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Evaluating cornification in the dog by characterizing canine transglutaminase 1 and defining the clinical, histologic and morphometric features of normal, thyroxine-treated hypothyroid and untreated hypothyroid dogs

presented by

Kelly Margaret Credille

has been accepted towards fulfillment
of the requirements for

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Robert W Wilkerson DVM, MS
Major professor

Date 5-8-00

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EVALUATING CORNIFICATION IN THE DOG BY CHARACTERIZING CANINE TRANSGLUTAMINASE I AND DEFINING THE CLINICAL, HISTOLOGIC AND MORPHOMETRIC FEATURES OF NORMAL, THYROXINE-TREATED HYPOTHYROID AND UNTREATED HYPOTHYROID DOGS

By

Kelly Margaret Credille

A DISSERTATION

Submitted to
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for the degree of

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Department of Pathology

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ABSTRACT

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By

Kelly Margaret Credille

Cornification is the process in which epithelial cells, called keratinocytes, mature to form the life-sustaining outer-most layer of the skin, the stratum corneum, and several components of the hair follicle including the hair shaft. Although cornification is a complex and highly regulated process, it always requires three elements: 1) structural proteins such as keratins, keratin-associated proteins, and the proteins that form the cell envelope, 2) lipids which act as a glue to aid in annealing proteins and 3) the action of enzymes known as transglutaminases that cross-link proteins and stabilize cell membranes.

In the first objective of the research described herein, a gene essential for normal cornification, the canine *transglutaminase 1* (*tgm1*) gene, was characterized by determining the sequence of its coding region, its putative amino acid sequence and its location within the canine genome using the polymerase chain reaction, fluorescence in situ hybridization and radiation hybrid mapping. This enzyme is central to the formation of the stratum corneum in all mammals and is a candidate cause for a number of inherited scaling skin diseases of dogs. The results show that canine *tgm1* has a marked similarity to its human ortholog and is located on canine autosome 8.

The second objective of this dissertation was to define the differences in follicular cornification and hair growth in normal, thyroxine-treated hypothyroid and untreated-

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hypothyroid Beagle dogs using morphologic and morphometric evaluation of the hair cycle and assessment of cell proliferation within the hair follicle by immuno-histochemistry. These investigations demonstrated that the hair cycle of Beagle dogs is unlike that of the scalp hair of humans. Normal Beagles have a telogen-predominant hair growth pattern, meaning the majority of the hair follicles are in telogen, the resting stage of the hair cycle. In states of thyroid hormone deficiency, this resting stage becomes prolonged and when a telogen hair is eventually shed, it is not replaced, resulting in mild hair loss that differs from the classically described alopecia found in hypothyroidism. In thyroxine-treated hypothyroid dogs, telogen is still the predominant phase of the hair cycle, but the presence of many more anagen hairs suggests, as breeders have long believed, that thyroid hormone supplementation stimulates hair growth.

The morphologic changes that occur during the transition from telogen to anagen in canine hair follicles after hair shaft plucking were also examined in the three groups. Differences in the regeneration of plucked anagen and telogen follicles depended on the degree of injury that occurred at the time of plucking and the thyroid hormone state of the dog: hair follicles from untreated-hypothyroid dogs having the fewest hairs that regenerated. Importantly, unlike human and murine follicles, canine follicles do not demonstrate a localized area of proliferation within the follicle during the initiation of hair growth.

DEDICATION

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So many thanks, of course, to Bob for mentorship that has spanned 10 years as a vet student, resident and graduate student.

I must thank the staff of the Clinical Center Histology Lab, Josselynn Miller, Amy Porter, Kathy Campbell and Rick Rosebury for their wonderful outlook and expert skill in immunohistochemistry.

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LIST OF ABBREVIATIONS

AP	activator protein
BDNF	brain derived neurotrophic factor
BMP	bone morphogenetic protein
BrdU	5-bromo-2 deoxy-uridine
cDNA	complementary DNA
CE	cornified envelope
CFA	*Canis familiaris* autosome
cR	centi-ray
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
FGF	fibroblast growth factor
FISH	fluorescence in situ hybridization
FT3	free triiodothyronine
FT4	free thyroxine
gDNA	genomic DNA
H&E	hematoxylin and eosin
HGF/SF	hepatocyte growth factor, scatter factor
^{3}H-TdR	tritiated labeled thymidine
IFAP	intermediate filament associated protein
IFN g	interferon gamma
IL-1	interleukin 1
ILGF	insulin like growth factor
kb	kilobase
kD	kilodalton
KGF	keratinocyte growth factor
LEF-1	lymphoid enhancer factor-1
LI	lamellar ichthyosis
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
nm	nanometer
NT	neurotrophin
PCR	polymerase chain reaction
RH	radiation hybrid
rT3	reverse triiodothyronine
RT-PCR	reverse transcription PCR
SHH	sonic hedgehog

SPR	small proline rich proteins
T3	triiodothyronine
T4	thyroxine
TBG	thyroid hormone binding globulin
TBPA	thyroxine binding prealbumin
TGM 1	transglutaminase 1
Tgase	transglutaminase
TGF a	transforming growth factor alpha
TGF B	transforming growth factor beta
Tm	temperature of melting
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone
TT3	total triiodothyronine
TT4	total thyroxine
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
VEGF	vascular endothelial growth factor

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

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EPIDERMIS AND STRATUM CORNEUM

Structure and Function

The skin's first line of defense and protection is provided by a few layers of cells known collectively as the epidermis. The main cell type comprising the epidermis is an epithelial cell called a keratinocyte. The major function of this cell is to differentiate through a complex and carefully orchestrated set of morphologic and biochemical steps, to form an outer, life sustaining barrier that prevents the loss of water from the body and withstands chemical, microbial, immunological and ultraviolet/solar assault. This process is known as cornification.

As keratinocytes differentiate, they lose mitotic activity, increase in volume, flatten, synthesize new structural and enzymatic proteins and lipids, produce new organelles, express new surface antigens and undergo breakdown and loss of specific molecules and organelles. Although a continuum, the morphology of the epidermis has been subdivided into layers (from inside to outside): the basal cell layer, spinous cell layer, granular cell layer (variably present in dogs and cats) and stratum corneum or cornified layer, which provides most of the epidermal barrier function. Once keratinocytes fully cornify, they are called corneocytes or sometimes "squames" (Figure 1). In Beagle dogs, as in humans, this journey from the basal layer to corneocyte requires about 22 days (Kwochka and Radermakers 89).

In the normal epidermis, only the cells in the basal layer undergo DNA synthesis and mitosis. Basal keratinocytes represent a single layer of cuboidal cells that are anchored to the basement membrane via hemidesmosomes. Although the basal layer is

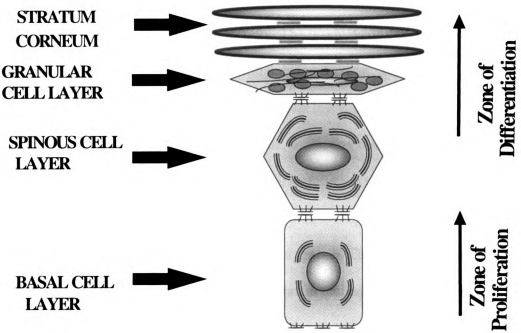

Figure 1. Diagram of the morphologic continuum of keratinocyte differentiation.

The basal cell layer is the progenitor cell layer and therefore is the zone of proliferation. The spinous cell layer, granular cell layer, and stratum corneum represent the morphologic progression of the basal cell progeny.

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the only mitotically active layer, most basal cells are reversibly arrested in the G0 phase of the cell cycle and function primarily to attach to the basement membrane. These anchoring cells often have a more serrated basal surface. A small subpopulation of the basal cells, known as stem cells, provide the supply of new keratinocytes and repopulate the epidermis. Although previously believed to have a high mitotic rate, stem cells rarely divide. When they do, they give rise to daughter cells that have a short burst of high mitotic activity, known as transient amplification. The products of these cells then migrate from the basal layer and undergo terminal differentiation, ultimately forming the stratum corneum. Once cells transit into the suprabasal layer, they irreversibly lose their ability to progress through the cell cycle and divide, indicating that losing the ability to undergo mitosis is an early and important step in differentiation (Clausen and Potten 90, Jetten and Harvat 97).

The cells of the spinous cell are large and polygonal. These cells appear "spinous" because during tissue processing with paraffin embedding, they shrink, resulting in a stretching of the desmosomal intercellular attachments. The elongated desmosomes appear as "spines" surrounding the cells of this layer.

In addition to serving as points of adhesion between cells, desmosomes are also the sites where the protein filaments of the cytoskeletal network anchor to the plasma membrane. In the basal cell layer, desmosomes are less common than in the spinous cell layer and express different sets of adhesion molecules. Modified desmosomes, known as corneodesmosomes, persist in the cornified layer and play a role in corneocyte adhesion in the lower and mid levels of the stratum corneum. Corneodesmosin is a protein synthesized very late in keratinocyte differentiation at the junction of the cornified layer

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and living epidermis. This newly discovered protein binds to the pre-existing desmosomes and is progressively proteolysed into smaller fragments in more superficial layers of the stratum corneum. In this way, corneodesmosin may protect the intercellular adhesion proteins of the corneodesmosomes from early proteolytic degradation (Haftek et al 97, Guerrin et al 98).

The granular cell layer is composed of thin, flattened keratinocytes with variably prominent blue granules. These granules, visible by light microscopy and known as keratohyaline granules, contain products of keratinocyte differentiation used to assemble components of the stratum corneum. A second structure synthesized in this layer is the lamellar body, which is also critical to the formation of the stratum corneum.

The stratum corneum is composed of extremely thin, flattened, protein-filled and anucleate corneocytes. In humans, the stratum corneum averages 15 cells thick but this varies with age, sex and body location.

The Process of Cornification

Cornification results in a structural barrier through which neither hydrophilic nor hydrophobic molecules can easily pass. This barrier is the result of the convergence of three biochemical processes, followed by desquamation. These three pathways are 1) the formation and organization of keratin intermediate filaments, 2) the formation and dispersion of an intercellular lipid glue or mortar that anneals the fully cornified cells together, and 3) the synthesis of the cornified envelope, the toughest portion of the stratum corneum, that also functions to interconnect the intracellular keratin matrix with the intercellular lipid glue.

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Formation and Organization of Keratin Intermediate Filaments

The cytoskeleton of epithelial cells is composed of three systems of filamentous proteins, microfilaments (6 nm in diameter), intermediate filaments (10 nm in diameter) and microtubules (25 nm in diameter). The keratin proteins are intermediate filaments and are synthesized specifically in epithelial cells, including the cells that comprise the epidermis and hair follicles. Keratin provides the scaffold supporting the intracellular architecture of the epithelial cell, imparting resilience and defining the cell's shape. This function has become better understood as diseases caused by mutations in keratin genes have been defined, disorders usually characterized by mechanically induced blisters within stratified epithelial tissues, including the cornea, skin, esophagus and hair (Fuchs et al 97, Hutton et al 98, Dunnill 98).

Although "keratin" is often described as a protein in the singular form, over 30 different keratins have been recognized and it is likely more will be identified, especially those in the hair shaft (Fuchs et al 97, Suter et al 97, Powell et al 97). These proteins are subdivided into 2 major families, the type 1 and type 2 keratins, based on their size, pH, and amino acid sequence (Figure 2). All of the keratins, however, share a highly conserved, largely helical secondary structure with a central rod-like alpha helical domain subdivided into 4 segments by nonhelical linkers and bracketed by nonhelical amino and carboxy terminal end domains. It is believed that the linker regions add flexibility to the molecules, the central region is responsible for the features common to all keratins, such as interaction with other proteins, and the two variable end domains impart each keratin with its unique characteristics. These head and tail domains are divided into subdomains based on sequence similarities to other intermediate filaments (Figure 3) (Albers 96).

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Keratin Type	pH	Size	Molecularly Distinct Forms
Type I	Acidic	Smaller (40-60kD)	15-30 genes
Type II	Neutral to basic	Larger (50-70kD)	15-30 genes

Figure 2. Differentiating characteristics of type I and II keratins.

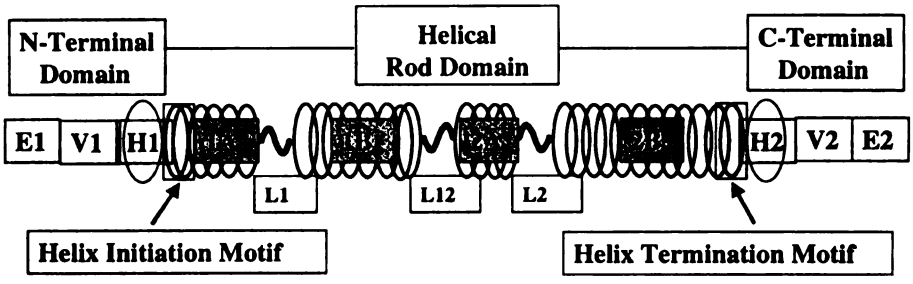

Figure 3. Molecular structure of a (type II) keratin.

The alpha-helical segments are labeled 1A, 1B, 2A and 2B and inbetween the helices are linker sequences L1, L2 and L12. H1 and H2 denote subdomains of high DNA sequence homology between members of the type I and II families. The V regions contain variable numbers of peptide repeats and the end regions labeled E are highly charged.

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Glycine rich termini are characteristic of epidermal keratins, as this small amino acid forms a compact protein, while many cysteines are found in hair keratins, resulting in tougher ("hard") keratins through extensive disulfide bridges (Smack et al 94, Blumenberg and Tomic-Canic 97).

Keratin filaments are obligate heteropolymers, meaning that the filaments are composed of a specific pairing of type I (or acidic) and type II (or basic) keratin molecules. Initially, the type I and type II keratins align to form a heterodimer. Two heterodimers then assemble in a staggered fashion to form a protofilament. Four protofilaments bind to form the 10nm intermediate filament (Suter et al 97). The initial pairing of keratins is not a random process: a type I keratin will only bind with a specific type II molecule. Remarkably, the intermediate filaments can assemble without the aid of helper proteins or enzymes.

The different keratin pairs result in variations in the cytoskeleton that are adapted to different anatomical locations (Table 1) (Suter et al 97, Hutton et al 98). Within the basal layer of the epidermis, keratins K5 and K14 are characteristically expressed. These keratins are relatively small and because they are serine-rich, are prone to phosphorylation. Phosphorylation is essential for the breakdown of the keratin cytoskeleton during cell division. In contrast suprabasal keratinocytes primarily express the pair K1/K10. The keratins of terminal differentiation, K1/K10, have long glycine-rich terminal domains. Glycine is the smallest amino acid, having no side chains; it is also extremely hydrophobic. This allows K1/K10 to pack tightly into paracrystalline arrays that are resistant to proteolysis making this keratin pair ideal for providing a

Table 1.

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K1	
K6	
K2e	
K9	

Table 1. Epidermal keratin pairs based on anatomic location.

Type I Keratin	Type II Keratin	Location	Comments
K5	K14, 15	Basal to lower spinous layers	More malleable than K1-K10
K1	K10	Mid spinous to stratum corneum	Mid spinous to stratum corneum
K6	K16	Throughout epidermis	Mid spinous to stratum corneum
K2e	K1?	Mid spinous to stratum corneum	Replaces K1-K10 during hyperproliferation
K9	K10?	Mid spinous to stratum corneum	Expressed on palms,soles (humans) foot pads (mice)

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protective barrier (Blumenberg and Tomic-Caninc 97). K6 and K16 are not expressed in the normal epidermis; rather they are synthesized when the epidermis is proliferating more quickly than normal. Thus, as this pair is synthesized, production of the differentiation-specific keratins, K1/K10 in the epidermis and K3/K12 in the cornea, is turned off. Lastly, for some keratins, such as K2e and K9, the type I keratin to which it pairs is still unknown (Blumenberg and Tomic-Canic 97, Suter et al 97).

In both humans and mice, the acidic (type I) and basic (type II) keratin gene families (including epithelial and hair keratins) have been localized to two chromosomal regions. The acidic keratins are located on human autosome 17q and murine autosome 11. The basic cluster is found on chromosome 12q in humans and chromosome 15 in mice. This gene clustering is also true for dogs, as recently several canine keratin genes were cloned and their chromosomal locations were identified on autosome 9 (acidic gene cluster) and autosome 27 (basic gene cluster) using fluorescent *in situ* hybridization (FISH) (Miller et al 99).

As keratinocytes reach the upper levels of the epidermis, the keratin filaments undergo a final step in processing that requires an intermediate filament associated protein (IFAP) called filaggrin. The precursor protein of filaggrin, profilaggrin, is a 450 to 600 Kd, heavily phosphorylated protein that is synthesized in the granular cell layer where it is the major component of keratohyaline granules (Smack et al 94). Profilaggrin is poorly soluble and has a distinctive amino acid composition rich in histidine, serine, glycine, glutamine and arginine. As keratinocytes enter the transitional zone between the granular and cornified layers, profilaggrin undergoes dephosphorylation and proteolysis to form filaggrin. Mature filaggrin forms a matrix that packs keratin into parallel

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macrofibrils and facilitates disulfide cross-linking within the stratum corneum. This organization of keratin by filaggrin increases the strength and stability of the cornified layer (Smack et al 94, Albers 96). After organizing keratin intermediate filaments, filaggrin is no longer required and undergoes rapid breakdown. Some of the amino acids released are modified and function as regulators of stratum corneum homeostasis. Pyroglutamic acid, derived from glutamine and glutamic acid, retains water within the superficial cornified cells. Urocanic acid, derived from histidine, acts as a natural sunscreen (Jackson et al 93). At the end of the process of keratinization, approximately 85% of the total protein volume of a corneocyte is composed of keratin (Albers 96).

Synthesis and Role of the Stratum Corneum Lipids

In the nucleated layers of the epidermis, desmosomes function to hold keratinocytes together. In the stratum corneum, corneodesmosomes play a subordinate role to intercorneocyte lipids in annealing cells together. The composition of lipids is also critical for the impermeability of the skin; keeping it "waterproof" and protected from desiccation.

The stratum corneum is rich in nonpolar (hydrophobic) lipids- ceramides, fatty acids and cholesterols. Most lipid researchers find ceramides to be the most abundant (varying from 33-55% of total), with cholesterols next at 25-33% and free fatty acids present at roughly 10-33% (Downing 92, Menon and Ghadially 97). Both the precursors of these lipids (glucosylceramides and phospholipids) and the enzymes that convert them (glycosidases and lipases) are synthesized and packaged within modified lysosomes known as "lamellar bodies" (also known as Odland bodies and keratinosomes) within the

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epidermis. Lamellar bodies are generated from a widely dispersed tubulo-reticular system arising from the Golgi apparatus, known as the trans Golgi network, that forms an intricate interconnecting system for the transportation of lipids within the apical cytoplasm of the outermost granular cell (Elias et al 98). Lamellar bodies appear as small, 0.2 micron in diameter, ovoid organelles composed of stacks of compressed lipids that are membrane-bound (Wertz 97). Synthesis of these organelles originates in the spinous cell layer, but is most abundant in the granular cell layers where they aggregate beneath the cell membrane of the outermost granular layer cell. The compressed lipids within the lamellar bodies are secreted into the space between the epidermis and the first corneocyte layer when the bounding membrane of the lamellar body fuses with the keratinocyte plasma membrane. The lipid contents are released as round vesicles that elongate into bilayered disks and then fuse end-to-end to form sheets or lamellae (Menon and Ghadially 97). These multilamellar sheets of lipid fill most of the extracellular space of the stratum corneum, annealing corneocytes together and comprising much of the water barrier of the cornified layer. When examined ultrastructurally, the intercellular spaces are filled by a regular and alternating pattern of electron dense and electron lucent bands. The dense bands are thought to be the polar head groups of the lipid layers, while the lucent regions are those containing non-polar hydrocarbon chains (Wertz 97).

The most unusual of these lipids is acylglucosylceramide, which is localized to the membrane portion of the lamellar granule. Once released this lipid is further intercellularly catabolized into a long hydroxyceramide consisting of hydroxyacids linked to sphingosine bases. The hydroxyceramide molecules become covalently attached to the glutamine residues of proteins located on the outer surface of corneocytes (called the

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cornified envelope), producing a complete lipid coating around each cornified cell. This monomolecular lipid structure is 5 nm thick and is referred to as the "lipid envelope" (Marekov and Steinert 98). The lipid envelope interdigitates with other intercellular lipids within the stratum corneum in an highly organized manner, forming a unified lipid layer that contributes to the cohesiveness of the cornified layer after the disintegration of the corneodesmosomes (Wertz 97). Paradoxically, lamellar bodies are also where corneodesmosin, the protein that modifies and may protect corneodesmosomes from breakdown, is synthesized (Guerrin et al 98).

In addition to the enzymes needed to remodel the lamellar body lipids into functional barrier lipids, lamellar bodies also contain a complement of catabolic hydrolytic and proteolytic enzymes that become activated in the upper stratum corneum. These enzymes actively mediate the process of desquamation by degrading desmosomes and intercellular lipids (Proksch et al 93, Suter et al 97).

Finally, a surprising but potentially important role for the intercellular lipids has been proposed, that of messenger molecules. A sphingolipid called sphingosine that is abundant in human epidermis acts as a modulator of cell growth and differentiation by inhibition of protein kinase C in vitro and in vivo and by affecting other kinases, cytochrome-c oxidase and the thyrotropin releasing hormone receptor (Wertz 90). It has been proposed that a sphingosine gradient within the stratum corneum and epidermis may serve as a common pathway to coordinate mitotic activity within the basal layer, differentiation within the upper layers and desquamation of cornified cells (Wertz 90).

Ceramides are also included in lipid signaling in the example of increasing ceramide concentration causes decreased keratinocyte proliferation but promotes

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differentiation (i.e. cornified envelope formation, transglutaminase activity) in cultures of human and murine keratinocytes (Jung et al 98).

Another lipid, cholesterol sulfate, has been shown to activate transcription of the transglutaminase 1 (*tgm1*) gene (which encodes for an enzyme critical for normal cornification) by binding to a specific upstream regulatory region of the *tgm1* gene (Kawabe et al 98).

Formation of the Cornified Envelope

Although the majority of the corneocyte is composed of keratin, the most impermeable portion is just below the cell membrane, a tough, insoluble protein structure known as the cornified envelope (CE). Synthesis of the CE originates in the most superficial granular cell layer. Ultrastructurally, the fully developed CE appears as a strikingly uniform 15 nm thick band. The CE is formed by the crosslinking of a variety of proteins: loricrin, involucrin, cystatin A (keratolinin), S100 proteins and small proline rich peptides into a molecular syncytium (Jarnik et al 98, Nemes and Steinert 99). The crosslinking requires the action of a family of enzymes known as transglutaminases, the most important of which in the epidermis is transglutaminase 1 (Yamamoto et al 98). These enzymes, in the presence of calcium, link the CE proteins by forming a bond between the ε-NH_2 group of a lysine and the γ–amide group of a glutamine, creating an isodipeptide N^{ε}-(γ–glutamyl)lysine cross-link, one of the least soluble in organic chemistry (Nemes and Steinert 99). These bonds provide the stratum corneum with mechanical strength, chemical insolubility and, as they interact with the intercellular lipids, contribute to its impermeability. Despite its importance, the structure and

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assembly of the CE are poorly understood. Once the components of the CE are crosslinked, the bonds cannot be broken down into individual proteins. Morphologically, study of the CE is also hindered by artifactual changes related to processing (Jarnik et al 98). The best defined of the CE constituents are outlined below.

Cornified Envelope Components

Involucrin comprises only 2-5% of the total volume of the CE but is important because it is the first protein deposited and functions as a foundation for the attachment of the other proteins that make up this structure (Steinert and Marekov 97, Yamamoto et al 98). The elongated rod shape of this protein suggests its function is to connect smaller, more widely spaced proteins within the CE and its abundance of glutamine and glutamic acid residues provides many substrates for transglutaminase crosslinking (Yamamoto et al 98, Nemes and Steinert 99). The gene for involucrin is located within the cluster of genes known as the epidermal differentiation complex, found on human chromosome 1q21, along with several other CE precursors, including loricrin, small proline rich proteins, profilaggrin, and S-100 (Mischke et al 96).

Loricrin is the most abundant component of epidermal CEs and accounts for > 70% of the CE protein mass (Nemes and Steinert 99). Loricrin is rich in glycine, serine and cysteine and has the highest glycine content of any protein known in biology. Glycine functions to form unique, highly flexible structures known as "glycine loops" (Yamamoto et al 98). Interspersed between the glycine loops are glutamine rich regions and the terminal domains contain abundant glutamine and lysine. These amino acids are targeted for crosslinking by transglutaminase enzymes in CE assembly (Candi et al 95).

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Loricrin is expressed late in epidermal differentiation and can be found distributed diffusely in superficial granular layer cells in human epidermis.

Small proline rich proteins (SPRs or cornifins or pancornulins) are a family of small, closely related proteins that function to produce multiple cross-links between other CE components, with each SPR molecule participating in as many as four cross-links (Steinert et al 98, Candi et al 99). Epithelia that require greater resistance to mechanical trauma (palms and soles, esophagus and gingiva) contain a greater amount of SPRs (comprising from 15 to 50% of the CE proteins) arguably because SPR rich sites have enhanced stability (Nemes and Steinert 99, Candi et al 99).

Cystatin A or keratolinin, although a minor constituent of the CE when in a phosphorylated form, is a cysteine protease inhibitor. This feature may contribute to the bacteriostatic properties of the skin by inhibiting the activity of cysteine protease in Staphylococcus aureus (Takahashi et al 96, Nemes and Steinert 99). The human gene is located on chromosome 3cen-q21 (Yamamoto et al 98).

Profilaggrin is a major product of differentiation of the epidermis. Profilaggrin consists of numerous filaggrin units flanked by amino and carboxy terminal domains. As discussed with the keratins, filaggrin is released via proteolysis and functions to hold keratin in tight arrays within the corneocyte (Nemes and Steinert 99). Although most filaggrin is broken down, some is crosslinked into the CE, with keratin intermediate filaments. Keratin filaments become crosslinked through a single well-conserved lysine located in the amino terminal end of the type II keratins 1, 2e, 5,and 6. In this way, the keratin matrix becomes integrated in a stable manner with the CE (Candi et al 98).

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proteins are also incorporated into the CE. Desmoplakin, a major protein of the intracellular plaque of desmosomes and two structural homologues, envoplakin and periplakin, become crosslinked components of the CE. Envoplakin may mediate linkages between desmoplakin and the keratin intermediate filaments (Ruhrberg et al 96, Ruhrberg et al 97).

The *S100 family* of proteins are small calcium binding proteins and are similar to the amino terminal end of profilaggrin. Some of these proteins are expressed in the epidermis, while S100A10 and S100A11 are constituents of the CE (Mischke et al 96, Nemes and Steinert 99).

A number of other proteins have been implicated as minor CE precursors. Elafin is a serine proteinase inhibitor, a potent inhibitor of elastase and proteinase 3 found in inflammatory cells. Its precursor form, proelafin, is found in fragments and in crosslinkages in proteolytically digested CEs. Very little proelafin is expressed in normal epidermis, however, high levels are produced in pathological conditions, such as psoriasis. Proelafin is also transiently expressed in fetal epidermis. Elafin may serve a dual role as both a CE protein and as an anchored enzyme inhibitor that can protect the corneocytes from damage in inflammation. Its gene has been mapped to human chromosome 20q12-q13 (Yamamoto et al 98).

Annexin 1 is a member of a family of proteins identified in all eukaryotic kingdoms, except fungi. Annexins are defined by a conserved, ancient domain that controls ion channels by binding to phospholipids, the cytoskeleon and extracellular matrix. Annexin 1 is expressed in most tissues and has been found to be a component of CEs in cultured keratinocytes, but has not been found in vivo (Nemes and Steinert 99).

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Transglutaminases

In humans, transglutaminases (Tgases) are a related family of calcium dependent enzymes that function in stabilizing cell membranes. These enzymes catalyze the formation of insoluble bonds between glutamine and lysine residues, (N-epsilon [gamma glutamyl] lysine bonds). Seven members of the Tgase family have been identified in humans. Tgases 1 and 3 are expressed in epithelia and are important in CE assembly (Yamamoto et al 98). Tgase 2, known as tissue transglutaminase, is ubiquitous and functions in the crosslinking of membranes in apoptosis. Tgase 4 is expressed in the prostate and catalyzes clotting of seminal plasma. The catalytic subunit "a" of factor XIII, found in platelets, is a fifth Tgase and is important in blood clotting. Another Tgase is Band 4.2, an inactive, structural protein located in the membrane of erythrocytes. Recently, a new transglutaminase was isolated in the epidermis, called Tgase X, and its function remains to be defined (Nemes and Steinert 99).

Transglutaminase 1 (TGM 1, also known as keratinocyte Tgase) is the transglutaminase that plays the major role in CE synthesis. The enzyme in humans is approximately 92kD and consists of 817 amino acids (Yamanisihi et al 92). Characterizing the enzyme is difficult as its protein is very unstable and is difficult to isolate or purify in quantities for biochemical studies. The majority of TGM 1 is bound to the plasma membranes via its amino terminal region by acylated fatty acids but it is also present in a number of cleaved, soluble forms in the cell cytoplasm (Kim et al 95). The enzyme is activated by an influx of calcium into the cytoplasm when cell membranes lose their integrity during the final stages of maturation. TGM 1 is most active when

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anchored to the cell membrane after undergoing proteolytic processing, rather than in its full-length form. During terminal differentiation, TGM 1 is cleaved into 3 fragments, which remain held together on the membrane. This cleaved membrane complex has a 200 fold higher activity based on in vitro assays (Kim et al 94). Finally, a portion of the largest fragment, which contains the catalytic site, may detach from the plasma membrane and contribute to crosslinking in the cytoplasm (Nemes and Steinert 99).

The human *tgm1* gene consists of 15 exons separated by 14 introns, covering 14.2kb of genomic DNA on chromosome 14q11.2. Transcription of *tgm1* is controlled by a variety of signaling systems including the transcription factor AP-1, glucocorticoids, calcium, and retinoids (Yamanishi et al 92). Although low levels of *tgm1* mRNA can be detected in basal cells, it is primarily expressed in the upper spinous and granular cell layers of the epidermis. It is also expressed in the adnexa, including in the outer sheath, inner sheath, cortex and medulla of hair follicles and in sebaceous glands (Yoneda et al 98).

The extreme toughness and insolubility of the cornified envelope is due to the polymerization of the CE precursor proteins (involucrin, loricrin, SPRs, etc.) by the isopeptide bonds created by TGM 1, and to a lesser extent, Tgase 3 (Candi et al 95). The action of TGM 1 on the formation of the CE is central to the development of the stratum corneum. The keratin cytoskeleton is attached to the CE by Tgase crosslinking (Candi et al 98) and Tgases are responsible for the formation of the lipid envelope by covalent attachment of the hydroxyceramides of the lipid coat to glutamine residues in the protein envelope via esterification (Nemes and Steinert 99).

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Order of Cornified Envelope Assembly

The observation that the CEs of different epithelia contain differing proteins and peptides led to the hypothesis that CE was formed by the random cross-linking of waste proteins of cornification by transglutaminases, a concept called the "dustbin hypothesis". Recent evidence suggests that the formation of the CE is an organized process (Eckert et al 93, Steinert and Marekov 95 and 97). Presently, the formation of the CE is thought to be initiated at the cell membrane. The CE then becomes thicker by attaching new proteins toward the cell interior (i.e. the "outer" portion of the CE is deposited first). CE assembly appears to start with the deposition of involucrin and cystatin A, possibly initiating at desmosomal sites (Eckert et al 93, Rice et al 94, Steinert and Markeov 97). More peripheral proteins may also include those specific for desmosomes: desmoplakin, envoplakin and periplakin. These proteins form radiating, interconnecting networks with each other, the desmosome constituents and the keratin cytoskeleton (Nemes and Steinert 99). Current models suggest that the deposition of involucrin in the epidermis is used as a scaffold for the attachment of reinforcing proteins, including loricrin and SPRs, which together comprise 85% of the total mass of the CE (Steinert and Marekov 97). Loricrin and SPRs are first crosslinked together to form small oligomers by cytosolic Tgase 3 and subsequently, the membrane anchored form of TGM 1 attaches this complex to the developing CE structure (Candi et al 99). Loricrin, SPRs and possibly elafin comprise the middle third of the CE, while loricrin, SPRs and filaggrin make up the innermost portion. Keratin may be crosslinked throughout the CE (Steinert and Marekov 95 and Steinert et al 98).

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crosslinking. This explains why the lack of loricrin in the knock-out mouse model results in a relatively mild phenotype. Affected newborn mice have shiny, erythematous translucent skin with diminished barrier function, but this improves after the first five days of life, suggesting CE proteins can compensate for missing members of the envelope (de Viragh et al 97). Most of the CE proteins mentioned above, including involucrin and the desmosomal proteins, are common to the CEs of the epithelia of all mammals. At the same time, there are marked species differences in the proportion of these proteins based on anatomic location, such as mucosae, footpads, and lip. For example, loricrin, so abundant in skin, does not appear to be expressed in most internal epithelia (Hohl et al 93, Steinert et al 98).

Control of Keratinocyte Proliferation and Differentiation

As in the hair follicle, growth factors are important regulators of keratinocyte proliferation and differentiation. Some factors, such as the insulin-like growth factor (ILGF) system, consisting of insulin-like growth factors I and II and the ILGF binding proteins 1-6, affect proliferative activity. ILGF I acts on cells primarily by binding to an ILGF specific trans membrane receptor that initiates a phosphorlyation cascade of intracellular proteins. Both ILGF I and II are potent epidermal growth regulators, stimulating migration and proliferation of keratinocytes. The ILGF binding proteins modulate this activity. ILGF I also acts through other signal transduction pathways, including epidermal growth factor (EGF) and transforming growth factor a (TGFa), both of which enhance keratinocyte migration and proliferation. ILGFs I and II are mainly produced by dermal fibroblasts, indicating the dermis is involved in regulating epidermal

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EGF, TGFa, heparin-binding EGF and the closely related factor, amphiregulin, all bind to the epidermal growth factor receptor. Except for EGF, which is produced by fibroblasts, all are produced by keratinocytes and act in an autocrine fashion by binding to the EGF receptor located primarily on basal keratinocytes. In vitro these factors stimulate keratinocyte proliferation and migration, but not differentiation and are needed for the transition from the G1 S phase of the cell cycle (Kobayashi et al 98). Experiments with transgenic mice have found these factors do affect keratinocytes in vivo. Neonatal mice overexpressing TGFa have a scaly, thickened epidermis with twice the rate of proliferation. Interestingly, these skin alterations normalize by 5 weeks of age (Suter et al 97). In contrast, mice with homozygous disruption of the TGFa gene have altered hair follicles (see following section) but no epidermal abnormalities, suggesting compensatory growth factors are present (Mann et al 93). However, mice generated without functional EGF receptors have a thin, poorly organized epidermis with premature differentiation and a decreased proliferation rate. These mice die a few weeks after birth (Suter et al 97).

A number of growth factors have been identified that control epidermal growth and differentiation. For example, the transforming growth factor B (TGF B) family of cytokines suppresses keratinocyte proliferation in vitro, and induces differentiation. Keratinocyte growth factor (KGF), a member of the fibroblast growth factor family stimulates the proliferation of cultured cells, while promoting keratinocyte differentiation. KGF is produced by fibroblasts and its receptor is located on keratinocytes. Transgenic mice with alterations in the gene for KGF and the KGF receptor display abnormalities in epidermal proliferation and differentiation. As with

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TGFa, mice with homozygous disruption of the KGF gene have a normal appearing epidermis, again suggesting redundancy for KGF action in the skin. Interferon gamma (IFNg) suppresses keratinocyte proliferation, but promotes expression of markers of differentiation such as small proline rich proteins and transglutaminase 1 (Eckert et al 97).

Retinoids, modified forms of vitamin A, are potent regulators of keratinocyte growth. These agents bind to a group of receptors and form complexes that influence keratinocyte gene expression by acting as direct or indirect transcription factors. Although forms of retinoic acid are currently used to treat diseases of the epidermis by normalizing maturation, specifically how retinoids function is unclear. What makes retinoids difficult to study is their ability to have opposite effects depending on the environment where they act. For example, retinoids can suppress differentiation in vitro, while stimulating many aspects of differentiation in vivo, such as increasing expression of cornified envelope precursors and TGM 1 (Eckert et al 97).

Vitamin D3, which is synthesized in the epidermis, also affects keratinocytes by inhibiting their proliferation and differentiation through decreased DNA synthesis. Vitamin D nuclear receptors are expressed in all layers of the epidermis (Suter et al 97).

In addition to growth factors, levels of extracellular calcium have profound effects on keratinocyte differentiation in culture. Only with increased calcium concentration can the morphologic and biochemical changes associated with differentiation occur.

Apoptosis (programmed cell death) plays an important role in the epidermis. "Classical apoptosis" in which a dying cell condenses and falls apart like "petals off a flower" (Kerr et al 72) is biochemically and morphologically obvious in the embryonal

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development of the epidermis and adnexa, during the transition from anagen to telogen hair follicles and in some diseases like erythema multiforme in which there is individual necrosis of keratinocytes, however, whether apoptosis is the mechanism of cell death cornification is a current debate. All keratinocytes are programmed to die, assuming their most important function in the stratum corneum. In addition, many of the biochemical hallmarks of apoptosis, such as transglutaminase activation and the formation of a stable membrane, internucleosomal cleavage of DNA and activation of caspases all occur during the formation of the stratum corneum; however, because the fragmentation of cells (the original definition of apoptosis) does not occur in cornification, epidermal maturation is, at minimum, an outlier in the traditional view of apoptotic cell death (McCall and Cohen 91, Weil et al 99).

Regulation of Keratinocyte Gene Expression

The dramatic morphologic and biochemical changes each keratinocyte undergoes as it traverses from the basal cell layer to the stratum corneum indicate that a wide variety of genes must be turned on and off with precise and coordinated timing. Much of the control growth factors and hormones exert is mediated at the level of gene transcription, largely through the action of proteins known as transcription factors. Transcription factors can control gene expression by their location within the epidermis, activity level (phosphorylation state), and concentration (Eckert et al 97).

Members of the activator protein 1 (AP-1) transcription factor family (c-jun, c-fos, etc.) have a major role in epidermal gene expression and differentiation. AP-1 factors and the signal transduction pathways that lead to their activation have been found

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in the epidermis and in cultured keratinocytes. These factors modulate transcription by binding to complementary motifs in the regulatory regions of target genes. Although essentially all AP-1 family members are expressed in the epidermis, some may not be necessary for epidermal differentiation and the AP-1 factors that regulate each gene are thought to vary with exposure to drugs, ultraviolet light and trauma to the skin (Crish et al 97, Rossi et al 98).

Activator protein 2 (AP-2) is also implicated in the regulation of keratinocyte genes. This protein transcription factor recognizes a 9 nucleotide consensus sequence found in upstream regulatory regions of many genes. AP-2 messenger RNA is expressed in keratinocytes (Eckert et al 97).

Several members of a family of proteins, known as POU domain proteins, are expressed in the suprabasal epidermis in neonatal and adult murine skin. These include Skn-1i and Skn-1a, which are thought to be activators and suppressors of gene activity in suprabasal keratinocytes, respectively (Eckert et al 97).

How transcription factors work can be further demonstrated in the regulation of the expression of transglutaminase 1. A 2.9-kb upstream promoter region has been shown to contain regulatory elements for tissue-specific and differentiation-specific control of TGM 1 synthesis. Binding sites for transcription factors, including an AP-1 binding site are present in this region (Jetten and Harvat 97).

Desquamation

The final step in the process of cornification is the shedding of corneocytes from the skin surface. Desquamation results in a constant thickness of the stratum corneum by

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ensuring the number of corneocytes lost balances the number produced. Desquamation was once thought to simply be a passive process, in which adhesion between corneocytes eventually broke down away. Now there is evidence this is an active, highly regulated process, in which specific enzymes are produced that destroy corneodesmosomal attachments and intercellular lipids.

The breakdown of corneodesmosomes is associated with progressive morphologic changes. In the upper stratum corneum, the normally rounded contours of the extracellular portions of the corneodesmosomes become pointed and asymmetrical. Next, the laminated extracellular plaque becomes a homogeneous plug, and finally, this swells and becomes mottled and amorphous (Fartasch et al 93, Haftek et al 97). Stratum corneum trypsin-like, chymotrypsin-like proteases and less well-defined enzymes, produced in and secreted from lamellar bodies into the intercorneocyte spaces, are important in corneodesmosome breakdown (Suzuki et al 94, Guerrin et al 98). In vitro, corneodesmosome degradation within sheets of stratum corneum is decreased when inhibitors to these enzymes are present (Suzuki et al 94).

The breakdown of intercellular lipids is due in part to the activity of steroid sulfatase, an enzyme that cleaves intercorneocyte cholesterols. The importance of this enzyme is demonstrated in recessive X-linked ichthyosis of humans, a disease in which steroid sulfatase activity is diminished or absent. Affected individuals have prominent dark, thick scaling over the extremities, face, neck, trunk and buttocks due to decreased stratum corneum exfoliation and persistence of desmosomes in the outer stratum corneum (Ishida-Yamamoto and Iizuka 98, Ammirati and Mallory 98).

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Abnormalities in Cornification

When there is a defect in any of the pathways needed for production of the stratum corneum or in desquamation, abnormalities in cornification occur. Because of the many pathways in cornification these are heterogeneous diseases; however they all have excessive scaling in common as a clinical feature. Because the scaling is often present in layers, it appears somewhat like "fish skin" and the term "ichthyoses" is applied generically to these disorders. Most of these disorders are inherited, with signs appearing at or shortly after birth. The ichthyoses can be divided into epidermolytic and non-epidermolytic forms based on the microscopic appearance.

The epidermolytic ichthyoses are due to defects in the synthesis of one or more of the superficial keratins and therefore represent the only true "keratinization disorders". Because keratins are cytoskeletal structural proteins, disruptions in their synthesis are accompanied by fragility and collapse of keratinocytes. This results in the defining histologic feature of these diseases: epidermolysis, an alteration of the granular and superficial spinous layers that is characterized by swollen and vacuolated keratinocytes with large keratohyaline granules. Because of the inherent fragility of these cells, a common secondary event is lysis, and with minimal trauma, vesicles can develop. A loose scale overlies affected areas. At least eight forms of epidermolytic hyperkeratosis have been described in humans, corresponding to mutations in different keratin genes (Ishida-Yamamoto and Iizuka 98).

The non-epidermolytic ichthyoses represent the "everything else" category, including diseases associated with defects in the synthesis or breakdown of stratum corneum lipids or the formation of the cornified envelope. Lamellar ichthyosis (LI) is the

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best defined of the non-epidermolytic ichthyoses in humans. One of the most severe forms, many cases are associated with mutations in *tgm1* (Russell et al 95). However, mutations in this gene are not responsible for all cases of lamellar ichthyosis and at least two other gene loci have been incriminated (Huber et al 95, Parmentier et al 96, Hennies and Kuster 98).

LI is one of two clinical subtypes of autosomal recessive ichthyosis in humans. The second form is known as congenital ichthyosiform erythroderma (CIE) (Williams et al 85). Patients with this disease are often born as collodion babies and it is not until the scales develop that clinical differences become apparent. CIE is characterized by prominent erythroderma and fine white scaling, rather than large, plate-like scales (Ammirati and Mallory 98). Both forms of autosomal recessive ichthyosis have marked variation in severity and because of this variability, some propose that these two forms represent "poles" in between which there is a spectrum of scaling and erythema (Bale et al 96). Currently, these two phenotypes are generally described as separate diseases.

Infants with LI are often born preterm and may be encased in a taut membrane that resembles collodion, and therefore are called "collodion babies". This membrane fissures easily, putting these infants at a high risk for sepsis and dehydration. The membrane exfoliates within the first weeks of life and the underlying phenotype emerges. LI is characterized by the presence of large, thick, dark plate-like scales. These scales are present over the entire body, but in most cases the forehead, flexures and lower extremities are most severely affected. Erythroderma may be present but is not a striking feature. Patients with LI have severe hypohidrosis and are predisposed to hyperthermia. Ectropion is also a common feature. These clinical signs do not alter with age and the

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Histologically, both LI and CIE have marked laminated to compact hyperkeratosis with mild epidermal hyperplasia. The hyperkeratosis is primarily orthokeratotic in LI, while CIE often has areas of parakeratosis. Autosomal recessive congenital ichthyosis has also been classified into 4 forms based on ultrastructural features (Niemi et al 91, 92, 93, and 94). In this classification, LI corresponds to EM type II and CIE to EM type I.

The inability to identify TGM 1 in the epidermis of some patients with LI using immunohistochemistry suggested this enzyme was involved in the disease (Hohl et al 93). Assays to measure the activity of the TGM 1 enzyme were then developed. One such assay measures enzyme activity in well differentiated keratinocytes cultured from skin biopsies and is considered the "gold standard", while a newer *in situ* assay can be performed on frozen biopsy sections of affected skin and provides more rapid results (Hohl et al 98). Abnormalities in TGM 1 as a cause for LI was supported, when, using strict phenotypic criteria for LI, a locus for the disease was mapped to chromosome 14q, in the region where the gene for TGM 1 resides (Russell et al 94). Subsequently, point mutations were identified in the *tgm1* genes of the patients in the linkage study (Russell et al 95).

A second locus associated with LI, on chromosome 2q33-35, has also been identified in a region which currently lacks any candidate genes. Pedigrees from other affected families have failed to show linkage to chromosome 14 or 2, suggesting at least a third disease-causing gene (Parmentier et al 96).

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mutations result in a variety of single amino acid changes, premature termination codons, and splice site alterations leading to deletions or premature terminations that affect either the activity or expression of the transglutaminase 1 enzyme (Huber et al 97, Laiho et al 97, Petit et al 97, Hennies et al 98, Kuster et al 98, Pigg et al 98). Many of the amino acid changes are thought to destabilize the protein structure, rendering it susceptible to proteolytic degradation. Patients can either be homozygous for a single mutation or can be compound heterozygous for two different mutations (Candi et al 98). Recently, a number of *tgm1* mutations were expressed in baculovirus vectors in keratinocyte cultures. The consequences of these mutations on the enzyme was determined by comparison with a three dimensional model of the structure of TGM 1 based on the known structure of the related factor XIIIa enzyme. Many of the mutations resulted in partial protein mis-folding and/or domain rearrangements causing a marked reduction in enzyme activity. Two of the mutant enzymes produced at first appeared to result in a gain of function as the enzymes were significantly more active than wild type when expressed under similar experimental conditions. Both mutants, however, could not be proteolytically processed to the highly active membrane-bound form and therefore, the mutations ultimately resulted in insufficient enzyme activity. This work suggests that LI could also be caused by other adverse effects on TGM 1 processing and membrane anchorage (Candi et al 98).

Recently, fourteen families with LI were analyzed using clinical phenotype, linkage analysis, TGM 1 enzyme activity assay, and *tgm1* gene sequencing to try to determine if a correlation between phenotype and genotype could be established. The results demonstrated that the group of LI patients with no mutations in *tgm1* had erythematous and non-erythematous skin clinically and presented with a range of scaling

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from fine, white to large and dark brown. These clinical differences could still be explained by different genes as a cause. However, a similar spectrum of phenotypes was seen in the patients with confirmed *tgm1* mutations. In addition to the classical LI presentation, patients with erythema and finer, white scaling were also identified. There was also the surprising finding of clinical heterogeneity when comparing two patients with identical genotypes. Two patients were homozygous for the same splice site mutation, but the first case had moderate erythema with brownish scales on the lower limbs, while the second had no erythema and fair scaling over the entire body. The size and color of the second patients scaling had changed over time, however, even in the absence of retinoid therapy. This study defined that there are no conclusive criteria for the clinical differentiation TGM 1 defective and non-TGM 1 defective LI (Hennies et al 98).

In summary, the major function of keratinocytes is to form the stratum corneum, the life-sustaining outer layer of the skin. The stratum corneum is often compared to a brick wall in which the corneocyte bricks are held together with lipid mortar. We believe this can be more accurately described as a wall constructed of Mentos candies held together with rubber cement. The center of the candy is filled with a tough material that corresponds to the fibrous keratin protein matrix that comprises most of the corneocyte. The hardest part of the candy is the outer shell. This corresponds to the most insoluble region of the corneocyte, the cornified envelope. Finally, the rubber cement that holds the candy in place is a better representation of the mortar of the intercellular lipid layers because, like rubber cement, these lipids are easily broken down, resulting in continuous exfoliation of old corneocytes as new ones replace them. A major component of this dissertation is to examine the molecular

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features of the canine TGM 1 enzyme associated with the synthesis of the CE- the shell of the corneocyte that links the inner keratin matrix to the intercellular lipids.

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THE HAIR FOLLICLE

Structure and Function

The hair follicle, a defining characteristic of mammals, is a deceptively simple structure. Although on first consideration, producing a hair shaft should not be very difficult, in reality it is a dynamic process requiring complex interactions between epithelial and connective tissue elements and alternating phases of quiescence and dramatic growth. The easiest way to begin to understand the hair follicle is to view it when the hair follicle is actively growing (a period called anagen). When in anagen, the follicle consists of five major components: the outer sheath, the inner sheath, the hair matrix, the hair shaft and the follicular papilla. The anagen follicle consists of a tube within a tube and growing through the center of these tubes is the hair shaft. The outer tube is known as the outer sheath, the inner tube is the inner sheath and the swelling that supports the growing hair follicle at its base is called the hair bulb. Growing within the hair bulb is a nubbin of connective tissue known as the follicular papilla. The outer sheath is contiguous with the epidermis and the hair shaft and inner sheath are produced by the hair matrix, the proliferating cells present in the bulb (Figure 4).

Outer Sheath of the Hair Follicle

The cells of the outer sheath arise from a downgrowth of surface epithelium in early skin development. In an anagen hair, the outer sheath surrounds the entire follicle from its opening to the hair bulb where it thins and gradually stops. The outer sheath is thickest, up to several layers, at mid follicle. The upper third of the outer sheath is biochemically distinct but morphologically indistinguishable from the epidermis and like

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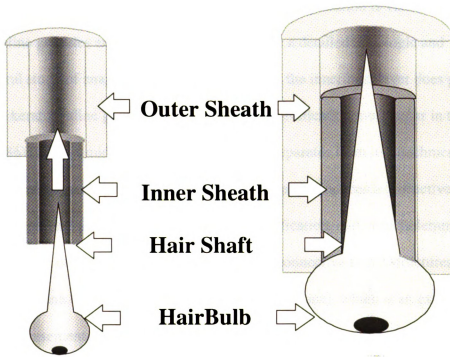

Figure 4. A hypothetical depiction of an anagen hair follicle.

This structure consists of a tube (the inner sheath) surrounded by a tube (the outer sheath) and growing through the center of both tubes is the hair shaft. Note that in reality the inner sheath arises from the hair bulb, the same anatomical region that produces the hair shaft.

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the epidermis, the more superficial cellular layer contains basophilic keratohyaline granules that produce a cornified layer with a basket-weave appearance. In the lower two thirds of the anagen follicle, the outer sheath keratinocytes are polygonal and pale or clear due to abundant cytoplasmic glycogen. Here, no cornification is visible and keratinohyaline granules are rarely identified; however a detailed histologic and ultrastructural study of anagen follicles has shown that the innermost layer does produce a few small keratohyaline granules, indicating some cornification must occur in this region (Ito 86). Above mid follicle, the inner sheath separates from its attachment to the outer sheath and fragments. At this point, the outer sheath produces a distinctive, wavy, eosinophilic, compact to parakeratotic pattern of cornification, called tricholemmal cornification. The outer sheath is surrounded by two connective tissue structures. The basement membrane (also called glassy or hyaline membrane), which is an extension of the epidermal basement membrane zone and a surrounding fibrous root sheath, consisting of a layer of dense connective tissue.

Inner Sheath of the Hair Follicle

The inner sheath is present between the outer sheath and the hair shaft. It consists of three concentric layers: Henle's layer is outer-most, then Huxley's layer and innermost is the cuticular layer. The inner sheath originates from matrix cells and, at its inception, appears as several elongate cell layers containing prominent red granules, known as trichohyaline granules. As the inner sheath grows upward (note that the outer sheath is thought to grow from the epidermis downward and the inner sheath and hair shaft grow upwards from the hair bulb), the outermost cells (Henle's layer) begins to cornify with

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the inner layer, Huxley's layer, continuing to produce trichohyaline granules. Approximately one third of the way up the follicle, Huxley's layer cornifies and inner sheath cornification is complete. Inner sheath cornification appears glassine and opaque. Located internal to Huxley's layer is the cuticular layer of the inner sheath, a layer that when cornified is distinguished on light microscopy from the inner sheath cornification only by the presence of serrations that correspond and interlock with those present on the cuticle of the hair shaft. The inner sheath and its cuticle are believed to serve as a means of supporting and molding the hair shaft.

The formation of the inner sheath requires trichohyalin, a major component of the trichohyaline granules. Trichohyalin is a keratin-associated protein that functions as the inner sheath equivalent of keratohyalin to aggregate and align the bundles of keratin fibrils in the inner sheath cells parallel to the direction of hair growth. The gene structure of trichohyalin is similar to several of the epidermal structural proteins, including involucrin, profilaggrin and loricrin. Because the genes encoding all of these proteins are located in proximity to each other on human chromosome 1q21, they are thought to be part of an epithelial protein superfamily. In addition, these four proteins are all substrates for transglutaminases that crosslink them to keratins (Manabe et al 92, Fietz et al 93, O'Keefe et al 93). Subsequent to the covalent crosslinking of trichohyalin to itself or to keratin, a second important enzyme, peptidyl-arginase deiminase, converts many of the arginines found in trichohyalin to citrulline, accounting for the abundance of this rare amino acid in the hair (Bertolino and O'Guin 92). Due to this unusually high content of charged residues, trichohyalin is proposed to assume an elongated, single stranded alpha helical conformation that is subsequently post-translationally modified. The elongate

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conformation allows trichohyalin to form a highly aligned matrix with itself and with keratin, imparting rigidity to the inner sheath cells. Trichohyalin is also found in the medulla of the hair shaft, which contains few, if any, keratin filaments. Here, the trichohyalin becomes dispersed as amorphous, denatured protein, forming vacuolated deposits within the hair shaft. By entrapping air in the hair, these vacuoles may help to maintain body temperature in mammals (Tarsca et al 97).

Hair Matrix and Hair Shaft

The hair matrix of the hair follicle is composed of small, deeply staining cells that form the expanded base of the follicle, or bulb. These cells have a high nuclear to cytoplasmic ratio, are monomorphic and are very mitotically active with a proliferation rate second only to the cells within the bone marrow. As the matrical cells differentiate, they migrate to the upper portion of the bulb, produce cells with more abundant, paler cytoplasms, and are known as supramatrical cells. This theoretical line dividing the proliferating matrical cells and supramatrical cells that terminally differentiate is drawn across the widest point of the bulb and is known as the line of Auber. The supramatrical cells then differentiate into tightly packed keratinocytes that are narrow, oval and are arranged perpendicular to the skin surface and parallel to each other. Eventually, the more centrally located matrical cells lose their organelles and nuclei and become the cornified cells or "trichocytes" of the hair shaft. The peripheral matrix cells form the three layers of the inner sheath.

When maturing into the hair shaft, trichocytes follow one of three pathways- to form the medulla, cortex or cuticle. The medulla or innermost portion is usually present

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in larger diameter hairs only and is formed by compact longitudinal columns of flattened cells. The medulla does not extend the full length of the shaft and often becomes discontinuous distally. The cortex or middle layer is the main component of the hair and is composed of fully cornified, spindle-shaped cells whose long axis runs parallel to the hair shaft. The cortex is responsible for both the bulk of the shaft and the chemical and physical properties of the hair, including the color. Cortical cells incorporate melanosomes by clipping off melanocytic dendrites that contain melanosomes. Hair that is dark brown or black have large melanosomes containing eumelanin, whereas red hair contains has spherical melanosomes containing phaeomelanin. In blond hair, the follicular melanocytes produce few or incompletely melanized melanosomes. The pattern of cornification that comprises the cortex is called trichogenic or trichocytic cornification and is characterized by retention of nuclear outlines within the trichocytes ("ghost" or "shadow" cells). The cuticle is the outermost layer and is formed by flat, partially overlapping anucleate cells. This serrated cuticle interdigitates with the cuticle of the inner sheath, locking the hair in the follicle.

Hair Shaft Keratins

Several large families of proteins are involved in the formation of hair and an estimated 50-100 individual proteins comprise the hair fiber. Many of these proteins, particularly those that are cysteine-rich, have much compositional similarity and are closely related. The two major groups of proteins that make up the bulk of the hair are 1) hair-specific keratin intermediate filaments that are also referred to as "low sulfur" or "hard" keratins to distinguish them from the epidermal keratins and, 2) the proteins that

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organize the keratins into a matrix, called keratin-associated proteins or intermediate filament-associated proteins. As in the epidermis, the follicular keratin filaments are composed of obligate heterodimers of an acidic type I and a neutral to basic type II protein, that polymerize to form 8-10 nm diameter filaments. The 8-10 nm keratin intermediate filaments are estimated to contain 32 keratin protein chains (Powell and Rogers 97). The stability of the keratin filaments depends on a number of interactions, including covalent cystine cross-links, interactions between side group chains, hydrogen bonds and hydrophobic interactions (Bertolino and O'Guin 92). At the DNA level, the hair keratin genes are very similar to the epidermal genes: the type I genes of both contain 6 introns and are 4-5 Kb in size and the type II genes have 8 introns and are 7-9Kb. However, the hair keratins differ structurally from the epidermal keratin molecules by lacking long runs of glycine residues in the end domains and by containing many cysteine residues that facilitate extensive disulfide bind crosslinking with other keratin filaments and with the surrounding matrix proteins. Currently, 13 hair keratins have been identified from humans, mice or sheep and it is likely more will be characterized (Powell and Rogers 97). In humans, partial sequences of seven type I and four type II hair keratins have been described and their differential expression in the hair cortex, medulla and matrix have been shown (Rogers and Powell 93, Powell and Rogers 97, Bowden et al 98).

The keratin-associated proteins originally were classified in three large families, the "high sulfur" group, the "ultra high sulfur" group and the "high glycine/tyrosine" group, each containing many sub-families. These categories are now inadequate as more of these proteins are identified. The naming of this large number of proteins is still

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cumbersome and controversial (Powell and Rogers 97, Dunn et al 98) and a "simplified" summary is shown in Table 2. These proteins are known to form a complex meshwork or matrix surrounding the keratin filaments, but how they function or interact with keratin is largely undefined. An interesting feature of the keratin-associated proteins is that the genes that encode them are small (0.6-1.5 Kb) and lack introns- a rare occurrence- and this is the largest group of intron-less genes known (Powell and Rogers 97). The genes for the hair keratins and many of the keratin-associated proteins are clustered together in families. This close location within the genome suggests their organization and structure have been conserved during evolution (Rogers et al 95, Powell and Rogers 97).

Follicular Papilla

The last anatomic structure of the hair follicle to be discussed is the follicular papilla, which is of mesenchymal/dermal origin and controls the growth of the hair shaft. During embryonic development, the primitive follicular papilla appears as a small group of condensed fibroblasts in the superficial dermis that induces the overlying epidermis to develop into hair follicles. In fully formed anagen follicles, the papilla is an elliptical invagination within the hair bulb. In telogen, the senescent stage of the hair cycle, the papilla is smaller and is situated beneath a region of basilar epithelial cells called the hair germ. The variation in the size of the papilla over the hair cycle is mostly the result of changes in the amount of extracellular matrix (glycosaminoglycans) within it (Taylor et al 92). During anagen, the entire papilla is rich in basement membrane proteoglycans such as heparan sulfate proteoglycan, and a chondroitin sulfate proteoglycan species distinct from those found in the general dermis. The chondroitin sulfate proteoglycan

Table

Hair Keratin
Keratin A

Table 2. Summary of hair proteins in humans, mice and sheep.

This simplified classification scheme is adapted from Powell and Rogers 97.

	Group	Other Names	Number Reported
Hair Keratins			
	Type I		9
	Type II		4
Keratin-Associated Proteins			
	Family 1	High Sulphur B2	4 to 7
	Family 2	High Sulphur BIIIA	11 or 12
	Family 3	High Sulphur BIIIB	4 or 5
	Family 4	Cortical- newly found	3
	Family 5	Ultra High Sulphur Cuticle	5 to 15
	Family 6	High Gly/Tyr Type II	2 to 20
	Family 7	Gly/Tyr rich Type I C2	1
	Family 8	Gly/Tyr rich Type I F	1
	Family 9	Ultra High Sulphur Cortex	3
	Family 10	Cuticle proteins	1
	Family 11	Hacl-1	1
	Trichohyalin		1

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diminishes through catagen and is difficult to detect in the telogen papilla (Messenger et al 93). The follicular papilla has little of the type I collagen that predominates in mature dermis and even type III collagen is relatively sparse, although type IV collagen which forms the lamina densa layer of basement membranes is present (Couchman et al 90, Hardy 92). The function of the proteoglycans and collagens of the follicular papilla is more than just structural. Growth factors that play a role in hair follicle growth and development, such as transforming growth factor B and fibroblast growth factor b, bind to the extracellular matrix, localizing these peptides to sites of action in the follicle (Messenger et al 93). The follicular papilla also contains capillary loops in anagen that diminish in telogen, although little is known about how the vasculature remodels in the papilla during the hair cycle (Messenger et al 93).

The interaction of the mesenchymally-derived papilla with the epithelial cells of the matrix is absolutely necessary for hair growth. The importance of these mesenchymal-epidermal interactions have been studied for over 50 years. Historically, the papilla was simply thought to be a plug of dermis that supported the vasculature of the hair bulb. In the early 1950s, experiments showed that the papilla was essential for hair follicle development. Subsequently, Oliver demonstrated if the papilla- containing bulb and up to one third of the lower follicle were amputated, a new papilla could develop from the mesenchymal sheath surrounding the follicle. In addition, once the papilla was regenerated, the amputated follicle would produce a new bulb and hair shaft. Contact of papilla cells with afollicular epidermis can also stimulate the de novo development of a hair follicle (Oliver 66, Jahoda and Reynolds 93).

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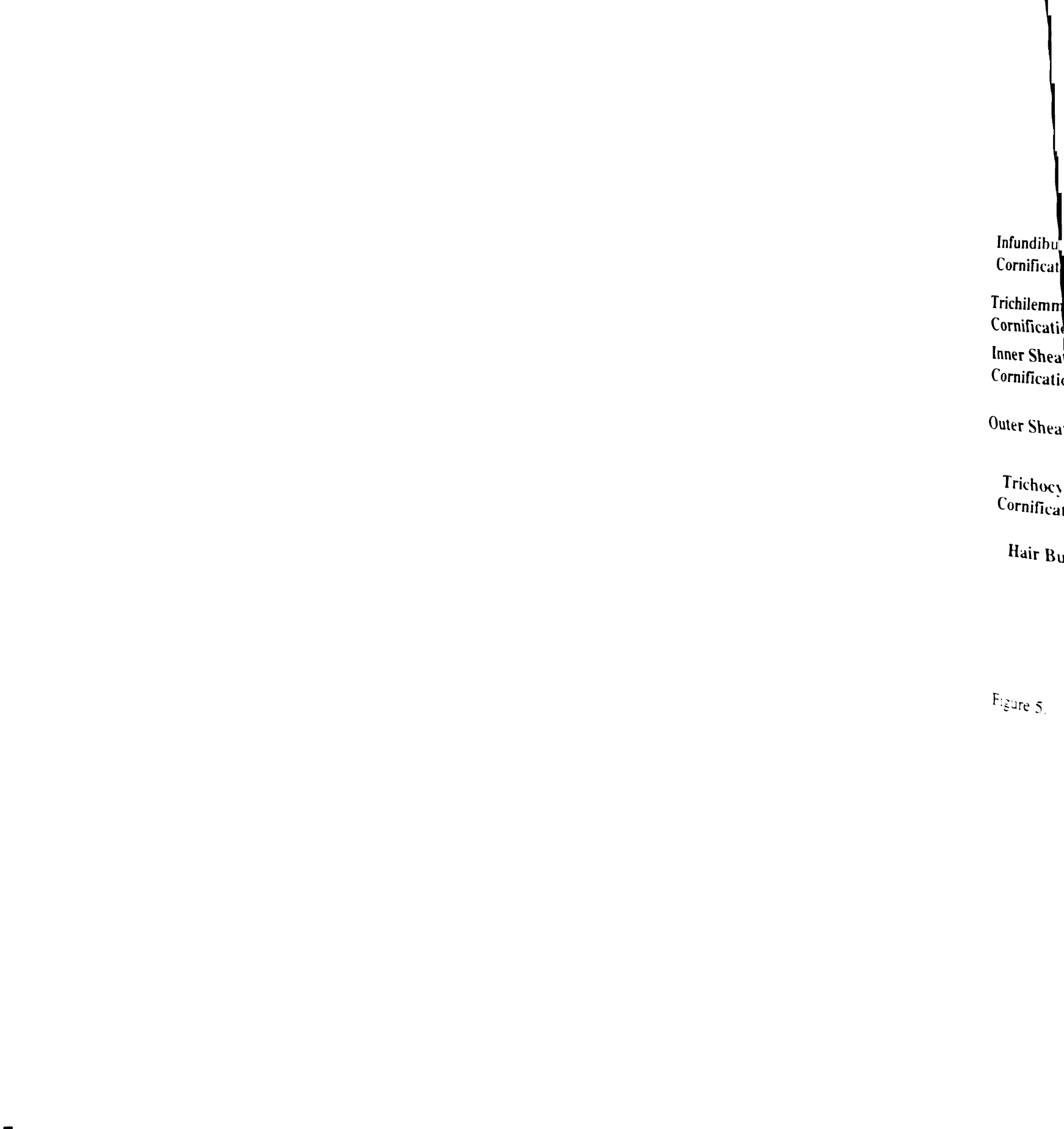
Anatomy of the Canine Compound Hair Follicle

Extending from top to bottom, the anagen hair follicle can be divided into three major anatomic regions. The uppermost portion of the follicle is called the infundibulum. This region extends from the opening of the follicle (the follicular os) to the beginning of tricholemmal cornification. The infundibulum is lined by epithelium of the outer sheath and is continuous with the epidermis. Like the epidermis, the infundibular epithelium has a granular cell layer with characteristic intracytoplasmic blue keratohyaline granules and produces a loose, basket-weave stratum corneum.

The mid portion of the follicle is called the isthmus. This region extends from the beginning of tricholemmal cornification to the insertion of the arrector pili muscle, or more accurately, to the point at which the inner sheath is fully cornified.

The third and bottom-most portion of the follicle is called the inferior region. This region extends from the first non-cornified cell of Huxley's layer to the base of the follicle (Figure 5).

There are several important differences in the structure of hair follicles across species. Most omnivores and herbivores have "simple" follicles. This means that each infundibulum contains a single hair shaft that exits through the "os". Exceptions include fiber-bearing herbivores like goats, sheep or llamas, which may have two to three hairs per infundibulum. Carnivores, in contrast, have "compound" follicles. That is, there are multiple hair follicles growing closely together that unite at the upper isthmic region and share a common infundibulum that then contains multiple hair shafts. By convention, the largest hairs in a compound follicle are called primary or guard hairs and the smaller hairs that make up the majority of the hair shafts in a compound follicle are called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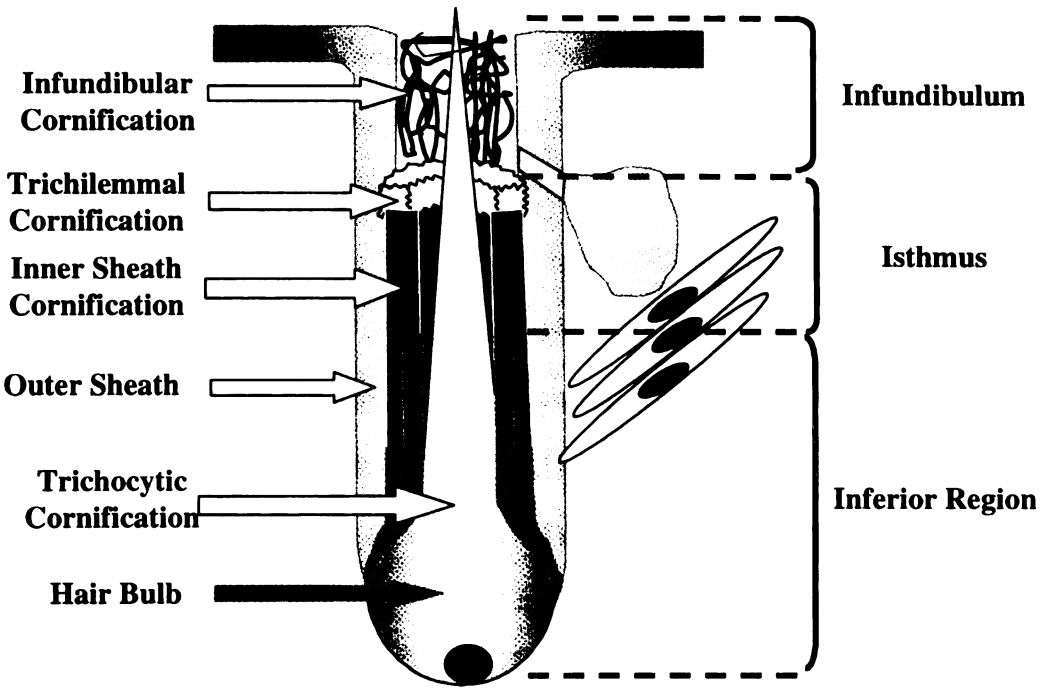

Figure 5. Diagram of the three major anatomic divisions of the anagen hair follicle and the different patterns of cornification that define them.

The infundibular region extends from the opening of the hair follicle to the beginning of trichilemmal cornification. The isthmic region extends from the beginning of trichilemmal cornification to where the inner sheath is fully cornified. The inferior region extends from the point where the inner sheath is fully cornified to the base of the hair bulb. The dotted region of the inner sheath represents the presence of trichohyaline granules.

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secondary or undercoat hairs. However, based on diameter, there is no way to differentiate a primary from a secondary hair. This is because there is a continuum of diameters from largest to smallest. It should also be noted that an old telogen hair shaft retained concurrently with a newly emerging hair (a feature common in rodents) does not constitute a compound follicle even though two shafts may be found in the infundibulum (Figure 6).

In most mammals, the follicles begin to develop prenatally in one location, such as the crown of the head and develop in a wave over the body. In species with a dense haircoat, such as dogs, the primary follicles emerge first, followed by additional waves of smaller, secondary follicles that grow from the primary follicle (Hardy 92). In dogs, the compound follicles are arranged in groups of three. We call this anatomic grouping of compound follicles a "follicular unit". Thus, if one looks closely at the skin of a dog whose hair has been clipped, the follicular ostia will typically appear as three openings close together, then a space, and then another three openings close together. In addition, there is also a distinctive orientation of the primary and secondary hairs. Primary hairs are always cranial with the largest primary hair in the center and somewhat in front of the two flanking primary hairs. Secondary hairs are caudal and the secondary hairs closest to the primary hairs are the largest in diameter and become progressively smaller the more caudally they are located. The arrector pili muscles segregate the three compound follicles within the group. In this way, the hair follicles are designed so that the hairs will lie down smoothly, with the guard hairs lying on top of the fine undercoat (Figure 7). The common concept is that dog hair consists of two distinct hair types, primary and secondary. This is not true. In reality, there is a continuum of diameters and the only

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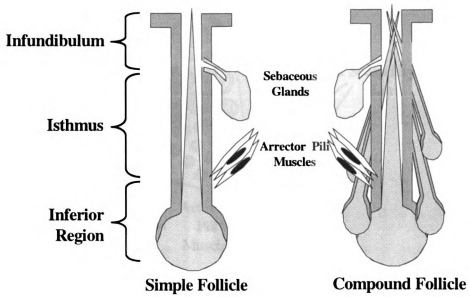

Figure 6. Histologic differences between a simple and compound hair follicle.

A simple follicle has only a single hair shaft exiting through the follicular os. A compound follicle consists of multiple follicles that share a common infundibulum. As a result, in compound follicles, multiple hair shafts exit through the follicular os.

Figure 7.

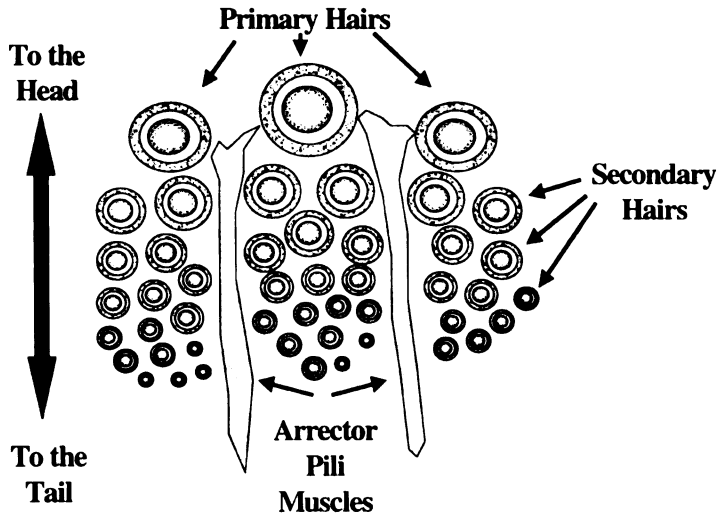

Figure 7. The canine follicular unit examined by transverse sectioning.

Follicular units usually consist of three groupings of hair follicles, characterized by a large, anterior primary hair and associated secondary hairs that become smaller caudally. Each one of the three groupings constitutes a compound follicle with its own follicular os. Because some of the secondary hair shafts can have diameters that approximate the diameter of primary hairs, we refer to the anterior follicle in each compound follicle as an anatomic primary hair follicle and the posterior hairs as anatomic secondary hairs when we want to denote location of the follicles rather than diameter.

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way to separate a primary from a secondary hair is to either establish an arbitrary cutoff point diameter or to define primary hairs anatomically: the most anterior hair in each group of follicles can be considered a primary hair regardless of its diameter. Finally, the ratio of secondary to primary hairs is breed-related but can be greater than 10:1.

Adnexa

Structures that are associated with the hair follicle are the sebaceous gland, apocrine gland and arrector pili muscle. Sebaceous glands are poorly to non-innervated, lobular, foamy holocrine glands (the entire content of the epithelial cell is secreted) that drain into the base of the follicular infundibulum via a short duct lined by cornifying squamous epithelium. The oily secretion produced by these glands is called sebum and consists primarily of lipids. Sebum coats the hair shaft, providing much of the sheen of the pelage. In addition, sebum may act as a lubricant to allow the cornified debris within the lumen of the infundibula to be extruded. Sebum has also been reported to have an antibacterial function (Kligman et al 63).

Apocrine glands are well-innervated coiled or tubular glands connected to the follicle by a long duct that enters the upper (herbivores and humans) or mid (dogs and cats) infundibulum. The secretory portion of apocrine glands is composed of multiple acini lined by a layer of cuboidal epithelium that secrete via apical "decapitation". There is considerable species variability in the presence and function of these glands. For example, in domestic animals, all follicles have an attached apocrine gland. The skin of rodents, such as mice, are devoid of apocrine glands and humans have apocrine glands only in axillary and inguinal areas. The major function of these glands in most species is

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to produce a viscous secretion associated with body odors that are dispersed by the hair shafts. In horses and cattle, these glands are responsible for perspiration and function in thermoregulation. The last structure associated with the follicle is the arrector pili muscle. This is a variably sized band of smooth muscle that originates in the superficial dermis and inserts on the follicle at the isthmus. In primates, the attachment site of the arrector pili muscle is associated with an evagination of the outer sheath- a structure called the "bulge". These arrector pili muscles receive cholinergic innervation and function in piloerection (elevating the hairs) to assist in thermoregulation.

Hair Follicle Immune System

Unlike the epidermis, the hair follicle has few intra-epithelial immune cells. In Paus' recent immunohistomorphometric study of murine skin, Langerhans cells, so important in epidermal surveillance for antigen, are present only in the infundibulum in murine skin- the portion of the follicle most like the epidermis. In addition, murine dendritic T lymphocytes of the gamma/delta subtype (alpha/beta T cells are the major subtype in human hair follicles) are present in low numbers in the infundibulum, but lymphocytes are found only rarely lower in the hair follicle. In humans, CD4 and CD8 cells are numerous in the infundibulum, but are scarce below this point. During embryological development, both Langerhans cells and intraepithelial lymphocytes migrate to the epidermis and do not extend into the follicular epithelium until later, approximately when the sebaceous gland duct is formed. The number of these cells in murine skin detectable via immunohistochemistry fluctuates with the hair cycle, although this varies with the method used to modulate the cycle. Depilation induced anagen was

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associated with increased follicular Langerhans cells and gamma/delta T lymphocytes, while these cells were decreased in cyclosporine-induced anagen (Paus 98, Tobin 99).

In mice, both mast cells and macrophages appear very early in the dermis around developing hair follicles and increase until adulthood. Interestingly, the number of perifollicular mast cells detected by immunohistochemistry also changed with the hair cycle in mice. There was a sharp decline of mast cells that correlated with their degranulation in early anagen. In addition, mast cell secretory products induced anagen after intracutaneous injection, while antagonists of these products, as well as inhibitors of degranulation slowed the induction of anagen *in vivo*. Overall, this suggests that mast cells products play an important role in the hair cycle of mice. A previous hypothesis that catagen is induced by macrophages attacking the hair bulb could not be confirmed in this study as perifollicular macrophages did not increase in number in catagen (Paus 94, Paus 98).

A feature of the portion of the anagen follicle below the isthmus is that it typically lacks an antigen critical to self-recognition, MHC class Ia. Because cells in other organs that do not express this antigen are immunologically privileged (testes, eye, brain and placenta), the lower portion of the follicle is thought to be similarly privileged. In mice the follicular papilla expresses MHC class I in anagen, but not in catagen or telogen (Tobin 99).

Hair Follicle Development

With limited exceptions, all of the hair follicles a mammal will ever develop are formed during embryogenesis. Hair follicles arise from both the ectoderm and the

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underlying mesoderm and their morphogenesis depends on a regulated sequence of messages between these two tissue types (Hardy 92, Panaretto 93). The first message in hair follicle formation defines where each follicle will arise. This message comes from the mesenchyme (dermis), and stimulates the overlying ectoderm (epidermis) to form regularly spaced invaginations into the dermis, known as placodes. This is a highly conserved message that is apparently common to all vertebrates, as murine dermis can initiate scale placode formation in lizard epidermis. The second message arises from these epidermal in-growths and signals the cluster of dermal cells to become a follicular papilla. This signal is species-specific as tissue recombination experiments have shown that only mouse dermal cells will respond to mouse epithelial cells. The third message originates from the newly formed papilla and stimulates the epithelial cells to proliferate rapidly, forming a small follicular germ that develops into a larger follicular peg and finally, the hair matrix that produces the hair follicle. Epithelial buds derived from the follicle will become the associated sebaceous and apocrine glands (Moore et al 91, Hardy 92).

The factors that control development are known, generically, as "morphogens". The concept of morphogens is less than a decade old and the molecular signals that control hair development remain largely undefined. What is known is that morphogens can be produced systemically or within the local environment of the hair follicle. Morphogens identified so far include growth factors that stimulate hair growth embryologically and the hair cycle post-natally, adhesion proteins that are important in epithelial/mesenchymal interactions, and homeotic genes and their products that are important in determining how developing embryos are subdivided. Recent studies have

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shown that homeotic genes found in Drosophila, such as sonic hedgehog (SHH) and bone morphogenetic proteins 2 and 4 (BMP 2 and 4), have homologs associated with feather and hair follicle formation in vertebrates, demonstrating the evolutionary conservation in morphogenesis (Stenn et al 96).

Recently, BMPs 2 and 4 (members of the Transforming Growth Factor-B family), homeobox genes, and factors in the fibroblast growth factor (FGF) family have been shown to be expressed very early in follicle development. All are secreted proteins whose binding to receptors in appropriate cells results in transcription of target genes. The specific role of these genes in development is still unknown; however, experimental mis-expression of these proteins results in growth of highly abnormal follicles (Tobin 99). Recent work has shown that FGF-4 and SHH induce placode formation of feather follicles while the cells surrounding the placode are inhibited from undergoing similar changes by BMPs 2 and 4 (Jung et al 98).

A transcription factor, known as lymphoid enhancer factor-1 (LEF-1) has also been identified as important in the initiation of hair follicle development. This factor is expressed in the ectoderm and genes involved in hair follicle development have binding sites for it in their promoters. *In situ* hybridization shows that LEF-1 mRNA is concentrated at the sites of future hair follicles in the ectoderm of embryonic mice before placodes become visible (Zhou et al 95). Transgenic mice which carry a homozygous mutation in the LEF-1 gene lack hair and whiskers, in addition to teeth, mammary glands and the mesencephalic nucleus of the trigeminal nerve, the only neural crest derived neuronal population (van Genderen et al 94).

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dermal papilla begin to divide and differentiate. The regulation of this proliferation is still poorly understood. Two morphogenetic proteins, FGF-2 and epidermal growth factor (EGF) have been studied *in vivo* and are implicated. For example, when injected into neonatal mice, both FGF-2 and EGF significantly delay hair follicle development (du Cros 93). Recently, SHH was shown to be essential for follicle development past the hair germ stage in mice (St. Jacques et al 98, Chiang et al 99).

The Hair Cycle

The growth of hair is cyclical and most of the morphologic changes associated with the cycle occur in the lower half of the hair follicle. The active growth phase of the hair cycle is called "anagen" and is characterized histologically by a hair bulb surrounding an inverted follicular papilla and a fully formed inner sheath. The involutionary phase between active and no growth is known as "catagen". In catagen, there is loss of the hair bulb and separation from the follicular papilla. Although a thickened follicular sheath and increased numbers of apoptotic cells in the outer sheath are present, the most defining feature of catagen is the partial replacement of the inner sheath by tricholemmal cornification as the hair bulb is lost and the follicular papilla evaginates. Telogen is the stage of senescence and begins when there is complete replacement of the inner sheath with tricholemmal cornification. Telogen follicles are approximately one third the length of anagen follicles. The portion of the outer sheath at the base of the telogen hair is composed of a small, compact nest of basaloid cells known as the hair germ. This cluster of cells sits on the follicular papilla, which is poorly defined when not invaginated within the hair bulb. Telogen is a physiologic, not

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pathologic process and is as much a part of the hair cycle as anagen. Telogen only becomes pathologic when a new anagen hair does not soon replace a telogen hair and the follicle continues to involute. The length of time required to complete the hair cycle varies between different species, and is different among dog breeds. In human scalp hair, anagen is the longest phase of the cycle; thus the hair grows almost constantly, resulting in the need for hair cuts. In most mammals, telogen is the longest phase; the hairs grow to a preordained length and then enter a long period of inactivity in which the telogen hair follicle firmly retains the hair shaft. A good example of telogen-predominant hair cycles occurs in quilled mammals. In 1996 we described the morphologic features of quill follicles in porcupines, hedgehogs and echidnas. We found that after the quills had grown to a set length, they entered into a "haired telogen" state in which the quill was tightly annealed to the outer sheath. These quills were neither shed nor did they grow. In hedgehogs, the quills could remain intact for years, perhaps for the life of the animal. The value of growing and then retaining hair is likely in the conservation of protein and the energy required for its synthesis (Winn Elliot et al 96). We believe that the hair cycle in most canine breeds is more like that of quilled mammals than humans. In short, dogs have telogen-predominant hair cycles in which the hair shafts are retained in telogen follicles for long periods of time. How long the follicle remains in telogen is a breed-specific phenomenon. In some canine breeds such as the Nordic breeds, the hair appears to be held in a telogen state sometimes for years. In other breeds of dogs, such as Poodles, the hair cycle is anagen-predominant and these dogs, like humans, need to have haircuts (Figures 8 and 9).

To begin anagen, germinative epithelial cells in the telogen follicle undergo

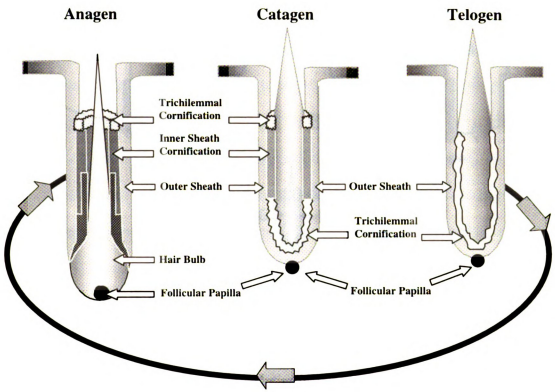
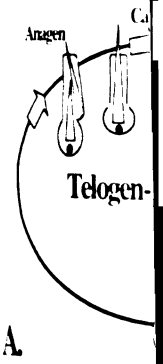


Figure 8. The major morphologic changes associated with the hair cycle.



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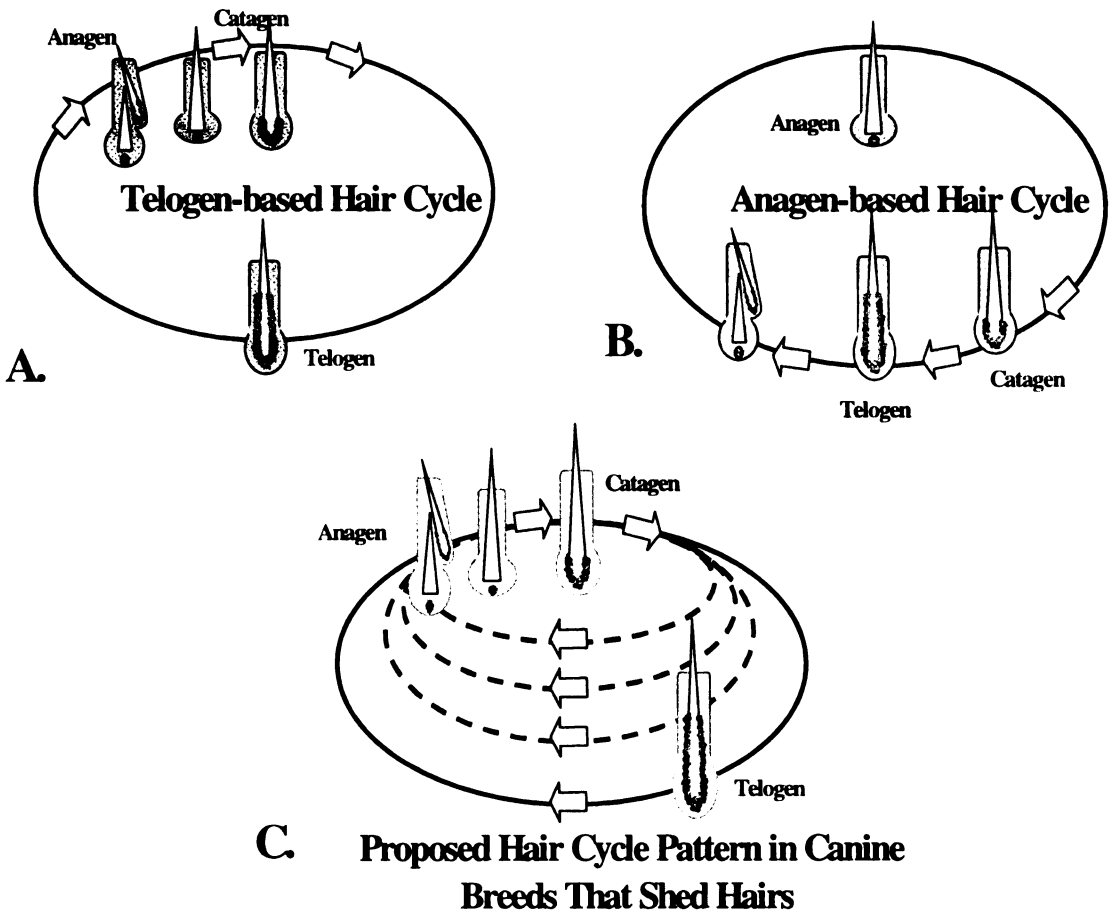

Figure 9. Species and canine breed differences in the mammalian hair cycle.

Stereotypically, there are two major hair cycle patterns. One in which the predominant phase of the hair cycle is telogen (A) and one in which the predominant phase of the hair cycle is anagen (B). Any species that requires a haircut (humans, Poodles) are examples of anagen-based hair cycles. Quilled mammals that grow their quills and retain them for years are an example of a telogen-based hair cycle. Although we believe most dogs have telogen-based cycles, the length of the anagen phase appears to have considerable breed variability (C).

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mitosis, grow downwards to surround the re-forming follicular papilla and differentiate into the hair bulb. These germinative epithelial cells have long been assumed to be the follicular stem cells. In 1990, Cotsarelis et al. challenged this view by suggesting that the stem cells of the follicle reside in a localized area called the "bulge", a knob-like evagination of the upper portion of the outer sheath associated with the site of attachment of the arrector pili muscle.

To appreciate the importance and controversy of Cotsarelis' proposal, a brief discussion of stem cells is order. Stem cells are the source of renewal in regenerating tissues such as stratified squamous epithelium, hematopoietic tissue and intestinal epithelium. At one time these cells were thought to act as "queen bees" producing thousands of offspring. Now most believe that stem cells are an undifferentiated cell population with a relatively low turnover rate to reduce the chance for a mutation that could occur during replication. Stem cells are located in well-protected, well-innervated and nourished sites so the chance of damage is minimized. Although stem cells have a low mitotic rate, they produce "transiently amplifying" cells that turnover rapidly for a limited period of time. These transiently activated cells give rise to a large population of terminally differentiating cells that form the mature tissue. Because the stem cells are slow cycling, they are only rarely labeled by a pulsed administration of a marker of proliferation such as tritiated thymidine. However, once these cells incorporate the marker, they retain it for a long period, as the marker is not diluted through subsequent cell division. This is why stem cells are known as "label retaining cells". Biochemically and ultrastructurally these cells are not distinctive. The nuclei of are often described as convoluted or crenulated.

Utilizing tritiated thymidine labeling on the skin of mice, Cotsarelis et al. reported label-retaining cells in a localized area of the outer sheath at the insertion of the arrector pili muscle. These cells had a convoluted nuclear outline and ultrastructurally appeared undifferentiated. Based on these observations, Cotsarelis and workers hypothesized that in the hair follicle, the slow cycling stem cells are located in the bulge region, the transiently amplifying cells are the matrix cells of the hair bulb and the terminally differentiated cells are the cells that form the inner sheath and hair shaft. (Cotsarelis et al 90, Lavker et al 93). As part of their "bulge activation hypothesis" the authors pointed out that there are a number of advantages for the follicular stem cells to be located in the bulge. This site is not removed when hairs are plucked from the follicle so these cells are protected, it is well vascularized and in telogen, the bulge region is situated so that it can interact with the follicular papilla (Cotsarelis et al 90). The idea that the stem cells are located in the upper portion of the follicle rather than in the matrix also helps to explain the problems posed by the results of the transection experiments of Oliver, who surgically removed the lower follicle of the rat vibrissa and found that as long as a new follicular papilla was supplied, the follicle could regenerate (Oliver 66).

The bulge activation hypothesis remains a subject of controversy. Although the bulge may be the stem cell site for humans and primates, this may not be so for rodents and other mammals, as they do not have this follicular structure (Dunstan et al 95). A detailed morphologic study of the follicles of eight domestic species, including mice and rats, failed to reveal a true bulge in any of them. Rather, what appears to be a bulge in rodent follicles was always found to be a "pseudobulge", a protuberance of the outer sheath that contains a retained "club" hair or telogen hair in an anagen follicle.

Recently, another labeling study of the hair follicles of mice questioned the importance of the bulge cells. While confirming the presence of persistently labeled cells within the bulge region, the study also demonstrated that these cells were not the first to proliferate after plucking to stimulate the follicles into anagen and neither label retaining cells nor their progeny were found to have migrated to the hair germ. Rather, the persistently labeled cells remained in the bulge region as the anagen hair follicle regrew a new shaft (Morris et al 99). That hair germinative epithelium can proliferate without migration of cells from the bulge region is also supported by the microdissection studies of Reynolds and Jahoda. These studies demonstrated that if germinative epithelial cells were microdissected from follicles and were cultured with follicular papilla cells, the keratinocytes proliferated and recombined with the papilla cells to form primitive follicular structures (Reynolds and Jahoda 91). A more recent study by Reynolds and Jahoda brought into question not only the bulge activation hypothesis, but also the need to have stem cells at all (Reynolds et al 99). In this investigation, hair was removed from the scalp of a human and all epithelial elements were removed. What was left was the surrounding connective tissue sheath. Cells from these sheaths were transplanted into the arm of a female volunteer. Several weeks later, large coarse hair shafts similar to the scalp hair of the donor began growing from the transplantation site. Genetic analysis defined that the epithelium of the hair follicles had the XX karyotype indicating the follicles were from the cells of the recipient, yet the connective tissue sheaths were of XY karyotype, indicating they were from the donor. In addition to demonstrating that hair follicles may be immunologically protected, these results suggest that the connective tissue sheath may be all that is required for the development of a new hair follicle. Even

if the bulge activation hypothesis is true, it neither explains the factors that control the initial epithelial- follicular papilla interaction at the start of anagen, nor the controls that govern the cyclical transitions into catagen and telogen.

As with many other organs that are remodeled during development, apoptosis is believed to be important in catagen, the involutionary stage of the hair cycle (Weedon and Strutton 82). The concept that apoptosis (from Greek meaning "a falling off" as petals from a flower) is a separate form of cell death from necrosis was described in 1972 (Kerr et al 72). Since then, apoptosis has become a focus of biomedical research and after nearly 30 years of study, many of the pathways, proteins and signals involved in apoptosis are now defined, such as Fas and caspase activation (McConkey 98). It is also becoming evident that rather than being a distinct process, apoptosis shares many overlapping pathways with both necrosis and, arguably, terminal differentiation (Lemasters 99).

In 1982 Weedon and Strutton studied murine follicles in catagen using electron microscopy and concluded the reduction in follicle size was the result of cell loss through apoptosis. More recently, murine and human catagen follicles have been examined for cleavage of the cell's DNA at regular internucleosomal intervals, a molecular feature of apoptosis. Utilizing an immunohistochemical technique known as the terminal deoxy-nucleotidyl transferase-mediated dUTP nick end labeling (or TUNEL technique) and electrophoresis of DNA, DNA cleavage characteristic of apoptosis was demonstrated in the lower portions of the catagen follicles by both techniques (Matsuo et al 98). *In vivo* work on human hair follicles has also shown that in catagen, proliferation of epithelial cells is no longer observed and TUNEL positive cells increase dramatically in the outer

sheath and in the epithelial cells surrounding the follicular papilla. (Soma et al 98).

Factors Controlling the Hair Cycle

All hair follicles have an intrinsic rhythm that can be altered by systemic factors. The interplay between local (intrinsic) and systemic (extrinsic) factors in hair cycles control has been illustrated by grafting studies in rats. Flaps elevated on the flanks of rats and rotated 90-180 degrees and then replaced continue to shed in the rhythms of their original sites for long periods. Similarly, grafts between syngeneic mice of different ages also retain the shedding pattern of the donor. Eventually, however, the hair cycles in the grafts and flaps synchronize with the surrounding skin (Ebling 90). The cycles of the hair follicles that comprise the pelage or hairs within a body region may be synchronized or may cycle in a mosaic pattern, in which a mixture of anagen, catagen and telogen follicles are present at any time. The human scalp is a good example of follicles cycling in a mosaic pattern. The hair coats of most rodents are synchronized and shed in waves. In mice, the first and second hair cycles are highly synchronized, starting in a wave-like manner on the ventrum and progressing from the head to the tail, over a 24 hour period. A very short catagen-telogen period of 3-4 days is followed by a second anagen lasting about 9 days. The follicles enter telogen and remain quiescent for at least 40 days. In the third anagen, there is a breakdown of the cycle synchronization, with patches of hair follicles entering anagen randomly over the body (Wilson et al 94).

In the hair follicle, intrinsic factors (known as growth factors or cytokines) are produced by and act on a variety of different cell types: epithelial cells including the cells of the hair matrix, inner sheath and outer sheath, mesenchymal cells of the follicular

papilla and fibroblasts surrounding the follicle and within its connective sheath, and endothelial cells of the blood vessels that supply the follicles. Extrinsic factors affecting the hair follicle are produced by organs other than the skin. Both sets of factors work together to control hair development, growth and the hair cycle, which largely determines the length of the hair. Still, the molecular basis for the morphologic changes that occur in the hair follicle cycle is largely undefined and there are still no regulator molecules identified that are unique to the hair follicle.

Genes Controlling Hair Growth

The number of genes identified as being important for hair growth is still growing. Recently, linkage analysis- the technique of linking the inheritance pattern of a certain trait to the segregation of a unique chromosomal marker or position- has been used to identify several genes associated with hair growth in mice. The mouse *agouti* locus was isolated this way. Mutations in this gene produce a number of abnormalities, including alterations in hair pigmentation. Also, the *nude* gene of mice and rats, which causes fragile hair shafts and thymic dysfunction when mutated, has been identified and shown to be a member of the winged helix domain family of transcription factors. Lastly, the *hairless* gene in mice was identified and cloned, taking advantage of the fact that the mutation in this gene was caused by the insertion of an endogenous murine leukemia virus. This gene codes for a transcription factor of the zinc finger family (Cachon-Gonzalez et al 94, Sundberg 94).

Intrinisc Factors

Growth factors (interleukins, cytokines) are peptides that have stimulatory and/or inhibitory effects on cell proliferation in culture and *in vivo*. Whether a growth factor stimulates or inhibits follicular cell growth depends upon their interaction with other growth factors, the state of cell differentiation, the surrounding environment and when the growth factors and cells are exposed to each other.

Most growth factors mediate their effects by binding to specific cell surface receptors, a process of cell activation known as signal transduction. The cell receptors become activated when a growth factor binds two neighboring receptors, inducing conformational changes in intracellular portions of the receptors and eliciting a cascade of intracellular reactions (often phosphorylation of cell proteins) that result in a number of cell responses such as activation of specific genes, DNA synthesis and cell division. Also, an increasing number of cell adhesion molecules such as integrins have been identified as important in cell-to-cell and cell-to-matrix communication within the follicle. Growth factors may also act on the same cell that produced them (autocrine control) or on neighboring cells within the same or adjacent tissue (paracrine control). Another form of regulation that may play a role in the follicle is "intracrine" control, in which the growth factor is synthesized but is not secreted from the cell, acting on intracellular receptors to produce its effects (Peus and Pittelkow 96).

There are an ever-growing number of intrinsic factors known or suspected to have effects on hair follicles (Table 3). Prominent among them are growth factors produced from four major growth factor families: the epidermal growth factor family, the fibroblast growth factor family, the transforming growth factor B (TGF B) family, and the insulin-

Table 3. Summary of intrinsic factors/cytokines known to affect hair growth.

Intrinsic Factor	**Site(s) of Production**	**Site(s) of Receptor(s)**	**Effect on Hair Follicle Growth**
Epidermal growth factor	Outer sheath	Outer sheath, hair bulb	*In vitro*- -Stimulates *In vivo* --Inhibits
Transforming growth factor-a	Outer and inner sheath	Outer sheath, hair bulb	*In vitro* --Stimulates *In vivo* --Inhibits
Fibroblast growth factors FGF-1,2	Outer sheath, inner sheath, hair bulb basement membrane	Outer sheath, inner sheath, hair bulb basement membrane	*In vitro* --Stimulates *In vivo* --Inhibits
Keratinoctye growth factor (FGF-7)	Follicular papilla	Outer sheath, hair bulb	*In vitro* -- Stimulates *In vivo* -- Stimulates
Hepatocyte growth factor/ scatter factor	Follicular papilla	Hair bulb	*In vitro* --Stimulates
Transforming growth factor-B	Entire follicle	Entire follicle?	*In vitro*- -Inhibits
Insulin-like growth factor	Follicular papilla	?	*In vitro* --Stimulates
Interleukin 1	Follicular papilla	Entire follicle?	*In vitro* --Inhibits
Vascular endothelial growth factor	Follicular papilla, hair bulb	?	*In vitro* --Stimulates

like growth factor (ILGF) family (Danilenko et al 96, Peus and Pittelkow 96, Stenn et al 96).

EGF was one of the first growth factors to be isolated and purified and has a stimulatory effect on the proliferation of many cell types in culture, including follicular keratinocytes. In the hair follicle, EGF is produced by the outer sheath; its receptor is expressed on the outer sheath and matrix cells in the hair bulb. *In vitro*, EGF stimulates proliferation of epidermal keratinocytes, dermal sheath cells and follicular papilla cells (Pisansarakit et al 91), however, when injected systemically into mice, EGF delays the hair cycle and retards hair growth. (Moore et al 81, Danilenko et al 95). These contradictory effects remain to be explained but may be related to the higher concentration of the growth factor in culture and the stage of the hair cycle the follicles are in when EGF is injected *in vivo*. Overall, EGF is thought to induce follicle regression and slow hair growth.

Transforming Growth Factor alpha (TGFa), which shares homology with EGF and binds to the EGF receptor, also delays hair growth when given *in vivo*. Interestingly, mice in which the TGFa gene has been disrupted have eye abnormalities and pronounced waviness to the hair and whiskers that are very similar to spontaneous murine mutants known as *waved 1* and *waved 2*. Thus it is not surprising that *waved 1* and TGFa have been shown to be allelic (Mann et al 93). Subsequently, *waved-2* was mapped to the vicinity of the EGF/TGFa receptor gene on mouse chromosome 11 and was shown to be a point mutation in one domain of the EGF receptor, resulting in poor receptor activation when EGF binds (Luetteke et al 94). When the EGF receptor gene is completely inactivated in mice through a knock-out mutation, the phenotype is much more severe

and results in impaired epithelial development in many organs including the epidermis, hair follicles, intestine and lung and is associated with embryonal or neonatal lethality. Animals that survive have rudimentary whiskers and wavy hair with a decreased follicle density (Miettinen et al 95, Hansen et al 97).

Although *FGFs* were named for their mitogenic effect of fibroblasts, they are now known to have a wide range of biological activities, including wound healing, angiogenesis, and morphogenesis. To date, four members of the FGF family have effects on both epithelial and mesenchymal cells on the hair follicle: FGF-1, FGF-2, FGF-5 and FGF-7. The follicular production of both acidic and basic FGFs (FGF 1 and 2) and the expression of their receptors changes with the stage of the hair cycle based (Rosenquist and Martin 96). FGF-1 has been identified in the anagen inner sheath and FGF-2 has been found in the basement membrane of the outer sheath and in the hair matrix, while their receptors are found in these sites and also in the follicular papilla during anagen, but disappear as the follicle approaches telogen. Like EGF, these factors can induce proliferation of some follicle constituents in culture, but retard hair growth and cycling when given *in vivo* (Danilenko et al 95). FGF 5 has been reported to induce the transition from anagen to catagen (Hebert et al 94). In 1994, knockout mice deficient in FGF 5 were created and their phenotype was identical to a naturally occurring mutant called *angora*, which has an abnormally long haircoat due to the prolongation of anagen. It was further shown that the natural *angora* mouse is caused by a mutation in FGF 5`(Hebert et al 94). A fourth member of the FGF family is FGF-7 (also known as keratinocyte growth factor or KGF). In contrast to the inhibitory effects of the three previous FGFs, FGF-7 stimulates proliferation of hair follicles and even induced hair growth in athymic nude

mice (Danilenko et al 95). When given systemically, FGF-7 appears to prolong anagen and can significantly reduce hair loss in a rat model of chemotherapy-induced alopecia (Danilenko et al 95). FGF-7 is expressed by fibroblasts within the dermal papilla and its receptor is located on epithelial cells of the outer sheath and hair matrix. FGF-10 has been recently identified and is highly homologous with KGF. Preliminary evidence indicates this factor is expressed in the follicular papilla *in vitro* and has been found in the connective tissue sheath surrounding the follicle *in vivo* (Hamada et al 1999).

There are at least three isoforms of *TGF B* and all are critically involved in embryogenesis, carcinogenesis and immunomodulation. TGF B inhibits the growth of most epithelial cells but stimulates proliferation of many mesenchymal cells and promotes the deposition of extracellular matrix. In hair follicles, TGF B is produced just before catagen where it is expressed in the inner sheath, outer sheath, matrix and follicular papilla- and the receptors are also widely distributed. These growth factors are thought to inhibit follicular proliferation (Philpott et al 94, Danilenko et al 95). Recently, when isolated, intact cultured anagen follicles were exposed to TGF-B, this cytokine induced catagen morphology (Soma et al 98).

Insulin-like growth factors represent peptides with substantial amino acid homology to insulin. There are two members of this family that affect hair growth. ILGFs are produced by follicular papilla cells, although the location of their receptors is unknown. Both ILGFs stimulate follicle growth at physiologic concentrations and suppress hair follicle entry into catagen (Danilenko et al 95).

Interleukin 1 (IL-1) was originally identified as a component of the cellular immune response and as a pro-inflammatory cytokine. As with so many cytokines, IL-1

is produced by many different cell types and has a wide range of functions in both immunologic and non-immunologic processes. IL-1 exists in two forms, IL-1a and IL-1b, that are encoded by separate genes with little homology, yet both bind to the same type I and type II receptors. Currently it is unclear which cells within the hair follicle produce IL-1 or contain IL-1 receptors, but when cultured hair follicles were grown with IL-1b and then treated with IL-1b specific inhibitors and stimulators, IL-1b inhibited the growth of the hair (Hoffmann et al 97). The authors hypothesized that IL-1b effects occur through an inhibition of matrix cell differentiation and proliferation mediated by cAMP. IL-1b has been shown to be produced by cultured follicular papilla cells when a phorbol ester was added to the culture. As would be expected, the IL-1b exerted a potent inhibitory effect on whole hair follicles grown in culture (Xiong and Harmon 97).

The initiation of anagen and sustaining hair growth requires a complex and highly developed system of blood vessels. This vascularization remodels and diminishes as the follicle enters catagen. *Vascular endothelial growth factor (VEGF)* is a growth factor that is critical for angiogenesis because it stimulates the activation, proliferation and migration of endothelial cells. VEGF is expressed in the bulb of anagen hairs, in mesenchymal cells such as fibrous sheath fibroblasts, and most strongly, in follicular papilla cells. For these reasons, it is believed that VEGF is responsible, at least in part, for angiogenesis related to the hair cycle (Kozlowska et al 98).

Hepatocyte growth factor/scatter factor (HGF/SF) is a multifunctional polypeptide that was originally identified as a mitogen for hepatocytes. In 1994, Jindo et al reported that HGF/SF stimulated the growth of mice vibrissae and human scalp hairs within a whole follicle culture system. HGF/SF acts directly on the matrix region by

stimulating DNA synthesis of hair bulb-derived keratinocytes (Shimaoka et al 95). Currently, it is thought that this factor is expressed by follicular papilla cells and binds to receptors on the epithelial cells of the hair matrix. To clarify the stimulatory effect of HGF/SF on the follicle, HGF/SF was injected intradermally into the dorsal skin of mice with follicles in all stages of the hair cycle over consecutive days. In these experiments, HGF/SF caused enlargement of anagen follicles and prolonged the anagen phase of the cycle, delaying the transition into catagen. Injection into skin containing telogen hairs resulted in inducement of a low number of hairs into anagen (Jindo et al 98). Because this factor appears to promote hair growth in a dose dependent manner, further studies are underway to examine the potential clinical use of HGF/SF as a hair growth stimulant.

Neurotrophins are a family of four related polypeptides that control the development of peripheral tissue innervation. They are also expressed and have receptors in the skin. In recent *in vitro* and *in vivo* work, the expression of two neurotrophins, brain derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) and their receptor, was tightly linked with the hair follicle cycle and peaked during follicle regression. When BDNF and NT-4 knockout mice were examined, the catagen stage was lengthened, delaying shedding, whereas, mice overexpressing BDNF displayed a shortened catagen, with earlier hair loss (Botchkarev et al 99).

Finally, a number of other compounds are reported to have profound effects on the hair cycle. In 1987, a fungal metabolite called cyclosporin A, used as an immuno-suppressant in the treatment of organ transplant rejection and some autoimmune diseases, was shown to induce hair growth in nude mice (Sawada 87). Minoxidil, a potent arteriolar vasodilator originally developed as an antihypertensive agent, was observed to

stimulate hair growth. Currently, 2-3% Minoxidil solutions are being used topically to treat androgenetic alopecia in humans.

Extrinsic Factors

Extrinsic factors affecting hair growth are hormones produced by organs other than the skin that can stimulate or inhibit production of the hair shaft, alter the length of the hair cycle or affect the size of the follicles. The major hormones that have an influence on the follicle are melatonin, prolactin, the gonadal (estrogen, progesterone and testosterone) and adrenocortical hormones, and thyroid hormones.

Melatonin and Prolactin: These hormones are implicated in seasonal shedding of mammals. Although the mechanisms are still not totally understood, there is an inverse relationship between melatonin, produced in the pineal gland, and prolactin, produced in the pituitary, that changes with the day length. As day length shortens in the winter, melatonin levels from the pineal gland rise, resulting in lower prolactin levels and development of the winter pelage. This situation reverses with increasing daylight, leading to shedding in the spring. Experimentally, pinealectomy prevents the development of a winter coat in Siberian hamsters (Badura and Goldman 92).

Gonadal and Adrenocortical Hormones: Sex hormones and adrenal steroids also affect the hair cycle. In rats and humans, estrogen and adrenocorticoids are associated with a prolonged telogen state, while the effects of androgens are much more complicated. The importance of androgens in human hair growth has been known for more than half a century, when Hamilton observed that men castrated before puberty neither grew beards nor went bald unless they were treated with testosterone (Hamilton

42). The mane in lions is also stimulated by androgens and several primate species have a mature-onset anagen associated scalp balding, however, neither castration nor treatment with testosterone has any effect on the rate of hair growth in rats (Scott et al 95).

The wide range of response to androgens depending on body site indicates the specificity of the response is determined locally at the level of the follicle. This is illustrated by the success of hair transplants to balding scalps in which the donor follicles retain the behavior of the donor (Messenger et al 93). Androgens circulate in the blood either freely or bound to proteins, especially sex hormone-binding globulins. Androgens, like other steroids, diffuse into the cell and bind to a specific nuclear receptor. The hormone-receptor complex interacts with the DNA to activate specific genes. Understanding how a specific androgen affects hair growth is complicated by the extensive intracellular conversion to more or less potent steroids in target tissues (Messenger et al 93).

The type of androgen can positively or negatively affect the size of the follicle, the diameter of the shaft and the hair cycle, although the exact mechanism is unknown. The volume of the follicular papilla has long been thought to determine the size of the follicle (Van Scott and Ekel 58). If follicle size is dependent on the papilla, then the primary action of androgen-related follicular changes may be on the papilla, rather than on the follicular epithelium. If true, this would mean the papilla has androgen receptors. In 1992, androgen receptors were identified on papilla cells using immunohistochemistry (Choudhry et al 92) and binding studies on cultured papilla cells (Randall et al 92). How androgens act on the papilla to affect the follicle is unknown. One hypothesis is that they might simply change the volume of the papilla, altering the area that interacts with the

matrix cells (Messenger et al 93).

Adrenal Glucocorticoids: The effects of glucocorticoids are best appreciated by the changes induced by hormone excess. Cortisol decreases the mass of dermal connective tissue through direct effects on fibroblasts. Glycosaminoglycan synthesis is decreased and the composition of these molecules is altered. Cortiocsteroids slow hair growth possibly by hindering the initiation of anagen (Freinkel 93). Like androgens, corticosteroids also appear to alter the size of follicles, with excess hormone associated with miniaturization of follicles. High affinity nuclear receptors for these hormones have been identified in both fibroblasts and keratinocytes; however, the role of steroids in the skin is complicated by the many effects that occur in states of hormone excess. There are surprisingly few discernible changes when glucocorticoids are absent (Freinkel 93).

Thyroid Hormones: The thyroid hormones affect many fundamental mechanisms of the metabolism. They are critical for fetal development, particularly development of the neural and skeletal systems. Thyroid hormones stimulate calorigenesis, protein synthesis, and virtually all aspects of carbohydrate and lipid metabolism and affect myriads of biosynthetic and degradative cellular processes. They have a large number of sites of action at the level of the cell membrane, mitochondria, and gene transcription. In the skin, thyroid hormones appear to be important for the initiation and maintenance of hair growth and for the secretion of sebum (Freinkel 93). Epidermal mitotic activity, oxygen consumption and protein synthesis are increased by thyroid hormones. They also affect the production of collagen and mucopolysaccharides by dermal fibroblasts (Feldman and Nelson 87, Freinkel 93).

Thyroid hormones are synthesized in the thyroid follicle, a sphere formed by a

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single layer of cuboidal to columnar cells. The lumen of the follicle contains colloid, a gel like substance secreted by the thyroid cells and the storage site for thyroglobulin, the large glycoprotein precursor of all thyroid hormones. Adequate ingestion of iodide is necessary for normal hormone synthesis and it is incorporated into the precursors in the follicle. For thyroid hormone to be released, thyroglobulin must re-enter the follicle cells and undergo proteolysis into thyroxine (T4) and, to a lesser degree, triiodothyronine (T3), which diffuse into the blood. The synthesis and secretion of thyroid hormones are controlled by the hypothalamic- pituitary- thyroid gland axis. Thyrotropin or thyroid stimulating hormone (TSH), produced in the anterior pituitary, is the major modulator of thyroid activity, by affecting secretion of thyroid hormones. The feedback loop between the thyroid and pituitary glands is modulated by the hypothalamus and thyrotropin-releasing hormone (TRH). TRH stimulates production of TSH in the pituitary gland by mechanisms that are poorly understood. TSH increases the secretion of thyroid hormones. Circulating levels of these hormones are detected by receptors in the hypothalamus which modulates the amount of TRH that is released.

The majority of the thyroid hormones in plasma are bound to proteins, including thyroid hormone binding globulin (TBG), thyroxine binding prealbumin (TBPA), albumin and plasma lipoproteins. Less than 1% of T4 and T3 circulate freely, entering cells to exert their effects and then be metabolized. The major pathway of T4 catabolism is the progressive deiodination of the molecule. Depending upon which initial iodine group is removed, either the biologically active T3 or the less active reverse T3 (rT3) is produced. Once in the cell, thyroid hormones bind to receptors. This resultant hormone-receptor complex enters the nucleus and binds to the DNA, initiating transcription of

certain genes.

Structural and/or functional abnormalities of the thyroid gland can lead to deficient production of thyroid hormone. Primary hypothyroidism is the most common form in the dog, resulting from pathology within the gland. Inflammation, leading to destruction of the gland, is the usual cause, although rarely congenital defects in thyroid hormone production have been reported. Secondary hypothyroidism, also rare, occurs with dysfunction (malformation, neoplasia, or iatrogenic suppression or destruction) within the pituitary gland leading to impaired secretion of TSH. Tertiary hypothyroidism is defined as deficient TRH secretion by the hypothalamus and has not been reported in the dog.

Primary hypothyroidism accounts for 95% of the cases of naturally occuring thyroid failure in dogs. The two main morphologic patterns of thyroid destruction in dogs are lymphocytic thyroiditis and thyroid gland atrophy. Lymphocytic thyroiditis is characterized by patchy to diffuse infiltrates of lymphocytes with fewer plasma cells and macrophages, resulting in necrosis of follicles and fibrotic replacement. Clinical signs associated with this destruction do not become apparent until at least 75% of the gland has been ablated. In colony-bred beagles, lymphocytic thyroiditis has been shown to be genetically transmitted (Fritz et al 70 and 76). In the second form, thyroid gland atrophy, there is loss of the gland parenchyma and replacement with adipose tissue associated with scant inflammation. It is still unknown if atrophy of the gland represents the end stage of lymphocytic thyroiditis (Feldman and Nelson 87).

In dogs, hypothyroidism is the most commonly diagnosed endocrinopathy (Feldman 87). Because thyroid hormones are needed for normal cellular metabolic

functions in many organs, deficiencies have been postulated to produce a wide range of clinical signs, including lethargy, weight gain, cold intolerance, reproductive dysfunction, neuromuscular signs (particularly megaesophagus and laryngeal paralysis), cardiac abnormalities, gastrointestinal disorders, hemostatic changes and dermatologic signs including alopecia, scaling, hyperpigmentation, seborrhea, and myxedema. The most common hematologic changes are a nonregenerative anemia and hypercholesterolemia (Feldman and Nelson 87, Kemppainen and Clark 94, Merchant and Taboada 97). As controlled studies of hypothyroidism have been undertaken to further examine these anecdotal signs, many of these manifestations cannot be attributed to hypothyroidism. In August 1996, an international veterinary symposium on hypothyroidism reached the consensus that lethargy, weight gain, and some skin changes were the only common signs of hypothyroidism, while neuromuscular signs, female infertility, myxedema and ocular disorders were uncommonly seen and there was no proof that male infertility, coagulopathies, cardiovascular disorders, gastrointestinal disease and behavioral disorders are caused by hypothyroidism in dogs (Nelson 97, Johnson et al 99).

That thyroid hormones have profound effects on the skin is clear. Ablation of the thyroid gland of sheep in utero retards the formation of hair follicles and other adnexa, as well as the development of the epidermis and dermis (Chapman et al 74). In humans, a deficiency of thyroid hormones is reported to produce cool, dry, pale skin. The hair is dry, coarse and grows slowly. There is also loss of scalp and eyebrow hair and the percentage of telogen follicles is increased. The most obvious clinical change in the skin is the accumulation of dermal mucopolysaccharides, a condition known as myxedema. The cause of myxedema is still unclear. As thyroid hormones are believed to slow the

synthesis and increase the breakdown of glycosaminoglycans, decreased thyroid function could cause their accumulation in the dermis (Heymann 92, Freinkel 93).

The published history of hypothyroidism and skin disease in the dog is long. A description of a case of myxedema and hypothyroid dermatosis can be found as long ago as 1940, as well as an article on therapy with thyroid hormones in 1937 (Witzigmann 37, Ojemann 40). In a 1953 review of endocrinopathies, the association of hair loss with hypothyroidism was described as a commonly recognized finding: "the fact that hypothyroidism alters the character of the hair in both animals and man is so well known that no special documentation is required..." Clinically, the authors reported a gradual loss of luster of the hair, a bilaterally symmetrical truncal wear pattern of hair loss and failure of clipped hair to regrow. They also described the skin in alopecic areas as smooth and hyperpigmented (Coffin and Munson 53). In 1958, one of the first descriptions of the histology of hypothyroidism was published. The changes included hyperkeratosis in an otherwise normal epidermis, an increased number of hair follicles in telogen and atrophic sebaceous glands (Clark and Meier 58). In 1965, similar changes were reported in a textbook on veterinary dermatology. By 1969, when the first edition of "Small Animal Dermatology" by Muller and Kirk was published, hypothyroidism was a well-established canine disease. This text highlights that not all hypothyroid patients show skin lesions and alopecia. They describe hypothyroidism histologically as an "atrophic dermatosis" with a thin epidermis, washed out appearance to the collagen fibers of the dermis, and atrophy with keratin plugging of follicles.

In 1982 Danny Scott published a review article on endocrine skin disorders of the dog. In this review, hypothyroidism was identified as the most commonly occurring

endocrine dermatosis. As before, clinical signs included a bilaterally symmetrical alopecia, a dull haircoat, and failure to regrow after clipping. Scott also described clinical features not previously recognized, including seborrhea, comedones, poor wound healing, bruising, pyoderma and myxedema. Histologically, the characteristic alterations change. Features not mentioned previously are epidermal hyperplasia and perivascular and periadnexal inflammation.

By 1995, three major dermatologic textbooks all describe hyperkeratosis, and follicular atrophy with a predominance of telogen follicles as characteristic histologic changes of hypothyroidism (Gross et al 92, Yager and Wilcock 94, Scott et al 95). Vacuolation of arrector pili muscles, although not consistent, is cited in two of three texts, as is a thickened dermis or increased dermal mucin. Yager and Wilcock report myxedema in 33% of cases, however, this alteration can be missed on routine H&E stains and requires alcian blue stains for detection. In contrast to earlier reports, the epidermis is now described as hyperplastic and the majority of cases contain prominent inflammation. Inflammation is reported in 50% of cases by Scott et al, is "common" in cases seen by Gross et al and is "extremely common" in those cases examined by Yager and Wilcock. This inflammatory component is believed to be secondary to bacterial infections. All three authors state that skin biopsy is not a sensitive test for hypothyroidism and all concur that this condition is overdiagnosed. The recent development of an assay for canine TSH that can be paired with total or free T4 will greatly help to improve the accuracy of the diagnosis and should distinguish those dogs with low T4 values due to sick euthyroidism. (Dixon et al 96).

In summary, the hair follicle consists of a downgrowth of the epidermis that

interacts with an aggregate of modified, dermal fibroblasts known as the follicular papilla to form a hair shaft. Hair shaft formation is cyclical in all mammals, with periods of active hair growth (anagen), senescence (catagen) and inactivity (telogen). How the hair cycle is regulated remains unclear, but it is now known from human and rodent studies that numerous growth factors, systemic hormones and their receptors influence both hair follicle development and cycling. A major goal of this dissertation is to define the effects of thyroid hormones on the canine hair follicle cycle.

Chapter 2

CHARACTERIZATION AND PHYSICAL MAPPING OF THE CANINE TRANSGLUTAMINASE 1 GENE

Introduction

Cornification is the process by which keratinocytes, the predominant cell of the epidermis, mature to form the outermost layer of the skin, the stratum corneum. This layer is analogous to a "wall" of flattened, anucleate keratinocyte bricks (called corneocytes) that are held together by a mortar composed of extracellular lipids and remnants of desmosomes. Together, the corneocytes and the molecules that bind them serve as a life-sustaining barrier that protects against external chemical, microbial, and ultraviolet injury and prevent the loss of internal body fluids. As new corneocytes are formed in the lower regions of the stratum corneum, older cells move toward the surface and are eventually sloughed.

Critical to the formation of the stratum corneum is the cornified envelope. Located just beneath and eventually incorporating the plasma membrane of corneocytes, it appears ultrastructurally as a 15nm thick, uniform, electron-dense band. On closer examination, this band actually consists of two parts: the internal protein envelope (~10nm thick) that is composed of cross-linked structural proteins and the external lipid envelope (~5nm thick) that is composed of lipids that are covalently bound to the protein envelope. The importance of the cornified envelope is two-fold: 1) it is the most insoluble region of the stratum corneum and 2) it is the site where the intercellular lipids and keratinocytes are joined into a functional syncytium. These features provide the stratum corneum with much of its mechanical strength and impermeability.

Formation of the protein and the lipid portions of the cornified envelope is largely due to the action of transglutaminase 1 (TGM1). TGM1 is one of a family of evolutionarily-related, calcium-dependent enzymes that include the blood clotting factor

XIIIa and a number of other tissue transglutaminases (Polakowska et al 92). All function in stabilization of membranes. These enzymes catalyze the formation of a bond between the ε-NH_2 group of lysine and the γ–amide group of glutamine, forming an isodipeptide N^{ε}-(γ–glutamyl)lysine cross-link that stimulates aggregation of the target proteins of which the linked amino acids were a component. Because nature has not designed a method for the cleavage of the N^{ε}-(γ–glutamyl)lysine crosslink, transglutaminase activity results in the formation of insoluble macromolecules. In the epidermis, TGM1 acts with calcium to bind a group of sequentially expressed proteins to the intracellular surface of the corneocyte plasma membrane (Steinert and Marekov 95). Included in this group of proteins are the keratin intermediate filaments, which upon being integrated with the cornified envelope results in a linking of the surface and internal cytoskeletons of the corneocytes (Candi et al 98). In addition, TGM1 is also thought to participate in lipid envelope formation by covalent attachment through esterification of ω-hydroxyceramides to glutamines of the protein envelope (Nemes and Steinert 99).

TGM1 in humans is approximately 92kD and consists of 817 amino acids (Yamanishi et al 92). The majority of the active form of TGM1 is bound to the plasma membranes via its amino terminal region by acylated fatty acids, but it is also present in a number of cleaved, soluble forms in the cell cytoplasm (Kim et al 95). Analysis of the enzyme has been problematic as the protein is very unstable and is difficult to isolate and purify in quantities necessary for biochemical studies. The human *tgm1* gene is 14.2kb, consists of 15 exons separated by 14 introns, and is located on chromosome 14q11.2. Transcription of this gene is controlled by a variety of signaling systems including the

transcription factor AP-1, glucocorticoids, calcium, and retinoids (Yamanishi et al 92, Medvedev et al 99).

We are interested in characterizing canine *tgm1* to better define the process of cornification in the dog and because this gene is a candidate for one or more inherited canine skin diseases that clinically, histologically and ultrastructurally have much in common with human lamellar (recessive) ichthyosis, a disease often associated with a mutation in human *tgm1* (Russell et al 95). A second objective is to provide another gene for placement on the integrated linkage-radiation hybrid map of the canine genome. Reported here are the entire nucleotide and putative amino acid sequences for the coding region of canine *tgm1*, its chromosomal localization by fluorescence *in-situ* hybridization (FISH) and physical mapping of canine *tgm1* on the canine radiation hybrid (RH) map.

Materials and Methods

All procedures dealing with animal care, handling and treatment in this study were reviewed, approved and subjected to continuous monitoring by the All-University Committees for Animal Use and Care, Michigan State University and Texas A&M University.

Amplification, subcloning and sequencing: Sequence data were obtained from canine epidermal cDNA and canine genomic DNA. Tissue samples to be used for isolation of mRNA for subsequent synthesis of cDNA were obtained from either 6mm skin punch biopsy or by post-mortem sampling of the skin of mixed breed, unrelated dogs using a surgical dermatome. From these samples, the epidermis was excised with a scalpel blade, if needed, and RNA was isolated using Trizol Reagent (Gibco BRL,

Rockville, MD) following the manufacturer's protocol. Reverse transcription of total RNA was performed using random primers provided in an RT-PCR kit (Stratagene, La Jolla, CA). Canine genomic DNA was isolated from the buffy coat of peripheral blood samples using the Wizard Genomic DNA Purification kit (Promega, Madison, WI). Primers designed to amplify canine *tgm1* from cDNA or genomic DNA templates were derived from conserved nucleotide regions of the human and rat or human and rabbit TGM1 genes (Yamanishi et al 92, Phillips et al 90, Saunders et al 93). Primer sets and amplification conditions are summarized in Appendix A. The PCR products were resolved by electrophoresis through 1.4% agarose gels and were visualized following staining with ethidium bromide. PCR products with bands of appropriate length, as predicted from human *tgm1*, were purified directly or by gel extraction (Qiagen, Valencia, CA). Products were either sequenced directly or were subcloned into pCR2.1-TOPO and transformed into Top 10 cells provided as part of the Topo TA cloning kit (Invitrogen, Carlsbad, CA). All sequencing was accomplished using the Perkin Elmer ABI 373 or 377XL system. Sequence data were aligned and the putative amino acid sequence was determined using Sequencher software, version 3.1.1 (Gene Codes Co., Ann Arbor, MI).

Fluorescence *in situ* hybridization: A 5 kb fragment representing part of *tgm1* was amplified from canine genomic DNA and subcloned into pCR2.1 using the Topo TA cloning kit (Invitrogen, Carlsbad, CA). The identity of the insert was verified by sequencing. The insert was excised by digestion with Eco R1 and served as the probe for FISH. The following FISH techniques were performed by the laboratory of Dr. Matthew Breen, Genetics Section, Animal Health Trust, Suffolk UK. Metaphase chromosomes of

the dog were prepared by mitogenic stimulation of peripheral lymphocytes from a karyotypically normal individual. Conventional harvesting procedures of colcemid arrest and hypotonic treatments, followed by repeated fixation in (3:1) methanol:glacial acetic acid were used. Slides were dehydrated through an ethanol series (70%, 90%, 100%), and the chromosomes were then denatured in 70% formamide: 2x SSC for 2 min at 65C, passed through the ethanol series a second time, and air dried before use. The probe was labeled with digoxigenin-11-dUTP a nick translation reaction in which the DNase-I concentration had been optimized to result in labeled fragments 100-300 bp in size. Labeled DNA (150ng) was mixed with 10μg of sonicated canine genomic DNA, precipitated and resuspended in 15μl of a hybridization buffer comprising 50% formamide, 2x SSC and 10% dextran sulfate. The DNA mixture was denatured at 70C for 10 min and pre-annealed at 37C for 30 min prior to being added onto the denatured chromosomes under a 22mm x 22mm cover slip and sealed with cowgum. The hybridization was continued for 16-19hr at 37C in a humidified chamber. Post-hybridization stringency washes and immunocytochemical detection of the probe were as described by Breen and colleagues (1992). Chromosomes were counterstained in 80 ng/ml 4', 6-diaminidino-2-phenylindole (DAPI) and mounted in antifade solution (Vectashield, Vector Laboratories). Images were acquired and processed using a FISH workstation comprising a fluorescence microscope (Axiophot, Zeiss) equipped with an FITC/Texas Red/DAPI excitation filter set, and a cooled CCD camera (KAF 1400, Photometrics, Tuscon, AZ) both driven by dedicated software (SmartCapture, Vysis Inc.). The digital image of each DAPI stained metaphase spread was processed using a

high-pass spatial filter to reveal enhanced DAPI bands and the hybridization-sites were identified by reference to the DAPI banded dog ideogram of Breen et al (1999a).

Screening of canine radiation hybrid panel: The *tgm1*-specific fragments were mapped by Ms. Jennifer Lowe in the laboratory of Dr. Elaine Ostrander, Fred Hutchison Cancer Research Center, Seattle, WA using the 126 canine-rodent hybrid cell lines currently being used to develop a comprehensive canine RH map (Mellersh et al 2000, Priat et al 1998). The construction and characterization of the panel (RHDF5000) has been described previously (Priat et al 1998, Vignaux et al 1999). Further information regarding the cell line panel may be obtained through the web site http://www-recomgen.univ-rennes1.fr/doggy.html). PCR conditions for the three *tgm1* markers (*tgm1*rhm1, *tgm1*rhm2 and *tgm1*rhm3) examined were as follows: Initial denaturation at 95C for 1 min, followed by 35 cycles at 94C for 30 secs, Tm for 30 secs (marker 1 at 62C, marker 2 at 58C or marker 3 at 62C), and 74C for 40 secs, with a final extension of 74C for five mins. The resulting 1100, 580 or 720 base pair (respectively) PCR products were resolved by electrophoresis on a 1.2% agarose gel and visualized following staining with ethidium bromide. Radiation hybrid data from the markers were merged with data from 600 markers published previously (Priat et al 1998, Mellersh et al 2000). Analysis was done using the MultiMap package (Matise et al 1994) and markers were assigned to linkage groups using the find-all-linkage-groups function of MultiMap; markers were linked to at least one other marker with a Lod score of > 8.0. Multipoint analyses were carried out as described previously (Mellersh et al 2000, Priat et al 1998). Distances are expressed in centiRays 5000 (cR5000) in which 5,000 is the radiation dose used to generate the canine RH panel. Images in this dissertation are presented in color.

Results and Discussion

The sequence of the coding region of canine *tgm1* was determined for at least two unrelated mixed-breed dogs and each PCR product or cloned insert was sequenced in opposite directions at least twice. Sequence data for the dog are presented and aligned with the cDNA nucleotide and amino acid sequences reported for the human (Figure 10) (Phillips et al 90, Yamanishi et al 92). The canine coding region is comprised of 2,453 nucleotides distributed over 15 exons, an arrangement similar to the human. The canine coding region has 90% identity to human *tgm1*. The deduced canine TGM1 protein is 816 amino acids long and is 92% identical to human TGM1. The canine cDNA sequence has been deposited in GenBank (accession number AF262219).

The ATG initiation codon for canine *tgm1* is located in exon 2, and can be aligned with sequence for the rat. The human sequence contains two ATG sites in succession; the second is aligned with both rat and dog, but the first is believed to be the likely translation start in humans (Polakowska et al 92). The greatest number of amino acid differences between the human and canine sequences were found in exons 2 and 12, with a similarity of 84% and 83%, respectively. Exon 2 shows the highest divergence between species and among other members of the transglutaminase enzyme family, suggesting this region confers a specialized and/or species-specific function to the enzyme (Phillips et al 92, Kim et al 94).

Several predicted sites of post-translational modification important in enzyme function were examined and compared with the reported human sequence. Within the amino acid sequence of exon 2 in humans and rats, approximately 45 residues from the N terminus, there is a cluster of five cysteine residues among seven amino acids. This

Figure 10. Alignment and comparison of the nucleotide and predicted amino acid sequences of the coding regions of canine and human *tgm1*.

Translation initiation and stop codons are in boldface. Differences in the human nucleotide or amino acid sequences are underlined. Regions important in enzyme function are indicated with an asterisk (membrane anchorage site at nucleotide position 144 and enzyme active site at nucleotide position 1130). Hyphens indicate gaps which were added to maximize sequence similarities. Nucleotide positions are numbered on the right.

Exons begin at the following nucleotide positions:

Exon 2	1
Exon 3	326
Exon 4	515
Exon 5	764
Exon 6	883
Exon 7	991
Exon 8	1166
Exon 9	1305
Exon 10	1409
Exon 11	1498
Exon 12	1652
Exon 13	1934
Exon 14	2095
Exon 15	2232

```
          M  D  G  P  R  S  D  V  G  R  W  G  G  N  P  W  Q  P  P
Dog   GTGATGGATGGTCCTCGTTCAGATGTGGGCCGCTGGGGCGGGAACCCCTGGCAGCCCCCT     60
Human ATGATGGATGGGCCACGTTCCGATGTGGGCCGTTGGGGTGGCAACCCCTTGCAGCCCCCT
        M  M  D  G  P  R  S  D  V  G  R  W  G  G  N  P  L  Q  P  P

        T  T  P  S  P  E  P  E  P  E  P  E  P  E  R  R  S  R  R  G
Dog   ACCACACCTTCGCCAGAGCCAGAGCCAGAGCCAGAGCCAGAGAGACGGTCTCGTCGAGGA    120
Human ACCACGCCATCTCCAGAGCCAGAGCCAGAGCCAGAC------GGACGCTCTCGCAGAGGA
        T  T  P  S  P  E  P  E  P  E  P  D  -  -  G  R  S  R  R  G

                                  *  *     *  *     *
        -  G  R  S  F  W  A  R  C  C  G  C  C  S  C  R  N  R  A  D
Dog   ---GGCCGTTCCTTCTGGGCTCGCTGCTGTGGCTGCTGCTCCTGCCGAAACAGGGCAGAT    180
Human GGAGGCCGTTCCTTCTGGGCTCGCTGCTGTGGCTGCTGTTCATGCCGAAATGCGGCAGAT
        G  G  R  S  F  W  A  R  C  C  G  C  C  S  C  R  N  A  A  D
                                  *  *     *  *     *

          D  D  W  G  P  E  P  -  -  H  R  D  R  G  S  G  S  G  R  R
Dog    GATGACTGGGGACCTGAGCCC------CACCGAGACCGAGGGTCTGGCTCTGGGAGGCGA    240
Human  GACGACTGGGGACCTGAACCCTCTGACTCCAGGGGTCGAGGGTCCAGCTCTGGCACTCGA
          D  D  W  G  P  E  P  S  D  S  R  G  R  G  S  S  S  G  T  R

        R  P  D  S  R  G  S  D  S  R  R  P  G  S  R  A  S  G  V  N
Dog   AGACCCGACTCCCGGGGCTCAGATTCCCGCCGGCCTGGCTCCCGGGCCAGCGGTGTGAAC    300
Human AGACCTGGCTCCCGGGGCTCAGACTCCCGCCGGCCTGTATCCCGGGGCAGCGGTGTCAAT
        R  P  G  S  R  G  S  D  S  R  R  P  V  S  R  G  S  G  V  N

        A  A  G  D  G  T  I  R  E  G  M  L  V  V  T  G  V  D  L  L
Dog   GCAGCTGGAGATGGCACCATCCGAGAGGGCATGCTGGTGGTGACTGGTGTGGATCTGCTG    360
Human GCAGCTGGAGATGGCACCATCCGAGAGGGCATGCTAGTAGTGAACGGTGTGGACTTGCTG
        A  A  G  D  G  T  I  R  E  G  M  L  V  V  N  G  V  D  L  L

        S  S  R  S  D  Q  N  R  R  E  H  H  T  D  E  Y  E  Y  D  E
Dog   AGTTCGCGGTCAGACCAGAATCGCCGAGAGCACCACACAGATGAGTTTGAATACGATGAG    420
Human AGCTCGCGCTCGGACCAGAACCGCCGAGAGCACCACACAGACGAGTATGAGTACGACGAG
        S  S  R  S  D  Q  N  R  R  E  H  H  T  D  E  F  E  Y  D  E

        L  I  I  R  R  G  Q  P  F  H  M  V  L  H  F  S  R  P  Y  E
Dog   TTGATTATACGCCGTGGGCAGCCTTTTCACATGGTTCTGCACTTCTCTCGCCCCTATGAA    480
Human CTGATAGTGCGCCGCGGGCAGCCTTTCCATATGCTCCTCCTCCTGTCCCGGACCTATGAA
        L  I  V  R  R  G  Q  P  F  H  M  L  L  L  L  S  R  T  Y  E

        S  S  D  R  V  A  L  E  L  L  I  G  N  N  P  E  V  G  K  G
Dog   TCCTCTGATCGCGTCGCCCTGGAGCTGCTTATTGGAAACAACCCTGAGGTGGGAAAGGGC    540
Human TCCTCTGATCGCATCACCCTTGAGTTACTCATCGGAAACAACCCCGAGGTGGGCAAGGGC
        S  S  D  R  I  T  L  E  L  L  I  G  N  N  P  E  V  G  K  G
```

Figure 10

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          T  H  V  I  I  P  V  G  K  G  G  S  G  G  W  K  A  Q  V  T
Dog       ACCCACGTGATCATCCCAGTGGGCAAGGGGGGCAGTGGAGGCTGGAAAGCCCAGGTGACC     600
Human     ACGCACGTGATCATCCCAGTGGGCAAGGGGGGCAGTGGAGGCTGGAAAGCCCAGGTGGTC
          T  H  V  I  I  P  V  G  K  G  G  S  G  G  W  K  A  Q  V  V

          K  A  S  G  Q  N  L  N  L  R  V  H  T  S  P  N  A  I  I  G
Dog       AAGGCCAGTGGGCAGAATCTGAACCTCCGAGTCCACACCTCCCCCAATGCCATCATCGGC     660
Human     AAGGCCAGTGGGCAGAATCTGAACCTGCGGGTCCACACTTCCCCCAACGCCATCATCGGC
          K  A  S  G  Q  N  L  N  L  R  V  H  T  S  P  N  A  I  I  G

          K  F  Q  F  T  V  R  T  H  S  E  A  G  E  F  Q  L  P  F  D
Dog       AAGTTTCAGTTCACTGTCCGCACACACTCAGAGGCTGGCGAGTTCCAGCTGCCTTTTGAC     720
Human     AAGTTTCAGTTCACAGTCCGCACACAATCAGACGCTGGGGAGTTCCAGTTGCCCTTTGAC
          K  F  Q  F  T  V  R  T  Q  S  D  A  G  E  F  Q  L  P  F  D

          P  H  N  E  I  Y  I  L  F  N  P  W  C  P  E  D  I  V  Y  V
Dog       CCCCACAACGAGATCTACATCCTCTTTAATCCTTGGTGCCCAGAGGACATCGTGTACGTG     780
Human     CCCCGCAATGAGATCTACATCCTCTTCAACCCCTGGTGCCCAGAGGACATTGTGTACGTG
          P  R  N  E  I  Y  I  L  F  N  P  W  C  P  E  D  I  V  Y  V

          D  H  E  D  W  R  Q  E  Y  V  L  N  E  S  G  R  I  Y  Y  G
Dog       GACCATGAGGACTGGCGACAGGAGTACGTGCTTAATGAGTCTGGGAGAATCTACTACGGG     840
Human     GACCATGAGGATTGGCGGCAGGAGTATGTTCTTAATGAGTCTGGGAGAATTTACTACGGG
          D  H  E  D  W  R  Q  E  Y  V  L  N  E  S  G  R  I  Y  Y  G

          T  E  A  Q  I  G  E  R  T  W  N  Y  G  Q  F  D  H  G  V  L
Dog       ACTGAAGCACAGATTGGTGAGCGGACCTGGAACTATGGGCAGTTTGATCATGGGGTGCTG     900
Human     ACCGAAGCACAGATTGGTGAGCGGACCTGGAACTACGGCCAGTTTGACCACGGGGTGCTG
          T  E  A  Q  I  G  E  R  T  W  N  Y  G  Q  F  D  H  G  V  L

          D  A  C  L  Y  I  L  D  R  R  G  M  P  Y  G  G  R  G  D  P
Dog       GATGCTTGCCTGTACATTCTGGACCGGCGGGGCATGCCATATGGAGGCCGTGGGGACCCT     960
Human     GATGCCTGCTTATACATCCTGGACCGGCGGGGGATGCCATATGGAGGCCGTGGAGACCCA
          D  A  C  L  Y  I  L  D  R  R  G  M  P  Y  G  G  R  G  D  P

          V  S  V  S  R  V  I  S  A  M  V  N  S  L  D  D  N  G  V  L
Dog       GTCAGTGTCTCCCGGGTCATCTCTGCCATGGTGAACTCCTTGGATGACAATGGGGTCCTG    1020
Human     GTCAATGTCTCCCGGGTCATCTCTGCCATGGTGAACTCCCTGGATGACAATGGAGTCCTG
          V  N  V  S  R  V  I  S  A  M  V  N  S  L  D  D  N  G  V  L

          I  G  N  W  S  G  D  Y  S  R  G  T  N  P  S  A  W  V  G  S
Dog       ATTGGAAACTGGTCTGGTGATTACTCTCGAGGCACCAACCCTTCAGCGTGGGTGGGCAGC    1080
Human     ATTGGGAACTGGTCTGGTGATTACTCCCGAGGCACCAACCCATCAGCGTGGGTGGGCAGC
          I  G  N  W  S  G  D  Y  S  R  G  T  N  P  S  A  W  V  G  S
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                                                          *  *  *  *  *
          V  E  I  L  L  S  Y  L  R  T  G  Y  S  V  P  Y  G  Q  C  W
Dog   GTGGAGATCCTACTCAGCTACCTCCGCACTGGCTATTCTGTCCCCTATGGCCAGTGCTGG   1140
Human GTGGAGATCCTGCTTAGCTACCTACGCACGGGATATTCCGTCCCCTATGGCCAGTGCTGG
          V  E  I  L  L  S  Y  L  R  T  G  Y  S  V  P  Y  G  Q  C  W
                                                          *  *  *  *  *

          V  F  A  G  V  T  T  T  V  L  R  C  L  G  L  A  T  R  T  V
Dog   GTCTTCGCCGGTGTGACCACCACAGTGCTGCGTTGCCTGGGCCTGGCTACCCGCACTGTC   1200
Human GTCTTTGCTGGAGTGACCACCACAGTGCTGCGCTGCCTGGGTCTGGCCACCCGTACTGTC
          V  F  A  G  V  T  T  T  V  L  R  C  L  G  L  A  T  R  T  V

          T  N  F  N  S  A  H  D  T  D  T  S  L  T  M  D  I  Y  F  D
Dog   ACCAACTTCAACTCGGCACATGACACAGACACATCCCTCACCATGGACATCTACTTTGAC   1260
Human ACCAACTTCAACTCCGCCCACGACACAGACACATCCCTTACCATGGACATCTACTTCGAC
          T  N  F  N  S  A  H  D  T  D  T  S  L  T  M  D  I  Y  F  D

          E  N  M  K  P  L  E  H  L  N  H  D  S  V  W  N  F  H  V  W
Dog   GAGAACATGAAGCCCCTGGAGCACCTGAACCATGATTCTGTTTGGAACTTCCACGTGTGG   1320
Human GAGAACATGAAGCCCCTGGAGCACCTGAACCATGATTCTGTCTGGAACTTCCATGTGTGG
          E  N  M  K  P  L  E  H  L  N  H  D  S  V  W  N  F  H  V  W

          N  D  C  W  M  K  R  P  D  L  P  S  G  F  D  G  W  Q  V  V
Dog   AATGACTGCTGGATGAAGAGGCCAGACCTGCCCTCTGGCTTTGACGGGTGGCAAGTGGTA   1380
Human AACGACTGCTGGATGAAGAGGCCGGATCTGCCCTCGGGCTTTGATGGGTGGCAGGTGGTG
          N  D  C  W  M  K  R  P  D  L  P  S  G  F  D  G  W  Q  V  V

          D  A  T  P  Q  E  T  S  S  G  I  F  C  C  G  P  C  S  V  E
Dog   GACGCCACGCCCCAGGAGACCAGCAGCGGGATCTTCTGCTGTGGCCCCTGCTCTGTGGAA   1440
Human GATGCCACACCCCAAGAGACTAGCAGTGGCATCTTCTGCTGCGGCCCCTGCTCTGTGGAG
          D  A  T  P  Q  E  T  S  S  G  I  F  C  C  G  P  C  S  V  E

          S  I  K  N  G  L  V  Y  M  K  Y  D  T  P  F  I  F  A  E  V
Dog   TCCATCAAGAATGGCCTAGTCTACATGAAGTATGACACACCCTTCATTTTTGCCGAGGTC   1500
Human TCCATCAAGAATGGCCTGGTCTACATGAAGTACGACACGCCTTTCATTTTTGCTGAGGTG
          S  I  K  N  G  L  V  Y  M  K  Y  D  T  P  F  I  F  A  E  V

          N  S  D  K  V  Y  W  Q  R  Q  D  D  G  S  F  K  I  V  Y  V
Dog   AACAGTGACAAAGTTTACTGGCAGCGACAGGACGATGGCAGCTTCAAGATCGTGTATGTG   1560
Human AATAGTGACAAGGTGTACTGGCAGCGGCAGGATGATGGCAGCTTCAAGATTGTTTATGTG
          N  S  D  K  V  Y  W  Q  R  Q  D  D  G  S  F  K  I  V  Y  V

          E  E  K  A  I  G  T  L  I  V  T  K  A  V  G  S  N  M  Q  D
Dog   GAAGAGAAGGCCATTGGCACGCTCATTGTCACAAAAGCTGTTGGATCCAACATGCAGGAC   1620
Human GAGGAGAAGGCCATCGGCACACTCATTGTCACAAAGGCCATCAGCTCCAACATGCGGGAG
          E  E  K  A  I  G  T  L  I  V  T  K  A  I  S  S  N  M  R  E
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          D  V  T  H  I  Y  K  H  P  E  G  S  E  A  E  R  K  A  V  E
Dog   GATGTCACCCACATCTATAAACACCCAGAAGGCTCAGAAGCAGAGCGCAAGGCGGTGGAG   1680
Human GACATCACCTACCTCTATAAGCACCCAGAAGGCTCAGACGCAGAGCGGAAGGCAGTAGAG
          D  I  T  Y  L  Y  K  H  P  E  G  S  D  A  E  R  K  A  V  E

          T  A  A  A  H  G  S  K  P  N  V  Y  T  N  R  D  S  A  E  D
Dog   ACAGCAGCTGCGCATGGCAGCAAACCCAACGTGTACACCAACCGCGACTCGGCTGAGGAT   1740
Human ACAGCAGCAGCCCACGGCAGCAAACCCAATGTGTATGCCAACCGGGGCTCAGCGGAGGAT
          T  A  A  A  H  G  S  K  P  N  V  Y  A  N  R  G  S  A  E  D

          V  A  L  Q  V  E  A  Q  D  A  V  M  G  Q  D  L  T  V  S  V
Dog   GTGGCCCTGCAGGTGGAGGCCCAAGACGCAGTGATGGGGCAGGACCTGACAGTCTCCGTA   1800
Human GTGGCCATGCAGGTGGAGGCACAGGACGCGGTGATGGGGCAGGATCTGATGGTCTCTGTG
          V  A  M  Q  V  E  A  Q  D  A  V  M  G  Q  D  L  M  V  S  V

          V  L  T  N  R  G  S  S  T  R  T  V  K  L  H  L  Y  L  S  V
Dog   GTGCTGACCAATCGCGGCAGCAGCACCCGCACTGTGAAGCTGCATCTCTACCTCTCAGTC   1860
Human ATGCTGATCAATCACAGCAGCAGCCGCCGCACAGTGAAACTGCACCTCTACCTCTCAGTC
          M  L  I  N  H  S  S  S  R  R  T  V  K  L  H  L  Y  L  S  V

          T  F  Y  T  G  V  T  G  P  V  F  K  E  S  K  K  E  V  V  L
Dog   ACCTTCTACACTGGTGTTACAGGGCCTGTCTTCAAGGAGAGCAAGAAGGAAGTGGTGCTC   1920
Human ACTTTCTATACTGGTGTCAGTGGTACCATCTTCAAGGAGACCAAGAAGGAAGTGGAGCTG
          T  F  Y  T  G  V  S  G  T  I  F  K  E  T  K  K  E  V  E  L

          A  P  G  A  T  E  R  V  S  M  P  V  A  Y  K  E  Y  R  P  Q
Dog   GCGCCAGGGGCCACGGAACGCGTGTCCATGCCTGTGGCCTACAAGGAATACCGGCCCCAA   1980
Human GCACCAGGGGCCTCGGACCGTGTGACCATGCCAGTGGCCTACAAGGAATACCGGCCCCAT
          A  P  G  A  S  D  R  V  T  M  P  V  A  Y  K  E  Y  R  P  H

          I  V  D  Q  G  S  M  L  L  N  V  S  G  H  V  K  E  N  G  Q
Dog   ATCGTAGACCAGGGGTCCATGCTGCTCAATGTCTCAGGCCACGTCAAGGAGAATGGACAG   2040
Human CTTGTGGACCAGGGGGCCATGCTGCTCAATGTCTCAGGCCACGTCAAGGAGAGCGGGCAG
          L  V  D  Q  G  A  M  L  L  N  V  S  G  H  V  K  E  S  G  Q

          V  L  A  K  Q  H  T  F  R  L  R  T  P  D  L  S  L  T  L  L
Dog   GTGCTGGCCAAACAGCATACCTTCCGTCTGCGCACCCCAGATCTCTCCCTCACATTATTG   2100
Human GTGCTGGCCAAGCAGCACACCTTCCGTCTGCGCACCCCAGACCTCTCCCTCACGTTACTG
          V  L  A  K  Q  H  T  F  R  L  R  T  P  D  L  S  L  T  L  L

          G  A  A  V  V  G  Q  E  C  E  V  Q  I  V  F  K  N  P  L  P
Dog   GGGGCAGCTGTGGTTGGCCAGGAGTGCGAAGTACAGATTGTCTTCAAGAACCCCTTGCCT   2160
Human GGAGCAGCAGTGGTTGGCCAGGAGTGTGAAGTACAGATTGTCTTCAAGAACCCCCTTCCC
          G  A  A  V  V  G  Q  E  C  E  V  Q  I  V  F  K  N  P  L  P
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          V  T  L  T  N  V  V  F  R  L  E  G  S  G  L  Q  R  P  K  I
Dog     GTCACCCTCACCAATGTCGTCTTCCGGCTTGAGGGCTCCGGGCTACAGAGACCCAAGATC   2220
Human   GTCACCCTCACCAATGTCGTCTTCCGGCTCGAAGGCTCTGGGTTACAGAGGCCCAAGATC
          V  T  L  T  N  V  V  F  R  L  E  G  S  G  L  Q  R  P  K  I

          L  N  V  G  D  I  G  G  N  E  T  V  T  L  H  Q  K  F  V  P
Dog     CTCAATGTGGGGGACATTGGGGGCAACGAGACAGTGACACTACACCAGAAGTTTGTGCCT   2280
Human   CTCAACGTTGGGGACATTGGAGGCAATGAAACAGTGACACTGCGCCAGTCGTTTGTGCCT
          L  N  V  G  D  I  G  G  N  E  T  V  T  L  R  Q  S  F  V  P

          V  R  P  G  P  R  Q  L  I  A  S  L  D  S  P  Q  L  S  Q  V
Dog     GTGCGGCCAGGCCCCCGCCAGCTCATCGCCAGCTTGGACAGCCCACAGCTCTCCCAGGTG   2340
Human   GTGCGACCAGGCCCCCGCCAGCTCATTGCCAGCTTGGACAGCCCACAGCTCTCCCAGGTG
          V  R  P  G  P  R  Q  L  I  A  S  L  D  S  P  Q  L  S  Q  V

          H  G  V  I  Q  V  D  V  A  P  A  P  G  G  G  G  F  F  S  N
Dog     CATGGTGTCATCCAGGTGGACGTGGCTCCAGCCCCTGGGGGTGGGGGCTTCTTTTCAAAT   2400
Human   CACGGTGTCATCCAGGTGGATGTGGCCCCAGCCCCTGGGGATGGGGGCTTCTTCTCAGAC
          H  G  V  I  Q  V  D  V  A  P  A  P  G  D  G  G  F  F  S  D

          A  G  G  N  S  P  L  G  E  T  I  P  M  A  S  R  G  G  A
Dog     GCTGGAGGCAATAGTCCCTTGGGGGAGACCATCCCTATGGCATCTCGAGGTGGGGCTTAG   2460
Human   GCTGGAGGTGACAGTCACTTAGGAGAGACCATCCCTATGGCATCTCGAGGTGGAGCTTAG
          A  G  G  D  S  H  L  G  E  T  I  P  M  A  S  R  G  G  A
```

region has been shown to serve as the site of fatty acid esterification that results in membrane anchorage for the enzyme (Phillips et al 93). These five cysteines are conserved in the canine sequence as well (see Figure 10, asterisks highlight residues beginning at nucleotide position 144). Exon 2 also contains a high number of serines located in close proximity to arginine residues that are thought to be targets for phosphorylation by protein kinase C, potentially affecting enzyme activity and substrate recognition (Rice et al 96). A similar serine/arginine motif is present in the dog, although the canine sequence contains slightly fewer serine residues. The reported active region of the enzyme, Tyr-Gly-Gln-Cys-Trp, is in exon 7 and this region is perfectly conserved between the dog and the human (see Figure 10, asterisks indicate residues beginning at nucleotide position 1130).

Approximately 700 base pairs of untranslated 5' sequence for canine *tgm1* was determined (Appendix B), which could not be aligned to human or rabbit orthologs of *tgm1*. The 5' flanking region has been examined by computer analysis (Yamanishi et al 92) and in expression studies (Saunders et al 93, Mariniello et al 95) in the human and rabbit for sequence elements that may be involved in the control of gene expression. In the 5' untranslated region of canine *tgm1*, the following sequence elements identical to human putative or confirmed regulatory elements were found: a TATA box –like sequence (CATAA), a pair of reverse complemented Sp1 binding motifs approximately 20 nucleotides apart (CCGCCC) and an AP2-like site (TCCCTGGGC).

Eight of the fourteen canine *tgm1* introns were completely or partially sequenced (introns 1, 2, 9, 10,11, 12, 13, and 14) (Appendix C). All of the introns examined were conserved in size in comparison to the human gene and conformed to the GT/AG rule for

5' and 3' intron borders.

The chromosomal localization of canine *tgm1* by FISH is shown in Figure 11. Chromosome assignments were made with reference to the DAPI banded ideogram of Breen et al (1999a). When used as a probe, the 5 kb subcloned-PCR product hybridized only to a single *Canis familiaris* autosomal region, CFA 8q11.2-12. This result was expected as human *tgm1* maps to *Homo sapiens* autosome (HSA) 14q11 and HSA 14 and CFA 8 have been shown to be syntenic by reciprocal chromosome painting (Breen et al 1999b).

The position of canine *tgm1* on the integrated linkage–radiation hybrid map is shown in Figure 12. Marker assignments were made with reference to the current canine map (Priat et al 1998, Mellersh et al 2000). Canine *tgm1* mapped to CFA 8 in an interval 15.4cR from FH2149 and 8.9cR from MYH7, a region corresponding to human chromosome 14q11-12.

Characterizing the coding region of canine *tgm1* is a first step in examining the role of this enzyme in several inherited cornification diseases in dogs. Comparison of the sequence shows that canine *tgm1* shares a high degree of nucleotide (90%) and amino acid (92%) identity with its human ortholog. Also, physical mapping places canine *tgm1* on CFA 8, further demonstrating conservation of synteny with this canine chromosome and HSA 14.

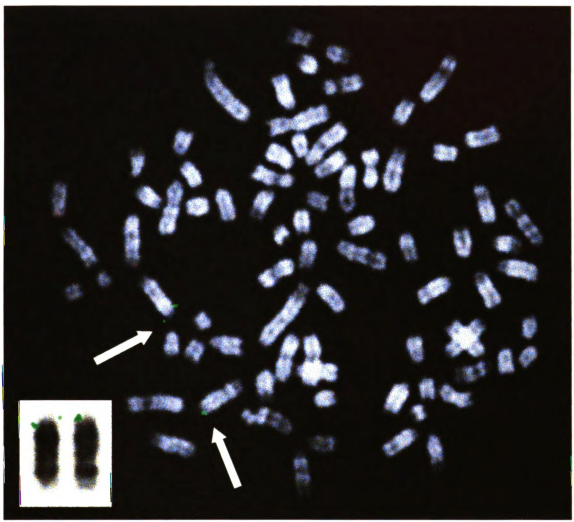

Figure 11. Chromosomal localization of *tgm1* by fluorescence *in situ* hybridization.

Localization of 5 kb genomic segment of canine *tgm1* shows a single hybridization signal to CFA 8q11.2-12.

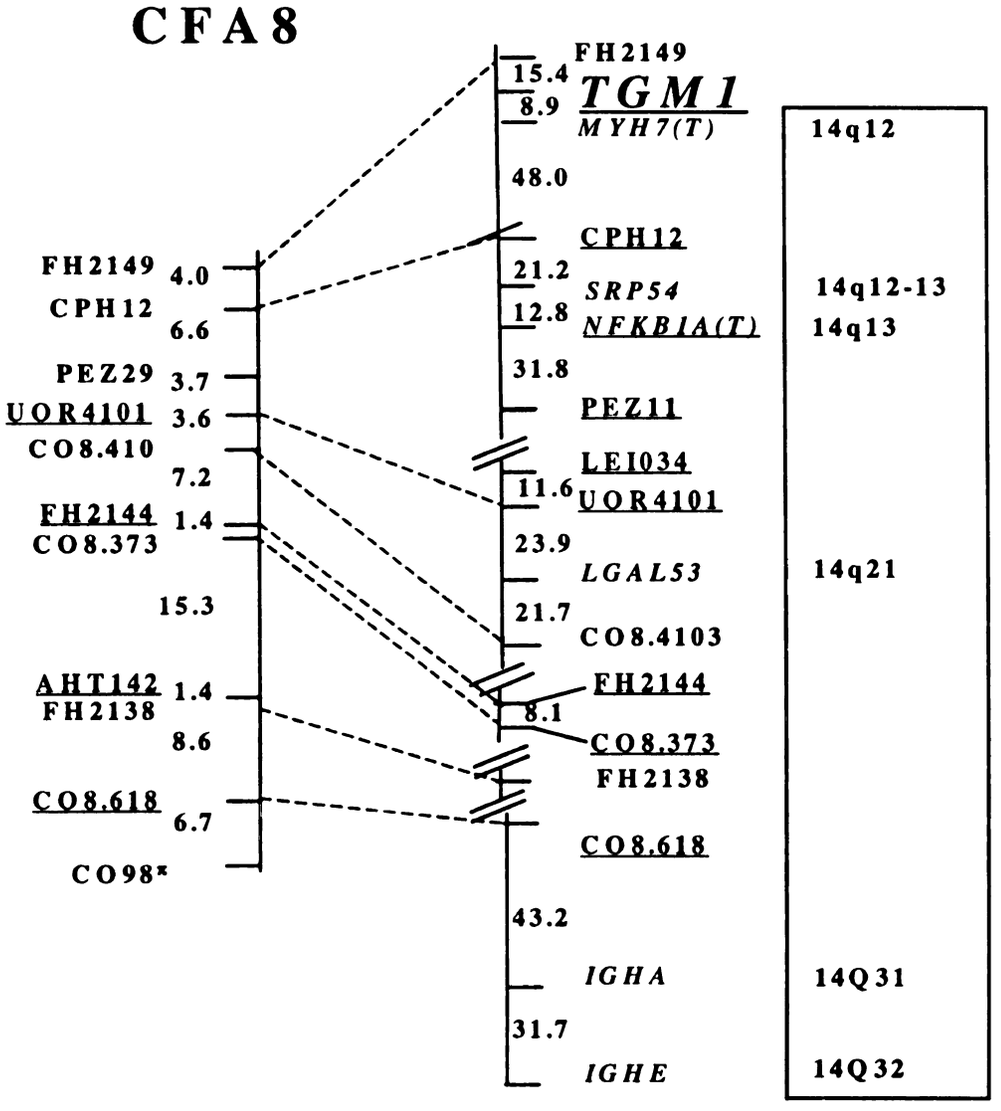

Figure 12. Integrated linkage-radiation hybrid map of CFA 8.

The radiation hybrid map is represented by the vertical bar to the right with the corresponding linkage map to the left. Canine *tgm1* is located on the radiation hybrid map at the distal end of CFA 8. The dotted lines connect markers that have been placed on both RH and linkage maps. The underlined markers were ordered with statistical support higher than 1000:1. The markers listed in italics are gene-based. The chromosome specific marker is indicated with an asterisk and markers corresponding to traced orthologous amplified sequence tags (TOASTs) are indicated with the suffix (T). The rectangle on the right demonstrates the syntenic relationship with HAS 14.

Chapter 3

THE EFFECTS OF THYROID HORMONES ON THE SKIN OF BEAGLE DOGS

Introduction

Although hypothyroidism is the most commonly diagnosed endocrinopathy of dogs, there is still much to be learned about the clinical features that define this disorder. In a 1996 International Symposium on Canine Hypothyroidism, a consensus was reached that the most common clinical manifestations of hypothyroidism were lethargy, weight gain, alopecia, pyoderma and seborrhea--all changes that can occur in a large number of diseases unrelated to hypothyroidism.

The fact that there was general agreement that the canine skin appeared to be particularly susceptible to a deficiency of thyroid hormones is not surprising. A relationship between thyroid hormone levels and the skin of dogs has been described for over 50 years. In a 1953 review of canine endocrinopathies, Coffin and Munson stated: "the fact that hypothyroidism alters the character of the hair in both man and animals is so well known that no special documentation is required."

However, reports in the literature on the effects of thyroid hormones on the skin are not consistent. In humans, skin changes attributed to hypothyroidism are generally mild and may take years to develop. The skin is cold, xerotic and pale. The most characteristic site of hair loss is the outer third of the eyebrows (Heymann 92). In mice, two hypothyroid mutant strains have been discovered in the past decade: the *hyt/hyt* hypothyroid mouse that has a mutation in thyroid stimulating hormone receptors and the *cog/cog* mouse, a model for congenital goiter (Stein 94, Adkison et al 90). Neither model has skin lesions.

In dogs, the literature is also inconsistent regarding the cutaneous manifestations of hypothyroidism, especially concerning the location and severity of hair loss. Some

authors report that hypothyroid alopecia is the most common endocrine-induced cause of hair loss, others contend alopecia in hypothyroidism is uncommon. Some state the site of hair loss is over the flanks, others report that hair loss occurs over areas of wear (dorsum of the tail, ventral thorax, lateral thorax/abdomen) (Scott et al 95, Rosychuk 97). Histologic criteria for the diagnosis of the disease by a skin biopsy sample are equally ambiguous and sometimes contradictory (Scott 89, Gross and Ihrke 90, Rosychuk 97).

We contend that part of the confusion regarding the clinical and microscopic changes of hypothyroidism in canine skin is that studies to date have been performed on spontaneous cases and have not taken breed differences into consideration. Using spontaneous cases to define hypothyroidism is problematic because the duration and degree of thyroid hormone deficiency are difficult to establish. In addition, different breeds appear to have markedly different hair cycles and follicular morphology, factors that also may influence the clinical and histologic features of the disease.

In this study we seek to define the effects of hypothyroidism on canine skin by comparing morphologic, morphometric and hair cycle differences in three groups of age- and sex-matched Beagle dogs: 1) euthyroid dogs; 2) dogs made hypothyroid by administration of ^{131}I; 3) dogs made hypothyroid and maintained in a euthyroid state by the treatment with synthetic thyroxine (Soloxine).

Materials and Methods

All procedures dealing with animal care, handling and treatment in this study were reviewed, approved and subjected to continuous monitoring by the All-

University Committee for Animal Use and Care, Michigan State University.

Dogs: Nine male intact Beagles, 11-17 months of age, weighing 10.9-15kg were used for this study. All were housed in separate kennels with 12-hour light-dark cycles. The dogs were randomly assigned to 2 groups. Group 1 consisted of 3 dogs that were used as untreated, healthy controls throughout the study. Group 2 consisted of 6 dogs that were made hypothyroid by the oral administration of 15mCi of ^{131}I (Johnson et al 99). The effectiveness of ablation by isotopes was confirmed by a panel of thyroid hormone assays (serum total thyroxine [TT4], free thyroxine [FT4], total triiodothyronine [TT3], free triiodothyronine [FT3], and endogenous thyroid stimulating hormone [TSH]). After demonstrating the six ^{131}I-treated dogs were hypothyroid by comparison with reference standards, they were randomly divided into treated and untreated groups. Three of the hypothyroid dogs were not treated and three were treated with oral thyroxine supplementation (Soloxine, Daniels Pharmaceuticals) at 0.1mg per 4.5kg once daily. In order to study the long-term effects of both deficiency and treatment, these dogs were followed for 10 months prior to sampling. Every two months and a week before the skin of the dogs was sampled, the panel of thyroid hormone assays was repeated to confirm the control dogs had normal circulating thyroid levels, the untreated dogs were hypothyroid and the hypothyroid-treated dogs were maintained in an euthyroid state.

Sampling: Prior to obtaining biopsy specimens, each dog was photographed and the pelage was examined. The dogs were given general anesthesia and two 6mm punch biopsy samples were obtained from clipped sites over the left and right dorsal thoracic area 3cm lateral to and parallel with the dorsal midline.

Processing of tissues: Tissues were fixed in zinc formalin for 24 hours. Samples were

then post-fixed in 70% ethanol. One biopsy sample from each of the two pairs was trimmed vertically from the epidermis though the dermis and into the subcutaneous fat, parallel with the flow of the hair coat. The second biopsy sample was trimmed horizontally (through the dermis on a plane parallel with the epidermis) as described by Headington (1984). Tissues were processed routinely, embedded in paraffin, sectioned at 6 microns and stained with 1) hematoxylin and eosin and 2) alcian blue (pH 2.5) with a periodic acid Schiffs counter stain. Horizontally-trimmed samples were generally step-sectioned because quantitative analysis could only be performed when the sections were made at the level of the follicular isthmus.

Examination of samples: Both vertically- and horizontally-trimmed samples were examined for changes in the epidermis, adnexa, and dermis and for evidence of inflammatory skin disease. Morphometric analysis was performed on horizontally-trimmed samples using a "follicular unit" as the basis for quantifying results. We defined a follicular unit as the anatomic aggregation of compound hair follicles that occurs in dogs. Most follicular units consist of three compound follicles adjacent to each other with the middle compound follicle containing the largest (primary) hair shaft (Figure 7). For each dog, five follicular units were identified, the number of hair follicles they contained were counted, the diameters of hair shafts were measured and the stage of the hair cycle (anagen, haired telogen, or hairless telogen) for each of the follicles was determined. Hair diameter measurements were performed on a DAGE MTI VE1000 Image Analyzer with NIH Image Software and Microsoft Excel to organize the data. Determining the stage of the hair cycle was based on an adaptation of the staging methods of Headington and Nixon (Headington 84, Nixon 93). Briefly, follicles were

considered in anagen when the hair shaft was surrounded by an inner root sheath and in haired telogen when the hair shaft was surrounded by tricholemmal cornification. When the follicular lumens contained only a rosette of tricholemmal cornification or if the follicles had no lumenal cornification, they were counted as "hairless telogen". Because catagen follicles are difficult to identify on transverse section and because they comprise such a small component of the total number of hair follicles, they were not counted (Nixon 93).

Using the data obtained from the staging of the hair follicles, the following were evaluated in five follicular units for each dog: 1) the number of hair follicles, 2) the number of anagen follicles, 3) the total number of follicles in telogen including both telogen follicles with hair shafts and hairless telogen follicles, 4) the number of hairless telogen follicles, 5) the number of primary follicles in anagen, 6) the total number of primary follicles in telogen including those with hair shafts and those that are hairless, 7) the number of primary follicles in hairless telogen, 8) the number of secondary follicles in anagen, 9) the total number of secondary follicles in telogen including those in hairless telogen, 10) the number of secondary follicles in hairless telogen, 11) the median diameter of all hair shafts, 12) the median diameter of primary hair shafts and 13) the median diameter of secondary hair shafts.

The data were summarized in three tables. In the tables, the number of hair follicles counted for each parameter or hair shaft diameter measured in five follicular units was given for each dog. A percentage was calculated by dividing the count by the total number of hair follicles found in five follicular units for each dog. Each treatment group was summarized by calculating the median value of each parameter examined.

Medians rather than means were because a normal distribution was not found.

A Kruskal Wallis one way nonparametric analysis of variance was used to detect differences between the three groups. If a $P<0.05$ was obtained, then post-hoc comparisons using the Bon Ferroni multiple comparisons test were performed to determine if a significant pairwise difference among the groups could be found. These parameters were analyzed using Statistix for Windows. Images in this dissertation are presented in color.

Results

Thyroid hormone value determination: TT4, FT4, TT3, FT3 and TSH concentrations determined immediately prior to the study are presented in Figure 13. They are representative of the values obtained after ^{131}I administration: TT4, FT4, TT3, and FT3 were low and TSH was elevated for the untreated-hypothyroid dogs (with values consistent with hypothyroidism based on reference standards), TT4, FT4, TT3, FT3 and TSH were normal in controls, and TT4, FT4, TT3 and FT3 were normal to elevated and TSH was normal in treated-hypothyroid dogs.

Clinical features: On clinical examination, there was little to distinguish untreated-hypothyroid dogs from euthyroid or treated-hypothyroid animals. None of the untreated-hypothyroid beagles had a symmetrical truncal alopecia. One untreated-hypothyroid dog had partial alopecia over the sternum. With this exception, the hair over pressure points and sites exposed to friction was not thinned. Two untreated-hypothyroid dogs had excessive scales and a greasy texture to their coats and one developed myxedema. A clinically distinctive feature observed in two of the untreated-hypothyroid dogs was marked alopecia over the bridge of the nose. A feature not noted until after this study

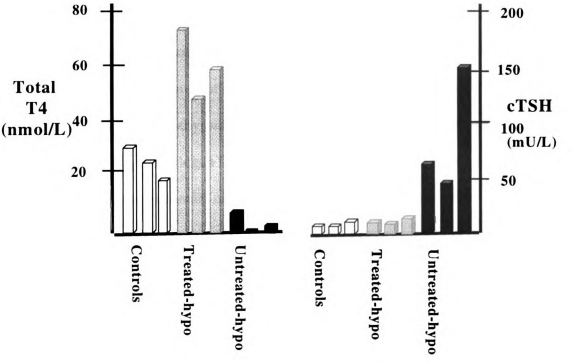

Figure 13. Graph of T4 and TSH values in the control, treated-hypothyroid and untreated-hypothyroid Beagles confirming their status prior to initiating the study.

was completed was a difference in the rate of hair re-growth. In the untreated-hypothyroid Beagles, hair clipped on the dorsum on day 1 of the study had minimal re-growth two months later. This was in sharp contrast to the hair re-growth in the control and treated-hypothyroid Beagles. For these dogs, the clipped areas could not be distinguished from the unclipped areas after two months (Figure 14).

Histologic evaluation: Histologic differences were subtle. Mild keratin plugging of follicular infundibula was present in all untreated-hypothyroid dogs (Figure 15). Control, treated- and untreated-hypothyroid dogs all had a substantial number of follicles in telogen. Flame follicles (telogen follicles containing excessive tricholemmal cornification) were occasionally noted in all three groups and could not be used as a diagnostic criterion for hypothyroidism. No differences were identified in the size of the adnexal glands or in the degree of hypertrophy and/or vacuolation of arrector pili muscles among the three groups. In spite of the clinical observation of scaling and greasy coats in two of the untreated-hypothyroid dogs, neither acanthosis nor hyperkeratosis was noted that was severe enough to distinguish these animals. There was no evidence of hyperpigmentation or abnormal dispersion of melanin around hair follicles although the coat color of the dorsum from all dogs in the study was either black or dark brown. The dog with clinical myxedema had more dermal mucin than any of the other dogs, including the other two untreated-hypothyroid dogs, but this could be only confirmed with alcian blue staining. None of the dogs had inflammatory skin disease.

Horizontally-trimmed sections defined that Beagle hair is similar to that of other dogs: follicular units varied from 2-5 compound follicles, each compound follicle having a large anterior primary follicle flanked by smaller primary follicles. Associated with

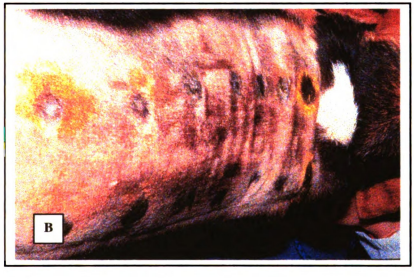

Figure 14. Clinical photographs of A) a control Beagle and B) an untreated-hypothyroid Beagle taken 64 days after their dorsums were clipped.

There is a distinct lack of hair regrowth in the untreated-hypothyroid dog without loss of hair. This is indicative of hair that is in a haired telogen stage.

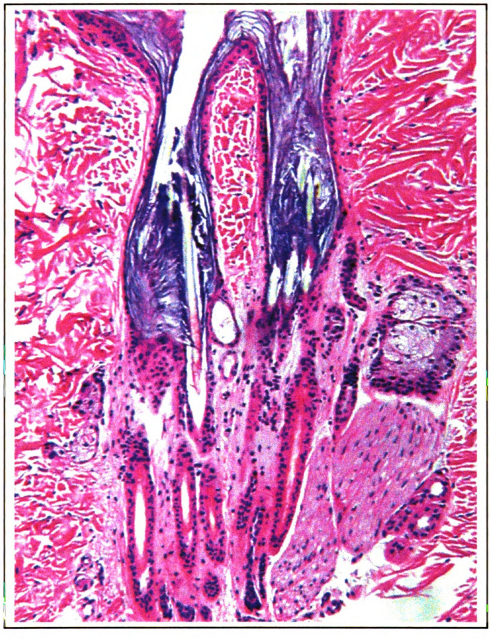

Figure 15. Photomicrograph of a vertically-sectioned biopsy from an untreated-hypothyroid Beagle (10x H&E).

There is keratin plugging of the follicular infundibula and sebaceous glands that are of normal size. The prominence of the arrector pili muscles is because the biopsy is from the dorsum.

each of the primary hairs were secondary hairs that became progressively smaller caudally (Figure 16).

Morphometric evaluation: The results of the morphometric analyses are presented in Tables 4, 5A and 5B. The total number of hair follicles/five follicular units was similar in the control, untreated-hypothyroid and treated-hypothyroid dogs. Significant differences were noted overall in the number of hair follicles in anagen ($p=0.016$), telogen ($p=0.007$) and the number of hairless telogen follicles ($p=0.034$). However, significant differences between the three groups were only noted in the number of follicles in telogen in the untreated-hypothyroid and treated-hypothyroid dogs. This inability to distinguish the different groups even when the overall p value was significant was likely due to small sample size and moderate differences between the groups leading to a lack of statistical power. Statistically significant differences were not noted in the median diameter of the hair shafts; however, the graph in Figure 17 suggests, by inspection, that the loss of hair shafts in the untreated-hypothyroid dogs occurred largely in medium to large diameter anatomic secondary hairs.

As defined in Table 5A, no significant differences in the number of hairs in anagen, telogen or hairless telogen between the groups were noted in the primary follicles. However, in Table 5B, differences in the hair cycle of secondary hair follicles were identified. There were significant differences in the number of secondary follicles in anagen ($p=0.010$)--the untreated-hypothyroid dogs were significantly different from the treated-hypothyroid dogs. The untreated-hypothyroid dogs had the fewest anagen follicles and the treated-hypothyroid dogs had the most, but neither group was significantly different from the control group. There were also significant differences in

Figure 16. Morphologic features of horizontally-sectioned skin demonstrating a follicular unit in A) control Beagle; B) untreated-hypothyroid Beagle; C) treated-hypothyroid Beagle (10x H&E).

Although all dogs have the same approximate number of hair follicles, the stage of the hair cycle is different. This is most apparent in the untreated-hypothyroid dog where most of the secondary hairs are in a "hairless telogen" stage. Note that most of the follicles with hair shafts have an eosinophilic, serrated border separating the hair shaft from the outer root sheath. This pattern of cornification is a feature of hairs in the "haired telogen" stage.

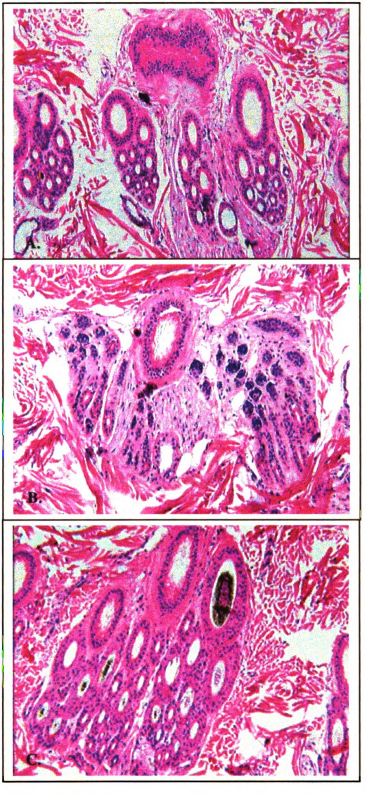

Figure 16.

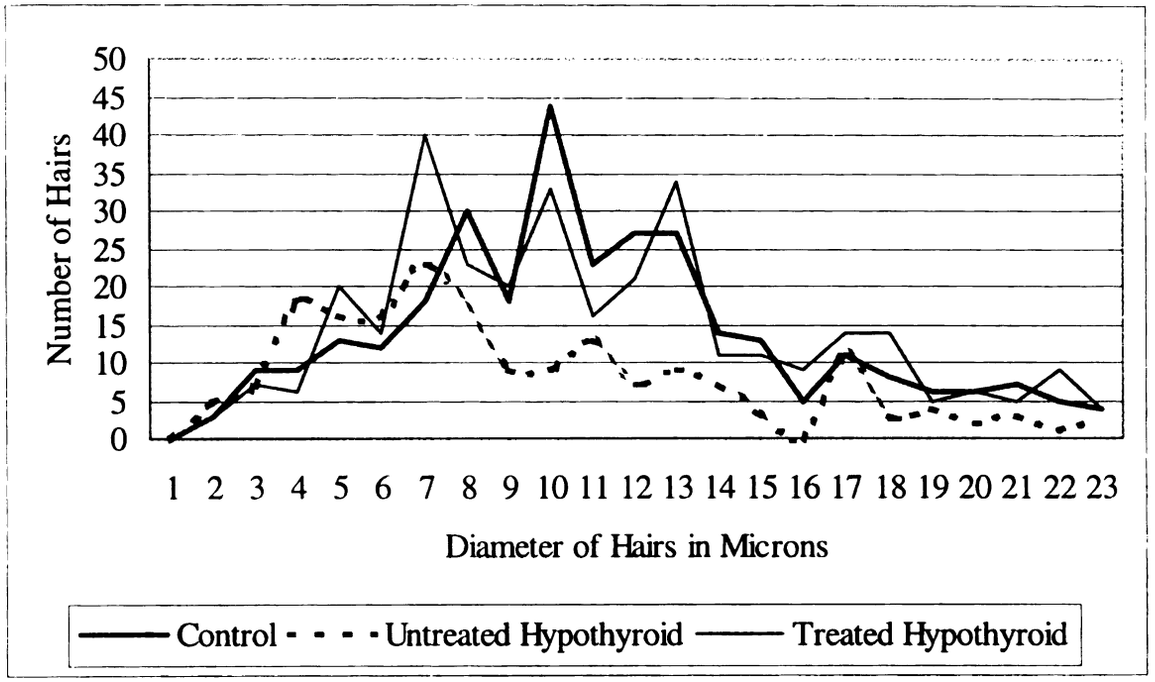

Figure 17. Graph of hair shaft diameters between 1 to 23 microns for control, untreated-hypothyroid, and treated-hypothyroid Beagles.

This graph demonstrates that the hair loss in the untreated-hypothyroid dogs appears to occur in hairs between 8 and 16 microns.

Table 4. Morphometric evaluation of normal, untreated-hypothyroid and treated-hypothyroid Beagles.

The first four columns contain the total number of hair follicles counted in five follicular units for each dog in each treatment group. The percentages in the middle columns are calculated by dividing each count by the total number of follicles found in the 5 follicular units for that dog (data shown in column 1). The last column contains the median diameter in microns of all the hair shafts measured in 5 follicular units for each dog. Each treatment group is summarized by determining the median value for each parameter with its corresponding percent of follicles per five follicular units, if applicable.
*This analysis had an overall significant p value, but there was insufficient power to determine which groups were different from the others.
**This analysis had an overall significant p value with the untreated-hypothyroid group significantly different from the treated-hypothyroid group.

Table 4. Morphometric evaluation of normal, untreated-hypothyroid and treated-hypothyroid Beagles.

	Number of Hair Follicles	Number of Follicles in Anagen	Number of Follicles in Telogen	Number of Follicles in Hairless Telogen	Median Diameter of Hair Shafts (um)
Controls					
Dog 1	153	9 (5.9%)	144 (94.1%)	28 (18.3%)	10.4
Dog 2	145	35 (24.1%)	110 (75.9%)	45 (31%)	14.1
Dog 3	184	56 (30.4%)	128 (69.6%)	22 (12%)	11.6
Median	153	35 (24.1%)	128 (69.6%)	28 (18.3%)	11.6
Untreated Hypothyroid					
Dog 1	155	4 (2.6%)	151 (97.4%)	61 (39.4%)	13.2
Dog 2	165	4 (2.4%)	161 (97.6%)	107 (64.8%)	10.7
Dog 3	209	0 (0%)	209 (100%)	128 (61.2%)	6.7
Median	165	4 (2.6%)	161 (97.6%)	107 (64.8%)	10.7
Treated Hypothyroid					
Dog 1	181	123 (68%)	58 (32%)	52 (28.7%)	12.8
Dog 2	127	50 (39.4%)	77 (60.6%)	31 (24.4%)	11.5
Dog 3	157	46 (29.3%)	111 (70.7%)	11 (7%)	11.6
Median	157	50 (39.4%)	77 (60.6%)	31 (24.4%)	11.6
p value	p=0.65	p=0.02*	p=0.007**	p=0.03*	p=0.79

Tables 5 A and B. Morphometric evaluation of anatomic primary and secondary hair follicles of normal, untreated-hypothyroid, and treated-hypothyroid Beagles.

The first three columns represent the total number of primary (Table 5A) or secondary (Table 5B) hair follicles counted in five follicular units for each dog in each treatment group. The percentages are calculated by dividing the count by the total number of hair follicles found in five follicular units for each dog (data shown in column 1 of Table 4.) The last column contains the median diameter in microns of either the primary or secondary hair shafts measured in five follicular units for each dog. Each treatment group is summarized by determining the median value for each parameter with its corresponding percent of follicles per five follicular units, if applicable.

*This analysis had an overall significant p value, but there was insufficient power to determine which groups were different from the others

**This analysis had an overall significant p value with the untreated-hypothyroid group significantly different from the treated-hypothyroid group.

Table 5A. Morphometric evaluation of anatomic primary hair follicles of normal, untreated-hypothyroid, and treated-hypothyroid Beagles.

	Number of Primary Follicles in Anagen	Number of Primary Follicles in Telogen	Number of Primary Follicles in Hairless Telogen	Median Diameter of Primary Hairs (um)
Controls				
Dog 1	0 (0%)	15 (9.8%)	4 (2.6%)	39.8
Dog 2	4 (2.8%)	13 (9.0%)	1 (0.7%)	46.2
Dog 3	8 (4.3%)	8 (4.3%)	0 (0%)	63.2
Median	4 (2.8%)	13 (9.0%)	1 (0.7%)	46.2
Untreated Hypothyroid				
Dog 1	1 (0.6%)	14 (9.0%)	0 (0%)	34.9
Dog 2	0 (0%)	16 (9.7%%)	1 (0.6%)	60.6
Dog 3	0 (0%)	16 (7.7%)	4 (1.9%)	22.8
Median	0.0 (0%)	16 (7.7%)	1.0 (0.6%)	34.9
Treated Hypothyroid				
Dog 1	12 (6.6%)	3 (1.7%)	1 (0.6%)	40.6
Dog 2	2 (1.6%)	13 (10.2%)	1 (0.8%)	43.2
Dog 3	1 (0.6%)	14 (8.9%)	0 (0%)	44.2
Median	2 (1.6%)	13 (10.2%)	1.0 (0.6%)	43.2
p value	p=0.23	p=0.13	p=0.89	p=0.56

Table 5B. Morphometric evaluation of anatomic secondary hair follicles of normal, untreated-hypothyroid, and treated-hypothyroid Beagles.

	Number of Secondary Follicles in Anagen	Number of Secondary Follicles in Telogen	Number of Secondary Follicles in Hairless Telogen	Median Diameter of Secondary Hairs (um)
Controls				
Dog 1	9 (5.9%)	120 (78.4%)	25 (16.3%)	11.9
Dog 2	31 (21.4%)	97 (67%)	44 (30.3%)	9.3
Dog 3	48 (26%)	120 (65.2%)	22 (12%)	11.5
Median	31 (21.4%)	120 (65.2%)	25 (16.3%)	11.5
Untreated Hypothyroid				
Dog 1	3 (1.9%)	137 (88.4%)	61 (39.4%)	11
Dog 2	4 (2.4%)	145 (87.9%)	106 (64.2%)	8.3
Dog 3	0 (0%)	193 (92.3%)	124 (59.3%)	5.9
Median	3 (1.9%)	145 (87.9%)	106 (64.2%)	8.3
Treated Hypothyroid				
Dog 1	111 (61.3%)	55 (30.4%)	51 (28.2%)	11.3
Dog 2	48 (37.8%)	64 (50.4%)	30 (23.6%)	10.4
Dog 3	45 (28.7%)	97 (61.8%)	11 (7.0%)	10.4
Median	48 (37.8%)	64 (50.4%)	30 (23.6%)	10.4
p value	p=0.01*	p=0.002**	p=0.03*	p=0.28

the total number of secondary follicles in telogen (p=0.002)--the untreated-hypothyroid dogs had the most secondary follicles in telogen and the treated-hypothyroid dogs had the fewest. Once again, neither of these groups differed significantly from the euthyroid controls. When comparing the number of secondary follicles in hairless telogen, significant differences were found for the overall ANOVA (p=0.034); however there was not enough power to distinguish pairwise differences. Significant differences were not noted in the median diameters of the primary or secondary hair shafts.

Discussion

In this report, we attempted to characterize the normal histology and hair follicle morphometry specific to a canine breed and to compare these data with age-, breed- and sex-matched dogs affected with hypothyroidism, a disease that purportedly has a profound effect on the canine hair follicle (Scott et al 95, Rosychuk 97). To our knowledge, this study represents the first time such an approach has been used to define any disease affecting the canine hair follicle.

We performed this study, in part, to better define the morphologic changes associated with canine hypothyroidism, but also because the organization of a compound follicle (a follicle that has a common infundibulum into which grow multiple hair follicles) appears to be ideal for studying hair growth and cycling. Rather than having hair shafts of variable diameters in a random pattern (humans, sheep, mice), the organization of the compound follicle of the dog has the largest hair shafts located cranially and follicles that become progressively smaller are arranged caudally. This orientation allows for the examination of changes in hair growth and cycling based on

direction (anterior to posterior) and hair shaft diameter. It also allows primary follicles (the human analogue of terminal follicles) to be distinguished from secondary follicles (the human analogue of vellus follicles) using anatomic criteria rather than selecting an arbitrary diameter above which all hair shafts are considered to be primary hairs. In the dog, an anatomic primary hair is the follicle that is the most anterior in a compound follicle and produces the largest hair shaft.

In order to assess the degree of hair loss, we divided telogen hair follicles into those that retained hair shafts (haired telogen) and those that did not contain hair shafts (hairless telogen). We counted the haired telogen, hairless telogen and anagen follicles in five follicular units, each follicular unit containing three to four compound follicles. Five follicular units represented the number routinely found on a horizontally-trimmed 6mm punch biopsy sample. There were 29 to 36 hair follicles/follicular unit in the control dogs meaning each compound hair of the normal Beagle has approximately 10-12 hair follicles.

Obtaining sections to allow morphometric analysis requires sectioning horizontally-trimmed biopsy samples at the level of the follicular isthmus. This was confirmed by identifying completely traversed follicular units that contained sebaceous glands and/or arrector pili muscles. Sections were considered optimal when they were obtained from the upper isthmic region at the level of the sebaceous glands but below the site of tricholemmal cornification. Every effort was made to determine haired from hairless telogen by examining sections from optimal regions; however, occasionally, sections were evaluated slightly below this anatomic plane. This may have skewed the data slightly by counting a haired telogen follicle as a hairless telogen follicle, but should

not have influenced the number of follicles/follicular unit nor should it have influenced the number of follicles in telogen. In a preliminary study, we determined that for biopsy samples obtained from the dorsum, the base of secondary hairs in hairless telogen did not involute above the uppermost site of the insertion of the arrector pili muscle, even when the primary hair remained in anagen. That the level of sectioning at the isthmus did not influence hair follicle numbers is indicated by comparing the mean number of hair follicles/5 follicular units in the untreated-hypothyroid dogs with the other two groups. The untreated-hypothyroid dogs had on average the largest number of hairless telogen follicles yet similar follicles/follicular unit as the other two groups.

That each of the three groups had a similar number of follicles/follicular unit confirmed the opinion of many authors that hair loss in hypothyroidism is due to atrophy and not destruction of hair follicles (Gross et al 92, Heymann 92, Yager and Wilcock 94).

The majority of the hair follicles in the Beagle dog are in telogen irrespective of the group examined. Only one dog, a treated-hypothyroid animal, had greater than 50% of its follicles in anagen (68%). This observation suggests that, at least for Beagle dogs, the hair cycle is telogen-predominant. In other words, the majority of hair follicles are in telogen at any given time. Thus, telogenization cannot be used as a criterion for the diagnosis of hypothyroidism (Gross et al 92, Scott et al 95). Whether all canine breeds that do not require frequent hair clipping remains have telogen predominant hair cycles remains to be determined; however, clinical observations suggest that this is true. Only dogs that require frequent hair clipping become alopecic when treated with chemotherapeutic agents such as doxorubricin that target actively dividing cells. Because the epithelia of telogen follicles have minimal/no mitotic activity, they are spared the

effects of doxorubricin therapy.

Normal dogs with the majority of their hair follicles in telogen have no propensity to become alopecic because telogen hairs can be retained for long periods (months to years) without falling out. The tricholemmal cornification that surrounds the base of a telogen hair shaft tightly adheres the hair to the outer sheath of the hair follicle. Studies in humans have demonstrated that as much force is required to extract a telogen hair as a hair in anagen (Chapman 92). What appears to occur in the canine hair cycle is that the anagen follicle produces a hair shaft that grows a set length. The follicle then enters telogen and remains in a prolonged state of quiescence in which the hair shaft is firmly affixed to the outer sheath. Retaining telogen hairs is a means of maintaining the pelage and conserving protein with very little investment of energy. Anagen in a normal dog occurs only when the hair is plucked (a potent stimulator of anagen) or there is some stimulus to produce an anagen hair that eventually pushes out the old telogen hair by mechanical pressure.

This study confirms that the lack of circulating thyroid hormones has a profound effect on the hair follicle, but this effect is different than previously described. None of the untreated-hypothyroid dogs had a discernable alopecia and the most striking difference between these dogs and those in the other two groups was the inability of the untreated-hypothyroid Beagles to regrow their hair after clipping. That the untreated-hypothyroid dogs had a mean of 98.9% of their follicles in telogen explains why their hair did not regrow. Although no clinical alopecia was noted, the untreated-hypothyroid dogs had a mean of 56.3% of their hair follicles devoid of hair shafts. When compared to the Beagles used as controls (mean of 19.7% of follicles devoid of hair shafts) the

untreated-hypothyroid dogs had, on average, approximately one third fewer hair shafts than the control group. Why this difference could not be discerned on gross examination may be explained by Table 5B and Figure 17. Most of the hair loss in hypothyroidism occurred in anatomic secondary hairs with a diameter between 9-25 um--hairs that are difficult to visualize with the naked eye.

Differences were not identified in the number or stage of the hair cycle of primary hairs in the three groups. This is in part due to the variability in diameter of primary hairs and the relatively small number of primary follicles measured (each follicular unit had approximately 3 primary hair follicles). Untreated-hypothyroid dogs had a smaller number of secondary follicles in anagen and a larger number of secondary follicles in telogen and secondary follicles in hairless telogen; however, these differences could not be shown to be significant when compared to the control group.

What appears to distinguish untreated-hypothyroid dogs from the control group is that when telogen hairs from untreated-hypothyroid dogs are lost, they are not replaced. Although we did not observe areas of hair loss in any of our untreated-hypothyroid dogs we could imagine that areas of alopecia could develop over pressure point regions and body sites exposed to friction. In our experience, such alopecia would most likely take years rather than months to develop. We also speculate that hypothyroid dogs would not go through seasonal shedding, as retention of hair in a prolonged telogen state may serve as an energy-conserving measure.

Although there is no doubt that a decrease in circulating thyroid hormones affects the hair follicle, other features described as characteristic of hypothyroidism were difficult to confirm. None of the untreated-hypothyroid dogs had clinical or histologic

evidence of a pyoderma and seborrhea (defined here as hyperkeratosis in any pattern) was an inconsistent finding.

The data from the treated-hypothyroid dogs indicate thyroxine has a profound stimulatory effect on the canine hair follicle. Treated-hypothyroid dogs had a larger number of follicles in anagen and a smaller number of follicles in telogen than the control group. In other words, thyroid hormone replacement therapy appears capable of stimulating telogen follicles into the anagen stage, perhaps even in euthyroid dogs. Thus, the long-held belief by breeders that thyroid hormone supplementation improves the hair coat of a dog may have merit. The stimulatory effects of thyroxine on hair growth that we identified correspond to a study by Gunartanam (1986) in which it was determined that application of thyroxine topically stimulated hair growth in a population of cross-bred male dogs. That the number of hairless telogen follicles was the same on average for the control and treated hypothyroid groups suggests their may be a population of telogen hairs that remain in a permanent telogen state or at least are resistant to the effects of thyroid hormones.

A frustration of this study was an inability to consistently confirm statistical significance in the results within the three populations examined, even though there were often large mean differences between the groups. We believe this problem is largely due to the small sample size used in this investigation. Because the literature lacks a similar study, determining how many dogs would be required for statistical differentiation of the populations had to be estimated. Still, the trends in the data are quite compelling: untreated-hypothyroid dogs had a greater number of follicles in telogen and fewer hair shafts than the control population. The control population had a greater number of

telogen follicles but the same number of hair shafts as the treated-hypothyroid group. This study also indicates that future morphometric evaluation of canine hair follicles must take breed into consideration because of the potential of profound interbreed variability.

Chapter 4

THE EFFECTS OF HAIR PLUCKING ON FOLLICLE REGENERATION IN NORMAL, UNTREATED-HYPOTHYROID AND THYROXINE-TREATED HYPOTHYROID BEAGLE DOGS

Introduction

Hair follicles are characterized by cyclical growth that requires an interaction between epithelial cells and the mesenchymal cells of the follicular papilla. The cycle of the follicle is divided into three successive phases: anagen, catagen and telogen. Anagen represents the growing phase of the hair cycle and is defined by the formation of the matrix, inner sheath, hair shaft and cuticular layers. During catagen, there is a progressive loss of the inner sheath and a cessation of hair growth. In telogen, the inner sheath is lost completely and the follicle is in a state of quiescence.

Telogen represents a break in the hair cycle, the equivalent of the G_0 state of the cell cycle (Paus 98, Wilson et al 94). Because the transition from this period of epithelial hibernation into the metabolic awakening that results in the next anagen phase is one of the main targets of hair cycle control, understanding the mechanisms associated with this process is critical to defining how hair grows.

Examining this telogen-anagen transition has provided evidence for the bulge activation hypothesis. According to this theory, stem cells of the follicle are not located in the germinative epithelial cells immediately adjacent to the follicular papilla as was long believed but rather, are present in a region of the hair follicle known as the bulge: an evagination of the outer sheath into the dermis that corresponds to the insertion of the arrector pili muscle. In the bulge activation hypothesis, these cells give rise to a population of transiently activated cells that undergo a period of intense but temporally limited mitotic activity. The offspring of these transiently activated cells become terminally differentiated, producing the structures that define the anagen phase: a hair shaft, an inner sheath and the cuticular layers of the follicle. Evidence that stem cells are

located in the bulge is based on their similarity with keratinocyte and corneal stem cells: 1) the cells in the bulge region are morphologically undifferentiated; 2) labeling these undifferentiated cells requires repeated administration of a cell proliferation marker, an indication they are slowly cycling; 3) when labeling does take place, the label is retained for long periods of time indicating these cells are "label-retaining"; 4) in early anagen, these cells are the first to become labeled indicating they become activated in the transition from telogen to anagen; 5) bulge cells express cytokeratin 15—a marker for hair follicle stems cells (Lyle et al 98, Wilson et al 94, Cotsarelis et al 90). The major reason why follicular stem cells are located in such an anatomically out-of-the-way site is because they are so important to follicular regeneration that they need to reside in a site that is protected from injury.

The bulge activation hypothesis, although conceptually elegant, remains a major controversy in dermatology. An example of the debate surrounding this hypothesis lies in conclusions drawn from two hair plucking studies. Hair plucking is the standard mechanism of investigating the telogen-anagen transition and anagen hair repair. When plucking occurs in telogen and there is traumatic removal of the club hair (i.e., mild wounding of follicular epithelium) in rats, mice and humans, there is induction of anagen and the start of a new cycle (Gharzi et al 99, Paus 98, Wilson et al 94). When an anagen hair is plucked, a number of different breakage patterns occur; however, after a brief period of repair, hair re-growth occurs with the follicle remaining in the anagen phase (Bassukas and Hornstein 89). The first hair plucking study was performed on 45 day old SENCAR mice whose hair was in a non-growing (telogen) phase of the cell cycle (Wilson et al 94). The hair was plucked and the site injected with tritiated thymidine (^{3}H-

TdR) immediately after plucking and at intervals from one to ten days afterwards. Autoradiography and routine histopathology performed on skin obtained after labeling demonstrated that the bulge region cells were the first to express the ^{3}H-TdR label, indicating the normally slow-cycling bulge cells underwent a transient proliferation during early anagen. These results supported the bulge activation hypothesis. In the second study, mid-anagen vibrissa follicles were plucked successively from inbred PVG hooded rats and the plucked follicles were examined histologically and immunohistochemically immediately after plucking and at intervals from six hours to nine days afterwards (Gharzi et al 99). 5-bromo-2-deoxy-uridine (BrdU) was used as a cell proliferation marker whose presence was defined using anti-BrdU antibody. In contrast to the telogen pelage hairs of mice, the anagen vibrissae of rats did not demonstrate labeling of cells at the outer sheath adjacent to the insertion of the arrector pili muscle after they were plucked. Instead, BrdU staining was restricted to germinative epithelial cells of the hair bulb with almost no labeling observed in the upper part of the follicle. This pattern was repeated for each of the subsequent pluckings, indicating the germinative epithelial cells were the source of the new matrix each time. Because the bulge activation hypothesis considers germinative epithelial cells transiently amplified cells, they should have a limited mitotic potential. Finding that germinative cells could continuously renew the hair matrix and regrow a hair shaft without drawing from a new supply of cells from the bulge region contradicted a basic tenet of the bulge activation hypothesis.

In this study, we describe the effect of plucking hair shafts on age- and sex-matched control, untreated-hypothyroid and thyroxine-treated hypothyroid Beagle dogs.

The dog model was chosen for several reasons. First, dogs have larger follicles than either the mouse or rat, facilitating the visualization of morphologic changes associated with hair plucking and regeneration. Second, dogs do not have an anatomic bulge but do have a more prominent arrector pili muscle than humans, rats or mice (Dunstan and Linder 95). Thus, if there is an association of the insertion of the arrector pili muscle and the presence of stem cells, this should become apparent as telogen follicles enter into anagen post-plucking. Third, dogs have compound follicles, meaning they have multiple follicles that share a common infundibulum. The follicles that make up a compound follicle have a distinctive orientation with a progression from the largest follicles present cranially to the smallest follicles caudally. The dog model is useful, therefore, to define if size and location of the follicle can be associated with follicular renewal and whether the regeneration that occurs with hair plucking is limited to the injured follicle or induces stimulation of adjacent follicles as well. We chose the Beagle dog for this study because it is the breed most widely used for investigations and because Beagles dogs have a telogen-predominant hair cycle having, on average, greater than 70% of their hair follicles in telogen, and of these, approximately 20% are in telogen without hair shafts. At the same time, they also have anagen hairs. Thus in Beagles, the effects of plucking can be examined on both anagen and telogen hairs concurrently. In a previous study, we demonstrated that untreated-hypothyroid dogs had a diminished hair growth compared to controls, whereas hair growth was stimulated for treated-hypothyroid dogs. We included these three groups of dogs in this investigation because we were unable to identify a report in the recent literature in which the effect of hair plucking on the hair cycle was defined for follicles with inhibited or stimulated hair growth.

Materials and Methods

All procedures dealing with animal care, handling and treatment in this study were reviewed, approved and subjected to continuous monitoring by the All-University Committee for Animal Use and Care, Michigan State University.

Dogs: Nine male intact Beagles, 11-17 months of age, weighing 10.9-15kg were used for this study. All were housed in separate kennels with 12-hour light-dark cycles. The dogs were randomly assigned to 2 groups. Group 1 consisted of three dogs that were used as untreated, healthy controls throughout the study. Group 2 consisted of 6 dogs that were made hypothyroid by the oral administration of 15mCi of ^{131}I (Johnson et al 99). A panel of thyroid hormone assays (serum total thyroxine [TT4], free thyroxine [FT4], total triiodothyronine [TT3], free triiodothyronine [FT3], and endogenous thyroid stimulating hormone [TSH] confirmed the effectiveness of ablation by isotopes. After demonstrating the six ^{131}I-treated dogs were hypothyroid by comparison with reference standards, they were randomly divided into treated and untreated groups. Three of the hypothyroid dogs were not treated and three were treated with oral thyroxine supplementation (Soloxine, Daniels Pharmaceuticals) at 0.1mg per 4.5kg once daily. In order to study the long-term effects of both deficiency and treatment, these dogs were followed for 10 months prior to sampling. Every two months and a week before the study began, the panel of thyroid hormone assays was repeated to confirm the control dogs had normal circulating thyroid levels, the untreated dogs were hypothyroid and the treated-hypothyroid dogs were maintained in an euthyroid state.

Sampling: Prior to plucking hair, all dogs were anesthetized and two "pre-plucking" biopsy samples were obtained from sites on either side of the dorsal midline. Along

parallel lines from the first biopsies, separated by six cm intervals, the hair within 14 (7 pairs) 1 cm circles was plucked using tweezers. These paired sites were then biopsied using lidocaine with epinephrine as an anesthetic at day zero (approx. 1 hr. post-plucking) and 24 hours, 72 hours, 8 days, 16 days, 32 days, and 64 days post-plucking. Six mm punch biopsies were used throughout this study. For each of the dogs, the plucked hair shafts were placed in plastic bags to be examined later. Hair shafts were also plucked from the forearm of one of the investigators (RWD) and stored. The plucked sites were examined for hair re-growth and photographed at each time point before biopsy samples were taken.

Processing of samples: The first biopsy of the paired samples was trimmed vertically and the second, horizontally at the level of the mid-dermis parallel with the epidermis. Each of the sectioned samples was injected with and then immersed in RPMI-1640 culture media containing 100mM BrdU. Immersed samples were then incubated at 37°C and 5% CO_2 for two hours. After incubation, tissues were fixed in 10% zinc formalin for 4 days and then transferred to 70% ethyl alcohol prior to routine processing and paraffin embedding and sectioning at six microns. Standard morphologic evaluation and morphometric assessment of the hair follicles was performed on sections stained with hematoxylin and eosin (H&E). Horizontally trimmed samples were generally step-sectioned because quantitative analysis could only be performed when the sections were made at the level of the follicular isthmus. Standard morphologic evaluation was performed on all dogs at each sampling period. Morphometric evaluation was performed on Day 0, Day 32 and Day 64 of the study. BrdU staining to define cells in the S phase of the cell cycle employed a variation of the methods described by Tezuka et al (1991).

Mouse monoclonal anti-BrdU (Becton-Dickinson) was used as the primary antibody, with equine biotinylated anti-mouse (Vector Laboratories) as the second antibody, followed by incubation with avidin/biotin alkaline phosphatase (KPL Laboratories). Vector Substrate Kit 1 (Fast Red) was used as the chromagen and slides were lightly counterstained with hematoxylin. BrdU staining was performed on all samples obtained from all dogs at each sampling period. Finally, for each of the dogs, plucked hair was mounted on glass slides to evaluate the pattern of hair shaft breakage.

Examination of samples: Morphometric analysis was performed on horizontally-trimmed samples using a "follicular unit" as the basis for quantifying results. We defined a follicular unit as the anatomic aggregation of compound hair follicles that occurs in dogs. Most follicular units consist of three compound follicles adjacent to each other with the middle compound follicle containing the largest (primary) hair shaft (Figure 7). For each dog, five follicular units were identified, the number of hair follicles they contained were counted and the stage of the hair cycle (anagen, haired telogen, or hairless telogen) for each of the follicles was determined. Determining the stage of the hair cycle was based on an adaptation of the staging methods of Headington and Nixon (Headington 84, Nixon 93). Briefly, follicles were considered in anagen when the hair shaft was surrounded by an inner sheath and in haired telogen when the hair shaft was surrounded by tricholemmal cornification. When the follicular lumens contained only a rosette of tricholemmal cornification or if the follicles had no lumenal cornification, they were counted as "hairless telogen". Because catagen follicles are difficult to identify on transverse section and because they comprise such a small component of the total number of hair follicles, they were not counted (Nixon 93).

Using the data obtained from the staging of the hair follicles, the following were evaluated in five follicular units for each dog at Day 0, Day 32 and Day 64: 1) the number of hair follicles, 2) the total number of anagen follicles, 3) the number of primary follicles in anagen, 4) the number of secondary follicles in anagen, 5) the total number of follicles in telogen with hair shafts ("haired telogen"), 6) the number of primary follicles in telogen with hair shafts, 7) the number of secondary follicles in telogen with hair shafts, 8) the total number of hairless telogen follicles, 9) the number of primary follicles in hairless telogen, and 10) the number of secondary follicles in hairless telogen.

The data were summarized in Tables 6-8. In each table, the number of hair follicles counted for each parameter in five follicular units for each dog were given. A percentage was calculated by dividing the count by the total number of hair follicles found in five follicular units for each dog. Each treatment group was summarized by calculating the median value of each parameter examined. Medians rather than means were used since a normal distribution was not found for our data.

A Kruskal Wallis one way nonparametric analysis of variance was used to detect differences between the three groups. If a $P<0.05$ was obtained, then post-hoc comparisons using the Bon Ferroni multiple comparisons test were performed to determine if a significant pairwise difference among the groups could be found. These parameters were analyzed using Statistix for Windows.

Both vertically- and horizontally trimmed H&E stained samples were examined for changes in the epidermis, adnexa, and dermis and for evidence of inflammatory skin disease.

Evaluation of BrdU-stained sections was also performed on vertically- and

horizontally- trimmed sections. Attention was paid to the site at which labeled cells were first identified and any association with the outer sheath adjacent to the insertion of the arrector pili muscle.

Evaluation of plucked hairs concentrated on morphologic differences in the three groups of dogs in the study and differences, if any, between plucked hairs in dogs and humans. Images in this dissertation are presented in color.

Results

Clinical features: The re-growth of hair at the plucked sites was assessed at 24 and 72 hours after plucking, and on Days 8, 16, 32 and 64 of the study. Hair was first clinically apparent between Days 16 and 32 for the dogs in the control and treated-hypothyroid groups. Hair was first noted in the untreated-hypothyroid dogs between Days 32 and 64. At the end of the study, the quantity and length of hair at the plucked sites approximated that of adjacent, non-plucked areas in the control and treated-hypothyroid dogs. For the untreated hypothyroid dogs, the hairs at the plucked sites were less abundant and shorter than in adjacent non-plucked sites. (Figure 18).

Morphometric evaluation: The results of the morphometric analyses are presented in Tables 6-8. The total number of hair follicles per five follicular units in the control, untreated-hypothyroid and treated-hypothyroid dogs was relatively similar throughout the study with medians ranging from 143 to 179 follicles per five follicular units. Significant differences were noted overall in the total number of follicles in anagen and the number of secondary anagen follicles for each of the periods of measurement (Days 0, 32 and 64) with significant differences noted between the untreated- and treated-hypothyroid dogs at

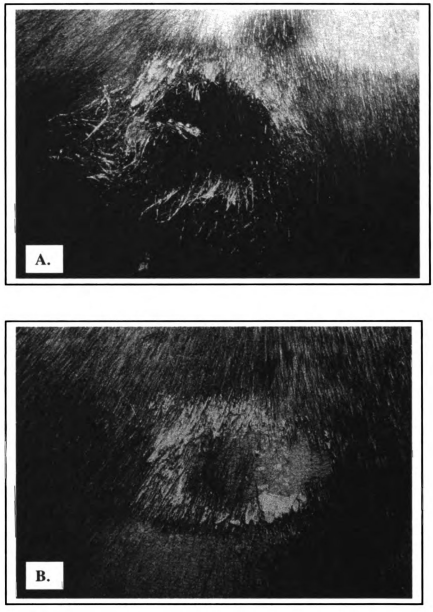

Figure 18. Clinical photographs of A) a control Beagle and B) an untreated-hypothyroid Beagle taken 64 days after the sites were plucked.

Hair re-growth is sparse and many hairs are shorter in the untreated-hypothyroid dog compared to the control.

Tables 6-8. Morphometric evaluation of normal, untreated-hypothyroid and treated-hypothyroid Beagles at Days 0, 32 and 64.

Each column contains the total number of hair follicles counted in five follicular units for each parameter examined for each dog in each treatment group. The percentages in columns 2-6 are calculated by dividing each count by the total number of follicles found in the 5 follicular units for that dog (data shown in column 1). Each treatment group is summarized by determining the median value for each parameter with its corresponding percent of follicles per five follicular units, if applicable.
*This analysis had an overall significant p value, but there was insufficient power to determine which groups were different from the others.
**This analysis had an overall significant p value with the untreated-hypothyroid group significantly different from the treated-hypothyroid group.

Table 6. Morphometric evaluation of normal, untreated-hypothyroid and treated-hypothyroid Beagles at Day 0.

	Number of Hair Follicles	Number of Follicles in Anagen	Number of Primary Follicles in Anagen	Number of Secondary Follicles in Anagen	Number of Follicles in Telogen with Hair Shafts	Number of Follicles in Hairless Telogen
Controls						
Dog 1	153	9 (5.9%)	0 (0%)	9 (5.9%)	116 (75.8%)	28 (18.3%)
Dog 2	145	35 (24.1%)	4 (2.8%)	31 (21.4%)	65 (44.8%)	45 (31%)
Dog 3	184	56 (30.4%)	8 (4.3%)	48 (26%)	106 (57.6%)	22 (12%)
Median	153	35 (24.1%)	4 (2.8%)	31 (21.4%)	106 (57.6%)	28 (18.3%)
Untreated Hypothyroid						
Dog 1	155	4 (2.6%)	1 (0.6%)	3 (1.9%)	90 (58.1%)	61 (39.4%)
Dog 2	165	4 (2.4%)	0 (0%)	4 (2.4%)	54 (32.7%)	107 (64.8%)
Dog 3	209	0 (0%)	0 (0%)	0 (0%)	81 (38.8%)	128 (61.2%)
Median	165	4 (2.6%)	0.0 (0%)	3 (1.9%)	81 (38.8%)	107 (64.8%)
Treated Hypothyroid						
Dog 1	181	123 (68%)	12 (6.6%)	111 (61.3%)	6 (3.3%)	52 (28.7%)
Dog 2	127	50 (39.4%)	2 (1.6%)	48 (37.8%)	46 (36.2%)	31 (24.4%)
Dog 3	157	46 (29.3%)	1 (0.6%)	45 (28.7%)	100 (63.7%)	11 (7%)
Median	157	50 (39.4%)	2 (1.6%)	48 (37.8%)	46 (36.2%)	31 (24.4%)
p value	p=0.65	p=0.02*	p=0.23	p=0.01*	p=0.28	p=0.03*

Table 7. Morphometric evaluation of normal, untreated-hypothyroid and treated-hypothyroid Beagles at Day 32.

	Number of Hair Follicles	Number of Follicles in Anagen	Number of Primary Follicles in Anagen	Number of Secondary Follicles in Anagen	Number of Follicles in Telogen with Hair Shafts	Number of Follicles in Hairless Telogen
Controls						
Dog 1	132	50 (37.9%)	8 (6.1%)	42 (31.8%)	6 (4.5%)	76 (57.6%)
Dog 2	147	27 (18.4%)	8 (5.4%)	19 (12.9%)	14 (9.5%)	106 (72.1%)
Dog 3	200	62 (31%)	11 (5.5%)	51 (25.5%)	29 (14.5%)	109 (54.5%0
Median	147	50 (37.9%)	8 (5.4%)	42 (31.8%)	14 (9.5%)	106 (72.1%)
Untreated Hypothyroid						
Dog 1	207	8 (3.9%)	2 (1.0%)	6 (2.9%)	20 (9.7%)	179 (86.5%)
Dog 2	138	5 (3.6%)	0 (0%)	5 (3.6%)	6 (4.3%)	127 (92%)
Dog 3	178	19 (10.7%)	12 (6.7%)	7 (3.9%)	1 (0.6%)	158 (88.8%)
Median	178	8 (3.9%)	2 (1.0%)	6 (2.9%)	6 (4.3%)	158 (88.8%)
Treated Hypothyroid						
Dog 1	267	86 (32.2%)	12 (10.5%)	35 (30.7%)	125 (46.8%)	56 (21%)
Dog 2	114	47 (41.2%)	5 (1.9%)	81 (30.3%)	19 (16.7%)	48 (42.1%)
Dog 3	179	72 (40.2%)	11 (6.1%)	61 (34.1%)	30 (16.8%)	77 (43%)
Median	179	72 (40.2%)	11 (6.1%)	61 (34.1%)	30 (16.8%)	56 (21%)
p value	p=0.9	p=0.02*	p=0.68	p=0.02*	p=0.16	p=0.07**

Table 8. Morphometric evaluation of normal, untreated-hypothyroid and treated-hypothyroid Beagles at Day 64.

	Number of Hair Follicles	Number of Follicles in Anagen	Number of Primary Follicles in Anagen	Number of Secondary Follicles in Anagen	Number of Follicles in Telogen with Hair Shafts	Number of Follicles in Hairless Telogen
Controls						
Dog 1	122	64 (52.5%)	13 (10.7%)	51 (41.8%)	6 (4.9%)	52 (42.6%)
Dog 2	152	60 (39.5%)	7 (4.6%)	53 (34.9%)	36 (23.7%)	56 (36.8%)
Dog 3	172	27 (15.7%)	2 (1.2%)	25 (14.5%)	102 (59.3%)	43 (25%)
Median	152	60 (39.5%)	7 (4.6%)	51 (41.8%)	36 (23.7%)	52 (36.8%)
Untreated Hypothyroid						
Dog 1	201	2 (1.0%)	0 (0%)	2 (1.0%)	10 (5.0%)	189 (94.0%)
Dog 2	143	17 (11.9%)	3 (2.1%)	14 (9.8%)	7 (4.9%)	119 (83.2%)
Dog 3	138	12 (8.7%)	8 (5.8%)	4 (2.9%)	3 (2.2%)	123 (89.1%)
Median	143	12 (8.7%)	3 (2.1%)	4 (2.9%)	7 (4.9%)	123 (89.1%)
Treated Hypothyroid						
Dog 1	219	110 (50.2%)	10 (4.6%)	55 (25.1%)	54 (24.7%)	55 (25.1%)
Dog 2	158	65 (41.1%)	9 (5.7%)	101 (63.9%)	39 (24.7%)	54 (34.2%)
Dog 3	166	116 (69.9%)	15 (9%)	101 (60.8%)	23 (13.9%)	27 (16.3%)
Median	166	110 (50.2%)	10 (4.6%)	101 (60.8%)	39 (24.7%)	54 (34.2%)
p value	p=0.49	p=0.001**	p=0.14	p=0.001**	p=0.18	p=0.03*

Day 64 for the total number of follicles in anagen and the number of secondary follicles in anagen. Although statistical differences were not noted over time, the overall trend in each of the three groups was an increase in the number of anagen follicles. However, throughout the study the median number of anagen follicles in the untreated-hypothyroid group was much lower than the other two groups and the median number of anagen follicles increased the most for the treated-hypothyroid dogs. No significant differences between the three groups were found for the primary anagen follicle counts. The median number of primary anagen follicles for all three groups increased at Day 32 and remained at the same level at Day 64. For secondary anagen follicles, there was a significant difference between the treatment groups at Day 0 that continued to the end of the study. At Day 64, a significant difference was found between the untreated and treated-hypothyroid groups. There was a steady rise in the median number of secondary anagen follicles for the control and treated-hypothyroid dogs, while the median number for the untreated-hypothyroid group increased slightly at Day 32 and then decreased at Day 64, approximating Day 0 levels.

The follicles in telogen were divided into telogen follicles with hair shafts and telogen follicles without hair shafts. Significant differences were not identified in the number of haired telogen follicles between each group or over time. Still, the trends are worth noting. The median number of haired telogen follicles dropped precipitously from Day 0 to Day 32 in the control dogs with a moderate increase at Day 64. Although not as marked, a similar pattern was noted for the treated-hypothyroid dogs. For the untreated-hypothyroid dogs, the median values decreased at Day 32 with no change at Day 64. For both periods, the median values were much lower than those in the other two groups.

Data were calculated for primary and secondary telogen follicles but are not shown because they mimicked precisely the data for the total haired and hairless telogen follicles.

Significant differences were noted in the number of hairless telogen follicles at Days 0, 32 and 64 with significant difference between the untreated-hypothyroid and treated-hypothyroid dogs at Day 32. Significant differences were not noted over time. The trend in the median values for the control dogs was an increase in hairless telogen follicles at Day 32 with a decrease at Day 64. A similar but less exaggerated distribution was noted for the untreated-hypothyroid dogs. For the treated-hypothyroid dogs there was a slight increase at Day 32 and this decreased moderately by Day 64.

Histologic evaluation: Although telogen follicles predominated, anagen follicles were present in the pre-plucking biopsies (Day 0) in all three groups. These were most abundant in the treated-hypothyroid dogs and least numerous in the untreated-hypothyroid dogs (Table 6). When these follicles were plucked, three patterns of damage to anagen follicles were noted (Figure 19). In the first pattern, the hair shaft was broken above the hair bulb, sparing the matrix epithelial cells. In the second, epilation resulted in breakage in the upper half of the hair bulb, disrupting the matrix keratinocytes in this region but leaving most of these cells in place (Figure 20A). Lastly, and seen least often, there was breakage at the base of the follicular papilla resulting in most of the hair bulb being extruded with plucking. In this pattern, the follicular papilla was occasionally prominently invaginated into the follicular lumen and small collections of attached matrix cells around the base of the follicular papilla remained after plucking. Typically, the inner sheath was disrupted with extraction of the shaft and occasionally, curled remnants

Figure 19. Morphologic changes in plucked anagen follicles.

Plucking the hair shaft of an anagen follicle (A) results in 3 patterns of damage. The shaft can break above the bulb (B). The break can occur within the bulb (C). The shaft and much of the bulb keratinocytes can be extracted, leaving small clusters of epithelium around the follicular papilla (D). Patterns B and C resume anagen (E). The most damaged follicles (D) also regenerate in anagen or theoretically undergo involution (F).

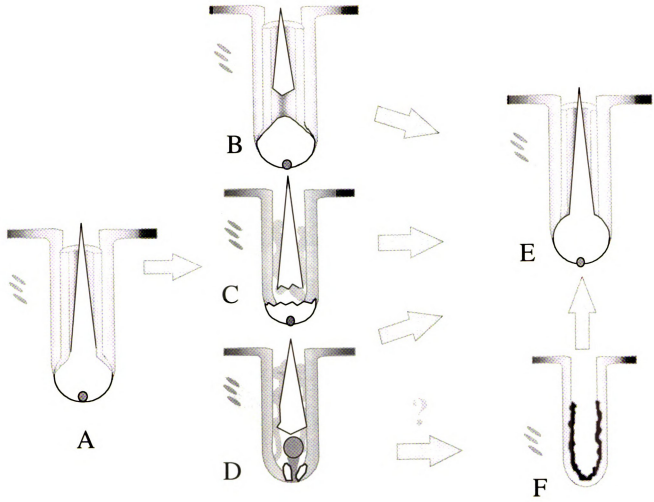

Figure 19. Morphologic changes in plucked anagen follicles.

Figure 20. Histology and BrdU immunohistochemistry of plucked anagen (A and B) and telogen follicles (C and D).

Photomicrographs A and B illustrate an anagen follicle with the second pattern of injury in which there is breakage in the upper half of the hair bulb, disrupting matrix keratinocytes but leaving the lower half of the hair bulb in place. BrdU labeling at 24 hours after plucking shows that many of the matrix cells surrounding the follicular papilla are proliferating.

Photomicrographs C and D illustrate a telogen follicle with the second pattern of injury in which the tricholemmal cornification surrounding the hair shaft was removed, resulting in multifocal necrosis of the outer sheath epithelium and accumulation of fibrin in the follicular lumen. BrdU labeling at 72 hours after plucking shows that intact epithelial cells are proliferating to repair the outer sheath and are actively dividing just above the follicular papilla.

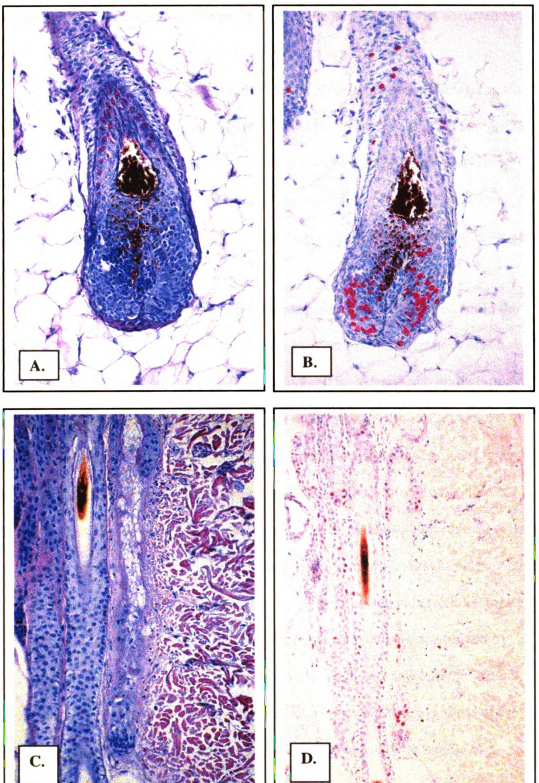

Figure 20. Histology and BrdU immunohistochemistry of plucked anagen (A and B) and telogen follicles (C and D).

of it were found within the follicle lumen. Unlike the injury patterns that occurred in telogen follicles, in anagen, damage to the outer sheath was not noted.

Anagen follicles with the first two patterns of plucking-induced damage appeared to repair the hair bulb and resume hair shaft re-growth immediately after plucking by proliferation of keratinocytes within the matrix, without first entering an intervening telogen phase (Figure 20A). Although some of the more severely disrupted follicles did undergo regeneration without entering into telogen, we could not determine this for all such follicles.

Plucking of telogen follicles produced three patterns of damage that could be identified one hour post-plucking (Figure 21). In the first pattern, extraction of the shaft resulted in minimal damage to the supporting follicle. The outer sheath epithelium and lining tricholemmal cornification remained intact with some collapse of the empty follicle lumen. In the second pattern, the tricholemmal cornification surrounding the hair shaft was removed with the hair shaft resulting in focal necrosis of the outer sheath epithelium and accumulation of fibrin and necrotic debris in the follicular lumen (Figure 20C). Small collections of intact outer sheath epithelium often remained lining the base of the follicle or along the dermal sheath. The third pattern of plucking-related injury of telogen follicles was the most severe but least common. This was characterized by extensive necrosis of the outer sheath cells from the follicle base through the isthmus with no outer sheath epithelial cells attached to the dermal sheath. Regardless of the pattern, damage to the dermal sheath was not noted and the follicular papilla was markedly enlarged compared to its typical appearance in telogen.

Beginning at 24 hours and continuing through 72 hours and eight days, plucked

Figure 21. Morphologic changes in plucked telogen follicles.

Plucking the hair shaft of a telogen follicle (A) results in 3 patterns of damage. The shaft can be removed without damage to the tricholemmal cornification or outer sheath (B). The tricholemmal cornification and regions of the outer sheath may be removed or injured (C). All of the tricholemmal cornification and outer sheath can be lost (D). Plucking of telogen follicles results in the induction of anagen (E).

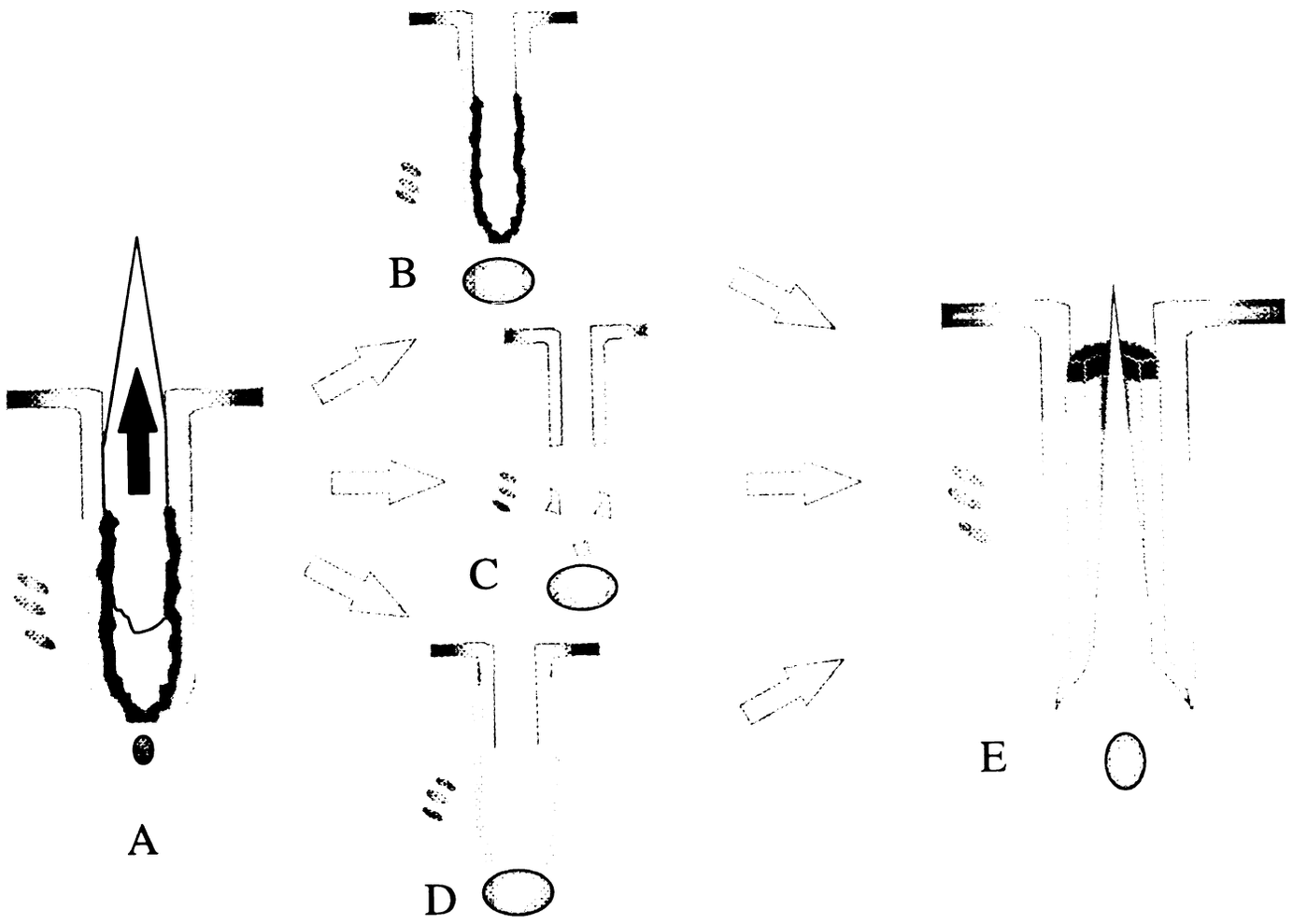

Figure 21. Morphologic changes in plucked telogen follicles.

telogen follicles underwent a period of regeneration as defined by replacement of necrotic outer sheath epithelium and thickening of the outer sheath from the base of the follicle to the infundibulum. The regeneration for the first pattern of plucked telogen follicles mimicked the normal transition from telogen to anagen with swelling of the follicular papilla and increased basophilia of the adjacent follicular epithelium as a new hair bulb formed. For the second pattern, there was replacement of the outer sheath by the remaining collections of outer sheath epithelial cells as defined first by increased basophilia of these cells and morphologic evidence of mitotic activity (Figure 20C). If cells in the location of germinative epithelium remained, they would initiate outer sheath and hair bulb regeneration. For the pattern in which there was necrosis of the entire lower isthmic region, replacement was by migration of intact, upper isthmic outer sheath cells down the dermal sheath. By 72 hours post-plucking, the outer sheath of the isthmus, infundibulum and to some extent, the epidermis, were thicker than samples obtained at Day 0 in all groups of dogs. At approximately 8 days week post-plucking, early anagen bulbs began to form at the base of plucked telogen follicles.

Features of regeneration could be identified in all three groups of dogs; however the untreated-hypothyroid Beagles had fewer hairs entering anagen and the process of hair shaft re-growth was slower than for the control and treated-hypothyroid dogs. These differences became more apparent as the study progressed. At 16 and 32 days after plucking, the control and treated-hypothyroid dogs had the maximum number of new anagen follicles, with robust, large diameter bulbs producing new shafts. At 64 days after plucking, more anagen follicles were found than in the pre-plucking samples, but for most follicles the hair bulb was not as large and more ovoid than the majority of hair

bulbs identified at Day 32, resembling the anagen follicles present in the pre-plucking biopsies. In contrast, the samples from the untreated-hypothyroid Beagles at 16 and 32 days after plucking still contained many empty telogen follicles. Anagen follicles were increased in number from pre-plucking samples, but were still in early anagen with little hair shaft production. At 64 days after plucking in this group, anagen follicles were producing shafts, however empty telogen follicles occasionally remained. Other than having more plucked telogen hairs failing to enter the anagen phase and a slower rate of growth for those which did, the morphology of the untreated-hypothyroid follicles was the same as the other two groups.

Evaluation of BrdU labeled sections: The labeling of anagen follicles in the pre-plucked biopsy samples was limited to the follicular matrix epithelium that surrounded the base of the follicular papilla and to isolated cells within the outer sheath from the isthmus to the infundibulum. No change in this pattern could be detected at one hour after plucking. In those anagen follicles in which epilation produced damage from the mid-bulb and above, BrdU cell labeling within the matrix epithelium continued and was mildly increased (2-3 cells per follicle) in the outer sheath from 24 hours post-plucking until the shaft was reformed for most follicles, by Day 32 (Figure 20B). Labeling by BrdU also demonstrated that intact matrix cells left after most of the hair bulb was extruded, retained their ability to proliferate and gradually reformed the bulb around the follicular papilla by 16-32 days after plucking, while cell labeling also occurred in low numbers of individual scattered outer sheath cells; however, in some severely injured plucked anagen follicles no such labeling occurred, suggesting rare anagen follicles can be damaged to the degree that they may enter telogen before producing a new shaft.

BrdU labeling of unplucked-telogen follicles demonstrated only rare uptake of the label (1-2 cells in occasional follicles) within the outer sheath epithelium, follicular papilla and infundibular epithelium. At one hour after plucking, no increase in labeling of plucked telogen follicles could be observed. 24 hours after plucking, there was a mild increase in labeled cells in the outer sheath (1-3 cells per follicle) up to the level of the infundibulum. A similar mild increase in labeled cells also occurred in the infundibulum and in the epidermis. At 72 hours after plucking, the number of labeled cells in the outer sheath up to the level of the infundibulum was increased 3 to 10 fold over the 24 hour samples (Figure 20D). Again, there was a similar but milder increase in labeling in the infundibulum and epidermis, which corresponded to the slight thickening of these layers noted on standard microscopy (Figure 22). At 8 days after plucking, a low number of BrdU labeled cells (2-6) were individually scattered at all levels within the outer sheath, infundibulum and interfollicular epidermis. As new anagen hair bulbs began to form, the predominant location of the labeled cells was in the germinative epithelium immediately above the follicular papilla where labeled cells were present in small clusters. At no time during this regenerative process was there a focus of BrdU labeling in the outer sheath associated with or in the region of the insertion of the arrector pili muscle.

Evaluation of plucked hair shafts: No morphologic differences in plucked hairs were found between the three groups of dogs. The plucked telogen hairs were identical to club hairs in humans. Morphologic differences noted between humans and dogs in the plucked anagen hair follicles were subtle but definitive. In approximately 60% of the plucked anagen hairs in humans, the inner sheath and occasionally, portions of the outer sheath were attached to the plucked hair shaft. In contrast, none of the plucked anagen

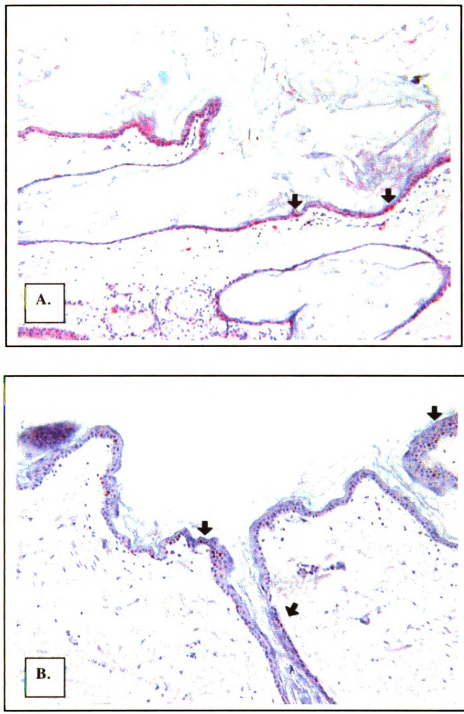

Figure 22. BrdU immunohistochemical labeling of the epidermis and infundibulum of A) a control dog before plucking and B) a control dog 72 hours after plucking (10X hematoxylin).

Note the thickness of the epidermis as well as the increased number of labeled cells in the slide from the dog 72 hours after plucking.

hairs from dogs had attached debris from the inner and outer sheaths (Figure 23).

Discussion

In this study, we describe the effects of hair plucking on follicular regeneration in normal, untreated-hypothyroid and treated-hypothyroid dogs. At the same time, we wanted to validate the conclusions drawn from a recent paper in which we examined the effects of thyroid hormone on the skin of Beagle dogs (Credille et al, manuscript in progress).

The morphometric assessment of the effects of plucking hair from follicles in the three groups of dogs analyzed in this study corresponded to results obtained in other mammalian species: plucking hair stimulates follicles to enter or remain in the anagen phase of the hair cycle (Oliver 66, Ibrahim and Wright 78, Bassukas and Hornstein 89, Wilson et al 94;Gharzi et al 99). This being noted, there were still differences between the three groups analyzed. The median number of anagen follicles in the untreated-hypothyroid dogs was much lower and the median number of anagen follicles was much greater in the treated-hypothyroid dogs than the controls at Day 64. The increase in anagen follicles in the untreated-hypothyroid dogs over the course of the study differed by only a few follicles from the Day 0 value and this increase was due to a transition of primary follicles from the telogen to the anagen phase, as the median number of secondary anagen follicles in this group actually decreased over the course of the study. These data suggest that in the dog, the post-plucking anagen phase is subject to regulatory mechanisms and that trauma-induced follicular renewal can be inhibited by decreased circulating thyroid hormones while the administration of thyroid hormones can

Figure 23. Morphologic differences between plucked human (A) and canine (B) anagen hair shafts.

In approximately 60% of the plucked anagen hair shafts in humans, the outer sheath remained attached to the hair shaft. In contrast, none of the plucked anagen hairs from dogs had attached debris from the outer sheath.

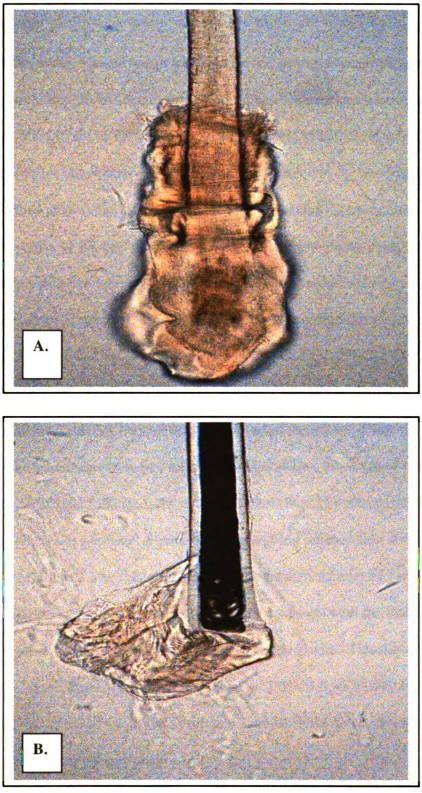

Figure 23. Morphologic differences between plucked human (A) and canine (B) anagen hair shafts.

stimulate this process.

In a previous study, we defined that the beagle has a telogen-predominant hair cycle. For the normal Beagle this means that ~66% of hair follicles are in telogen. In addition, telogen follicles can be broken down into two types: 1) haired telogen follicles in which the hair shaft is annealed by tricholemmal cornification to the outer root sheath, often for long periods of time and 2) hairless telogen follicles that do not contain hair shafts. These two types of telogen need to be defined for morphometric analysis because only hairless telogen follicles serve as an indicator of the presence of hair loss. Using this method to separate the two types of telogen follicles, the median number of haired telogen follicles decreased at Day 32 and increased at Day 64 especially for the control and treated-hypothyroid dogs. In none of the three groups was the median number of haired telogen follicles greater at Day 64 than at the start of the study. These data suggest that the decrease in the number of haired telogen follicles at Day 32 occurred either because the plucked telogen follicles (now devoid of a hair shafts) were counted as hairless telogen follicles or because follicles were undergoing regeneration and were counted as anagen follicles. The minimal change in the median number of haired telogen follicles in the untreated-hypothyroid dogs at Day 64 corresponds with the inability of these follicles to generate substantive numbers of new anagen hairs. The slightly larger rebound effect for the treated-hypothyroid dogs than the control dogs at Day 64 indicates that follicles from the treated-hypothyroid dogs are more likely to enter anagen and hypothetically, subsequent telogen phases.

Corresponding results were obtained for the median numbers of hairless telogen follicles that increased at Day 32 and then decreased at Day 64. The increase in hairless

telogen follicles for all dogs at Day 32 can be correlated with the decrease in haired telogen follicles as plucked follicles not undergoing regeneration would be counted as hairless telogen follicles. In none of the three groups did the number of hairless telogen follicles decrease below the Day 0 baseline values. This suggests the transition from hairless telogen into anagen did not occur during the time frame of this study. These data also suggest that at least for the canine compound follicle, plucking does not result in stimulation of the compound follicle as a unit. Rather, each plucked follicle enters into anagen individually. This observation is further supported by histologic evaluation of horizontally-trimmed sections where the onset of post-plucking anagen occurred in a piecemeal pattern in the follicles that comprise the compound follicle.

On histologic evaluation of vertically-trimmed sections, three morphologic patterns were associated with plucked anagen hairs:1) the hair shaft was broken above the hair bulb, sparing the matrix epithelial cells; 2) epilation resulted in breakage in the upper half of the bulb hair bulb, disrupting the matrix keratinocytes in this region but leaving most of these cells in place; 3) breakage at the base of the follicular papilla resulting in most of the hair bulb being extruded with plucking. These patterns correspond to three of the four break patterns identified in a study that analyzed the effects of plucking on the anatomy of the human hair bulb (Bassukas and Hornstein 89). The only pattern identified in humans not found in the dog is a pattern of breakage in which only the dermal sheath remains and the entire hair bulb is extruded. Another morphologic difference between plucking canine and human anagen follicles is the incidence of the different break points. In dogs, >60% of hair breakage in anagen hairs occurs above the follicular papilla. In humans ~50% of hair breakage in anagen hairs occurs at the base of

the follicular papilla, the least common breakage site in dogs (~10% of plucked follicles). Whether the differences in the pattern and frequency of location of the break sites in the two species is anatomic, based on different substages of the anagen cycle in the two species (Paus 99) or due to differing plucking techniques could not be determined. Hair regrowth in canine plucked anagen follicles with morphologic patterns 1 or 2 would invariably regenerate without entering into telogen. A similar regrowth without a telogen interlude was also noted in plucked anagen follicles with morphologic pattern 3; however, we could imagine variants of this pattern with such severe injury to the hair bulb and germinative epithelium that regrowth could require complete replacement of the hair bulb and consequently a telogen-like phase. We also considered the possibility of an injury so severe that a follicle might be permanently lost but did not give this much credence. In none of the plucked anagen follicles examined was there evidence of damage to the dermal sheath and because a follicle can regenerate de novo from just a dermal sheath, we thought it would be very unlikely that trauma due to plucking an anagen hair could result in a permanent destruction of a hair follicle (Reynolds et al 99).

Three morphologic patterns were also associated with plucked telogen hairs: 1) extraction of the shaft with retention of tricholemmal cornification in the follicular lumen; 2) removal of tricholemmal cornification and part of the outer sheath cells with extraction of the hair shaft; 3) removal of the entire outer sheath of the follicular isthmus with extraction of the shaft. That plucking a haired telogen follicle can be so destructive should not be surprising. There is an accumulating body of evidence to indicate that tricholemmal cornification can tightly anneal the hair shaft to the outer sheath and plucking such a firmly affixed shaft should be extremely damaging to the outer sheath.

In humans, chest hairs in telogen have the same anchoring strength as those in anagen indicating that telogen hairs are not easy to remove (Chapman 92). In quilled mammals such as hedgehogs, the quills are retained in telogen for long periods of time, perhaps for life and are extremely difficult to remove (Winn-Elliott et al 96). The different morphologic patterns associated with plucking telogen hairs most likely correspond to the stage of the hair cycle the follicle was in when the shaft was removed. Telogen hairs that are in the "G_0" stage of the hair cycle should be tightly annealed to the hair follicle and removal would be associated with destruction of the outer sheath. Telogen hairs in a follicle that is soon to enter anagen and shed its old shaft would be easy to remove, leaving most of the tricholemmal cornification in the follicle.

The rapidity of regeneration of a plucked haired follicle depended on the severity of the injury to the follicle and whether the dog was in the control, treated-hypothyroid or untreated-hypothyroid groups. The untreated-hypothyroid dogs lagged behind the other two groups not only in the number of plucked follicles that were undergoing transition into anagen but also the time required to regrow a new hair shaft. For some of the untreated-hypothyroid dogs, newly forming anagen hairs were identified Day 32 post plucking. Based on morphometric analyses, the treated-hypothyroid dogs should have had a more rapid induction of post-plucking anagen and possibly, more rapid hair growth; however, such differences could not be confirmed clinically.

A feature noted in the histologic assessment of the plucked biopsies was that the epithelial regenerative process extended beyond the lower outer sheath and matrix to also involve the infundibulum and interfollicular epidermis. This was found in all groups of dogs from 24 hours to eight days after plucking and was characterized by mild thickening

of the infundibulum epithelium and epidermis with slightly increased basophilia of the cells in the basal layers. This was supported by a corresponding 2-3 fold increase in BrdU labeled cells when compared with unplucked sections from the same dogs. Although this suggested that follicular regeneration is associated with an up-regulation in mitotic activity of all epithelial cells, including those in the infundibulum and epidermis, it may have simply represented a transient nonspecific response to the trauma caused by plucking.

The conclusions drawn from combining the post-plucking morphologic changes with the distribution of BrdU labeled cells in the hair follicle are difficult to reconcile with the bulge activation hypothesis. Within twenty-four hours post plucking of anagen hairs, there was evidence of proliferation in the damaged outer sheath and at the level of the germinative epithelium. Although occasional outer sheath cells in the inferior and isthmic portions of the hair follicle were labeled at this time, there was no localized proliferation in the outer root sheath that could have been considered a "bulge region." Even if such a localized proliferation did occur, we had a difficult time conceiving how these cells could migrate to the hair bulb and give rise to a population of transiently activated cells. That plucked anagen hairs can regrow a hair shaft without the need for proximity to a putative bulge region for a new "fix" of transiently activated cells contradicts a major tenet of the bulge activation hypothesis. Our conclusions are similar to those drawn by Gharzi et al 99, where germinative epithelium of rat vibrissae in anagen was shown to continue to provide a source for matrix cells after five successive pluckings.

A similar conundrum was found for the plucked telogen follicles. This study was

patterned after the wok by Wilson et al 94, where plucking murine telogen hairs and labeling proliferating cells one day later with ^{3}H-TdR, defined that outer sheath cells at the insertion site of the arrector pili muscle underwent a transient proliferation at the onset of early anagen. No such focus of proliferation was noted in the canine plucked telogen follicles. Instead, the earliest labeled follicular cells were randomly dispersed in small numbers in the outer sheath, representing outer sheath cells adjacent to trauma-induced necrosis or in the location of germinative epithelium. In addition, in some of the most severely damaged telogen hairs, the entire lower isthmus was lost, a region that corresponds to the location of stem cells in murine telogen follicles. Still, these severely damaged follicles were capable of repair and assumedly, new hair shaft production.

Based on these observations, hair shaft formation can apparently occur in Beagle dogs from a damaged anagen follicle or from a damaged telogen follicle without the need of a bulge region. This conclusion does not invalidate the bulge activation hypothesis, but does suggest a bulge region is not needed for hair shaft formation in all mammalian species. On microscopic evaluation, none of the plucked anagen hairs from dogs had attached keratinocytes from the outer sheath. This is in contrast to humans where ~60% of plucked anagen hairs have outer root sheath components attached (Bassukas and Hornstein 89). Speculatively, if one of the major reasons for an anatomic bulge is because stem cells need to be in a protected site (Costarelis et al 90), dogs may not have a bulge or "bulge region" simply because they do not need one as, in contrast to humans, the canine outer sheath remains intact after plucking. Without the need for a localized site for stem cells, one would expect an advantage would be obtained if, as was suggested in early anagen in the canine follicle, stem cells were more diffusely located throughout

the outer sheath. A recent study demonstrating that mesenchymal cells from the dermal sheath alone are capable of inducing epithelial-mesenchymal interaction and producing new anagen follicles suggested another possibility for hair follicle renewal (Reynolds et al 99). Perhaps the dermal sheath, morphologically intact in all post-plucking canine follicles regardless of the stage of the hair cycle, induced an environment for follicular regrowth without the need for a traditional stem cell population.

Because of the low number of animals used in this study, these observations need to be considered preliminary. More detailed evaluation of post-plucking anagen by defining the expression of β_1 integrin, a marker for proliferative potential and cytokeratin 15, a putative follicular stem cell marker, would be helpful in determining if and how the canine hair cycle differs from rodents and humans (Lyle et al 98). In addition, repeating this study using different canine breeds, such as poodles, that have anagen-predominant cycles analogous to human scalp hair would also be of interest. Finally, this study indicates that studying models for hair growth other than laboratory rodents may be of benefit, if not a necessity, in truly understanding the complex process of hair growth.

SUMMARY

A principle of the discipline of pathology is that differences in form mean differences in function. The preceding four chapters are united in the fact that the stimulus to perform the investigations within them was initiated during the daily practice of veterinary dermatopathology--as histologic alterations were recognized, hypotheses were formed around them. These chapters are also united through a single cell: the keratinocyte and its pathway to maturation as a corneocyte or trichocyte, in a process known as cornification. This process is essential to the protective function of the skin in all species and to the production of hair in all mammals. Finally, these chapters are united through the belief that keratinocyte biology can be studied with the dog as its focus, to better define diseases of the epidermis and hair follicle in both man and animals.

A large number of disorders of epidermal cornification exist in the dog, some such as traumatic-induced hyperkeratosis are acquired, others such as the ichthyoses are congenital. Although similar diseases occur in humans, they are more easily studied in the dog. For example, ichthyosis is found at a much higher frequency in dogs than humans due to the practice of inbreeding to produce purebreds. In addition, no good murine models for most of these conditions have been discovered or created through transgenic technology. All of these factors contributed to the desire to characterize a gene central to cornification, *transglutaminase 1*, in the dog.

The health and beauty of a pet's coat is an essential link in the human- animal bond, as any veterinary dermatologist or dermatopathologist can attest. Because the process of cornification is central to formation of the hair shaft, alopecias can be viewed broadly as cornification defects. In the second part of the research in this dissertation, the morphologic, morphometric and proliferative response of the hair follicle in

hypothyroidism, the most commonly diagnosed endocrine disease of dogs is described. This work demonstrates the canine follicle is both similar to and yet different from the follicles of the two most widely studied mammalian species: humans and mice.

Therefore, the main value of this work lies not in the individual data points generated, but rather in demonstrating that canine skin represents a unique and useful model for dermatology research that begs for further investigation.

The first chapter of this dissertation is a review of the current literature and summary of the processes of epidermal cornification and hair growth. Cornification is the process by which keratinocytes terminally differentiate to become either corneocytes, the building block of the stratum corneum, or trichocytes, the primary component of hair shafts. Cornification results in a structural barrier through which neither hydrophilic nor hydrophobic molecules can easily pass. This barrier is the result of the convergence of three biochemical processes, followed by desquamation. These three pathways are 1) the formation of structural proteins such as keratins, keratin-associated proteins, and the proteins that form the cell envelope, 2) the synthesis of lipids which act as a glue to aid in annealing proteins and forming a hydrophobic barrier and 3) the action of enzymes such as transglutaminases that cross-link proteins, stabilize cell membranes and form a tough "cornified envelope" around cells. When there is a defect in any of the pathways needed for production of the stratum corneum or in desquamation, abnormalities in cornification occur. Because of the many pathways in cornification these are heterogeneous diseases; however they all have excessive scaling in common as a clinical feature. The scaling often appears layered, simulating the appearance of scales, hence the term "ichthyoses" is

applied generically to these disorders. Most of these disorders are inherited, with signs appearing at or shortly after birth.

The hair follicle consists of a downgrowth of the epidermis that interacts with an aggregate of modified, dermal fibroblasts known as the follicular papilla to form a hair shaft. Hair shaft formation is cyclical in all mammals, with periods of active hair growth (anagen), senescence (catagen) and inactivity (telogen). How the hair cycle is regulated remains unclear, but it is now known from human and rodent studies that numerous growth factors, systemic hormones and their receptors influence both hair follicle development and cycling. Thyroid hormones are known to have a profound effect upon the skin and their deficiency is postulated to cause alopecia, seborrhea and pyoderma in dogs.

The transition period from the epithelial hibernation of telogen into the next anagen phase is one of the most critical in the hair cycle. Traditionally, during this period it was believed that germinative epithelial cells in the telogen follicle undergo mitosis, grow downwards to surround the re-forming follicular papilla and differentiate into the hair bulb. These germinative epithelial cells were assumed to be the follicular "stem cells", the source of renewal in regenerating tissues such as stratified squamous epithelium, hematopoietic tissue and intestinal epithelium. In 1990, Cotsarelis et al. challenged this view by suggesting that the stem cells of the follicle reside in a localized area called the "bulge", a knob-like evagination of the upper portion of the outer sheath associated with the site of attachment of the arrector pili muscle. In this study the authors pointed out that there are a number of advantages for the follicular stem cells to be located in the bulge. This site is not removed when hairs are plucked from the follicle so

these cells are protected, it is well vascularized and in telogen, the bulge region is situated so that it can interact with the follicular papilla. The hypothesis that this site contains the stem cells that become activated and proliferate in order to renew the hair follicle at each cycle is known as the "bulge activation hypothesis" and has remained a controversial one in dermatology over the past decade.

In chapter 2, the canine *transglutaminase 1 (tgm1)* gene is characterized. Mutations of this gene in humans cause altered cornified envelope synthesis, resulting in a disease known as lamellar (recessive) ichthyosis. Clinical and histologic analogs of human lamellar ichthyosis have been recognized in the dog, but because so little is known about the mechanisms of epidermal cornification in species other than humans or laboratory rodents, the pathogenesis of this and other disorders of the canine stratum corneum remain largely undefined. In this chapter, the complete coding region of canine *tgm1*, its chromosomal localization and its map position in the integrated canine linkage-radiation hybrid map is reported. Canine *tgm1* consists of 2,453 nucleotides distributed over 15 exons. The nucleotide sequence has 90% identity to human *tgm1*. The deduced canine TGM1 protein is 816 amino acids long and is 92% identical to human TGM1. Using fluorescence *in situ* hybridization (FISH), the chromosomal localization of canine *tgm1* was determined to be on *Canis familiaris* chromosome 8 (CFA 8q11.2-12). Canine *tgm1* localized to CFA 8 on the integrated linkage-radiation hybrid map in the interval FH2149 -MYH7. This characterization of the coding region of canine *tgm1* is a first step in examining the role of this enzyme in normal and defective cornification in the dog.

The third and fourth chapters describe the effects of hypothyroidism on the canine hair follicle. In chapter three, the effects of hypothyroidism on canine skin are defined by

comparing morphologic, morphometric and hair cycle differences in skin biopsy samples from three groups of age- and sex-matched Beagle dogs: 1) euthyroid dogs; 2) dogs made hypothyroid by administration of ^{131}I; 3) dogs made hypothyroid and maintained in a euthyroid state by the treatment with synthetic thyroxine (Soloxine). After ten months, there was little to distinguish untreated-hypothyroid dogs from euthyroid or treated-hypothyroid animals on clinical examination and none of the untreated-hypothyroid beagles had a symmetrical truncal alopecia. A marked difference in the rate of hair re-growth after clipping was found in the three groups, however, as the untreated-hypothyroid Beagles had minimal re-growth of clipped hair after two months. The morphologic and morphometric results show that the majority of the hair follicles in the Beagle dog are in the resting stage of the hair cycle (telogen) irrespective of the group examined, suggesting that, at least for Beagles, the hair cycle is telogen-predominant. Yet, normal dogs with the majority of their hair follicles in telogen have no propensity to become alopecic as their telogen hairs can be retained in the follicles for long periods (months to years) without falling out. In states of thyroid hormone deficiency, this resting stage becomes prolonged and when a telogen hair is eventually shed, it is not replaced, resulting in mild hair loss that differs from the classically described alopecia found in hypothyroidism. In thyroxine-treated hypothyroid dogs, telogen is still the predominant phase of the hair cycle, but the presence of many more anagen hairs suggests, as breeders have long believed, that thyroid hormone supplementation stimulates hair growth. These results indicate different canine breeds may have markedly different hair cycles and follicular morphology, factors that may influence the clinical and histologic features of hypothyroidism.

Chapter 4 contains the results of a study in which the effects of plucking hair shafts from age and sex-matched normal, untreated hypothyroid Beagle dogs and thyroxine-treated hypothyroid Beagle dogs were defined. The dog model was chosen for several reasons. First, dogs have larger follicles than either the mouse or rat, facilitating the visualization of morphologic changes associated with hair plucking and regeneration. Also, dogs do not have an anatomic bulge but have a more prominent arrector pili muscle than humans, rats or mice. In addition, Beagles dogs have a telogen-predominant hair cycle having, on average, greater than 70% of their hair follicles in telogen, and of these, approximately 20% are in telogen without hair shafts. At the same time, they also have anagen hairs. Thus in Beagles, the effects of plucking can be examined on both anagen and telogen hairs concurrently. The three groups of dogs were included in this investigation as no previous reports could be found in which the effect of hair plucking on the hair cycle was defined for follicles with inhibited or stimulated hair growth.

The study was conducted by plucking multiple sites on the dorsum of the Beagles dogs and taking skin biopsy samples from these sites for a number of time periods over 64 days. Routine descriptive histology, morphometry of the hair follicles, ,immunohistochemistry to define the cell populations proliferating after plucking and comparisons of the extracted hair shafts between dog and humans were performed.

The histologic evaluation of the skin biopsy samples demonstrated that there were three morphologic patterns associated with plucking of both anagen and telogen follicles. The patterns associated with plucked anagen hairs were:1) the hair shaft was broken above the hair bulb, sparing the matrix epithelial cells; 2) epilation resulted in breakage in the upper half of the bulb hair bulb, disrupting the matrix keratinocytes in this region but

leaving most of these cells in place; 3) breakage at the base of the follicular papilla resulting in most of the hair bulb being extruded with plucking. Hair re-growth in canine plucked anagen follicles with morphologic patterns 1 or 2 would invariably regenerate without entering into telogen. A similar re-growth without a telogen interlude was also noted in plucked anagen follicles with morphologic pattern 3; however, it is possible follicles with this degree of injury to the hair bulb and germinative epithelium could require complete replacement of the hair bulb and consequently a telogen-like phase. Three morphologic patterns were also associated with plucked telogen hairs: 1) extraction of the shaft with retention of tricholemmal cornification in the follicular lumen; 2) removal of tricholemmal cornification and part of the outer sheath cells with extraction of the hair shaft; 3) removal of the entire outer sheath of the follicular isthmus with extraction of the shaft. Plucking of telogen follicles resulted in regeneration of the follicle and production of a new hair shaft.

The rapidity of regeneration of a plucked haired follicle depended on the severity of the injury to the follicle and whether the dog was in the control, treated-hypothyroid or untreated-hypothyroid groups. The untreated-hypothyroid dogs lagged behind the other two groups not only in the number of plucked follicles that were undergoing transition into anagen but also the time required to regrow a new hair shaft.

The morphometric assessment of the effects of plucking hair from follicles in the three groups of dogs analyzed corresponded to results obtained in other mammalian species: plucking hair stimulates follicles to enter or remain in the anagen phase of the hair cycle. This being noted, there were still differences between the three groups analyzed. The median number of anagen follicles in the untreated-hypothyroid dogs was

much lower and the median number of anagen follicles was much greater in the treated-hypothyroid dogs than the controls at Day 64. The increase in anagen follicles in the untreated-hypothyroid dogs over the course of the study differed by only a few follicles from the Day 0 value and this increase was due to a transition of primary follicles from the telogen to the anagen phase, as the median number of secondary anagen follicles in this group actually decreased over the course of the study. These data suggest that in the dog, the post-plucking anagen phase is subject to regulatory mechanisms and that trauma-induced follicular renewal can be inhibited by decreased circulating thyroid hormones while the administration of thyroid hormones can stimulate this process.

The cell proliferation labeling studies showed that there was no localized site of cell turnover in the outer sheath (ie. bulge region) of the plucked canine follicles, regardless of the stage of the cycle in which the follicle was plucked. In anagen follicles, plucking left a population of rapidly proliferating cells at the bottom of the follicle that could rapidly regenerate the hair bulb and shaft. In telogen follicles, the majority of the cell proliferation occurred as needed to regenerate the damaged epithelium with the follicles.

Based on these observations, hair shaft formation can apparently occur in Beagle dogs from a damaged anagen follicle or from a damaged telogen follicle without the need of a bulge region. This conclusion does not invalidate the bulge activation hypothesis for humans or mice, but suggests a bulge region is not needed for hair shaft formation in all mammalian species. On microscopic evaluation, none of the plucked anagen hairs from dogs had attached keratinocytes from the outer sheath. This was in contrast to humans where ~60% of plucked anagen hairs have outer sheath components attached. Therefore,

if one of the major reasons for an anatomic bulge is because stem cells need to be in a protected site, dogs may not have a bulge or "bulge region" simply because they do not need one as, in contrast to humans, the canine outer sheath remains intact after plucking. One might expect an advantage would be obtained if, as was suggested in early anagen in the canine follicle, stem cells were more diffusely located throughout the outer sheath, without the need for a traditional stem cell population.

Appendices

Appendix A

Summary of primers and conditions used for PCR amplification of canine *tgm1*

Sizes of expected genomic DNA and cDNA bands listed in base pairs (bp).

Temperature of melting (Tm) given in degrees Celsius.

Conditions for PCR amplification:

A: 10x buffer contains 200mM Tris, 500mM KCl, and 15mM MgCl.

10x Mix of four dNTPs is 1mM.

Working primer concentration is 5uM.

Taq polymerase is added at 1 unit per 25ul reaction.

B: Epicentre Fail Safe PCR system.

Optimal buffer premixes (also contains dNTPS) used listed in notes.

Epicentre Taq polymerase blend is added at 0.6 units per 25ul reaction.

Source of Primers- primers were designed initially to human TGM1 sequence (H), and later to canine sequence (C).

Appendix A

Table 9A. Summary of primers and conditions for PCR amplification of canine *tgm1*.

Primer	Name	g DNA	c DNA	Tm	Conditions	Region amplified	Source	Notes
AGC CAG AGC CAG AGC CAG A TGG TGC TCT CGG CGG TTC T	TGK 1D TGK 1U	485bp	335bp	64 67	A	Exon 2- Exon 3	Human (H) Sequence	RD 11
TCA ATG CAG CTG GAG ATG G GGA CTG TGA ACT GAA ACT TGC	TGK 2D TGK 2U	1525bp	385bp	61 57	A	Exon 2- Exon 4	H	
ACG CAC GTG ATC ATC CCA GT CCG CTC ACC AAT CTG TGC TT	TGK 3D TGK 3U	725bp	325bp	64	A	Exon 4-Exon 5	H	
CCG CAC ACA CTC AGA GGC T ACT CCA TTG TCA TCC AGG G	TGK 4Dz TGK 4U	1320bp	340bp	62 58	A	Exon 4- Exon 7	H	
GCT GGA TGC CTG CTT ATA CAT GGA GTT GAA GTT GGT GAC AG	TGK 5D TGK 5U	1170bp	320bp	59 56	A	Exon 6-Exon 8	H	
GAT ATT CCG TCC CCT ATG G GTA GAC GAG CCC ATT CTT GA	TGK 6D TGK 6U	3105bp	355bp	57	A	Exon 7-Exon 10	H	
CTG CCC TCG GGC TTT GAT G GTC TGA GCC TTC TGG GTG CT	TGK 7D TGK 7U	NA	310bp	66 62	A	Exon 9-Exon 11	H	7D crosses exon/intron boundary
AAG GCC ATC AGC TCC AAC AT CTT CCT TCT TGG TCT CCT TGA	TGK 8D TGK 8D	430bp	320bp	62 58	A	Exon 11-Exon 12	H	Does not amplify well
GAC GAT GGC AGC TTC AAG A CCT GAG ACA TTG AGC AGC AT	TGK 9Dz TGK 9U	750bp	490bp	59	A	Exon 11-Exon 13	H	
GGA GTG CGA AGT ACA GAT TGT C TCC ACC TCG AGA TGC CAT AG	TGK 10D TGK 10U	4935bp	335bp	59 61	A	Exon 14-Exon 15	H	RD 10
GCT ACC TCC GCA CTG GC TAT ATC ATG GTT CAG GTG CTC CA	TGK 6ND TGK 11U	600bp	200bp	53	A	Exon 7- Exon 8	Canine (C) Sequence	
CTG TCA CCA ACT TCA ACT CGG CTA CCA CTT GCC ACC CGT C	TGK 12D TGK 12U	340bp	190bp	52	A	Exon 8- Exon 9	C	

Appendix A

Table 9B. Summary of primers and conditions for PCR amplification of canine *tgm1*, continued.

Primer	Name	g DNA	c DNA	Tm	Conditions	Region amplified	Source	Notes
TTT GCA CCA GTC TGG CAG G CTC CTT GCA GAA GAC AGG TGG	TGK exon 1-1d TGK exon 1-1u	640bp	NA	63	B	5' Untrans-Intron 1	C	Fail Safe Buffer D or F
TGG TCA GGT TCC CAG ACA CA AGT CCC ATC CAG CAG ACT GAG	TGK exon 1-2d TGK exon 1-2u	520bp	NA	62	B	Intron 1	C	Fail Safe Buffer D or F
GGG AGA CGA TTT CTC GGC ATT C GCT GCG TTC ACA CCG CTG G	TGK exon 1-3d TGK exon 2u	550bp	NA	68	B	Intron 1-Exon 2	C	Fail Safe Buffer E or F
GGC AAC GAG ACA GTG ACA CT ATC TTG GGG CAA TGT CCT T	TGK exon 15-1d TGK exon 15-1u	280bp	NA	59	A	Exon 15- 3' Untrans	C H	
GTC GCC CTG GAG CTG CTT AT CCA CTG GCC TTG GTC ACC T	Rhmap 1d Rhmap 1u	1100bp	120bp	63	A	Exon 3- Exon 4	C	Radiation-hybrid map marker
GTG GGC AGA ATC TGA ACC TC CTC ATG GTC CAC GTA CAC GA	Rhmap 2d Rhmap 2u	580bp	180bp	60	A	Exon 4- Exon 5	C	Radiation hybrid map marker
CAT GCA GGA CGA TGT CAC C GAC GGA AGG TAT GCT GTT TGG	Rhmap 3d Rhmap 3u	720bp	460bp	61	A	Exon 11- Exon 13	C	Radiation hybrid map marker
AGA ACC GCC GAG AGC ACC A TTG AAG AGG ATG TAG ATC TC	RD 1 RD 2	1370bp	375bp	66 50	A	Exon 3-Exon 4	H	
AAC TCC CTG GAT GAC AAT GG CAC CAC CTG CCA CCC ATC AA	RD 3 RD 4	930bp	380bp	60 67	A	Exon 7- Exon 9	H	
TGT GGA GTC CAT CAA GAA TG TTC CTT CTT GGT CTC CTT GA	RD 5 RD 6	1100bp	470bp	57	A	Exon 10-Exon 12	H	
CAA GGA GAC CAA GAA GGA AGT AGA GCC TTC GAG CCG GAA GA	RD 7 RD 8	840bp	310bp	58 66	A	Exon 12- Exon 14	H	
ACA CTG CGC CAG TCG TTT GT TCC ACC TCG AGA TGC CAT AG	RD 9 RD 10	195bp	195bp	64 61	A	Exon 15- Exon 15	H	

Appendix B

5' Untranslated sequence data for canine *transglutaminase 1*

CCTGGCTGCTGATGTCACCAGTTGGCAGCCTAGGATCCCAGGACTCCCTGGGCAGGACCAGGAAA
GGGGGCCTGGGAGTGGGGAACACTGCATAGGGGCGTGGGTGGAAGGCACCCTGGCCCCTTGCCCA
CGGGCTTCTCAGCCAGGAGAGGGGCCCTTTTTGCACCAGTCTGGCAGGGAGTGGGGTCCATCTGT
GGGGCAGGCTCACCAAAGCCTTCCACTTTCCCCTCAGCTTTGAGTCCTGACAGACCTTGGCCCCA
CCCTGTATCATCACTTTTCCACAGTTCCTCGAAATGGGAAAGGGGGACGTGCCTAGGTCCCATTT
CCTGCCCCTTACCTTCAGAGACAGCCCCAAGAGGCCGGCCTTTGTGTGGGGGGGTGGAACAATCA
GGCTCCCCCTTCTCTGCGCCTCAGCCCCGCCCTCTCCCCCTCATAAACCACGTACCGCCCCGGCC
CCCACACCTTCCACTTGCCAGCCGCCAGCCCCGTCCCAGCAGTCCCGACCTGGCTGCTGGACTCA
GCGCTGCTGTCGCCAGCACCGCCACCAGGTGAGGCTCTGTCGGGGATGGTGGTGCTGAGCCCAGG
GGGCCGAGGTCAGGCCCCAGCAAGGTGGGGTGTAGGCCAGCCCGAGCCTGCCAGCCCTGGTCAGG
TTCCCAGACACAGAAGCTGTGCGCCCCGGGGACCACGGGCTCCCGCACCTGCAGCACCCCACCAC
TCCTGCAGCTCTGTGTGGGTGCTTGACCCAATGTGAAGAATGCCAAGGACCCCGAGTCCCACATG
TCACCCACCTGTCTTCTGCAAGGAGCCTTGGATGGGGTATCTGCTAAGAATGGGTCCTAGAGGCA
GGCAGCTCCTACATCCCAAACTGAGTAGCTGTCAGTTTGTCAGATGGTGGGAGCATCTCCCAGGG
ATGGTCTGTGTTCTGGGTCCTGAGGCTGTGACAAGTGCCACTCCTTTCTGGGATGGGTCCTTGAG
AGCAGGTTTCTCAATGGGGCATCTGGTTTGGTGGATGTCACTGTGGGGGTAGGAGGGAGACGATT
TCTCGGCATTGTGGGTAGTTTCTCTAGGTGAACCTGAAAATAATCTTTGGGGAGGATCTCTGGTT
GGGTCTGGGGGAATCTCCAGGGCAAAAGATCATCTCAGTCTGCTGGATGGGACTGAGTCAGCTCT
CTGGATGGAGGGAGGGCTTCTGTGTGTGCCTGCCTGGGGCATTCGAAAGTACAGGAGACCTGAGT
GAGTGATCAGAGACTCACTGCTTTTTCCACTCAG

Sequence data determined at least twice except for the underlined segment which was sequenced once.

Appendix C

Sequence data for canine *transglutaminase 1* introns

Intron 1

GTGAGGCTCTGTCGGGGATGGTGGTGCTGAGCCCAGGGGGCCGAGGTCAGGCCCCAGCAAGGTGG
GGTGTAGGCCAGCCCGAGCCTGCCAGCCCTGGTCAGGTTCCCAGACACAGAAGCTGTGCGCCCCG
GGGACCACGGGCTCCCGCACCTGCAGCACCCCACCACTCCTGCAGCTCTGTGTGGGTGCTTGACC
CAATGTGAAGAATGCCAAGGACCCCGAGTCCCACATGTCACCCACCTGTCTTCTGCAAGGAGCCT
TGGATGGGGTATCTGCTAAGAATGGGTCCTAGAGGCAGGCAGCTCCTACATCCCAAACTGAGTAG
CTGTCAGTTTGTCAGATGGTGGGAGCATCTCCCAGGGATGGTCTGTGTTCTGGGTCCTGAGGCTG
TGACAAGTGCCACTCCTTTCTGGGATGGGTCCTTGAGAGCAGGTTTCTCAATGGGGCATCTGGTT
TGGTGGATGTCACTGTGGGGGTAGGAGGGAGACGATTTCTCGGCATTGTGGGTAGTTTCTCTAGG
TGAACCTGAAAATAATCTTTGGGGAGGATCTCTGGTTGGGTCTGGGGGAATCTCCAGGGCAAAAG
ATCATCTCAGTCTGCTGGATGGGACTGAGTCAGCTCTCTGGATGGAGGGAGGGCTTCTGTGTGTG
CCTGCCTGGGGCATTCGAAAGTACAGGAGACCTGAGTGAGTGATCAGAGACTCACTGCTTTTTCC
ACTCAG

Unknown if intron complete as exon/intron boundaries are estimated from human sequence.
Sequence data determined once.

Intron 2

GTGAGGCCCTCCTATCCACCTTCTCTGAGAGCTGAAAGGCCTTAATGGGGCCTGAGACTCATCCT
GACATCTCTGGGGGGCCTGGGAGGCTAAGGATGGGGGCTTTGCTGACCTAGTGCCGTGGCCTTGC
AG

Intron is 132bp, sequence data determined at least twice.

Intron 9

GTGARCCGGGGTCCTGCCTTCTCCTGTGCCCCACCGTGGCCCCATGCCCGTCTCCCAGCCTGAGG
GACAGACCCCGGGGGTCAGTGCCAGTCTGGCCTACAGAGCCAGGCCCTTTCACCCCTATGTTTCT
GTAAACAGGCTTACTGGCAGCATCACTGATTGGACTTTAGCTGATTTTCACCTTTCTGTGTAACT
CTTTGCCTAGATTTTCTTTATTTATGATTTATTATGATTGTTATATTGAGATGGCTCAAAAGTCA
AAAGATTCAAGAGAGTTCAGTGAGAGGTTTCCCTCCCTAAGACACCCACGCTTCTCATTTGCTTT
TTTGCTTACATATCCCAAAGATAATTAATTGAAGGATCCTTTGATTTATGTATAGTTTTAAAGCT
AGAAAGGTCATTATAATAAGTAATACATGCCTGCAAGGAGGGAAAGAGTATAGAATTGAATAACA
CAAAATGTAAAATTTTCCCTATTCTCTCAGGACGCTTTTGTTTTGTTTTGTGTAAATGTCTAGGT
CCCATCCCAGACCTGACACATCAGGCACTCCAGGGATAGACTGATGTGAAGAGTCTATGTTTCTG
AAAGTCTGAAAATTCCCCTGGTGATGCTGAAGATCAGCTGGGATGGGGACCACTGCAAGCCTTTG
TGGGACCCATGGGTACTGCTAGCCCAGAGAAGTGGAGTGGCTCTCACTAGGGCACAGAGCATTAG
GTCAGCGGCAGAGCCCCTCCCGTCCCTGACTCCCTCCCTTCACACATTCTCACTTTCACCCAAGT
TCCCCTCTTGACTGTTTCGTTGCTCATTTTACGTTGTATGCATARTATGGAATGAAACCAGGGTT
CAAATAGTTCGAAGAAGAAAATAGCCCCCATTACCCAGCCCTCAGAAATCATCGCTGACAACATC
ACCTTTTCCCCAGTGCACACATTTCTGCAAAAACTAGACYTGCTATCCACTCTATTCTCAAATAG
CCCTCCCTTCCCGCCTCAACATTCCAGCTCCAGGCAGCCAGACTGTGCTCCCTGCTGCCTTCTTC
CCCACCTCTCCCTTTTCTGGGTTTGTCTTGATGCAGGATGGTCCTGACCTCGGGGGGTCACAGAC
AGCCAACATCCAGCTTCAGACAGTGGAGGAAGGATGTGCTGATGATTTGGTTATCAAGGCAGGCT
TGTCCCACATTTAGCCCCGCCCTGGTTGGCTGAGGCCCAAATCGACCTGAGCTCTTCCTTGTGCT
ACTTCCCTGGGTTGGACCAGGTGGCACCTGCCCCTGAGGCAGTGGGCACTGCACCTGGCTGGGTG
GGCCTCTCCCCAGGATCACAGGGTCCCCTTGGCCTCCTTGGATCTTTCACTTCTGTCCTGCTCCC
CCAG

Intron is 1369bp, sequence data determined at least twice.

Intron 10

GTGAGGGCCATGCTCCAGATGCTTTCTTGGGCTGTCTGGTAGAACCACAGCACTGGTGTCAGGCC
TGGGCTCTCTGGTCCTGATTTCACTGCTGACTGACTAGYTGTGTGACCTTGGGCAAGTCACTTAT
TCTAGAGTCTAGGTTTCCTCAACTGTAAAAGGGTGATAATAATGGCTGCTGTGCTTCCTGTACTC
AGGATCAAAAGAAATAATGGGTACGAACCTGCTTTATAAGTTGTAAAGTCCGTGAAGGAGCATAA
GGGATTACTGTTATTAATGCTAAAACGCACTGCAAGGACCCTCCCAGTTTCTTCTCTGCTCTCTG
GCTTCATCTGGCTCARCTTGGAAAGTCTCCCGGTTTGGCTGTGCCAGGAAGTTCCCAACCTTTGG
ATCCTGCCCTCCCTCCTGCTGGAGGTCAGGAGAAGGCAGCTGGGGGTTAGGCCTACACTCAGACC
CTCTGGCCTGCTCATTCTTGGCTTCCACCCCCTACACTCCCCCAACAG

Intron is 503bp, sequence data determined at least twice.

Intron 11

GTACCATCNTTCCAGCNTCATCAAGTGTGGCATATACCAAGGGTTTCCAGTGAGCACGTGCCCAC
CCCGGAAGTGGCTATAACCTCACCTTGACCCTTAACCCCCAG

Intron is 107bp, sequence data determined once.

Intron 12

GTGCTTCTTCCCCAGCCCTGCCCCACTGATGGCCAGGCATCCCATGGCCATGGGCCTGGAGACCC
CAGGAGGCCGTGCCTGTGTAAGCAGGTCCTGGTGACACCTTGAGTCTCCTCCCCAG

Intron is 121bp, sequence data determined once.

Intron 13

GTGAGTGCTGGTTGCTGGATGTGCGGGANGCTCTANGGCTGANGGAAATACTAGGGGAAGGGGCG
AAANCANTAAGCTGGGAAAAGCCTGGTCTGGCGAACAGTTCCAGGANAANGAAGTCAGAAACTGG
CTTTGGGCTCTTGGGCCTGTTTCCTCATCTGTTAAATTGGATCTCATATTCCTCAACCCTTCCAA
AACTTTT.....GGNTCTAGGGCTGAGGGAAAANACTAGGGGAAGGGGGCGAGAGCAGTAAGCTGG
GAGAGNGNTGGTCTGGCGAGCAGTTCCAGGAGAAGGAGGTCAGAGACTGGCTTTGGGCTCTTGGG
CCTGTTCCTCATCTGTAAAATTGGATCTCATATTCTCAACCCTGCAGAAGCTTTGGTGGGGGAGC
AGGTTGGAGTTGCTTCTCTGGCTTTTGTGGAGCCCCATTTCTCTCAGTGACAGAAATGTGGGACT
TGGGGAGGTTTGGATGCAGAGAGTTTTCCCTTGTATGGTGGAGTGTGTGGTTCCACTGTATCTGG
CGGGGGTCTCTGATGTGGAGGTGGGCCTCTTTTTCTCCTAG

Intron not complete, sequence data determined once.

Intron 14

GTGAGTCTCGCCTCTCCCCTGCCCCTATCCTTTCCTGCCTCCCAGACTGCCCCGGGTCCCACACT
GCTTTCTCTCTTGGTCAGGACAGTAGTCAGCCAGGACCCAGTAGGGCCAGCCAGCAATACCTCCT
GACCCAGTTCAGGGCCAACAGCATTTAAGGAGACCTGCCCTGTGCCTACGGCTGGGCTGGTCCTT
CCTCACTAGGGAGCCAACACTTCACTTAGGAATCTTAGCAACTCATCACCAACACGTGCCCAGCA
CTCAGGAACCGACTGGGCACTTGAGGATCATTATCTCCTTTGCAGACCACAAAAAACCTTTAAAG
TACAGTCACGATCATCATGTGATTAAAAGCAAAGCTTTGGGATCCTGAANACTTGGGTTCAAGTC
CTAGCTCCAGCACTCACTGGCTCTGTGCCCTGGAACAAATGACTTGCTCTTTTAAGTCTATTTTC
TTACCTGGAAAGTGGGGGGNGATAACTGTTCCGGCTCCAAA.....

Intron not complete, sequence data determined once.

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