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Hair follicle differentiation and regulation

GEORGE E. ROGERS*

School of Molecular and Biomedical Sciences, University of Adelaide, South Australia

ABSTRACT Ten years ago, Hardy (1992) wrote a timely review on the major features of hair follicle development and hair growth which she referred to as a secret life. Many of these secrets are now being revealed. The information discussed in this brief review comprises the structure of the hair and hair follicle, the continuing characterisation of the genes for keratin and keratin associated proteins, the determination of the location of their expression in the different cell layers of the hair follicle, molecular signals which control keratin gene expression and post-translational events in the terminal stages of hair formation.

KEY WORDS: *hair, follicle layer, keratinocyte, keratin gene, gene expression*

A hair arises from the integrated activities of several keratinocyte layers in the hair follicle

Keratinocytes differentiate into several distinct cellular layers of the follicle during the growth phase (anagen) of the hair cycle (Figs. 1,2). From the outermost aspect of the follicle the histological structures are: the outer root sheath (ORS) consisting of several cell layers and the innermost adjacent to the inner root sheath (IRS) is called the companion layer (Orwin, 1971). Adjoining the ORS on the dermal side is a basket-like arrangement of two orthogonally arrayed layers of collagen fibres, the glassy layer (Rogers, 1957) now known as the dermal sheath.The Henle, Huxley and cuticle layers of the IRS; the IRS cuticle layer adjoins the cuticle of the presumptive hair fibre.The presumptive hair shaft comprises an outer layer of overlapping cuticle cells surrounding a cellular cortex and sometimes a central medulla.

The primary activities in anagen hair follicle to produce a hair involve proliferation of the germinative epithelial cells in the bulb region, the determination of cell lineages for all the follicle layers (Fig. 2)and terminal differentiation (keratinisation). The differentiation products of the temporally regulated processes consist of structural proteins within the cells and adhesion proteins between the cells that hold them tightly packed together within the cylindrical-shaped hair.

The region in the bulb where keratinocytes proliferate rapidly is called the critical region or hair matrix zone (Auber, 1950, Orwin, 1979) (see Fig. 2); it surrounds the dermal papilla separated by a basement membrane. Since the seminal experiments of (Cohen, 1961) and (Oliver, 1966) it has been known that the dermal papilla provides essential stimuli for both follicle induction and hair growth. The molecular factors and receptors that are expressed during follicle induction and in the anagen growth phase are becoming increasingly well-defined and the fluctuations of their activity are discussed in reviews (Botchkarev and Kishimoto, 2003, Fuchs *et al.*, 2001). Prominent regulatory proteins in both developing and anagen follicles include the BMPs, Sonic hedgehog and several WNT proteins and the receptors, BMPR1A, EGFR, FGFR and TGFR.

A daughter cell from a mitotic event might move out of the matrix zone of the follicle and differentiate or could remain in the zone and continue dividing. Whether a cell moves or stays within the critical region might be controlled by the level of β1-integrin expression since the expression of β1-integrin is greater in epidermal stem cells and is reduced when they enter a differentiation phase (Zhu *et al.*, 1999). The importance of β1–integrin for the maintenance of proliferating cells in the hair bulb perhaps as a stem cell population was also evident from observations made on the hair follicles of β1-integrin null mice (Brakebusch *et al.*, 2000). The importance of BMPs in restricting a normal proliferating population to the bulb region was demonstrated by the over expression of the BMP inhibitor Noggin, in transgenic mice. In such mice, dividing cells were then observed in the hair shaft distal to the bulb (Kulessa *et al.*, 2000).

The synthesis and intracellular deposition of keratin structural proteins in the spindle-shaped keratinocytes of the cortex leads to assembly of a composite of intermediate filaments (IFs)

Abbreviations used in this paper: IF, intermediate filament; IRS, inner root sheath; KAP, keratin associated protein; ORS, outer root sheath.

***Address correspondence to:** Dr. George Rogers. School of Molecular and Biomedical Sciences, University of Adelaide, South Australia 5005, Australia. Fax: +61-8-8303-4262. email: george.rogers@adelaide.edu.au

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and a matrix of keratin associated proteins (called either IFAPs or KAPs) (Powell and Rogers, 1997), often referred to as the IF-matrix complex. During keratinisation this composite is finally stabilised mainly by the formation of inter- and intra-molecular disulfide bonds. Presumptive cuticle cells that surround the cortex undergo flattening as they emerge from the bulb region and are interlocked with the IRS during their passage up the follicle anchoring the hair in the follicle.

The keratin and keratin-associated proteins expressed in the cortex and cuticle

The term "keratin" is now generally restricted to designating the intermediate filament proteins of the fibre cortex. The proteins of two large families, Type I and Type II, form the IFs of the "hard" keratins of hair (also the "soft" keratins of the epidermis). The two keratin families are distinguished by their isoelectric points (Type I, acidic; Type II, basic/neutral). Equimolar amounts of the two types are required to form the IFs (Steinert and Roop, 1988). Studies of the keratins of sheep (Powell and Rogers, 1997) indicated that the families of chains consist of four Type I chains and four Type II chains. However, investigation of human hair keratin gene families have strikingly revealed that the Type I family has nine members and the Type II has six members (Langbein *et al.*, 1999, Langbein *et al.*, 2001). The genes are respectively on human chromosomes 17q12-21 and 12q13 (Rogers *et al.*, 1995).

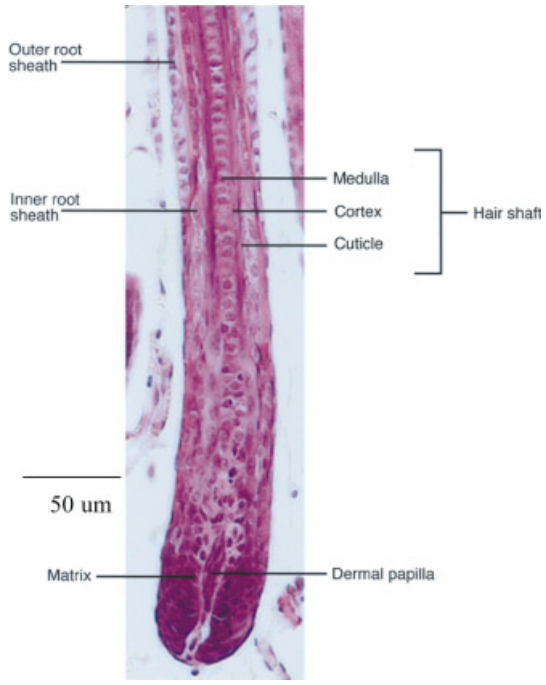
The keratin-associated (KAP) proteins that constitute the matrix of the keratin composite of wool are a large group of possibly up to 100 different proteins. Originally, they were divided into three main families according to their amino acid composition and molecular size. The proteins containing 35-60 mol% of glycine and tyrosine (now KAPs 6, 7 & 8, (Powell and Rogers, 1997)) were originally referred to as the high-glycine/tyrosine group whereas the sulphur-rich (cysteine-rich) proteins (KAPs 1-5 (Powell and Rogers, 1997)) were divided into a high-sulphur KAP group with less than 30 mol% of cysteine and an ultra-high sulphur group with cysteine contents above that value (Gillespie, 1991). Sequence comparisons of the genes for wool proteins showed that there are at least eight families of cysteine–rich proteins and two main groups with more than twenty glycine/tyrosine-rich proteins (Powell and Rogers, 1997). Studies of the human genome for equivalent genes encoding glycine-tyrosine rich proteins identified a domain of 17 genes on chromosome 21q22.1 (Rogers *et al.*, 2002). Furthermore, on the same domain seven KAP genes for high-sulphur proteins were located which extends an earlier study that revealed a cluster of 37 genes for the sulphur-rich KAP group (Rogers *et al.*, 2001) on chromosome 17q12-2 interestingly within a domain of Type I IF genes and could be grouped into seven gene families.

The formation of IF and KAP proteins in hair follicles

There is a high rate of protein synthesis in the hair follicle. For a hair fibre of diameter 100 μm and length growth rate of about 20 μm per h, 5-10μg of protein are produced in a single follicle every 24 h. The large families of *IF* and *IFAP* genes may be necessary for the high rate of synthesis in enabling transcription of mRNAs at a level commensurate with demand.

Since the zone of synthesis and maturation in the approximately lower third of the follicle (called the keratinisation zone) through which a cortical cell passes is about 1000 μm long, it follows that the cells are completely filled with the keratin complex and cross-linked over a period of 48 h as they pass through the zone.

A notable feature is the orderly expression of the IF and KAP proteins in the developing hair. The temporal sequence with which they are laid down has been determined by *in situ* hybridisation detection of specific mRNAs for the different gene families using cRNA probes (Powell *et al.*, 1992, Powell and Rogers, 1997). These studies (Fig. 3) and a more recent and detailed investigation



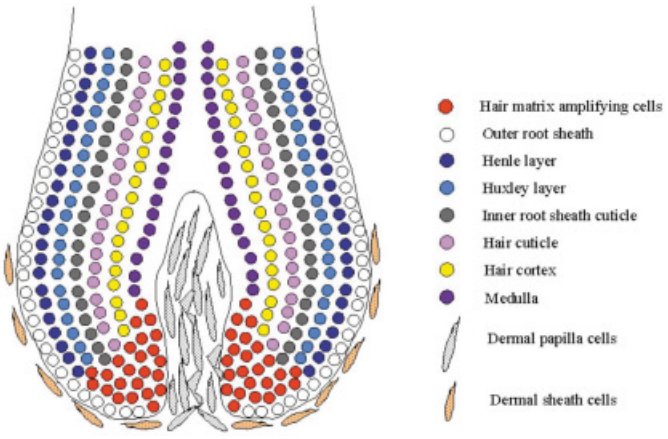


Fig. 1 (Left). Light micrograph of the different layers of the hair follicle. *In the bulb region, a proliferating epithelial matrix surrounds the mesenchymal dermal papilla. The hair shaft of cortex, medulla and cuticle layers enclosed by the inner root sheath move outwards within the outer root sheath which is continuous with the epidermis. (From Millar, 1999; reproduced with permission from Elsevier).*

Fig. 2 (Right). Cartoon of the follicle bulb. *The diagram illustrates the several lineages of cells which are determined in their differentiation pathway and leave the follicle forming the different layers of the hair and surrounding follicle.*

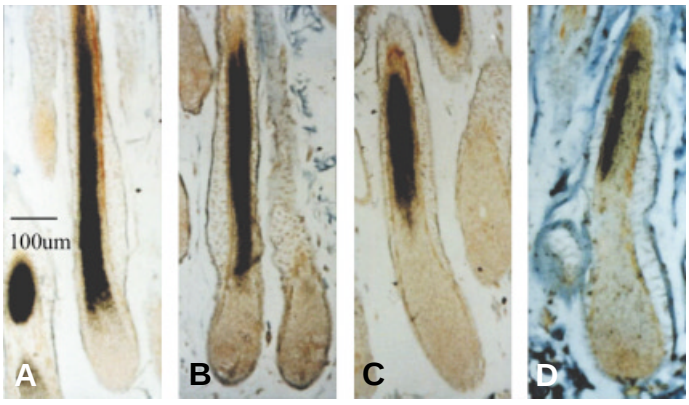


Fig. 3. Localisation of expression of mRNAs encoding the keratin IF and KAP proteins of wool fibre cortex by *in situ* hybridisation. *Specific 3'-cRNA probes were used to localise the sequential expression of* **(A)** keratin IF; **(B)** Glycine-tyrosine rich KAPs; **(C)** Cysteine –rich KAPS (< 30 mol%) and **(D)** Cysteine-rich KAPs (>30 mol%), the last to be expressed.

(Langbein *et al.*, 2001) have conclusively shown that IF genes are the first to be expressed followed by the high glycine/tyrosine genes and then the cysteine-rich protein families.

Hair cortex

In situ hybridisation experiments with cRNA probes (Powell *et al.*, 1992) revealed that IF genes *K2.12, K2.9, K2.10* and *K2.11* for wool keratins are expressed in that order in the cortical cells beginning with K2.12 just above the critical zone of the follicle bulb and the later expression of *K2.11* in the upper bulb region. The expression is coincident with Type I gene expression. Extensive studies of human genes (Langbein *et al.*, 1999, Langbein *et al.*, 2001) have shown that the pattern of IF gene expression is also complex and as to which Type I and II proteins out of some twelve different members specifically pair to form the keratin IFs remains unclear. *In vitro* experiments, similar in kind to those conducted with epidermal IFs (Coulombe *et al.*, 1990, Coulombe and Fuchs, 1990) to determine the relative affinities of different combinations of IF proteins would appear to be a major direction for elucidating the most likely combinations of Type I and Type II chains for hair keratin IF formation. Such investigations are in progress but so far inconclusive (Herrling and Sparrow, 1991, Thomas *et al.*, 1986, Wang *et al.*, 2000) and an important improvement is the utilisation of recombinant IF proteins in such recombination experiments (Hofmann *et al.*, 2002).

Ultra-high sulphur KAP families including those of the cuticle are the last KAPs to be expressed. It should be noted that the expression of the different gene families are not distinct but merge one into the other and the translation of their mRNAs continue until the last stages of hair formation. There is no precise evidence so far of down-regulation of one set of genes and up-regulation of another compared to the transcription of epidermal genes (Fuchs *et al.*, 1989). Biochemical events are reflected in structural changes visualised by transmission electron microscopy (TEM) of the keratinised hair fibre cortex at high resolution. When the cortex is forming the IFs are seen to be aggregated into fibrils and in conjunction with the *in situ* expression data it can be concluded that as the matrix proteins are expressed they migrate into the spaces within the IF aggregates. The synthesis and insertion of matrix proteins become coincident processes especially in the late phases of cortical cell differentiation and aggregation of the IFs. Evidence for this is that in the keratinising zone the matrix proteins appear to aggregate as "blocks" (Fig. 4) that subsequently disperse between the filaments as differentiation advances (Fig. 5). The matrix proteins and IFs interact further to produce either the typical quasi-hexagonal or cylindrical packing of the keratinised hair cortex (Fig. 6) (Fraser *et al.*, 1972, Rogers, 1959b). The relative abundance of the two major KAP groups and possibly the IFs as well may be structural factors responsible for these organisational patterns.

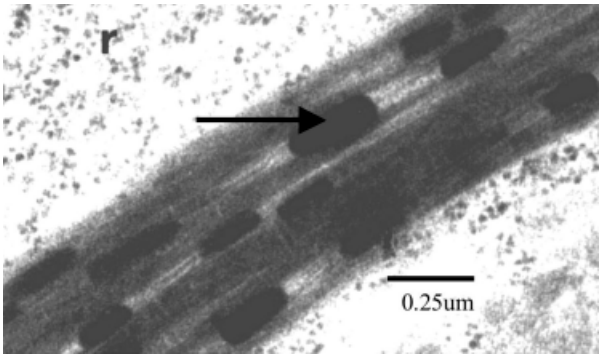


Fig. 4 (Left). Electron microscopic image (TEM) of loose bundles of keratin IFs in a longitudinal section of a wool follicle. *The TEM shows the presence of dense aggregates (arrow) within bundles of IFs (macrofibrils) and are assumed to be KAP proteins. They are also readily observed in cross sections at a later stage of follicles, in which it can be seen that the granules begin to disperse and interact with the IFs (see Fig. 5).*

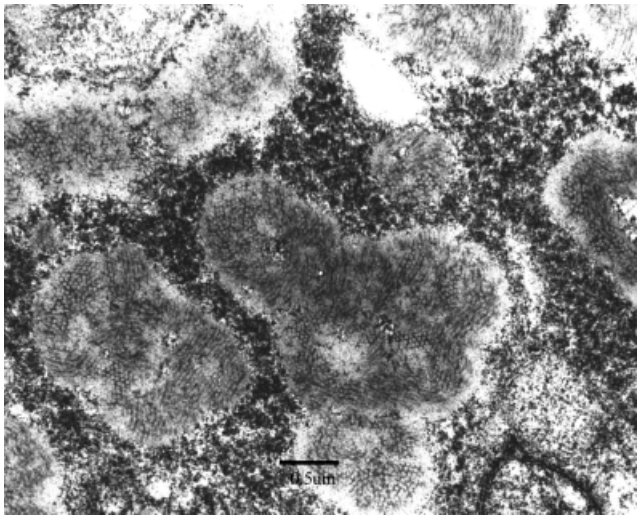
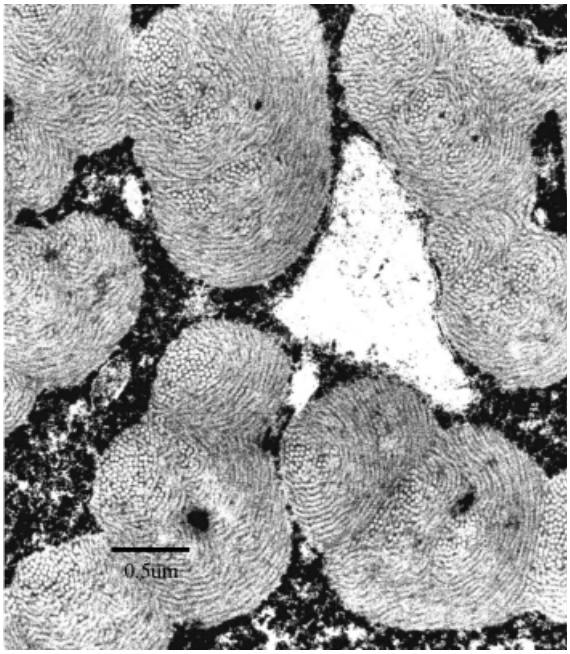


Fig. 5 (Right). TEM of part of a cortical cell in an osmium-fixed follicle. *The aggregates seen as "blocks" in Fig. 4 appear as electron-dense matrix incompletely distributed between the IFs which constitute macrofibrils. Dense cytoplasmic material is abundant between the macrofibrils. This differentiation process occurs in cortical cells at mid level of the keratinisation zone of a hair follicle.*



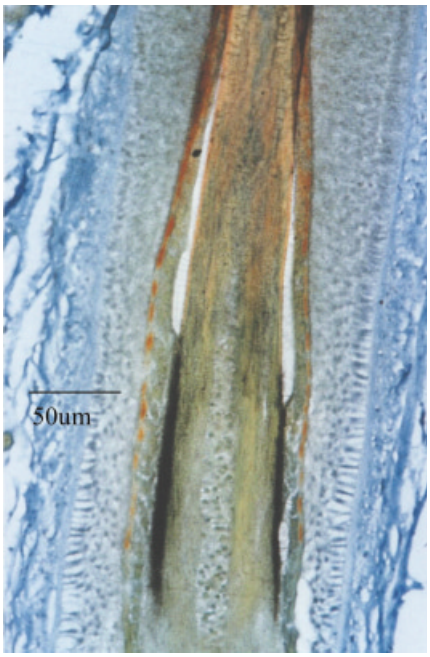


Fig. 6 (Left). Cross-section of macrofibrils of the developing cortex at a late stage of differentiation. *The keratin IF in the macrofibrils are completely separated by the electron-dense matrix consisting of the KAP proteins which have dispersed between them. The IFs display the cylindrical mode of packing. The cytoplasmic material between the macrofibrils has markedly decreased in abundance.*

Fig. 7 (Right). Localisation of the expression of *KAP 5* mRNA by *in situ* hybridisation. *The expression of the* KAP 5 *gene was detected using a 3'- cRNA probe. Strong expression signal appears in the cuticle at a late stage of hair formation in a human follicle.*

From X-ray diffraction studies and TEM studies (Jones *et al.*, 1997, Strelkov *et al.*, 2003) it has been deduced that the organisation of keratin chains within the IFs consists of a regular array of dimer units and an average of 32 chains in the IF cross-section. However the precise location of covalent links between IFs and especially between the IFs and matrix proteins have yet to be mapped in a detail that would more precisely explain the physical properties of hair.

Hair cuticle

The amorphous cystine-rich contents of scale cells (Bradbury, 1973, Fraser *et al.*, 1972) consist of at least two unique families of proteins (KAP5 and KAP10) of the cuticle (Fig. 7) (Jenkins and Powell, 1994, MacKinnon *et al.*, 1990, Rogers *et al.*, 2004). The evidence for the expression of specific IF proteins, Type I (hHa2 and hHa5) and Type II (hHb2 and hHb5) in the developing cells was unexpected (Langbein *et al.*, 2001, Rogers *et al.*, 1996). These chemical findings appear to be in conflict with the microscopic evidence from TEM that IFs are not prevalent in developing cuticle cells. Some "tufts" of IFs can be visualised in the developing scale cells in the bulb region but the prominent structures that are produced are globular masses that fuse to form the exocuticle (Orwin, 1979, Powell and Rogers, 1997, Rogers, 1959a, Rogers, 1959b). The KAP 5 and KAP10 proteins are major components of the exocuticle (unpublished observations). An explanation might be that the IF proteins are degraded in the later phases of differentiation. Other proteins yet to be confirmed in scale cells are those related to keratinocyte cell envelopes such as involucrin and loricrin (Kalinin *et al.*, 2002, Steinert and Marekov, 1995) that are cross-linked by isopeptide bonds (Rice *et al.*, 1994). An additional feature of the hair cuticle is the presence of a group of long-chain fatty acids that is responsible for the hydrophobicity of the hair surface. Chemical evidence indicates that the fatty acids are linked by thioester bonds to protein(s) that are probably components of the scale cell envelope (Jones and Rivett, 1997). Unexpectedly more than 50% of the fatty acids is 18-methyleicosanoic acid (MEA) and a genetic defect affecting the synthesis of MEA produces structural defects in the intercellular layers of the cuticle. The details of the site and synthesis of these cuticular proteolipids are unknown. A summary of the structure of the cuticle of hair in shown in Fig. 8.

Inner root sheath

The IRS is adjacent and adherent to the growing hair and is responsible for the surface topography of the fibre. It is degraded and sloughed as the hair emerges from the follicle. The establishment of the IRS cell lineage is dependent on the activities of several factors and recently the transcription factor GATA-3 has been identified as playing a central role. The IRS is not formed in *GATA-3* null mice, the normal coat is absent and aberrant hairs are produced from embryonic skin grafted onto nude mice (Kaufman *et al.*, 2003, Kobielak *et al.*, 2003).

The IFs in the IRS cells are morphologically indistinguishable from IFs of the cortex but markedly different in protein composition. The expressed genes for a Type I in sheep have been identified and localised in the IRS (Fig. 9), one Type II in mice (Aoki *et al.*, 2001) and four in human (follicles) (Langbein *et al.*, 2003). It is not known whether they specifically pair but the deficiency of one IF in the IRS through a spontaneous mutation in mice, causes collapse of the IF network and interrupts normal IRS formation (Peters *et al.*, 2003). It would seem likely that there are more members of the IRS intermediate filament family to be found.

Differentiation of the IRS is characterised by the synthesis of trichohyalin a specific precursor matrix material that finally binds the filaments into a cross-linked composite analogous to that of the cortex. As the cells move upward with the hair the trichohyalin that is present as cytoplasmic aggregates, undergoes a post-translational modification of arginine residues to citrulline causing the protein to disperse between the filaments (Rogers *et al.*, 1997, Rothnagel and Rogers, 1986). The final IF-matrix composite does

not have the quasi-crystalline packing seen in cortical keratinocytes and instead of disulphide bonds it is extensively cross-linked by isopeptide bonds produced by transglutaminase activity present in the follicle.

Other differentiation products reported in the cells of the IRS include μ-crystallin, a protein primarily found in the eye. It possesses both enzymic and structural properties (Aoki *et al.*, 2000). Several proteins are calcium-binding proteins, namely trichohyalin itself and the enzymes, peptidylarginine deiminase and transglutaminase

Why are the proteins of the IRS different from those of the hair shaft? The answer surely lies in the requirement for IRS cells to be finally sloughed as the anagen hair emerges from the skin surface and is released from the supporting layers of the IRS. This change is achieved by the proteins of the IRS being readily degradable by proteases (Rogers, 1964a) whereas the molecular organization of hair keratin makes it resistant to proteolysis. Proteolytic activity has been observed to be a central feature of epithelial desquamation and is present in the hair follicle distal to the opening of the sebaceous duct (Ekholm and Egelrud, 1998).

Outer root sheath

The outer root sheath (ORS) is continuous with the epidermis but the layer of cells immediately adjacent to the IRS Henle layer has some features that differentiate it as a distinct entity (Rogers, 1964b)]. Notable are tufts of intermediate filaments located at the ORS/IRS junction complex oriented so that they encircle the follicle. This layer was later named the companion layer (Orwin, 1971) but it's role in the dynamics of hair follicle function is unclear. Certainly the junction adjoining the Henle layer is different in lacking desmosomes and that could indicate that the IRS moves relative to the companion layer during outward growth of the hair. The alternative is for the layer to move with the IRS as that layer differentiates with the growing hair. Families of K6 proteins together with K16 (Rothnagel and Roop, 1995, Takahashi *et al.*, 1995, Winter *et al.*, 1998) are expressed in the companion layer and presumably they correspond to the keratin IFs seen in the cells.

Signals which regulate cell specificity and gene expression

The regulatory molecules and their networks that control differentiation of hair keratinocytes are becoming increasingly defined and indeed complex. Notch has been identified as a factor in the determination of cell type (Kopan and Weintraub, 1993, Lin *et al.*, 2000). BMP signalling inhibits follicle development (Botchkarev, 2003) and when the abundance of BMPs was reduced by over-expressing Noggin the differentiation of keratinocytes into mature cortical and cuticle cells was severely impaired demonstrating the key role of BMPs in the formation of the hair layers (Kulessa *et al.*, 2000). The central role of BMP and its linkage to the WNT pathway has been strikingly substantiated (Kobielak *et al.*, 2003) by knocking out the gene for the BMP receptor BMPRIA resulting in hairless mice with malformed follicles. Follicle growth was inhibited and the follicles lacked an IRS although those features are not necessarily causally related.

The regulation of gene activity in the anagen hair follicle shares a significant degree of commonality with the keratinocytes of the epidermis for which a large number of transcription factors for both positive and negative regulation have been recognised (Eckert *et al.*, 1997, Fuchs *et al.*, 2001, Nakamura *et al.*, 2001). The involvement of LEF1 in hair keratin gene expression indicated the participation of this factor in hair follicle development through the WNT regulatory pathway (DasGupta and Fuchs, 1999). The importance of that pathway has been demonstrated by numerous findings that when it is dysfunctional through natural or experimental mutations, hair follicle development is affected and produces a variety of hair and follicle phenotypes. It is also essential for the maintenance of inducing activity of the dermal papilla (Kishimoto *et al.,* 2000; Shimizu and Morgan, 2004). The central molecule in the WNT pathway is β-catenin which has dual

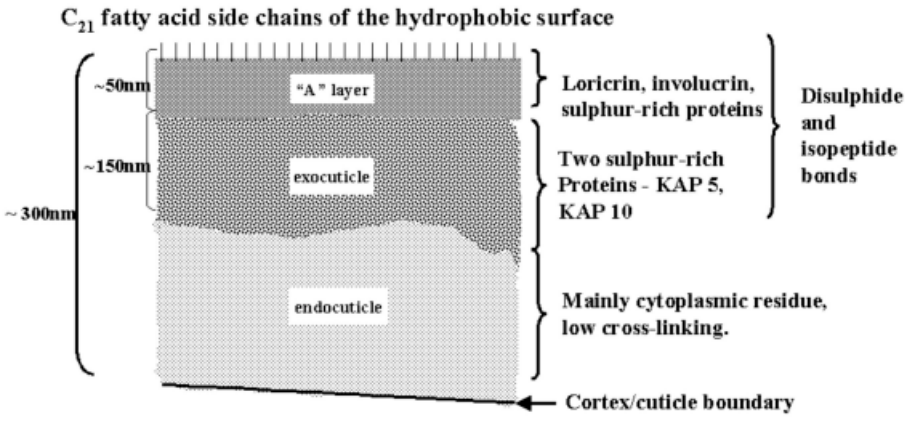


Fig. 8 (Left). Diagrammatic representation of a scale cell of the hair cuticle. *The major intracellular layers, hydrophobic layer, A layer, exocuticle and endocuticleand protein components of a scale cell are indicated in cross-section.*

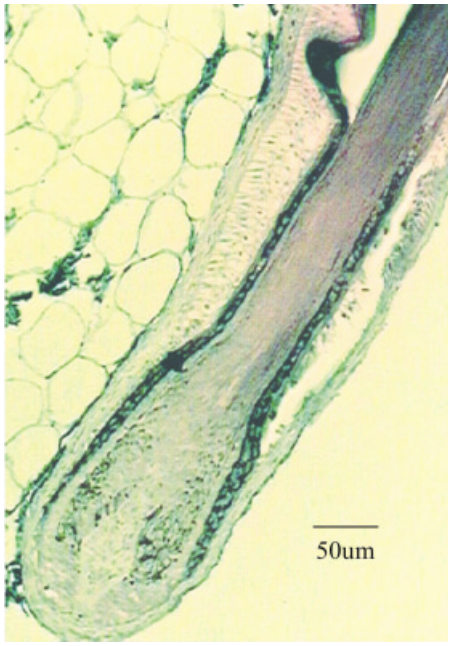


Fig. 9 (Right). Localisation by *in situ* hybridisation of the expression of the mRNA encoding a Type I IF protein. *The mRNA detected by a 3'-probe is highly expressed in the IRS of a hair follicle. The dark material in the bulb region is melanin.*

roles of being part of the cadherin complexes of cell junctions or acting within the nuclei of cells after transportation from the cytoplasm (DasGupta and Fuchs, 1999, Merrill *et al.*, 2001) and forming a transcription complex with the LEF1/TCF DNA binding family of proteins. This complex activates genes involved in hair follicle development and presumably, hair keratin genes as well, given that the *LEF1* consensus is present in the proximal promoters referred to earlier.

An early survey for control sequences in the 5' promoter region of several hair keratin genes (Powell *et al.*, 1992, Powell *et al.*, 1991) revealed several binding sites that are commonly active in the control of gene expression. A sequence *CTTTGAAGA* was found to be common to some 15 hair keratin genes and located between 180 and 240 bp upstream of the transcription start sites (Powell *et al.*, 1991). This sequence was later recognised as the site for LEF1 binding (Zhou *et al.*, 1995) An investigation using a *K2.10 –lacZ* transgene expressing in transgenic mice, demonstrated that all the regulatory elements for expression appear to be located in 400 bp of the promoter of the *K2.10* gene (Dunn *et al.*, 1998). Reduction of the promoter to 200 bp including deletion of the LEF1 site resulted in no expression of the transgene. Site-directed mutagenesis of the LEF1 binding site in the 400 bp transgene allowed patchy expression and indicates that a different factor(s) is required for follicle cell specificity. The presence of trans-acting regulatory factors that bind to the promoter of the *K2.10* keratin gene was demonstrated in hair follicle extracts by DNAse-1 foot printing.

It has been suggested (Powell and Beltrame, 1994) that the coordination of keratin gene expression could be under the control of locus control regions (*LCRs*) that open up chromatin domains and thereby direct the activation of keratin genes as found for the globin gene loci (Trimborn *et al.*, 1999). The regions for control of expression would include Type I and Type II gene families of hair and epidermis keratin IF linked into separate large clusters (Powell and Rogers, 1997) and *KAP* genes that are linked to the Type I keratin *IF* domain an organisation that has been more recently extensively revealed (Langbein *et al.*, 1999, Langbein *et al.*, 2001, Rogers *et al.*, 2004). At the present time it is presumed that the establishment of loci of different families of homologous genes arose by gene duplication but the elements controlling the activity of these loci have yet to be elucidated.

The final events of keratinisation

As the regulated expression of the keratin genes progresses to the last stages and the IF and KAP proteins are laid down, the keratinocytes of the differentiating hair and the accompanying IRS cells undergo other structural changes.

Desmosomes, gap junctions and tight junctions are established between differentiating keratinocytes of the hair fibre and the IRS to varying extents on their upward journey in the follicle. According to electron microscopic studies (Orwin *et al.*, 1973a, Orwin *et al.*, 1973b, Orwin *et al.*, 1973c) gap junctions and desmosomes cover about 10% of the plasma membrane surface of cortical cells in the bulb region and then gradually degenerate. Desmosomes are more abundant in the IRS compared to the developing cortex. Tight junctions (zonula occludens) are also established between Henle and Huxley layers and between Henle cells and those of the apposed companion layer of the outer root sheath (ORS). These junctions probably alter the movement of small molecules (signalling molecules and metabolites) between the cells.

In the differentiation of both hair and the IRS keratinocytes, the junctions are replaced with a new cell membrane complex (CMC) that gradually develops as a continuous layer between the cells. This complex consists of an electron-dense central (δ) layer about 15nm thick surrounded by β-layers that are approximately 5nm wide (Jones and Rivett, 1997, Rogers, 1959a, Rogers, 1959b). Once the growing hair has passed through the keratinisation zone in the lower third of the follicle, morphological changes occur in the nuclei and cytoplasm of all the cells. Although nuclei remain in the cells the chromatin is degraded and mostly resorbed. Several markers have shown that apoptosis participates in the mechanisms that occur during morphogenesis of the hair follicle and in catagen and anagen of the hair cycle (Magerl *et al.*, 2001, Müller-Röver *et al.*, 1998). A low degree of apoptosis continues in the outer root sheath of the anagen follicle but not in the keratinising hair shaft above the bulb region. Instead the nuclear membrane becomes insoluble and remains in the keratinised cell as an elongated structure in the cortex. The changes that make it insoluble are probably isopeptide links (Rice *et al.*, 1994).

The aqueous milieu that supports the biochemical processes of differentiation disappears in the late stages of hardening. The loss of water is probably aided by the rapid disulfide cross-linking of sulfhydryl groups in the newly synthesised keratin proteins (1650 μmoles cysteine/g reduces to about 30 μmoles/g; see Gillespie (1991). This event occurs at the upper region of the keratinisation zone over a distance approximating the length (100 μm) of a cortical cell. How these events are catalysed and regulated is not known although copper in some biochemical form has been implicated in wool growth (Gillespie, 1991, Marston, 1946, Marston, 1949).

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