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The canine hair cycle – a guide for the assessment of morphological and immunohistochemical criteria

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Short running head: The canine hair cycle

Abstract

The hair follicle has a lifelong capacity to cycle through recurrent phases of controlled growth (anagen), regression (catagen), and quiescence (telogen), each associated with specific morphological changes. A comprehensive classification scheme is available for mice to distinguish the cycle stages anagen I-VI, catagen I-VIII, and telogen. For dogs, such a classification system does not exist although alopecia associated with hair cycle arrest is common. We applied analogous morphological criteria and various staining techniques to subdivide the canine hair cycle stages to the same extent as has been done in mice. Of all the staining techniques applied, haematoxylin and eosin stain (H&E), Sacpic, Masson Fontana and immunohistochemistry for vimentin and laminin proved to be most useful. To evaluate the applicability of our criteria, we investigated skin biopsies from healthy beagle dogs (n=20, biopsies from shoulder and thigh) kept under controlled conditions. From each biopsy, at least 50 hair follicles were assessed. Statistical analysis revealed that 30% of the follicles were in anagen (12% early, 18% late), 8% in catagen (2% early, 5% late, 1% not determinable) and 27% in telogen. Thirty five percent of hair follicles could not be assigned to a specific cycle stage because not all follicles within one biopsy were oriented perfectly. In conclusion, this guide will not only be helpful for the investigation of alopecic disorders and possibly their pathogenesis, but may also serve as a basis for research projects in which the comparison of hair cycle stages is essential, *e.g.* comparative analysis of gene expression patterns.

Introduction

The hair follicle is a fascinating miniorgan, which provides the body with protection, insulation, camouflage and the means for social and sexual communication.[1]

Hair follicles cycle during the entire life span of a mammal. This requires intimate interactions between epithelium and mesenchyme[2] in which extrinsic and intrinsic factors such as growth factors and cytokines, hormones, genetic background, age, sex, location (region of the body), neurogenic stimulation, nutrition, status of health, numerous environmental factors (photoperiod, grooming, ambient temperature), friction and trauma are involved.[2,3,4]

The hair cycle consists of tightly regulated alternating phases of controlled growth (anagen), regression (catagen), and relative quiescence (telogen). Hair shafts are produced during anagen only. The anagen phase is inevitably followed by a relatively rapid phase of regression (catagen) during which the inferior segment of the hair follicle is removed, both by cessation of the mitotic activity of the matrix cells and by well coordinated apoptosis in the inferior portion of the follicle. Simultaneously, the dermal papilla (DP), which releases the key inductive signals for hair-shaft synthesis, is devaginated and moves upward.[2] Histologically, an increased number of apoptotic cells is visible in the outer root sheath (ORS), the bulb, and the suprabulbar region. During catagen the club hair is formed.[5] At the end of the involution phase, the follicle attains its smallest dimensions and is considered to be resting in telogen.[2]

In telogen, the inner root sheath (IRS) has been replaced by trichilemmal keratin, anchoring the club hair in the follicle, and the DP lays at the base of the telogen follicle.[5] After a variable time in telogen, initiation of a new anagen phase occurs through activation of stem cells, likely in response to inductive signals from the DP and the surrounding mesenchyme. The follicle thus enters the growth phase known as anagen that is characterized by active keratinocyte proliferation and differentiation. The previously involuted portion of the hair follicle is reconstructed and a new hair fibre is synthesized.[2]

Recently, shedding of the hair shaft has been described as the fourth distinct cycle phase named *exogen*.[6]

Morphology as well as the most basic control mechanisms of the cycling hair follicle seem to be broadly similar in different mammalian species .[2] However, it is well known that laboratory animal studies might not translate directly to humans and to domestic animals, as there are important species differences that limit the extrapolation of data from mice to dogs. In contrast to mice and humans, dogs have compound follicles consisting of a primary and several secondary hairs which exit through the same orificium.[7] The primary hairs are equipped with an arrector pili muscle, sebaceous, and apocrine glands.[8] One to six compound follicles are grouped together and form follicular units.[7] In these follicular units the central primary hair follicle and several lateral primary hair follicles can be distinguished.[8] The different hair follicles of one compound cycle independently of each other.[9] In addition to seasonal variations, large differences exist between different dog breeds, mainly in regards of the duration of the anagen and telogen cycle phase.[10] This is in contrast to mice in which, at least in young animals, the hair follicles cycle in a wave pattern.

For the mouse, in addition to the well known cycle phases anagen, catagen and telogen, a comprehensive classification scheme is available for more detailed division of the hair cycle, describing even subtle hair cycle changes.[5] In this scheme, morphological and immunohistochemical criteria are applied to distinguish eight substages each in anagen and catagen.

Limited research has been conducted on canine hair cycle phases. When cycle phases were determined and described, they were limited to the main categories anagen, catagen and telogen.[11,12,13,14] This is unfortunate since primary alopecia, a common problem in dogs, is histologically characterized as hair cycle arrest, and distinction of the different diseases in this group is currently not possible based on these available morphologic criteria.

The purpose of this study is to establish criteria that allow for a more accurate classification of the canine hair cycle phases, similar to the one for mice which has proven to be a helpful tool in murine hair research.[5]

Materials & Methods

Skin biopsy specimens were obtained from nine female and eleven male, healthy, adult beagle dogs at the age of 14-18 months (n=18) and twelve years (n=2). These dogs had been used as control animals in toxicological studies in a pharmaceutical company. They had been kept indoors and fed a defined maintenance diet. The biopsies were collected at the euthanasia of the dogs from June to July 2009.

Biopsies were taken from shoulder and thigh, fixed immediately in 10% neutral buffered formalin for 24 hours, embedded in paraffin, sectioned at 4µm, deparaffinized and rehydrated and routinely stained with haematoxylin and eosin (H&E) according to standard procedures. Additional histochemical and immunohistochemical stainings were performed on serial sections to evaluate if they facilitated assignation to a specific hair cycle stage. Staining characteristics of these and their suitability for the characterization of specific canine hair cycle stages are depicted in Table 1. The immunohistochemistry protocols are listed in Table 2. In addition to the listed procedural steps, endogenous peroxidase was blocked with 3% H_2O_2 in methanol for the anti-cytokeratin 14, anti-Ki-67 and anti-CAM5.2 antibodies. For anti-CAM5.2, unspecific staining was blocked with normal goat serum, diluted 1:20 in phosphate buffered saline with Tween (PBST). All sections were counterstained with Ehrlich's haematoxylin. Control sections for immunohistochemistry were incubated with normal mouse or rabbit IgG (both Dako, Zug, Switzerland), respectively, in the same concentration as the primary antibody. The following histochemical stains were used: the modified Sacpic Method[15], Rhodamin and Toluidine blue[16], Masson Fontana[17], toluidine blue and Safranin[18,19], and orcein-giemsa[20] as described in the literature.

For both skin sites (shoulder and thigh) an exact hair cycle stage was assigned to at least 50 consecutive hair follicles, including primary and secondary follicles. If this was not possible, cycle stages were classified into broader categories. These broader categories were early anagen (anagen I - anagen IIIb), late anagen (anagen IIIc - anagen VI), early catagen (catagen I - IV), late catagen (catagen V - catagen VIII), telogen with hair and telogen without hair. Moreover, the number of compound follicles consisting of a primary hair follicle surrounded by secondary hair follicles was

determined for each skin site. Subsequently, for each dog and skin site, the percentage of follicles in the respective categories was calculated. Mean percentages for the various outcomes (follicle stages, primary and secondary follicles) were compared between gender and location using a 2-way repeated (over localization) measure ANOVA with Geisser-Greenhouse adjustments. P-values < 0.05 were considered statistically significant.

Results

We identified similar morphological features of the different hair cycle stages to those described for mice.[5,21,22] Key characteristics, necessary for the assignment of a hair follicle to one of these stages in the dog are illustrated in detail in Figures 1-3.

As depicted in Figures 1-3, the most important and easily visible feature of all the criteria used for the classification of the cycle stages, was the position of the DP in relation to the follicle, and its absolute location in the dermis or the subcutaneous fat (the latter demonstrated in Figure 4). Although the DP was often visible in H&E stained sections, double labeling with anti-vimentin and anti-laminin antibodies proved to facilitate its recognition. While anti-Vimentin stains the mesenchymal DP, anti-laminin marks the follicular basement membrane. With this labeling, the exact position of the DP, its form and its relation to the follicle was easily determined.

For the subdivision of the late anagen stages, the length of the hair shaft was another important additional criterion (Figure 4).

The use of serial sections proved to be essential for the identification of hair cycle stages. A slightly paramedian cut hair follicle might mimic other hair cycle stages and, for example, could be misinterpreted as the shape of the DP and the length of the hair shaft was not shown ideally.

We applied additional immunohistochemical and histochemical staining techniques which we expected to facilitate the assignment of follicles to specific cycle stages. The specific staining characteristics are summarized in Table 1 and Figure 5. However, with the exception of the vimentin and laminin labeling, Sacpic and Masson Fontana, none of these stains were specifically helpful in identifying specific hair cycle stages.

The modified Sacpic Method[15], a differential stain like H&E, highlighted both the non-keratinized (bluish with yellowish granules) and the keratinized (bright red) IRS. Thus Sacpic was helpful to determine early anagen stages when the inner root sheath develops.

Masson Fontana stains melanin granules and is thus helpful for the differentiation of anagen from catagen stages in hair follicles with pigmented hair shafts. In anagen IIIa first melanin granules were visible in the bulbar area above the DP (Figure 1e).[5] Their number increased towards the late anagen stages (Figure 1i) and then decreased again in early catagen (catagen II) until they finally disappeared in catagen III (Figure 2a, d).

Mostly due to the orientation of the hair follicles in the sections, not all hair follicles could be assigned to a specific cycle stage. A set of broader categories was used characterizing these follicles as follows: early and late anagen, early and late catagen, and telogen. The most important features of early anagen (anagen I – IIIb; Figure 1a - h) were the onset of growth and the movement of the DP downwards into

the subcutaneous fat. At the end of early anagen, the new hair shaft was clearly visible and reached up to 2/3 of the follicle length. In late anagen (anagen IIIc – VI; Figure 1i - p), the DP was fully enclosed by the bulb, and reached the deepest location in the subcutaneous fat where it remained. The hair shaft was fully developed and reached the epidermal surface in anagen VI. In early catagen (catagen I – IV; Figure 2a - f) involution of the inferior portion of the hair follicle began. It was best identified by evaluating the DP and the bulb. The DP changed its form and position, and in the bulb mitotic activity stopped and an increasing number of apoptotic keratinocytes were visible. In late catagen (catagen V – VIII; Figure 2g – n), the hair follicle was remarkably shortened and the club hair formed. The DP continuously moved towards the dermis, leaving behind a tail of trailing connective tissue sheath (CTS) cells. In telogen (Figure 3a, b), the DP was positioned in the dermis and no trailing CTS cells remained.

Evaluating the biopsies from both thigh and shoulder of 20 beagle dogs the following results were obtained: 30.1% of the hair follicles were in anagen, 7.6% in catagen, and 27.6% in telogen. Of the telogen follicles 14.4% contained a hair while 13.2% did not. These hair follicles were either allocated to a specific cycle stage or were classified into one of the broader categories, defined in the materials and methods. Statistical analysis was only possible for the broader categories. In 34.7% of the follicles, allocation to a specific hair cycle stage was not possible. The mean percentage of the different cycle stages is listed in Table 3. Interestingly, the percentage of follicles that could not be assigned to a specific cycle stage was significantly higher in the skin biopsy specimens obtained from the thigh (38.5%) than from those from the shoulder (30.2%, p=0.004).

Investigating the influences of gender and location, only one significant difference was detectable: among the classified 50 follicles the proportion of primary to secondary follicles was greater in females (primary follicles: females: 22.4%, males: 18.3%, p=0.001; secondary follicles: females: 75.3%, males: 80.4%, p=0.002). In females, the 50 specified follicles were arranged in 13.0 compounds while in males, they were arranged in only 10.7 compounds (p=0.005). The 12-year-old age group contained only two animals and was thus too small for statistical analysis. However, we did evaluate if their data influenced the values for gender and location, which was not the case (data not shown). The effect of age was thus not further investigated. Significant interactions between gender and localization were not found.

Discussion

On the basis of the criteria for the subdivision of anagen[5,21] and catagen[22] hair follicle stages in human, and in analogy to the classification guide for murine hair follicles of Müller-Röver et al.[5], we established criteria that are well suited to determine the canine follicle stages on formalin fixed material.

These classification criteria for the canine hair cycle phases will allow for better standardization of studies investigating, for example, the specific morphology of canine hair cycle disorders or gene and protein expression patterns during specific cycle phases.

The accurate classification is, however, dependent on optimally oriented hair follicles in the paraffin block. In reasonably sized skin biopsy specimens of dogs which encompass at least 50 hair follicles it is not possible to orient all the hair follicles optimally. This is because the dog has compound follicles and primary and

secondary hairs exit through the same orificium but do not lay in the dermis exactly in parallel. Therefore, approximately one third of the hair follicles investigated in this study could not be assigned to a cycle stage.

Three factors are essential for the classification of the hair cycle stages: the position of the DP, the use of serial sections, and selected stainings to visualize important morphological structures. The position of the DP marks the follicle as being in a certain phase of the cycle (Figure 4). During the anagen stages, the length of the growing hair shaft is another important feature. The use of serial sections allows the assessment of the main characteristics in hair follicles that are embedded suboptimally and thus facilitates a more accurate classification. Of all the stains evaluated, double labelling for vimentin and laminin proved to be most helpful. It allowed for an exact assessment of the DP in relation to the bulb. Of great help were the histochemical stains Sacpic and Masson Fontana.

Even with these aids, some stages are difficult or impossible to differentiate. These are the transition from anagen VI to catagen I and from catagen VIII to telogen. The difficulties arising with these stages will be discussed later.

Due to the delicacy of skin tissues and their susceptibility to damage caused by the pretreatment procedures necessary for immunohistochemistry, the quality of the anti-Ki-67 and anti-cleaved lamin A stained sections was variable. This made the interpretation of the sections often difficult and serial sections were not always comparable. Given the fact that the assignment to a specific cycle stage did not absolutely require the use of proliferation markers, we concluded that for follicle staging Ki67 stain is not necessary.

In mice, the number of apoptotic cells is an important criterion to determine the different catagen stages.[5] This is particularly true for the transition from anagen VI to catagen I, which does not entail morphologic changes in H&E, and is only discernable by staining of apoptotic cells in the bulb.[5] We chose to use immunohistochemistry for cleaved lamin A as a straightforward method to detect apoptotic cells instead of the technically more demanding and not always reliable TUNEL assay.[23] The antibody recognizes a neoepitope of lamin A generated through its cleavage by caspases. With anti-cleaved Lamin A we could detect apoptotic cells in the ORS and the epithelial strand. However, the number of labeled cells was very low and none were in the bulb region. It was impossible for us to differentiate the early catagen stages according to the number of apoptotic cells in the bulb as suggested for the murine hair follicles.[5] The low number of apoptotic cells detected with anti-cleaved lamin A in the inferior portion and the absence of marked cells in the bulb of catagen may be due to the fact, that the early catagen stages are very short[5] and thus the probability of observing them in a section is very low.

Toluidine blue/safranin[18,19] staining stains the nucleus and nuclear debris of dead cells deeply blue, and is therefore considered suitable for detection of apoptotic cells in hair follicles. In our hands it stained presumably apoptotic cells slightly darker red than the background. Additionally, the darker stained cells seemed to be contracted. However, their identification was ambiguous. When comparing the staining results obtained with toluidine blue/safranin with the results of the anti-lamin A labeling in serial sections, some cells were stained with both stains. However, with toluidine blue/safranin a higher number of cells were stained. Since we had doubts about the

reliability of toluidine blue/safranin, we did not use this stain to determine apoptotic cells.

Stains that we applied but that proved to be unnecessary were rhodamin/toluidine blue, acid orcein-giemsa, and Anti-Cytokeratin 14. Rhodamin/Toluidine blue and acid orcein-giemsa are very picturesque differential stains, but they are not superior to H&E for the determination of cycle stages. Anti-Cytokeratin 14 stains the ORS, which is also easily visible by H&E.

As mentioned earlier, the transition from anagen VI to catagen I does not entail morphologic changes but assignment in mice depends on the presence of apoptotic cells. Since we could not rely on our apoptotic markers, we searched for mitotic figures in H&E stained sections, and melanin granules to dispel any doubts on whether a follicle was still in anagen or already in catagen. If there were mitoses present in the bulb, we assigned the follicle to late anagen. A decrease of melanin granules in the bulbar area of pigmented hair shafts points to catagen.

The differentiation between catagen VIII and telogen is difficult in some cases. In both stages the DP resides at the same level in the dermis, the only difference being a tail of trailing CTS cells present in catagen VIII. Müller-Röver et al. [5] stained these trailing cells with a NCAM antibody which requires frozen sections. Since it is our goal to apply the classification criteria to archive material from dogs with alopecia or hypotrichosis, we chose to work with formalin fixed tissue only. As there is, to our knowledge, no alternative to the NCAM antibody available to stain specifically trailing CTS cells, we relied on morphology for classification. In some cases, the trailing cells were also visible in H&E sections. When in doubt, we opted for telogen, since this is the longer-lasting stage of the two, despite the risk of misclassifying a few catagen VIII follicles.

Primary alopecia, histologically characterized as hair cycle arrest, is caused by either an endocrine imbalance or yet unknown factors. This common problem in dogs causes much concern for owners. The current opinion is that these hair-cycle disorders lack anagen induction as suggested for hypercorticism, have an impaired anagen promotion as suggested for hypothyroidism, or are prematurely forced into catagen as suggested for alopecia X and seasonal flank alopecia.[10] However, this view is based on data from mice and humans which differ in some aspects from dogs.

We hope that, with the help of this guide, a first step towards understanding the pathogenesis of these hair cycle disorders will be possible, allowing a more detailed analysis of the hair follicle stages involved. In addition, the guide will be helpful in the standardization of further studies investigating, for example, gene and protein expression patterns of canine hair follicles.

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Figure Legends:

Figure 1: Key features of the canine anagen hair cycle stages modified from the classification system used in mice.[5] Figure in analogy to the classification guide for murine hair follicles by Müller-Röver et al. (2001).[5] In the first column the basic features of the anagen stages are depicted in schematic drawings. The second column lists the most important morphological features of each stage that are visualized in histological photograph in the third column. Photomicrograph a: Orcein Giemsa, a differential stain. Photomicrographs b, c, e, k, l, n and p: haematoxylin and eosin (H&E). Photomicrograph d: Immunohistochemistry for Vimentin and Laminin. The anti-Vimentin antibody marks mesenchymal tissue – in this case the dermal papilla - visualized by permanent Red (pink) and the anti-Laminin antibody labels the basement membrane visualized by diaminobenzidine (brown). Photomicrographs f and g: Immunohistochemistry for CAM5.2. The Anti-CAM5.2 antibody recognizes keratin 8 which is expressed by the non-keratinized part of the IRS, visualized in red by AEC (3-amino-9-ethylcarbazole). Photomicrograph h: example of Rhodamin Toluidine blue staining which shows the IRS brightly pink. Photomicrographs i and j: Masson Fontana, depicting the melanin granules black. Photomicrographs m and o: Sacpic, a differential stain. Abbreviations: DP: dermal papilla; IRS: inner root sheath; ORS: outer root sheath; SG: sebaceous gland; APM: arrector pili muscle. Bar = 100µm.

Figure 2: Key features of the canine catagen hair cycle stages modified from the classification system used in mice.[5] Figure in analogy to the classification guide for murine hair follicles by Müller-Röver et al. (2001).[5] In the first column the basic features of the catagen stages are depicted in schematic drawings. The second column lists the most important morphological features of each stage that are visualized in histological photographs in the third column. Photomicrograph a: Masson Fontana, staining melanin granules black. Photomicrographs b, d and n: Immunohistochemistry for vimentin and laminin. The anti-vimentin labels mesenchymal tissue – in this case the dermal papilla - visualized by permanent Red (pink) and the anti-Laminin antibody marks basement membrane shown by diaminobenzidine (brown). Photomicrographs c and i: Orcein Giemsa, a differential stain. Photomicrographs e, g and k: haematoxylin and Eosin (H&E). Photomicrographs f and h: Immunohistochemistry for Cytokeratin 14 which stains the ORS and is visualized by AEC (3-amino-9-ethylcarbazole, red). In contrast to catagen IV, in catagen V a club hair is formed which is entirely surrounded by the secondary hair germ. The secondary hair germ is part of the ORS and expresses Cytokeratin 14. Photomicrographs j and l: Immunohistochemistry for cleaved Lamin A

which marks apoptotic cells in the epithelial strand and trailing CTS cells and is visualized by permanent Red (pink). Photomicrograph m: example of Rhodamin Toluidine blue staining which stains the club hair blue and the keratinized trichilemmal keratin dark blue to purple. Abbreviations: CTS: connective tissue sheath; DP: dermal papilla; IHC: Immunohistochemistry; ORS: outer root sheath; SG: sebaceous gland; APM: arrector pili muscle. Bar = 100µm.

Figure 3: Key features of the canine telogen hair cycle stage modified from the classification system used in mice.[5] Figure in analogy to the classification guide for murine hair follicles by Müller-Röver et al. (2001).[5] In the first column the basic features of the telogen stage are depicted in a schematic drawing. The second column lists the most important morphological features that are visualized in two histological photographs in the third column. Photomicrograph a: haematoxylin and Eosin (H&E). Photomicrograph b: Immunohistochemistry for Vimentin and Laminin. Anti-Vimentin antibodies mark mesenchymal tissue – in this case the dermal papilla - with permanent Red (pink) and Anti-Laminin marks the basement membrane with diaminobenzidine (brown). Abbreviations: CTS: conntective tissue sheath; DP: dermal papilla; IHC: Immunohistochemistry; IRS: inner root sheath; ORS: outer root sheath; SG: sebaceous gland; APM: arrector pili muscle. Bar = 100µm.

Figure 4: Schematic drawing of the position of the dermal papilla and hair shaft during the hair cycle stages. Note that the dermal papilla moves from the dermis (telogen through anagen II) into the subcutaneous fat (anagen III through catagen VIII; it has its deepest position during anagen IIIc through catagen II) and up into the dermis again.

Figure 5: Schematic drawing of the anatomical structures stained with the different immunohistochemical stains used to characterize the canine hair cycle. The follicle in the drawing represents a late anagen stage. Immunohistochemistry for Cytokeratin (CK) 14 stains the ORS as well as the basal layer of the epidermis. CAM5.2, an antibody for keratin 8, labels the non-cornified part of the IRS. Ki-67 marks proliferating cells, which are most commonly encountered in matrical cells. Vimentin is expressed by the mesenchymal dermal papilla, while Laminin depicts the basement membrane of the hair follicle. Lamin A is a marker for apoptotic cells, some of which can be encountered in the ORS of anagen follicles. An increase of Lamin A-positive cells occurs in catagen especially in the epithelial strand and the trailing CTS cells. Abbreviations: CTS: conntective tissue sheath; DP: dermal papilla; IRS: inner root sheath; ORS: outer root sheath, SG: sebaceous gland, APM: arrector pili muscle

Table 1: Staining characteristics of the histochemical and immunohistochemical stains applied to characterize the canine hair cycle

ANTIBODY/STAIN	Helpful for…	Epi-dermis	BM follicle	ORS	IRS	HM	TK	DP	ES	tCTS	SwG	M.ap
Anti-Cytokeratin 14	Transition catagen IV-V	+		+++					+++			
Anti-CAM 5.2	Anagen IIIa				+++ (non keratinized)						+++	
Anti-Ki-67	Anagen stages	+		++ (anagen)	++ (anagen)							
Anti-Vimentin	Position and shape of DP							++ +		+ / -		++
Anti-Laminin	Position and shape of DP		+++									+++
Anti-cleaved Lamin A	Limited help for catagen stages			+ (catagen)					+ (catagen)	+ (catagen)		
Sacpic	Anagen IIIa; late catagen stages; transition catagen-telogen				+++ (keratinized and non keratinized)		++ +					
Masson Fontana	AnagenIIIa; transition catagenII-III					++ (pre-cortical)						
Toluidine blue/Safranin	Questionable reliability			++ (catagen)			++					
Rhodamin/ Toluidine blue	Differential staining like HE			++	+++	++	++ +					
Acid Orcein-Giemsa	Differential staining like HE				+++		++ +					

BM: basement membrane; ORS: outer root sheath; IRS: inner root sheath; HM: hair matrix ; DP: dermal papilla ; ES : epithelial strand ; tCTS : trailing connective tissue sheath cells ; SwG: sweat gland; M.ap: M. arrector pili; TK: trichilemmal keratin

Table 2: Antibodies and protocols of the immunohistochemical stains applied to characterize the canine hair cycle

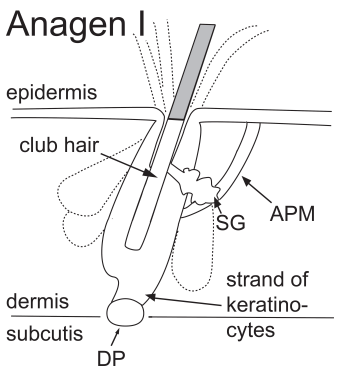
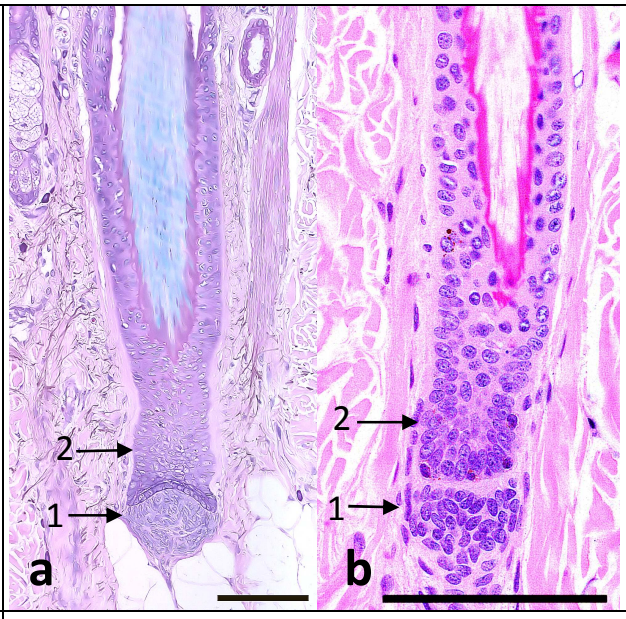
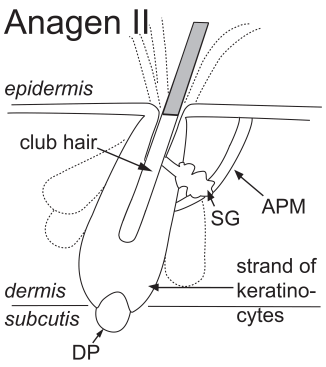
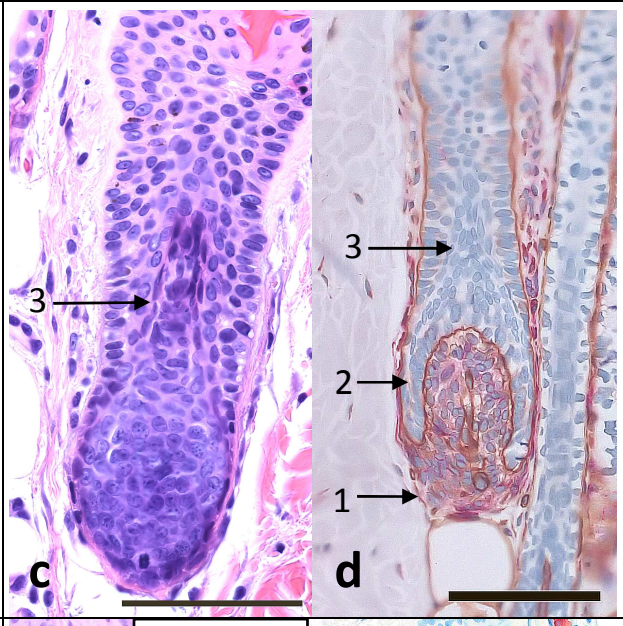
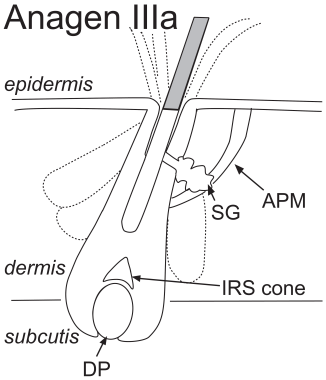
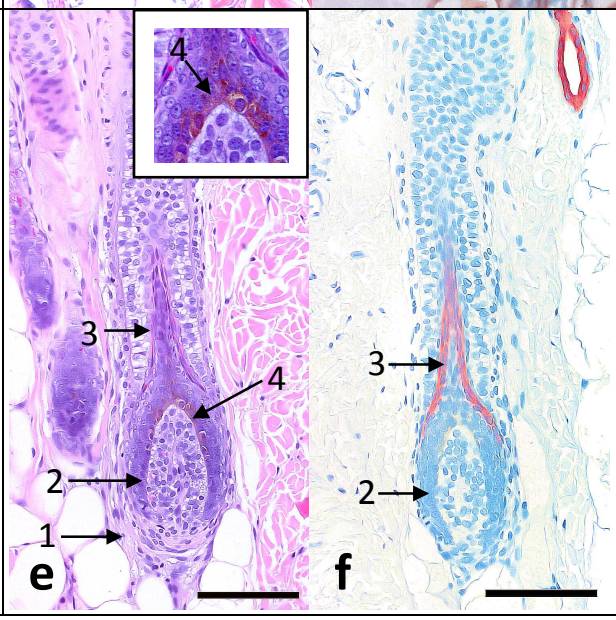
1°Antibody	Supplier	Clone	Species	Dilution	Pretreatment	Incubation	2°Antibody	Supplier	Visualization
Anti-Cytokeratin 14	BioGenex[A]	MU146-UC	Mouse	1:50	HIER citrate buffer pH6 in microwave, 15min	ON, 4°C	Goat-anti-mouse	Jackson ImmunoResearch[H]	Streptavidin HRP-conjugate, Aminoehylcarbazole (Sigma)[F]
Anti-CAM 5.2	Becton Dickinson[B]	Monoclonal	Mouse	RTU	Trypsin 0.25% (w/v)in Tris-HCl-buffer, 20min, 37°C	40min, RT	Goat-anti-mouse	Jackson ImmunoResearch[H]	Streptavidin HRP-conjugate, Aminoehylcarbazole (Sigma)[F]
Anti-Ki-67	Invitrogen[C]	7B11	Mouse	1:75	HIER citrate buffer pH6 in microwave, 15min	ON, 4°C	Goat-anti-mouse	Jackson ImmunoResearch[H]	Streptavidin HRP-conjugate, Aminoehylcarbazole (Sigma)[F]
Anti-Vimentin	Dako[D]	Vim 3B4	Mouse	1:500	Proteinase K 1%, 15min, 37°C	60min, RT	EnVision G/2 Doublestain System	Dako[D]	EnVision G/2 Doublestain System (Dako)[D]
Anti-Laminin	Dako[D]	Polyclonal	Rabbit	1:1400		60min, RT			
Anti-cleaved Lamin A (small subunit)	Cell signaling[E]	Polyclonal	Rabbit	1:100	Heating in cell conditioning solution CC1, 4 x 8min	60min, RT	Discovery Universal 2° Antibody	Roche[G]	RedMap™ detection kit (Roche)[G]

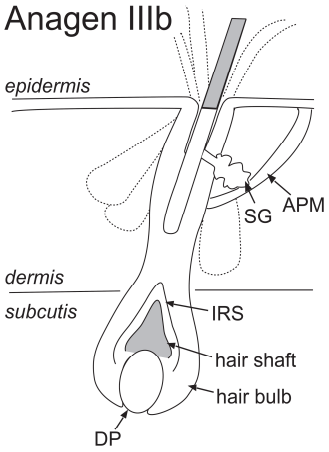
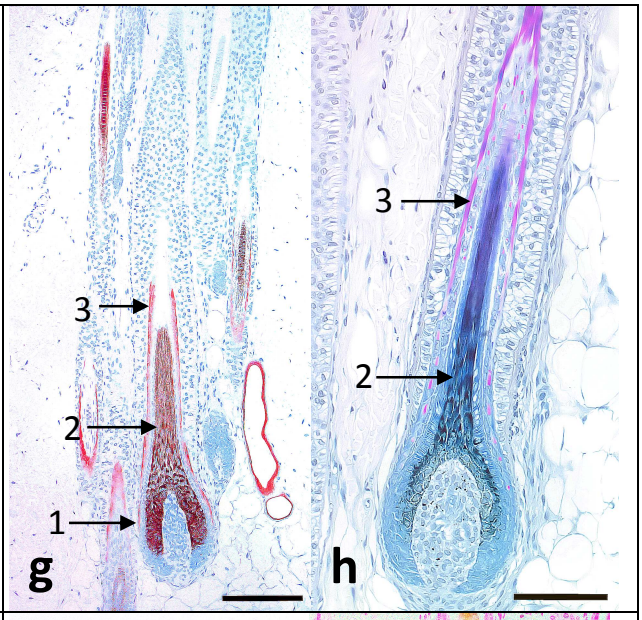
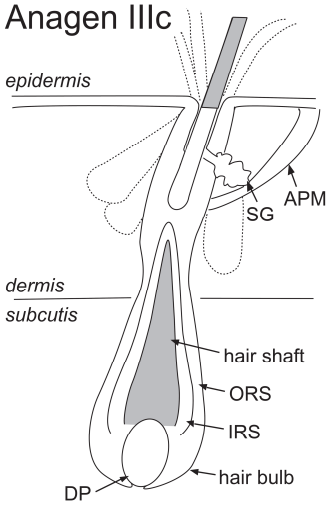
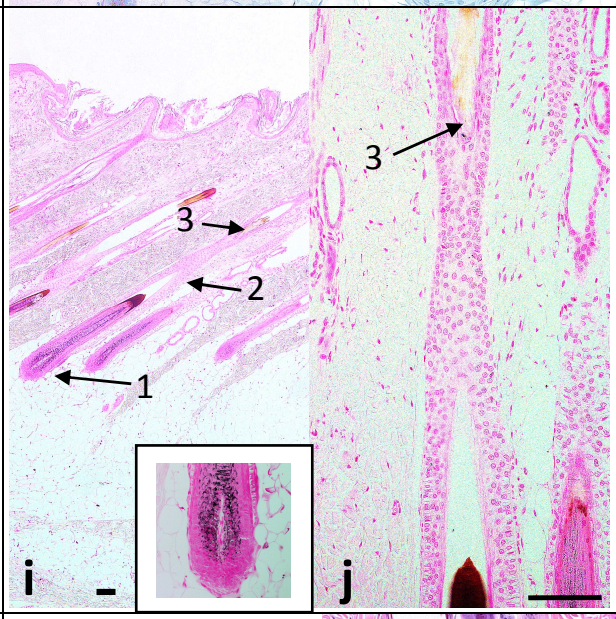
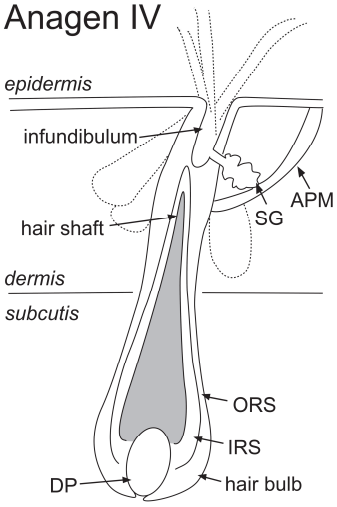
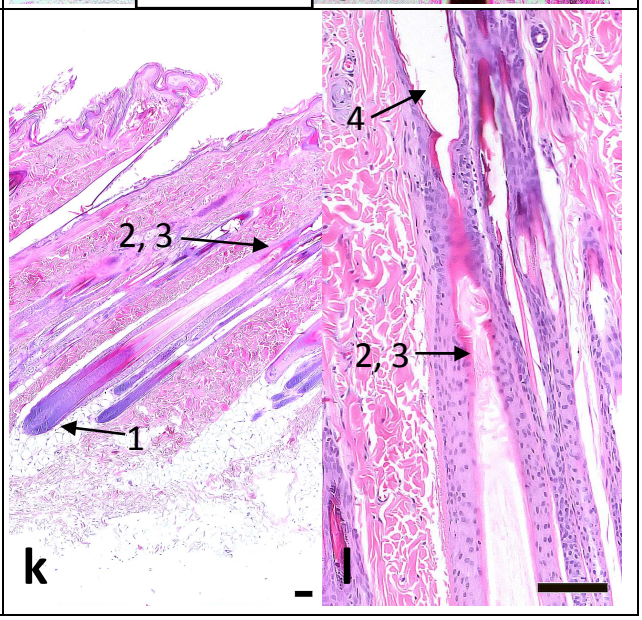
HIER: heat induced epitope retrieval; RTU: ready to use; RT: room temperature; ON: overnight
[A] BioGenex Laboratories Inc., San Ramon, California, USA; [B] Becton Dickinson AG, Allschwil, Switzerland; [C] Invitrogen AG, Basel, Switzerland; [D] Dako Schweiz AG, Baar, Switzerland; [E] Cell signalling technology-BioConcept, Allschwil, Switzerland; [F] Sigma-Aldrich Chemie GmbH; [G] Roche Basel, Switzerland; [H] Jackson ImmunoResearch Europe Ltd., Newmarket, Suffolk, UK

Table 3: Mean percentage of the different cycle stages obtained from skin biopsies from shoulder and thigh of 20 beagle dogs. Not all hair follicles could be assigned to an exact cycle stage and thus could only be placed into one of the broader categories

Main category	Percentage	Broader category	Percentage	Exact cycle stage	Percentage
Anagen	30.1	early anagen	11.8	anagen I	4.4
				anagen II	1.0
				anagen IIIa	1.5
				anagen IIIb	3.9
		late anagen	17.8	anagen IIIc	1.1
				anagen IV	1.5
				anagen V	1.1
				anagen VI	0.5
		anagen not determinable	0.5		
Catagen	7.6	early catagen	1.9	catagen I	0
				catagen II	0.4
				catagen III	0.4
				catagen IV	0.2
		late catagen	4.9	catagen V	0.2
				catagen VI	0.4
				catagen VII	1.7
				catagen VIII	1.1
		catagen not determinable	0.8		
Telogen	27.6	telogen with hair	14.4		
		telogen without hair	13.2		
Not determinable	34.7				

Figure 1b

	1. Bulb and DP still reside in dermis 2. Thickening and prolongation of keratinocyte strand between DP and club hair	
	1. DP at border dermis/subcutis 2. Matrical cells partially enclose the DP 3. Matrical cells above DP begin to differentiate towards hair shaft and IRS	
	1. Bulb at border dermis/subcutis 2. The DP is further enclosed by matrical cells than in anagen II 3. IRS-cone appears above DP 4. First melanin granules visible	

Anagen IIIb  	1. Bulb and DP reside half way down in the subcutaneous fat tissue 2. Fully developed hair shaft above the DP 3. Hair shaft entirely enclosed by IRS	
Anagen IIIc  	1. Bulb and DP have reached deepest position in subcutaneous fat tissue 2. Hair shaft reaches approximately the middle of the isthmus 3. The old club hair may still be present *Inset:* large amount of melanin granules in the bulb	
Anagen IV  	1. Bulb and DP reside deep in the subcutis 2. Distal end of IRS and tip of hair shaft reach the infundibulum 3. Hair shaft has not yet entered the infundibulum 4. The old club hair is gone	

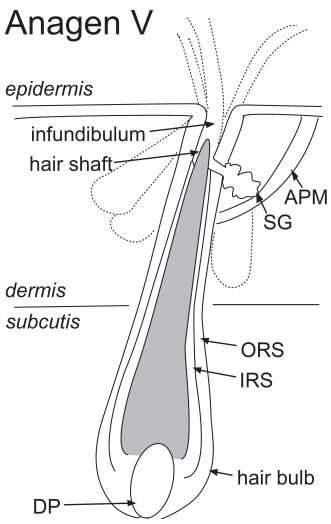
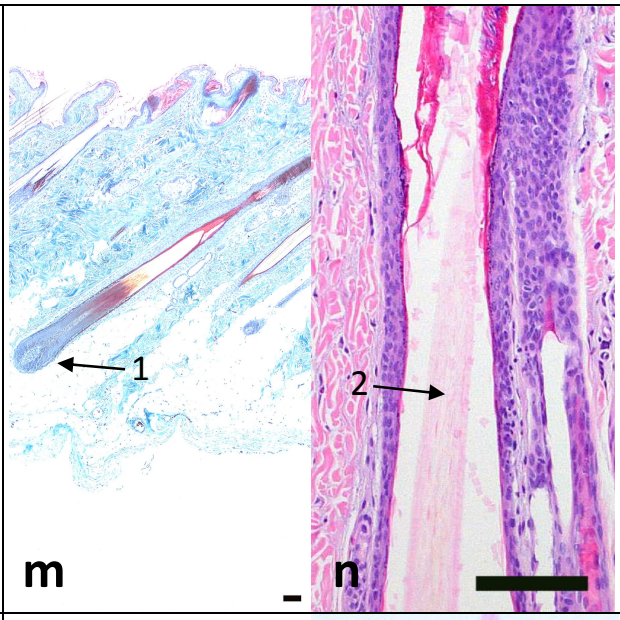
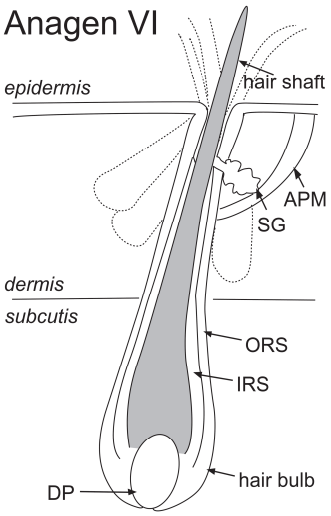
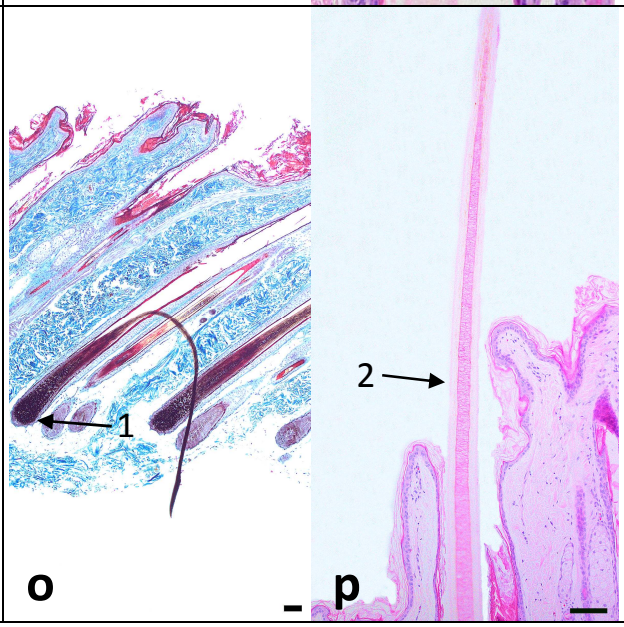
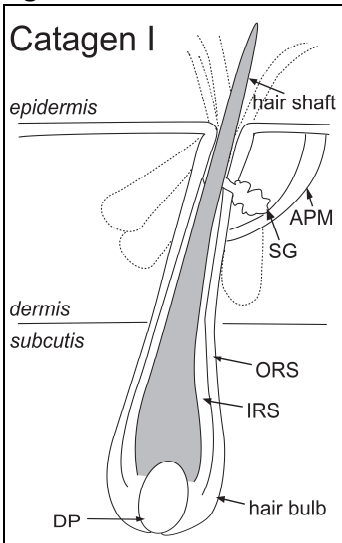
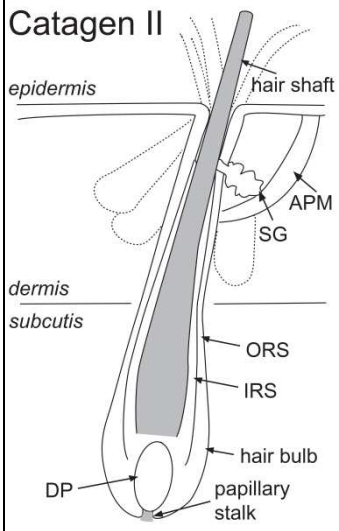
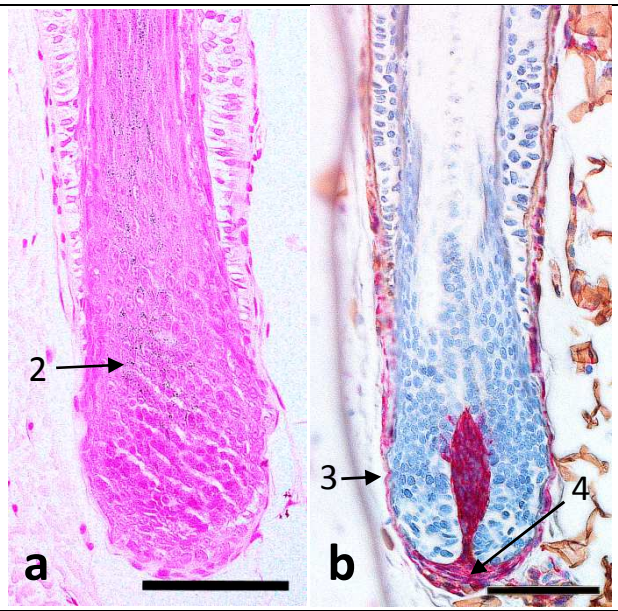
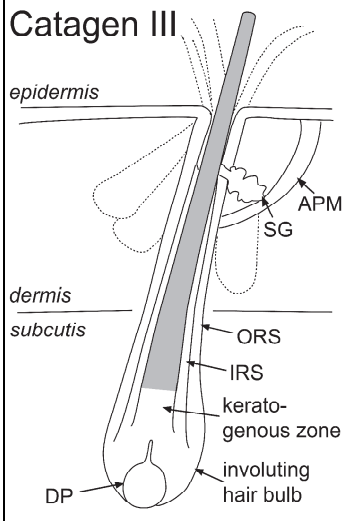
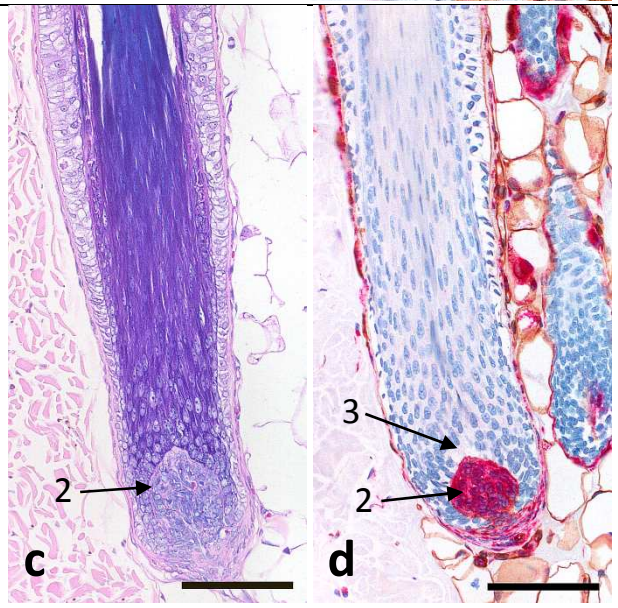
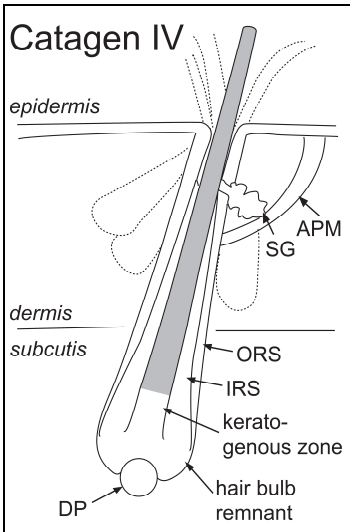
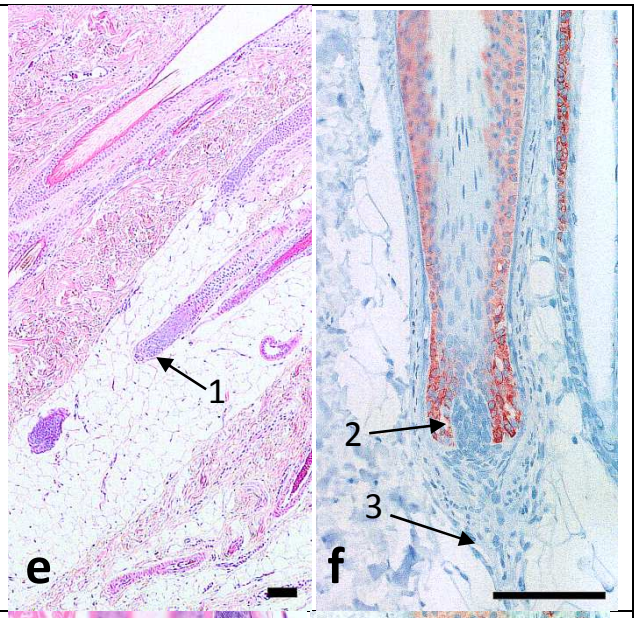
Anagen V  	1. Bulb and DP reside deep in the subcutis 2. Tip of hair shaft enters the infundbulum	
Anagen VI  	1. Bulb and DP reside deep in the subcutis 2. Tip of hair shaft emerges through the ostium	

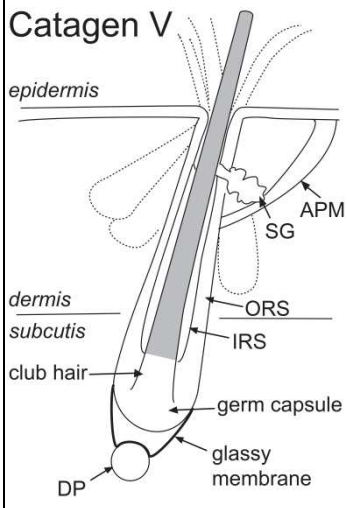
Figure 1c

	1. „theoretical stage“ (Straile, 1961) 2. No morphological alterations compared to anagen VI	
	1. Bulb and DP reside deep in the subcutis 2. Decreased number of melanin granules above the DP 3. Bulb is narrower than in anagen VI 4. Papillary stalk of fibroblasts between DP and CTS > 3 cell layers	
	1. Hair follicle is shorter than in catagen II 2. DP has a characteristic onion shape 3. No melanin granules above the DP	

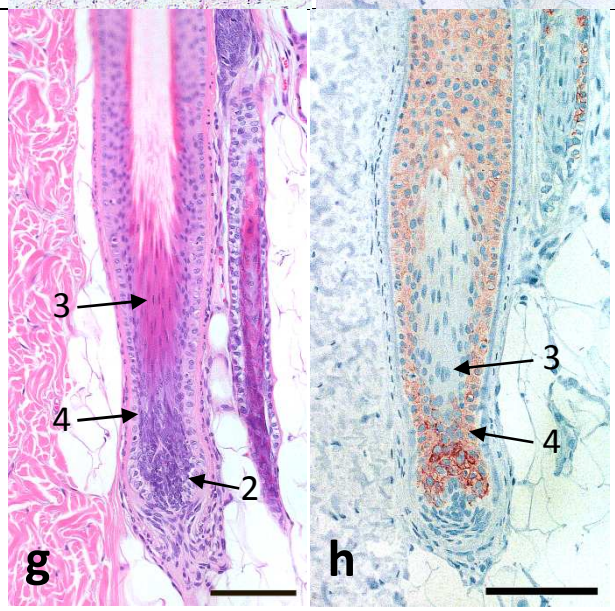


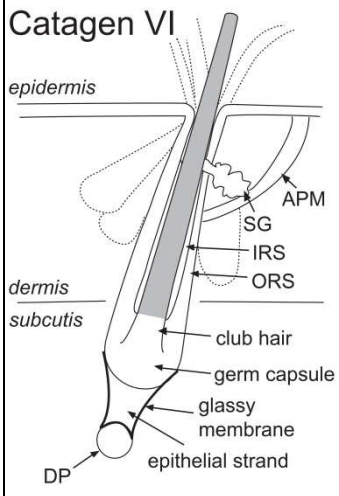
1. Hair follicle is shorter than in catagen III
2. DP forms compact ball and is < 50% enclosed by the bulb
3. Trailing CTS cells are now visible



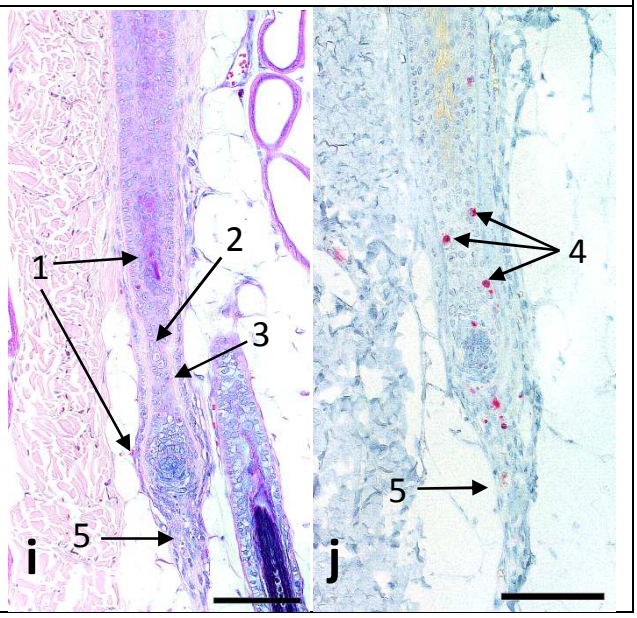


1. Hair follicle is shorter than in catagen IV
2. DP still located in subcutaneous fat tissue
3. Proximal end of hair shaft develops into club hair, surrounded by 2° hair germ of the ORS
4. Constriction of developing epithelial strand between DP and 2° hair germ





1. Club hair and DP reside still in subcutis
2. Epithelial strand > DP diameter
3. Glassy membrane around epithelial strand
4. Many apoptotic cells in the epithelial strand
5. Trailing CTS cells



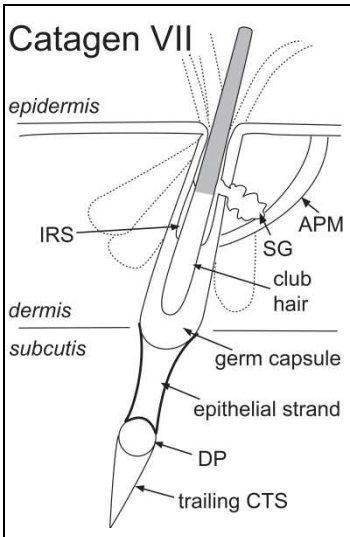
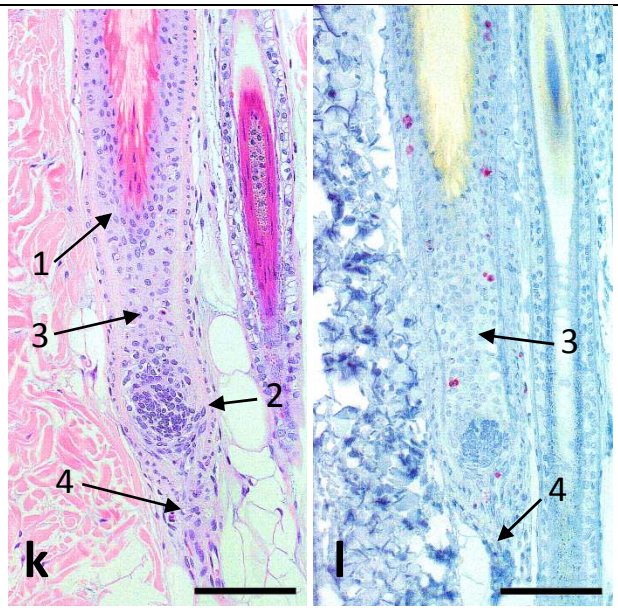
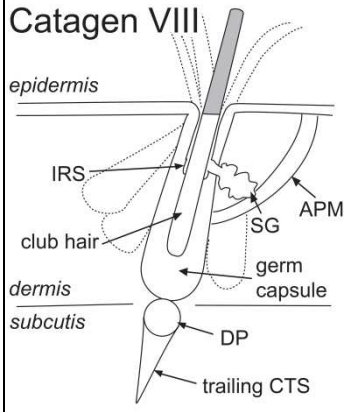
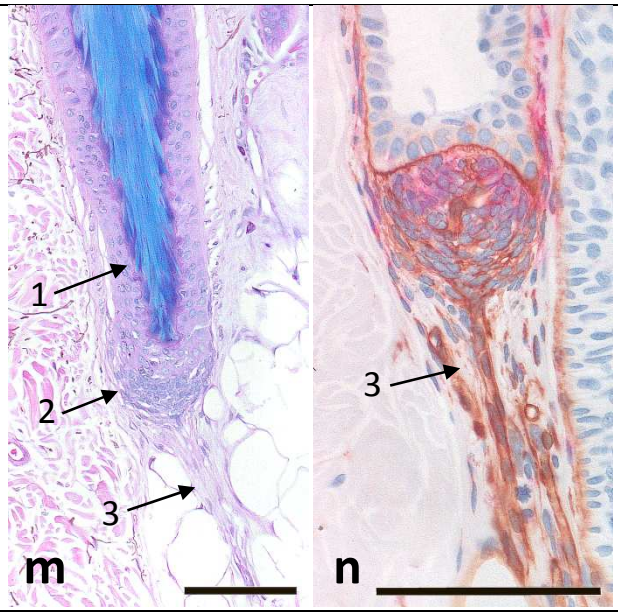
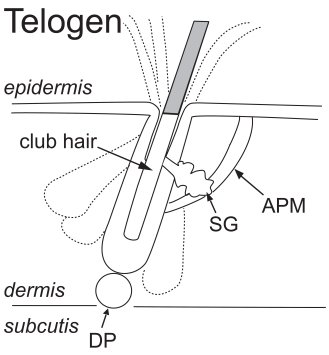
	1. Club hair and 2° hair germ at border dermis/subcutis 2. DP resides still in subcutis 3. Epithelial strand > twice DP diameter 4. Prominent trailing CTS cells	
	1. Club hair and 2° hair germ in the dermis 2. DP resides at the border dermis/subcutis 3. Trailing CTS cells	

Figure 1a



1. Hair follicle and DP reside in dermis
2. Club hair anchored in 2° hair germ in the ORS
3. DP compact and ball-shaped
4. No IRS
5. No trailing CTS cells

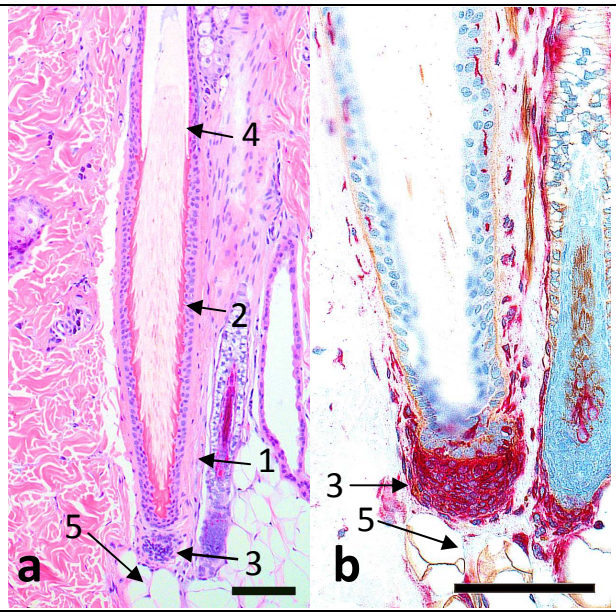


Figure 2

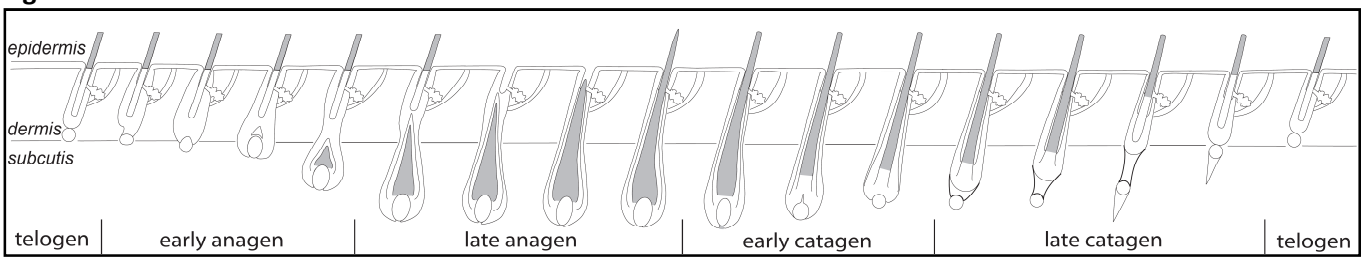
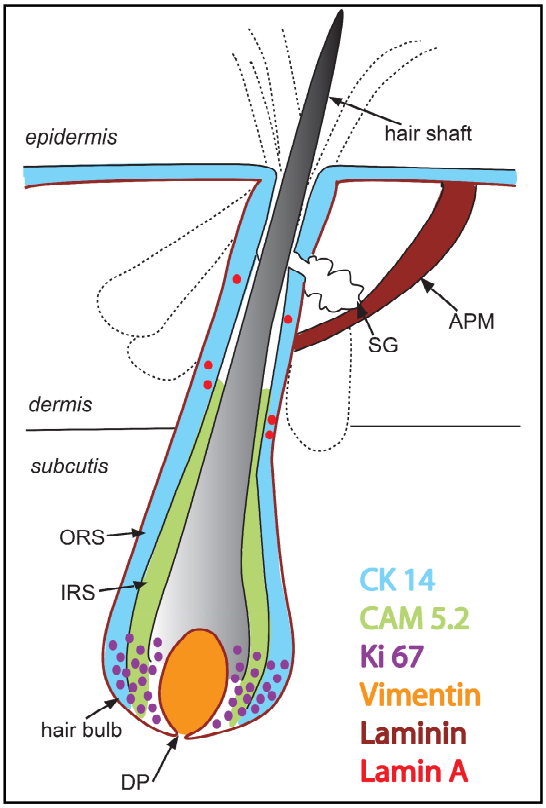

epidermis
dermis
subcutis
telogen
early anagen
late anagen
early catagen
late catagen
telogen

Figure 3


epidermis
hair shaft
APM
SG
dermis
subcutis
ORS
IRS
hair bulb
DP
CK 14
CAM 5.2
Ki 67
Vimentin
Laminin
Lamin A