K6irs1, K6irs2, K6irs3, and K6irs4 Represent the Inner-Root-Sheath-Specific Type II Epithelial Keratins of the Human Hair Follicle[1]

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In this study we report on the cloning of two novel human type II keratin cDNAs, K6irs3 and K6irs4, which were specifically expressed in the inner root sheath of the hair follicle. Together with the genes of two previously described type II inner root sheath keratins, K6irs1 and K6irs2, the *K6irs3* and *K6irs4* genes were subclustered in the type II keratin/hair keratin gene domain on chromosome 12q13. Evolutionary tree analysis using all known type II epithelial and hair keratins revealed that the K6irs1–4 formed a branch separate from the other epithelial and hair keratins. RNA *in situ* hybridization and indirect immunofluorescence studies of human hair follicles, which also included the K6irs2 keratin, demonstrated that both K6irs2 and K6irs3 were specifically expressed in the inner root sheath cuticle, but showed a different onset of expression in this compartment. Whereas the K6irs3 expression began in the lowermost bulb region, that of K6irs2 was delayed up to the height of the apex of the dermal papilla. In contrast, the K6irs4 keratin was specifically expressed in the Huxley layer. Moreover, K6irs4 was ideally suited to further investigate the occurrence of *Flügelzellen*, i.e., Huxley cells, characterized by horizontal cell extensions that pass through the Henle layer, abut upon the companion layer, and form desmosomal connections with the surrounding cells. Previously, we detected *Flügelzellen* only in the region along the differentiated Henle layer. Using the Huxley-cell-specific K6irs4 antiserum, we now demonstrate this cell type to be clearly apposed to the entire Henle layer. We provide evidence that *Flügelzellen* penetrate the Henle layer actively and may play a role in conferring plasticity and resilience to the otherwise rigid upper Henle layer. ***Key words: cytoskeleton/differentiation/gene expression/intermediate filaments/keratin. J Invest Dermatol 120:512–522, 2003***

The hair follicle consists of a central hair forming compartment, which is surrounded by three concentric epithelial structures, the outer root sheath (ORS), the companion layer, and the inner root sheath (IRS). Numerous investigations on the keratin composition of the hair follicle revealed that the epithelial keratins K5, K6, K14, K16, and K17 represent the main keratins of the ORS (Lynch *et al*, 1986; Stark *et al*, 1987; Heid *et al*, 1988a; 1988b; Coulombe *et al*, 1989; Kopan and Fuchs, 1989; Winter *et al*, 1998; Langbein *et al*, 2002b) whereas the members of the recently elucidated hair keratin multigene family are specifically but differentially expressed in the matrix, cuticle, and cortex of the hair forming compartment (Langbein *et al*, 1999; 2001). Recently, a human epithelial keratin, termed K6hf, as well as its murine ortholog, have been identified as specific constituents of the companion layer, a compartment that also expresses the keratins K6, K16, and K17 (Winter *et al*, 1998; Wojcik *et al*, 2001). In contrast, the characterization of the keratins expressed in the IRS, consisting of the Henle and Huxley layers as well as the IRS cuticle, has not kept this pace. Although previous immunohistochemical and gel electrophoresis studies suggest the IRS expression of a wide range of keratins normally found in both 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), only very recently a number of papers have appeared in which hitherto unknown keratin members were described as being specifically located in this follicular compartment. Thus, Bawden *et al* (2001) reported on the characterization of three type I sheep keratins oIRSa1, oIRS2, and oIRS3.1, as well as two human orthologs hIRSa1 and hIRSa3.1, whose mRNAs were all expressed in the IRS of wool and hair follicles. In the same year, Aoki *et al* (2001) described a novel type II keratin, mK6irs, which appeared to be expressed in the Henle and Huxley layers of mouse hair follicles. In addition, Porter *et al* (2001) reported on a new human type II keratin, which they suggested to be the human ortholog of mK6irs. Subsequently, our laboratory detected another human type II keratin, which was expressed in all three compartments of the IRS. Its gene could be localized next to the *K5* gene at one end of a BAC clone, AC055736, which was contiguous with two PAC clones harboring the entire set of type II hair keratin genes (Rogers *et al*, 2000; Langbein *et al*, 2002b). As this keratin clearly exhibited a distinctly higher sequence homology to mK6irs than the keratin

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[1]This article is dedicated to Harald zur Hausen on the occasion of his retirement as head of the German Cancer Research Center (Deutsches Krebsforschungszentrum) in Heidelberg with gratitude and appreciation for his 20 years of leadership.

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Abbreviations: ORS, outer root sheath; IRS, inner root sheath.

described by Porter *et al* (2001), it was named hK6irs1, whereas the designation of the keratin reported by Porter *et al* (2001) was changed to hK6irs2 (Langbein *et al*, 2002b).

In this study we characterize two additional and novel human type II IRS-specific keratins, K6irs3 and K6irs4. We describe the organization of their genes relative to that of the *K6irs1* and *K6irs2* genes, as well as their expression profiles in the IRS, including new findings on K6irs2 expression. In addition, we present new data on the previously rediscovered *Flügelzellen* (Langbein *et al*, 2002b) in the Huxley layer.

MATERIALS AND METHODS

Isolation of the human K6irs3 and K6irs4 cDNAs EMBO/Genebank database analysis was performed using the recently described K6irs1 cDNA sequence as a probe (Langbein *et al*, 2002b). This led to the identification of three novel type II keratin gene loci on human BAC clone AC055715, termed *K6irs2*, *K6irs3*, and *K6irs4*, one of which encoded the recently described type II IRS-specific keratin, *K6irs2* (Porter *et al*, 2001). 3′-noncoding fragments of the *hK6irs3* and *K6irs4* genes were generated by genomic polymerase chain reaction (PCR) (see **Table I**) and used to screen both an arrayed (K6irs4) as well as an unarrayed (K6irs3) human scalp cDNA library by methods previously described (Rogers *et al*, 1995; 2001). Positive clones were isolated and characterized.

DNA sequencing and database analysis The isolated K6irs3 and K6irs4 cDNA clones were sequenced using a fluorescent chain termination DNA sequencing kit (Big Dye, Applied Biosystems, Weiterstadt, Germany) and analyzed on an ABI-310 sequencing apparatus (Applied Biosystems). DNA sequence correction and assembly was performed using the STADEN software program. Genome-wide database searching was accomplished using the BLASTN program. DNA and protein homology comparisons were carried out using the CLUSTAL program. All programs were contained in the Heidelberg Unix Sequence Analysis Resource (HUSAR). In order to establish an evolutionary tree, an amino acid homology analysis of a highly conserved 296 amino acid region encompassing two-thirds of the central rod domain of all known human type II keratins was performed using the CLUSTAL program. Evolutionary tree analysis was accomplished using the CLUSTREE program.

Extraction of keratins, gel electrophoresis, and Western blots Both the ORS and IRS from freshly plucked beard hairs of several volunteers were mechanically removed from the lower part of the hair follicle using a micro glass Potter homogenizer (size 10–25 µl; Carl Roth, Karlsruhe, Germany), and extracted for keratins as described previously (Winter *et al*, 1998; Langbein *et al*, 2002b). In parallel, keratin extracts from footsole epidermis, taken for medical reasons, were prepared (Langbein *et al*, 1993). The extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide) as previously reported (Winter *et al*, 1998; Langbein *et al*, 2002b). For Western blots, gels were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by semidry blotting. After staining with Ponceau Red, destaining, and blocking (5% nonfat milk powder in Tris-buffered saline), membranes were incubated for 1 h with the respective primary antibodies (see *Antibodies*). Thereafter, the blots were washed and the keratins were detected using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions.

Antibodies Primary mouse monoclonal antibodies used were specific for K6 [KS6.KA12; Progen, Heidelberg, Germany; indirect immunofluorescence microscopy (IIF), dilution 1 : 100; ECL, dilution 1 : 10,000], hair keratin hHa2 (clone LHTric17, kindly provided by Dr. I. M. Leigh, Center for Cutaneous Research, Royal London Hospital, London, U.K.; IIF, dilution 1 : 50; see Langbein *et al*, 1999), and ezrin (clone 3C12, Sigma, Deisenhofen, Germany; IIF, dilution 1 : 100). Primary polyclonal antibodies used were specific for keratin K6irs2 (serum designation T3-2; antigen peptide SYKTAAADVKTKGSC; IIF, dilution 1 : 2000; ECL, dilution 1 : 20,000), keratin K6irs3 (serum designation T2-1; antigen peptide YSMLPGGCVTGSGN; IIF, dilution 1 : 2000; ECL, dilution 1 : 5000; for ECL, this serum was purified using an affinity column coated with K6irs3 peptide), and keratin K6irs4 (serum designation T4-1; antigen peptide CGKSTPASIPARKATR; IIF, dilution 1 : 2000; ECL, dilution 1 : 10,000). A cysteine residue was added to the K6irs4 peptide before coupling to keyhole limpet protein (all peptides were prepared by Peptide Specialty Laboratories, Heidelberg, Germany). The antisera were produced in guinea pigs by injection of the above-mentioned peptides (*underlined* in **Fig 2**). Moreover, a rabbit antiserum specific for mouse K6irs (Aoki *et al*, 2001) and cross-reacting with its human ortholog K6irs1 (Langbein *et al*, 2002b) was used for double IIF labeling studies (IIF, dilution 1 : 2000). The secondary antibodies (IgG or IgG + IgM used for IIF at a dilution of 1 : 200) were goat antiguinea pig, antimouse or antirabbit, coupled to Alexa 568 (red fluorescence) or Alexa 488 (green fluorescence) (Molecular Probes, Leiden, The Netherlands). For chemiluminescence detection (ECL), horseradish-peroxidase-coupled rabbit antimouse or antiguinea pig IgG (H + L) (Dianova, Hamburg, Germany) was used at a dilution of 1 : 10,000.

Table I. Primers and PCR conditions for IRS-specific keratin screening and ISH probes

Probe	Size (bp)	Oligonucleotide sequence	PCR annealing temperature
K6irs2–3′	194	tgttttgcctgagccagtattg cccatctttctgcctccatc	55°C
K6irs3–3′	263	acaatcccaatcagaagatgaa gatgcaaggagtccagtcag	55°C
K6irs4–3′	254	ggaaatagatgctgccattctt ggctgtcaaagtcaccattct	55°C

Indirect immuno fluorescence microscopy (IIF) The procedure was carried out essentially as previously described (Langbein *et al*, 2002b). Briefly, after rinsing in phosphate-buffered saline (PBS), cryostat sections of both human scalp (taken for medical reasons and kindly provided by Dr. B. Cribier, Dermatological Hospital, Strasbourg, France) and plucked beard hairs were fixed in methanol (−20°C; 5 min). The sections were subsequently permeabilized with 0.1% Triton-X100/PBS for 5 min and blocked with 5% normal goat serum in PBST (0.001% Triton-X100, PBS). The primary antibodies were applied for 1 h. After washing in PBS, they were followed by the application of secondary antibodies (1 h). After washing in PBS, the slides were rinsed in ethanol, dried, and mounted in fluoromount-G (Southern Biotechnology Associates, Birmingham, AL). Visualization and documentation were performed with a photomicroscope (Axiophot II; Carl Zeiss, Jena/Oberkochen, Germany). For confocal laser scanning microscopy, a Zeiss LSM 510 UV microscope (Carl Zeiss) operating with an argon ion laser (488 nm) and an HeNe laser (543 nm) was used.

***In situ* hybridization (ISH)** ISH on cryostat sections of human scalp or plucked beard hairs was carried out in parallel as described previously in detail (Langbein *et al*, 1999; 2001; 2002b). For ISH, the following probes were used: a 194 bp PCR fragment of the 3′-noncoding region of the K6irs2 gene, a 263 bp PCR fragment of the 3′-noncoding region of the K6irs3 gene, and a 254 bp PCR fragment of the 3′-noncoding region of the K6irs4 gene cloned into the plasmid pCR4:1 (see **Table I**) (Rogers *et al*, 1997; Langbein *et al*, 2002b). Using these plasmids, ^{35}S-radiolabeled K6irs2, K6irs3, and K6irs4 probes were generated by *in vitro* transcription and used for overnight hybridization at 42°C. Sections were washed with 2 × sodium citrate/chloride buffer (SSC)/50% formamide/20 mM dithiothreitol (DTT), 1 × SSC/50% formamide/20 mM DTT, and 1 × SSC/50% formamide/0.1% SDS at 50°C for 30 min each, digested with RNaseA (10 mg per ml, 30 min at 37°C), and washed with 0.5 × SSC/50% formamide/20 mM DTT at 50°C. Sections were then dehydrated in an ethanol series and dried. After dipping in photoemulsion (NTB-2; Kodak) and drying, sections were mostly exposed for 2–3 d, stained with hematoxylin, and embedded in fluoromount. For the recording of the ISH signals by reflection microscopy, the confocal laser scanning microscope LSM 510 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, i.e., IHS signals, in red).

Electron microscopy Conventional electron microscopy was performed essentially as described previously (Langbein *et al*, 2002a). Briefly, plucked hair follicles were rinsed with PBS and fixed in 2.5% glutaraldehyde in sodium cacodylate buffer (pH 7.2) for 30 min. After three 5 min washes in sodium cacodylate buffer, they were postfixed in 2% OsO_4 for 2 h on ice, followed by washes with distilled water. The specimens were then block-stained overnight in 0.5% uranyl acetate in water, dehydrated in an ethanol series and propyleneoxide, followed by

embedding in Epon. Micrographs were taken with an electron microscope EM900 (LEO, Oberkochen, Germany).

Accession numbers The accession numbers for the K6ir3 and K6irs4 cDNA sequences are AJ508776 and AJ508777, respectively. The clone for human K6irs3 can be obtained from the authors. The clone for K6irs4 is available from the German Human Genome Resource Center (RZPD) under the number dkfzp636J1720Q4.

RESULTS

Isolation of cDNAs encoding K6irs3 and K6irs4 and characterization of K6irs2–4 We have recently characterized an approximately 350 kb DNA contig on chromosome 12q13, consisting of two PAC clones, isolated in this laboratory (Rogers *et al*, 2000; Langbein *et al*, 2002b), and BAC clone AC055736, characterized by the Human Genome Sequencing Consortium. The genes present on this domain comprised the entire set of type II hair keratin genes/pseudogenes, the *K6hf* gene, three *K6* gene isoforms (*K6a*, *K6h*, *K6b*), as well as keratin gene *K5*. In addition, the *K7* gene bordered one end of this contig whereas the recently described IRS-specific keratin gene *K6irs1* occupied the other (**Fig 1*A***). Further homology search of the EMBO/Genebank database led to the identification of another completely sequenced BAC clone AC055715, which overlapped AC055736 in the vicinity of the *K6irs1* gene (**Fig 1*A***). Screening for keratin rod domain sequences revealed the presence of four keratin genes on clone AC055715. At one end, we identified the *K2e* gene (Smith *et al*, 1998). In addition, the clone contained the gene for a second, recently described IRS-specific keratin gene, *K6irs2* (Porter *et al*, 2001; Langbein *et al*, 2002b), as well as two other novel keratin genes, which could be shown to also encode IRS-specific keratins (see below) and which were therefore termed *K6irs3* and *K6irs4* (**Fig 1*A***). Using specific 3′-sequences of *K6irs3* and *K6irs4*, we initially screened an arrayed human scalp cDNA library for these novel keratins. This resulted in the identification of a full-length cDNA clone for K6irs4, but yielded only partial clones for K6irs3. We therefore re-screened K6ir3 using the larger, originally nonarrayed library, which led to the isolation of a full-length cDNA for this keratin. The amino acid sequences derived from the K6irs3 and K6irs4 cDNAs are indicated and compared with the previously published K6irs1 and K6irs2 sequences in **Fig 2**. The four keratins shared highly conserved rod domains as well as the flanking non-α-helical H1 and H2 subdomains, whereas their remaining head and, in particular, their tail domains were fairly unrelated. Homology analysis of a 296 amino acid region encompassing essentially the latter two-thirds of the rod domain of the four IRS-specific keratins (**Fig 2**, *dotted line*), and concomitant evolutionary tree analysis with similar regions of all known type II epithelial and hair keratins, not only confirmed the high relationship of the IRS keratins between each other, but also showed their divergence as a group from the other type II keratins (**Fig 1*B***).

Identification of the K6irs2–4 proteins by Western blotting ORS/IRS fractions, isolated from freshly plucked human beard anagen follicles, were extracted for keratins as described previously (Winter *et al*, 1998; Langbein *et al*, 2002b).

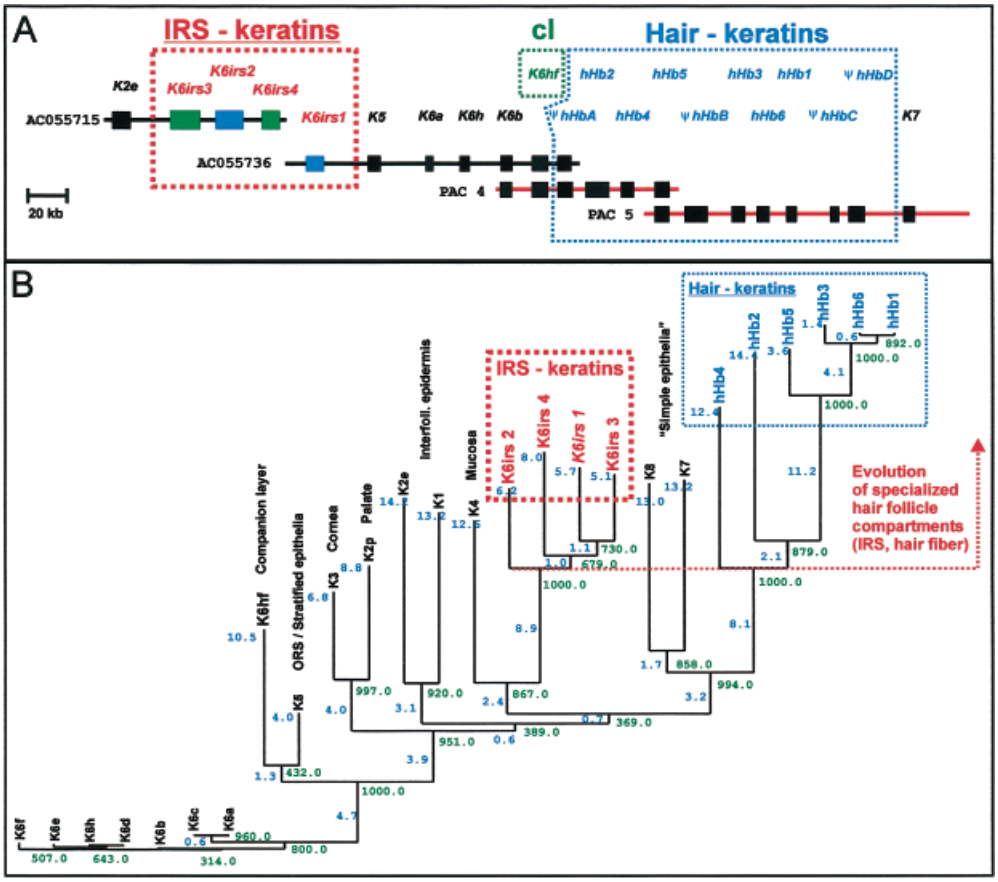

Figure 1. Physical map of the human *K6irs1-K6irs4* gene region and evolutionary analysis of human type II epithelial keratins. (*A*) Shown is the cluster of the IRS-specific type II keratin genes *K6irs1–4* (previously described *K6irs1* and *K6irs2* genes in *blue*, *K6ir3* and *K6irs4* genes in *green*) and its flanking keratin/hair keratin genes (*black boxes*). *Black horizontal lines*, BAC clones characterized by the Human Genome Project Consortium. *Red horizontal lines*, PAC clones characterized in this laboratory (Rogers *et al*, 2000). (*B*) Evolutionary tree of human type II epithelial and hair keratins. Distance values are shown in *blue*; bootstrapping values are indicated in *green*. Note that the IRS-specific keratins and the hair keratins form individual branches, which divided nearly at the same time during evolution.

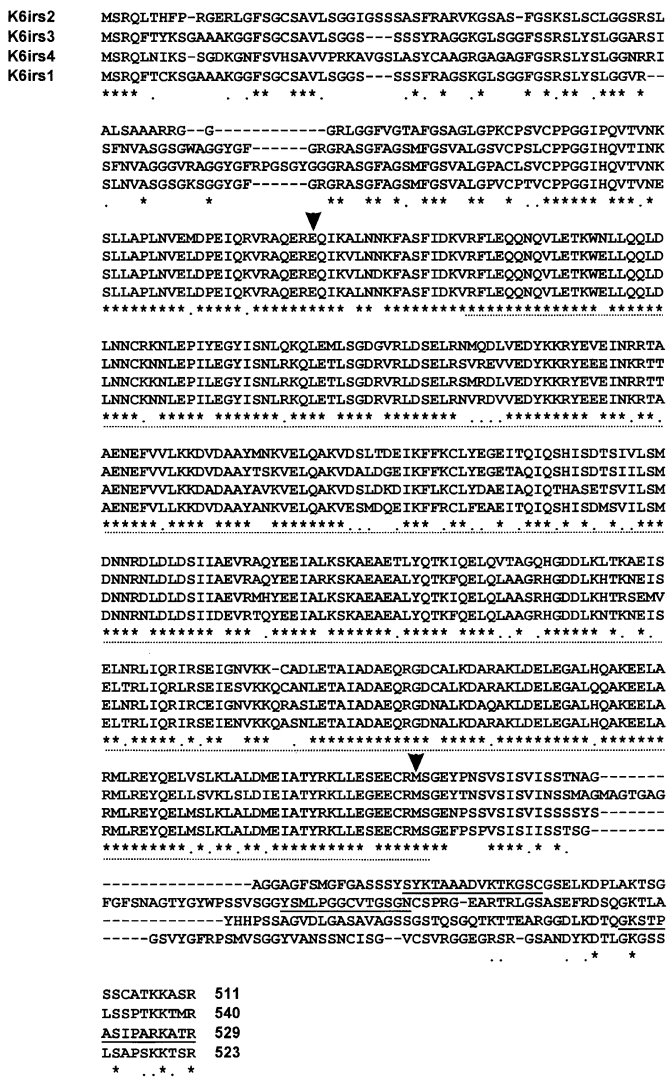

Figure 2. Sequence comparison of the human type II IRS keratins K6irs1-K6irs4. *Arrowheads* indicate the central rod domains of the keratins. *Asterisks* below the sequences indicate amino acid identities. *Underlined* sequences in the tail domains of the K6irs2–4 keratins denote peptide sequences used as antigens for the generation of specific antisera. Sequences underlined by a *dotted line* were used for evolutionary tree construction.

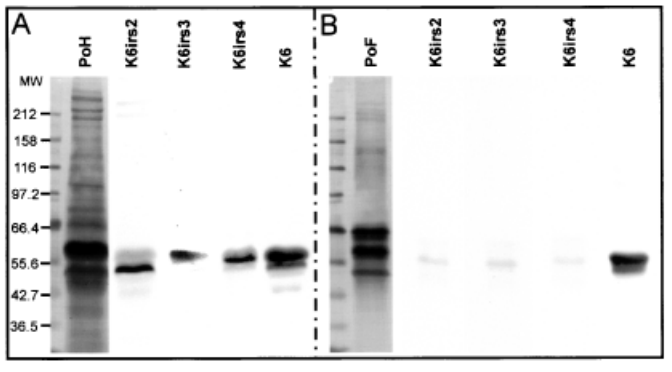

Figure 3. SDS-PAGE and Western blot analysis. Keratin extracts from (*A*) the ORS/IRS fraction removed from plucked beard hair follicles and (*B*) a cytoskeletal extract from human footsole epidermis were resolved by SDS-PAGE electrophoresis (10% gel), transferred to a nitrocellulose membrane, and stained with Ponceau Red (*lanes PoH*, *ORS/IRS*, and *PoF*, footsole epidermis, respectively). Western blot analyses were performed with the antibody/antisera against the indicated keratins. Lanes at the left-hand side of (*A*) and (*B*) represent marker proteins.

In parallel with a cytoskeletal extract of human footsole epidermis, the ORS/IRS keratin extracts were separated by one-dimensional gel electrophoresis and blotted onto nitrocellulose membranes. The membranes were stained with Ponceau Red and subjected to Western procedure using polyclonal antisera directed against specific oligopeptides derived from the tail domains of K6irs2–4 (**Fig. 2**, *underlined*). For comparison, a monoclonal antibody against human K6 was used. As shown in **Fig 3**, each of the K6irs2, K6irs3, and K6irs4 antisera recognized a single protein band in ORS/IRS extracts (**Fig 3*A***) but not in footsole extracts (**Fig 3*B***), whereas K6 could be demonstrated in both extracts. The migration of the individual K6irs2–4 proteins was in good agreement with their calculated molecular weights (K6irs2, 55.9 kDa; K6irs3, 59.0 kDa; K6irs4, 57.9 kDa) and fairly similar to that of K6 (approximately 60.0 kDa) (**Fig 3*A***, ***B***).

Expression of the K6irs2–4 keratins in the hair follicle The expression of the K6irs2–4 keratins was investigated by both ISH with specific 3′-probes and IIF using the antibodies described above. Throughout, cryostat sections of plucked beard hair follicles and human scalp were scored in parallel. As the results were identical in both types of follicles, only those obtained in beard hair follicles are reported. Both detection methods demonstrated that K6irs2 and K6irs3 were exclusively expressed in the cuticle of the IRS. As shown in **Fig 4**(***A***)**–**(***E***), the K6irs3 mRNA synthesis in the IRS cuticle started in the lowermost bulb region and terminated in a region where Henle cells underwent their abrupt terminal differentiation. This is best seen in **Fig 4**(***A***), (***C***), in which the terminally differentiated Henle cells show up as an ascending whitish but clearly discontinuous band. Use of the K6irs3 antiserum confirmed this expression pattern, except that the K6irs3 protein could be followed up to the level of the uppermost cortex region, where IRS cuticle cells were terminally differentiated (**Fig 5*A–D***).

Compared to K6irs3 expression, the onset of K6irs2 mRNA and protein synthesis in the IRS cuticle was clearly delayed, initiating approximately at the apex of the dermal papilla (**Figs 4*F***, ***G***, ***I***, **5*E***, ***G***, ***H***). Moreover, the K6irs2 mRNA expression terminated later at the level of the mid-cortex region (**Fig 4*F***, ***G***), but similar to K6irs3 the K6irs2 protein was demonstrable up to the height of the uppermost cortex region (**Fig 5*E***).

Double label IIF, using the K6irs2 antiserum (*green*) in combination with the antibody against the type I hair keratin hHa2 of the adjacent hair cuticle (*red*) (Langbein *et al*, 1999), not only confirmed the rather late onset of K6irs2 expression in the IRS cuticle (**Fig 5*I***, ***L***), comparable to that of hHb2 in the hair cuticle (Langbein *et al*, 2001), but also indicated that the terminal differentiation of IRS cuticle cells occurred slightly prior to that of hair cuticle cells (**Fig 5*I***, ***K***). **Figure 5**(***M***) represents a double label IIF study with the K6irs2 antibody (*green*) and an antibody against mK6irs (Aoki *et al*, 2001), i.e., the murine ortholog of K6irs1 (*red*), which is expressed in all three IRS compartments (Langbein *et al*, 2002b). The merged yellow staining of the IRS cuticle demonstrates that these keratins are coexpressed in this compartment.

Unlike the IRS-cuticle-specific keratins K6irs2 and K6irs3, both ISH and IIF demonstrated that the expression of the remaining keratin K6irs4 was selectively restricted to the Huxley layer. The expression of K6irs4 at the mRNA (**Fig 6*A–C***) and protein level (**Fig 7*A***, ***D***, ***G*)** began approximately at the height of the line of Auber. Similar to the K6irs2 mRNA, K6irs4 transcripts occurred up to the level of the mid-cortex region (**Fig 6*A–C***), whereas the protein was demonstrable up to the zone of terminal differentiation of Huxley cells (**Fig 7*A***, ***B***). These findings are in contrast to the expression pattern of the previously described K6irs1 keratin in the Huxley layer, in which both its transcripts and the protein could be detected along the complete living portion of the Huxley layer (**Fig 6*H*** and Langbein *et al*, 2002b).

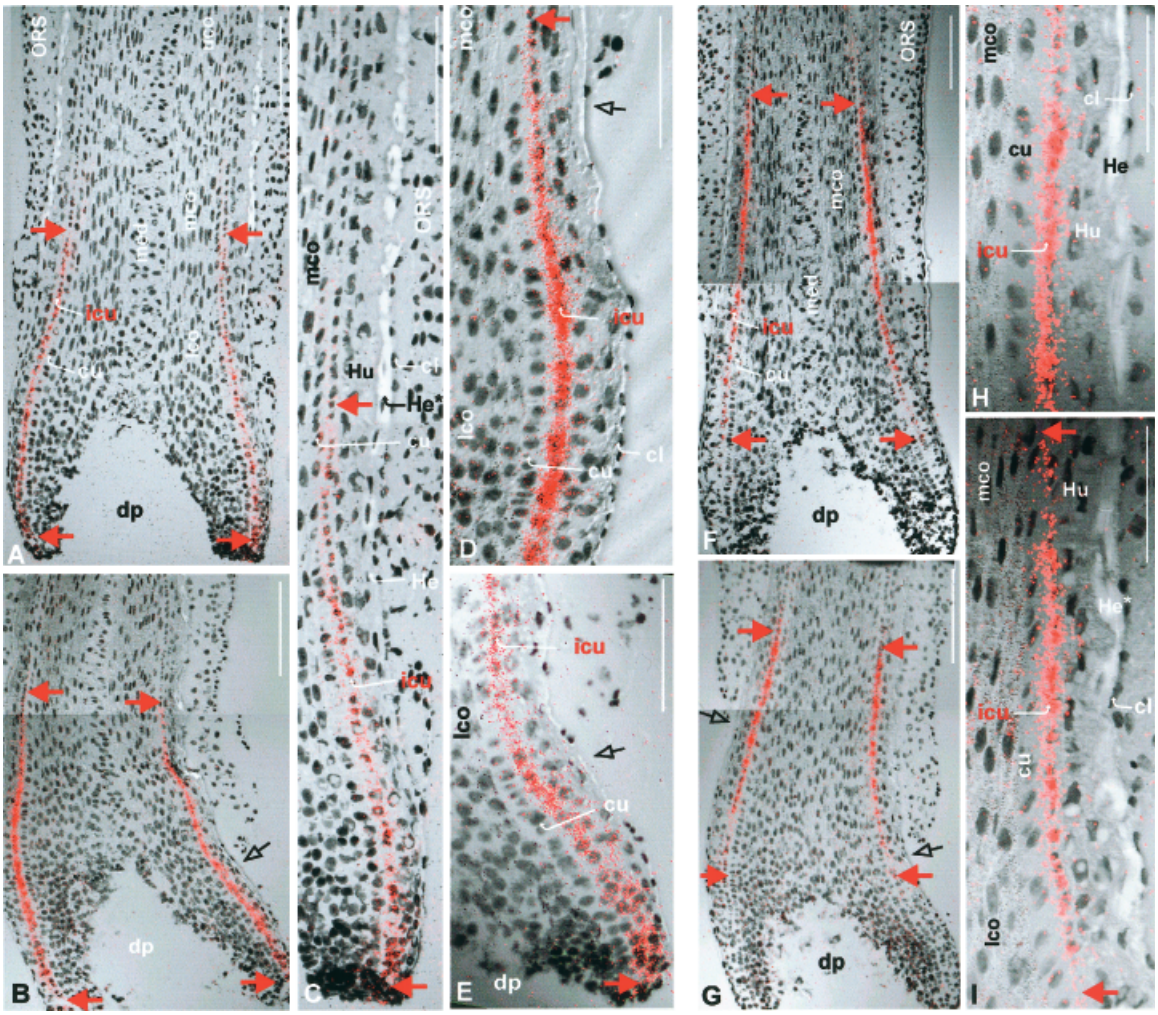

Figure 4. K6irs3 and K6irs2 mRNA expression in hair follicles. ISH with a specific 3′-probe for K6irs3 (*A–E*) and hK6irs2 (*F–I*) on cryostat sections of plucked beard hair follicles. K6irs3 mRNA occurs in the IRS cuticle (*icu*) in which its expression begins in the lowermost bulb region (*red arrows* in *A–C*, *E*) and terminates at the level of the lower cortex region (*red arrows* in *A–D*), more precisely, at the height of terminal differentiation of Henle cells (He* in *C*). Note that in (*B*), (*D*), (*E*) and (*G*), the ORS has been partially lost; therefore the outermost cells of these follicles below the *black open arrows* represent companion layer cells. K6irs2 mRNA is also synthesized in the IRS cuticle but starts only at the height of the apex of the dermal papilla (*red arrows* in *F*, *G*, *I*) and ends at the level of the mid-cortex region (*red arrows* in *F–I*). He, Henle layer; Hu, Huxley layer; cu, hair cuticle; cl, companion layer; dp, dermal papilla; med, medulla; lco, lower cortex; mco, mid-cortex. *Scale bars*: 150 μm.

Presence of *Flügelzellen* in the entire Huxley layer Our previous K6irs1 expression studies had shown that this keratin was expressed in all three compartments of the IRS. In each layer, however, the K6irs1 antibody was accessible and clearly detected its antigen only up to the respective zones of terminal differentiation (Langbein *et al*, 2002b). Typically, Henle cells reached terminal differentiation much earlier than Huxley cells and could then be identified as an ascending whitish band of terminally differentiated cells between the still viable Huxley and companion layer cells (Langbein *et al*, 2002b; see also **Fig 4*A*, *C***). It was along this region that, by both ISH and IIF, we observed numerous K6irs1-positive Huxley cells with lateral, outwardly directed cellular extensions that passed between differentiated Henle cells and abutted on companion layer cells (Langbein *et al*, 2002b). Due to the occurrence of K6irs1 in both living Henle and Huxley cells, these *Flügelzellen*, or "winged Huxley cells", could not clearly be demonstrated by IIF or ISH in the lower portion of the hair follicle, although preliminary electron microscope studies did not exclude their presence in this area (Langbein *et al*, 2002b). The discovery of the new, Huxley-layer-specific keratin K6irs4 enabled us to investigate this area in more detail. As shown in **Fig 6(*D*)**, (***E***), ISH with a specific K6irs4 probe confirmed the presence of *Flügelzellen* along the area of differentiated Henle cells, but unambiguously demonstrated this cell type below the site of Henle cell differentiation (**Fig 6*F***), as well as in the area below the line of Auber (Auber, 1952; see **Fig 6*G***). This encloses the entire Henle layer. An even better demonstration of ubiquitous *Flügelzellen* was obtained by means of the K6irs4 antibody, which, besides high level *Flügelzellen* (**Fig 7*B*, *E*, *C*, *F***), clearly revealed this cell type along the entire Huxley layer below the site of Henle cell differentiation. Obviously, the foot processes occurred between all Henle cells, thus giving the K6irs4-stained Huxley layer the appearance of a saw-blade (**Fig 7*B–G***). Moreover, double label IIF using antibodies against K6irs1 (*green*) and K6irs4 (*red*) also revealed yellow-stained broad and slender foot processes of *Flügelzellen* above and below the level of Henle cell differentiation (**Fig 7*H***).

DISCUSSION

In this paper we describe the elucidation of two new human type II IRS-specific keratins, K6irs3 and K6irs4. Together with the genes of two previously described IRS-specific keratins, K6irs1 (Langbein *et al*, 2002b) and K6irs2 (Porter *et al*, 2001; Langbein

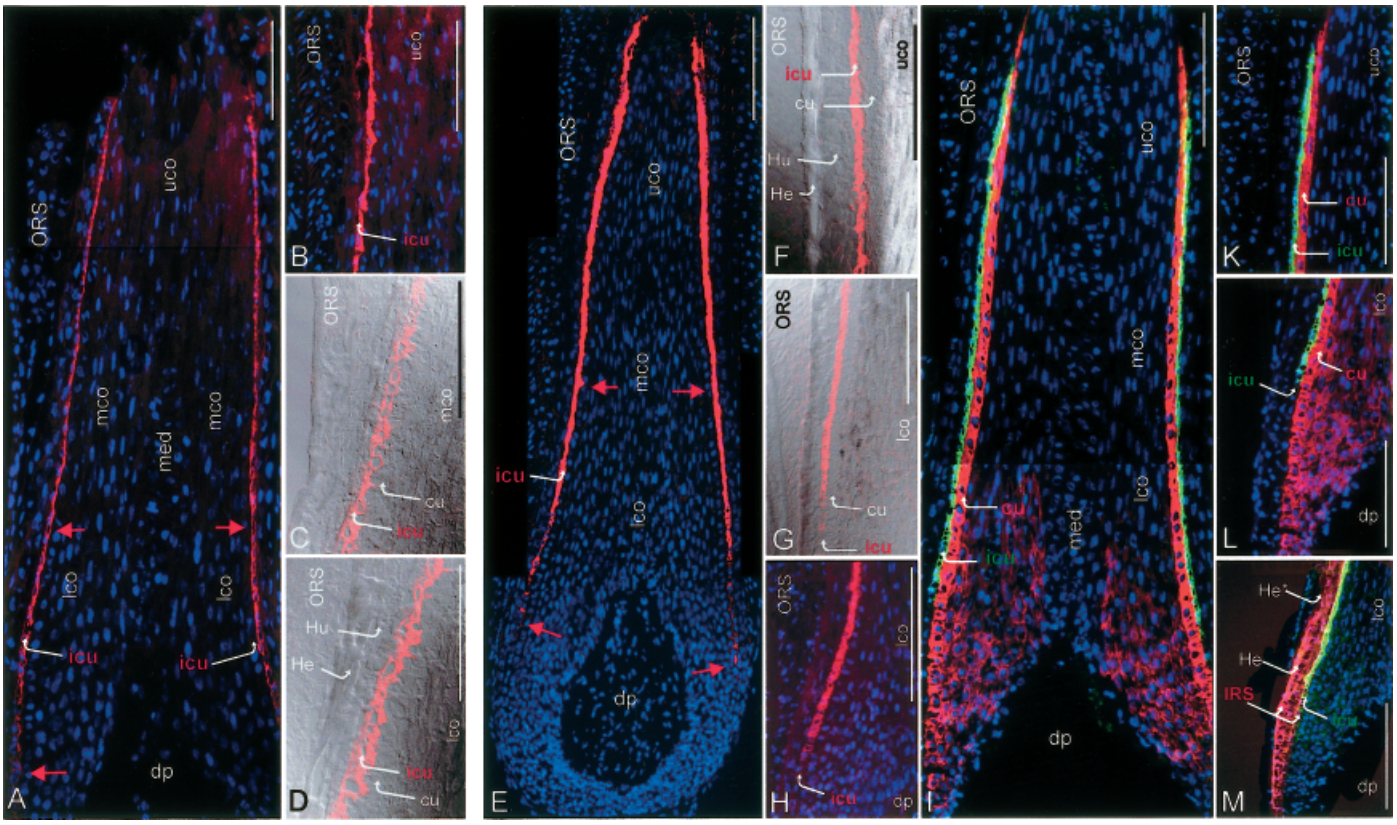

Figure 5. K6irs3 and K6irs2 protein expression in hair follicles. IIF on cryostat sections of plucked beard hair follicles using antisera against K6irs3 (*A*–*D*), K6irs2 (*E*–*H*), K6irs2/hHa2 (*I*–*L*), and K6irs2/mK6irs1 (*M*). Both K6irs3 and K6irs2 proteins occur specifically in the IRS cuticle (icu). K6irs3 synthesis begins in the germinative cell pool (*A*), whereas that of K6irs2 starts later approximately at the height of the apex of the dermal papilla (*E*, *H*). Both proteins can be followed up to the terminal differentiation of IRS cuticle cells (*A*, *E*). Higher magnifications of the K6irs3 and K6irs2 expression patterns are shown at the level of the lower (*D*, *H*), mid- (*C*, *G*), and upper cortex region (*B*, *F*) of the follicle. (*I*)–(*L*) Double labeling study using antisera against K6irs2 (*green*) and the type I hair cuticle keratin hHa2 (*red*). (*M*) Double label IIF using antisera against the previously described mK6irs and cross-reacting with hK6irs1 (*red*) (Aoki *et al*, 2001; Langbein *et al*, 2002b) and K6irs2 (*green*). Whereas K6irs1 is located in all three compartments of the IRS, K6irs2 is seen only in the IRS cuticle (*merged greenish color*). Throughout, *red arrows* indicate the beginning and end of mRNA expression. DAPI nuclear staining (*A*, *B*, *E*, *H*, *I*–*M*); differential interference contrast (DIC) microscopy (*C*, *D*, *F*, *G*). uco, upper cortical region. For further abbreviations, see Fig 4. *Scale bars*: 150 μm.

et al, 2002b), the genes of the novel K6irs3 and K6irs4 keratins form an approximately 75 kb cluster located between the *K2e* and *K5* genes on chromosome 12q13 (Langbein *et al*, 2002b). A preliminary survey of the gene domain upstream of the *K2e* gene (**Fig 1*A***) did not provide evidence for further IRS-specific genes (unpublished data), thus indicating that the *K6irs1–4* gene cluster most probably comprises the entire complement of human type II IRS keratin genes. The construction of an evolutionary tree, comprising all presently known human type II keratins/hair keratins, revealed that both the four structurally related IRS keratins and the six hair keratins form individual, highly bootstrap-supported branches that obviously emerged simultaneously during evolution (**Fig 1*B***).

Our expression studies of the new K6irs3 and K6irs4 keratins included also the K6irs2 keratin, recently described as human K6irs by Porter *et al* (2001). Initially, these authors assumed an orthologous relationship between this keratin and the murine keratin K6irs (Aoki *et al*, 2001). However, our laboratory later found the hK6irs1 keratin to be the true ortholog of mK6irs and therefore we renamed K6irs into K6irs2 (Langbein *et al* 2002b). Unlike these authors, we were unable to raise a specific antiserum against this human keratin and show by both ISH and IIF that K6irs2 as well as K6irs3 are specifically expressed in the cuticle of the IRS, but exhibit a different onset of expression in this compartment. Whereas the K6irs3 expression begins in the lowermost bulb region, that of K6irs2 begins later at the height of the apex of the dermal papilla. In contrast, the K6irs4 keratin exhibits a restriction of its expression to the Huxley layer.

Figure 8(*A*), in which the mRNA expression profiles of the K6irs2–4 keratins are schematically given together with those of the previously described K6irs1 keratin (Langbein *et al*, 2002b), reveals that different numbers of type II keratins are expressed in each of the IRS layers with the outer Henle layer expressing only one (K6irs1), the central Huxley layer two (K6irs1, K6irs4), and the inner cuticle three members (K6irs1–3). Whereas the expression of the two hitherto known human type I keratins hIRSa1 and hIRSa3.1 in the IRS was shown by ISH (Bawden *et al*, 2001), a possibly layer-specific localization has not yet been explicitly demonstrated. Based on our data on type II IRS keratins, it is interesting that, compared to Henle and Huxley cells, the distinctly smaller IRS cuticle cells seem to express the highest number of IRS keratins. This appears plausible considering that IRS cuticle cells are subject to considerable mechanical constraints when tightly interacting with the cells of hair cuticle during the upward journey of the growing hair.

Our previous K6irs1 expression studies had shown that both its mRNA and protein synthesis started in two adjacent cells in the lowermost hair bulb, whose descendants initially ascended as parallel, single layered cell rows, with the outer forming the Henle layer and the inner giving rise to the Huxley layer (Langbein *et al*, 2002b). In this study, the bulbar origin of the Huxley layer from a single cell could be confirmed by the expression profile of the Huxley-layer-specific keratin K6irs4. Previous expression studies of both the companion-layer-specific keratin K6hf (Winter *et al*, 1998) and the hair-cuticle-specific keratins hHa2 and hHb2 (Langbein *et al*, 1999; 2001) consistently identified the origin of

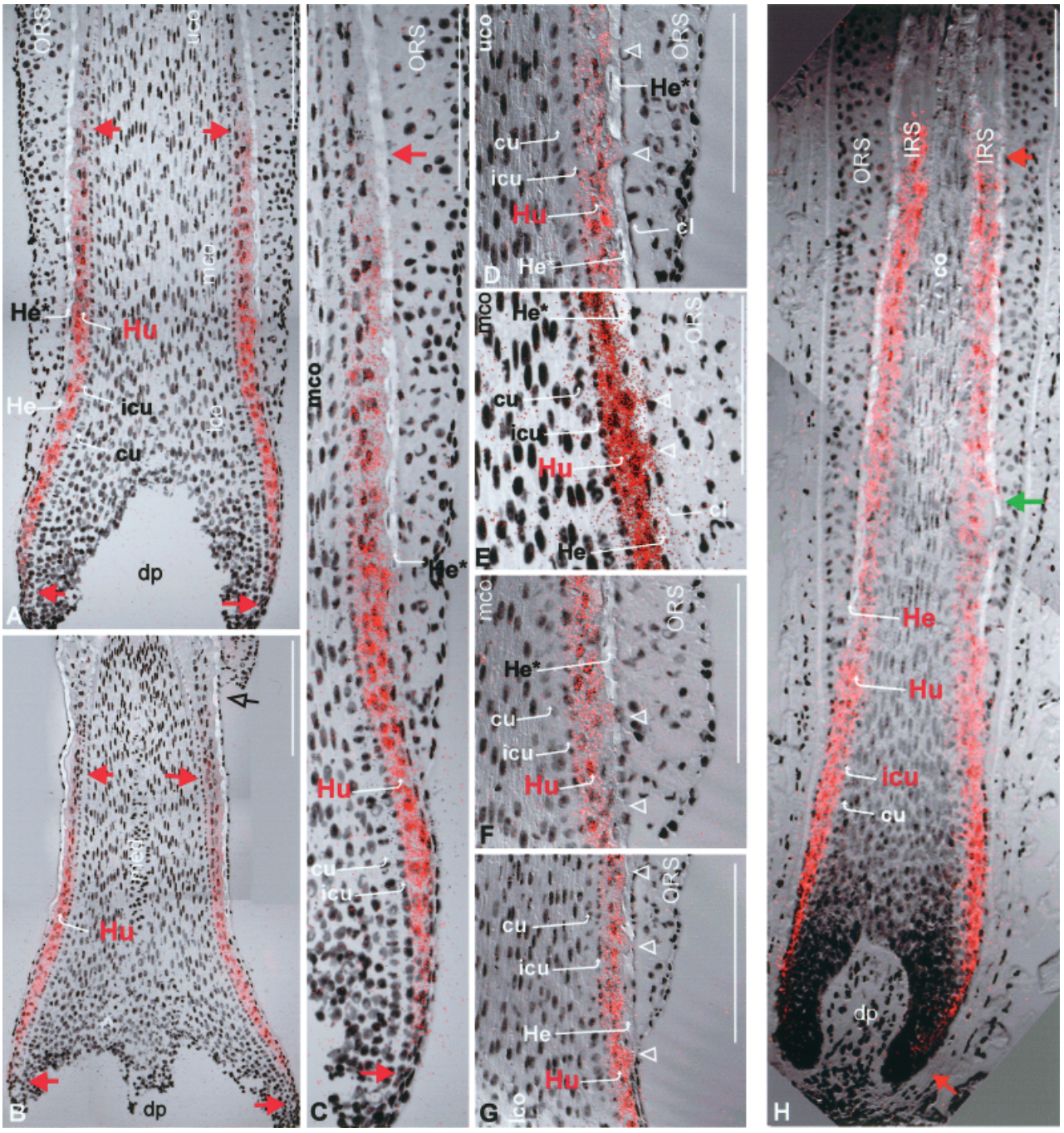

Figure 6. K6irs4 mRNA expression in hair follicles and identification of *Flügelzellen* in the Huxley layer. ISH with a specific 3′-probe for K6irs4 (*A*–*G*) on cryostat sections of plucked beard hair follicles. The K6irs4 mRNA synthesis is restricted to the Huxley layer in which its expression begins approximately at the height of the line of Auber (1952;*red arrows* in *A*, *B*, *C*) and terminates at the level of the mid-cortex region (*red arrows* in *A*–*C*). Note that in (*B*) the ORS has been lost below the level of the mid-cortex region (*open black arrow*). Higher magnifications at the level of the upper (*D*), mid- (*E*) and lowermost cortex region (*F*, *G*) reveal the presence of *Flügelzellen* with pseudopodal processes in the Huxley layer (*white open arrowheads*). (*H*) mRNA expression profile (*red arrows*) of the previously published keratin K6irs1 (Langbein *et al*, 2002b) in all IRS compartments of a scalp follicle. The *green arrow* in (*H*) indicates the site of termination of K6irs4 mRNA expression. He* in (*A*), (*C*), (*D*)–(*F*) denotes the site of abrupt terminal differentiation of Henle cells. For further abbreviations, see Fig 4. *Scale bars*: 150 μm.

these vertical layers as a single bulbar cell located either external to the Henle or internal to the Huxley precursor cells. Due to the very weak expression of K6irs1 in the IRS cuticle (Langbein *et al*, 2002b), we were previously not able to reliably demonstrate a correspondingly low bulbar IRS cuticle precursor cell for this follicular compartment. This gap could now be closed based on the expression pattern of the new K6irs3 keratin. Our data demonstrate that the various compartments of the hair follicle internal to the ORS arise from differentially committed progenitor cells that are generated from pluripotential cells at defined sites in the germinative cell pool at the base of the hair bulb (**Fig 8*B***). Moreover, it is now possible to define each compartment of the hair follicle at the molecular level through specific keratin/hair keratin members (indicated in *red* in **Fig 8*B***).

The previous expression studies with the K6irs1 antiserum had also shown that Henle cells could only be stained up to the level of their abrupt transition into fully keratinized cells, whereas the adjacent Huxley cells, which undergo terminal differentiation

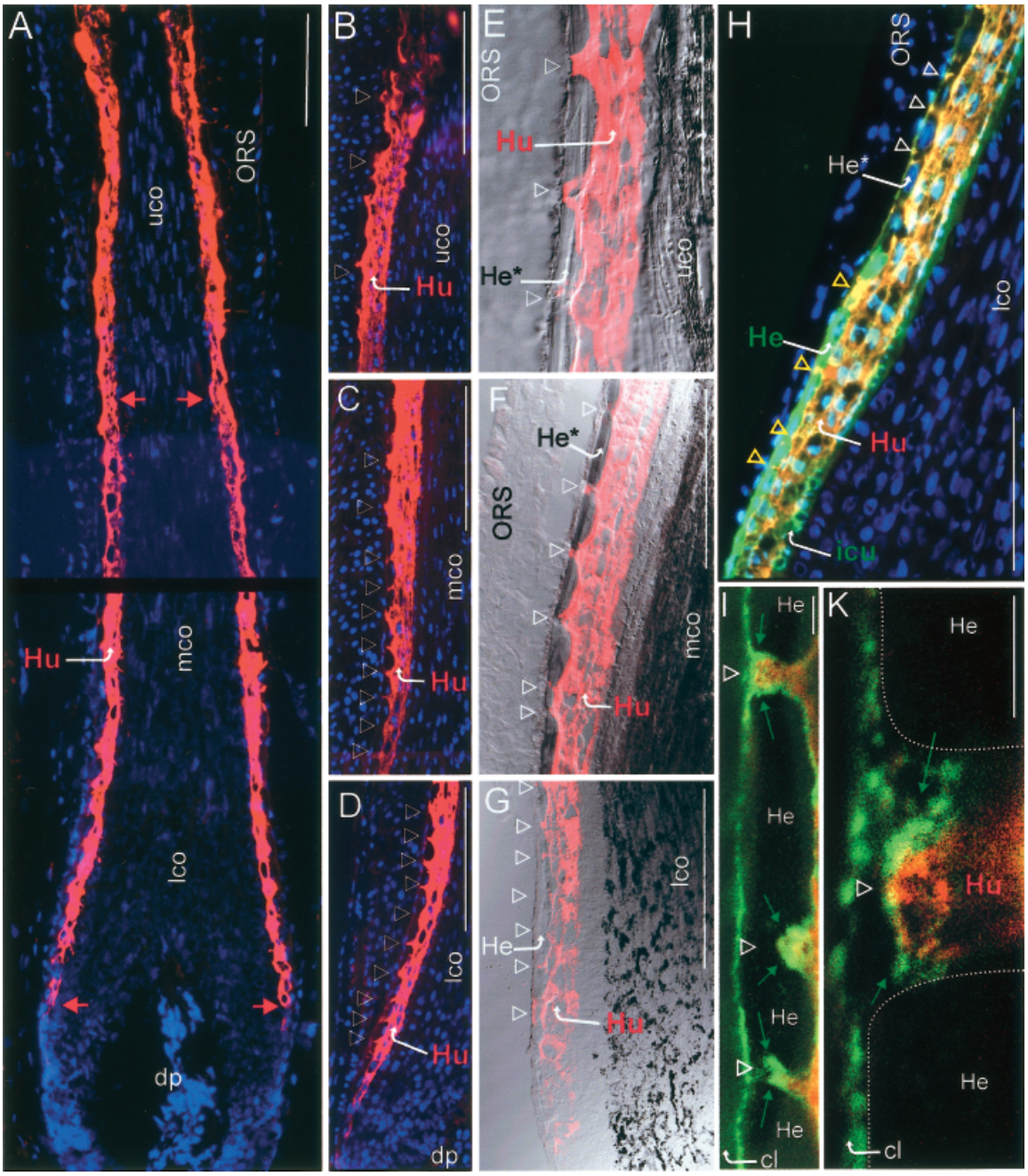

Figure 7. K6irs4 protein expression in hair follicles and identification of *Flügelzellen* in the Huxley layer. IIF on cryostat sections of plucked beard hair follicles stained for K6irs4 (*A*–*G*), K6irs4/mK6irs1 (*H*), and K6irs4/ezrin (*I*, *K*). K6irs4 is specifically expressed in the Huxley layer starting approximately at the line of Auber (Auber, 1952) and terminating at the level of Huxley cell terminal differentiation (*A*, *red arrows*). Higher magnifications of the K6irs4 expression pattern shown at the level of the lower (*D*, *G*), mid- (*C*, *F*) and upper cortex region (*B*, *E*) of the follicle reveal the presence of *Flügelzellen* with pseudopodal processes in the Huxley layer (*open arrowheads*). He*, region of terminally differentiated Henle cells; He, region of viable Henle cells. (*H*) Double label IIF using antisera against K6irs4 (*red*) and mK6irs1 (*green*). Note K6irs1-stained undifferentiated Henle cells (He, *green*), unstained differentiated Henle cells (He*), K6irs4/K6irs1 coexpressing Huxley cells (*merged yellow/greenish staining*), part of which form foot processes passing through the Henle layer (*white* and *yellow open arrowheads*), and K6irs1-stained IRS cuticle cells (icu, *green*). (*I*), (*K*) Double label IIF using antisera against K6irs4 (*red*) and ezrin (*green*) showing ezrin concentration at the leading edges (*green arrows*) of *Flügelzell* protrusions (*white arrowheads*). The *dotted line* in (*K*) demarcates boundaries of undifferentiated Henle cells. DAPI nuclear staining (*A*–*D*, *H*); DIC microscopy (*E*–*G*). For further abbreviations, see Fig 4. *Scale bars*: (*A*)–(*H*) 150 μm; (*I*) 10 μm; (*K*) 5 μm.

considerably later, were still accessible to the K6irs1 antiserum (Langbein *et al*, 2002b). Within the differentiated and unstained Henle layer, we detected differently shaped, K6irs1-positive, horizontal cell extensions that clearly originated from Huxley cells and abutted upon cells of the companion layer. This observation led to the conclusion that the terminally keratinized Henle layer represents a porous cylinder (Langbein *et al*, 2002b).

In the literature, we have noticed that as early as 1840 Henle, in his first description of the IRS, reported on "holes and slits" (*Löcher und Spalten*) in an isolated sheet of the differentiated portion of the outermost IRS layer (Henle, 1840). Although not excluding a preparation artefact, he suggested that this structure might resemble a "fenestrated membrane" (*gefensterte Membran*), as illustrated later, for instance, by Koelliker (1889) (**Fig 9*A***).

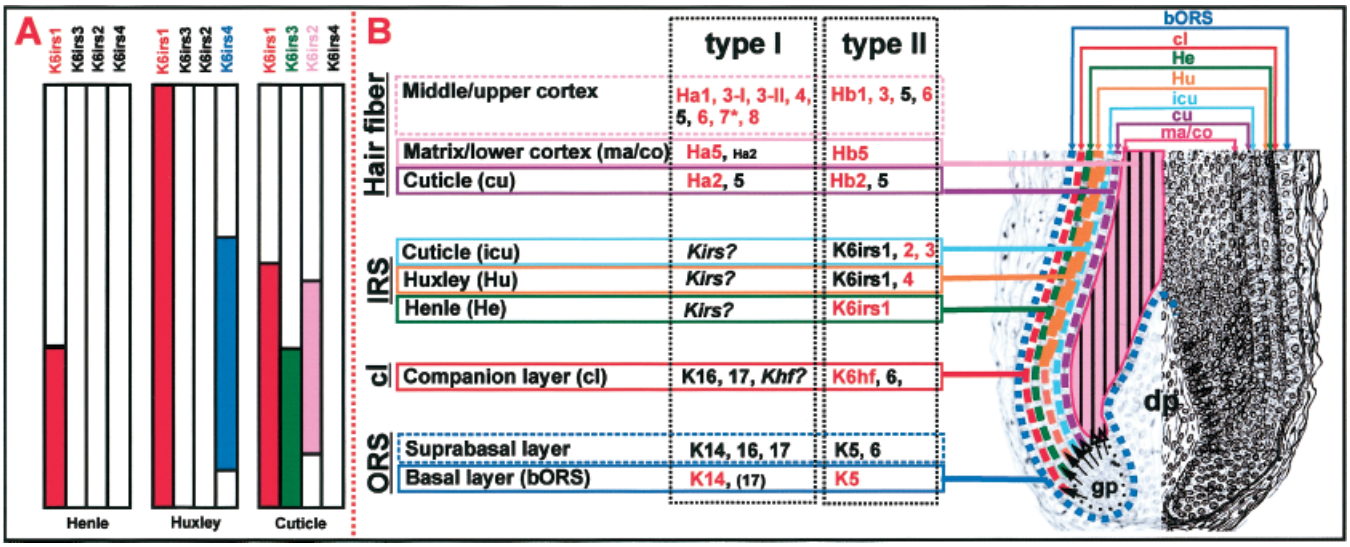

Figure 8. Schematic presentation of expression patterns of presently known keratins/hair keratins in the various hair follicle compartments. (*A*) K6irs1–4 mRNA expression profiles in the IRS of the hair follicle. (*B*) Defined tissue compartments of the hair follicle and associated keratin/hair keratin expression patterns. With the exception of the ORS (*blue*), each of the various follicular compartments arises from cells in the germinative cell pool (gp) at the base of the hair bulb (*closed arrows*, epithelial sheaths; *open arrows*, hair forming compartments) and expresses specific keratin/hair keratins. Keratins/hair keratins indicated in *red* represent members unique for a given tissue compartment. 7*, up to now only found in vellus hairs (Langbein *et al*, 1999). The designation "K6irs?" in the column of type I keratins indicates that the exact location of the recently described human hIRSa1 and hIRSa3.1 (Bawden *et al*, 2001) in the different IRS compartments has not yet been determined. Similarly "Khf?" means that it is still unknown whether the companion-layer-specific type II keratin K6hf possesses a distinct type I partner.

Subsequently, the presence of gaps between cells of the upper Henle layer was confirmed in longitudinal hair follicle sections, in which von Ebner noticed for the first time "eleidin [now trichohyalin] granules-containing cell extensions of Huxley cells which squeeze into these openings and end on the outer root sheath" (Ebner, 1876). Illustrations of these granulated Huxley cell extensions (see **Fig 9*B***, Brunn, 1894) led Waldeyer to emphasize the morphologic resemblance of their cell protrusions with those of the "winged cells of Langerhans" (*Flügelzellen von Langerhans*, now Langerhans cells) (Waldeyer, 1882). In 1927, Hoepke referred to this similarity when coining the name *Flügelzellen* for these Huxley cells (**Fig 9*C***, Hoepke, 1927). Later, ultrastructural investigations of *Flügelzellen* suggested the formation of gap junctions (Clemmensen *et al*, 1991) and demonstrated the presence of desmosomal connections of their foot processes with the differentiated Henle cells between which they pass (Langbein *et al*, 2002b). Recent immunohistochemical studies revealed that, in contrast to previous conceptions, the foot processes did not abut upon ORS cells but on companion layer cells, to which they are also connected by numerous desmosomes (Langbein *et al*, 2002b).

In both the early and more recent studies, the question whether the occurrence of *Flügelzellen* was restricted to the region of terminally differentiated Henle cells or whether they were also present further below remained unanswered. Recently, by electron microscopy, we provided first evidence that *Flügelzellen* already exist immediately below the site of Henle cell terminal differentiation. Our immunohistochemical analysis of the lower follicular regions with the K6irs1 antiserum was hampered by the expression of K6irs1 in both living Henle and Huxley cells, however, including *Flügelzellen*, so that their ubiquitous labeling did not allow the detection of possible foot processes within the lower Henle region (Langbein *et al*, 2002b). In this study, this handicap could be overcome by the Huxley-layer-specific K6irs4 antiserum, which, in longitudinal follicular sections, clearly revealed the presence of the characteristic horizontal protrusions of *Flügelzellen*, most probably between all lower Henle cells. Moreover, K6irs4-stained sections obtained by weakly oblique cuts along the surface of a hair follicle showed a fairly homogeneous cellular staining as long as the cut went through the Huxley layer (**Fig 9*D***, ***E***). Once traversing the Henle layer, however, the homogeneous staining was abruptly replaced by a multitude of mostly spindle-shaped, K6irs4-positive structures of varying size, obviously representing the differently sectioned foot processes of *Flügelzellen* within unstained Henle cells (**Fig 9*D–E′***). It appears obvious that these structures could fit into the gaps within the isolated sheet of the differentiated Henle layer shown in the early drawing of **Fig 9(*A*)**. In retrospect, these data fully confirm Henle's more than 160-y-old notion that the layer that now carries his name resembles a "fenestrated membrane" (Henle, 1840).

What is the function of *Flügelzellen*? We have previously proposed that the companion layer and the three IRS compartments form a functional tissue unit, surrounding the hair forming compartment like the "hoops of a barrel" (Ito, 1989), aimed at properly molding and guiding the growing hair (Winter *et al*, 1998; Langbein *et al*, 2002b). As such, this unit has to be strong and resistant enough to fulfill this task, but has also to be flexible enough to respond to, and to withstand, both vertical and lateral forces constantly exerted on the skin, which are transmitted to the adnexal organs. Due to its comparatively early and strong terminal differentiation, the Henle layer would theoretically be composed in its entire upper portion of heavily keratinized, rigid cells, with the surrounding companion layer as well as the Huxley and IRS cuticle layers still containing fully viable cells. It is evident that such a rigid central layer would substantially reduce the overall flexibility of the proposed unit. We therefore hypothesize that the multiple intercalation into the differentiated Henle layer of living cell protrusions as "joints" provided by *Flügelzellen*, which are firmly anchored by desmosomes to their neighboring cells, is ideally suited to restore the plasticity and resilience of the entire tissue unit and thus to optimally adapt it to its supposed functions.

The question arises whether the formation of gaps in the Henle layer is an inherent property of this layer or whether the gaps are formed by the active penetration of *Flügelzellen*. In general, cell motility and the formation of cellular processes are based on activities of local assemblies of actin and actin-binding proteins, such as ezrin, profilin, and drebrin, which accumulate at the leading edges of lamellipodia and filopodia as well as in cytoplasmic processes of a variety of other cells (Peitsch *et al*, 2001, and references therein). **Figure 7(*I*)**, (***K***) shows a double

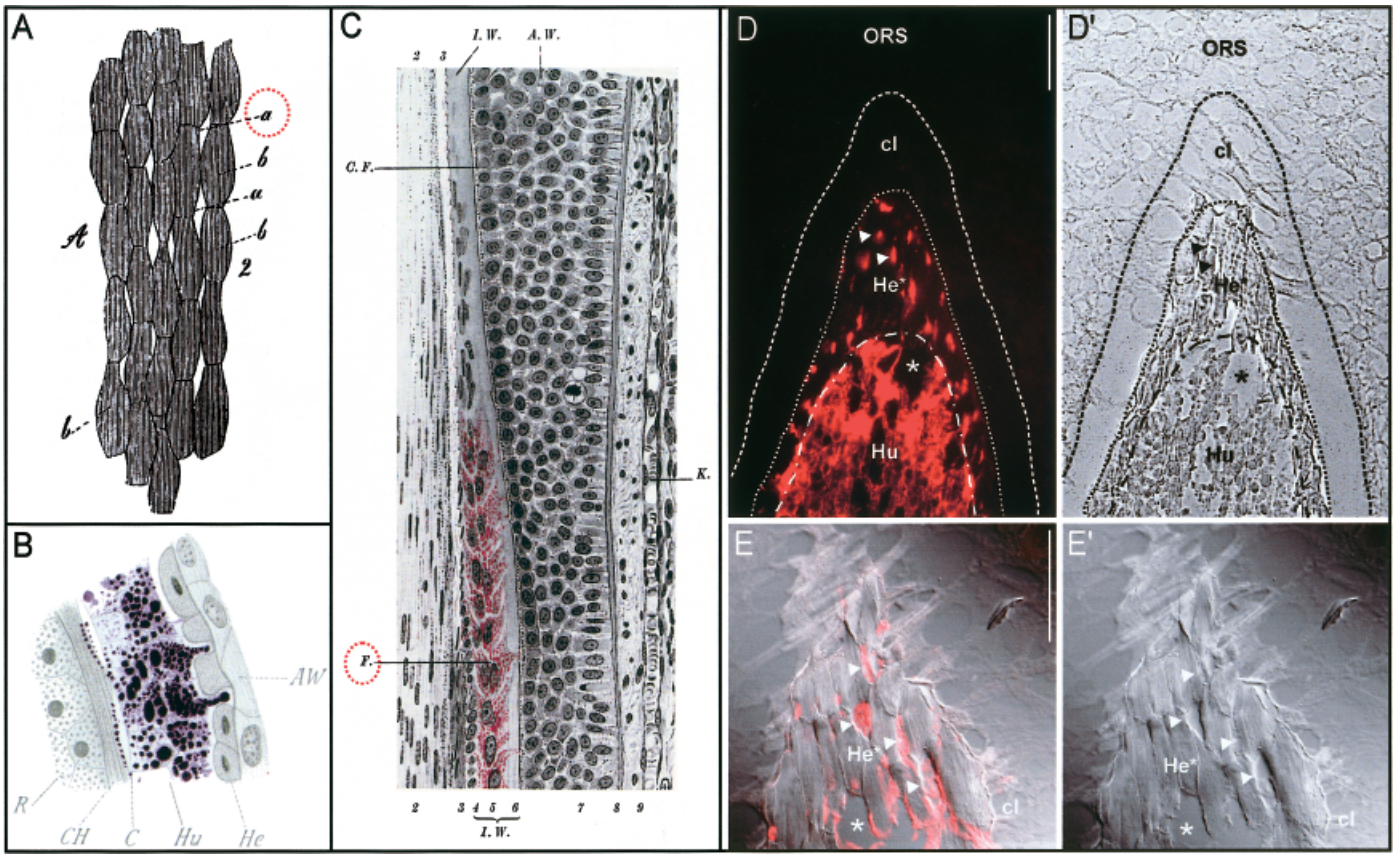

Figure 9. History of *Flügelzellen*. (*A*) Drawing of an isolated sheath of the differentiated portion of the Henle layer (Koelliker, 1889) with "holes and slits" (encircled *a*) between the Henle cells (*b*). (*B*) Drawing of a longitudinal section of the upper portion of a hair follicle, showing trichohyalin-containing Huxley cells and their extensions through the Henle layer (Brunn, 1894). R, hair cortex, CH, hair cuticle, C, IRS cuticle, Hu, Huxley cells, He, Henle cells, AW, outer root sheath. (*C*) Drawing of a longitudinal section of the upper portion of a hair follicle, in which Huxley cells, exhibiting outwardly directed cell extensions (*red*) through the Henle layer, were for the first time designated *Flügelzellen* (encircled *F*) (Hoepke, 1927). 2, cortex; 3, hair cuticle; 4, IRS cuticle; 5, Huxley layer; 6, Henle layer; 7, ORS; 8, glassy membrane; 9, fibrous sheath. Note that in (*B*) and (*C*) *Flügelzellen* are still thought to abut upon cells of the ORS. (*D*) Oblique section through a hair follicle, stained with an antiserum against the Huxley-cell-specific K6irs4 keratin. (*D′*) Phase contrast of (*D*). Note the cytoplasmic staining of all Huxley cells (Hu) in (*D*) and the occurrence of multiple K6irs4-positive, oval structures in the differentiated Henle layer (He), representing extensions of *Flügelzellen*. Some of the structures in (*D*) (*white arrowhead*) are discernible in (*D′*) as white vertical spots (*black arrowhead*). cl, companion layer. (*E*), (*E′*) Oblique section through the differentiated portion of the Henle layer (He), either stained with the K6irs4 antiserum (*E*) or phase-contrasted (*E′*; DIC microscopy). Note that most of the K6irs4-positive *Flügelzellen* appear as clearly visible deepenings in the phase contrast picture (*white arrowheads* in *E*, *E′*). *Asterisks* in (*D*)–(*E′*) indicate a partial preparative loss of Huxley and Henle cells, respectively. *Scale bars*: (*D*), (*E*) 50 μm.

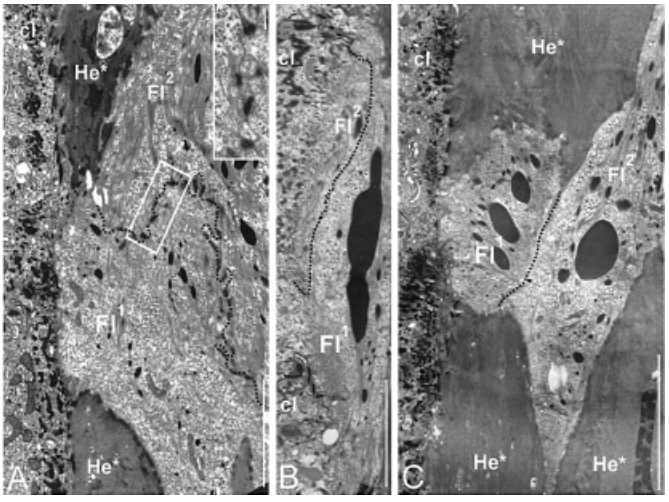

Figure 10. Electron microscope study of extensions of *Fügelzellen*. (*A*), (*B*), (*C*) show various situations of at least two individual trichohyalin-containing extensions of *Flügelzellen* (Fl^1, Fl^2) between differentiated Henle cells (He^*). The *dotted lines* denote the boundaries between the individual foot processes. The upper inset in (*A*) represents a higher magnification of the boxed area and reveals numerous desmosomal connections between the foot processes. cl, companion layer. *Scale bars*: (*A*)–(*C*) 2 μm; *insert* in (*A*) 0.5 μm.

label IIF study of *Flügelzellen* in the lower, undifferentiated Henle layer with the K6irs4 antiserum (*red*) and an antibody against ezrin (*green*). It can easily be seen that ezrin is most strongly expressed at the very tips of the foot processes (**Fig 7*I*, *K***). Moreover, we have previously noticed that in some foot processes, which have already formed desmosomes with the Henle cells through which they pass but not yet with cells of the companion layer, the normally vertical orientation of the keratin intermediate filament network of Huxley cells changes into a stream of horizontally organized intermediate filament bundles (Langbein *et al*, 2002b). Together with the ezrin data, this observation strongly suggests that an active penetration of *Flügelzellen* represents the driving force for the formation of gaps in the Henle layer. It is conceivable that the penetration of *Flügelzellen* occurs easiest as long as Henle cells are still undifferentiated and probably give less resistance to their separation. Remarkably, we

frequently noticed foot processes of two or even more *Flügelzellen* that participated in the creation of intercellular openings in the Henle layer (**Fig 10*A–C***). As we have evidence that, in those composed cellular joints, distinct foot processes may originate from Huxley cells not directly in touch with Henle cells but located more internally towards the IRS cuticle, we do not exclude that the propensity to develop these structures may be a general feature of all Huxley cells. In this respect, the previous notion of *Flügelzellen* as "specialized" Huxley cells (Clemmensen *et al*, 1991; Langbein *et al*, 2002b) would become obsolete.

It should be emphasized that the particular scenario of interactions between *Flügelzellen* and Henle and companion layer cells outlined here represents a highly dynamic system that is subject to a continual opening and reformation of cell connections, probably resulting in changes in the shape of both the foot processes and the gaps during the ascendance of its constituent cells. Provided that foot processes of *Flügelzellen*, as part of this system, also serve to enable the diffusion of nutrients from the outer to the inner cells of the upper part of the hair shaft (Zaun, 1968; Clemmensen *et al*, 1991), then these structures undoubtedly represent one of the most remarkable devices of the hair follicle.

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