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Active Hair Growth (Anagen) is Associated with Angiogenesis

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After the completion of skin development, angiogenesis, i.e., the growth of new capillaries from pre-existing blood vessels, is held to occur in the skin only under pathologic conditions. It has long been noted, however, that hair follicle cycling is associated with prominent changes in skin perfusion, that the epithelial hair bulbs of anagen follicles display angiogenic properties, and that the follicular dermal papilla can produce angiogenic factors. Despite these suggestive observations, no formal proof is as yet available for the concept that angiogenesis is a physiologic event that occurs all over the mature mammalian integument whenever hair follicles switch from resting (telogen) to active growth (anagen). This study uses quantitative histomorphometry and double-immunohistologic detection techniques for the demarcation of proliferating endothelial cells, to show that synchronized hair follicle cycling in adolescent C57BL/6 mice is associated with substantial angiogenesis, and that inhibiting angiogenesis *in vivo* by the intraperitoneal application of a fumagillin derivative retards experimentally induced anagen development in these mice. Thus, angiogenesis is a physiologic event in normal postnatal murine skin, apparently is dictated by the hair follicle, and appears to be required for normal anagen development. Anagen-associated angiogenesis offers an attractive model for identifying the physiologic controls of cutaneous angiogenesis, and an interesting system for screening the effects of potential antiangiogenic drugs *in vivo*. ***Key words: C57BL/6/PECAM-1/skin/TNP-470. J Invest Dermatol 114:909-916, 2000***

In contrast to embryonal vasculogenesis, angiogenesis is defined as the growth of new capillaries from pre-existing blood vessels (Hudlická *et al*, 1998). In the skin, angiogenesis is a common feature during embryonal and fetal development (Detmar, 1996), whereas secondary angiogenesis in adult skin is widely held to occur only under pathologic conditions (Folkman, 1995), such as wound healing (Arnold and West, 1991), hyperproliferative inflammatory skin diseases like psoriasis (Detmar *et al*, 1994), and in association with a wide range of tumors (Folkman, 1987, 1990, 1996). In fact, one authoritative view holds that, in adult mammalian tissues, there is essentially no physiologically occurring angiogenesis at all, with the notable exception of the ovarian and endometrial cycles and the lactating mammary gland (Folkman and Shing, 1992; Matsumoto *et al*, 1992).

There has long been circumstantial evidence, however, to suggest that there is also at least one phenomenon of physiologically occurring angiogenesis in adult mammalian skin. Namely, it has been noted that there are prominent rearrangements of the skin vasculature and skin perfusion associated with hair follicle cycling (Durward and Rudall, 1958; Ellis and Moretti, 1959; Forbes, 1967; Montagna and Parakkal, 1974). For example, dye-injection studies in rat and rabbit skin have revealed dramatic changes in the appearance and arrangement of perifollicular and interfollicular blood vessels during synchronized switches of large groups of hair follicles from the growth stage of the hair cycle (anagen) to resting (telogen) and vice versa (Durward and Rudall, 1958). Also, during human hair follicle regression (catagen), some degeneration of the capillary loops within the dermal papilla has been noted (Ellis and Moretti, 1959). Furthermore, the dermis around anagen hair follicles is more vascular than that around telogen follicles, and only during anagen have proliferating endothelial cells (PEC) been noted inside the follicular dermal papilla (Sholley and Cotran, 1976). Finally, in the rabbit corneal pouch assay, the epithelial hair bulb of rat anagen follicles possesses angiogenic properties (Stenn *et al*, 1988), and both outer root sheath keratinocytes and dermal papilla fibroblasts reportedly are sources of at least one key angiogenic factor [vascular endothelial growth factor (VEGF)] *in vivo* and *in vitro* (e.g., Goldman *et al*, 1995; Lachgar *et al*, 1996, 1998; Kozlowska *et al*, 1998).

Taken together, these observations are quite suggestive for the occurrence of angiogenesis whenever a hair follicle traverses from telogen to anagen. Yet, none of the above studies has provided formal proof for the concept that anagen-associated angiogenesis is a physiologic event that occurs in vast regions of the mammalian integument, and as long as hair follicles are cycling, i.e., during the entire lifespan of the mammalian organism (Paus and Cotsarelis, 1999). The challenge is to provide convincing evidence of angiogenesis during anagen development, as distinct from mere hair-cycle-associated changes in vessel calibers and perfusion, which

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Abbreviations: HGF/SF, hepatocyte growth factor/scatter factor; MVD, microvessel density (microvessels per microscopic field); p.d., post-depilation (day after anagen induction by depilation); PEC, proliferating endothelial cells; PECAM-1, platelet/endothelial cell adhesion molecule-1; TNP-470, O-chloroacetylcarbamoyl-fumagillol; VEGF, vascular endothelial growth factor (vascular permeability factor).

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must not be confused with genuine angiogenesis (Hudlická *et al*, 1998).

This study attempts to provide such evidence by combining morphometric and functional assays in a mouse model of highly synchronized hair follicle cycling that is uniquely suited for addressing this question. Anagen was induced by depilation in the back skin of adolescent C57BL/6 mice (Paus *et al*, 1990). Changes in standard morphologic criteria of angiogenesis, namely microvessel density (MVD), vessel calibers, and the number of endothelial cell nuclei [all assessed with the help of immunohistologic staining of endothelial cells with an antibody against CD31 (platelet/endothelial cell adhesion molecule-1, PECAM-1) (Horak *et al*, 1992; Couffinhal *et al*, 1998)], and in the number of proliferating (Ki-67-immunoreactive) endothelial cells were assessed qualitatively and quantitatively in defined reference areas throughout the hair cycle. High resolution light microscopy and transmission electron microscopy (TEM) were also used to search for mitotic endothelial cells. In addition, the effect of a widely used standard inhibitor of angiogenesis, the fumagillin derivative O-chloroacetylcarbamoyl-fumagillol (TNP-470) (Castronovo and Belotti, 1996), on anagen development *in vivo* was assessed in these mice. Collectively, these studies provide evidence that anagen development in mice is truely associated with angiogenesis.

MATERIALS AND METHODS

Animals and tissue collection Female C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany) and were housed in community cages under standard conditions (12 h light/dark cycle, water and mouse chow *ad libitum*). Active hair growth was induced as previously described (Paus *et al*, 1990; 1997; Slominski *et al*, 1991) by depilation in the back skin of 6- to 9-wk-old mice with all back skin hair follicles in telogen. Animals were sacrificed by cervical dislocation at all key stages of the depilation-induced hair cycle; back skin was dissected at the level of the subcutis just below the panniculus carnosus and was embedded by using a special technique for obtaining longitudinal cryosections through the hair follicles from one defined site (Paus *et al*, 1999).

Immunohistochemical staining for CD31 (PECAM-1) Cryosections were stained for CD31 (PECAM-1) immunoreactivity using a rat monoclonal antibody 1:2000 in Tris-buffered saline (clone 13.3, Pharmingen, San Diego, CA). CD31 is currently considered to represent one of the most specific markers for endothelial cells (Horak *et al*, 1992; Couffinhal *et al*, 1998). Immunostaining was performed according to our previously published protocols (cf. Eichmüller *et al*, 1996; 1998; Paus *et al*, 1998) employing the ABC technique (Vectastain Elite-Kit, Vector, Burlingame, CA).

Immunofluorescent staining for CD31 (PECAM-1) To count nuclei of endothelial cells, we performed double immunofluorescent staining for CD31 and 4′,6-diamidin-2′-phenylindol-dihydrochloride (DAPI; Boehringer Mannheim, Mannheim, Germany). Following previously published protocols (Botchkarev *et al*, 1997) cryosections were incubated with the monoclonal antibody against murine CD31, 1:2000 in Tris-buffered saline, employing a fluorescein isothiocyanate-labeled goat antirat-IgG antibody (1:20; Dianova, Hamburg, Germany). Sections were then incubated with DAPI (1 μg per ml) and mounted with embedding medium (Vector). The procedure results in a green cytoplasmatic staining of CD31-immunoreactive cells and a blue staining of all cell nuclei, allowing nuclei of CD31-immunoreactive cells to be counted.

Immunofluorescent double-staining for CD31 (PECAM-1) and Ki-67 antigen For the detection of proliferating endothelial cells, cryosections were double-stained for Ki-67, a widely used proliferation marker, maximally expressed during the S-phase of the cell cycle (cf. Vermeulen *et al*, 1995, 1997; Christenson and Stouffer, 1996; Creamer *et al*, 1997; Gerdes *et al*, 1997), and CD31 (PECAM-1). Sections were treated as described above to stain for CD31 immunoreactivity. After incubation with the secondary antibody, sections were pretreated again with goat normal serum and finally were incubated with a polyclonal rabbit antimouse Ki-67 antibody diluted 1:100 (Dianova), followed by a Cy3-labeled goat antirabbit-IgG antibody (1:200). Double-immunoreactive cells were identified as cells that reveal a red intranuclear staining (Ki-67) that is surrounded by a green cytoplasmatic staining (CD31), resulting in a small yellow-appearing zone around the nucleus, where both colors superimpose.

Controls All staining procedures were performed with negative controls (section without primary antiserum and section with primary antibody to an irrelevant antigen). As positive controls, the observed immunoreactivity patterns were compared with the published immunoreactivity patterns for CD31 and Ki-67, respectively, in murine skin (Gerdes *et al*, 1997; Couffinhal *et al*, 1998).

Morphometry In general, each parameter (immunoreactivity pattern) was scrutinized by investigating three sections per mouse, and three mice were assessed per hair cycle stage.

Assessment of MVD Sections stained for CD31 with the immunperoxidase technique were investigated at 400× magnification. Thirty microscopic fields per animal were evaluated for MVD, i.e., the number of microvessels per microscopic field (cf. Vartanian and Weidner, 1994; Hudlická *et al*, 1998). The microscopic fields were positioned distal to the subcutaneous muscle layer (panniculus carnosus), so as to evaluate major parts of the subcutaneous and dermal level 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 and 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 cycle, as the thickness of all skin compartments and the size of the hair follicles fluctuate significantly during the murine hair cycle (Chase *et al*, 1953; Paus *et al*, 1990). 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*, 1994). This compartment correction did not reveal any major changes in the hair-cycle-dependent MVD, however, compared with the original data (data not shown).

Assessment of mean vessel diameter and mean vessel length Employing CD31-stained mouse skin sections and a digital imaging system (Openlab, Improvision, Coventry, U.K.), the diameter of 150 transversally cut vessels and the length of 150 longitudinally cut vessels (derived from three different animals) was measured in sections of telogen skin [day 0 post-depilation (p.d.)] and anagen VI skin (day 12 p.d.).

Assessment of endothelial cell nuclei Sections stained for CD31 with the immunofluorescence technique and double-stained with DAPI were used to count endothelial cell nuclei in the interfollicular dermis and subcutis between two neighboring longitudinally cut hair follicles. The interfollicular region was chosen as reference area because the distance between two hair follicles remains relatively constant throughout the entire murine hair cycle. Endothelial cell nuclei were counted in 20 interfollicular areas per mouse in telogen (day 0 p.d.) and anagen VI (day 12 p.d.) skin.

Assessment of proliferating endothelial cells Sections stained for CD31 with the immunofluorescence technique were investigated at 400× magnification. Twenty-five microscopic fields per mouse were evaluated for the number of double-immunoreactive cells. The microscopic fields were positioned distal to the panniculus carnosus.

High resolution light microscopy and TEM Representative tissue samples of all depilation-induced hair cycle stages were fixed in Karnovsky's fixative (Karnovsky, 1965), postfixed in 2% osmium tetroxide and uranyl acetate, and embedded in resin as previously described (Tobin *et al*, 1991). Semithin sections were cut, stained with toluidine blue/borax, and examined by light microscopy. Ultra-thin sections were stained with uranyl acetate and lead citrate and then examined using a Joel 100CX electron microscope (Tobin *et al*, 1998).

Angiogenesis inhibition *in vivo* To investigate the effect of angiogenesis inhibition on the hair growth cycle, mice were treated with the fumagillin derivative TNP-470 (Schering AG, Berlin, Germany). TNP-470 is a very effective angiogenesis inhibitor with no major side-effects that has already been used in clinical trials (Castronovo and Belotti, 1996). TNP-470 was administered intraperitoneally once a day beginning with the day of depilation (day 0 p.d.) until animals were sacrificed for skin

harvesting. Dosages of 3, 6, and 12 mg TNP-470 per kg, dissolved in peanut oil with 0.24% ethanol, were used in three independent experiments. First, a group of seven mice was treated with TNP-470 (6 mg per kg) for 6 d, and a control group of seven mice received the vehicle only. Back skin was harvested after 6 d. Second, three different concentrations were tested in a total of three mice per tested dosage or vehicle alone, and back skin was harvested after 6 d. Third, a group of three mice treated with TNP-470 (6 mg per kg) and a group of control animals were sacrificed after 8 d of treatment. Harvesting of caudal back skin, tissue processing, and preparation of cryostat sections were performed as described above.

Cryostat sections were stained for endogenous alkaline phosphatase activity that is known to be expressed in the dermal papilla (Handjiski *et al*, 1994), and that can serve as a standard morphologic criterion for assessment of the exact hair cycle stage. The hair cycle stage of at least 60 hair follicles per mouse was assessed and recorded for statistical analysis.

Statistical analysis To evaluate statistical significance, the mean and the standard error of the mean as well as the p-values were calculated, using the Mann–Whitney U test.

RESULTS

This study provides evidence for the existence of hair-cycle-dependent angiogenesis in murine skin, using morphometric and functional assays in the well-characterized C57BL/6 mouse model for hair research (Paus *et al*, 1990). Cutaneous microvessels were visualized by immunohistochemical staining with a monoclonal antibody against PECAM-1/CD31, considered to be a highly specific marker for endothelial cells (Horak *et al*, 1992). This marker allows arteries, capillaries, and venules alike to be detected (Braverman, 1999). Multiple transversally cut vessels with a distinct central lumen, located just above the panniculus carnosus, represent the lower horizontal plexus. The upper horizontal plexus is located in the upper half of the dermis, in close proximity to the follicular isthmus and the sebaceous glands. Ascending vessels connect both plexuses and are seen as longitudinal structures within the dermis and subcutis. They connect to multiple small and short vessels that are distributed equally within the whole dermal tissue (Braverman, 1997) (**Fig 1*A–D***).

The cutaneous microvasculature undergoes substantial rearrangements during the murine hair cycle Vessels in unmanipulated telogen mouse skin (day 0 p.d.) appear much smaller in diameter and length and more tortuous than vessels in anagen VI skin (day 12 p.d.) (**Fig 1*A–D***). They are mainly located in the proximal part of the dermis and the subcutis, giving a somewhat condensed appearance. Endothelial cell nuclei are of flat, elongated shape (**Fig 1*E***, *insert*). In contrast, vessels in anagen skin have a straight course, many branches, and often show a distinct central lumen (**Fig 1*C*, *D***). Their nuclei are ovoid in shape, and appear to be larger than those in telogen skin (**Fig 1*G***, *insert*).

Vessels have a significantly larger median diameter ($p \leq 0.05$) and measurable length ($p \leq 0.05$) in anagen compared with telogen skin (**Fig 2*A*, *B***). These morphologic changes in the arrangement of the cutaneous microvasculature, however, are associated with very substantial remodeling of the entire dermis and subcutis, which is increased threefold in thickness (Chase *et al*, 1953; Paus *et al*, 1990)

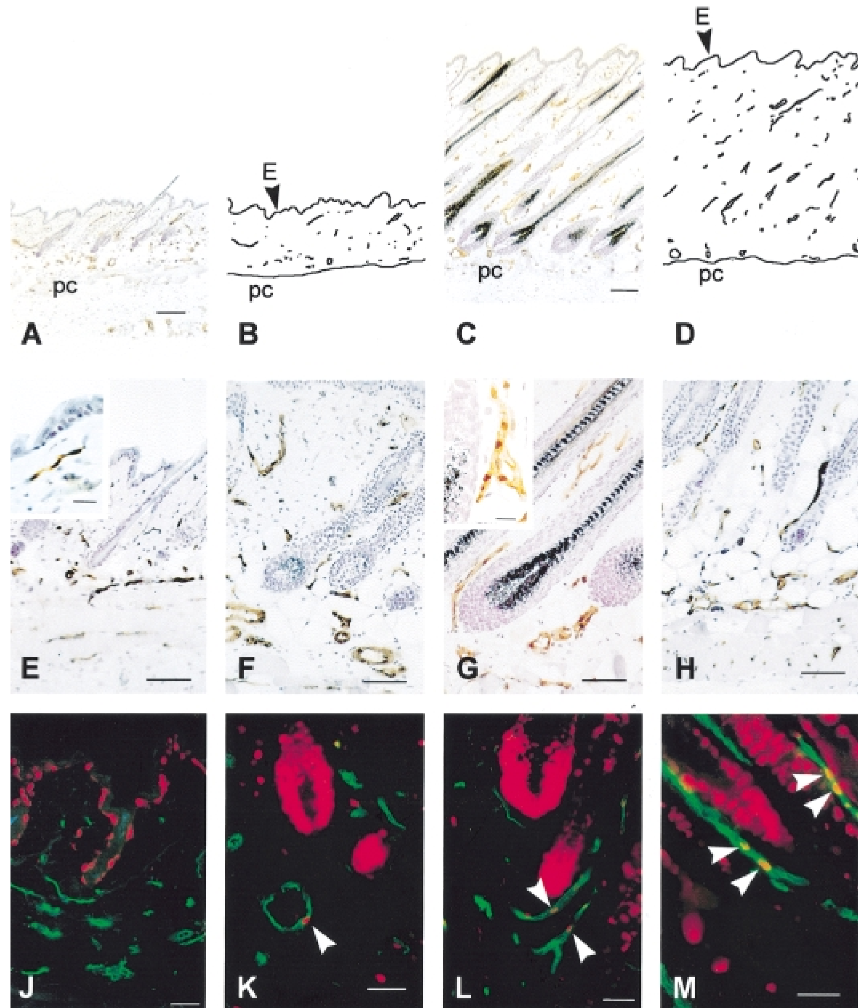

Figure 1. Hair-cycle-dependent rearrangement of the cutaneous microvasculature. (*A*)-(*D*) Histologic sections of mouse skin, which were stained immunohistochemically for the endothelial-cell-specific marker CD31 (PECAM-1), and schematic drawings of the CD31-immunoreactive cell aggregates reveal differences in the arrangement of the cutaneous microvasculature between telogen skin (day 0 p.d.) (*A*, *B*) and anagen VI skin (day 12 p.d.) (*C*, *D*); the cutaneous microvasculature was evaluated between the epidermis (*E*) and the panniculus carnosus (pc). (*E–H*) Histologic sections, stained immunohistochemically for CD31 (PECAM-1), reveal hair-cycle-dependent changes in vessel diameter, vessel length, MVD, and in the morphology of endothelial cell nuclei. In telogen skin (day 0 p.d.) (*E*) dermal vessels are small in diameter and length, and capillaries show flattened endothelial cell nuclei (*insert*); dermal vessels increase in diameter and length in anagen IV skin (day 5 p.d.) (*F*); they reach maximal length and diameter in anagen VI skin (day 12 p.d.), where vessels show many branches (*G*) and endothelial cell nuclei have an oval shape (*insert*); during hair follicle regression (catagen, day 19 p.d.) cutaneous vessels retain their elongated course, but become condensed as the surrounding dermal and subcutaneous tissue diminishes in size (*H*); hair-cycle-dependent changes in the dermal and subcutaneous tissue become very obvious by comparing (*E*)-(*H*), which are of the same magnification. (*J*)-(*M*) Double-immunofluorescent staining for CD31 (green) and Ki-67 antigen (red) demonstrates the presence of PEC in murine skin. No PEC are detectable in most sections of anagen I skin (day 1 p.d.) (*J*); in anagen IV skin (day 5 p.d.), single PEC become detectable, mostly in small ascending vessels, rarely in vessels of the lower horizontal plexus (*arrow*) (*K*); the number of PEC increases dramatically in anagen V (day 8 p.d.) (*L*) and anagen VI skin (day 12 p.d.) (*M*), where many PEC can be detected in branching dermal vessels (*L*, *M*). *Scale bars* represent 200 μm in (*A*)–(*D*), 80 μm in (*E*)-(*L*), 32 μm in the inserts of (*E*) and (*G*), and 50 μm in (*M*).

(**Fig 1*A*-*D***). Therefore, it cannot be excluded with certainty from these data alone that the observed morphologic changes of the vascular bed are simply related to stretching and widening of pre-existing, basically unaltered vessels.

To further evaluate hair cycle-dependent changes in the cutaneous vascular pattern, we assessed the MVD, i.e., microvessels per microscopic field (Vartanian and Weidner, 1994; Hudlická *et al*, 1998), during the entire depilation-induced murine hair cycle, by counting CD31-immunoreactive cell aggregates that show distinctive formation of a central lumen or that form longitudinal, occasionally branching, structures with the obvious appearance of blood vessels (**Fig 1*E*-*H***). The data clearly indicate a significant increase in MVD during anagen development, especially during early anagen stages II (day 3 p.d.) to IV (day 5 p.d.) ($p \leq 0.05$) (**Fig 3**). MVD increases constantly during anagen development from day 0 p.d. on and does not decline significantly until day 25 p.d., when all hair follicles have spontaneously re-entered the telogen stage. By day 34 p.d. MVD has reached similar values to those in unmanipulated telogen skin, suggesting that new blood vessels that had been constructed during anagen have regressed again (**Fig 3**).

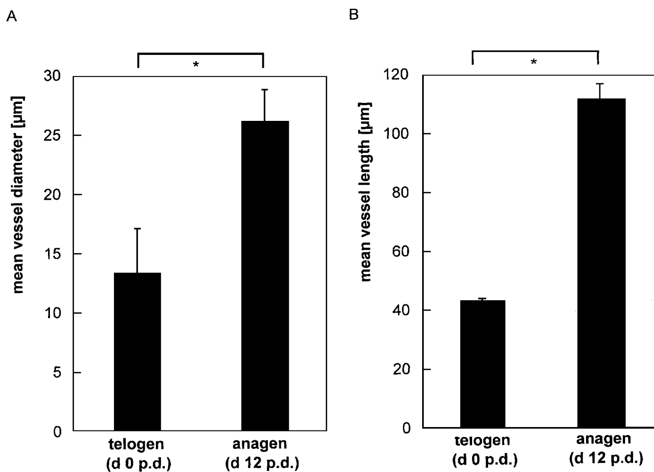

Figure 2. Differences in the mean vessel diameter and the mean vessel length between telogen and anagen VI mouse skin. (*A*) Mean vessel diameter; (*B*) mean vessel length (measured in µm). Each was assessed in 150 vessels derived from three different animals; mean ± SEM; $^*p \leq 0.05$.

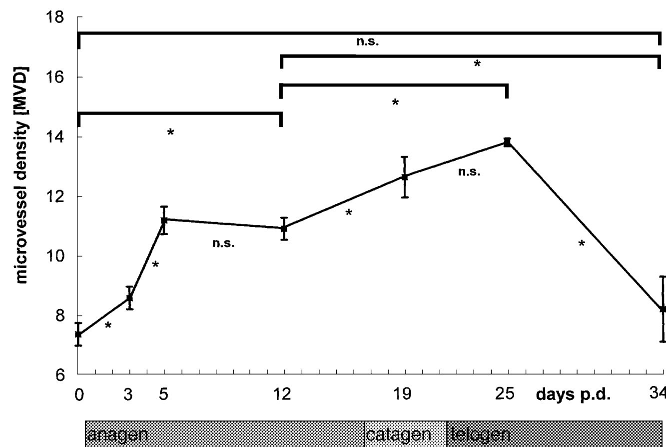

Figure 3. Cutaneous MVD during the depilation-induced murine hair cycle, assessed by immunohistochemical staining of CD31. The significant increase in MVD between anagen VI (day 12 p.d.) and telogen skin (day 25 p.d.) is probably due to quantitative changes of the dermal and subcutaneous tissue; microvessels were counted in 60 microscopic fields, derived from three different mice per hair cycle stage; means ± SEM; n.s., not significant; $^*p \leq 0.05$.

The number of endothelial cell nuclei increases during depilation-induced anagen development To confirm whether the morphologic changes observed in the cutaneous microvasculature really reflect anagen-associated angiogenesis, we next counted endothelial cell nuclei to explore whether the number of endothelial cells really increases during anagen development, as would be expected if angiogenesis were occurring. The interfollicular region was chosen as reference area because the space between two hair follicles remains relatively unaltered during the entire hair cycle. A significant difference ($p \leq 0.05$) in the number of endothelial cell nuclei could indeed be detected between telogen skin (day 0 p.d.) and anagen VI skin (day 12 p.d.), as additional suggestive evidence for endothelial cell proliferation (**Fig 4**).

Large numbers of proliferating endothelial cells (PEC) can be detected only during anagen To further confirm these data, sections were double-stained for CD31 (endothelial cells) and Ki-67 (nuclear antigen, maximally expressed during the S-phase of the cell cycle) (Hudlická *et al*, 1998). Very few double-immunoreactive cells, referred to as PEC, can be detected in telogen and early anagen skin (**Figs 1*J*, 5**). The number of PEC rises significantly ($p \leq 0.05$) from anagen II skin (day 3 p.d.) to anagen V skin (day 8 p.d.) (**Figs 1*K*, 5**) and reaches maximum levels at anagen V (day 8 p.d.) (**Figs 1*L*, *M*, 5**). PEC are mainly located in the mid-portion of the dermis, associated with long ascending cutaneous vessels (**Fig 1*L*, *M***). Rarely PEC are detectable in larger vessels of the lower and of the upper horizontal plexus. The number of PEC decreases significantly ($p \leq 0.05$) already in late anagen (between day 8 and day 12 p.d.) and in early catagen (day 17 p.d.) to almost the value of telogen skin, suggesting that cessation of endothelial cell proliferation is an early marker of hair follicle regression (**Fig 5**).

High resolution light microscopy of toluidine-blue-stained semithin sections was employed to count endothelial cells that undergo mitosis, i.e., that show chromatin separation (**Fig 6**). These were absent in telogen skin. Mitotic endothelial cells were only found between days 3 and 12 p.d., i.e., during anagen II to anagen VI. The number of mitotic endothelial cells (counted in two to five blocks, derived from two to three animals per time point) peaked on day 8 p.d. (no statistical analysis was performed, in view of the limited data). TEM analysis confirmed the light

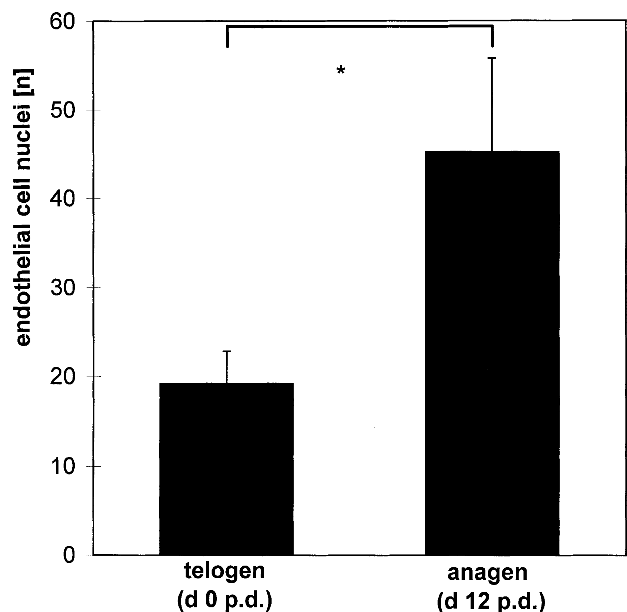

Figure 4. Differences in the number of endothelial cell nuclei per interfollicular area between telogen and anagen VI skin. The number of endothelial cell nuclei [n] was assessed in 60 interfollicular areas of three different animals per hair cycle stage; means ± SEM; $^*p \leq 0.05$.

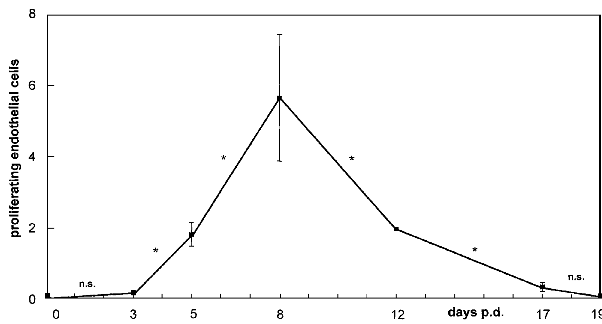

Figure 5. Changes in the number of PEC per microscopic field (MF) during the depilation-induced murine hair cycle. PEC were counted in 75 microscopic fields per hair cycle stage (derived from three different animals), using 400× magnification and double-immunofluorescent staining for CD31 and Ki-67 antigen; mean ± SEM; $^{*}p \leq 0.05$.

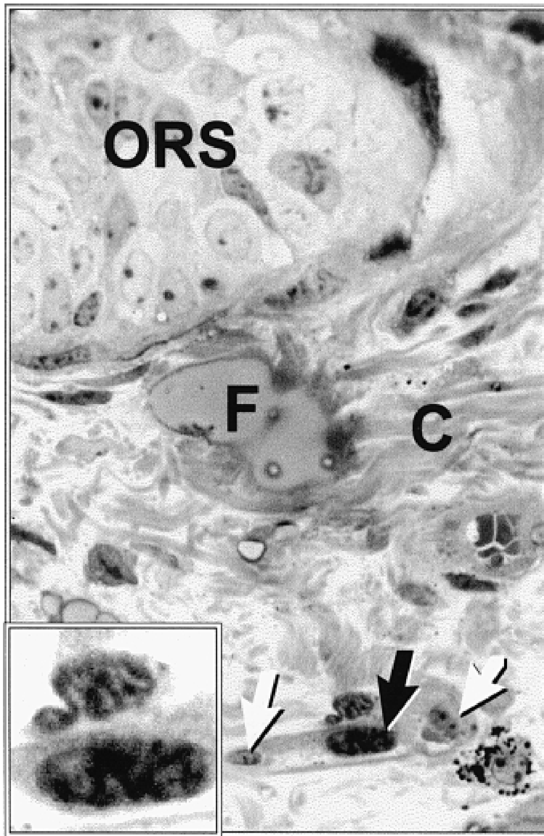

Figure 6. Mitotic endothelial cell with separated chromatin (black arrow, inset) in early anagen. Compare with the nonmitotic endothelial cells (*white arrows*); high resolution light microscopy of a toluidine-blue-stained semithin section of mouse skin on day 3 p.d.; ORS, follicular outer root sheath; F, fat; C, collagen fibrills; *scale bar*: 20 µm.

microscopy appearance of mitotic endothelial cells in anagen skin (**Fig 7**).

The angiogenesis inhibitor TNP-470 retards anagen development To investigate the functional significance of angiogenesis in the murine hair cycle, C57BL/6 mice were depilated to induce anagen and were treated daily 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; 1994), could already be observed at day 6 p.d. Whereas the skin of control mice was slightly gray in the caudal parts of the back, reflecting massive

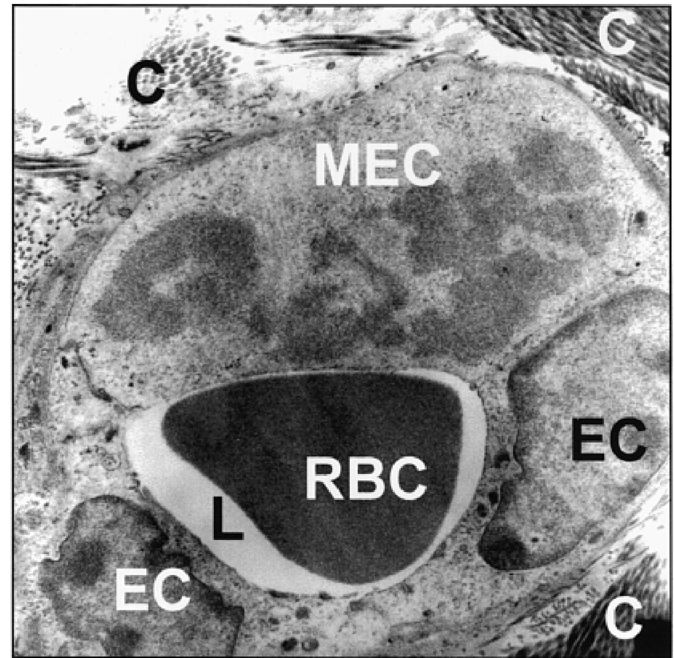

Figure 7. TEM of an activated endothelial cell in anagen skin (day 8 p.d.), highly suggestive for endothelial cell mitosis. C, collagen fibrills; EC, endothelial cell; L, capillary lumen; MEC, mitotic endothelial cell; RBC, red blood cell; *scale bar*: 2 µm.

anagen-associated follicular melanization (Slominski and Paus, 1993), TNP-470-treated mice retained a pink skin color. This difference in skin color remained obvious on day 8 p.d., when the skin of TNP-470-treated animals became slightly gray, reflecting the onset of follicular melanogenesis, whereas the skin of control mice was almost completely black, indicating the presence of far-advanced stages of anagen development (**Fig 8**).

These macroscopic observations demonstrate a significant retardation in anagen development by TNP-470. This was confirmed by quantitative histomorphometry. At day 6 p.d., control mice exclusively showed hair follicles in anagen III, whereas mice treated with TNP-470 had follicles in different stages of early anagen development (anagen I to anagen III). Statistical analysis revealed a highly significant ($p \leq 0.009$) retardation in anagen development after TNP-470 administration (**Fig 9**). Hair follicles of TNP-470-treated mice were in anagen stages I to IV at day 8 p.d., compared with hair follicles in anagen stages III to VI in control animals (**Fig 10**). No significant differences could be observed between the three different concentrations of TNP-470 used (3, 6, 12 mg per kg, data not shown).

DISCUSSION

Here, we show that synchronized hair follicle cycling in mice is associated with a substantial remodeling of the perifollicular and interfollicular cutaneous microvasculature, that anagen development is associated with angiogenesis, and that inhibiting angiogenesis leads to a retardation of anagen development.

To demarcate endothelial cells for morphologic analysis we used an antibody directed against CD31 (PECAM-1), a transmembrane protein of endothelial cell adherens junctions (Albelda *et al*, 1991). Though expression of CD31 on endothelial cells is reported to be downregulated in the case of malignant transformation (Ohsawa *et al*, 1995) or under the influence of interferon-γ (Bujan *et al*, 1999), and though we cannot with certainty exclude its downregulation in distinct cell cycle phases, CD31 has been widely used as a marker for endothelial cells even in mouse tissue (e.g., Couffinhal *et al*, 1998; Detmar *et al*, 1998; Suri *et al*, 1998) and has also been used in double-immunohistologic staining with Ki-67 to determine proliferating endothelial cells (e.g., Vermeulen *et al*,

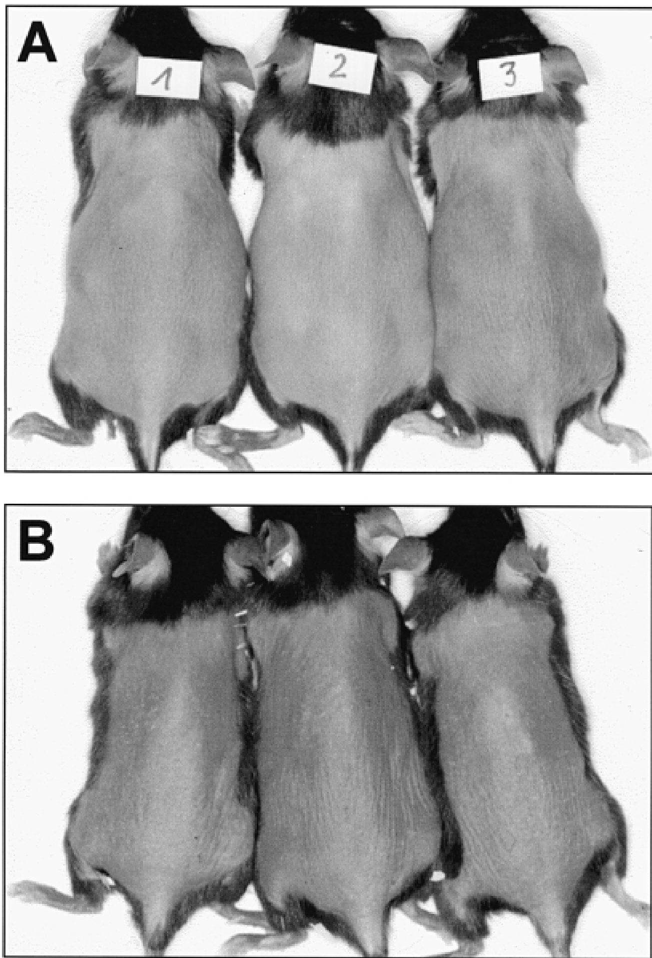

Figure 8. Inhibition of angiogenesis retards induced anagen development. C57BL/6 mice after treatment with the fumagillin derivative TNP-470 for 8 d (*A*), compared with controls (*B*). All mice were depilated and treated daily with TNP-470 (6 mg per kg) intraperitoneally. The difference in anagen development 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 gray (early anagen) to dark gray-black (anagen VI). Whereas untreated controls show a dark gray skin color, TNP-470-treated animals retain the pale to slightly gray skin color of early anagen.

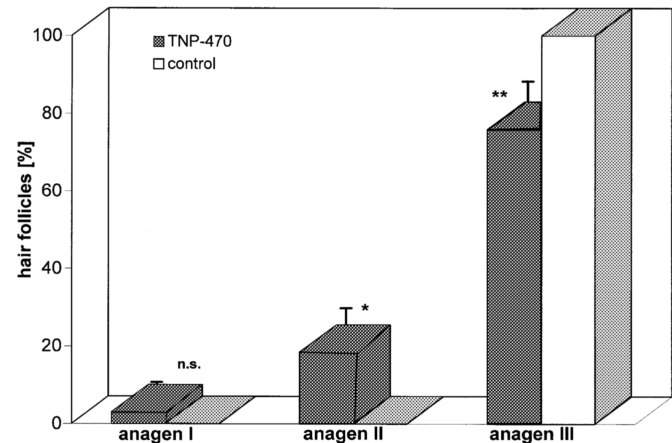

Figure 9. Treatment with TNP-470 over 6 d retards anagen development. Hair follicles (%) in different stages of the depilation-induced hair cycle after 6 d of TNP-470 application (6 mg per kg) compared with controls; seven mice were investigated in each group and the hair cycle stage of at least 60 hair follicles was assessed; mean ± SEM; $*p \leq 0.05$; $**p \leq 0.01$.

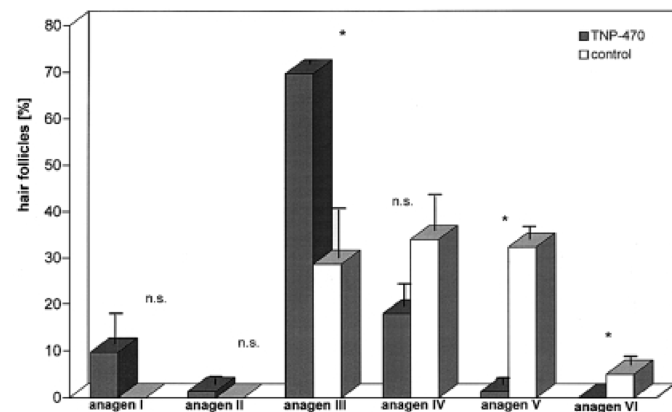

Figure 10. Treatment with TNP-470 over 8 d retards anagen development. Hair follicles (%) in different stages of the depilation-induced hair cycle after 8 d of TNP-470 application (6 mg per kg) compared with controls; three mice were investigated in each group and the hair cycle stage of at least 60 hair follicles per mouse was assessed; mean ± SEM; n.s., not significant; $*p \leq 0.05$.

1995, 1997; Christenson and Stouffer, 1996; Creamer *et al*, 1997). CD31 has been shown to be expressed on newly formed vessels as well as mature vessels (Gariano *et al*, 1996) and seems to be suited for assessment of changes in the mouse cutaneous microvasculature during the hair cycle.

In theory, the dramatic changes in MVD, caliber, and arrangement that are associated with hair follicle cycling (**Figs 1–3**) might still be explainable by hair-cycle-dependent modulations of vessel diameter, vessel course, and spatial arrangement rather than by angiogenesis. The observed anagen inhibition by a reference angiogenesis-inhibitory drug (**Figs 8–10**) suggests that the *de novo* formation of new blood vessels is functionally important for normal anagen development, even though we cannot exclude the theoretical possibility of a direct toxic effect of TNP-470 on anagen hair follicles. Though effects on cells of the immune system and inhibition of the proliferation of normal fibroblasts are reported (cf. Ingber *et al*, 1990; Castronovo and Belotti, 1996; Wong *et al*, 1994; Antoine *et al*, 1996), to the best of our knowledge the available literature on fumagillin derivatives does not report any toxic effects of this agent on keratinocytes (Castronovo, personal communication). TNP-470 and its toxicities have been extensively investigated in several preclinical studies and phase I to III clinical trials (cf. Castronovo and Belotti, 1996). Hair loss/alopecia/effluvium has not been reported as a side-effect of therapy in any of the published clinical trials, however, and one would definitely expect such an effect in the case of any significant hair follicle toxicity of the drug. Moreover, morphologic evidence of drug-induced hair follicle toxicity [i.e., sensitive indicators of discrete follicle dystrophy such as characteristic abnormalities in the hair follicle pigmentary unit (cf. Slominski *et al*, 1996; Tobin *et al*, 1998)] were absent in all examined TNP-470-treated mice, as assessed by light microscopy.

We present two mutually confirmatory pieces of evidence, however, that can only be explained by the occurrence of genuine angiogenesis. (i) 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 (**Fig 4**), demonstrating a significant increase in the pool of endothelial cells during anagen. (ii) There is a massive increase of *proliferating* endothelial cells in anagen (as evidenced by the presence of Ki-67 and PECAM-1 double-immunoreactive cells, by high

resolution light microscopy and by TEM) (**Figs 1*J–M*, 5–7**) compared with the very low, basal rate of endothelial cell proliferation in telogen skin, which is probably required to maintain blood vessel homeostasis even in telogen and early anagen skin (**Fig 1*J***). In the light of these data, it is reasonable to speculate that many of the hair-cycle-dependent fluctuations in blood vessel morphology and arrangement, reported above, indeed reflect changes in hair-cycle-dependent angiogenesis.

In view of the significant catagen/telogen-associated decline in cutaneous MVD (**Fig 3**), it is reasonable to ask whether apoptosis of endothelial cells occurs during synchronized catagen/telogen development in murine skin. Preliminary observations from our laboratories indicate that apoptosis of perifollicular endothelial cells can indeed be demonstrated during late catagen and telogen, using TEM and double-immunofluorescence (CD31/TUNEL staining) (Mecklenburg, Tobin, Paus, manuscript in preparation).

The demonstration that anagen is associated with angiogenesis is interesting in several respects. First, this shows that angiogenesis in adult mammalian skin is not exclusively associated with pathologic phenomena (e.g., wound healing, chronic inflammation, tumor growth). Second, as morphologic and immunohistologic evidence of angiogenesis can only be detected long after hair follicles have visibly switched from resting (telogen) to growth (anagen), the hair follicle seems to be able to dictate angiogenesis in normal murine skin. And third, the apparent dependence of hair cycle progression on angiogenesis offers an ideal *in vivo* model system for the screening of candidate angiogenesis-inhibitory compounds (cf. Auerbach *et al*, 1991): it is simple, easily reproducible, quantifiable, and biologically highly relevant.

The factors required for promoting angiogenesis (e.g., VEGF, fibroblast growth factor 2) can differ substantially between tissues and biologic settings (Neufeld *et al*, 1999). Therefore, it is not trivial to determine which putative key angiogenic factors are present in the hair follicle (cf. Goldman *et al*, 1995; Lachgar *et al*, 1996; 1998; Kozlowska *et al*, 1998) and whether their distribution and concentration as well as the expression of their cognate receptors shift during hair follicle cycling. Also it is important to clarify which angiogenic factors represent really critical elements in the control of *follicular* angiogenesis: is VEGF the relevant key factor (cf. Lachgar *et al*, 1996; 1998), or are platelet-derived growth factor (Wang *et al*, 1999), fibroblast growth factor 2 (Seghezzi *et al*, 1998), hepatocyte growth factor/scatter factor (HGF/SF) (Gille *et al*, 1998; Wojta *et al*, 1999), and angiopoietin (Suri *et al*, 1998) even more important? Also it needs to be identified what relative significance in the control of hair-cycle-associated angiogenesis must be attributed to locally generated angiogenesis inhibitors [e.g., angiostatin (O'Reilly *et al*, 1994), endostatin (Dhanabal *et al*, 1999)].

Most recently, we have shown that the expression of HGF/SF and its receptor is hair cycle dependent and that HGF/SF is exclusively expressed in the dermal papilla of anagen hair follicles (Lindner *et al*, 2000). In view of the angiogenesis-promoting effects of HGF/SF and its stimulatory effect on VEGF expression (Gille *et al*, 1998; Wojta *et al*, 1999), it is reasonable to speculate that both factors play an important role in anagen-associated cutaneous angiogenesis.

Although angiogenesis may be required for the normal speed of anagen development (**Figs 8–10**), it is not essential, as anagen also develops in skin organ culture after the severance of the skin vasculature (Li *et al*, 1992). Nevertheless, it is reasonable to explore whether topically applied angiogenesis inhibitors can be exploited to suppress unwanted hair growth in man, i.e., hypertrichosis and hirsutism.

Taken together, anagen-associated angiogenesis in C57BL/6 mice offers an excellent model for dissecting and manipulating physiologic angiogenesis in the adult mammalian organism in general, and in skin- and hair-follicle-related blood vessel formation in particular.

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