## FUTURE EXPERIMENTS

Building on this experience, three larger microarray experiments are underway. These are directed at identifying key genes involved in the formation of ovine primary follicles using foetal skin collected between days 47-67 of pregnancy and during follicle regression and reactivation in adult sheep. The experimental designs allow direct comparison of skin samples representing the principal transition states while allowing indirect comparisons between any pair of RNA samples from the same or other trials (Churchill, 2002; Yang & Speed, 2002). Genes that show altered expression at key stages of follicle development, but which are also differentially expressed during follicle regrowth, are likely to have causative roles in cellular functions common to both processes. The combined analysis of these experiments will provide a wealth of information on the specific genes and networks involved. For example, results from the follicle cycling experiments will be relevant to an understanding of the controls determining fibre length across the surface of the body (Scobie *et al*. 2006). However, different tissue comparisons will be required to access mechanisms controlling other attributes such as fibre diameter or crimp. Genes associated with wool follicle development and cycling could, in turn be compared with recent expression profiling of separated epithelial and dermal cells from mouse hair follicles (Rendl *et al*., 2005) to confirm general mechanisms of hair growth in mammals and assist in finding useful leads for improving wool production in sheep.

Following identification of putative control genes by microarray, the candidates are progressively screened through a series of analyses, each contributing further functional information. Differential expression is confirmed using Northern blot hybridisation and quantitative real-time PCR. Localisation of expression to skin structures can be described using *in situ* hybridisation or immunocytochemistry (if appropriate antibodies are available). Previously unidentified ESTs can be characterised through sequence extension and ongoing bioinformatic analysis. Ultimately, tests of gene function must be conducted using *in vitro* and *in vivo* assays to determine relevance to wool growth.

The most promising gene candidates are then prioritised for the development of new products and technologies that will support increased profitability of sheep farmers.

## ACKNOWLEDGEMENTS

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# Wool keratins – the challenge ahead

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## ABSTRACT

Sheep wool is predominantly composed of keratin proteins. These comprise keratin intermediate filament proteins embedded in a matrix of smaller keratin associated proteins. As a result of polymorphisms and post-translational modifications, these proteins display many more family members than there are genes. Recent studies using improved gene expression, electron tomography and proteomic techniques have provided useful preliminary data on the association of keratins with certain fibre properties. Current research is quantifying keratin gene expression in the skin and the consequent wool protein compositional differences between breeds of divergent fibre and fleece characteristics. A detailed understanding of the control of keratin gene expression and fibre protein composition arising from these and other studies will provide the foundation for the development of novel high-value wool types, solubilised keratins for specialised cosmetic applications and reconstituted keratin polymers for biomedical applications.

**Keywords:** sheep breed; genome; proteome; keratin.

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## INTRODUCTION

Keratin proteins form the major component of wool, making up some 98% of the fibre. They are distributed between the inner region, known as the cortex, and the overlapping scales of the cuticle, the latter constituting approximately 10% of the fibre volume. Mature cortical cells of a growing wool fibre are spindle shaped, about 100 μm long and 1-10 μm wide at their widest point, arranged in an overlapping and interdigitating fashion along the length of the cortex. The cortical cells are largely comprised of keratin intermediate filament proteins (KIFPs) and keratin associated proteins (KAPs), the main structural components of wool fibre. Following their sequential expression, commencing in the follicle bulb, the Type II KIFPs interact with their Type I counterparts, creating a filament scaffold. Many smaller KAPs, found in the matrix around the intermediate filaments, are expressed in the later stages of cortical cell differentiation and are believed to be cross-linked to the keratin intermediate filament proteins to strengthen the fibre (Powell *et al*., 1992). Differences in dye uptake between some fibre types are thought to be linked to the bilaterally distributed populations of structurally and compositionally- distinct cortical cells (the ortho- and paracortex) (Fraser & Rogers, 1955). In general, the paracortex follows the inside of the crimp wave while orthocortex is located on the periphery.

Wool has generally been thought to contain four abundant Type I and II keratin intermediate filament proteins, though the existence of a fifth has been postulated, based on evidence that five of each type have been reported in other mammalian hairs (Powell *et al*., 1992; Powell, 1996). More recently, nine human Type I and six Type II KIFP genes have been identified in human hair (Langbein *et al*., 1999; Langbein *et al*., 2001). In the case of wool, only two complete protein sequences for the Type I and three for the Type II keratin intermediate filament proteins are known (Powell *et al*., 1992; Powell, 1996). Likewise, the use of extensive bioinformatic analysis and gene expression approaches have revealed a total of 85 KAPs in 23 families in human hair (Rogers and Schweizer 2005), but only 30 ovine KAPs have been identified, of which 26 have been sequenced (Powell & Rogers, 1997). The number of potential proteins becomes even higher when polymorphisms are taken into account in both species (Rogers *et al*., 1994; Rogers & Schweizer, 2005). Recent research at Canesis Network Ltd and AgResearch Ruakura into gene and protein expression in the wool follicle and fibre has provided evidence in support of the existence of more keratin intermediate filament and associated proteins in wool (Z-D.Yu, unpublished data), although some appear to be relatively minor components. Differential expression of KIFP mRNA and proteins across and within sheep breeds is becoming increasingly apparent, with implications for the development of new wool genotypes.

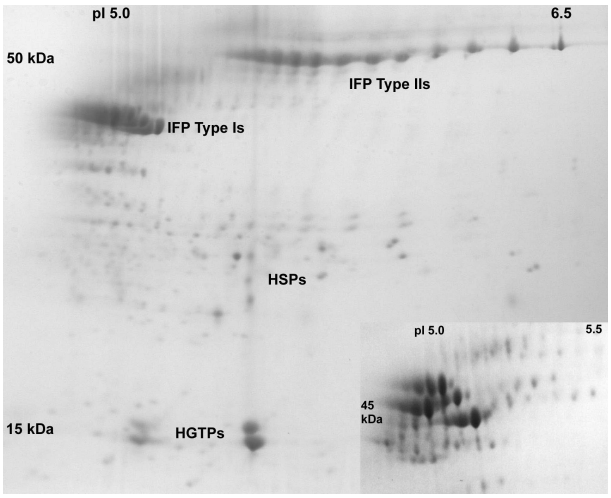
The process of fibre formation is further complicated by differential expression of KAPs within the fibre, with the high glycine-tyrosine proteins (HGTPs) being initially expressed on one side of the developing fibre and the high sulphur proteins (HSPs) and ultra-high sulphur proteins (UHSPs) being found initially on the other side of the fibre (Powell & Rogers, 1994). As the fibre elongates, most of the proteins in the matrix become more uniformly distributed across the fibre. However, some of the KAPs are confined to the half of the fibre that includes the paracortex (Powell & Rogers, 1994). It is also known that high crimp wools have higher cysteine content than low crimp wools (Kaplin & Whiteley, 1978) and that this increased cysteine content is concentrated in the paracortex (Gillespie *et al*., 1964). While the distribution of matrix proteins in differentiated cortical cells is thought to have an influence on fibre properties, such as crimp and curvature, how they affect these properties has not yet been determined.

Recent attempts to engineer changes in the wool fibre via genetic manipulation have had mixed results. Alteration of the level of the KIFP K2.10 in transgenic sheep resulted in wool with a high lustre and minimal crimp. However, there was almost complete loss of ordered IF structure in the cortical cells and the resulting fibres were also weaker than the wild-type (Bawden *et al.*, 1998; Bawden *et al.*, 1999). Subsequently, various attempts have been made to improve the intrinsic strength of fibres using this approach. One approach, involving increasing the levels of cysteine-rich keratin associated proteins such as the UHSPs KAP4.2 and KAP5.1, was found to lead to a decrease in fibre strength. Over-expression of the KAP6.1 HGTP was found to reduce the load bearing capacity and extensibility of the fibres (Bawden *et al.*, 2000). Increasing the number of isopeptide cross-links by expressing the human keratinocyte transglutaminase in the cortex of the fibre was also found to reduce the extensibility and break force of the fibres. All of which tends to suggest that application of transgenesis first requires a detailed understanding of the influence of individual proteins on fibre structure.

The development of medical devices from wool keratins is an example of the emerging opportunity for wool keratins to be used as a basis of new biopolymer materials (Kelly *et al.*, 2003). Commercial applications of individual wool keratin protein classes have also been developed due to their demonstrated ability to influence cosmetically important characteristics of human hair (Roddick *et al.*, 2004). Greater understanding of differences in the specific proteins expressed between different sheep breeds, within a breed or on different parts of the body of the animal could be used to influence the composition and properties of soluble proteins isolated for cosmetic and biopolymer end-uses.

## MAPPING THE KERATIN COMPOSITION OF WOOL

**FIGURE 1:** A two-dimensional electrophoretic map of proteins from Merino wool, from pI 4 to 7 and molecular weight 15 to 50 kDa showing the relative positions of the main protein classes: keratin intermediate filament proteins (KIFPs), high sulfur proteins (HSPs) and high glycine-tyrosine proteins (HGTPs). Inset a high resolution map of the Type I KIFPs between pI 4.75 and 5.05, and molecular weight 35 to 50 kDa. Protein extraction, 2D gel electrophoresis and protein analysis were conducted as described in the following references (Woods & Orwin, 1987; Plowman *et al*., 2002).




Our knowledge of the composition of expressed proteins in wool is incomplete and hence there are still many questions unanswered as to how these proteins influence fibre structure. One application that has the potential to provide this information is that of two-dimensional electrophoresis (2DE), where the proteins are separated by their overall charge in a pH gradient using isoelectric focusing in the first dimension and by their molecular weight using gradient polyacrylamide gel electrophoresis (PAGE) in the second dimension. Previous studies (Plowman *et al*., 2002) have shown that 2DE is capable of resolving the two major types of keratin intermediate filament proteins, with the Type Is clustered into four major "trains" around pI 5.0 and 37 kDa, while the Type IIs are in two long trains, of similar molecular weight, between pI 5.0 and 6.5 at 50 kDa (Figure 1). The use of mass spectrometry, in conjunction with peptide mass fingerprinting, has enabled the identification of some of these spots on the gels as coming from either of two of the three known Type II sequences (Plowman *et al*. 2002). The situation has not been so straightforward for the Type Is. Unlike the Type IIs, there are only two known sequences for the Type I proteins. Hence, this has placed constraints on both the peptide mass fingerprinting approach and peptide sequencing by mass spectrometry and has resulted in the current situation where the four major trains of spots in the Type I keratin intermediate filament protein region have only been tentatively identified as belonging to either of the two known Type Is (Plowman *et al*., 2002).

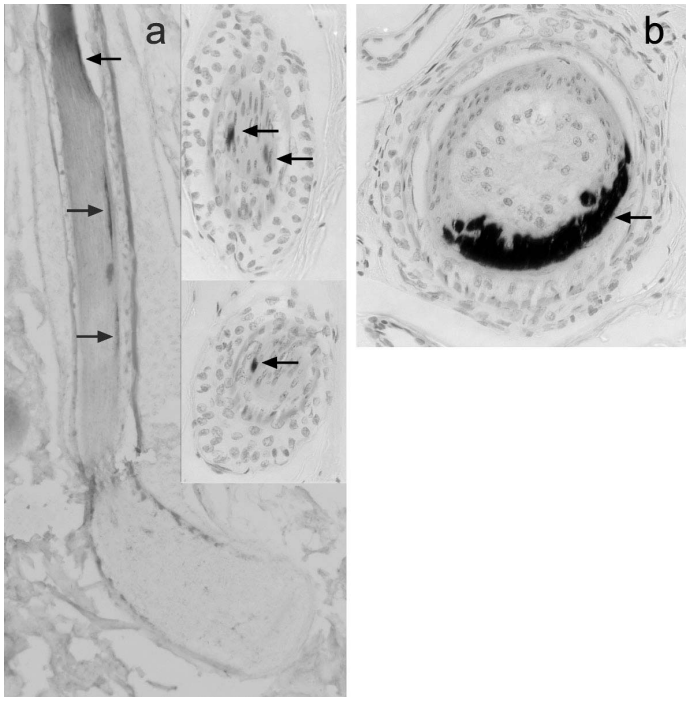
This lack of KIFP cDNA sequences is in the process of being overcome by the assemblage of sequences from skin cDNA libraries derived from New Zealand Wiltshire and Romney sheep and continued progress in the sequencing of the bovine and ovine genomes (Kijas *et al*., 2006). Some preliminary analyses have identified four KIFP cDNA sequences which are labelled as oHa1, oHa5, oHa8 and oHb5 (Z-D. Yu, unpublished data) where 'o' stands for ovine, 'H' for hair, 'a' for proteins from the acidic region (Type I) and 'b' for proteins from the neutral-basic region (Type II). The assignment of this nomenclature has been based on BLAST searches and sequence alignment studies with human hair protein sequences (Table 1) (Langbein *et al*., 1999; Langbein *et al*., 2001). Further sequence alignment studies with known wool intermediate filament proteins have demonstrated that the sequence of oHa1 is almost identical to K1.1 and oHb5 to K2.12 (Table 1), with slight differences in the sequences suggestive of a degree of polymorphism in these protein families. In contrast, oHa5 and oHa8 do not show as strong a similarity to any of the known Type I KIFP sequences, indicating that we are dealing with new sequences.

**TABLE 1:** Amino acid sequence identity between wool KIFPs and human hair counterparts based on protein sequence alignment of sequences in web-based databases, reported as percentage sequence identity. The highlighted figures represent strong sequence identities between the ovine and human proteins and matches to the known ovine sequences in protein databases. Amino acid sequences of oHa1, oHa5, oHa8 and oHb5 were translated from respective cDNA sequence. Other protein sequences were derived from the Swiss-Prot Database on the (ExPASy server), and the sequence comparison studies were conducted using BLAST searches on the Swiss-Prot website and aligned using the ALIGN tool.

| KIFP | hHa1 | hHa5 | hHa8 | hHb5 | K1.1 | K1.2 | K2.10 | K2.12 |
|---|---|---|---|---|---|---|---|---|
| **oHa1** | 85.9 | 71.6 | 64.7 | 28.8 | 99.3 | 91.5 | 27.7 | 31.0 |
| **oHa5** | 73.0 | 87.7 | 58.7 | 29.7 | 73.0 | 72.8 | 28.3 | 28.6 |
| **oHa8** | 64.0 | 62.3 | 75.8 | 28.4 | 64.8 | 64.4 | 27.4 | 28.4 |
| **oHb5** | 28.9 | 31.3 | 27.7 | 91.3 | 30.4 | 30.4 | 81.5 | 99.7 |

## DIFFERENTIAL KERATIN EXPRESSION BETWEEN WOOL FOLLICLE TYPES

**FIGURE 2:** *In situ* hybridisation expression of the oHa8 in sheep follicles: where (a) represents a longitudinal section of a Romney fleece follicle (inserts are transverse sections of two Wiltshire fleece follicles) and (b) represents the expression of a Wiltshire follicle on the lower leg in transverse section. Arrows point to the sites of oHa8 mRNA expression. The oHa8 sequence was derived from rapid amplification of cDNA ends (RACE) using primers derived from an ovine skin cDNA sequence. The KAP4.3 sequence was isolated as part of a Lambda clone from an EMBL3 sheep genomic DNA library and the KAP6.1 sequence was from Genbank (M95719.1). Short sequences from the 3' non-coding region of these genes were cloned for the synthesis of probes for *in situ* hybridisation.




Adding to the complexity of the numerous KIFPs and their associated proteins are the spatial and temporal expression patterns observed across the follicle growth cycle and between different types of wool follicle. For example, in fleece follicles, the expression of oHa8 is restricted to a small number of cortical cells, usually on one side of the cortex (Figure 2a). This finding corresponds to the low expression measured in fleece-bearing skin by quantitative PCR (Z-D. Yu, unpublished data). However, the expression of oHa8 in the follicles from face and lower legs is much more abundant, although the mRNA is still restricted to one side of the keratinising cortex (Figure 2b). The expression of oHa8 commences in the precortex, where cortical cells are in transition from cuboidal to elongated cells. Distally, oHa8-positive cells lose their nuclei and elongate. Different gene expression patterns of other wool keratin intermediate filament and associated proteins between fleece and non-fleece follicles are common (Z-D. Yu, unpublished data), implying some association of expression with the characteristics of these different fibre types.

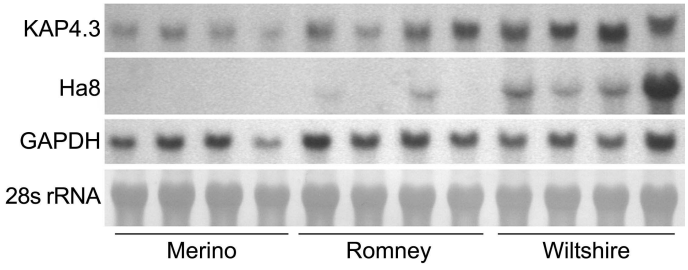
## DIFFERENTIAL KERATIN EXPRESSION BETWEEN BREEDS

Interestingly, there is no previous report of breed differences in the expression of KIFPs. Considering the critical roles of these proteins in the attributes of wool fibres (Parry & Steinert, 1995) and the difference of the attributes between breeds (Meadows, 1997; Pearson *et al.*, 1999), differential expression ought to be expected. The much higher level of oHa8 observed in Wilshire sheep (Figure 3) provides the first evidence that these proteins are differentially expressed between breeds, assuming that this expression, measured at the mRNA level, is indicative of protein composition in the wool.

In proteomic studies to date, no major differences have been observed between the intensity of protein spots in the major horizontal

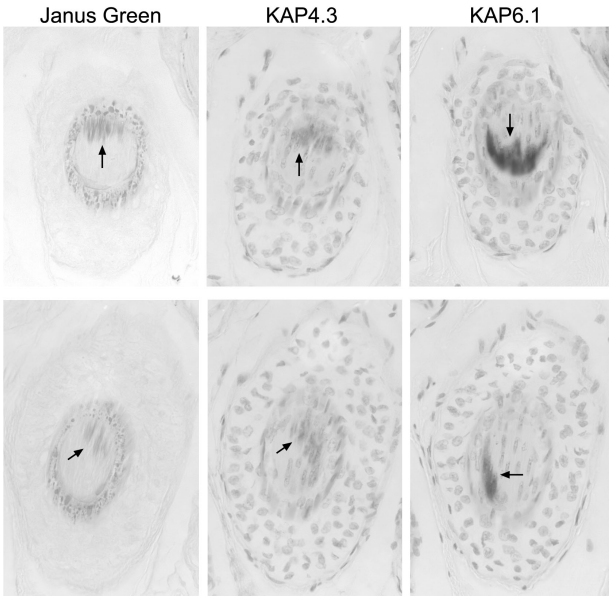
trains of keratin intermediate filament proteins (in particular the Type Is) between or within breeds of sheep, in particular Merino, Romney and Corriedale (Flanagan *et al.*, 2002). However, more recent efforts to improve the resolution of these proteins on 2DE gels have shown a number of other minor trains of spots appearing at lower molecular weight and lower concentration to the major four trains of spots in the gel (Figure 1, inset). This suggests that oHa8 could be a candidate for one set of these protein spots on the 2DE gel. Further work, utilising the recently developed resources of ovine skin cDNA sequences, is warranted to identify more novel ovine KIFPs corresponding to other spots on the 2DE gels.

**FIGURE 3:** Comparative breed expression of oHa8 and oKAP4.3 detected by Northern blot in skin collected from Merino, Romney and Wiltshire sheep grazed together in the same environment. Ten mg of total RNA derived from skin containing growing wool follicles was separated in a denatured agarose gel and blotted on nylon membrane. oHa8, oKAP4.3 and oGAPDH fragments derived from the 3' untranslated region were labelled with $P^{32}$ and used as hybridisation probes. The 28s band of ribosomal RNA indicates the RNA loading.



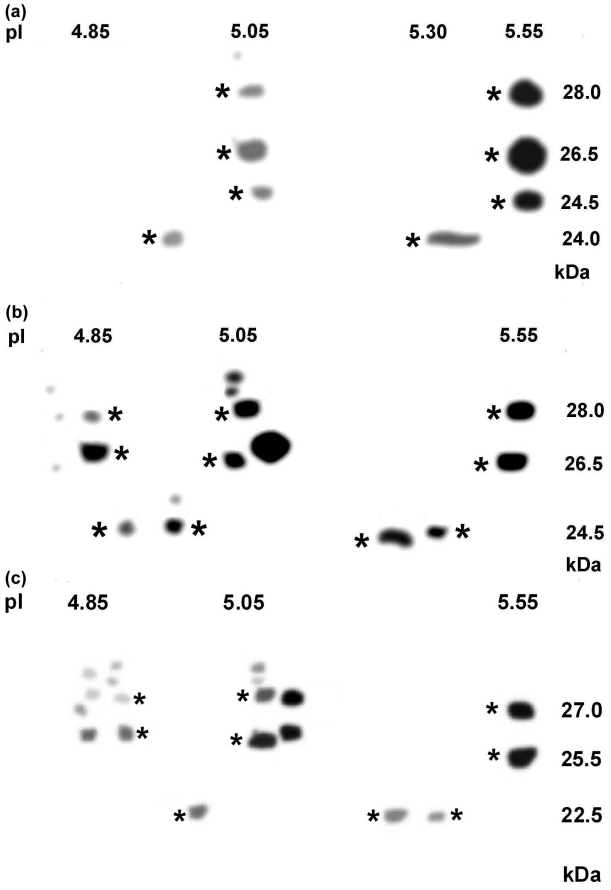

In the case of the matrix proteins, it has been reported that the expression of KAP4.3 UHSP in wool follicles is localised to the paracortex, co-localising with Janus Green staining, while KAP6.1 HGTP mRNA is restricted to the orthocortex (Fratini *et al*., 1993; Fratini *et al*., 1994). This relationship is demonstrated in Figure 4 using adjacent skin sections. The expression pattern changes along the follicle reflecting changes in the orientation of the underlying cortical cell types. Across breed differences in the expression of matrix proteins are also seen at the mRNA level for KAP4.3 and other genes (Figure 3). In comparison to the similar levels observed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a commonly used reference gene, between Merino, Romney and Wiltshire sheep, higher levels of KAP4.3 mRNA were found in Wiltshire sheep by Northern blot analysis (Figure 3). KAP4.3 is a new member of the KAP4 protein family, highly homologous to KAP4.1 and KAP4.2 in both the N- and C-terminal sequences. However, the intermediate sequence of KAP4.3 is longer and more abundant with the di-cysteine-containing pentapeptide. This could suggest strengthened interaction with intermediate filament proteins or other matrix proteins, or it may be associated with the high elasticity of the matrix (Liff, 1996).

**FIGURE 4:** The relationship of Janus Green staining of the paracortex with KAP4.3 and KAP6.1 expression, detected by *in situ* hybridisation, in fleece follicle sections approximately 35 μm apart (compare upper and lower panels). Adjacent paraffin sections (8 μm) were used for *in situ* hybridisation and Janus Green staining. To visualise the paracortex the sections were oxidised in freshly made formic acid for 1 hour and then rinsed in water. They were then stained in 0.1% Janus Green (Sigma, St Louis, Missouri) for 45 minutes, followed by rinsing in water, decolourising in 10% acetic acid for 15 minutes and counterstaining in 0.2% eosin for 1 minute (Dick & Sumner, 1995).




In the case of the wool proteome, the greatest variation between breeds and within breeds was found to occur in the major KAP1 HSP family (Plowman *et al.*, 2000; Flanagan *et al.,* 2002), which are found between the keratin intermediate filament proteins and the HGTPs on the 2DE gel (Figure 1). Early studies (Plowman *et al.*, 2000) were conducted on wools from chimaeric sheep (in which fertilised Merino and Lincoln embryos were fused at the blastomere stage) to neutralise the effects of physiology, age, nutrition and weather by expressing two genotypes in one animal (Scobie *et*

**FIGURE 5:** Composite protein maps of the HSP region of (a) Merino, (b) Romney and (c) Corriedale sheep derived from twelve 2DE gels, constructed using two-dimensional gel comparison software of gels visualised using autoradiography. Members of the KAP1 HSP family are marked by asterisks.




*al.*, 1999b). This early study provided some evidence for a correlation between the KAP1 HSP expression and fibre curvature, however it was limited by the small size of the population available. In a more extensive study (Flanagan *et al.*, 2002) four major pairs of spots were observed at pI 5.05 and 5.55, in the region between 22.5 and 27 kDa in Merino sheep, with the pairs at 22.5 and 27 kDa always being present (Figure 5a). Electrophoretic studies have shown that three different sub-groups exist within Merino sheep: those with all four pairs of spots present, those with the 25.5 kDa pair absent and those with the 23.5 kDa pair absent. The situation with Romney and Corriedale sheep was a little more complicated. Generally, in these sheep, the 23.5 kDa pair was absent and additional spots were observed at 4.85 and 5.10 kDa (Figures 5b and 5c). No distinctive sub-groupings of animals based on spot patterns were observed for the Romney sheep, while in the case of the Corriedales there appeared to be a sub-population related to animals with high crimp wool and another with low crimp wool, though this needed to be confirmed with a larger population (Flanagan *et al.*, 2002). The KAP1 HSP family is notable for having a common repeating sequence motif of 10 amino acid residues, ranging from two of these decapeptide repeats in KAP1.3 to five in KAP1.4. This variation in sequence length could affect such things as the volume occupied by the protein in the fibre or its extensibility, thus pointing the way towards some relationship between the observed presence or absence of these proteins (Flanagan *et al.*, 2002) and physical properties of the fibre.

## FUTURE DIRECTIONS

Past breeding programmes have shifted the average attributes of a fleece towards a desired goal without any underlying knowledge of how the component proteins contribute to the characteristics of the wool fibre. On the other hand modern transgenic approaches have introduced drastic and mostly deleterious changes without any understanding of why these have occurred. Studies of wool keratins undertaken in the last 50 years have resulted in many insights into wool composition and structure. However, this is complicated by the multiplicity of KIFP and KAP gene families, the post-translational modifications and the interactions of these components to form the complex structures of wool. What is required now is a more comprehensive study on how keratin proteins contribute to wool fibre traits, if we are to introduce improvements or desired traits in a systematic manner. Recent developments in genomic and proteomic technologies will enable a marked acceleration of progress. The combination of improved 2DE, mass spectrometry, extensive databases of ovine genomic and protein sequence data and orthologous sequence data from other species will enable the development of a comprehensive catalogue of wool keratins and linkages to economically important fibre attributes. Keratin-relevant, gene functional testing capabilities are now also available, including transgenic models, and the capability to undertake targeted knockout or over-expression of keratin genes in sheep skin or in cultures of follicle-derived cells. The ultimate aim of future studies using these tools is to develop a detailed knowledge of keratin expression and structural organisation that will facilitate the development of new wool types and biopolymer materials.

A knowledge-based ability to produce new, more consistent wool types would underpin the establishment of direct grower to end-user linkages and facilitate the diversification of wool production

on New Zealand farms. For the foreseeable future, the volume requirements for raw wool as a feedstock for keratin extraction are likely to be modest as a proportion of the total wool clip. Nevertheless, new products based on specific keratins are likely to provide an incentive for the development of specialised flocks producing wool matched to the requirements of the processing biotechnology and hence of higher value to the grower. Both of these outcomes would contribute to a future where diversification, direct supply chains and production to specification were the rule rather than the exception.

**ACKNOWLEDGEMENTS**

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# Gene-markers for wool fibre traits

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**ABSTRACT**

Selective breeding has been used for thousands of years to improve the quality and weight of wool, but surprisingly there is still a large amount of genetic variation present at the loci involved in the "blueprint" for wool synthesis. This genetic heterogeneity results in considerable variation within, and between individual fleeces. This has both positive and negative implications. Where a wool processor requires uniformity in raw stock, both to gain efficiency in processing and to make a product to specification, it has a negative effect. Where specific traits convey unique properties it opens up opportunities for differentiated products. Markers for genes or knowledge of individual gene effects may be useful for identifying sheep with desirable wool traits that are difficult or expensive to measure under field conditions. The discovery of genetic variation in valuable fibre traits could allow the development of gene-marker tests which would assist breeding for more consistent wool.

**Keywords**: gene-markers; fibre characteristics; sheep breeding.

## INTRODUCTION

The wool fibre consists of three distinct cell types; an external cuticle, a mass of cortical cells and, in coarser wool, a medulla (Höcker, 2002). The cortex comprises approximately 90% of the wool fibre and consists of microfibrils of intermediate filament keratins (KIFs), embedded in a protein matrix of keratin intermediate filament-associated proteins (KAPs) as reviewed by Plowman *et al.* (2006). Both the structure of the fibres, and the mean and variation in their properties affect processing performance and the suitability of any given wool for a particular end-use. In this review, we focus on some of the genes that have an impact on the structure and colour of the wool fibre and how variation in these genes may be developed into gene-markers to assist in the breeding of sheep that produce wool better suited to a processor's requirements.

## GENES ASSOCIATED WITH THE WOOL FIBRE

### Fibre protein composition

The genetic control of the protein composition of wool is complex with several distinctive multigene families encoding each group of matrix components. Type I and Type II KIFs are encoded by the KRT1.n and KRT2.n gene families respectively (Powell & Rogers, 1994). The *KRT2.10* and *KRT2.13* genes have been mapped to chromosome 3 and found to be tightly linked (McLaren *et al.*, 1997). One member of the KRT1.n family, *KRT1.2,* has been mapped to chromosome 11 and is linked to the cluster of high sulphur KAP genes (KAP1.n and KAP3.n) found at that location (McLaren *et al.*, 1997).

KAPs are divided into three broad groups based on their amino acid compositions; high-sulphur, ultra-high sulphur and high-glycine-

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