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ORIGINAL ARTICLE

K25 (K25irs1), K26 (K25irs2), K27 (K25irs3), and K28 (K25irs4) Represent the Type I Inner Root Sheath Keratins of the Human Hair Follicle

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The recent elucidation of the human type I keratin gene domain allowed the completion of the so far only partially characterized subcluster of type I keratin genes, *KRT25–KRT28* (formerly *KRT25A–KRT25D)*, representing the counterparts of the type II inner root sheath (IRS) keratin genes, *KRT71–KRT74* (encoding proteins K71–K74, formerly K6irs1–K6irs4). Here, we describe the expression patterns of the type I IRS keratin proteins K25–K28 (formerly K25irs1–K25irs4) and their mRNAs. We found that K25 (K25irs1), K27 (K25irs3), and K28 (K25irs4) occur in the Henle layer, the Huxley layer, and in the IRS cuticle. Their expression extends from the bulb region up to the points of terminal differentiation of the three layers. In contrast, K26 (K25irs2) is restricted to the upper IRS cuticle. Apart from the three IRS layers, K25 (K25irs1), K27 (K25irs3), and K28 (K25irs4) are also present in the hair medulla. Based on previous, although controversial claims of the occurrence in the IRS of various "classical" epithelial keratins, we undertook a systematic study using antibodies against the presently described human epithelial and hair keratins and show that the type I keratins K25–K28 (K25irs1–K25irs4) and the type II keratins K71–K74 (K6irs1–K6irs4) represent the IRS keratins of the human hair follicle.

Journal of Investigative Dermatology (2006) **126,** 2377–2386. doi:10.1038/sj.jid.5700494; published online 27 July 2006

INTRODUCTION

In the past 2 years, a broad-based *Keratin Nomenclature Committee* that included active investigators in the keratin field and members of the human and mouse genome committees worked out a novel consensus nomenclature system for keratin genes and proteins, which has been seen and approved by further researchers in the field of intermediate filament proteins (Schweizer *et al.*, 2006; see also Table 1). In the following, this new nomenclature system will be used for the designation of the keratins, with the hitherto used designations indicated in parentheses.

Regarding the keratins of the human hair follicle, a decade ago, only the outer root sheath (ORS) keratins K5, K14, K6, K16, and K17 were known, while with one exception (Yu *et al.*, 1993), virtually no molecular and genetic data existed for the keratins specifically expressed in the central hair forming compartment or in the inner root sheath (IRS), let alone the companion layer, which at that time was still considered the innermost layer of the ORS (Ito, 1989). Since then, this situation has dramatically changed. The unexpectedly complex human type I and type II hair keratin gene families and the expression patterns of the respective proteins in the hair follicle have been explored in great detail (Rogers *et al.*, 1998, 2000, 2005; Langbein *et al.*, 1999, 2001; Langbein and Schweizer, 2005). Moreover, a type II epithelial keratin gene, *KRT75* (*KRT6hf),* which flanks one side of the type II hair keratin gene cluster on chromosome 12, has been found to be specifically expressed in the companion layer of body hair follicles (Winter *et al.*, 1998) and, additionally, in the medulla of sexual hairs (Wang *et al.*, 2003). Recently, a cluster of four epithelial keratin genes on chromosome 12q13.1 turned out to encode the human type II IRS keratins K71–K74 (K6irs1–K6irs4), which were differentially expressed in the three IRS layers (Langbein *et al.*, 2002, 2003).

The elucidation of the type I counterparts of the human K71–K74 (K6irs1–K6irs4) keratins took a convoluted path. In 2001, Bawden *et al.* (2001) reported on four novel type I sheep wool keratin cDNAs, oIRSa1, oIRSa2, oIRSa3-1, and oIRSa3.2 (Table 2). Using the full oIRSa1 nucleotide sequence in a BLASTN search, the authors were able to identify a human chromosome 17 BAC genomic clone, which harboured the orthologous human genes *hIRSa1*, *hIRSa2*, and *hIRSa3.1*, as well as a further gene, called "*Gene 4*" (Table 2). Remarkably, this human gene did not encode the ortholog of oIRSa3.2, given that it was structurally

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Abbreviations: IIF, indirect immunofluorescence; IRS, inner root sheath; ISH, in situ hybridization; ORS, outer root sheath; PBS, phosphate-buffered saline; TBST, Tris buffered saline with Tween

Received 4 May 2006; revised 12 June 2006; accepted 14 June 2006; published online 27 July 2006

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Table 1. Current and new human keratin nomenclature (Schweizer *et al.*, 2006)

Human keratin proteins							
Type I				Type II			
Current name	New name	Current name	New name	Current name	New name	Current name	New name
K9	K9	K25irs1	K25	K1	K1	K6hf	K75
K10	K10	K25irs2	K26	K2e	K2	K2p	K76
K12	K12	K25irs3	K27	K3	K3	K1b	K77
K13	K13	K25irs4	K28	K4	K4	K5b	K78
K14	K14	Ha1	K31	K5	K5	K6l	K79
K15	K15	Ha2	K32	K6a	K6a	Kb20	K80
K16	K16	Ha3-I	K33a	K6b	K6b	Hb1	K81
K17	K17	Ha3-II	K33b	K6e/h	K6c	Hb2	K82
K18	K18	Ha4	K34	K7	K7	Hb3	K83
K19	K19	Ha5	K35	K8	K8	Hb4	K84
K20	K20	Ha6	K36	K6irs1	K71	Hb5	K85
K23	K23	Ha7	K37	K6irs2	K72	Hb6	K86
K24	K24	Ha8	K38	K6irs3	K73	—	—
—	—	Ka35	K39	K6irs4	K74	—	—
—	—	Ka36	K40	—	—	—	—

Note that with only few exceptions, the ''classical'' epithelial keratins maintain their designations according to Moll *et al.* (1988).

Table 2. Designations of the type I IRS keratins and their genes

Reference	Type I IRS keratins and their genes			
Sheep				
Bawden *et al.* (2001)	oIRSa1	oIRSa3.2	oIRS3.1	oIRSa2
Human				
Bawden *et al.* (2001)	hIRSa1	—	hIRSa3.1	hIRSa2
	hIRSa1	*Gene 4*[1]	*hIRSa3.1*	*hIRSa2*
Hesse *et al.* (2001)	K10C	K10D	K12B	*Not identified*
	KRT10C	*KRT10D*	*KRT12B*	—
Hesse *et al.* (2004)	Ka38	Ka39	Ka40	Ka41
	KRTa38	*KRTa39*	*KRTa40*	*KRTa41*
Rogers *et al.* (2004)	K25irs1	K25irs2	K25irs3	K25irs4
	KRT25A	*KRT25B*	*KRT25C*	*KRT25D*
Schweizer *et al.* (2006)	K25	K26	K27	K28
	KRT25	*KRT26*	*KRT27*	*KRT28*
Mouse				
Porter *et al.* (2004)	mIRSa1	Not identified	mIRSa3.1	mIRSa2
	mIRSa1	—	*mIRSa3.1*	*mIRSa2*

Indicated are the various current designations in different species as well as the new designation for the human keratins according to Schweizer *et al.* (2006).
[1]The human ''*Gene 4*'' is not the ortholog of the *oIRSa3.2* gene, but exhibits the closest similarity to hIRSa3.1 (unpublished data).

more related to oIRSa3.1, when the sequence data of Bawden *et al.* (2001) and Rogers *et al.* (2004) were compared (see below). In a concurrent study, Hesse *et al.* (2001) also identified bioinformatically these three human genes, but designated them *KRT10C*, *KRT10D*, and *KRT12B*, respectively (Table 2). Furthermore, Porter *et al.* (2004) have characterized the murine orthologs of the *hIRSa1*, *hIRSa3.1*, and *hIRSa2* genes (Table 2).

Recently, two groups reported on the elucidation of the complete human type I keratin gene domain on chromosome 17q21.2 (Hesse *et al.*, 2004; Rogers *et al.*, 2004). This not only allowed the definite positioning of the four type I IRS genes (protein and gene designations by Hesse *et al.*, 2004; Rogers *et al.*, 2004, respectively; see Table 2) between the *KRT10* gene and a keratin pseudogene (Figure 1a), and resulted in the isolation of cDNA sequences for the four genes, but also demonstrated that, similar to the hair keratins, the encoded IRS keratins form a separate branch when compared with all known human type I keratins in an evolutionary tree (Figure 1b, data based on Rogers *et al.*, 2004).

The rational of our study was to provide a detailed description of the expression patterns of all four type I human IRS keratins, K25 (K25irs1), K26 (K25irs2), K27 (K25irs3), and K28 (K25irs4), relative to that of their type II counterparts in the human hair follicle. In addition, we took the opportunity to re-examine previous investigations, claiming the presence in the IRS of several keratins of both types that are normally found in stratified and simple epithelia (Lynch *et al.*, 1986; Heid *et al.*, 1988b; Kopan and Fuchs, 1989; Stark *et al.*, 1990; Wilson *et al.*, 1994; Krüger *et al.*, 1996; Schirren *et al.*, 1997).

RESULTS

Expression of the four type I IRS keratins in the human hair follicle

The expression of the K25–K28 (K25irs1–K25irs4) mRNAs and proteins was investigated in cryostat sections of human chin and scalp skin as well as freshly plucked beard hairs by both *in situ* hybridization (ISH) with specific 3′ nontranscribed probes of the various mRNAs and indirect immunofluorescence (IIF) studies using antibodies raised against specific peptides of the individual keratins (see Materials and Methods).

Figure 2a shows that K25 (K25irs1) transcripts can clearly be seen in all three layers of the IRS. The mRNA expression starts in the lowermost bulb region and can be followed up to the point of terminal differentiation of the Huxley layer (see also, red arrows in Figure 2b) and, slightly below, the IRS cuticle, while transcripts in the Henle layer disappear much earlier at the site of terminal differentiation of this layer (open white arrowhead). The same expression pattern is observed for the K25 protein (Figure 2b). In the lower portion of the

follicle, this keratin is present in all three layers (Figure 2c). Higher up, K25 staining ceases abruptly in the Henle layer, thus allowing the visualization of K25-positive *Flügelzellen* of the Huxley layer, whose extensions pass between differentiated Henle cells to reach the companion layer (Figure 2d). In almost all aspects, this expression profile holds true for K27 (K25irs3) (Figure 2e and f) and, in principle, also for K28 (K25irs4), although the overall expression of this mRNA appears to be lower than that of K25 and K27 (Figure 2g and h). In contrast, K26 (K25irs2) transcripts (Figure 2i) and protein (Figure 2j, k and l, with the latter showing a double label study with an antibody against the hair cuticle keratin K32 (Ha2) (green), Langbein *et al.*, 1999) are clearly restricted to the IRS cuticle, in which their synthesis begins slightly above the apex of the dermal papilla. As a rule, mRNA expression in the Huxley layer and the IRS cuticle ceases slightly earlier than protein synthesis. This is in accordance with previous findings for other hair follicle-specific epithelial and hair keratins (Langbein and Schweizer, 2005).

Completely unexpected, we noticed that, in addition to all three IRS layers, K25 (K25irs1), K27 (K25irs3), and K28 (K25irs4) proteins occurred also in the medulla of beard hairs (Figure 2b, f and h, yellow arrows). The most prominent staining, which extended up to the mid-cortex region, was seen for K25 (K25irs1) (Figure 2b). It can, however, not be excluded that the expression of this keratin proceeds even further, as its tapering cessation may indicate that the upper portion of the medulla ran out of the section plane. Likewise surprising was the conspicuous downward branching of the K25 (K25irs1) staining at the apex of the dermal papilla, thus decorating the single-layered cell row apposed to almost the entire upper part of the dermal papilla (Figure 2b). Variants of

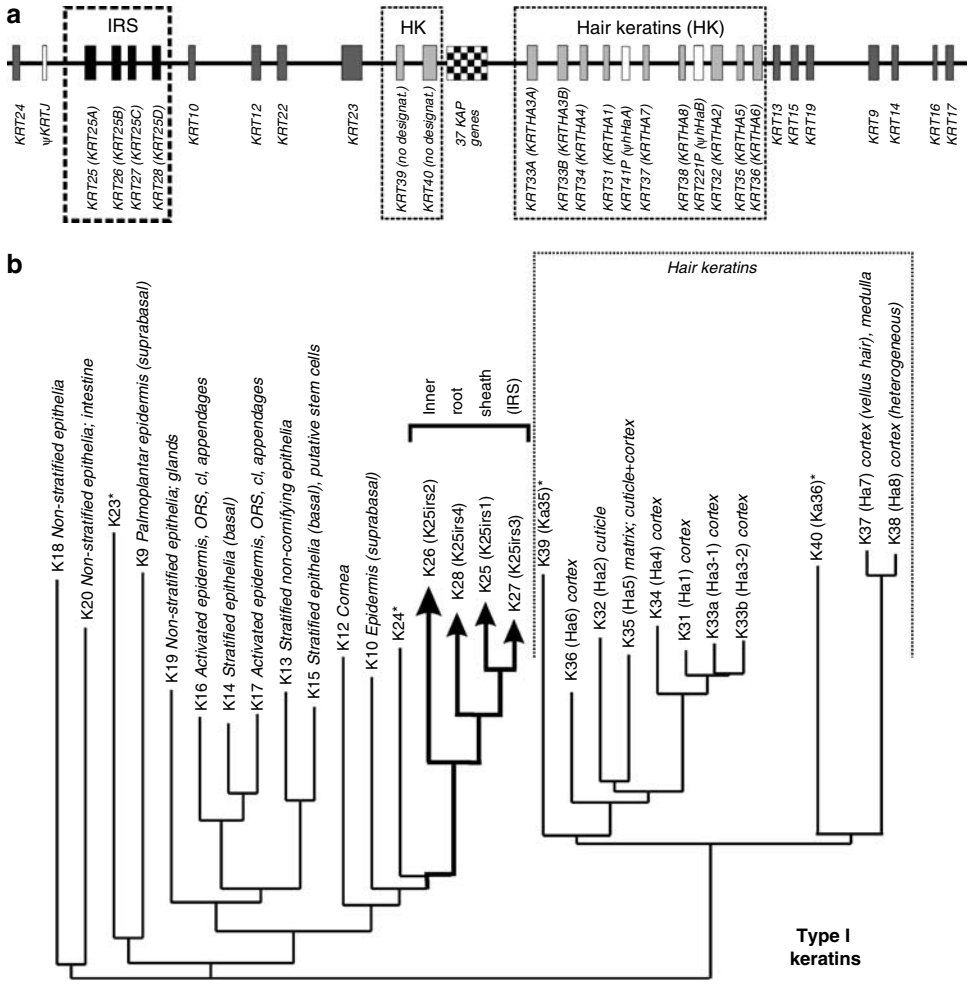


Figure 1. Schematic physical map of the human *KRT25–KRT28* (*KRT25A–KRT25D*) gene region and evolutionary tree analysis of human type I keratins. The keratin genes and their proteins are designated by the new nomenclature (Schweizer *et al.*, 2006) with their current names given in parentheses. (**a**) Shown is the cluster of the IRS-specific type I keratin genes (*IRS*, black boxes) and its flanking keratin (dark gray boxes)/hair keratin (*HK*, light gray boxes) genes or pseudogenes (*P*, formerly *Ψ*; white boxes). The scheme illustrates the clustering of keratin genes in the genome but compared to Rogers *et al.* (2004) does not reflect the authentic intergenic distances. (**b**) Evolutionary tree of human type I epithelial and hair keratins with the typical expression sites of their genes. Note that both the IRS-specific keratins and the hair keratins form individual branches, which divided nearly at the same time during evolution (the data are mainly based on Rogers *et al.*, 2004). *Expression profiles are currently being investigated.

this pattern were also seen for the K27 (K25irs3) and K28 (K25irs4) proteins. While both proteins were visible around the upper dermal papilla, K28 (K25irs4) seemed to exhibit a punctual medullary expression much higher than K25 (K25irs1) (Figure 2f and h). Out of the sections of plucked beard hairs used for ISH, only those of Figure 2a and c revealed a medulla, albeit only in the upper cortex, which in both cases was free of label. In contrast, the beard hair section shown in the inset of Figure 2a displayed the beginning of the medulla, which clearly contained K25 (K25irs1) transcripts in the lowermost portion as well as in the cell row lining the upper portion of the lost dermal papilla.

Expression of other keratins in the IRS?

In earlier IIF studies on paraffin sections of human skin, several antibodies or antisera against a variety of epithelial keratins have repeatedly been reported to decorate the IRS of hair follicles present in the skin sections (Hosokawa *et al.*, 1984; Ito *et al.*, 1986; Lynch *et al.*, 1986; Ramaekers *et al.*, 1987; Stark *et al.*, 1987, 1990; Moll *et al.*, 1988; Heid *et al.*, 1988a, b; Imcke *et al.*, 1988; Kopan and Fuchs, 1989; Lane *et al.*, 1991; Limat *et al.*, 1991; Tatsuta and Tezuka, 1994; van Baar *et al.*, 1994; Watanabe *et al.*, 1994; Wilson *et al.*, 1994; Demirkesen *et al.*, 1995; Schirren *et al.*, 1997; Ahmed *et al.*, 2005). In particular, keratins K1, K4, K10, K13, K18, and K7 were said to be demonstrable either in the entire IRS or in one of its constituent layers (Ramaekers *et al.*, 1987; Stark *et al.*, 1987, 1990; Heid *et al.*, 1988a, b; Imcke *et al.*, 1988; Moll *et al.*, 1988; Limat *et al.*, 1991; van Baar *et al.*, 1994; Watanabe *et al.*, 1994; Wilson *et al.*, 1994; Demirkesen *et al.*, 1995; Schirren *et al.*, 1997). A common feature of these studies was, however, that consistently, findings obtained with a given antibody varied between laboratories and that the use of various antibodies recognizing distinct or several keratins, often led to contradictory results. In order to clarify this issue, we undertook a systematic keratin analysis using, where possible, more than one antibody against almost each of the classical type I and type II epithelial keratins as well as hair keratins (keratins investigated were: K1, K2 (K2e), K76 (K2p), K3, K4, K5, K6, K7, K8; K9, K10, K12, K13, K14, K15, K16, K17, K18, K19, K20, K75 (K6hf); K31–K38 (Ha1–Ha8), K81–K86 (Hb1–Hb6). For keratin nomenclature, see Table 1 and for the antibodies used, see Table 3). Our study also included an antibody against the type II corneal keratin K12, given that K12 transcripts have recently been said to occur in the IRS of mouse anagen hair follicles (Ishimatsu-Tsuji *et al.*, 2005). To avoid artefacts by formalin fixation and paraffin embedding, throughout, we used fresh cryostat sections of human scalp immediately fixed with methanol. As expected, some of the antibodies reacted with the ORS (K5, K14, K6, K16, K17), the companion layer [K75 (K6hf), K6, K16, K17], as well as with the hair forming compartment K31–K38 (Ha1–Ha8) and K81–K86 (Hb1–Hb6), but in no case did we observe an unambiguous staining in the IRS (results not shown). This held true also for K12, indicating that, most probably due to the absence of the gene of the normal K12 type II partner K3 in the mouse genome (Hesse *et al.*, 2004), the expression profile of mouse keratin K12 may substantially deviate from that seen in humans.

DISCUSSION

In this study we have shown that, unlike earlier evidence, the keratin spectrum of the human hair follicle IRS is apparently restricted to eight specific epithelial keratins, comprising the previously described four type II members K71–K74 (K6irs1—K6irs4) (Langbein *et al.*, 2002, 2003) and the four type I members K25-K28 presented here. While in earlier expression studies of K25 (K25irs1) and K27 (K25irs3) (Bawden *et al.*, 2001), it was rather difficult to decide whether or not the respective mRNAs were clearly located in all three IRS layers of the human hair follicle, we could show by both ISH and IIF that this is not only the case for these two keratins but also for K28 (K25irs4). This latter finding is in agreement with protein expression studies of the murine counterpart of K28 (K25irs4) (Porter *et al.*, 2004). In contrast, K26 (K25irs2), which has previously not been investigated in humans nor has the expression of its sheep and mice orthologs been analyzed (Bawden *et al.*, 2001; Porter *et al.*, 2004), is specifically localized in the mid- to upper IRS cuticle.

The expression pattern of the type I IRS keratins is strikingly different from that of the type II IRS keratins. Within the latter, K71 (K6irs1) is the only keratin that is found in all three IRS layers, while K72 (K6irs2) and K73 (K6irs3) are specific for the IRS cuticle and K74 (K6irs4) is restricted to the Huxley layer (Langbein *et al.*, 2003). The overall expression patterns of the mRNAs of the type I and type II IRS keratins are schematically illustrated in Figure 3a and b. The table in Figure 3c reveals that the highest number of keratins is encountered in the cells of the IRS cuticle (7), followed by those of the Huxley and Henle layers (5 and 4, respectively). We have previously shown that also the cells of the adjacent companion layer, cl, which forms a functional unit with the IRS (Langbein *et al.*, 2002, 2003), express at least four keratins (K6, K75 (K6hf), K16, K17; Winter *et al.*, 1998). Most probably, this high number of keratins in each of the single layered tissue compartments of the cl-IRS unit endows each cell type with the dense and stabilizing IF network that is required for the supposed function of the unit in the moulding and guidance of the growing hair. This seems to be particularly evident for the exceptionally small cells of the IRS cuticle (see Figure 2l), which are subject to considerable mechanical constraints when tightly interacting with the cells of the hair cuticle during the upward journey of the hair.

The table in Figure 3c also reveals that consistently, in the cells of each IRS layer, the number of type I keratins is larger than that of type II keratins. This numerical imbalance is particularly pronounced in Henle cells in which the three type I keratins K25 (K25irs1), K27 (K25irs3), and K28 (K25irs4) are opposed to only one type II keratin, K71 (K6irs1). This implies that, for filament formation, the three type I keratins must all compete for K71 (K6irs1), resulting in the active formation of three defined keratin pairs in Henle cells. In contrast, in lower Huxley cells multiple and non-predictable keratin pairing is possible between the two type II

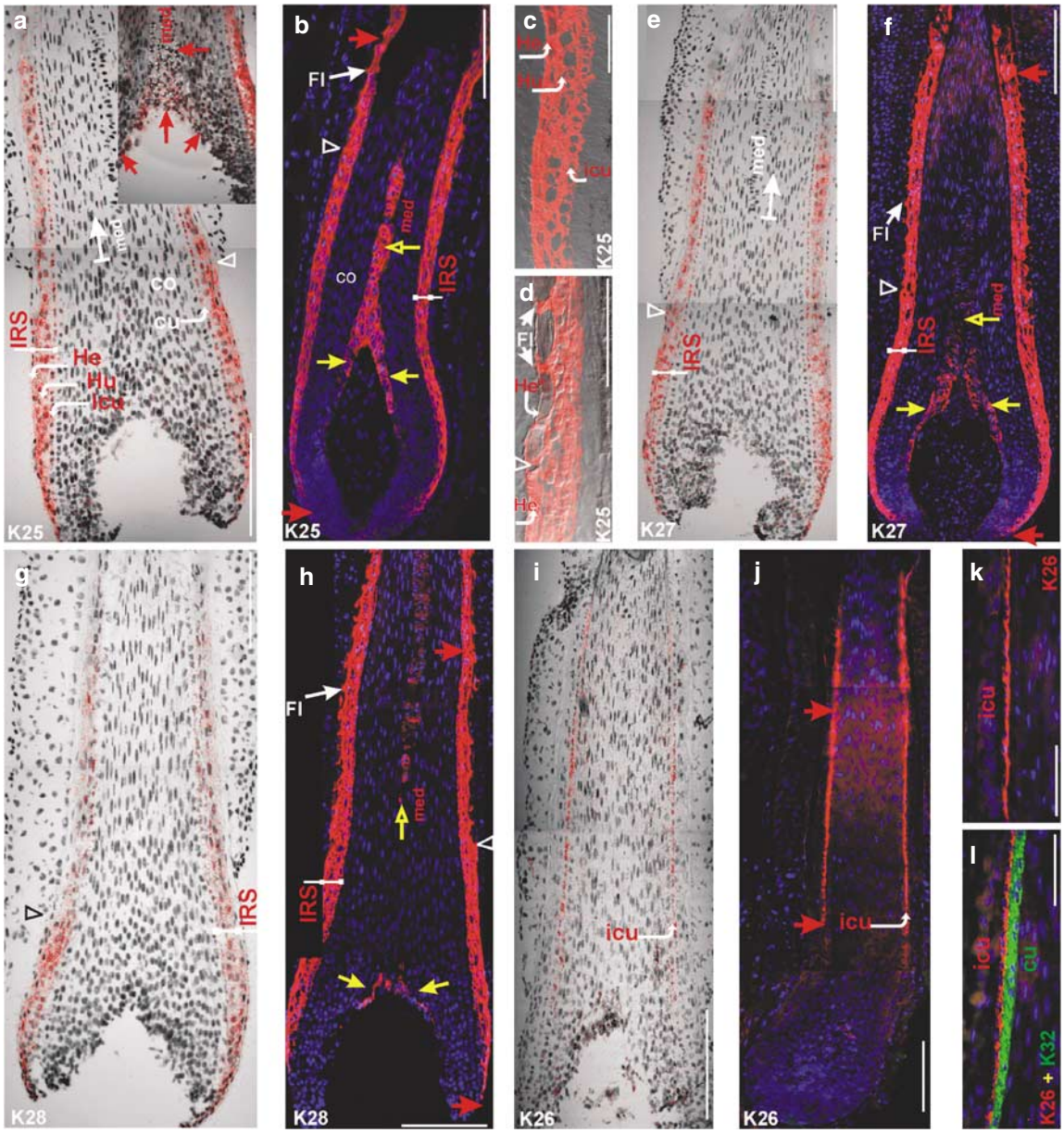


Figure 2. Expression of K25 (K25irs1), K26 (K25irs2), K27 (K25irs3), and K28 (K25irs4) at the mRNA (ISH) and protein (IIF) level in the hair follicle. (**a–d**) K25 (K25irs1). (Note that in the figure, keratins are only given according to the new nomenclature (Schweizer *et al.*, 2006).) Both (**a**) *ISH* and (**b–d**) *IIF* show a prominent staining of all compartments of the IRS: Henle- (*He*), Huxley (*Hu*) layer, and IRS-cuticle (*icu*) (for details see (**c**, **d**); IIF plus DIC microscopy) of plucked beard hairs. The mRNA and protein synthesis terminates first in the Henle layer at the point of terminal differentiation (triangle in (**a**/**b**), He* in (**d**)) but continues in the Huxley layer, including *Flügelzellen* (*Fl*), and (**b, d**) in the IRS cuticle. The hair medulla (*med*) is also positive for this keratin whose expression starts in the cell row flanking the upper part of the dermal papillae (inset in (**a**) for mRNA and yellow arrows in (**b**) for protein. Note that mRNA expression terminates earlier than the protein synthesis (horizontal red arrow in the inset in (**a**)). (**e–f**) K27 (K25irs3) and (**g, h**) K28 (K25irs4). (**a–d**) Both keratins show exactly the same expression pattern as K25 (K25irs1) although the K28 (K25irs4) mRNA expression appears considerably weaker and seems to terminate slightly earlier (red open arrow in (**g**)). (**i–l**) K26 (K25irs2). Both (**i**) the mRNA and (**i–k**) the protein are restricted to the IRS cuticle (*icu*). (**i** and red arrows in **j**) The mRNA expression begins at the apex of the dermal papillae and terminates at the level of the mid-cortex, (**j**) whereas the protein can be detected above this zone. Double staining for K26 (K25irs2). (*icu* in **h**, red staining) and hair cuticle keratin K32 (Ha2) (*cu* in **h**, green staining). Nuclear counterstaining by 4′,6 diamidino-2-phenylindole (DAPI) in blue. *co,* hair cortex; *cu,* hair cuticle. Bars, 150 μm.

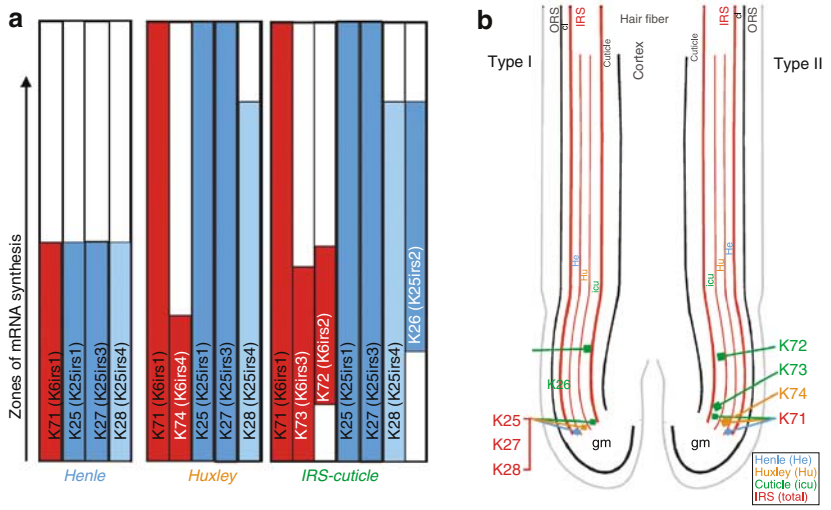
keratins K71 (K6irs1) and K74 (K6irs4) and the three type I keratins K25 (K25irs1), K27 (K25irs3) and K28 (K25irs4), while the keratin pairs occurring in upper Huxley cells correspond to those found in lower Henle cells. The number of potential keratin pairs is even more pronounced in the cells of the lower IRS cuticle, while higher up, the type II keratin K71 (K6irs1) is the only partner for four type I keratins (Figure 3c).

Considering the pivotal role played by the K71 (K6irs1) keratin in all three IRS layers, in particular the Henle layer, it

Table 3. Antibodies against keratins used in this study

Keratin	Clone/ antiserum	Species	Company	Dilution	Keratin	Clone/ antiserum	Species	Company	Dilution
K1	K1	mab	NCL Loxo	1:20	K17	E3	mab (su)	Progen	Undiluted
	K1rb	rb	Covance	1:2,000		GP-CK17	gp	Progen	1:2,000[1]
	GP-CK1	gp	Progen	1:4,000[1]	K18	Ks18.04	mab	Progen	1:10
K1/10	8.60	mab	Progen	1:4,000		*GP-CK18*	gp	Progen	1:100
K2e (K2)	Ks2.398.3.1	mab	Progen	1:100[1]	K19	*GP-CK19*	gp	Progen	1:200
	GP-CK2e	gp	Progen	1:2000[1]		Z105	mab (su)	Progen	Undiluted
K2p (K76)	GP-CK2p	gp	Progen	1:1000[1]		Ks19.10	mab (su)	Progen	Undiluted
K3/12	AE5	mab	Progen	1:10		A53BA4	mab (su)	Progen	Undiluted
K4	6B10	mab	Progen	1:10	K20	Ks20.3	mab	Progen	1:10
K5	GP-CK5	gp	Progen	1:1,000[1]		GP-CK20	gp	Progen	1:1,000[1]
	CK5	rb	Gift of T. Magin, Bonn, Germany	1:2,000	K6hf (K75)	GP-K6hf	gp	Progen	1:2,000[1]
K6	KA12	mab	Progen	1:100		GP-CK6hfprot	gp	Progen	1:1,000[1]
	K6rb	rb	Gift of P. Coulombe, Baltimore, MD	1:2,000	K6irs1 (K71)	GP-K6irs1	gp	Progen	1:2,000[1]
K7	RCK105	mab	Progen	1:10	K6irs2 (K72)	GP-K6irs2	gp	Progen	1:2,000[1]
	GP-CK7	gp	Progen	1:2,000[1]	K6irs3 (K73)	GP-K6irs3	gp	Progen	1:2,000[1]
K8	Ks8.7	mab	Progen	1:10	K6irs4 (K74)	GP-K6irs4	gp	Progen	1:2,000[1]
	GP-CK8	gp	Progen	1:2,000[1]	Ha1 (K31)	LH-Tric1	mab	Gift of I.M. Leigh	1:50
K9	GP-CK9	gp	Progen	1:3,000[1]		GP-Ha1	gp	Progen	1:5,000[1]
					Ha2 (K32)	LH-Tric17	mab	Gift of I.M. Leigh	1:10
	CK9 mix of Ks9.70/9.216	mab	Progen	1:10[1]		GP-Ha2	gp	Progen	1:1,000[1]
K10	RSKE60	mab	Progen	1:10	Ha3 (K33)	GP-Ha3	gp	Progen	1:500[1]
	DEK10	mab	DAKO	1:100	Ha4 (K34)	GP-Ha4	gp	Progen	1:300[1]
	K10	rb	Gift of D. Roop, Houston, TX	1: 100	Ha5 (K35)	GP-Ha5	gp	Progen	1:2,000[1]
K13	Ks13.1	mab	Progen	1:10	Ha6 (K36)	GP-Ha6	gp	Progen	1:1,000[1]
	GP-CK13	gp	Progen	1:3,000[1]	Ha7 (K37)	GP-Ha7	gp	Progen	1:1,000[1]
K14	GP-CK14	gp	Progen	1:2,000[1]	Ha8 (K38)	GP-Ha8	gp	Progen	1:5,000[1]
	CKB1	mab	Sigma	1:400	Hb1 (K81)	mou hHb1	mab	Gift of I.M. Leigh	1:50
	LL001	mab	Gift of I.M. Leigh, London, UK	1:40		GP-Hb1	gp	Progen	1:3,000[1]
	LL002	mab	NatuTec	1:2	Hb2 (K82)	GP-Hb2	gp	Progen	1:1,000[1]
K15	K15	mab	NCL Loxo	1:80	Hb3 (K83)	GP-Hb3	gp	Progen	1:500[1]
	LHK15	mab	Neomarkers	1:200	Hb4 (K84)	GP-Hb4	gp	Progen	1:1,500[1]
	GP-CK15	gp	Progen	1:2,000[1]	Hb5 (K85)	GP-Hb5	gp	Progen	1:1,000[1]
K16	K16	rb	Gift of P. Coulombe	1:2,000	Hb6 (K86)	GP-Hb6	gp	Progen	1:500[1]
	K16	mab	Labgen	1:40	—	—	—	—	—
	K16	mab	NCL Loxo	1:50	—	—	—	—	—

rb, rabbit; *gp*, guinea-pig; *su*, supernatant.
Keratins are indicated by their current names with their novel designations (Schweizer *et al.*, 2006) being added in parentheses.
[1]Indicated dilutions refer to the original serum samples generated in our laboratory, while the antibodies sold by Progen must be diluted as indicated by the company.



c

IRS-compartment	Total	Type I	Type II	Relation type I/II	Type I keratins	Type II keratins	Specific keratins
IRS (total)	8	4	4	1:1	K25 K26 K27 K28	K71 K72 K73 K74	8
Henle	4	3	1	3:1	K25 K27 K28	K71	none
Huxley	5	3	2	3:2	K25 K27 K28	K71 K74	1
IRS-cuticle	7	4	3	4:3	K25 K26 K27 K28	K71 K26 K73	3

Figure 3. Schematic presentation of the expression patterns of the IRS-specific type I and type II keratins. (Note that in (**a**) keratins are indicated by the current and new nomenclature (Schweizer *et al.*, 2006), while in (**b**) and (**c**) only the new designations are used]. (**a**) K25-K28 (K25irs1–K25irs4) and K71–K74 (K6irs1–K6irs4) mRNA expression profiles in the three IRS layers. The type I IRS-keratins are given in red and the type II IRS-keratins indicated in blue. The light blue colour is indicative for the low level expression of the K28 (K25irs4) mRNA. (**b**) Keratin protein expression in the various IRS compartments. Indicated is the start of synthesis of the individual keratins. IRS keratins of the Henle layer in blue, Huxley layer in orange, and IRS-cuticle in green. Keratins found in all three compartments are given in red. (*gm*, germinative matrix). (**c**) This summarizing table shows numbers, numerical relationships and distributions of the various keratins in the IRS. Keratins that are specific for a distinct IRS compartment are underlined and indicated in bold.

is evident that deleterious mutations in the corresponding gene should entail dramatic consequences regarding the integrity and function of the IRS as well as for the entire hair follicle. Although K71 (K6irs1) mutations have not yet been found in humans, two mouse mutants have recently been described. Peters *et al.* (2003) reported on an autosomal recessive mouse mutant RCO3, characterized by severe alopecia and easily extractable hairs in homozygotes, while heterozygous animals were phenotypically normal. Affected mice carried a 10-bp deletion in exon 1 of both alleles of the *mKRT71* (formerly *mKRT6irs1)* gene, which encoded a protein consisting of 58 amino acids of the mK71 (mK6irs1) head domain followed by 76 amino acids with no sequence homology to keratins and unable to participate in IF assembly. Ultrastructurally, normal IF bundles were absent from the severely disturbed Henle and Huxley layers; however, in line with the previously reported evidence that in the mouse, K71 (K6irs1) is not expressed in the IRS cuticle (Aoki *et al.*, 2001), the cells of the latter appeared normal (Peters *et al.*, 2003). A second, autosomal dominant mouse mutant, Ca^{Rin}, exhibited hairs that resembled those of the classical wavy coat mutation, caracul, *Ca*. Ultrastructurally, the mutant follicles showed severe disturbances of the IRS structure. The analysis of the *mKRT71* (*mKRT6irs1)* gene revealed two mutational hot spots either consisting of a 3-bp deletion in the first exon leading to an Asp deletion in the 1A helix, or a point mutation generating a Leu–Trp substitution in the 2B helix of the mK71 (mK6irs1) keratin (Kikkawa *et al.*, 2003). All in all, both studies emphasized that K71 (K6irs1) is indispensable for the proper formation of the IRS and that its loss or mutation compromises the correct moulding and growth of the hair.

It is noteworthy that, in addition to their order of complexity in terms of keratin expression, the various IRS

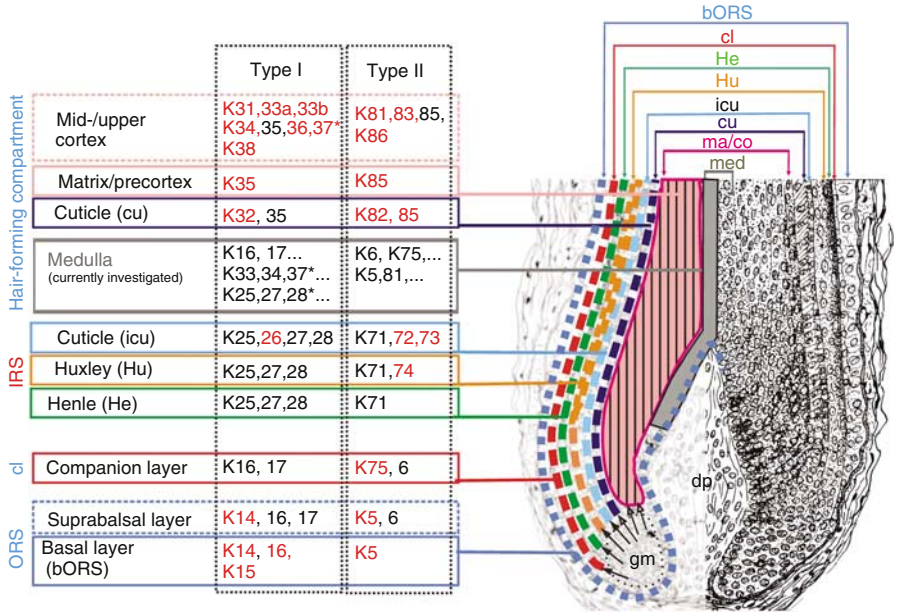


Figure 4. Schematic presentation of the expression patterns of epithelial/hair keratins presently identified in the various hair follicle compartments. Keratins are indicated according to the new nomenclature (Schweizer *et al.*, 2006). With the exception of the outer root sheath (*ORS*, blue), each of the further follicular compartments (companion layer, (*cl*, red); IRS with Henle layer (green), Huxley layer (orange), and cuticle (light blue) and the hair forming compartment with hair cuticle (blue), matrix/cortex (pink) and medulla (gray)) arises from cells in the germinative matrix (*gm*) at the base of the hair bulb (closed arrows, cl-IRS unit; open arrows, hair forming compartment). Epithelial-/hair keratins indicated in red at the left hand side of the scheme represent members that are unique for a given tissue compartment of the hair follicle. *Present in the cortex of vellus hair (Langbein *et al.*, 1999) and the medulla of sexual hairs (Jave-Suarez *et al.*, 2004). The mixed expression of epithelial and hair keratins in the medulla is incomplete and currently investigated in detail.

Table 4. Oligonucleotides used for PCR-amplification of the 3′-noncoding regions of K25–K28 (K25irs1–K25irs4) and PCR conditions as well as calculated molecular weights and isoelectric points of the proteins

Probe	Size (bp)	Oligonucleotide sequence	PCR annealing temperature (°C)	Molecular weight (kDa)	Isoelectric point (pH)
K25-3′ (K25irs1-3′)	143	gcaacagagaacgtatgccttcttctagatgaatggggaga	59	49.3	4.84
K26-3′ (K25irs2-3′)	171	ggaaaaagttatttggaaagaagcatagaacatgagaaaaggaa	49	51.9	4.73
K27-3′ (K25irs3-3′)	400 (233) (contains an intron)	ggcccaggaaatcaaacaaaaagcagaaaaataaggggacc	54	49.8	4.91
K28-3′ (K25irs4-3′)	147	agccctgggaattcatctaaagttctgtcttgccgttggtc	52	50.6	5.28

layers exhibit the same order regarding the expression of layer-specific keratins. Thus, cells of the IRS-cuticle contain three specific keratins, K71, K73, and K26 (K6irs2, K6irs3, and K25irs2), followed by Huxley cells with one specific keratin, K74 (K6irs4). In contrast, none of the keratins is specific for Henle cells (Figure 3c). In Figure 4, these data have been incorporated into a previously published scheme (Langbein *et al.*, 2001, 2003; Langbein and Schweizer, 2005) of both overall and layer-specific presently known keratins (i.e., ORS, cl, IRS, and hair forming compartment) of the human hair follicle. In this scheme, the medulla is the only follicular compartment whose constituent keratins have not been completely elucidated. We and others have previously shown that medullary trichocytes can be distinguished from cells of the other compartments by an unusual co-expression of a large number of both epithelial keratins (i.e., K6, K75 (K6hf), K16, K17) and hair keratins (i.e., K81, K83, K85, K86 (i.e., Hb1, 3, 5, 6) and K31, K33a, K34, K36, K37 (Ha1, 3-I, 4, 6, 7); Langbein and Schweizer, 2005). In this study we were able to demonstrate that also the three type I IRS keratins K25 (K25irs1), K27 (K26irs3), and K28 (K25irs4), expressed in all three IRS layers, are also cytoskeletal constituents of the medulla. As we suspect that this is not the full complement of medullary keratins, we plan to close this gap by submitting carefully prepared beard hair sections, containing the medulla in its entire length to IIF studies using not only the well-characterized antibodies against the four type II IRS keratins, but also against the collective of all recently known epithelial and hair keratins (keratins investigated were: K1, K2 (K2e), K76 (K2p), K3, K4, K5, K6, K7, K8; K9, K10, K12, K13,

K14, K15, K16, K17, K18, K19, K20, K75 (K6hf); K31–K38 (Ha1–Ha8), K81–K86 (Hb1–Hb6). For keratin nomenclature, see Table 1 and for the antibodies used, see Table 3) that were investigated for their putative presence in the IRS.

MATERIALS AND METHODS

Antibodies

Antisera against K25 (K25irs1), K26 (K25irs2), K27 (K25irs3), and K28 (K25irs4) were produced by injection into guinea-pigs (gp) of the synthetic peptides indicated below. In the absence of an internal cysteine, such a residue was added to some of the peptides for coupling to Keyhole limpet protein (peptide synthesis and coupling by Peptide Specialty Laboratories, Heidelberg, Germany); K25 (K25irs1) (C-PRPTTGSLRLYGG; amino acid (aa) pos. 13–26; 1:2,000), K26 (K25irs2) (KSKSTCYKSKGYRPV; aa pos. 397–411; 1:1,000), K27 (K25irs3) (GYGGPGNQTKDSS-C; aa pos. 404–416; 1:4,000), and (C-TVEEKSTKVNNKN; aa pos. 441–453; 1:4,000), and K28 (K25irs4) (C-HSIEEKTSKMTNGK; aa pos. 444–457; 1:1,000). These antisera were carefully checked for specificity and possible cross-reactivity on numerous types of epithelial and nonepithelial tissues. Antibodies against further keratins used in this study are listed in Table 3. The secondary antibodies (IgG or IgG + IgM used at a dilution of 1: 200 or 1:500, Cy3) were: Goat anti-guinea pig, -anti-mouse or -anti-rabbit, coupled to Cy3 or Alexa 568 (red fluorescence) or Alexa 488 (green fluorescence, Molecular Probes, Leiden, The Netherlands).

Indirect Immunofluorescence microscopy

This procedure was carried out essentially as described previously (Langbein *et al.*, 2003, 2004). Briefly, after rinsing in phosphate-buffered saline (PBS), cryostat sections of both human scalp and chin (obtained during surgery for medical reasons or from cadavers during pathological investigations, Institute of Pathology, University of Heidelberg and Dermatological Hospital, Strasbourg, France under institutional approval and included adherence to the Declaration of Helsinki Principles) and plucked beard hairs were fixed in methanol (−20°C; 10 minutes). The sections were permeabilized by dipping in Tris buffered saline with Tween (TBST) (0.001% Triton-X-100/PBS) 5 minutes and blocked with 5% normal goat serum in PBS. The primary antibodies were applied for 1 hour. After washing in PBS, secondary antibodies were applied for 30 minutes. The slides were washed in PBS then rinsed in ethanol, dried, and mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Control immunostaining was made by using the secondary antibody against Igs of the respective species only. 4′,6 Diamidino-2-phenylindole (DAPI) was added to the secondary antibodies for nuclear counterstaining. The respective phase contrast image was documented in parallel. Visualization and documentation was performed by means of a photomicroscope (Axiophot II) equipped with a digital imaging system (camera: AxioCam HR; software: AxioVision 4.4, all components from Carl Zeiss, Oberkochen, Germany).

In situ hybridization

ISH on cryostat sections of human scalp and chin skin or plucked beard hairs were carried out in parallel as described previously (Langbein *et al.*, 2003, 2004). For ISH, PCR fragments of the 3′-noncoding regions of K25–K28 (K25irs1–K25irs4) were amplified using the oligonucleotides and PCR conditions found in Table 4. The purified PCR products were cloned into the plasmid pCR4.1 and used to generate the respective antisense ^{35}S-radiolabeled cRNA probes by *in vitro* transcription for ISH. The probes were used for overnight hybridization at 42°C. Sections were washed with 2 × sodium chloride sodium citrate buffer (SSC) (5 minutes), 2 × SSC/50% formamide/20 mM DTT, 1 × SSC/50% formamide/20 mM DTT (30 minutes each), and 1 × SSC/0.1% SDS at room temperature for 5 minutes, digested with RNaseA (10 mg/ml in 1 × SSC, 30 minutes at 37°C), followed by washing with 0.5 × SSC/50% formamide/20 mM DTT at 50°C, dehydrated in an ethanol series, and dried. After dipping into photo emulsion (NTB-2; Kodak) and drying, sections were exposed for 2–3 days, developed through photochemical procedure, slightly stained with hematoxylin, and embedded. For the recording of the ISH signals by reflection microscopy, the confocal laser scanning microscope LSM 510Meta was used, which allows simultaneous visualization of ISH in epi-illumination for the detection of reflection signals and transmitted light in bright field for hematoxylin staining. The two signal channels were combined by an overlay in pseudocolor (transmission image in green, electronically changed into black/white using the ZEISS-LSMib software; reflection image, that is IHS signals, in red).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Herbert Spring for expert help with confocal laser microscopy and Werner W. Franke (both DKFZ, Heidelberg) for his continual interest and support. This work was supported by the Tumorzentrum Heidelberg/Mannheim (Grant LL/PS, TZ-FSP I/3).

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