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The role of the specific, profilaggrin-containing keratohyalin granules in the developing epidermis of the fetal horse hoof

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Summary

The adult equine hoof is subdivided into distinct segments with various keratinization modes. In the periople and bulbs of the heel, the epidermis forms a *Stratum granulosum* with basophilic keratohyalin granules during soft keratinization, whereas in the coronet, wall proper, sole, and frog, the epidermis undergoes hard keratinization by keratinizing and cornifying without forming keratohyalin granules. The present study tests the hypothesis that the presence of specific (profilaggrin-containing) keratohyalin granules in the hoof epidermis is correlated with the water-binding capacity and mechanical properties of the hoof horn. To identify these specific profilaggrin-containing keratohyalin granules, tissue samples of fetal hooves were studied with histochemistry, immunohistochemistry, and transmission electron microscopy. In a fetal hoof, a *Stratum granulosum* is formed in all hoof segments in the wake of the establishment of a segment-specific papillary body, but at differing developmental stages, starting in the coronet, then in the wall proper, and later in the sole and frog, and disappearing again in the same sequence. In the terminal part of the wall proper (i.e., *Zona alba*), the *Stratum granulosum* is retained at least until three days after birth. In the periople and bulbs of the heel, the *Stratum granulosum* appears last (and is retained in the adult) when the other segments have not yet completely lost theirs. The basophilic granules in the *Stratum granulosum* are specific profilaggrin-containing granules that were also described in the human skin. These observations are relevant for a better understanding of certain dyskeratotic processes in the hoof epidermis.

Keywords: Horse, hoof, development, horn, mechanical properties, epidermis, *Stratum granulosum*, keratohyalin, filaggrin, dyskeratosis, papillary body

Zur Funktion der spezifischen, Profilaggrin enthaltenden Keratohyalingranula in der Hufepidermis des Pferdefötus

Der Huf des adulten Pferdes wird in Segmente eingeteilt, die einen unterschiedlichen Verhornungsmodus aufweisen. Im Saum- und Ballensegment bildet die Hufepidermis ein Stratum granulosum, welches lichtmikroskopisch durch basophile Keratohyalingranula gekennzeichnet ist. Dagegen verhornt die Epidermis im Kronsegment, im Wandsegment, in der Sohle und im Strahl ohne ein solches Stratum granulosum auszudifferenzieren. Das Stratum granulosum enthält in der Epidermis des Menschen mindestens zwei verschiedene Arten von Keratohyalingranula, die „spezifischen" Keratohyalingranula, welche Profilaggrin für den Filament-Matrix-Komplex der verhornenden Zellen enthalten, und die „unspezifischen" Keratohyalingranula, welche Proteine für eine zusätzliche Zellhülle der verhornenden Zellen enthalten. Die spezifischen Keratohyalingranula werden im Zuge des Verhornungsprozesses in der verhornenden Epidermiszelle durch Enzyme abgebaut. Die bei diesem enzymatischen Abbauprozess aus den Profilaggrinmolekülen entstehenden Filaggrinmoleküle durchdringen die Keratifilamentbündel und bilden auf diese Weise einen Filament-Matrix-Komplex, der die verhornte Epidermiszellen festigt und so die mechanischen Eigenschaften des Hornzellverbandes massgeblich beeinflusst. Das Protein Profilaggrin, sowie das aus diesem durch enzymatische Spaltung entstehende Protein Filaggrin, weisen in der Epidermis ein hohes Wasserbindungsvermögen auf, welches die mechanischen Eigenschaften des Stratum corneum beeinflusst, wie dies für die Haut des Menschen nachgewiesen werden konnte. Die vorliegende Untersuchung prüft die Hypothesen, dass die Keratohyalingranula in der Hufepidermis von Pferdeföten und adulten Pferden spezifische Keratohyalingranula, die Profilaggrin enthalten, sind und dass das Vorhandensein dieser spezifischen Keratohyalingranula mit dem Wasserbindungsvermögen und den mechanischen Eigenschaften des Hufhornes von Pferdeföten und adulten Pferden zusammenhängt. Zudem wird die Hypothese geprüft, dass die fötale Entwicklung der Hufepidermis mit derjenigen des segmentspezifischen Papillarkörpers einhergeht. Um Profilaggrin in Keratohyalingranula nachzuweisen, wurden die Hufe von 53 Pferdeföten mit einer Scheitelsteißlänge von 52 mm bis 1050 mm (42 bis 253 Tage der Trächtigkeit), von zehn Fohlen (neugeboren bis zu 140 Tage alt), und von zehn adulten Pferden unbekannten Alters mit lichtmikroskopischen, histochemischen, immunhistochemischen und transmissionselektronenmikroskopischen Methoden untersucht. In der Hufepidermis von Pferdeföten erscheint ein Stratum granulosum in allen Hufsegmenten, aber in unterschiedlichen, segmentspezifischen Entwicklungsphasen und erst nachdem die Entwicklung eines segmentspezifischen Papillarkörpers begonnen hat. Die Entwicklung des Stratum granulosum beginnt im distalen Teil des Kronsegmentes, dehnt sich dann einerseits auf das gesamte Kronsegment und andererseits auch auf das Wandsegment aus, und erfasst schliesslich die Sohle und den Strahl. In diesen Hufsegmenten, mit Ausnahme des terminalen Teiles des Wandsegmentes, wird im Zuge der weiteren fötalen Entwicklung der Hufepidermis die Produktion der Keratohyalingranula eingestellt. Im terminalen Teil der Wandsegmentepidermis, welcher die weiße Linie des Hufes bildet, konnte auch bei drei Tage alten Fohlen noch ein Stratum granulosum nachgewiesen werden. In der Saum- und Ballenepidermis tritt ein Stratum granulosum zuletzt auf und bleibt auch im Hufe adulter Pferde erhalten. Immunhistochemische Untersuchungen identifizierten die im Stratum granulosum der fötalen Hufepidermis befindlichen basophilen Keratohyalingranula als sogenannte spezifische Keratohyalingranula, die Profilaggrin enthalten, wie sie auch für die Epidermis des Menschen beschrieben wurden. Da das Stratum corneum sowohl im weich-elastischen Saumsegment adulter Pferde als auch in der weich-elastischen hinfälligen Hufkapsel (Capsula ungulae decidua) von Pferdeföten Filaggrin enthält, liegt es nahe, die hohe Wasserbindungskapazität und weich-elastische Konsistenz des Saumhornes adulter Pferde und der hinfälligen Hufkapsel auf dieses Filaggrin zurückzuführen. Das sporadische Auftreten eines Stratum granulosum in der Epidermis von Hufsegmenten, die normalerweise im Hufe adulter Pferde nach dem Modus der „harten Verhornung" ohne Stratum granulosum differenzieren, kann als Reaktivierung von Keratingenen aus der fötalen Entwicklung verstanden werden. Diese Untersuchungsergebnisse sind für ein besseres Verständnis der Verhornung und deren Störungen im Pferdehuf, zum Beispiel bei Hufrehe und Hufkrebs, von Bedeutung.

Schlüsselwörter: Pferd, Huf, Entwicklung, Horn, mechanische Eigenschaften, Epidermis, Stratum granulosum, Keratohyalin, Filaggrin, Dyskeratosis, Papillarkörper

Introduction

At the organismal level, the epidermis can be classified into pliable structures characterized by soft keratinization (e.g., skin, oral mucosa) and more rigid, cornified structures with hard keratinization (i.e., cornified end organs, such as hooves and claws)[1]. The mechanical quality of softness and hardness of epidermal structures, however, also depends on the thickness of the *Stratum corneum* of the epidermis, irrespective of the type of keratinization. The thickness of the *Stratum corneum*, in turn, is correlated with the height of the underlying dermal papillary body (*Homberger* 2001, *Bragulla* 2003).

At the organ level, the "soft" and "hard" epidermis can be distinguished also by the configuration of the papillary body that is formed with the underlying dermis. The papillary body of cornified organs is generally more complex and forms a more extensive interface for mechanical anchoring and an increased exchange of substances than the papillary body of soft epidermis. However, the papillary body becomes more complex also in soft epidermis that generates a thickened *Stratum corneum* (*Homberger* 2001, *Bragulla* 2003). Cornified end organs, such as hooves and claws, however, are complex structures whose segments are characterized by different properties and degrees of hardness that are not necessarily tied to the type of keratinization and histological structure (*Budras* and *Huskamp* 1995).

At the tissue and cellular levels, the picture becomes still more complex with particular regions and segments of cornified end organs exhibiting certain histological characteristics of what has traditionally been associated with soft keratinization in the skin. In the adult mammalian skin, for example, the epidermis has traditionally been subdivided into three main layers, namely the basal, intermediate and superficial layers. The cells in the *Stratum basale* are mitotically active and generate continuously new epidermal cells. The cells in the *Stratum intermedium* form an elaborate cytoskeleton of keratin filament bundles. In the uppermost layers of the *Stratum intermedium*, the cells generate irregularly shaped, dense, basophilic keratohyalin granules that are attached to the keratin filament bundles and are visible through hematoxylin staining under light microscopy (*Tezuka* and *Takahashi* 1987). The layer of the *Stratum intermedium* that contains the cells with basophilic granules is called the *Stratum granulosum*. This *Stratum granulosum* is characteristic of the process of soft keratinisation in the epidermis of the hairy skin (*Banks* 1993) and modified epidermis of digital pads (*Künzel* 1990). At the transition between the *Stratum granulosum* and the *Stratum corneum*, these basophilic granules are disassembled through enzymatic processes. The proteins that are released form a filament-matrix complex in combination with the keratin filaments in the fully keratinized cells of the *Stratum superficiale*, thereby becoming a *Stratum corneum* of the skin (*Banks* 1993).

Table 1 Diversity of keratinization types within the adult horse hoof and synonymies of terms. (Dark grey = soft keratinization with keratohyalin granules in the *Stratum granulosum*; light grey = hard keratinization without keratohyalin granules)
Die verschiedenen Typen der Keratinisierung und der Verhornung in den Hufsegmenten des adulten Pferdes. (Dunkelgrau = weicher Verhornungstyp mit Keratohyalingranula im Stratum granulosum; hellgrau = harter Verhornungstyp ohne Keratohyalingranula)

Latin				English				German			
Main hoof parts	Hoof segments	Name of *Stratum corneum*		Main hoof parts	Hoof segments	Name of the cornified epidermal layer		Main hoof parts	Hoof segments	Name of the *Hornschicht*	
Paries corneus	*Perioplum*	*Eponychium (Stratum externum s. tectorium)*		Wall	Periople	Perioplic horn		Hufplatte	Saum	Saumhorn	
	Corona	*Mesonychium (Stratum medium)*			Coronet	Coronary horn			Krone	Kronhorn	
	Paries sensu stricto	*Hyponychium (Stratum lamellatum)*	*Laminae corneae Tubuli cornei terminales*		Wall proper	Horn of the wall proper	Cornified lamellae Terminal tubular horn		Wand	Wand-horn	Horn-blättchen Terminales Röhrchen-horn
Solea sensu lato	*Solea sensu stricto*	*Solea cornea*		Sole in the broad sense	Sole in the strict sense	Sole horn		Hufsohle im weiten Sinne	Sohle im engen Sinne	Sohlenhorn	
	Torus digitalis	*Cuneus*	*Cuneus corneus*	Digital pad	Frog	Frog horn		Ballen im weiten Sinne	Strahl	Strahlhorn	
		Torus ungulae	*Torus corneus*		Bulbs of the heel	Horn of the bulbs of the heel			Ballen im engen Sinne	Ballenhorn	

Table 2 The *Stratum granulosum* in the various segments of the digital end organ of adult mammals. (+ = *Str. granulosum* present; +* = *Str. granulosum* present only in the terminal epidermis of wall proper; +** = *Str. granulosum* incomplete or discontinuous; +*** = *Str. granulosum* only along the dorsal crest; --- = *Str. granulosum* absent; N.A. = not applicable or data not available).
Das Stratum granulosum in den verschiedenen Segmenten des Zehenendorganes von adulten Säugetieren. (+ = Str. granulosum vorhanden; + = Str. granulosum nur im terminalen Teil des Wandsegmentes vorhanden; +** = Str. granulosum unvollständig oder diskontinuierlich; +*** = Str. granulosum nur im Bereich des Rückenwulstes; --- = Str. granulosum fehlt; N.A. = nicht anwendbar oder Daten nicht vorhanden).*

Species (group) and Digital end organ	Author(s) (year)	Periople	Coronet	Wall proper	Sole	Frog	Bulbs of the heel
Horse hoof	Stoss (1906)	+	---	---	N.A.	N.A.	N.A.
	Trautmann and Fiebiger (1949)	+	---	N.A.	N.A.	N.A.	N.A.
	Ziegler (1954)	+	---	+*	---	---	N.A.
	Larsson et al. (1956)	+	---	N.A.	---	---	N.A.
	Bucher (1987)	+	---	---	---	---	+
	Budras and Huskamp (1995)	+	---	---	---	---	+
Cow hoof	Wilkens (1963)	+	---	---	+ **	+ **	+**
	Dirks (1985)	N.A.	---	---	N.A.	N.A.	N.A.
	Fürst (1992)	N.A.	N.A.	N.A.	N.A.	---	+
	Mülling (1993)	N.A.	N.A.	+ *	---	---	+
Sheep hoof	Deane et al. (1955)	+	+	+ **	+	+	+
	Wilkens (1963)	N.A.	---	---	+	+	+
	Korte (1987)	+	---	---	+	+	+.
	Rosskopf (1986)	+	N.A.	N.A.	+	+	+
Pig hoof	Giroud et al. (1934)	N.A.	---	---	---	---	+
	Kovacs and Somogyvari (1974)	+	+	---	+	+	+
	Geyer (1980)	+	---	---	N.A.	---	+
Dog claw	Stoss (1906)	N.A.	N.A.	---	N.A.	N.A.	
	Glückel (1922)	N.A.	N.A.	+***	N.A.	N.A.	
	Giroud et al. (1934)	N.A.	N.A.	N.A.	+	N.A.	
	Zietzschmann (1943)	N.A.	N.A.	N.A.	N.A.	+	
	Warner and McFarland (1970)	N.A.	N.A.	---	+	+	
	Seidel (1992)	+	---	+ *	+	N.A.	
Primate nail	Horstmann (1955)	+	---	---	N.A.	N.A.	
	Zaias and Alvarez (1968)	+	---	N.A.	N.A.	N.A.	
	Stöhr (1969)	N.A.	---	N.A.	N.A.	N.A.	
	Hashimoto (1971 b)	N.A.	---	N.A.	N.A.	N.A.	
	Stenn (1988)	N.A.	---	---	N.A.	N.A.	

Depending on the type of proteins they contain, at least two types of keratohyalin granules can be distinguished in the mammalian epidermis: (1) The specific keratohyalin granules, and (2) the non-specific keratohyalin granules. The specific keratohyalin granules contain profilaggrin, which is the precursor molecule of the keratin filament-aggregating protein filaggrin. Both profilaggrin and filaggrin are histidine-rich (*Lynley* and *Dale* 1983). Filaggrin is generated through enzymatic cleavage of profilaggrin as the cells cornifiy and eventually forms the filament-matrix complex via disulfide bonds in the fully keratinized cells (*Matoltsy* 1975, *Dale* et al. 1980). Filaggrin has been shown to be responsible for the characteristic water-binding capacity of the hairy skin (*Dale* 1985). The non-specific keratohyalin granules, in contrast, contain other sulphur-rich amorphous proteins, such as involucrin, loricrin, and keratolinin, which are precursors of proteins that form eventually the cornified cell envelope (i.e., marginal band) of fully keratinized cells (*Jessen* 1973). The marginal band is attached to the cytoplasmic surface of the cell membrane in keratinized epidermal cells (*Jessen* and *Behnke* 1986). Only the specific keratohyalin granules that contain profilaggrin and filaggrin are indicative of soft keratinization in the epidermis.

[1] The term "keratinization" (in German "Keratinisierung") as used in this study refers to the synthesis of keratin filaments and keratin filament-associated proteins in epidermal cells regardless of (1) whether a *Stratum corneum* with programmed cell death is formed, and (2) whether the process results in "soft" or "hard" horn material. The term "cornification" (in German "Verhornung") as used in this study refers to the formation of a layer of cells that have undergone programmed cell death to form the uppermost *Stratum corneum* of the epidermis regardless of whether the process results in "soft" or "hard" horn material. In order to distinguish structures and organs with hard keratinization from those with soft keratinization at the organ level, however, we follow general usage in English and restrict the term "cornified" to more or less rigid structures and organs, whose epidermis consists mostly of a relatively thick *Stratum corneum* and "hard" keratin. In contrast, the German term "verhornt" is generally applied to a thickened epidermis irrespective of whether it contains hard or soft keratin and, by extension, to the *Stratum corneum* in general. Therefore, to ensure clarity, we will use specific histological terms when discussing the epidermis at the tissue and cellular levels.

In the fetal mammalian skin, the developing epidermis contains so-called fetal keratohyalin granules in the *Stratum granulosum* (*Hayward* and *Kent* 1981). The fetal keratohyalin granules differ in their ultrastructure from the adult keratohyalin granules in the mature skin with respect to size and electron density (*Hayward* and *Kent* 1981).

In the adult cornified end organs of mammals, however, most of the keratinizing epidermis does not exhibit a *Stratum granulosum* with basophilic keratohyalin granules (*Budras* et al. 1989). Instead, the synthesized filament-associated proteins, rather than being stored in keratohyalin granules, are immediately attached to keratin filaments. The filament-associated proteins together with the keratin filaments then form a dense filament-matrix complex in the spongy network of the cytoskeleton of cornified cells (*Budras* et al. 2002, *König* and *Budras* 2003). In the epidermis of the coronet and of the wall proper (Table 1) of the adult horse hoof, for example, electrophoretic studies demonstrated sulphur-containing keratin filament-associated proteins that infiltrate the bundles of keratin filaments and stabilize the filament-matrix complex through disulfide bonds immediately after their synthesis (*Grosenbaugh* and *Hood* 1992).

However, in certain segments of the cornified end organs of adult mammals (Table 2), the epidermis does show a *Stratum granulosum* with basophilic keratohyalin granules that eventually form a filament-matrix complex to stabilize the keratinized cells in the same manner as in the soft epidermis of the skin. For adult horse hooves, for example, *Budras* and *Huskamp* (1995) described a *Stratum granulosum* that is characterized by keratohyalin granules in the periople and in the bulbs of the heel, and *Ziegler* (1954) mentioned a *Stratum granulosum* also in the epidermis of the terminal part of the wall proper, which forms the white line (i.e., *Zona alba*, formerly known as *Linea alba*). Interestingly, the *Zona alba* of adult bovine hooves always shows a *Stratum granulosum* (*Mülling* 1993). In canine claws, a *Stratum granulosum* is present not only in the epidermis of the terminal part of the wall proper, but also in the sole horn (*Budras* and *Seidel* 1992, *Süsskind* 2004). In adult human finger and toe nails, a *Stratum granulosum* is formed normally only in the perioplic epidermis (*Mörike* 1954).

In dyskeratotic and regenerating adult cornified end organs, a *Stratum granulosum* with keratohyalin granules is retained, or reappears, in those segments in which it is no longer present in the healthy state. In the dyskeratotic bovine hoof, for example, the keratohyalin granules remain almost intact instead of being broken down to form the filament-matrix complex that is typical for the *Stratum corneum* of healthy hooves (*Mülling* et al. 1999). As another example, if the nail plate is extracted from an adult human finger or toe nail, the regenerating epidermis that covers the nail bed forms a *Stratum granulosum* unlike the epidermis of a healthy nail (*Mörike* 1954).

In the cornified digital end organs of fetal mammals (Table 3), the hoof epidermis first generates fetal hoof horn in the *Stratum corneum*, which is also called a "deciduous hoof capsule" (*Bragulla* and *Budras* 1991, *Bragulla* et al. 1998). The fetal hoof epidermis of the horse also forms a *Stratum granulosum* with basophilic granules even in those hoof segments that lack one in the adult horse (*Möller* 1872, *Kunsien* 1877, *Mettam* 1896). At a certain stage of development, the fetal hoof epidermis gradually switches to a different mode of keratinization that is typical of the postnatal and adult stages. Comparable observations were made for the bovine fetal hooves (*Kunsien* 1877, *Bragulla* et al. 1994), for the ovine fetal hooves (*Korte* 1986), for the porcine fetal hooves (*Jorquera* and *Garrido* 1977), and for the fetal human finger nail (*Hashimoto* et al. 1966, *Zaias* 1966, *Runne* and *Orfanos* 1981). In addition, *Breathnach* (1981) described "atypical granules or dense homogeneous deposits" for an unspecified stage of the developing human finger nail without mentioning keratohyalin granules specifically.

Table 3 The *Stratum granulosum* in the various segments of the digital end organ in fetal mammals. (+ = *Str. granulosum* present; --- = *Str. granulosum* absent; N.A. = not applicable or data not available)
Das Stratum granulosum in den verschiedenen Segmenten des Zehenendorganes von Säugetierföten. (+ = Str. Granulosum vorhanden; --- = Str. granulosum fehlt; N.A. = nicht anwendbar oder Daten nicht vorhanden)

Species and digital end organ	Author(s) (year)	Periople	Coronet	Wall proper	Sole	Digital pad
Horse hoof	Kunsien (1882)	N.A.	N.A.	+	N.A.	N.A.
	Mettam (1896)	+	+	+	+	+
Cow hoof	Kunsien (1882)	N.A.	N.A.	+	N.A.	+
	Wilkens (1963)	+	+	+	+	+
	Bragulla (1994)	+	+	+	+	+
Sheep hoof	Korte (1987)	+	+	+	+	+
Pig hoof	Thoms (1896)	N.A.	N.A.	+	N.A.	N.A.
	Jorquera and Garrido (1977)	+	N.A.	+	N.A.	+
Dog claw	Süsskind-Schwendi (2004)	+	---	---	+	+
Cat claw	Kato (1977)	N.A.	N.A.	+	N.A.	N.A.
	Ernsberger (1998)	N.A.	N.A.	+	N.A.	N.A.
Human nail	Zaias (1963)	N.A.	+	+	N.A.	N.A.
	Hashimoto (1966)	N.A.	+	+	N.A.	N.A.
	Runne and Orfanos (1981)	N.A.	+	+	N.A.	N.A.

Hence, in adult mammals not only the skin but also certain segments of complex cornified end organs, such as horse hooves, are formed by an epidermis that retains a *Stratum granulosum* in which keratohyalin granules are made visible with hematoxylin staining of histological sections (Table 2). This raises several questions and generates hypotheses that our study tried to answer and test, respectively. First, the question arises whether the basophilic keratohyalin granules in the *Stratum granulosum* of the various segments of adult horse hooves are specific and contain profilaggrin, since (1) filaggrin is responsible for the water-binding capacity of at least the human skin (*Dale* 1985); and (2) the horn of the periople and the bulbs of the heel of the adult horse hoof has a high water-binding capacity and retains a *Stratum granulosum* with keratohyalin granules (*Budras* et al. 2002). Second, the question arises whether the keratohyalin granules in the fetal horse hoof are specific keratohyalin granules that contain profilaggrin and filaggrin, and whether these granules are attached to the keratin filaments of the cytoskeleton because of their role in forming the filament-matrix complex of cornified cells. Third, the question arises whether the development of the dermal papillary body and of the keratohyalin granules is correlated, because a papillary body increases the nutritional supply of the avascular epidermis, which might be necessary for the synthesis of the keratohyalin granules.

An elucidation of the development and identity of the keratohyalin granules and of the *Stratum granulosum* in the various segments of the horse hoof is likely to be of clinical interest, because dyskeratotic epidermis often retains keratohyalin granules instead of disassembling them in the course of the cornification process (*Mülling* et al. 1999).

Material and methods

Materials

The hooves of 53 equine fetuses (crown-rump lengths 52 - 1,050 mm, or 42 - 253 days of gestation), ten foals (new-born to 140 days old), and ten adult horses of unknown age were collected. [The gestational age of the fetuses was calculated based on their crown-rump length (CRL) using Keller´s formula published by *Schnorr* (1989)]. The hooves of the left fore and hind limbs were studied with light microscopy (i.e., histochemistry and immunohistochemistry), and the hooves of the right fore and hind limbs were studied with transmission electron microscopy.

Methods

Light microscopy: The left hooves were sagittally cut in halves and fixed in buffered formalin (pH 7.2) for several days at room temperature. From each segment of each half of the hooves, tissue samples (10 x 10 x 20 mm^3) were obtained, dehydrated in a graded series of ethanol, and embedded in paraffin. Sagittal and horizontal sections (5-7 μm thick) were prepared using a microtome (Reichert-Jung, Heidelberg, Germany). The sections were rehydrated and stained with hematoxylin and eosin or with the trichrom staining procedure according to Masson and Goldner (*Romeis* 1989) to reveal the cellular structure, including the keratin filaments.

Histochemistry

To identify and visualize the amino acid histidine in the specific keratohyalin granules, the histological sections were incubated according to *Kiszely* and *Posalaky* (1964) in potassium iodine (to prevent the amino acids tyrosine and tryptophan from binding to Fast-Blue B) and subsequently stained in a phosphate-buffered solution of Fast-Blue B to detect the amino acid histidine. After repeatedly rinsing the histological sections in phosphate buffer, the stain was stabilized by incubating the stained sections in H-acid (1-Amino-8-naphtol-3,6-bisulfonic acid). As a result, the specific keratohyalin granules were visible as dense, dark red or brown deposits.

Immunohistochemistry

To detect and identify the keratin filament-associated proteins filaggrin and its precursor profilaggrin, the mouse-anti-human-filaggrin antibody (Cat.-No. 5100193, Paesel & Lorei, Hanau/Germany) was applied as primary antibody to histological sections in a dilution of 1 : 100. Subsequently, the biotinylated secondary antibody, a Goat-anti-mouse-IgG-antibody, was applied to the histological sections in a dilution of 1 : 500. This secondary antibody was visualized in the histological sections with avidin-peroxidase and diaminobenzidine and hydrogen peroxide. The diaminobenzidine precipitated as deposits of brown pigments on the bound primary and secondary antibody chains. Specimens of human and equine skin were used as positive controls, and specimens of feline small intestine were used as negative controls.

Transmission electron microscopy

The right hooves were sagittally cut into 5 mm thick sections. From each section, some samples were obtained, trimmed to a size of 2 x 2 x 5 mm^3, and fixed in a cacodylate-buffered solution of 2 % glutaraldehyde for three days at 6° C. Subsequently, they were rinsed in cacodylate-buffer and post-fixed in 1% osmium tetroxyde. The specimens were then dehydrated in a graded series of ethanol and embedded in epoxy-resin (Epon®). Semi-thin sections (1 μm) were prepared and stained in a solution of 1 % methylene blue. Ultra-thin sections (40 - 60 nm) were cut from regions of interest as identified in the semi-thin sections and collected on nickel grids. The ultra-thin sections were subsequently stained with 5% uranyl acetate and 3 % lead citrate to enhance the contrast prior to studying them using a transmission electron microscope (Type EM 10, Zeiss AG, Oberkochen/Germany).

Chemistry

The proteins of the keratohyalin granules in the *Stratum granulosum* were extracted according to *Ugel* (1975) and *Ugel* and *Idler* (1972) from some of the samples (2 x 2 x 5 mm^3) that were obtained from the right hoof sections by incubating the specimens in a 1 molar solution of potassium phosphate (pH 7.0) for twelve hours. Subsequently, the specimens were rinsed in phosphate puffer and processed for the transmission electron microscopic study as described above.

The treated specimens were compared to the untreated ones, and the holes in the cells of the potassium phosphate-treated specimens were identified as the locations from which the proteins of the keratohyalin granules had been extracted.

Results

The morphology of the epidermis of the fetal horse hoof changes during development (Fig. 1) and differs significantly from the adult hoof (Figs. 2 and 3). These changes are the result of developmental modifications at the tissue and cellu-

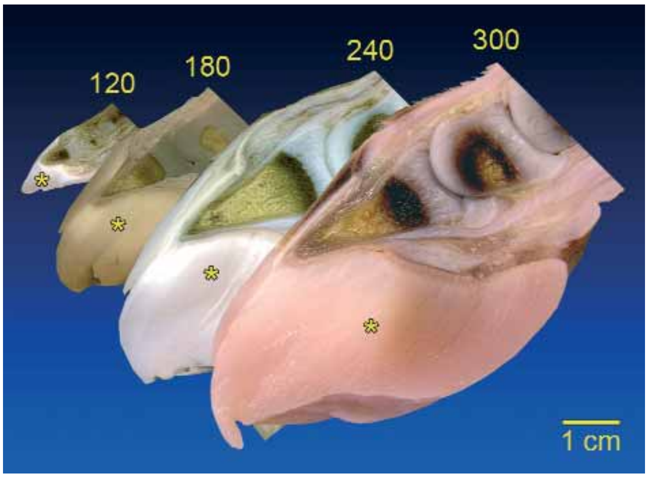


Fig 1 Developmental series of median sections of forelimb hooves of fetal horses to show the increase in thickness of the fetal Stratum corneum (i.e., deciduous hoof capsule) (*). From left to right: 120 days of gestation (CRL ca. 220 mm), 180 days of gestation (CRL ca. 550 mm), 240 days of gestation (CRL ca. 850 mm), and 300 days of gestation (CRL ca. 1050 mm).
Die Entwicklungsserie von fötalen Pferdehufen im Medianschnitt zeigt die Dickenzunahme des fötalen Stratum corneum (d.h. hinfällige Hufkapsel). Von links nach rechts: 120 Tage der Trächtigkeit (Scheitelsteißlänge ca. 220 mm), 180 Tage der Trächtigkeit (Scheitelsteißlänge ca. 550 mm), 240 Tage der Trächtigkeit (Scheitelsteißlänge ca. 850 mm), und 300 Tage der Trächtigkeit (Scheitelsteißlänge ca. 1050 mm).

lar levels. Our study reveals that specific, profilaggrin containing keratohyalin granules in a *Stratum granulosum* (Figs. 4 and 5, Table 4) are generated in all segments of the fetal hoof epidermis. However, the developmental stages at which the specific profilaggrin-containing keratohyalin granules appear, and in certain segments later disappear, are specific for each hoof segment.

The light microscopical observations revealed that prior to the appearance of the keratohyalin granules, the cells of the *Stratum basale* proliferate, which results in an interdigitation of the epidermal and dermal tissues along the basement membrane. This interdigitated tissue complex is called the papillary body (Fig. 6). Only after the onset of the formation of the segment-specific papillary body, the cells of the *Stratum intermedium* generate an increased amount of keratin filaments (Fig. 4). These cells also produce basophilic keratohyalin granules containing profilaggrin in the uppermost two to three layers of the *Stratum intermedium* next to the *Stratum corneum*, as revealed by immunohistochemistry (Fig. 5). [In the positive control (i.e., human and equine skin), the primary antibody used in our study also attached specifically to the keratohyalin granules, whereas the negative control (i.e., cat intestine) remained unstained.] The specific, profilaggrin-containing keratohyalin granules are dense, homogeneous, and variable in shape and size (i.e., 5 - 25 % of the size of cell nucleus, Fig. 4 inlay). The keratohyalin granules are attached to the cytoskeletal network of keratin filaments and are gathered around the cell nucleus as revealed by the histochemical

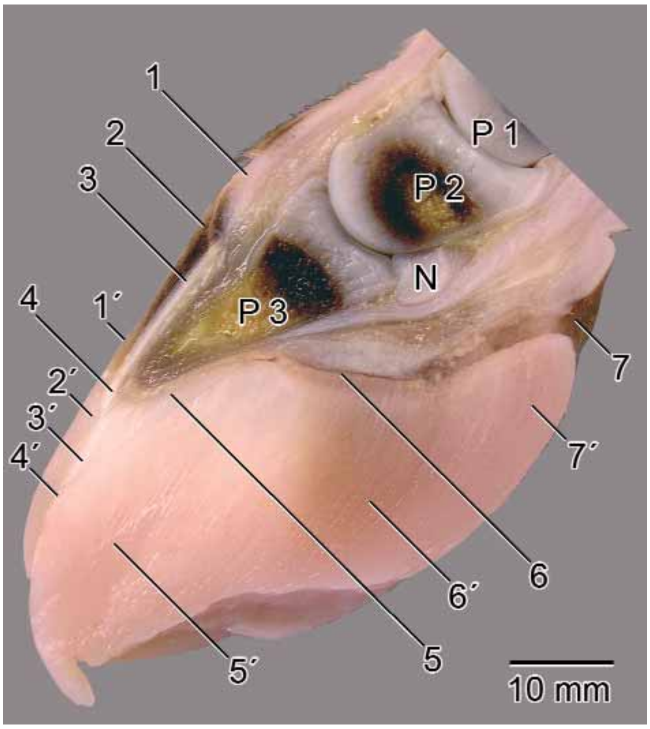


Fig 2 Median section through the left forelimb hoof of an equine fetus at 300 days of gestation to show the permanent hoof epidermis covered by the deciduous hoof capsule. 1 = permanent periople, 1' = perioplic horn of the deciduous hoof capsule, 2 = permanent coronet, 2´= coronary horn of the deciduous hoof capsule, 3 = permanent wall proper, 3´ = wall horn of the deciduous hoof capsule, 4 = permanent white line (*Zona alba*), 4´ = *Zona alba* of the deciduous hoof capsule, 5 = permanent sole, 5´ = sole horn of the deciduous hoof capsule, 6 = permanent frog, 6´ = horn of the frog of the deciduous hoof capsule, 7 = permanent bulbs of the heel, 7´ = horn of the bulbs of the heel of the deciduous hoof capsule. P 1 = proximal phalanx, P 2 = middle phalanx, P 3 = distal phalanx, N = navicular bone (distal sesamoid bone).
Der Medianschnitt durch den Huf der linken Vordergliedmaße eines 300 Tage alten Pferdefötus zeigt die permanente Hufkapsel, die von der hinfälligen Hufkapsel bedeckt ist. 1 = permanente Saumepidermis, 1' = Saumhorn der hinfälligen Hufkapsel, 2 = permanente Kronepidermis, 2´= Kronhorn der hinfälligen Hufkapsel, 3 = permanentes Wandsegment, 3´ = Wandhorn der hinfälligen Hufkapsel, 4 = permanente Weiße Linie (Zona alba), 4´ = Zona alba der hinfälligen Hufkapsel, 5 = permanente Sohlenepidermis, 5´ = Sohlenhorn der hinfälligen Hufkapsel, 6 = permanente Strahlepidermis, 6´ = Strahlhorn der hinfälligen Hufkapsel, 7 = permanente Ballenepidermis, 7´ = Ballenhorn der hinfälligen Hufkapsel. P 1 = Phalanx proximalis, P 2 = Phalanx media, P 3 = Phalanx distalis, N = Os sesamoideum distale.

stain of the amino acid histidine within the keratohyalin granules. The histidine content of these keratohyalin granules provides further support for identifying them as specific (i.e., profilaggrin-containing) keratohyalin granules. After the onset of the production of the specific keratohyalin granules, the newly cornifying cells exhibit a dense, more acidophilic cytoskeleton (Fig. 4). The development of specific, profilaggrin-containing keratohyalin granules in the wake of the formation of the papillary body, however, proceeds at different developmental stages in the various hoof segments (Table 4).

The ultrastructural observations revealed details of the structure of the keratohyalin granules in the epidermis of the fetal hooves (Figs. 6 and 7). These keratohyalin granules are not enclosed by any membrane and have a high affinity to osmium. Their content is a combination of amorphous, highly electron-dense material and of filaments that are significantly thinner than keratin filaments. The keratohyalin granules are located near the nucleus and are interspersed among

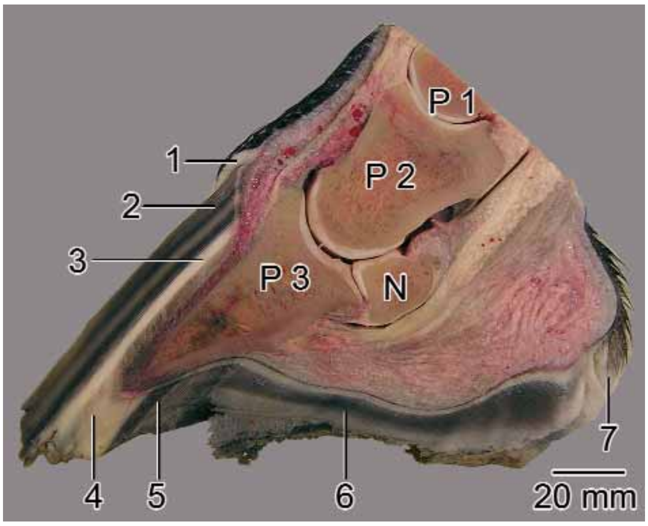


Fig 3 Median section of the left forelimb hoof of a 15 years old mare to show the permanent, adult segment-specific epidermis. 1 = periople, 2 = coronet, 3 = wall proper, 4 = white line (*Zona alba*), 5 = sole, 6 = frog, 7 = bulbs of the heel. P 1 = proximal phalanx, P 2 = middle phalanx, P 3 = distal phalanx, N = navicular bone.
Der Medianschnitt durch die linke Vordergliedmaße einer 15 Jahre alten Stute zeigt die Segmente im Hufe adulter Pferde. 1 = Saum, 2 = Krone, 3 = Wand, 4 = Weiße Linie (Zona alba), 5 = Sohle, 6 = Strahl, 7 = Ballen. P 1 = Phalanx proximalis, P 2 = Phalanx media, P 3 = Phalanx distalis, N = Os sesamoideum distale.

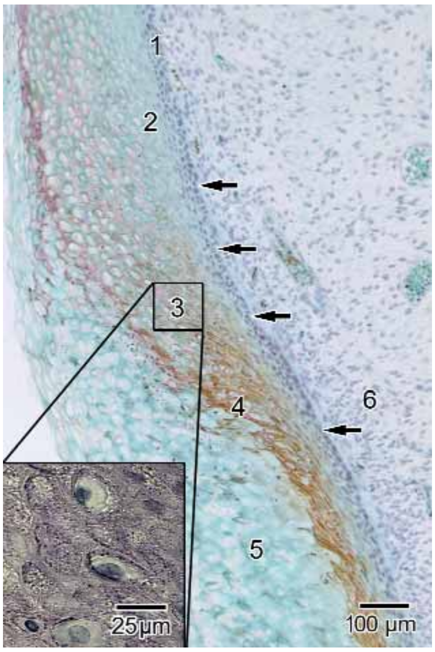


Fig 4 Histological median section through the coronet and wall proper of the left hoof of an equine fetus (120 days of gestation) stained with trichrom (Masson and Goldner) to show the difference between the newly formed and the "old" parakeratotic fetal *Stratum corneum*. Inlay (stained with hematoxylin and eosin) to show epidermal cells of the *Stratum granulosum* containing numerous basophilic keratohyalin granules of variable size and gathering preferentially around the cell nucleus. 1 = *Stratum basale* of coronary epidermis, 2 = *Stratum spinosum* of coronary dermis, 3 = *Stratum granulosum* of the coronary epidermis, 4 = newly formed layer of the *Stratum corneum* of the deciduous hoof capsule, 5 = parakeratotic "old" layers of the *Stratum corneum* of the deciduous hoof capsule, 6 = dermis of the coronary segment. Arrows point to developing dermal papillae.
Der histologische Medianschnitt, trichrom-gefärbt (Masson und Goldner), durch das Kron- und Wandsegment des Hufes einer linken Gliedmaße eines Pferdefötus (120 der Trächtigkeit) zeigt den Unterschied zwischen dem neu geformten und dem „alten" parakeratotischen fötalen Stratum corneum. Der vergrößerte Ausschnitt eines histologischen Schnittes desselben Präparates, gefärbt mit Hämatoxylin und Eosin, zeigt die epidermalen Zellen des Stratum granulosum mit ihren basophilen Keratohyalingranula, die von unterschiedlicher Größe sind und überwiegend um den Zellkern herum angeordnet sind. 1 = Stratum basale der Kronepidermis, 2 = Stratum spinosum der Kronepidermis, 3 = Stratum granulosum der Kronepidermis, 4 = neu gebildetes Kronhorn der hinfälligen Hufkapsel, 5 = parakeratotisches, „altes" Horn des Stratum corneum der hinfälligen Hufkapsel, 6 = Lederhaut des Kronsegmentes. Die Pfeile weisen auf die sich entwickelnden Lederhautpapillen.

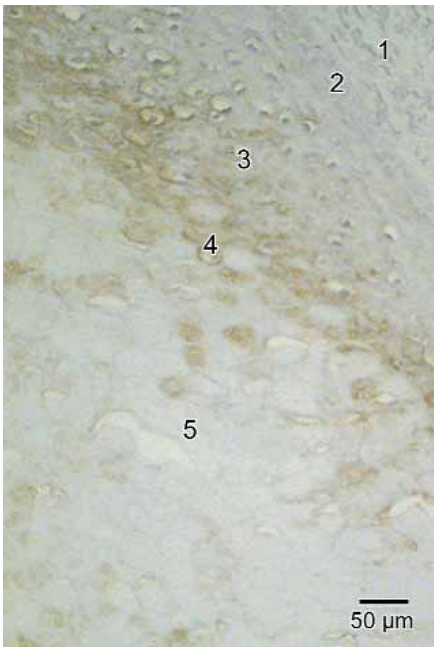


Fig 5 Histological sagittal section through the coronet of the hoof of an equine fetus (120 days of gestation) treated with anti-human filaggrin antibody to visualize specific keratohyalin granules (brown deposits) containing profilaggrin and filaggrin. 1 = Dermis, 2 = *Stratum basale*, 3 = *Stratum granulosum*, 4 = newly formed *Stratum corneum* of the deciduous hoof capsule, 5 = "old" parakeratotic *Stratum corneum* of the deciduous hoof capsule.
Immunhistochemischer Nachweis von Profilaggrin und Filagrin in den spezifischen Keratohyalingranula (braune Farbniederschläge) mit dem anti-human filaggrin-Antikörper im lichtmikroskopischen Sagittalschnitt durch das Kronsegment des Hufes eines 120 Tage alten Pferdefötus. 1 = Kronlederhaut, 2 = Stratum basale, 3 = Stratum granulosum, 4 = neu gebildetes Stratum corneum der hinfälligen Hufkapsel, 5 = „altes", parakeratotisches Stratum corneum der hinfälligen Hufkapsel.

Table 4 Timing (days of gestation) of the development of the papillary body and the *Stratum granulosum* in the various segments of the fetal horse hoof.
Zeitlicher Ablauf (in Tagen der Trächtigkeit) der Entwicklung des segmentspezifischen Papillarkörpers und des Stratum granulosum in den verschiedenen Hufsegmenten eines Pferdefötus.

Segment of the equine hoof		Development of the segment-specific papillary body		Development of the *Stratum granulosum* with basophilic, profilaggrin-containing keratohyalin granules		
		Onset	Completion	Appearance	Completion	Disappearance
Periople		147	191	156	215	no
Coronet		69	172	82	no	106
Wall proper	proximal 3/4	69	172	82	no	156
	distal 1/4	69	172	82	221	330 (i.e., birth)
Sole		78	191	106	no	208
Digital pad	Frog	115	172	130	no	220
	Bulbs of heel	172	208	215	224	no

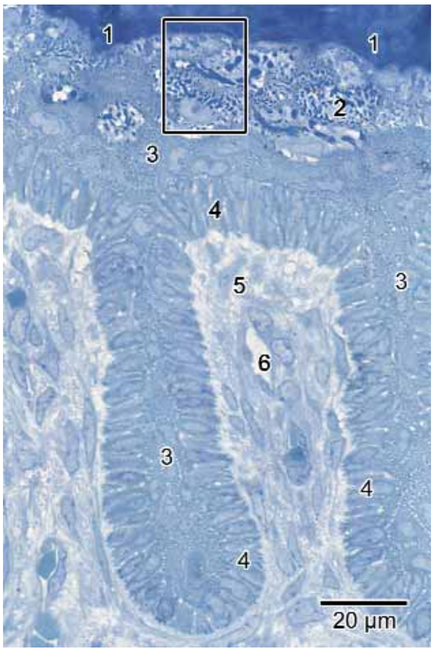


Fig 6 Histological semi-thin transverse section through the proximal one-third of the wall proper of the hoof of an equine foetus (120 days of gestation.), stained with methylene blue, to show the vascularized dermal papillary body and the avascular epidermis. 1 = newly formed *Stratum corneum*, 2 = *Stratum granulosum*, 3 = *Stratum spinosum*, 4 = *Stratum basale*, 5 = primary dermal lamina, 6 = capillary. The frame indicates the location of Figure 7.
Der transversale Semidünnschnitt, gefärbt mit Methylenblau, durch das proximale Drittel des Wandsegmentes des Hufes eines Pferdefötus (120 Tage der Trächtigkeit) zeigt den gefäßführenden Papillarkörper der Lederhaut und die gefäßlose Hufoberhaut. 1 = neu gebildetes Stratum corneum, 2 = Stratum granulosum, 3 = Stratum spinosum, 4 = Stratum basale, 5 = primäres Lederhautblättchen, 6 = Kapillare. Der Rahmen zeigt die Stelle der Abbildung 7.

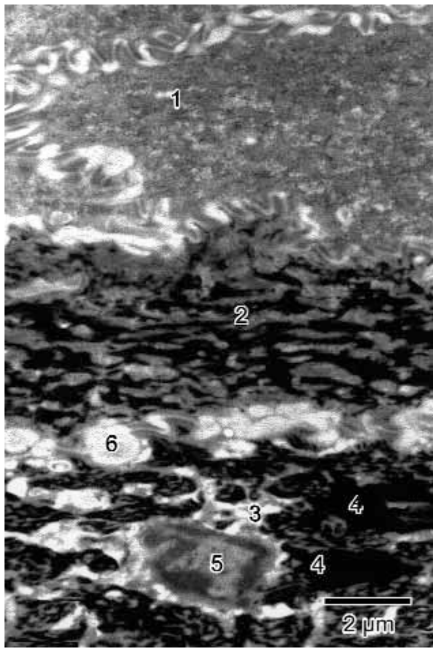
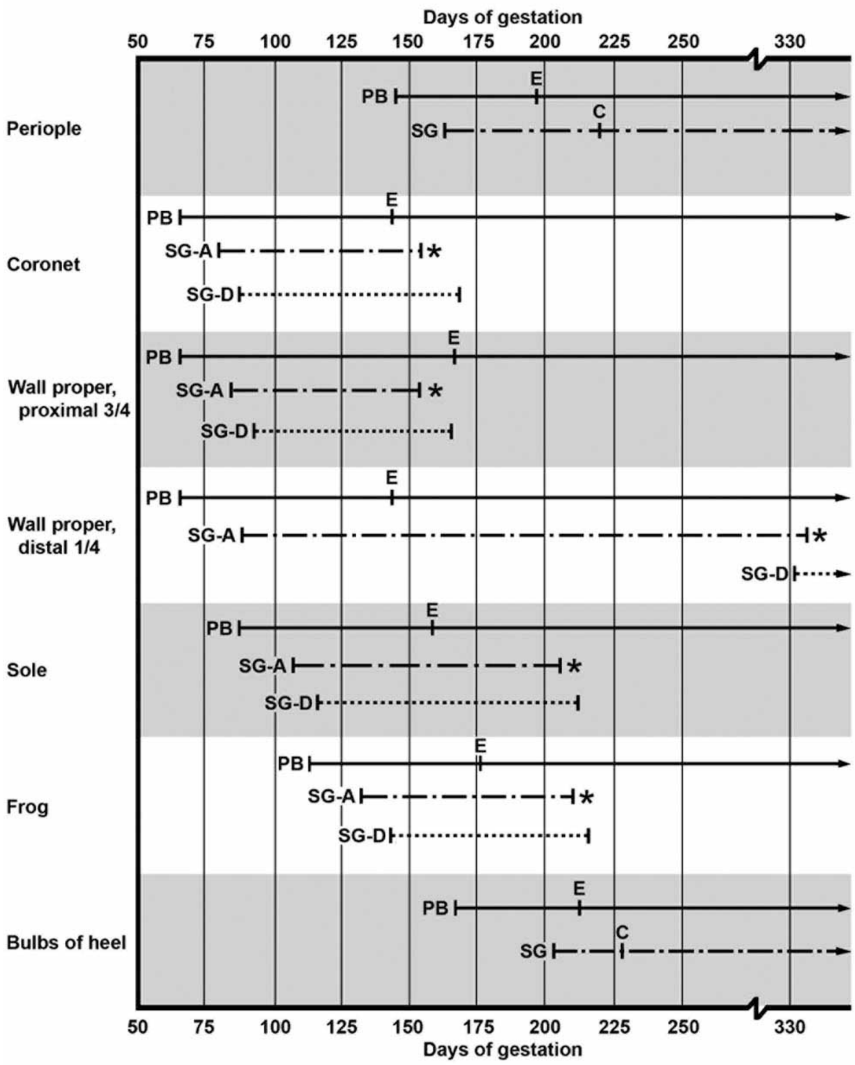


Fig 7 Transmission electron microscopic transverse section through the wall proper of the hoof of an equine fetus (120 days of gestation) to show the granular and transitional cells of the *Stratum granulosum* being denser and smaller than the newly formed cornified cell of the *Stratum corneum* of the fetal hoof epidermis. 1 = newly formed cornified cell with marble-like structure, 2 = transitional cell without a nucleus, 3 = granular cell of the *Stratum granulosum*, 4 = keratohyalin granule, 5 = nucleus of the granular cell, 6 = intercellular space.
Der transmissionselektronenmikroskopische Querschnitt durch das proximale Drittel des Wandsegmentes des Hufes eines Pferdefötus (120 Tage der Trächtigkeit) zeigt die dichten und vergleichsweise dünnen Zellen des Stratum granulosum mit den Übergangszellen und die neu geformten Zellen des Stratum corneum mit ihrer marmorierten Struktur in der fötalen Hufepidermis. 1 = neu geformte Hornzelle mit ihrer marmorierten Struktur, 2 = Übergangszelle ohne Zellkern, 3 = granulierte Zelle des Stratum granulosum, 4 = Keratohyalingranulum, 5 = Kern der Zelle im Stratum granulosum, 6 = Interzellularraum

the keratin filaments of the cytoskeleton (Fig. 7). The bundles of keratin filaments are of moderate electron density. Near the *Stratum corneum*, the cells of the *Stratum granulosum* show the first signs of programmed cell death, as indicated by their pycnotic nucleus. The keratohyalin granules show deep marginal indentations, and the electron-dense material of the keratohyalin granules partially infiltrates the cytoskeleton of the cells of the *Stratum granulosum*. In the *Stratum corneum*, the electron-dense material of the keratohyalin granules and the moderately electron-dense material of the cytoskeletal keratin filaments form a filament-matrix complex that almost completely fills the cornified cell. The heterogeneous electron density of the filament-matrix complex gives the cornified cells

Fig 8 Time table to show the segment-specific development of the papillary body and *Stratum granulosum* in the hoof of the fetal and newborn horse. (C = completion of the *Stratum granulosum* within an entire hoof segment, E = complete establishment of the segment-specific papillary body within an entire hoof segment, PB = papillary body, SG = *Stratum granulosum*, SG-A = appearance of the *Stratum granulosum*, SG-D = disappearance of the *Stratum granulosum*, * = onset of the segment-specific differentiation of the permanent hoof epidermis).
*Die Abbildung zeigt den zeitlichen Verlauf der Entwicklung des segmentspezifischen Papillarkörpers und des Stratum granulosum in den einzelnen Hufsegmenten des Pferdefötus und neugeborenen Fohlens. (C = Stratum granulosum im ganzen Hufsegment ausgebildet, E = segmentspezifischer Papillarkörper im ganzen Hufsegment ausgebildet, PB = Papillarkörper, SG = Stratum granulosum, SG-A = erstes Auftreten eines Stratum granulosum, SG-D = Stratum granulosum nicht mehr vorhanden, * = Beginn des segmentspezifischen Keratinisierungs- und Verhornungsmodus.*



a marble-like structure (Fig. 7) that resembles the ultrastructure of the cornified epidermal cells of the periople and skin of adult horses. The cornified cells of the fetal hoof epidermis possess also a thickened cell membrane (i.e., marginal band or cellular envelope), because the cytoplasmic surface of the cell membrane is lined by a band of homogeneous material of moderate electron density. This band is about as thick as the cell membrane.

The onset of the development of the segment-specific papillary body and the appearance of the *Stratum granulosum* is different for each of the hoof segments (Tab. 4, Fig. 8).

In the periople, the formation of a segment-specific papillary body starts in the hoof of a fetus of about 147 days of gestation (CRL 380 mm) and is established at 191 days of gestation (CRL 600 mm) (Table 4, Fig. 8). A *Stratum granulosum* starts to develop in the dorsodistal part of the perioplic epidermis at ca. 156 days of gestation (CRL 420 mm) and is completed in the entire periople at ca. 215 days of gestation (CRL 750 mm). After the *Stratum granulosum* has been completed (Fig. 9 and 10), the number of keratohyalin granules increases, and the *Stratum granulosum* thickens to about ten to twelve cell layers in the adult horse (Fig. 11) and extends to the hairy skin where the *Stratum granulosum* is only about three to six cell layers thick (Fig. 12). The *Stratum granulosum* is retained in the perioplic epidermis of the adult hoof.

In the coronet, the formation of a segment-specific papillary body starts to develop in the dorsodistal part of the coronary segment at 69 days of gestation (CRL 110 mm) (Table 4, Fig. 8). The segment-specific papillary body is established in the entire coronary segment at 172 days of gestation (CRL 500 mm). The first keratohyalin granules appear in the uppermost layers of the *Stratum intermedium* of the distal part of the coronary epidermis at 82 - 106 days of gestation (CRL 150 - 220 mm). The cells containing these keratohyalin granules form a thin *Stratum granulosum* that is overlain by a parakeratotically keratinized *Stratum superficiale* (Fig. 4). (Parakeratotic cells form only a sparse cytoskeleton consisting of thin keratin filament bundles.) After about 162 days of gestation (CRL ca. 450 mm), the keratohyalin granules are no longer synthesized in the coronary epidermis.

In the wall proper, a segment-specific papillary body starts to develop in the dorsoproximal part of the wall segment at 69 days of gestation (CRL 110), and the segment-specific papillary body of the wall proper is established at 172 days of gestation (CRL 500 mm) (Table 4, Fig. 8). The keratohyalin granules appear in the proximal part of the wall proper at 82 - 106 days of gestation (CRL 150 - 220 mm). The cells that contain these keratohyalin granules form a very thin (one to three cells thick) *Stratum granulosum* in the proximal three quarters of the epidermis of the wall proper at 82 - 156 days of gestation (CRL 150 to 420 mm). This *Stratum granulosum*

Fig 9 Histological sagittal section through the periople (with *Stratum granulosum*) and the coronet (without *Stratum granulosum*) of the hoof of a newborn foal (stained with hematoxylin and eosin) to show the segment-specific stratification of the epidermis. 1 = *Stratum corneum* of the periople, 2 = *Stratum granulosum* of the periople, 3 = papillary body of the periople, 4 = Subcutis of the periople, 5 = Subcutis of the coronet, 6 = papillary body of the cornet, 7 = *Stratum spinosum* of the coronet, 8 = *Stratum corneum* of the coronet.

Der histologische Längsschnitt, gefärbt mit Hämatoxylin und Eosin, durch das Saumsegment (mit Stratum granulosum) und durch das Kronsegment (ohne Stratum granulosum) im Hufe eines neugeborenen Fohlens zeigt den segmentspezifischen Aufbau der Hufepidermis. 1 = Stratum corneum der Saumepidermis, 2 = Stratum granulosum der Saumepidermis, 3 = Papillarkörper im Saumsegment, 4 = Subcutis im Saumsegment, 5 = Subcutis im Kronsegment, 6 = Papillarkörper im Kronsegment, 7 = Stratum spinosum in der Kronepidermis, 8 = Stratum corneum der Kronepidermis.

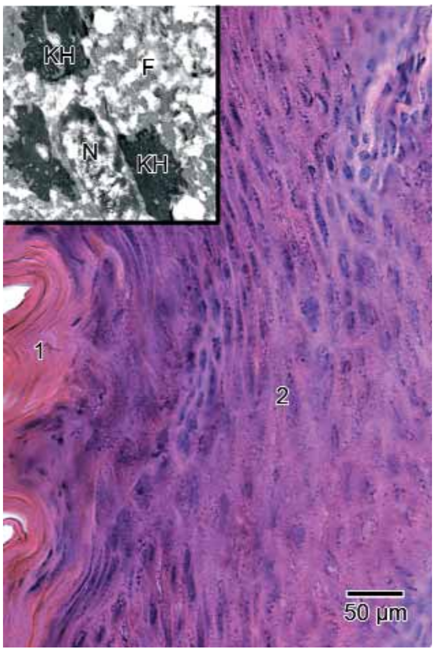


Fig 10 Histological sagittal section through the periople of the hoof of a newborn foal (stained with hematoxylin and eosin) to show a thin and pliable *Stratum corneum* covering the thick *Stratum granulosum* formed by several cell layers with basophilic keratohyalin granules. Inlay: Transmission electron microscopic section through the *Stratum granulosum* of the periople to show the electron-dense keratohyalin granules and the keratin filaments of the cytoskeleton. 1 = *Stratum corneum*, 2 = *Stratum granulosum*, F = keratin filaments, KH = keratohyalin granule, N = cell nucleus.

Der histologische Längsschnitt, gefärbt mit Hämatoxylin und Eosin, durch die Saumepidermis im Hufe eines neugeborenen Fohlens zeigt das dünne und flexible Stratum corneum, welches das dicke Stratum granulosum bedeckt, das aus zahlreichen Lagen von Zellen besteht, die basophile Keratohyalingranula enthalten. Ausschnitt: Der transmissionselektronenmikroskopische Ultradünnschnitt zeigt eine Zelle im Stratum granulosum der Saumepidermis mit den Keratohyalingranula und den Keratinfilamenten im Zytoskelett. 1 = Stratum corneum, 2 = Stratum granulosum. F = Keratinfilamente, KH = Keratohyalingranulum, N = Zellkern.

Fig 11 Histological sagittal section through the periople of the hoof of an adult horse treated with anti-human filaggrin antibody to visualize profilaggrin and filaggrin. 1 = cells of the *Stratum granulosum* with specific, profilaggrin-containing keratohyalin granules, 2 = obliquely sectioned dermal papillae.

Immunhistochemischer Nachweis von Profilaggrin in einem histologischen Längsschnitt durch das Saumsegment eines adulten Pferdes mittels anti-human Filaggrin-Antikörper. 1 = Zelle des Stratum granulosum mit den specifischen, Profilaggrin enthaltenden Keratohyalingranula, 2 = Schrägschnitt durch eine Lederhautpapille.

is overlain by a parakeratotically keratinized *Stratum superficiale*. In the terminal part (i.e., the distal one quarter) of the epidermis of the wall proper, which forms the white line (i.e., *Zona alba*), a *Stratum granulosum* is still maintained in the hooves of new-born foals (CRL ca. 1200 mm) and of foals of at least three days of age (Figs. 13, 14 and 15).

In the sole, a segment-specific papillary body starts to develop at 78 days of gestation (CRL 135 mm) and is completed at 191 days of gestation (Table 4, Fig. 8). The *Stratum granulosum* starts to develop at 106 - 115 days of gestation (CRL 220 - 250 mm) near the terminal part of the wall proper and proceeds towards the centre of the sole segment as well as towards the frog. In fetuses older than ca. 208 days of gestation (CRL >700 mm), the sole epidermis does not form a *Stratum granulosum* any longer.

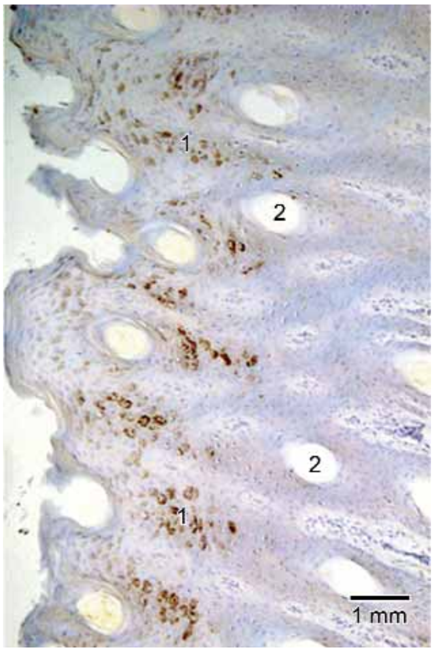


Fig 12 Histological transverse section through the skin proximal to the periople of the hoof of an adult horse, treated with anti-human filaggrin antibody to visualize profilaggrin and filaggrin. 1 = cells of the *Stratum granulosum* of the interfollicular epidermis with specific, profilaggrin-containing keratohyalin granules; 2 = hair follicle.
Immunhistochemischer Nachweis von Profilaggrin in einem histologischen Querschnitt durch die Haut oberhalb des Hufsaumes eines adulten Pferdes mittels anti-human Filaggrin-Antikörper. 1 = Zelle des Stratum granulosum in der interfollikulären Epidermis mit spezifischen, Profilaggrin enthaltenden Keratohyalingranula; 2 = Haarfollikel.

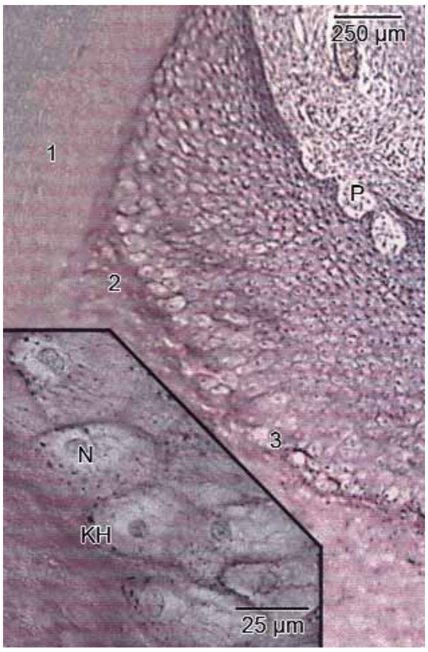


Fig 13 Histological sagittal section through the coronary horn and terminal part of the wall proper of the hoof of an equine fetus (115 days of gestation), stained with hematoxylin and eosin, to show the thin *Stratum granulosum* and a developing papillary body. Inlay shows basophilic keratohyalin granules. 1 = *Stratum corneum* of the coronet, 2 = *Stratum corneum* of the wall proper, 3 = thin *Stratum granulosum* in the terminal part of the wall proper. KH = keratohyalin granule, N = nucleus of a cell of the *Stratum granulosum*, P = developing terminal dermal papilla.
Der histologische Längsschnitt, gefärbt mit Hämatoxylin und Eosin, durch das Kronhorn und den terminalen Teil des Wandsegmentes des Hufes von einem Pferdefötus (115 Tage der Trächtigkeit) zeigt das dünne Stratum granulosum und die Entwicklung des Papillarkörpers. Der vergrößerte Ausschnitt zeigt die Keratohyalingranula. 1 = Stratum corneum der Krone, 2 = Stratum corneum des Wandsegmentes, 3 = dünnes Stratum granulosum im terminalen Teil des Wandsegmentes. KH = Keratohyalingranulum, N = Kern einer Zelle im Stratum granulosum, P = Terminalpapille in der Wandlederhaut.

Fig 14 Histological sagittal section through the coronary horn and terminal part of the wall proper of the hoof of an equine fetus (210 days of gestation), stained with hematoxylin and eosin, to show a thick *Stratum granulosum* with a well-developed segment-specific papillary body. 1 = tubular horn of the permanent *Stratum corneum* of the coronet, 2 = laminar horn of the permanent wall proper, 3 = *Stratum granulosum* of the terminal part of the wall proper, 4 = dermal laminae, 5 = Dermis. Arrows indicate a row of obliquely sectioned terminal dermal papillae. The frame indicates the location of Figure 15.
Der histologische Längsschnitt, gefärbt mit Hämatoxylin und Eosin, durch das Kronhorn und den terminalen Teil des Wandsegments im Huf eines Pferdefötus (210 Tage der Trächtigkeit) zeigt das dicke Stratum granulosum und den segmentspezifischen Papillarkörper. 1 = Röhrchenhorn des permanenten Stratum corneum der Krone, 2 = Blättchenhorn der permanenten Wandepidermis, 3 = Stratum granulosum im terminalen Teil der Wandepidermis, 4 = Lederhautblättchen, 5 = Dermis. Die Pfeile weisen auf eine Reihe von schräg angeschnittenen Terminalpapillen. Der Rahmen zeigt die Stelle der Abbildung 15.

In the frog and the bulbs of the heel, the formation of the papillary body starts in the abaxial grooves of the frog at 115 days of gestation (CRL 250 mm) and proceeds to the axial groove of the frog and to the bulbs of the heel at ca. 172 days of gestation (CRL ca. 500 mm) (Table 4, Fig. 8). The *Stratum granulosum* appears in the frog when the papillary body has achieved a certain height in the hoof of a fetus of 130 days of gestation (CRL 310 mm). The *Stratum granulosum* is thickest in the abaxial grooves and at the tip of the frog. It extends into the bulbs of the heel at ca. 215 days of gestation (CRL 750-780 mm). After birth, a *Stratum granulosum* is no longer formed in most of the frog, but is retained in the base of the frog and in the bulbs of the heel. The *Stratum granulosum* of the bulbs of the heel is continuous with that of the periople.

In conclusion, the timing of the appearance of the *Stratum granulosum* is dictated by the onset of the formation of the papillary body, but the subsequent timing of the completion and disappearance as well as the duration of the *Stratum granulosum* are specific to the individual hoof segments (Table 4; Fig. 8).

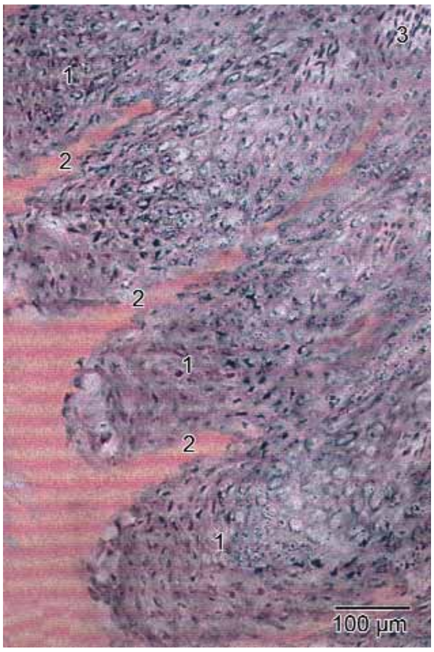


Fig 15 Histological sagittal section through the terminal part of the wall proper of the hoof of an equine fetus (210 days of gestation), stained with hematoxylin and eosin, to show a magnified view of the *Stratum granulosum* between cornified epidermal laminae. 1 = five to six cell layers of the *Stratum granulosum* with basophilic keratohyalin granules, 2 = cornified epidermal lamina, 3 = terminal dermal papilla.
Der histologische Längsschnitt, gefärbt mit Hämatoxylin und Eosin, durch das Kronhorn und den terminalen Teil des Wandsegmentes im Huf eines Pferdefötus (210 Tage der Trächtigkeit) zeigt eine Vergrößerung des dicken Stratum granulosum zwischen den Hornblättchen. 1 = Fünf bis sechs Zelllagen des Stratum granulosum mit den basophilen Keratohyalingranula, 2 = Hornblättchen, 3 = terminale Lederhautpapille.

Discussion

Although various earlier authors (*Möller* 1872, *Kunsien* 1882, *Mettam* 1896) observed basophilic granules in the epidermis of fetal horse hooves, our data differentiate and complete the picture of the fetal development of the equine hoof epidermis. A more complete knowledge of epidermal development is the prerequisite for a better understanding of dyskeratotic processes of the horse hoof, such as laminitis or hoof cancer (*Marks* and *Budras* 1985).

Whereas basophilic keratohyalin granules in the epidermis of fetal cornified end organs have been mentioned for the cow, sheep, pig, cat, and human (Table 3), they were mentioned for the horse only by early authors (e.g., *Möller* 1872, *Kunsien* 1882, *Mettam* 1896) and subsequently forgotten. The recent discovery of at least two types of keratohyalin granules in the human epidermis (*Jessen* 1973, *Jessen* and *Behnke* 1986) raised the possibility that such a differentiation also occurs in the skin, in the fetal hoof, and in the hoof segments with soft keratinization in the adult horse (i.e., the periople and bulbs of the heel). Our immunohistochemical experiments demonstrate that the basophilic granules in the epidermis of the entire fetal hoof and of the adult periople and bulbs of the heel are specific keratohyalin granules that contain profilaggrin.

The presence of specific, profilaggrin-containing keratohyalin granules in the epidermis of the entire fetal hoof and in the periople and bulbs of the heel of the adult hoof suggests a correlation between the presence of filaggrin and a high water-binding capacity of the keratinizing epidermis, as was already mentioned by *Dale* (1985) for the human skin. Our study on the fetal and adult horse hooves suggests in addition that the water-binding capacity may also be correlated with an increased flexibility of the epidermis. The fetal hoof epidermis is relatively soft in order not to injure the fetal membranes or damage the birth canal during parturition. In the adult horse hoof, the epidermis of the periople and of the bulbs of the heel is softer than that of the other hoof segments and may act as a shock absorber between the hard hoof horn and the soft hairy skin of the digit. The softer epidermis of the periople also protects the newly formed horn along the proximal margin of the coronet (*Budras* et al. 2002), and the softer epidermis of the bulbs of the heel supports the hoof mechanism (*Dyce* et al. 2002).

Our study shows for the first time that a *Stratum granulosum* is formed in all segments of the fetal horse hooves. It also provides an explanation for some of the contradictory observations by earlier authors. Because the onset, completion, and duration of the *Stratum granulosum* differ in the various segments of the fetal horse hoof (Table 4; Fig. 15), it would be easy to miss particular developmental stages of the *Stratum granulosum* unless large aged series are studied. Our observation that a *Stratum granulosum* is still present in the terminal part of the wall proper in a foal three days after its birth raises the possibility that it could occasionally also be still present in adult horses as was mentioned by *Spary* (1923) and *Ziegler* (1954). A *Stratum granulosum* in the terminal part of the wall proper was also described for the adult cow hoof (*Mülling* 1993) and the adult dog claw (*Budras* and *Seidel* 1992).

Our study also clarifies the cell composition of the thickened *Stratum corneum* of the fetal horse hoof (i.e., deciduous hoof capsule). The fetal cornified cells of the first generation, which develop in the first 90 days of gestation, resemble parakeratotic epidermal cells. They are pushed distally by the next generation of cells that differentiate in the same manner as the epidermal cells of the periople and bulbs of the heel in the adult horse hoof. This observation clarifies the significance of what *Mettam* (1896) stated, namely that the epidermal cells of the fetal hoof undergo a differentiation process that is similar to that in the hairy skin. In combination with our own observations, it means that the epidermal cells of the fetal hoof develop in a similar manner as the epidermal tissues (i.e., skin, periople, and bulbs of the heel) that undergo soft keratinisation in the adult horse. Hence, the epidermis of the fetal horse hoof undergoes soft keratinisation. This similarity between the fetal horn and the adult perioplic horn may explain why *Zietzschmann* and *Krölling* (1955) called the fetal horn "*Eponychium*", which in the Nomina Anatomica Veterinaria (2005) is used only for the perioplic horn.

Our study establishes a connection between the development of the papillary body and the appearance of the *Stratum granulosum* by showing for all the segments of the fetal horse hoof that a *Stratum granulosum* appears only after a certain height of the papillary body has been achieved. This development of a papillary body increases the interface between the vascularized dermis and the avascular epidermis and, thereby, increases the nutritional supply to the epidermis. This increased nutrition appears to be crucial for the production of the specific, profilaggrin-containing keratohyalin granules and other constituents of the cytoskeleton (i.e., the filament-matrix complex), as well as for processes related to keratinization and cornification, such as the formation of the marginal band. These cellular processes lead to the programmed death of the epidermal cells undergoing cornification and influence the mechanical properties of the hoof horn. Other differentiation processes, such as the synthesis and exocytosis of the intercellular substance (*Budras* and *Bragulla* 1991, *Mülling* et al. 1994, *Anthauer* et al. 2005), also influence the mechanical properties of the hoof horn.

In the epidermal hoof segments that retain a *Stratum granulosum* in the healthy adult hoof, the keratohyalin granules are broken down enzymatically as the cells cornify and become the most recent layer of the *Stratum corneum*. If these epidermal segments become dyskeratotic in an adult hoof, the keratohyalin granules are not broken down any longer, as was described for the ulcerated sole epidermis of bovines (*Mülling* et al. 1999). We hypothesize that at least in some cases of dyskeratosis of the horse hoof (e.g., laminitis, hoof cancer, etc.), the dyskeratotic epidermis may reactivate a *Stratum granulosum* and may also retain profilaggrin- or filaggrin-containing keratohyalin granules, or at least filaggrin, instead of disassembling them. Such a reappaerance of a *Stratum granulosum* has been described as hypergranulosis in the dyskeratotic matrix of the adult human nail (*Fanti* et al. 1994). The soft and cheesy consistency of dyskeratotic horn may be due to the persistence of keratohyalin granules that contain profilaggrin. Our hypothesis that the filaggrin content in the cells of the *Stratum corneum*, the degree of the cell hydration, and the relative softness and pliability of the horn are correlated is supported, on the one hand, by the observation that filaggrin has a high water-binding capacity as shown for the soft and pliable human skin (*Dale* 1985) and, on the other hand, by the observation that the soft periople consists of hydrated horn (*Budras* et al. 2002) and that the perioplic epidermis actually contains specific, profilaggrin-containing keratohyalin granules, as our data show. Our hypothesis that the mechanical properties of the hoof horn is influenced by the water-binding capacity and filaggrin content of the *Stratum corneum* is further supported by the observations that the degree of hydration and the fracture toughness are correlated in the adult horse hoof (*Bertram* and *Gosline* 1987, *Pütz* 2006) and in the human finger nail (*Finlay* et al. 1980, *Forslind* et al. 1980).

Any failure to break down proteins (including profilaggrin) that are stored in the specific keratohyalin granules could have various causes: (1) The enzymes responsible for the breakdown of the specific keratohyalin granules (*Dale* and *Holbrook* 1987) could be missing or altered; (2) the keratohyalin granules of dyskeratotic epidermis may contain aberrant proteins that cannot be broken down by normal enzymes; and (3) the specific keratin filaments of the cytoskeleton may be modified or missing and are, thereby, unable to bind to filaggrin and to form a filament-matrix complex. When the epidermis of adult cornified end organs is injured, a *Stratum granulosum* seems to be activated as part of wound healing or re-epithelisation processes even in the epidermal segments that normally do not maintain a *Stratum granulosum*, as was described by *Mörike* (1954) for the adult human finger nail. The possibility that such an adult *Stratum granulosum* is actually a re-activation of the fetal stage in the wake of a re-building of the papillary body will need to be tested in the future.

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