire outer root sheath and in the secondary hair germ cell cluster located directly above the follicular dermal papilla. Dermal papilla fibroblasts in murine telogen follicles were not VEGF-immunoreactive
(Figure 3.3.1 A). *Early anagen:* During early anagen development, the extending outer root sheath and the dermal papilla became immunoreactive, whereas the hair matrix keratinocytes and the inner root sheath of the new anagen hair follicle were non-immunoreactive (Figure 3.3.1 B-D). *Full anagen:* In mature anagen VI hair follicles, the outer root sheath stained moderately for VEGF, the dermal papilla and the infundibular epithelium stained very weakly, and the matrix keratinocytes as well as the inner root sheath were negative (Figure 3.3.1 E). *Catagen:* During catagen development, VEGF-immunoreactivity remained in the regressing epithelial strand, while the dermal papilla fibroblasts lost VEGF immunoreactivity (Figure 3.3.1 F). *Telogen:* Once hair follicles had spontaneously reached telogen, they showed a pattern of VEGF immunoreactivity comparable to pre-depilation telogen hair follicles (Figure 3.3.1 G). Many dermal and subcutaneous arteries, arterioles and capillaries exhibited VEGF immunoreactivity throughout the entire hair growth cycle (Figure 3.3.1 H,I).

This immunohistochemical analysis suggests that epithelial cells of the outer root sheath are a major source of VEGF for endothelial cells in the reticular dermis and the subcutis above the panniculus carnosus. Moreover, it suggests that part of the hair follicle epithelium is a constant source of VEGF throughout the entire hair cycle, while the dermal papilla appears to provide VEGF only during early and mid anagen. However, the immunohistochemistry data did not permit any distinction between various VEGF-isoforms, that have been shown to differ in their biological activity (ROBINSON and STRINGER 2001). Furthermore, immunohistochemistry left many unresolved questions relating to the exact timing and localization of VEGF synthesis during the hair cycle, since it might be detected long after its production, or in localizations where it is sequestered but not produced.

3.3.1.2 Cutaneous expression of VEGF mRNA-splice variants is hair cycle-dependent

Alternative splicing of mRNAs is an important component of cell–specific gene regulation that contributes to the fine-tuning of gene expression and the functional
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diversification of gene products (LOPEZ 1998). It is well known that the VEGF mRNA is alternatively spliced in a variety of human and animal tissues (SHIMA et al. 1996, ROBINSON and STRINGER 2001). However, there is no data clarifying, which splice variants of VEGF mRNA are expressed in mouse skin, and whether their pattern of expression varies according to the hair growth cycle. As a first step towards clarifying the above functionally important issues, RT-PCR analysis of VEGF mRNA in mouse back skin was performed throughout the depilation-induced hair growth cycle. For this, oligonucleotides matching at exons 1 and 8 of the VEGF gene were used to amplify all variants of VEGF-cDNA (Figure 2.4). Lung tissue, known to express VEGF mRNA on a high level, was used as a positive control. In line with previously published results in rats and mice (GORDEN et al. 1997, BURCHARDT et al. 1999), we were able to demonstrate four splice variants of VEGF mRNA in normal lung tissue, encoding for the four isoforms VEGF$^{188}$, VEGF$^{164}$, VEGF$^{144}$ and VEGF$^{120}$ (Figure 3.3.2). Since our results perfectly matched previously published observations and since bands of the expected sizes were detected, the PCR products were not sequenced. In normal lung tissue, splice variants for the long isoforms VEGF$^{188}$ and VEGF$^{164}$ are most prominent, whereas splice variants for the shorter isoforms VEGF$^{144}$ and VEGF$^{120}$ are expressed on a much lower level (BURCHARDT et al. 1999).

All splice variants of VEGF mRNAs could be detected in adult mouse skin (Figure 3.3.2). In contrast to lung tissue, however, the splice variant for the VEGF$^{188}$ isoform was hardly detectable in murine back skin, even with a high number of amplification cycles. Therefore, it was presumed that this variant does not play an important part in regulating vascular homeostasis in mouse skin and was not further analysed during the hair growth cycle. Instead, the most prominently expressed splice variant in mouse skin was the one encoding for VEGF$^{164}$. Splice variants encoding for VEGF$^{144}$ and VEGF$^{120}$ were only weakly expressed in telogen skin (Figure 3.3.2).

In order to compare the expression levels of different VEGF mRNA-splice variants, firstly a semiquantitative analysis was performed by digital image analysis–based densitometry. For comparison of steady-state mRNA levels between different hair cycle stages, VEGF mRNA levels were normalized according to the mRNA levels of
β-actin, an ubiquitous cytoskeleton protein (BUSTIN 2001). Although there is widespread evidence that its levels of transcription can vary widely between individuals and different experimental settings, β-actin still is widely used as an internal standard to exclude interindividual variances of template concentration (BUSTIN 2001). We compared the mRNA levels of β-actin and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) in our samples and did not detect any significant differences throughout the hair growth cycle, thus presuming that β-actin was a suitable housekeeping gene for semiquantitative RT-PCR analysis of mouse skin (data not shown).

In telogen skin, the most prominently expressed splice variant was the one encoding for VEGF\textsubscript{164}. Those encoding for VEGF\textsubscript{144} and VEGF\textsubscript{120} were much weaker expressed. After anagen induction, an increase in the steady-state level of the VEGF\textsubscript{120}–mRNA variant was detectable. However, throughout anagen–development, the level of VEGF\textsubscript{120} mRNA appeared to be lower than those of the VEGF isoforms VEGF\textsubscript{144} and VEGF\textsubscript{164} (Figure 3.3.2). After the onset of spontaneous catagen development (d 17 p.d.), mRNA expression of all VEGF isoforms declined markedly (Figure 3.3.2). The band, representative for the VEGF\textsubscript{120}–mRNA splice variant completely disappeared in early catagen (d 17 p.d.) samples already, while those representing VEGF\textsubscript{164} and VEGF\textsubscript{144} reduced in intensity in late catagen skin (d 19 p.d.) only (Figure 3.3.2). Interestingly, when hair follicles had spontaneously reached the telogen stage of the hair cycle (d 25 p.d.), the expression of all VEGF mRNA splice variants almost equaled that of telogen samples before depilation (Figure 3.3.2).

3.3.1.3 Quantitative real-time RT-PCR reveals marked changes in the hair cycle-dependent mRNA -levels of VEGF\textsubscript{164}, VEGF\textsubscript{120}, VEGFR-1 and VEGFR-2

The semiquantitative RT-PCR analysis suggested that cutaneous VEGF gene expression is strictly regulated qualitatively and quantitatively in a temporal manner. Therefore, quantification of gene transcription, i.e. steady-state mRNA levels,
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appeared to be crucial in order to dissect the function of various VEGF isoforms for regulating vascular homeostasis in mouse skin. Since conventional RT-PCR is at best semiquantitative and thus might not be accurate enough to detect important differences in mRNA levels, mRNA steady-state levels of two VEGF–isoforms (VEGF$_{164}$ and VEGF$_{120}$) were quantified by real-time PCR based on the TaqMan technique. Simultaneously, mRNA levels of both VEGF receptors were investigated throughout the hair growth cycle.

Total RNA was extracted from back skin of three mice in unmanipulated telogen skin (d 0 p.d.), anagen IV skin (d 5 p.d.), anagen VI skin (d 12 p.d.) and catagen VII skin (d 19 p.d.), and the same amount of total RNA (5 µg) was used for reverse transcription in all samples. Threshold cycle (C$_{t}$) –values were measured and converted into target copy numbers per µg RNA, after generating a standard curve (BUSTIN 2001). All samples were measured in triplicates and showed high reproducibility.

RT-PCR–specific errors in the quantification of mRNA transcripts are easily compounded by any variation in the amount of starting material between samples, especially when deriving from different animals (BUSTIN 2001). The accepted method for minimising these errors and correcting sample-to-sample variation is to amplify a cellular RNA that serves as an internal reference against which the target mRNA values can be normalized (KARGE et al. 1998). However, there is currently no ideal internal standard that clearly is unaffected by the experimental setting (BUSTIN 2001). We investigated mRNA levels of β-actin and porphobilinogen deaminase (PBDG), two frequently and widely employed housekeeping genes in all our samples, in order to verify their function as housekeeping genes for normalization (BUSTIN 2001, MAX et al. 2001, KIELAR et al. 2001, HASSELMANN et al. 2001). Surprisingly, we detected a statistically significant variation of these mRNA levels between different hair cycle stages, while the variation between individuals within one group, i.e. one time point of analysis, was not significant. Precisely, a statistically significant increase in β-actin mRNA steady-state levels could be detected between telogen and anagen IV and between anagen IV and anagen VI skin. There was no statistically significant difference between anagen VI and catagen VII skin, but a significant
difference could be observed between catagen VII and unmanipulated telogen skin (Figure 3.3.3). Steady-state levels of porphobilinogen deaminase (PBDG) mRNA revealed similar changes between the investigated hair cycle stages (data not shown). This observation clearly suggests a hair cycle–dependent regulation of β-actin and PBDG gene transcription, and thus would exclude them as suitable internal standards for the quantification of specific mRNA.

Since hair cycle–dependent variations in β-actin and PBDG–mRNA expression have not been reported so far, we wanted to verify whether β-actin-normalization of target RNA actually effects the results of quantitative RT-PCR analysis, and thus their interpretation. Therefore, we measured steady-state mRNA levels of tyrosin kinase receptor C (TrkC), a high affinity receptor for neurotrophin-3, in our samples. The hair cycle-dependent expression of TrkC had previously been demonstrated in our laboratory by semiquantitative RT-PCR analysis and had been correlated to protein expression and functional data. These analyses had shown that TrkC mRNA levels are rising during anagen–development and decline during catagen–development (BOTCHKAREV et al. 1998). Our analysis of steady-state levels of TrkC mRNA by TaqMan RT-PCR confirmed a statistically significant increase between telogen and anagen VI skin (p=0.004), when measuring copy numbers related to total RNA concentration (Figure 3.3.4). However, when the value of each sample was normalized to its β-actin mRNA level that - as mentioned above - clearly varied in a hair cycle–dependent manner, significant changes in the mRNA levels occurred, that now did neither correspond to the previously published data of mRNA expression, nor to protein expression or functional data obtained previously (BOTCHKAREV et al.1998) (Figure 3.3.4). This demonstrates that neither β-actin nor PBDG are suitable internal standards for quantitative RT-PCR analysis of mouse skin of different hair cycle stages. This is not a unique phenomenon, since variations in the levels of β-actin gene expression have also been reported in human breast epithelial cells (SPANAKIS 1993), various porcine tissues (FOSS et al. 1998) and canine myocardium (CARLYLE et al. 1996), demonstrating the difficulty of using housekeeping genes for normalization purposes in quantitative RT-PCR analysis (BUSTIN 2001). The accepted method to overcome this problem is to resign the
standardization to an internal standard and simply express mRNA values as the number of copies of target mRNA in relation to the total RNA concentration used for reverse transcription (BUSTIN 2001).

The steady-state levels of VEGF$_{164}$ mRNA were statistically compared to those of VEGF$_{120}$ mRNA by multiple t-tests, employing a Bonferroni alpha-adjustation (significance niveau: 0.015). In all hair cycle stages examined, steady-state levels of VEGF$_{164}$–mRNA were significantly higher than steady-state levels of VEGF$_{120}$ mRNA levels (p<0.001).

The mRNA steady-state levels of the four investigated time points of the hair growth cycle for VEGF$_{164}$ and VEGF$_{120}$ were statistically compared by analysis of variance and subsequent Scheffé-test. The VEGF$_{164}$ mRNA level declined significantly between telogen and anagen IV (p=0.01) and raised again between anagen IV and anagen VI (p=0.01). Thus mRNA levels of anagen VI skin were comparable to mRNA levels of unmanipulated telogen skin. A highly significant decline in the steady-state mRNA levels for VEGF$_{164}$ could be detected between anagen VI and catagen skin (p<0.001) (Figure 3.3.5).

The levels of VEGF$_{120}$ mRNA did not differ between unmanipulated telogen skin and anagen IV. They increased, however, significantly between anagen IV and anagen VI (p<0.001) and decreased significantly between anagen VI and catagen skin (p<0.001). There was a slightly significant difference in VEGF$_{120}$ mRNA levels between catagen skin and unmanipulated telogen skin (Figure 3.3.5).

Using the same samples of mouse skin, mRNA levels of the two VEGF receptors, i.e. VEGFR-1 and VEGFR-2, were analysed. VEGFR-1 mRNA levels were significantly higher than mRNA levels of VEGFR-2 (p<0.001) throughout the entire hair growth cycle.

No statistically significant difference was observed for VEGFR-1 mRNA levels between unmanipulated telogen skin and anagen VI skin. Between anagen VI and catagen, however, there was a highly significant decline of the VEGFR-1 mRNA levels (p=0.002) (Figure 3.3.5).
VEGFR-2 mRNA levels decreased between unmanipulated telogen skin and anagen VI skin ($p=0.016$). There appeared to be a further decline of VEGFR-2 mRNA levels between anagen VI and catagen skin. This, however, was not statistically significant, although comparison between catagen skin and unmanipulated telogen skin revealed a statistically significant difference ($p=0.001$) (Figure 3.3.5).

3.3.1.4 VEGFR-1 protein expression is downregulated in the perifollicular microvasculature during anagen to catagen transformation of hair follicles

We attempted to correlate the transcription data of the apparently decisive VEGFR-1 gene with the corresponding changes in VEGFR-1 protein levels in situ. Immunofluorescence double staining for PECAM-1 and VEGFR-1 protein demonstrated detectable expression of VEGFR-1-immunoreactivity is not constant throughout the hair growth cycle. In all examined hair cycle stages, the larger vessels of the lower horizontal plexus revealed marked VEGFR-1–immunoreactivity. In unmanipulated telogen skin, few perifollicular vessels could be detected that did not express VEGFR-1–immunoreactivity (Figure 3.3.6). During anagen-development, the density of perifollicular vessels increased, and so did the immunostaining for VEGFR-1. Anagen VI skin finally exhibited many perifollicular blood vessels with marked VEGFR-1-immunoreactivity. There was, however, still a significant amount of vessels non–immunoreactive for VEGFR-1 (Figure 3.3.6). During catagen–development of hair follicles, the amount of VEGFR-1 negative vessels increased. Almost all blood vessels that surrounded the regressing proximal portion of hair follicles were not stained for VEGFR-1. Finally, telogen skin, 25 days after anagen–induction by depilation, still contained more non–immunoreactive vessels, than unmanipulated telogen skin (Figure 3.3.6).
3.3.1.5 Neutralization of VEGF–bioactivity during early anagen-development decreases microvessel density but does not affect hair follicles

The mRNA and protein expression data described above, suggested a crucial role for VEGF in anagen skin, i.e. during anagen-development of hair follicles and in skin with mature anagen VI hair follicles.

To further investigate this role, mice in which anagen had been induced by depilation were treated with a VEGF–neutralizing antibody that is known to neutralize VEGF bioactivity in several experimental settings (CORNE et al. 2000).

Firstly, the antibody was applied subcutaneously between the day of depilation (d 0) and eight days after depilation, i.e. during the phase when the microvessel density in the skin is known to increase (Figure 3.2.6). Eight days after depilation, no difference in the skin color could be detected between anti-VEGF-treated animals and controls. Both groups showed a dark grey skin color, suggesting advanced anagen–development (data not shown). When back skin was investigated microscopically, no differences with regard to the cycle stage of hair follicles could be observed between both groups. Anti-VEGF–treated animals and controls had hair follicles in stage V and VI of anagen–development (Figure 3.3.7). The microvessel density, however, was significantly reduced in anti-VEGF–treated mice, demonstrating the effect of a VEGF–neutralizing antibody on anagen–associated angiogenesis (Figure 3.3.7). A study, that was later published, demonstrated a reduced diameter of hair follicle bulbs after treatment with a VEGF–neutralizing antibody for a longer period of time (YANO et al. 2001). We therefore measured the diameters of hair follicle bulbs in the back skin of our mice after 8 days of treatment, but were not able to demonstrate any statistically significant difference between the two groups (Figure 3.3.7).

In a second experimental setting, anagen was induced in mice by depilation, and some animals were treated intraperitoneally with the VEGF-neutralizing antibody between day 8 after depilation and the spontaneous initiation of catagen (day 16 p.d.), i.e. while all hair follicles in back skin are in anagen VI. No difference in the skin color could be detected at the time point of skin embedding (data not shown).
Histologically, both groups showed exclusively anagen VI hair follicles. No evidences of early catagen–development could be detected in either group (Figure 3.3.8). The microvessel density had not been altered by anti-VEGF–treatment, and the diameters of hair follicle bulbs did not differ between both groups (Figure 3.3.8).

3.3.1.6 VEGF^f/f K5-Cre^+^-mice exhibit a decreased microvessel density and accelerated spontaneous catagen–development of hair follicles

Neutralization of VEGF does not differentiate between keratinocyte–derived VEGF and VEGF that is produced or stored in other cutaneous compartments. In order to dissect the function of follicular–derived VEGF, we investigated effects of a conditional gene ablation in cells that express keratin 5, i.e. basal keratinocytes of the epidermis and keratinocytes of the follicular outer root sheath. The latter had been shown to be the predominant source of VEGF in mouse skin (3.3.1.1).

At birth, transgenic mice had a similar microvessel density as non-transgenic animals. Microvessel density rised more slowly in transgenic mice during hair follicle morphogenesis, and was much lower at day 12 p.p., i.e. when hair follicles had completed morphogenesis (Figure 3.3.9 G). Hair follicle morphogenesis itself was not affected by the decreased microvessel density (Figure 3.3.9 A,B), and no difference with regard to the diameters of hair bulbs could be detected between transgenic and non-transgenic animals (Figure 3.3.9 C). Microvessel density finally raised until day 16 p.p. and almost reached the value of non-transgenic mice. However, it declined faster after hair follicles had entered spontaneous catagen–development (Figure 3.3.9 G). This was associated with an accelerated catagen–development of hair follicles, which can be assessed by the larger amount of hair follicles in more advanced stages of catagen (Figure 3.3.9 D,E,H) and by a faster decline of the skin thickness (Figure 3.3.9 F). This data is based on observations from only 2 to 4 animals per time point and genotype. Thus, it has to be verified in a larger amount of animals, which is currently ongoing, and statistical analysis has not been performed so far.
3.3.1.7 **Treatment with recombinant VEGF during anagen VI does neither influence the perifollicular vasculature nor spontaneous catagen–development of hair follicles**

The highly significant downregulation of VEGF mRNA expression that is associated with catagen–development of hair follicles and the fact that lack of follicle–derived VEGF accelerates catagen–development, suggest a critical role for VEGF in mediating regression of the perifollicular vasculature. In order to verify this hypothesis, mice with hair follicles in anagen VI were treated with recombinant mouse VEGF until hair follicles had spontaneously entered catagen–development.

Morphometry on CD31-stained frozen sections from back skin, however, did not reveal any effect of the VEGF–treatment on the cutaneous microvascular density ([Figure 3.3.10](#)). No difference was observed when comparing the skin of VEGF-treated animals and the controls macroscopically. Histologically, both groups exhibited a similar wave of catagen–development of hair follicles ([Figure 3.3.10](#)).
Figure 3.3.1 (page 105): Immunolocalization of VEGF in mouse skin. Detection with the avidin-biotin-system. Developed with DAB. Counterstained with methylene green. (A) Strong immunoreactivity in the interfollicular epidermis, outer root sheath (ORS), the secondary hair germ (HG) and many dermal fibroblasts; note the lack of immunoreactivity in the dermal papilla (DP); telogen (d 0 p.d.). (B) Strong immunoreactivity in the developing outer root sheath (ORS) of an anagen III hair follicle (d 3 p.d.); note moderate immunoreactivity in the dermal papilla (DP) and lack of immunoreactivity in the matrix keratinocytes (MK). (C) Strong immunoreactivity in the outer root sheath (ORS) and the dermal papilla (DP); anagen IV (d 5 p.d.). (D) Moderate immunoreactivity of the outer root sheath (ORS) and the dermal papilla (DP); note immunoreactivity of the wall of a subcutaneous vessel (arrow); anagen V (d 8 p.d.). (E) Moderate to strong immunoreactivity in the interfollicular epidermis and the outer root sheath (ORS) of an anagen VI hair follicle (d 12 p.d.); note very weak immunoreactivity in the dermal papilla (DP) and absence of immunoreactivity in matrix keratinocytes; also note strong immunoreactivity in the wall of multiple dermal and hypodermal vessels (arrows). (F) Moderate to strong immunoreactivity in the outer root sheath and the epithelial strand (ES) of a catagen VI hair follicle (d 19 p.d.); note lack of immunoreactivity in the dermal papilla (DP). (G) Immunoreactivity in a telogen hair follicle, 25 days after depilation; the pattern of immunoreactivity is similar to that seen in telogen hair follicles before induction of the hair cycle (A). (H,I) Immunoreactivity of dermal (H) and hypodermal (I) vessels in anagen VI skin. Scale bars: A-C, F-H: 20µm, D-E: 30µm.
Figure 3.3.2: RT-PCR analysis of VEGF mRNA–splice variants at different time points of the murine hair cycle. Experiments were performed in triplicates, from three individual animals per time point. cDNA bands, representing different VEGF mRNA splice variants, are detectable at predicted sizes: 360bp (VEGF\textsubscript{120}), 432bp (VEGF\textsubscript{144}), 492bp (VEGF\textsubscript{164}), 564bp (VEGF\textsubscript{188}). Lung tissue exhibits VEGF\textsubscript{188} as the most prominently expressed splice variant, whereas in murine telogen skin VEGF\textsubscript{164} is the most prominently expressed splice variant. VEGF\textsubscript{188} is hardly detectable in murine skin at any time point of the hair growth cycle. The samples were normalized according to the expression of β-actin mRNA. The graph below shows the intensity of each band, evaluated by digital image analysis-based densitometry. Mean values from three animals at each time point.
Figure 3.3.3: Levels of β-actin mRNA in mouse skin are hair cycle-dependent. Copy numbers of β-actin mRNA/µg RNA in mouse skin of different hair cycle stages. The mRNA steady-state levels were quantified by real-time (TaqMan) RT-PCR in 3 samples (deriving from 3 individual animals) per hair cycle stage. Note the significant differences in mRNA levels between different hair cycle stages and the similarity of mRNA levels between individuals of the same hair cycle stage. *** = p<0.001. Analysis of variance and Scheffé-test.

Figure 3.3.4: Normalization to β-actin changes target mRNA levels. Copy numbers of TrkC mRNA/µg RNA in mouse back skin of different hair cycle stages. Original values (TrkC) and normalized values (TrkC norm.), according to β-actin mRNA levels in individual samples. The mRNA steady-state levels were quantified by real-time (TaqMan) RT-PCR in 3 samples (deriving from 3 individual animals) per hair cycle stage. Shown are mean values ± SD. Original values reveal a significant increase in the TrkC mRNA levels between telogen and anagen VI skin, whereas this is not apparent after normalization. * = p<0.05. Analysis of variance and Scheffé-test.
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Figure 3.3.5: Hair cycle–dependent mRNA levels of different VEGF splice variants and the two VEGF receptors. Boxplots of mRNA levels of VEGF\textsubscript{164}, VEGF\textsubscript{120}, VEGFR-1 and VEGFR-2 at selected time points of the murine hair growth cycle, assessed by quantitative real-time (TaqMan) RT-PCR. mRNA levels are shown in copy numbers/µg mRNA. Shown are maximum value, 75% quartil, median, 25% quartil, and minimum value. For each time point skin samples that derived from three individual mice were evaluated. Significant differences in mRNA levels between groups were evaluated by analysis of variance and subsequent Scheffé-test. * = p<0.05; ** = p<0.01; *** = p<0.001; n.s. = no statistically significant difference.
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**Figure 3.3.6** (page 110): Hair cycle–dependent VEGFR-1 protein expression in the cutaneous vasculature. Immunofluorescence for PECAM-1 and VEGFR-1 in murine skin at selected time points of the depilation–induced hair growth cycle. Both stainings were superimposed and double positive vessels revealed a yellow staining. This was superimposed again to a phase contrast image, demonstrating the anatomical structures of the section. All vessels, negative for VEGFR-1 are indicated with arrows. Note that the arrows do not point to vessels in the VEGFR-1 row (green). hf = hair follicle; pc = panniculus carnosus (subcutaneous muscle layer). Original magnification: 250x. Note the increase in perifollicular blood vessels during anagen–development. Also note the lack of VEGFR-1–immunoreactivity of the perifollicular vessels in catagen and telogen (day 25 p.d.) skin, compared to anagen and unmanipulated telogen (day 0 p.d.) skin.

**Figure 3.3.7:** Neutralization of VEGF during early anagen–development reduces microvessel density but does not exert any effect on hair follicles. (A,B) Histological analysis of back skin reveals hair follicles in anagen V and anagen VI in anti-VEGF–treated (A) and PBS–treated (B) animals. (C) The microvessel density, assessed by PECAM-1–staining is significantly lower in anti-VEGF–treated animals. (D) The diameter of hair follicle bulbs does not differ significantly between the anti-VEGF–treated animals and controls. Mean ± 95% confidence interval. ***=p<0.001. Two-sided student’s t-test for unpaired samples.
Figure 3.3.8: Neutralization of VEGF during anagen VI does not exert any effect on hair follicles. (A,B) Histological analysis of back skin reveals hair follicles in anagen VI in anti-VEGF–treated (A) and PBS–treated (B) animals. (C) The microvessel density, assessed by PECAM-1–staining is equal in both groups. (D) The diameter of hair follicle bulbs does not differ significantly between the anti-VEGF–treated animals and controls. Mean ± 95% confidence interval. Two-sided student’s t-test for unpaired samples.
Results

Figure 3.3.9: VEGF<sup>−/−</sup> K5-Cre<sup>+</sup> mice that lack VEGF in keratin 5-expressing cells display decreased microvessel density and accelerated catagen–development of hair follicles. (A,B) Histology of back skin from Cre-transgenic (A) and non-transgenic (B) mice. Both groups reveal hair follicles in different stages of morphogenesis. No anagen-retardation is evident, and hair bulbs at day 12 p.p. exhibit the same diameter in both groups (C). (D,E) Histology of back skin from Cre-transgenic (D) and non-transgenic (E) mice. Cre-transgenic mice exhibit hair follicles in more advanced stages of catagen–development, as can also be assessed by the accelerated decrease in skin thickness (F). (G) Microvessel density in Cre-transgenic animals and non-transgenic mice is similar at birth. In Cre-transgenic mice, it rises more slowly during anagen–development and declines faster during catagen–development of hair follicles. (H) Cre-transgenic animals have more hair follicles in advanced stages of catagen–development (catagen IV-VII) than non-transgenic mice. Shown are mean values, deriving from 2-3 animals. Due to the small number of investigated animals, no statistical analysis has been performed so far.
Figure 3.3.10: Administration of recombinant murine VEGF during anagen to catagen–transformation of hair follicles does not alter catagen–development. (A,B) Histological analysis of back skin reveals hair follicles in advanced stages of catagen–development in rmVEGF–treated (A) and PBS–treated (B) animals. (C) The microvessel density, assessed by PECAM-1–staining, is not altered by rmVEGF–treatment. Mean ± 95% confidence interval. Two-sided student’s t-test for unpaired samples. (D) Back skin of animals in both groups exhibits a similar wave of catagen–development of hair follicles. The graph shows the mean percentage of hair follicles in each hair cycle stage, evaluated in back skin from three animals per group. Note the similarity of both curves, indicating normal catagen–development of hair follicles in both groups.
3.3.2 Hair cycle–dependent expression of angiopoietins

The angiopoietins are the second most important family of growth factors, playing critical roles in angiogenesis, vessel remodeling and maturation (DUMONT et al. 1994, SATO et al. 1995). In fact, VEGF alone may not be a sufficient stimulus for the development and maintenance of normal blood vessels in the adult organism (GEVA and JAFFE 2000), and thus the angiopoietins act synergistically with VEGF (KOBLIZEK et al. 1998).

Simultaneously to VEGF, we therefore investigated the protein and mRNA expression of Angiopoietin-1, Angiopoietin-2 and their common receptor (Tie-2) in the same samples of mouse skin from selected time points of the depilation–induced hair growth cycle, as have been used for VEGF-analyses.

3.3.2.1 Angiopoietin-1 –immunoreactivity is prominent in outer root sheath keratinocytes of the bulge region

Angiopoietin-1 mRNA is known to be widely expressed in normal adult organisms, mainly by periendothelial cells. (MAISONPIERRE et al. 1997, GEVA and JAFFE 2000). However, expression of Ang-1 is not restricted to vascular pericytes but has also been detected in stromal cells of the dermis (KURODA et al. 2001), adipocytes (STACKER et al. 2000), macrophages (OTANI et al. 1999), astrocytes (ACKER et al. 2001), and retinal pigment epithelial cells (OTANI et al. 1999).

Ang-1 immunoreactivity was especially prominent in mesenchymal cells of the dermis and hypodermis throughout the entire hair growth cycle (Figure 3.3.11). A moderate immunostaining is furthermore detectable in the interfollicular epidermis. Telogen hair follicles exhibit a variable staining of follicular keratinocytes that ranges from moderate to strong immunoreactivity in all keratinocytes surrounding the club hair (Figure 3.3.11 G) to a very faint staining of these keratinocytes (Figure 3.3.11 A). In any case, the dermal papilla is non-immunoreactive. After anagen has been initiated, keratinocytes of the secondary hair germ, which is located at the insertion site of the arrector pili muscle, become Ang-1–immunoreactive (Figure 3.3.11 B). This
localization remains the most prominent site of Ang-1-immunoreactivity in hair follicles throughout the entire hair growth cycle (Figure 3.3.11). Keratinocytes of the bulge region still exhibit immunoreactivity in mature anagen VI hair follicles. In principle, immunoreactivity is detectable in the whole follicular outer root sheath, but becomes markedly weaker, the more proximal keratinocytes are located (Figure 3.3.11 D). Matrix keratinocytes or dermal papilla fibroblasts do not reveal Ang-1-immunoreactivity during any hair cycle stage. Catagen–development is still associated with strong Ang-1 immunoreactivity in keratinocytes of the bulge region. Again, the intensity of the staining fades, the more proximal the cells are located. Considerable immunoreactivity is still associated with the regressing epithelial strand in catagen, while the dermal papilla remains non-immunoreactive (Figure 3.3.11 E). Finally, hair follicles of early telogen show Ang-1 immunoreactivity in keratinocytes surrounding the club hair and in the germ cell cap of the hair follicle (Figure 3.3.11 G).

3.3.2.2 Angiopoietin-2–immunoreactivity is also prominent in outer root sheath keratinocytes of the bulge region

Angiopoietin-2 (Ang-2) probably represents the first example of an endogenous receptor antagonist, since it appears to block Ang-1–mediated signals (MAISONPIERRE et al. 1997). In contrast to Ang-1, Ang-2 is physiologically expressed only at sites of vascular remodeling, e.g. the ovary, uterus and placenta (MAISONPIERRE et al. 1997). It is mainly associated with blood vessels, especially strong with those of the theca interna of ovarian follicles (GEVA and JAFFE 2000) (Figure 3.3.12 A), but has also been demonstrated in macrophages (OTANI et al. 1999), smooth muscle cells (YUAN et al. 2000), natural killer cells (LI et al. 2001), glioma and carcinoma cells (ETOH et al. 2001, KOGA et al. 2001).

In mouse skin, Ang-2 immunoreactivity is restricted to keratinocytes of the hair follicles and to few mesenchymal cells in the dermal and hypodermal compartment. Similar to Ang-1, Ang-2 immunoreactivity can be detected in the germ cell cap above the dermal papilla of early anagen hair follicles (Figure 3.3.12 C). Strong Ang-2-
immunoreactivity remains in keratinocytes of the bulge region throughout anagen–development (Figure 3.3.12 D,F). Mature anagen VI hair follicles exhibit strong Ang-2-immunoreactivity in bulge–keratinocytes whereas immunoreactivity of the outer root sheath becomes markedly weaker the more proximal keratinocytes are located (Figure 3.3.12 F). In catagen hair follicles, Ang-2 immunoreactivity is evident in keratinocytes of the bulge-region and in the regressing epithelial strand, whereas it is still completely absent from the dermal papilla (Figure 3.3.12 E,G). Finally, telogen hair follicles exhibit Ang-2 immunoreactivity in all keratinocytes that surround the club hair (Figure 3.3.12 B,H).

3.3.2.3 Quantitative real-time PCR reveals differential changes in the hair cycle–dependent mRNA levels of Angiopoietin-1, Angiopoietin-2 and their common receptor Tie-2

Since angiopoietins act synergistically with VEGF in regulating vascular homeostasis, we quantified the mRNA levels of both angiopoietins and of their common receptor Tie-2. These mRNA levels were measured in the same samples that had been used to quantify mRNA levels for VEGF\textsubscript{164}, VEGF\textsubscript{120} and both VEGF receptors. Thus, a direct comparison of the mRNA profiles is possible.

Ang-1 mRNA levels decreased slightly between unmanipulated telogen skin and anagen IV skin (p=0.025) and dramatically between anagen IV and anagen VI skin (p=0.001). Thus, significantly lower Ang-1 mRNA levels were measured in mature anagen skin compared to telogen skin. There was a slight increase in the Ang-1 mRNA levels between anagen VI and catagen skin (Figure 3.3.14). This, however, could not be verified statistically yet.

Angiopoietin-2 mRNA levels showed a similar pattern of hair cycle–dependent changes compared to Ang-1 mRNA levels. Again, a decline was observed during anagen–development, and lowest mRNA levels were reached in mature anagen VI skin. Again, an increase in mRNA levels was evident between anagen VI and catagen skin, which, however, could not be verified statistically (Figure 3.3.14).
The means of Ang-1 and Ang-2 mRNA levels were compared at each investigated time point by student’s t-test (assuming that the values were normally distributed, which could not be proven with certainty, due to the small amount of investigated samples per time point). In telogen and anagen VI skin, no difference between Ang-1 and Ang-2 could be detected, whereas in anagen IV and catagen skin, Ang-2 mRNA levels were significantly higher than those of Ang-1 (p=0.037 and p=0.018, respectively).

Tie-2 mRNA levels raised significantly during early anagen–development, i.e. between unmanipulated telogen skin and anagen IV, and they declined subsequently thereafter, i.e. between anagen IV and anagen VI. Thus, in anagen VI skin, they had reached values comparable to unmanipulated telogen skin and these did not differ between anagen VI and catagen skin (Figure 3.3.14).
Figure 3.3.11 (page 119): **Angiopoietin-1–immunoreactivity in paraffin sections of mouse skin of different stages of the depilation–induced hair growth cycle.** Ang-1 protein is labeled brown. Counterstained with methylene green. (A) unmanipulated telogen skin; strong immunoreactivity of cells within the dermal mesenchyme, moderate immunoreactivity in the epidermis (E) and very faint immunoreactivity in the follicular epithelium around the club hair (CH). SG=sebaceous gland. Note lack of immunoreactivity in the dermal papilla (dp). (B) anagen II. Strong immunostaining in hair germ keratinocytes (arrow). Note lack of immunoreactivity in the matrix keratinocytes and the dermal papilla. (C) anagen V. Strong immunoreactivity in outer root sheath keratinocytes of the bulge area, where the arrector pili muscle inserts (arrows). (D) anagen VI. Immunoreactivity is mainly located to keratinocytes of the bulge region (arrow), and becomes weaker, the more proximal cells are located in the outer root sheath. (E) catagen VI. Moderate immunoreactivity in the epithelial strand (ES). (F) catagen VIII. Note lack of immunoreactivity in the dermal papilla. (G) telogen HF after depilation (day 25 p.d.). Moderate to strong immunoreactivity in keratinocytes surrounding the club hair (CH).

Figure 3.3.12 (page 121): **Angiopoietin-2–immunoreactivity in paraffin sections of mouse skin of different stages of the depilation–induced hair growth cycle.** Ang-2 protein is labeled brown. Counterstained with methylene green. (A) positive control: mouse ovary. Note immunostaining of the corpus luteum (cl) and lack of immunoreactivity in the granulosa cells of ovarian follicles (f). (B) unmanipulated telogen skin; APM=arrector pili muscle; dp=dermal papilla; note Ang-2 immunoreactivity in hair follicle keratinocytes (arrow) and in the wall of subcutaneous vessels (asterisk). (C) anagen II; M=hair follicle matrix; note immunostaining at the insertion site of the arrector pili muscle (arrow). (D) anagen V; SG=sebaceous gland; (E) catagen VI; ES=epithelial strand. (F) anagen VI. Note that the intensity of Ang-2–immunoreactivity declines in the outer root sheath from distal to proximal. (G) catagen VIII. CTS=connective tissue sheath. (H) telogen skin, 25 days after depilation.
Figure 3.3.13 (page 122): Schematic representation of immunoreactivity for VEGF, Ang-1 and Ang-2 during the hair growth cycle and its association with the perifollicular microvasculature. The color-intensity from orange to yellow represents the intensity of immunostaining for both angiopoietins. In telogen skin, the upper and lower horizontal blood vessel plexus and connecting vessels parallel to the hair follicles are most prominent. Compare to Figure 3.2.1. During mid-anagen–development, angiogenesis occurs in the perifollicular vasculature. Note the temporary immunoreactivity of the dermal papilla during this phase. The proximal portion of anagen VI hair follicles is surrounded by an extensive capillary network. After catagen has been initiated, the proximal portion of the hair follicle retracts and vascular regression occurs in the vascular network that is left behind.
**Figure 3.3.14:** Hair cycle–dependent mRNA levels of the two angiopoietins and their receptor. Boxplots of mRNA levels of Ang-1, Ang-2, and Tie-2 at selected time points of the depilation–induced murine hair growth cycle, assessed by quantitative real-time (TaqMan) RT-PCR. mRNA levels are shown in copy numbers/µg mRNA. Shown are maximum value, 75% quartil, median, 25% quartil, and minimum value. For each time point skin samples that derived from three individual mice were evaluated. Significant differences in mRNA levels between groups were evaluated by analysis of variance and subsequent Scheffé-test. * = p<0.05; ** = p<0.01; *** = p<0.001; n.s. = no statistically significant difference.
4. Discussion

This study reveals the step-wise development and remodeling of a distinct perifollicular vascular system in mouse skin, and demonstrates its close interrelation with defined stages of hair follicle morphogenesis and cycling. Growth factors (VEGF and Angiopoietin-1) that are known for playing an important role for vascular development, maintenance and remodeling are synthesized by hair follicles during advanced stages of their morphogenesis and thus seem to govern the follicular blood supply.

The cutaneous vascular system is modified subsequent to changes in the activity of hair follicles. Precisely, this study demonstrates that angiogenesis, i.e. the growth of new capillaries from pre-existing ones, occurs physiologically in murine skin during synchronized anagen–development of hair follicles. It is also shown that these vessels eventually regress again, temporally associated with catagen–development of hair follicles. These events are probably regulated by VEGF and the angiopoietins that derive from the hair follicle epithelium and that are temporally expressed in a hair cycle–dependent manner. Thus it appears that both during morphogenesis and cycling, the hair follicles dictate vascular remodeling processes in their surrounding mesenchyme, and it does not appear that changes in the perifollicular vasculature trigger key events in hair follicle biology.

Nevertheless, the importance of adequate hair cycle–dependent vascular remodeling is demonstrated by the fact that massive inhibition of angiogenesis leads to a retardation of anagen–development. However, the system of growth factors that dictate hair growth–associated vascular remodeling appears to be rather complex. The neutralization or lack of a single key regulatory factor (VEGF) over a short period of time leads to a reduction of the cutaneous vascular density but does not exert definite effects on hair follicle cycling.

Suitable model systems for physiological blood vessel remodeling are rare and urgently needed in order to dissect the underlying molecular events. It is therefore proposed that synchronized hair follicle cycling in mouse skin provides an easily
accessible, intriguing model for the study of physiologic blood vessel growth and regression. Finally, manipulation of the perifollicular vasculature might offer interesting new approaches to manipulate hair growth therapeutically in pathologic conditions such as unwanted hair growth (hirsutism) or hair loss (alopecia).

4.1 Development of a distinct perifollicular vascular system begins early in cutaneous development and is governed by the progress of hair follicle morphogenesis

Vasculogenesis, i.e. the formation of a primary embryonic vascular network, requires differentiation of angioblastic cells and vessel assembly (RISAU 1997). Moreover, angiogenesis, vascular remodeling, vessel maturation, and acquisition of vessel identity are necessary for generating a functional vascular system (AUGUSTIN 2001). All these processes crucially depend on two growth factors, i.e. vascular endothelial growth factor (VEGF) and Angiopoietin-1 (Ang-1), since lack of either growth factor, or absence of their receptors lead to severely disturbed vasculogenesis with subsequent embryonic lethality (FERRARA et al. 1996, CARMELIET et al. 1996, FONG et al. 1995, SHALABY et al. 1995, DUMONT et al. 1994, SURI et al. 1996). Thus the expression of vascular growth factors during tissue morphogenesis is essential for the formation of an organotypic vascular bed that fulfills the metabolic demands of the perfused tissue. The skin is a highly vascularized organ, because its vascular network not only functions as a nutritive system for the skin but also plays a role in thermal regulation (MONTAGNA and PARAKKAL 1974). It therefore requires development of an extensive vascular network which has to be adapted to the multiple functions of this organ (CHUONG et al. 2002).

This study demonstrates that in mice, development of the cutaneous vascular system is temporally and spatially coordinated with morphogenesis of hair follicles (Figure 3.1.1). It is further shown that the hair follicles appear to govern the development of
the vascular system by synthesizing key regulatory growth factors, i.e. VEGF and Ang-1 (Figure 3.1.5).

In the prenatal murine skin, hair follicle morphogenesis begins with the formation of an epithelial hair plug from the basal epidermal cell layer (PAUS et al. 1999). At this point of time, a single blood vessel plexus exists at the dermal-hypodermal interface (Figure 3.1.1), and immunoreactivity for VEGF and Ang-1 is restricted to suprabasal epidermal keratinocytes (Figure 3.1.5).

In the perinatal murine skin, the majority of pelage hair follicles has already extended into the dermal connective tissue and has started to build a keratinized hair shaft (stage 5 of morphogenesis). At this stage, there is a growing demand for blood supply. Due to this demand, the blood vessel plexus has extended into the hypodermal tissue (Figure 3.1.1). Simultaneously, there is marked immunoreactivity for both VEGF and Ang-1 in the suprabasal epidermis, the whole dermal connective tissue, and the outer root sheath of hair follicles in stages 5 and 6 of morphogenesis (Figure 3.1.5).

During postnatal development of the skin, the hair follicles further extend into the hypodermis, accompanied by a tremendous increase in the thickness of this compartment (CHASE et al. 1953, HANSEN et al. 1984). Simultaneously, the vessels, originally located at the dermal–hypodermal interface, spread inside the hypodermal tissue, following the hair follicles and surrounding them (Figure 3.1.1). This is temporally associated with a loss of VEGF- and Ang-1–immunoreactivity in the dermal compartment and with a gain of immunoreactivity in the entire outer root sheath of hair follicles (Figure 3.1.5).

These observations suggest that hair follicles substantially dictate the development of the cutaneous vascular system in hair follicle–bearing skin. This claim is further supported by the fact that the cutaneous vascular system finally is most intensive around the proximal outer root sheath of hair follicles, whereas the dermal compartment only is sparsly vascularized (Figure 3.1.1).

With regard to our observations about VEGF and Ang-1–immunoreactivity, it is most important to note that immunoreactivity does not allow differentiation between sites of
synthesis and sites of storage of the growth factor. We have demonstrated immunoreactivity for VEGF and Ang-1 both in epithelial cells (epidermis, outer root sheath) and in mesenchymal cells (dermal connective tissue). However, several experiments showed that VEGF expression is not detectable in any mesenchymal cell (KISHIMOTO et al. 2000, YANO et al. 2001). It is known that VEGF and Ang-1 are partially sequestered in the extracellular matrix (FERRARA et al. 1992, PARK et al. 1993, XU and YU 2001). Therefore, we assumed that the temporary VEGF and Ang-1-immunoreactivity seen in the dermal connective tissue (Figure 3.1.5) is due to sequestered growth factors, rather than to actual synthesis by these cells. This hypothesis is further supported by the fact that immunoreactivity for Angiopoietin-2 (Ang-2), which does not bind to the extracellular matrix (XU and YU 2001) could not be found in the dermal connective tissue in perinatal skin (Figure 3.1.5). It is assumed that Ang-2 does not have a comparably important role for vasculogenesis like Ang-1, although Ang-2-knockout mice do not exist and thus functional studies clarifying this aspect have not been performed so far (LOUGHNA and SATO 2001).

According to this hypothesis, the expression of Ang-2 is much more restricted than that of Ang-1 (MAISONPIERRE et al. 1997). Indeed Ang-2-immunoreactivity was not found in the hair follicle epithelium during any stage of cutaneous development in mice (Figure 3.1.4).

4.2 The cutaneous microvasculature in adult mouse skin is reorganized according to the activity of hair follicles

In the adult organism, blood vessels are generally believed to be quiescent (DETMAR 1996). This quiescence, however, only is maintained by the simultaneous activity of a multitude of stimulators and inhibitors of angiogenesis, which are strictly coordinated with regard to time and place of their expression (FOLKMAN 1997). They facilitate adjustment of the vascular bed according to the tissue’s changing metabolic demand, which occurs under many pathologic conditions like tumor growth or wound healing (FOLKMAN 1995). Some physiologic conditions, however, are also accompanied by vast changes in the metabolic demand of the tissue and thus in their
vascular arrangement. The ovarian corpus luteum, the endometrium, the placenta and the mammary gland are well known examples of cyclic tissue reconstruction, which is associated with vascular remodeling (FRASER and LUNN 2000).

Synchronized hair growth is another example for physiological cyclic tissue reconstruction, because hair follicles repeatedly undergo growth (anagen), regression (catagen), and rest (telogen). Since we had shown that hair follicles govern the development of a perifollicular vascular network during their morphogenesis (Figure 3.1.5), we speculated that synchronized hair follicle cycling was also associated with the remodeling of the surrounding microvasculature.

In order to verify this hypothesis, we used an antibody directed against CD31 (PECAM-1), a transmembrane protein of endothelial cell adherens junctions (ALBELDA et al. 1997), in order to mark endothelial cells for morphometric analysis. Though expression of CD31 on endothelial cells may be downregulated in the case of malignant transformation (OHSAWA et al. 1995) or under the influence of certain cytokines (BUJAN et al. 1999), and though we cannot exclude with certainty its downregulation in distinct cell cycle phases, CD31 has widely been used as a marker for endothelial cells including mouse tissue (e.g. COUFFINHAL et. al. 1998, DETMAR et al. 1998, SURI et al. 1998). Antibodies against CD31 have been used 1) in immunohistologic double-stainings with the Ki-67 antigen to determine proliferating endothelial cells (CHRISTENSON and STOUFFER 1996, CREAMER et al. 1997, VERMEULEN et al. 1997) and 2) in combination with in situ apoptosis detection to determine apoptotic endothelial cells (PARIS et al. 2001).

**Angiogenesis is a physiological event that occurs in the cutaneous vasculature during synchronized anagen-development of hair follicles**

Morphometric analyses have supported our hypothesis, since we were able to demonstrate significant morphological changes both in the microvessel density and in the caliber and arrangement of cutaneous blood vessels in mouse skin, that were associated with the cyclic growth and regression of hair follicles (Figure 3.2.2, 3.2.5, 3.2.6). Previous studies in rats and rabbits have also shown differences in the
arrangement of cutaneous blood vessels between anagen and telogen (DURWARD and RUDALL 1958). These studies, however, were not able to explain whether these changes are due to passive hair cycle-dependent modulations of perfusion or to active growth and regression of the vasculature. Our findings confirm that the observed morphological changes in the cutaneous vascular system can only be explained by the occurrence of genuine angiogenesis during anagen–development of hair follicles: Firstly, there is an increase in the number of nuclei of CD31-immunoreactive cells in the interfollicular dermis and subcutis that is unequivocally associated with anagen-development, demonstrating a significant increase in the pool of endothelial cells during anagen (Figure 3.2.7). Secondly, there is a massive increase of proliferating/mitotic endothelial cells in anagen (Figures 3.2.8, 3.2.9) compared to the very rare endothelial cell proliferation in telogen skin.

Our findings indicate that the observed angiogenesis includes proliferation of endothelial cells and thus is based, at least in part, on sprouting of vessels. Still, there are different forms of angiogenesis described, e.g. “intussusceptive” angiogenesis (RISAU 1997). The latter comprises the longitudinal splitting of vessels and thus does not require endothelial cell proliferation (RISAU 1997, CARMELIET and JAIN 2000). Nevertheless, the data in the current study strongly suggests that sprouting angiogenesis plays at least a partial role in the remodeling of the perifollicular vasculature during anagen–development of hair follicles, because significant numbers of proliferating endothelial cells have been detected in mouse skin during this hair cycle stage (Figure 3.2.8). However, we cannot exclude the parallel existence of intussusceptive angiogenesis during anagen-development, especially since this type of angiogenesis has been shown to be a major component of physiologic vascular remodeling in various organs, e.g. lung and endometrium (ROGERS et al. 1998, DJONOV et al. 2000).

**Angiogenesis is essential for anagen-development of hair follicles**

We were able to demonstrate that the anagen-associated angiogenesis is essential for development of anagen hair follicles. The systemic treatment with a potent anti-
angiogenic compound (TNP-470) not only resulted in a decreased microvessel density of anagen skin but also led to a retardation of anagen–development (Figure 3.2.10, 3.2.11, 3.2.12). This anagen-retardation could also be due to a direct toxic effect of TNP-470 on hair follicles, and thus must be discussed as a theoretical possibility for this observation. However, to the best of our knowledge, the literature available on fumagillin-derivatives does not report any toxic effects of this agent on keratinocytes (CASTRONOVO, personal communication). TNP-470 and its toxicity has extensively been investigated in several pre-clinical studies and phase I to III clinical trials (CASTRONOVO and BELOTTI 1996). During none of the published clinical trials hair loss/alopecia/effluvium has been reported as a side-effect of therapy, as one would definitely expect in case of any significant hair follicle toxicity of the drug. Moreover, in our study there was no morphological evidence of drug-induced hair follicle toxicity, i.e. characteristic abnormalities in the hair follicle pigmentary unit (SLOMINSKI et al. 1996, TOBIN et al. 1998). Therefore, the observed anagen-retardation in TNP-470–treated mice must be referred to angiogenesis-inhibition.

Cutaneous blood vessels eventually regress during catagen-development of hair follicles

Physiological angiogenesis is a multi-step process, comprising migration, proliferation and assembly of endothelial cells which eventually leads to either maturation of the vasculature or regression of the newly formed blood vessels (HANAHAN 1997, BENJAMIN 2000). The latter is typically associated with tissue involution/regression, as it is seen during corpus luteum regression (AUGUSTIN et al. 1995). We speculated that regression of cutaneous blood vessels occurs in association with the catagen-development of hair follicles, because catagen is also an example of tissue involution. Furthermore, vascular regression is likely to account for the maintenance of vascular homeostasis throughout the hair growth cycle, since new blood vessels are repeatedly formed during anagen.
Although morphological evidence of blood vessel regression in mammalian skin has indeed been described (MERKEL 1919, DURWARD and RUDALL 1958, ELLIS and MORETTI 1959), these observations did not differentiate between genuine blood vessel regression and a mere collapse of single blood vessels that are re-extended later on. The present study demonstrates that initiation of catagen—development of hair follicles truly is associated with a significant decline in the number of endothelial cells and subsequently the microvessel density (Figure 3.2.12). According to our morphological data, dissociation of capillary endothelial cell tight junctions occurs in perifollicular vessels in early catagen skin, and endothelial cells start to detach from their underlying basement membrane, protrude into the vessel lumen and eventually are shed, leaving areas devoid of a covering endothelial cell layer. These findings are accompanied by degenerative changes of endothelial cell nuclei. Classic morphologic findings of apoptosis (WYLLIE et al. 1980, WALKER et al. 1988) were also observed in endothelial cells of catagen skin, but appeared to be less frequent than other degenerative changes (Figure 3.2.14).

Eventually both degenerative and apoptotic endothelial cells are shed into the lumen to be removed by the blood circulation (Figure 3.2.14). This possibly explains why so few apoptotic endothelial cells are detectable using the histochemical detection of nucleosomal fragmentation products (TUNEL-technique) as a marker of apoptosis. In addition to this all such a DNA strand break labeling techniques detect only late phases of apoptosis, i.e. just before cell removal via the circulation or by phagocytosis. Accordingly, it has been shown that cultured bovine aortic endothelial cells only become TUNEL-positive after they have lost adherence to the tissue culture plastic (MODLICH et al. 1996). Detached apoptotic or degenerating endothelial cells could be found in the lumen of small vessels in catagen murine skin, and eventually occlusion of small capillaries by detached endothelial cells occurred during telogen (Figure 3.2.14). This is likely to promote further regression of the affected vessel, perhaps due to interruption of blood flow and resultant endothelial cell apoptosis (MEESON et al. 1996, DIMMLER and ZEIHER 2000). Notably, most apoptotic endothelial cells, detectable by the TUNEL-technique, occurred in clusters within small vessels (Figure 3.2.13), suggesting that simultaneous induction of
endothelial cell-apoptosis occurs in cutaneous vessels, possibly due to occlusion by microemboli and interrupted blood flow. Other potential mechanisms of endothelial cell loss, i.e. migration of endothelial cells away from the vascularized region and/ or transdifferentiation into mesenchymal cells (LATKER et al. 1986), have been discussed in the literature and deserve consideration here, but could not be verified accurately under the design of the present study.

On the cellular level, catagen–associated blood vessel regression in the skin is thus morphologically well comparable to other models of vascular regression, e.g. the regressing corpus luteum of the ovary (AUGUSTIN et al. 1995, MODLICH et al. 1996), regressing foreign body granulomas (HONMA and HAMASAKI 1998), and the regressing tunica vasculosa lentis (MITCHELL et al. 1998).

4.3 Vascular remodelling during the hair growth cycle is mediated by VEGF and angiopoietins

VEGF and its role in hair-cycle dependent remodeling of the perifollicular vasculature

VEGF not only is of major importance for vasculogenesis, but it is also a key regulator of vascular homeostasis (FERRARA and DAVIS-SMYTH 1997). VEGF-gene transcription, -mRNA and -protein expression have been demonstrated in mouse and human skin, and they have specifically been found in keratinocytes of the hair follicle (KOZLOWSKA et al. 1998, WENINGER et al. 1996, KISHIMOTO et al. 2000). Their expression throughout the hair growth cycle, however, has not been studied so far. The current study confirms by immunohistochemistry that the VEGF protein is predominantly detectable in keratinocytes of the interfollicular epidermis and in the outer root sheath of hair follicles throughout all stages of hair follicle cycling (Figure 3.3.1). Interestingly, there is also a window of VEGF immunoreactivity in the dermal papilla during anagen development (Figure 3.3.1 B,C). Expression of the VEGF gene in mesenchymal cells, however, is still controversial (SENGER and VAN DE WATER 2000). During the investigation of VEGF-immunoreactivity in cutaneous development, we already assumed that the temporary immunoreactivity
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seen in the dermis is actually based on sequestration of growth factors (4.1). We transferred this assumption to the temporary VEGF-immunoreactivity of dermal papilla fibroblasts during anagen-development. While considering this theory, another study described the detection of VEGF mRNA in mouse skin in situ. This investigation confirmed that VEGF mRNA is expressed in outer root sheath keratinocytes of anagen hair follicles, but failed to detect a hybridization–signal in the dermal papilla (YANO et al. 2001). Therefore, it is reasonable to assume that VEGF is synthesized and secreted by the hair follicle epithelium only, and that it is temporarily stored in the extracellular matrix of the mesenchymal dermal papilla during anagen III to anagen V. From this compartment it is liberated during late anagen–development, since it is not detectable in this compartment anymore in anagen VI (Figure 3.3.1, 3.3.13).

It is well known that different isoforms of the VEGF protein exist, and they mainly differ with regard to their capability to bind to the extracellular matrix and to cell membranes (ROBINSON and STRINGER 2001). Therefore, it is important to investigate which isoforms are expressed in murine skin and whether their expression changes during the hair growth cycle. RT-PCR analyses with a primer pair that allowed amplification of all known splice variants of VEGF mRNA (Figure 2.4, 2.5) have shown that there is indeed a distinct pattern of VEGF isoforms expressed in murine skin. Precisely, we have demonstrated that the isoform VEGF\textsubscript{188}, which is the most prominently expressed isoform in other highly vascularized tissues, e.g. the lung (BURCHARDT et al. 1999), is hardly detectable in murine skin. Actually, there is high expression of mRNAs encoding for the isoforms VEGF\textsubscript{164}, VEGF\textsubscript{144} and VEGF\textsubscript{120} (Figure 3.3.2). While VEGF\textsubscript{164} and VEGF\textsubscript{120} are widely distributed isoforms of VEGF, the 144aa–isoform has a much more restricted pattern of expression (NEUFELD et al. 1999). However, it was not surprising to find this isoform in murine skin, since it has mainly been localized in tissues that undergo substantial remodeling of the vasculature, e.g. the uterus and placenta (CHEUNG et al. 1995, POLTORAK et al. 2000).

Interestingly, the pattern of VEGF mRNA–splice variants in murine skin changes in accordance to the cyclic activity of hair follicles. The 164aa–isoform appears to be
prevailing in telogen and anagen VI skin. In contrast, the shorter isoform VEGF$_{120}$, that is known to lack heparan-binding capability and thus diffuses well through the tissue (NEUFELD et al. 1999), is rarely found in telogen skin but mainly expressed in late anagen skin (Figure 3.3.2, 3.3.5). This indicates that it is involved in anagen–associated vascular growth and maintenance. Quantitative (TaqMan) RT-PCR analysis showed a significant downregulation of VEGF$_{164}$-mRNA levels between telogen and anagen IV. This temporary decline in mRNA levels was not detected by semiquantitative analysis, and still has to be clarified by further studies.

During anagen–development, VEGF immunoreactivity is mainly localized in keratinocytes of the outer root sheath. This suggests that VEGF deriving from this compartment mediates proliferation of endothelial cells in the surrounding (i.e. perifollicular) vasculature (Figure 3.2.8). This is based upon the observation that experimental neutralization of VEGF bioactivity by antibody administration leads to a reduction in cutaneous microvessel density (Figure 3.3.8). The fact, that VEGF originates from the hair follicle epithelium, was confirmed by investigations in transgenic mice which lack endogenous VEGF production by outer root sheath keratinocytes. These animals display a markedly reduced microvessel density in the skin during postnatal development (Figure 3.3.9).

The biological and clinical consequences of this reduction in cutaneous vascular density for hair growth, however, are still unclear. A minimum of angiogenic capacity seems to be necessary to facilitate normal anagen–development of hair follicles, since massive pharmacological angiogenesis-inhibition does retard this developmental process (3.2.2.4). In contrast, the intermittent neutralization of VEGF during a relatively short period of time (3.3.1.5), or the lack of endogenous VEGF during hair follicle morphogenesis (3.3.1.6), apparently do not substantially affect hair follicle morphogenesis/cycling. However, the cutaneous microvessel density is reduced by these measures (Figure 3.3.7, 3.3.8, 3.3.9). One recent report suggests that the long-term neutralization of VEGF bioactivity reduces the diameter of hair bulbs and subsequently of the hair shaft that is produced by it (YANO et al. 2001). This indicates that hair follicle-derived VEGF may indeed affect the diameter of hair shafts, via reducing anagen–associated angiogenesis. It would be interesting to
study this process in details in the VEGF$^{f/f}$ K5-Cre$^+$ -mice, since these mice with their precisely targeted knockout of keratinocyte-derived VEGF do indeed exhibit reduced hair growth after repeated depilation (ROSSITER et al., personal communication). These mice represent an excellent model to investigate whether reduced endogenous production of VEGF can really induce disturbance of hair growth and might therefore be addressed to as a possible pathomechanism of alopecia.

With regard to the role of VEGF for the remodeling processes in the perifollicular vasculature, it is most interesting that VEGF is well known for inhibiting endothelial cell apoptosis (BENJAMIN and KESHET 1997, NOR et al. 1999, HOLASH et al. 1999). The high mRNA levels of VEGF$_{164}$ and VEGF$_{120}$ in late anagen skin suggest that VEGF is involved in maintenance of the perifollicular vasculature. Thus the decline in VEGF mRNA levels during anagen to catagen–transition (Figure 3.3.2, 3.3.5) might represent a relative withdrawl of an inhibitor of endothelial cell apoptosis, contributing to vascular regression which occurs during this hair cycle stage (Figure 3.2.12). This is supported by the fact, that mice which lack endogenous VEGF in outer root sheath keratinocytes exhibit an accelerated decline of the microvessel density during catagen-development of hair follicles (Figure 3.3.9). Most interestingly, catagen-development also appeared to be accelerated in these animals, and it might be speculated that this is due to the limited blood supply by the regressing vasculature (Figure 3.3.9). However, neutralization of VEGF bioactivity after hair follicles have reached anagen stage V did not result in a decline of cutaneous microvessel density (Figure 3.3.8), suggesting that other factors, like angiopoietins (c.f. Table 1.1), are also involved in mediating vascular stabilization and maintenance during anagen.

A highly significant decline of the transcript steady-state levels of all VEGF mRNA–splice variants is detectable immediately after the beginning of catagen–development (Figure 3.3.2, 3.3.5). Simultaneously, apoptosis and degeneration of endothelial cells occurs in the cutaneous microvasculature, finally resulting in partial regression of cutaneous blood vessels (Figure 3.2.12). Therefore, relative deprival of VEGF likely mediates the catagen–associated apoptosis and degeneration observed in endothelial cells of the perifollicular vasculature.
Immunohistochemistry has demonstrated that there is still considerable VEGF-immunoreactivity in the follicular outer root sheath associated with catagen–development of hair follicles, which could be explained by sequestration of VEGF in the extracellular matrix (Figure 3.3.1). Degeneration of endothelial cells and vascular regression, however, are mainly seen within the hypodermis, after the hair follicles have already retracted from this compartment (Figure 3.2.12). Thus, the fraction of VEGF protein that may be sequestered in the extracellular matrix of the hair follicle epithelium during catagen possibly is not available for maintenance of the hypodermal perifollicular vasculature (Figure 3.3.13).

If deprival of VEGF mediates vascular regression by the initiation of endothelial cell apoptosis/degeneration, then a continuous supply of VEGF during initiation of catagen would be expected to lead to a prolonged stabilization of the vasculature. Since VEGF-overexpressing mice (YANO et al. 2001) were not available for us, a provisional experiment was performed by treating mice with recombinant murine VEGF during spontaneous catagen-development. No effect on either the cutaneous microvessels or the hair follicles was observed (Figure 3.3.10). However, this certainly does not exclude that an extended, prolonged and continuous supply of VEGF would eventually have an effect on the vasculature, and corresponding experiments are currently ongoing. Indeed, a retardation of catagen–development was recently reported in transgenic mice that overexpress VEGF in the outer root sheath (M. Detmar, Montagna Symposium on Cutaneous Biology, July 2001, Snowmass, CO).

Effects of VEGF are predominantly mediated via two endothelial cell–specific tyrosine kinase receptors, VEGFR-1 and VEGFR-2 (NEUFELD et al. 1999). Both receptors are expressed in murine skin. In accordance with the report that VEGFR-2 mRNA but not VEGFR-1 mRNA was detectable by in situ-hybridization (DETMAR et al. 1998), a higher expression of VEGFR-2 mRNA than of VEGFR-1 mRNA was seen in murine skin by quantitative RT-PCR (Figure 3.3.5).

Stimulation of VEGFR-2 by VEGF mediates proliferation and survival of endothelial cells (NEUFELD et al. 1999, GILLE et al. 2001). Levels of mRNA of this receptor are
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highest in telogen skin, allowing survival of the vasculature during this hair cycle stage and simultaneously rapid induction of angiogenesis (Figure 3.3.5). Interestingly, mRNA levels for VEGFR-2 decline slightly during early anagen–development of hair follicles, possibly as a means of terminating endothelial cell proliferation. In contrast, mRNA levels of VEGFR-1 are upregulated in anagen VI skin, which may contribute to the stabilization of the vasculature during anagen, since this receptor has already been demonstrated to be crucially involved in vascular maintenance (GILLE et al. 2001). In accordance with this concept, downregulation of VEGFR-1 expression was seen in the regressing vasculature of the ovary during corpus luteum involution (GOEDE et al. 1998). We also observed a significant decline of VEGFR-1 protein expression in many perifollicular blood vessels during catagen (Figure 3.3.5, 3.3.6). This suggests that downregulation of VEGFR-1–expression on vascular endothelium contributes to the induction of endothelial cell apoptosis/degeneration and subsequent vascular regression in murine skin.

**Angiopoietins and their role in hair-cycle dependent remodeling of the perifollicular vasculature**

Since short time neutralization of VEGF bioactivity in anagen skin and substitution of VEGF during catagen–development do not affect the cutaneous vasculature (Figure 3.3.8, 3.3.10), other vascular–specific growth factors, e.g. angiopoietins, are possibly also important for maintenance and remodeling of perifollicular blood vessels.

The current study demonstrates that outer root sheath keratinocytes of the hair follicles are a source of Angiopoietin-1 and Angiopoietin-2. During cutaneous development, only Ang-1 is detectable in this compartment (Figure 3.1.5). Later on, in the adult skin, there is also Ang-2-immunoreactivity evident in these cells, and both angiopoietins are co-expressed during the entire hair growth cycle (Figure 3.3.13). Most interestingly, immunoreactivity was most prominent in keratinocytes of the bulge region, i.e. in the region where the stem cells of the hair follicle are presumed (STENN and PAUS 2001). It can only be speculated about the consequence of this finding, but it suggests that these cells are an important trigger of hair follicle biology.
with functions that go beyond the supply of transient amplifying cells which give rise to the new anagen hair follicle (COTSARELIS et al. 1990).

Ang-1 and Ang-2 share the same receptor, but Ang-1 stimulates it, while Ang-2 is an endogenous receptor antagonist. Thus Ang-1 mediates stabilization of the vasculature, while Ang-2 destabilizes it (DAVIS and YANCOPOULOS 1999, HOLASH et al. 1999). Therefore, the quantity of their expression must be of major importance for determining their biological functions. Interestingly, mRNA levels for both angiopoietins fluctuate in a similar pattern throughout hair follicle cycling, but there are distinct differences remaining which suggest how they are involved in regulating the remodeling of the perifollicular vasculature. (Figure 3.3.9, 3.3.10).

Ang-2 mRNA levels are significantly higher than Ang-1 mRNA levels in those hair cycle stages, that comprise vascular remodeling, i.e. during anagen- and catagen–development of hair follicles. Therefore, it is assumed that upregulation of Ang-2 transcripts in outer root sheath keratinocytes results in destabilization of the perifollicular vasculature and thus facilitates vascular remodeling, which results in either angiogenesis (during anagen–development) or vascular regression (during catagen–development of hair follicles). In contrast, no differences between Ang-1 and Ang-2 mRNA levels were found in mature anagen and in telogen skin, suggesting that these phases of the hair growth cycle are associated with significant stabilization of the vasculature, mediated via Ang-1. Similar functions of Ang-1 and Ang-2 have also been described in other model systems of vascular remodeling (MAISONPIERRE et al. 1997, HOLASH et al. 1999).

Tie-2 mRNA, the common receptor for both angiopoietins, was found to be upregulated in anagen skin (Figure 3.3.14), i.e. when there occurs endothelial cell proliferation in the cutaneous vasculature (Figure 3.2.8). This is perfectly in line with the literature that describes upregulation of the angiopoietin–receptor during angiogenesis in wound healing (WONG et al. 1997), and further supports the concept of Ang-2–involvement in anagen-associated angiogenesis.

The comparison of the mRNA levels of both angiopoietins with those of VEGF reveals obvious and fascinating differences (Figure 4.1): Telogen skin exhibits a high
level of both angiopoietins and VEGF. In anagen IV skin, mRNA levels of VEGF\textsubscript{120} and Ang-2 are still high, while those of VEGF\textsubscript{164} and Ang-1 have decreased. It can be assumed that the latter two contribute to stabilization of the vasculature and that their downregulation facilitates destabilization and subsequent growth of the perifollicular vasculature, which occurs during this hair cycle phase (Figure 3.2.8). In mature anagen skin, mRNA levels of both angiopoietins are similarly low, while levels of VEGF\textsubscript{120} and VEGF\textsubscript{164} are on their highest levels throughout the entire hair growth cycle. Since the VEGFR-1 is clearly upregulated during this hair cycle phase, it is assumed that VEGF mediates vascular maintenance via binding to this receptor. In contrast to this, catagen skin reveals the lowest mRNA levels of both VEGF isoforms, whereas those of both angiopoietins are clearly rising (Figure 4.1). Most interestingly, similar to anagen IV, Ang-2 mRNA levels are higher than those of Ang-1, suggesting that blood vessels are destabilized again, but opposed to anagen IV, they regress, possibly due to a lack of sufficient VEGF expression (Figure 4.1).

4.4 Hypothetical concept: How hair follicle–derived growth factors may influence the perifollicular vasculature

So far, it has been well appreciated that vascular homeostasis is regulated by the complex orchestration of a multitude of growth factors (HANAHAN 1997, DETMAR 1996, FOLKMAN 1997, YANCOPOULOS et al. 2000). The current study suggests that the molecular regulation of hair cycle–associated vascular remodeling is equally complex. However, the morphological, immunohistological, gene expression and functional data presented above, suggest to propose a hypothetical concept that can form the basis for the design of follow-up studies. This concept attempts to explain how secreted growth factors, deriving from the hair follicle epithelium, influence the perifollicular vasculature via their endothelial cell–specific receptors (Figure 4.2):

Telogen skin reveals a state of vascular quiescence, which is mediated by the balanced expression of many growth factors and their receptors. Comparable to other tissues of the adult organism, VEGF, Ang-1 and Ang-2 are the most important growth factors, and they are all expressed by outer root sheath keratinocytes of
telogen hair follicles. VEGF$_{164}$ is the most prominently expressed VEGF isoform in telogen mouse skin and mediates quiescence of the existing vascular system (CARMELIET et al. 1996, FERRARA et al. 1996). However, vascular quiescence is not based on VEGF$_{164}$ alone. Angiopoietins also contribute to vascular quiescence during this hair cycle stage, in as far as Ang-1, expressed by outer root sheath keratinocytes and perivascular cells, stabilizes cutaneous blood vessels (Figure 4.2).

Mid-anagen skin reveals a predominance of Ang-2 over Ang-1. This antagonization of Ang-1-binding to the upregulated Tie-2 receptor on endothelial cells results in destabilization of the perifollicular vasculature by inhibiting interactions between endothelial cells and pericytes (MAISONPIERRE et al. 1997). Since VEGF$_{120}$ and VEGF$_{164}$ are still considerably expressed in mid-anagen skin, VEGF stimulates proliferation of endothelial cells with subsequent growth of the perifollicular vasculature, i.e. angiogenesis (Figure 4.2).

In mature anagen skin, the angiogenic processes are eventually terminated, either by upregulation of VEGFR-1 or by downregulation of VEGFR-2 on endothelial cells. In any case, this leads to the stabilization of the existing, partially newly formed blood vessels, mediated by VEGF-binding to VEGFR-1. Angiopoietins apparently do not play an important role for maintaining the vasculature, especially since Ang-2 levels are similar to Ang-1 levels. However, it may be assumed that prolonged existence of mature anagen hair follicles, as it occurs in most mammals, is associated with eventual vascular quiescence, that equals the vascular state of telogen skin.

Catagen—induction of hair follicles is associated with a vascular switch in the murine skin, i.e. a tremendous change in the expression profiles of different vascular growth factors and their receptors. Vascular remodeling again is promoted by destabilization of the vasculature via relative upregulation of Ang-2. Moreover, VEGFR-1 is downregulated again, further supporting the destabilization of perifollicular blood vessels (GOEDE et al. 1998). Thus, with regard to the angiopoietins, a situation that is comparable to mid-anagen skin develops, which clearly allows vascular remodeling. However, there is a marked difference in the expression levels of all VEGF isoforms between anagen and catagen skin. VEGF—synthesis of outer root
sheath keratinocytes ceases, and thus deprives an important survival factor of endothelial cells. Apoptosis and degeneration consequently is induced in endothelial cells, and subsequently the blood vessels that surround the proximal portion of the hair follicle regress. Similar mechanisms of blood vessel regression have been described in other biological model systems (MAISONPIERRE et al. 1997, HOLASH et al. 1999).

Vascular regression is terminated by the upregulation of Ang-1, which stabilizes blood vessels, and by the eventual upregulation of VEGF\textsubscript{164}, that also contributes to vascular quiescence, once the hair follicles have reached the telogen stage of their cycle again.

Figure 4.1 (page 143): Summary of the cutaneous mRNA expression of vacular growth factors and their receptors, and association with remodeling–processes in the perifollicular vasculature. The mRNA levels derive from quantitative analysis by TaqMan RT-PCR and are represented in percentage of the telogen-values (compare to Figures 3.3.5, 3.3.9). This summary is hypothetical in as far, as telogen values after completion of the hair cycle were presumed to be similar to telogen values before cycling of hair follicles, although this has not been proven for all factors. Angiogenesis in the perifollicular vasculature is associated with upregulation of VEGF\textsubscript{120} and Tie-2, and temporary downregulation of VEGF\textsubscript{164} and Ang-1. Mature anagen skin is associated with high levels of VEGF\textsubscript{120} and VEGF\textsubscript{164} and low levels of both angiopoietins. VEGFR-1 is upregulated during this hair cycle phase (compare to VEGFR-2). This is probably associated with maintenance of the perifollicular vasculature (see Figure 4.2). Catagen –development of hair follicles and subsequent vascular regression is associated with low levels of VEGF\textsubscript{120} and VEGF\textsubscript{164} and rising levels of both angiopoietins. Note that Ang-2 mRNA levels are rising faster than those of Ang-1. VEGFR-1 is downregulated on endothelial cells during vascular regression.
Figure 4.2: Hypothetical concept of how hair follicle–derived growth factors influence the perifollicular vasculature during hair follicle cycling, via binding to their specific receptors on endothelial cells. The two time points of vascular remodeling are indicated by the background color. Vascular quiescence in telogen skin is achieved by the balanced expression of multiple growth factors, especially VEGF\textsubscript{164} and Ang-1. A relatively high expression of Ang-2 during anagen–development of hair follicles facilitates angiogenesis by destabilization of perifollicular blood vessels. VEGF\textsubscript{120} (relatively higher expressed than VEGF\textsubscript{164}) promotes vascular growth via VEGFR-2. In mature anagen skin, the perifollicular vasculature is stabilized by Ang-1 and VEGF\textsubscript{120}/ VEGF\textsubscript{164}, acting via VEGFR-1, which is upregulated on endothelial cells. It would be conceivable that prolonged anagen would finally result in a condition of vascular quiescence, similar to telogen skin. Catagen–development of hair follicles is accompanied by downregulation of VEGF and a simultaneous increase in Ang-2 expression. This leads to vessel destabilization and, in the absence of VEGF, facilitates vascular regression. Quiescence of the vasculature is finally achieved again by the balanced expression of growth factors after hair follicles have reached the telogen stage.
4.5 Hair follicle cycling in murine skin is an instructive model system for physiological blood vessel remodeling

Angiogenesis is a crucial event in many physiological and pathological scenarios (FOLKMAN 1995, CARMELIET and JAIN 2000). It has been investigated intensively during the last two decades, but our understanding of the cellular and molecular mechanisms of vascular remodeling under physiological circumstances is still very limited (YANCOPOULOS et al. 2000). This is primarily due to the lack of appropriate in vivo models in which the multiple steps of vascular morphogenesis and remodeling can be studied precisely (AUGUSTIN 2001). Therefore, model systems of physiological angiogenesis that encompass eventual maturation or regression of vessels, are essential research tools for investigating the balance of interacting angiogenesis stimulators and inhibitors (YANCOPOULOS et al. 2000). Today, only the female reproductive tract has been recognized as a suitable model in this regard (FRASER and LUNN 2000, GOEDE et al. 1998). However, in this study it is shown that murine skin also displays significant angiogenesis during synchronized anagen–development, and significant regression of blood vessels during synchronous catagen–development of the hair follicles. Comparable to luteolysis, catagen development is a tightly regulated physiological process of tissue involution (STENN and PAUS 2001). The current study demonstrates that, similar to the ovary (AUGUSTIN et al. 1995, MODLICH et al. 1996), blood vessel remodeling is a functionally important, essential event in the hair cycle-associated reorganization of the perifollicular mesenchyme.

Thus, hair cycle–dependent vascular remodeling in C57BL/6 mice offers an excellent model for dissecting and manipulating both physiological angiogenesis and physiological vascular regression in the adult mammalian organism in general, and of cutaneous and perifollicular blood vessel remodeling in particular.
4.6 Perspectives for future research

The data presented in this study and in resulting publications (MECKLENBURG et al. 2000) establishes that the vascular system in mouse skin is far from being quiescent. Instead it undergoes substantial cyclic remodeling processes, that are associated with and governed by the activity of hair follicles. Furthermore, we present evidence for the involvement of VEGF and angiopoietins in the regulation of these remodeling processes. However, many open questions remain that have to be addressed by subsequent experimental studies:

Which further investigations could contribute to the validity of the presented concept?

In the current study, determination of mRNA levels of VEGF and angiopoietins was limited to four time-points of the depilation-induced murine hair growth cycle. In order to obtain a continuous time-profile of the expression of these important growth factors during the hair growth cycle, more points of measurement would be necessary. In addition, investigation of a larger population of animals would facilitate the confirmation of the statistical significance of observed differences. Cell capture by laser microdissection would allow to study a clearly definable population of cells, i.e. outer root sheath keratinocytes of the bulge region, and would probably overcome the problem of normalization that we faced during our quantitative RT-PCR analyses.

More detailed investigation of Ang-1- and Ang-2–gene expression could help to clarify the mechanism of their interaction at each point of time during the hair cycle. Transgenic mice that express reporter genes under the control of either angiopoietin-promoter, e.g. Ang-2/LacZ-mice (YUAN et al. 2000), can serve as suitable models to answer this question, and are currently under investigation.

Furthermore, the effects of Ang-1 and Ang-2 on the regulation of remodeling of the perifollicular vasculature could be investigated in mice that overexpress Ang-1 in outer root sheath keratinocytes (MAISONPIERRE et al. 1997) and in mice, overexpressing Ang-2 in blood vessels (SURI et al. 1998). Overexpression of Ang-1
should lead to prolonged stabilization of the vasculature and would thus disturb both anagen-associated angiogenesis and catagen-associated vascular regression. In contrast, mice that overexpress Ang-2 in blood vessels should exhibit a destabilized vasculature, possibly leading to disturbed maintenance of the perifollicular blood vessels in mature anagen skin. Mice with targeted gene ablation, similar to those that lack VEGF in keratin 5-expressing cells, would provide further insight in the role of hair follicle-derived angiopoietins, but have not been generated so far.

**Which other factors are involved in the hair cycle-dependent vascular remodeling in the skin?**

As is demonstrated in Table 1.1, a multitude of factors participate in the regulation of vascular remodeling. Therefore, it is rather likely that, beside VEGF and the angiopoietins, other factors are also involved in regulating the hair cycle-dependent remodeling of the perifollicular vasculature.

Most of these factors are pleitrop, i.e. they mediate different biological effects in various tissues. PDGF-B, TGF-β1, FGF, HGF and prostaglandins (generated by cyclooxygenase-2 activity) are not only involved in angiogenesis but are also part of the epithelial-mesenchymal interactions that participate in hair follicle morphogenesis/cycling (MCELWEE and HOFFMANN 2000).

Furthermore, degradation of the extracellular matrix occurs both during anagen- and catagen-development of hair follicles (MESSENGER 1991, PAUS et al. 1994ab). An array of proteinases, e.g. proteinases of the plasminogen activator family and matrix metalloproteinases, is activated and makes it possible for the hair follicle to extend into and retract from the hypodermis (WEINBERG et al. 1990, GOODMAN and LEDBETTER, KARELINA et al. 1994, PAUS et al. 1994ab, SCANDURRO et al. 1995, YAMAZAKI et al. 1999, JENSEN et al. 2000, JAROUSSE et al. 2001). These proteinases might not only influence the mesenchymal remodeling during the hair growth cycle but also effect angiogenesis directly or indirectly via the liberation of several growth factors that are sequestered in the extracellular matrix, e.g. fibroblast...

**Is the long-term deprival of VEGF a pathogenetic mechanism of hair loss?**

It has been claimed that a reduced blood supply is a pathogenic factor of hair loss (CHIALE 1927, ELLIS 1958, MONTAGNA and PARAKKAL 1974). By pharmacological inhibition of angiogenesis we could indeed show that there is a reduction of the microvessel density in the skin, which leads to inhibition of anagen-development. However, nothing is known so far about the long-term effect of a reduced cutaneous vascularization on hair growth. We have demonstrated that short-time deprival of VEGF is one mechanism that leads to a reduction of the cutaneous blood supply, and it has been reported that lack of VEGF-bioactivity results in a decreased diameter of hair shafts (YANO et al. 2001).

Therefore, it is interesting to study the long-term effect of lack of VEGF on hair follicles. We started with studies in transgenic mice that lack endogenous VEGF in the follicular outer root sheath. In these mice, a reduced hair growth after repeated depilation has been reported (ROSSITER et al., unpubl. observation), but not carefully studied so far. Corresponding studies are on their way.

**Can hair growth be influenced by manipulating the perifollicular vasculature?**

Based on our findings that the lack of endogenous VEGF results in a destabilized cutaneous vasculature which regresses faster during catagen, it would be interesting to investigate whether maintenance of the vasculature (e.g. by prolonged administration of VEGF or Ang-1) would prolong-anagen and delay catagen-development of the hair follicles. Conversely, a reduction of the perifollicular vasculature might be able to induce catagen-development and would thus be exploitable as a therapeutical approach to treat excessive hair growth.
5. SUMMARY

Vascularization not only guarantees nourishment of the tissue, but is also of major importance for tumor growth and metastasis. This has led to a vast amount of investigations that elucidated the mechanisms and molecular controls of blood vessel growth and regression. While most of these studies were focussed on pathological conditions, it became clear that blood vessel remodeling actually is a physiological process, that only occurs temporarily limited in certain tissues of the female reproductive tract.

The current study aimed at verifying the hypothesis that physiological vascular remodeling also occurs in the skin. Speculating that hair follicles can actively modulate their surrounding vasculature by synthesizing blood vessel-specific growth factors in accordance to their metabolic demand, the depilation-induced hair growth cycle in mouse skin was analyzed to verify this hypothesis. It was investigated whether synchronized hair growth in mice is associated with growth and regression of cutaneous blood vessels, and whether vascular endothelial growth factor (VEGF) and angiopoietins, the two most important families of blood vessel-specific growth factors, are involved in their regulation. Furthermore, a pharmacological manipulation of VEGF and the lack of endogenous VEGF in transgenic mice were examined with regard to their effect on the cutaneous vasculature and hair growth.

Morphometric analyses on CD31-stained tissue sections revealed significant changes in the microvessel density and the number of endothelial cells in the cutaneous vasculature, implicating that growth and regression of blood vessels occurs throughout the hair growth cycle. Immunohistological double-staining techniques and electron microscopy were used to show that indeed angiogenesis, comprising proliferation of endothelial cells, occurs subsequent to anagen-development of the hair follicles. In contrast, catagen-development is associated with vascular regression, driven by apoptosis and degeneration of endothelial cells, as was demonstrated by *in situ*-apoptosis detection and electron microscopy.
Immunohistochemistry revealed that the outer root sheath keratinocytes of hair follicles are the predominant source of VEGF and angiopoietins. By qualitative and quantitative (TaqMan) RT-PCR analyses it was shown that the expression of these growth factors and their endothelial cell-specific receptors changes temporally in accordance to the activity of hair follicles.

The importance of adequate hair cycle-dependent vascular remodeling was demonstrated by the fact that massive inhibition of angiogenesis leads to a retardation of anagen-development. Investigations in transgenic mice that lack endogenous VEGF in outer root sheath keratinocytes suggest that VEGF in fact is of major importance for the homeostasis of the cutaneous vasculature. However, we demonstrate that the system of growth factors that dictate hair growth-associated vascular remodeling is very complex, since short-term manipulation of single growth factors does not exert definite effects on hair follicle cycling.

Based on these findings we present a hypothetical concept of how hair follicles modulate their blood supply according to their metabolic demand. It is concluded that hair follicle cycling in mouse skin provides an easily accessible and intriguing model for the study of physiological blood vessel remodeling, and it is suggested to explore manipulation of the perifollicular vasculature as a new therapeutical approach to manage hair growth disorders.
6. ZUSAMMENFASSUNG

Ein neues Modell der Blutgefäß-Umstrukturierung: Haarfollikel modulieren das perifollikuläre Gefäßnetz durch die Synthese von Schlüsselfaktoren für die Blutgefäß-Homöostase (Dr. Lars Mecklenburg, Hamburg)


Morphometrische Analysen an CD31-gefärbten Gewebeschnitten haben gezeigt, dass während des Haarzyklus signifikante Veränderungen in der Gefäßdichte und der Anzahl von Endothelzellen in der Haut auftreten. Dies deutet auf aktives Wachstum und anschließende Regression der kutanen Gefäße innerhalb eines

Immunhistochemische Untersuchungen haben gezeigt, dass die Keratinozyten der äußeren Wurzelscheide der Haarfollikel die hauptsächliche Quelle von VEGF und Angiopoietinen in der Haut darstellen. Mittels qualitativer und quantitativer (TaqMan) RT-PCR Techniken wurde gezeigt, dass die Expression dieser Wachstumsfaktoren und deren endothelzell-spezifischen Rezeptoren abhängig ist von der zyklischen Aktivität der Haarfollikel.

Die biologische Bedeutung dieser Gefäßumbauprozesse während des Haarzyklus wurde dadurch aufgezeigt, dass eine massive Hemmung der Angiogenese die Anagenentwicklung der Haarfollikel verzögern kann. Untersuchungen in transgenen Mäusen, denen endogenes VEGF fehlt, lassen vermuten, dass VEGF tatsächlich von entscheidender Bedeutung ist für die Gefäßhomeostase in der Haut. Darüber hinaus wird jedoch verdeutlicht, dass das System der Wachstumsfaktoren, die die haarzyklus-abhängigen Gefäßumbauprozesse steuern, sehr komplex ist. So hat die kurzzeitige Manipulation eines einzelnen Wachstumsfaktors keinen eindeutigen Effekt auf die Haarfollikelaktivität hervorgebracht.

Auf den Untersuchungsergebnissen dieser Studie basierend wird ein hypothetisches Modell vorgestellt, wie Haarfollikel, in Abhängigkeit von ihren metabolischen Ansprüchen, die sie umgebende Gefäßnetz aktiv modulieren. Der Haarwechsel in der murinen Haut stellt demzufolge ein leicht zugängliches, äußerst interessantes Modellsystem dar, an dem die physiologischen Prozesse des Gefäßumbaus untersucht werden können. Ferner erscheint es sinnvoll, die Manipulation des perifollikulären Gefäßnetzes als einen möglichen Ansatzpunkt für die Behandlung von Haarwachstumsstörungen zu untersuchen.
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parts of this study have been published in the following form:

**original articles:**


**abstracts:**

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ACKNOWLEDGEMENT

I am especially grateful to Ralf Paus, who initiated and enthusiastically supported this study.

Furthermore, I would like to thank the supervisor, Marion Hewicker-Trautwein, for her confidence and continuous support.

I deeply acknowledge the support of everybody, who actively contributed to this study. These are especially:


Last but not least, I am grateful to my wife Anja and my parents who contributed by proof-reading the thesis and by continuous support.