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Expression of keratin intermediate filament genes in wool follicles of Wiltshire sheep

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ABSTRACT

Co-ordinated expression of keratin intermediate filament genes in the wool follicle is essential for the formation of the wool fibre. The expression patterns of three acidic (oKRT31, oKRT35, oKRT38), one neutral-basic (oKRT85) and one acidic inner root sheath keratin (oKRT27) were localised by in situ hybridisation in the wool follicles of Wiltshire sheep. All five genes show distinct expression patterns in the primary wool follicles of the face and lower leg, but have comparable keratinisation zones to their human orthologues. During follicle regression, the expression of these genes is first attenuated distally, gradually becoming restricted to proximal regions before terminating in dormant follicles. The involvement of the expression of oKRT27, oKRT31 and oKRT85, but not oKRT35 and oKRT38, in brush-end formation indicates specialised regulation of keratin expression in the development of this anchoring structure. Intriguingly, significant differences in the expression of cortical oKRT31, oKRT35, oKRT38 and oKRT85 between primary and secondary follicles were identified. The association of asymmetrical expression of some keratins with follicle curvature raises the possibility of a role in fibre crimp formation. Future studies of these and other keratins will generate an understanding of the structural basis of fibre attributes that could contribute to development of novel wool fibres for specific applications.

Keywords: fibre curvature; hair follicle; in situ hybridisation; wool; follicle growth cycle.

INTRODUCTION

The growing wool follicle is an intricate organ composed of at least seven epithelially-derived cell layers arising from the germinal matrix, encased in a connective tissue sheath and surrounding the mesenchymally-derived dermal papilla (DP) (Figure 1).

During fibre production in the follicle growth phase (anagen), germinal matrix cells in the lower bulb undergo rapid proliferation under the control of the dermal papilla and migrate distally (away from the bulb), differentiating into the cell lineages which form the wool cortex, fibre cuticle and inner root sheath (IRS) (Powell et al., 1992). The finely orchestrated and sequenced production of wool proteins by follicle keratinocytes imparts upon the fibre its final attributes as these cortical cells become almost entirely composed of keratin and keratin associated proteins (KAPs). Keratins consist of the filamentous type I and type II keratin intermediate filaments which form the scaffold of keratin macrofibrils. These two sub-units are expressed in equimolar amounts to form coiled-coil heterodimers via hydrophobic interaction, then further assemble into protofibrils 4-5 nm in diameter (Fuchs & Cleveland, 1998). Grouped protofibrils form 7-8 nm intermediate filaments embedded in the often later-expressed KAPs which cross-link via the disulphide bonds of their abundant cysteine residues to harden the mature keratin macrofibril.

Considerable progress has been made recently in cataloguing the expression of keratin and KAP genes in human hair with the current recognition of nine acidic (type I) and six neutral-basic (type II) keratin genes in the hair shaft (Langbein et al., 1999; Langbein et al., 2001), four type I and three type II genes in the IRS (Langbein et al., 2006) and more than 85 KAP genes in 23 families (Rogers et
al., 2006). In comparison, their identification in the wool follicle has, for some time, stood at only four keratin and 30 KAP genes. We have recently identified cDNA representing new ovine keratin genes (Plowman et al., 2006; Yu et al., unpublished). Despite this progress, a number of questions regarding keratin gene expression remain unanswered. These include their dynamic expression across follicle cycling stages, differential expression in primary and secondary wool follicles and the association of their expression with wool attributes.

The New Zealand (NZ) Wiltshire sheep provides a useful model for studying these questions due to its readily manipulated wool follicle growth cycle (Parry et al., 1991; Parry et al., 1995; Pearson et al., 1996). Follicle regression is manifested by apoptosis in the lower follicle and termination of fibre growth which result in follicle atrophy and formation of the temporary anchoring structure known as the brush-end. The subsequent follicle reactivation is marked by proliferation of cells in the germinal matrix followed by their distal migration, differentiation and keratinisation. The sweat gland-associated primary follicles are relatively straight and generally medullated, while secondary follicles are finer and highly curved in this breed. The different fibre attributes derived from the two follicle types (Craven et al., 2007) provide a valuable tool for the study of crimp formation. Here we describe the spatial expression patterns of fibre component genes and their relationship to follicle cycling and fibre attributes, particularly curvature. Specification of wool at a molecular level is likely to become increasingly important for both traditional manufacturing and non-textile applications.

MATERIALS AND METHODS

Sheep skin samples

A cycle of fleece shedding and wool growth was induced in 12 NZ Wiltshire sheep by imposing an artificial short-day photoperiod regime, followed by a sudden increase in day length (Parry et al., 1995; Pearson et al., 1996). The resultant follicle shutdown, then cyclical return to anagen over approximately three months, allowed timed collection of skin samples accurately representing all cycle stages. Skin samples from different body sites were collected from animals sacrificed at specific cycle stages. Skin samples were fixed in phosphate-buffered 10% formalin and processed to paraffin for determining follicle activity (Nixon, 1993) and expression localisation. Longitudinal and transverse 8 µm sections from appropriate cycle stages were mounted and dried on RNase-free polysine slides for in situ hybridisation. Primary and secondary follicles were distinguished by their size, shape and presence of medulla.

Preparation of probes for keratin genes

Genes were selected on the basis of their distinct expression patterns in human hair follicles (Bawden et al., 2001; Langbein et al., 1999; Langbein et al., 2001). The human type I KRT27 is localised to the IRS, KRT38 only in the fibre cortex, KRT31 in both cortex and medulla, while type II KRT35 and type I KRT85 are found in both the cortex and fibre cuticle. In this paper we adopt the new keratin naming standard as proposed by Schweizer (Schweizer et al., 2006) with the prefix o (ovine) distinguishing the species. The full coding sequences for oKRT35, oKRT38 and oKRT27 have only recently been obtained (Yu et al., unpublished). Primers designed within, or close to, the 3’ untranslated regions (UTR) were used to amplify specific sequence for each selected gene by polymerase chain reaction (PCR). PCR products of expected size were cloned into pGEM-T (Promega, USA) to confirm their identities by DNA sequencing. Plasmids were then linearised to serve as templates for the synthesis of sense or antisense RNA probes using SP6 or T7 RNA polymerase with digoxigenin-labelled uracil triphosphate (Roche). The working concentration for each digoxigenin-labelled probe was determined empirically by testing dilutions ranging from 1:200 to 1:2400.

Digoxigenin in situ hybridisation

In this method the complementary nucleic acid hybridisation of antisense probes to the corresponding mRNA identifies cells and tissues where the gene is being transcribed. We essentially follow the method described by (Miller et al., 1993). Briefly, after dewaxing and rehydration, sections were incubated with proteinase K followed by post-fixation in 4% paraformaldehyde and acetylation with triethanolamine-buffered acetic anhydride. Hybridisation was conducted overnight at 65°C in buffer containing 50% formamide, followed by washing and treatment with RNase A and RNase T1 to remove non-specifically bound probe. After blocking with Roche blocking reagent, sections were incubated with alkaline phosphatase-coupled sheep Fab fragments to digoxigenin. Colour development proceeded at 37°C using 4-nitro blue tetraazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate substrates. When colour development

1 Synonyms from new and previous nomenclatures: KRT27=IRSa3.1, KRT31=KRTTHA1=KRT1.1 (ovine), KRT35=KRTTHA5, KRT38=KRTTHA8, KRT85=KRTTHB5=KRT2.12 (ovine).
was optimal the substrate was washed off and sections counter-stained with eosin or nuclear-fast red prior to mounting. Between 10 and 20 sections for each gene were hybridised with antisense probe in order to visualise the expression patterns in numerous follicles. Respective sense probes were used as negative controls for all genes. The term expression in this paper is generally limited to gene transcription, rather than translation into proteins.

RESULTS

oKRT31

The transcription of this type I keratin began in the cortex above the line of Auber (the widest part of the DP in longitudinal section, Auber, 1951), and extended up into the upper keratogenous zone in both primary and secondary follicles. Most primary follicles had symmetrical expression (Figure 2a), while a small number had weak or no expression on one side of the cortex (Figure 2b). Expression in the medulla was not observed in this or any other genes included in this paper. In contrast, secondary follicles exhibited a clear asymmetry of expression at both the most proximal and most distal expression zones (Figure 2c). Expression was first seen more proximally in the cortical cells of the inside curve of the follicle and terminated in one side in the distal expression zone. Expression of oKRT31 was also seen in late catagen primary follicles forming the brush-end (Figure 2d), but not in telogen follicles.

oKRT35

Compared to oKRT31, oKRT35 was expressed earlier and in both the cortex and fibre cuticle. The mRNA in the cortex was confined to the region in the mid- and upper bulb where cells retained their nuclei and rounded shape. Its distribution in the cuticle, however, extended to the lower keratogenous zone (Figures 2e - 2g). Both longitudinal (Figures 2f & 2g) and transverse sections (Figure 2g) of secondary follicles showed some evidence of asymmetrical expression proximal to the bend above the bulb. No expression was seen in the other follicular structures, or in catagen follicles forming the brush-end (not shown).

oKRT38

Expression for this type I keratin was restricted to the cortex of primary (Figure 2h) and secondary (Figure 2i) follicles. The lowest expression zone in the primary follicles was comparable to that of oKRT31 and extended from pre-cortex to the mid-keratogenous zone. Abundant, asymmetrical expression was also observed in transverse sections (Figure 2j). In secondary follicles, however, the expression of oKRT38 was confined to a few cells of the mid keratogenous zone (Figure 2i).

oKRT85

In primary follicles, expression of the type II oKRT85 began earlier in the cuticle and later in cortical cells of the lower bulb, continuing in both structures through to the mid-upper keratogenous zone. Expression was symmetrically distributed and terminated in both cell layers at the level where only a small number of the cortical cells still retained their nuclei (Figure 3a). Expression in secondary follicles, however, was seen earlier on the fibre’s inside curve and continued much further distally (Figure 3b). Asymmetrical signal distribution was seen in transverse sections of both the proximal (Figure 3c) and distal (not shown) expression zones. During follicle regression, expression gradually became restricted to the proximal region of the expression zone, and was present only in the cortical cells forming the brush-end (Figure 3d) at late catagen, before disappearing entirely in telogen follicles. Expression did not reappear until late proanagen and was maximal in anagen follicles.

DISCUSSION

We have shown for the first time the dynamic spatial distribution of multiple keratin gene transcripts through wool follicle regression from anagen to telogen. In anagen follicles, the ovine genes investigated showed patterns of expression that are broadly similar to their human orthologues (Langbein et al., 1999; Langbein et al., 2001). Keratinocytes first express both oKRT35 and oKRT85 shortly after leaving the germinal matrix,
Figure 2: Expression of three type I hair keratin genes. Presence of target mRNA exhibits a blue/purple signal, visible in these figures as localised areas of significant dark staining. Counterstain is either eosin (a-e, h, j) or nuclear fast red (f, g, i). Scale bars represent 100 µm.

-oKRT31 (a-d): (a) symmetrical and (b) asymmetrical expression in primary anagen follicles; (c) asymmetrical localisation at both the proximal and distal regions in the longitudinal plane and in the transverse planes (lower part of the picture) of curved secondary follicles; (d) expression in the brush-end-forming cortical cells of a primary follicle at late catagen.

-oKRT35 (e-g’): (e) expression in a primary anagen follicle; f to g’: asymmetrical localisation in curved secondary follicles in both longitudinal (f, g) and transverse (g’, inset) planes; (arrows) indicate the distally extended expression in the cuticle and block arrows point to earlier expression in the fibre cuticle.

-oKRT38 (h-j): asymmetrical expression in longitudinal (h) and transverse (j) sections of primary follicles; (i) asymmetrical expression in a longitudinally sectioned secondary follicle.
Figure 3: Symmetrical expression of the type II hair keratin oKRT85 gene. (a) symmetrical expression along the longitudinal plane of a primary follicle; (b) asymmetrical expression in proximal and distal regions of a secondary follicle; (c) asymmetrical expression in transverse section cut through the proximal expression zone of a secondary follicle; (d) expression in the proximal cortical cells forming the brush-end. Counterstaining is either with eosin (d) or nuclear fast red (a-c). Scale bars are 100 µm (a, b, d) or 10 µm (c).

Figure 4: Expression of the inner root sheath gene oKRT27. Expression in the IRS of primary (a) and secondary (b) follicles; (c) expression bordering the brush-end-forming cortical cells of a late catagen follicle. The arrowhead and block arrow in (a) indicate expression in Huxley’s and Henle’s layers respectively. Dashed arrow in (b) indicates the more proximal expression location on the follicle’s inner curve. Counterstaining is either with eosin (c) or nuclear fast red (a, b). Scale bars are 100 µm (a, b) or 10 µm (c).
well below the line of Auber, with expression first apparent in the fibre cuticle. In contrast, the mRNA of oKRT31 and oKRT38 is detectable only in the cortex above the line of Auber. In the distal expression zone, oKRT85 terminates at the same level in both structures, while oKRT35 extends further into the higher keratogenous zone in the cuticle, differing from its human orthologue. Relative to the restricted cortical expression of oKRT35 in the bulb and precortex, KRT85, KRT31 and KRT38 are expressed over a greater length and extend into the mid- to upper keratogenous zone of the cortex.

Bilateral distribution similar to that of oKRT35 and oKRT38 in primary wool follicles has been reported for KRT31 in the cortex of chimpanzee and gorilla hair follicles (Winter et al., 2001), although the biological significance and regulatory mechanisms remain unknown. The bilateral expression of oKRT38 in both the primary and secondary wool follicles differ from that of oKRT4.3 and oKRTAP6.1 which was observed only in the secondary follicles (Fratini et al., 1994; Fratini et al., 1993). The role of oKRT38 in wool follicle and fibre attributes may merit further study.

The IRS functions as a rigid mould for shaping and supporting the new fibre during growth. Additionally, the disintegration of the IRS at the zone of sloughing allows exit of the hair shaft without damage and loss of continuity with the epidermis (Alibardi, 2004). That the majority of the cells produced in the bulb are destined to become part of the IRS further indicates the importance of this structure (Wilson & Short, 1979). oKRT27 and trichohyalin (Fietz et al., 1993), another important IRS matrix protein, share similar expression patterns extending the length of the IRS, but are attenuated at the apex of the DP (Yu et al., unpublished data). The termination of oKRT27 expression in Henle’s layer at this level and its increase in Huxley’s layer further up the wool follicle IRS contrasts with expression in all three IRS layers of human follicles (Langbein et al., 2006) and in the Henle’s layer and fibre cuticle only of mouse follicles (Porter et al., 2004).

During follicle regression, keratin gene expression in the cortex is gradually restricted to the lower bulb, and shuts down completely upon entry into telogen. This is consistent with the cessation of keratinocyte proliferation and upwards-migration and hence earlier completion of keratinisation in more proximal cells. However, the presence of mRNA from some keratin genes (oKRT27, oKRT31, oKRT85), but not others (oKRT35 and oKRT38), in the brush-end-forming catagen follicle indicates that their involvement is not dictated by their expression site during anagen, but presumably by special requirements for the formation of this anchoring structure. In contrast to the gradually restricted, but high level expression during follicle regression, these genes are expressed only at very low levels in the early stages of follicle reactivation. No expression was detectable in early proanagen stages. By late proanagen (proanagen IV and V) (Nixon, 1993), however, their expression increases rapidly, becoming maximal in anagen (Yu et al., unpublished data).

The fundamental mechanisms of fibre crimp formation remain uncertain, although numerous hypotheses have been proposed. The bilateral segmentation of fine wool fibres into ortho- and para-cortical structures, and their associated differences in protein content (Fratini et al., 1994; Fratini et al., 1993), implies differential gene expression is likely to be a key underlying factor. In addition, the strong association of fibre crimp and follicle curvature suggests that they may derive from the same mechanism. The earlier expression of oKRT31, oKRT35 and oKRT85 in the precortex on the inner side of the major follicle bend illustrated in this study, and the bilateral expression of oKRTAP4.3 and oKRTAP6.1 in the secondary, but not primary follicles (Yu et al., unpublished data), provides further evidence of a structural basis for crimp formation. Asymmetrical patterns of expression similar to that of oKRT27 has also been observed for trichohyalin (Yu et al., unpublished data) which is also abundantly expressed in the IRS. If these differences in mRNA levels cause variations in fibre protein composition, they are likely to give rise to heterogeneous keratin cross-linking and hardening during fibre formation. Similar irregular expression of KRT82 and other genes has also been linked to the formation of curly human hairs (Thibaut et al., 2005). The more distal asymmetrical expression of oKRT31, oKRT85 and oKRTAP4.3 and oKRTAP6.1 (Fratini et al., 1994; Fratini et al., 1993) may further contribute to the consolidation and extension of fibre curvature. Ongoing quantitative studies in this laboratory have identified the differential expression of some genes between sheep breeds with varying fibre attributes, including curvature. Coupled with improved understanding of the underlying control mechanisms for keratin and KAP genes, this information could allow us to exploit existing variation or alter keratin composition in fibre producing animals. These studies are steps toward relating the complex, underlying chemical structure of wool to fibre attributes and product performance in apparel and interior furnishing.
markets, as well as specifying biopolymer properties in novel, non-textile applications.

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