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Interspecies Comparison of Morphology, Ultrastructure, and Proteome of Mammalian Keratin Fibers of Similar Diameter

Ancy Thomas,*,† Duane P. Harland,† Stefan Clerens, Santanu Deb-Choudhury, James A. Vernon, Gail L. Krsinic, Richard J. Walls, Charisa D. Cornellison, Jeffrey E. Plowman, and Jolon M. Dyer

AgResearch Ltd., Christchurch, New Zealand

ABSTRACT: Sheep wool has traditionally been viewed as the representative mammalian keratin fiber for the purposes of describing morphology and protein composition. We have investigated narrow fibers from the under-hairs of a range of species both closely and distantly related to sheep, comparing structure and protein composition. Within this group, curvature was negatively correlated with diameter for all but mohair. The cortical cell types present in alpaca, rabbit, and mohair fibers differed structurally from wool, primarily in terms of their macrofibril architecture. Except for rabbit, each species' fibers contained three cell types, and except for mohair, cell types were distributed asymmetrically across the cortex. In mohair, the cell types were distributed annularly, and each cell type had regions in which intermediate filaments were packed into highly aligned hexagonal mosaics, much like the mesocortex in wool. Coupled with this, were differences in the protein profiles; the rabbit fiber contained extra keratins and keratin associated proteins, while only subtle differences were noted between mohair and Merino fibers. In both rabbit and mohair fibers, the relative abundance of keratin K85 was lower than that of Merino. These results suggest that there may be links between relative protein composition and fiber morphology, albeit complex ones.

KEYWORDS: *alpaca, angora rabbit, Merino, mohair*

INTRODUCTION

Sheep wool research has traditionally and successfully provided the framework for observations and experimental studies on all trichokeratin fibers irrespective of breed or species.[1] However, there is a rising awareness that the wool framework is not universally applicable. Some mammalian fibers have ultrastructure that includes cell types not easily described using the wool cell-type terminology of orthocortex, paracortex, and mesocortex.[2] This is especially the case when both ortho- and para-like features appear within single cells.[3−5] In a textile-industry context, it is well known that fibers from nonsheep species differ considerably in such subjective textile-industry metrics as crimp, handle, prickle, and bulk.[6] At the same time, even less seems to be known about the protein composition of these fibers, and this situation is not aided by the relative lack of sequence information for other species. Therefore, one of the key questions in fiber science is the following: can fibers with similar morphology, such as diameter, scale pattern, curvature, stiffness, or strength, have a different internal architecture (e.g., very different from ortho/para) and protein composition? Precisely definitive relationships among proteomics, structure, and single-fiber properties have been difficult to pin down in wool, let alone in nonwool fibers where, with the exception of limited and isolated studies on some mammalian species,[7−10] the proteome and nanostructure of fibers are unknown. We believe there is a need to widen any such investigation of fiber properties to fibers from other species to acquire a better understanding of wool as well as how to use the wool gene−protein−structure relationships to refine our knowledge of all aspects of mammalian keratin fibers.

This study was initiated with the view of comparing the structural and protein composition of the well studied Merino wool (*Ovis aries*) with similar fibers from other animal species. Ultimately, the long-term aim of these studies is to determine how proteins influence the internal structure of the fiber and, in particular, macrofibrillar substructure. As such, this research represents the first step in this direction. The fibers selected for inclusion came from readily available agricultural wools: alpaca (*Vicugna pacos*), mohair (Angora goat, *Capra aegagrus hircus*), and from the underfurs of rabbit (German Angora rabbit, *Oryctolagus cuniculus*).

MATERIALS AND METHODS

Sample Origins. Wool was obtained in 2006 from a New Zealand Merino sheep run as part of a mixed Merino/Romney/Wiltshire flock on an AgResearch farm at Lincoln in Canterbury, New Zealand. Alpaca fibers were obtained from Dave Goulden of Glendou Farms, Weedons, Canterbury, while mohair fibers were obtained from Marnie Kelly of Touch Yarns (formerly in Canterbury but now located in Alexandra, Otago), and both were sourced in 2006. Fiber from a German Angora rabbit was obtained from The Shearing Shed in Waitomo, New Zealand, during the summer of 2008. All fiber samples were stored at −80 °C until analyzed.

Methods. *Microscopy.* Microscopy samples were scoured for two minutes twice at 60 °C with 0.15% nonyl phenyl ethoxylate (trade name Teric GN9) and then tap water, rinsed in ultrapure water (60 °C), air-dried, scoured again at room temperature for 30 s in heptane and tap water, then at 40 °C in 0.15% Teric, tap water, and ultrapure water, and air-dried again.

Curvature measurements were carried out using bright-field imaging (Zeiss Standard 14 light microscope fitted with an Olympus DP70 digital camera) and image analysis. Curvature was measured by fitting circle sectors to individual curves, and curvature (expressed in

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deg/mm) calculated by dividing arc length by sector angle. Curvatures for 20 fibers from each sample were measured by averaging five curvature measurements from each fiber.

For scanning electron microscopy (SEM), samples were prepared and imaged as described in a previous study.[2] For transmission electron microscopy (TEM), individual fibers were carefully selected to include fibers of representative diameter for each species and additional fibers that had been matched for diameter to Merino wool fibers, such that for each species it was possible to examine a similar-diameter Merino fiber.

The root-end regions of the selected individual fibers from each species were mounted for transverse sectioning as previously described.[2,11] Fibers were arranged so that sections would come from a region close to the fiber's original basal (root) end, at least 15 mm from any "club root". In all cases, a Merino wool fiber was included next to fibers from other species to provide a control for stain quality and intensity.

Fibers in frames were stained for TEM following a method given in detail elsewhere.[2,12,13] In brief, fibers were chemically reduced, stained with 1% osmium tetroxide (3 repeats), stained with 2% uranyl acetate, dehydrated, and embedded in acrylic resin (LR White). Ultrathin (100 nm) transverse sections of the fibers were enhanced with uranyl acetate and lead citrate, and examined with a Morgagni 268D TEM (FEI Company, Oregon, USA) operating at 80 kV. Images were captured with an SIS/Olympus (Tokyo, Japan) Megapixel III digital camera.

Proteomics. For proteomics, detipped samples were scoured according to the methods of Woods and Orwin[14] and then crushed to a powder in liquid nitrogen. The proteins from each fiber sample were extracted by shaking overnight on a Trident shaker (WRONZ Developments, Christchurch, NZ) in a solution of 7 M urea, 2 M thiourea, 30 mM Tris, Pharmalyte 2% (v/v), and 50 mM DTT, after which they were electrophoretically focused for 115 kVh in first dimension on either nonlinear pH 3−11 or linear 4−7 immobilized pH gradient strips (GE Healthcare Biosciences, Piscataway, USA) and then in the second dimension in 12.5%T and 7.5−12.5%T Tris-tricine polyacrylamide gels, respectively,[15] and then visualized with Blue Silver stain.[16] Selected spots were excised from the gels, destained, alkylated with acrylamide, and digested with sequencing-grade, TPCK-treated trypsin, before being analyzed on an Ultraflex III MALDI-TOF-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany), using α-cyano-4-hydroxy-*trans*-cinnamic acid as the matrix and a Q-Star Pulsar i (ABSciex, Foster City, CA, USA). The proteins were identified on a Mascot Server v2.2.0.6 (Matrix Science, London, UK) using the NCBI nr database (http://www.ncbi.nlm.nih.gov/),[17] with the taxonomy restricted to *Oryctolagus cuniculus*, *Capra* spp., or *Ovis aries*. ProteinScape v2.1 (Bruker) was used to store peak list data and for protein compilations.

RESULTS AND DISCUSSION

Figure 1a−d illustrates tufts of fibers from each species prior to scouring and close to the natural arrangement.

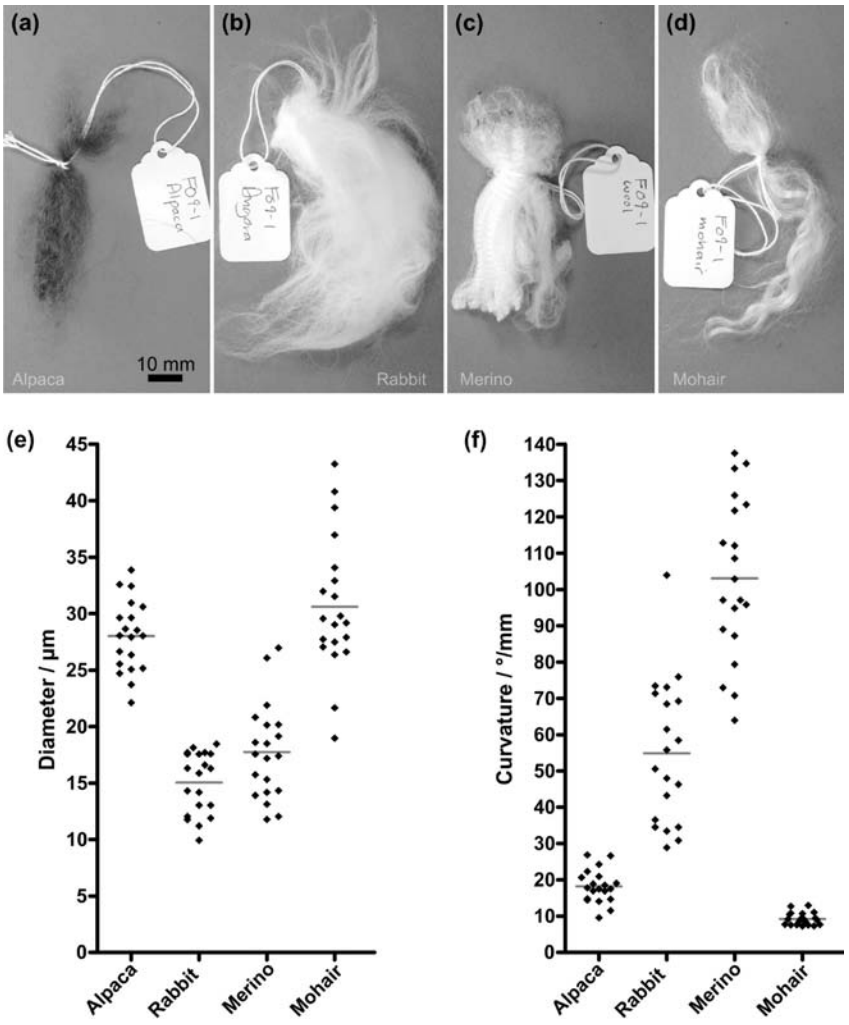

Figure 1. Samples used for structural analysis of fine-fiber species. Photographs a−d of staples of the same scale with the tip-most part of fibers at the top and that closest to the root at the bottom: (a) alpaca, (b) rabbit, (c) Merino sheep, and (d) mohair. (e) Plot of diameters of 20 fibers from each species. (f) Single-fiber curvature measurements from each of the same 20 fibers. Bars indicate mean diameter and curvature for each species.

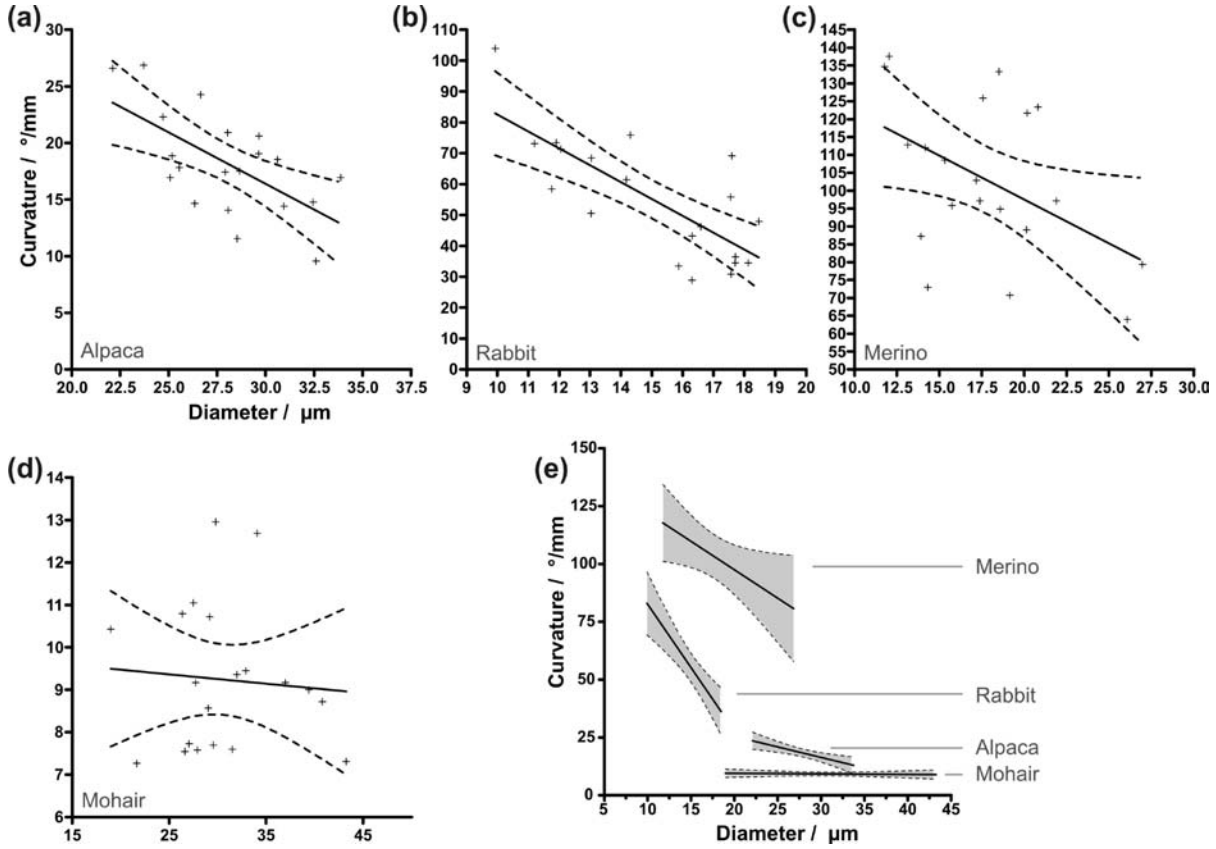

Figure 2. Curvature–diameter relationships for fine fibers: (a) alpaca, (b) rabbit, (c) Merino sheep, and (d) mohair. In each case, the solid line indicates linear regression, and the dashed line is the 95% confidence interval of the regression. All species except mohair show significant correlations and significant negative trends. (e) Regression lines and confidence intervals of regression for each species plotted together.

Fiber Diameter, Curvature, and Medullation. The fiber diameters ranged from as low as around 10 μm in the rabbit fibers to almost as high as 45 μm in mohair fibers; rabbit and Merino fibers had mean values of around 15 μm (Figure 1e). The species with the highest diameter fibers (alpaca and mohair) also had the straightest fibers with especially low and invariant single-fiber curvature measurements (Figure 1f). Merino wool had the highest mean curvature and, along with rabbit fiber, was notably variable. For Merino wool, there is a well-known negative correlation between curvature and diameter at the staple level[18,19] that holds at the single fiber level (Figure 2) for the underhairs of each species examined (two-tailed Spearman, $N = 20$ and $P < 0.05$ in each case) with the exception of mohair fibers, for which there was no appreciable relationship (Figure 2d; Table 1).

In general, medullation was found to be rare in the Merino and mohair fibers studied and, when observed, was of a simple form described as fragmented or interrupted medulla.[20−22] Alpaca fibers were typically medullated with simple or interrupted medulla, similar to those of high diameter (>30 μm) wool fibers. Rabbit fibers were all medullated, with the medulla consisting of a regular series of small chambers known as uniserial ladder medulla.

Table 1. Summary of Fiber Diameter and Curvature Data[a]

$N = 20$ fibers each	alpaca	rabbit	Merino	mohair
linear regression r^2	0.3953	0.5689	0.2187	0.005934
significantly nonzero (F; P)	11.77**	23.76***	5.038*	0.1074 NS
correlation	**	***	*	NS
mean diameter (μm)	28.01	15.06	17.75	30.62
SD diameter (μm)	3.146	2.723	4.200	6.047
mean curvature (deg/mm)	18.19	54.89	103.05	9.24
SD curvature (deg/mm)	4.546	19.740	22.092	1.725

[a]NS = nonsignificant; * = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.001$.

Morphology of the Fiber Surface. SEM observations of the scales on the surface of hairs were made (Table 2) near the root end of five or more fibers from each species. Scale patterns (Figure 3) conformed to those previously described for mammalian hairs.[20−22] Alpaca (Figure 3a) had an irregular mosaic scale pattern similar to wool, but with relatively lower scale edges (Figure 3e). Rabbit fibers typically had a single chevron scale pattern (Figure 3b), which is characterized by protruding angular scales (Figure 3f). Low diameter Merino fibers had a typical simple coronal pattern, and higher diameter fibers had a regular wave pattern (Figure 3c). Low diameter mohair fibers had a simple coronal pattern (Figure 3d) and an irregular mosaic pattern for higher diameter fibers. Scale-height in all mohair fibers was notably low (Figure 3h).

Scale patterns are formed as an impression of the follicle's inner-root sheath,[23] and scale edges occur at cuticle cell edges only when these cells match up closely with corresponding inner-root sheath cells during development. In alpaca, Merino, and mohair, scale edges were often sharp or jagged and close to perpendicular to the fiber surface. This suggests that in these cases cuticle cell boundaries often corresponded to scale edges. In contrast, rabbit fiber scale edges were often smoother and at an oblique angle to the fiber surface. What is more, thin lines interpreted as cell boundaries were observed not to correspond to scale edges (Figure 3f).

Table 2. Summary Comparison of Key Structural and Proteomic Observations

	alpaca (N = 6)	rabbit (N = 6)	Merino (N = 5)	mohair (N = 6)
number of cuticle layers	3−4	1	1	2−3
cuticle scale height	low	normal	normal	very low
cuticle scale pattern[a]	irregular mosaic to regular wave	single chevron	simple coronal to regular wave	simple coronal to irregular mosaic
medulla[b]	simple and interrupted	uniserial ladder	none or fragmental	none, fragmental, or interrupted
cortex cell types[c]	• ortho-like • human-hair-like • para-like	• light-staining ortho-like • dark-staining ortho-like • human-hair-like • meso-like	• orthocortex • mesocortex • paracortex	• ortho-like meso • human-hair-like meso • para-like meso
cell type distribution	• bilateral	• bilateral	• bilateral	• annular
2D-E KAPs	• no low-weight spots	• many low-weight spots • many high-weight spots	• no low-weight spots	• strong midweight spots
2D-E type I keratins	• two compact rows	• seven well separated rows	• four rows	• four rows starting higher weight
2D-E type II keratins	• three well separated rows • weak basic region	• two well separated rows • weak basic region	• one row • strong basic region	• two well separated rows • weak basic region
type I keratins identified	• K33b-like, K31/K33b/34-like		• K31, K33a, K33b, K34	• K31-like, K33a/33b-like
type II keratins identified	• K81/85/86-like		• K81, K83, K85, K86	• K81/83/86-like

[a]Nomenclature after Brunner and Coman,[22] and Wildman.[20]. [b]Nomenclature after Brunner and Coman,[22] N = 5 fibers per species. [c]On the basis of macrofibril architecture and staining density using TEM.

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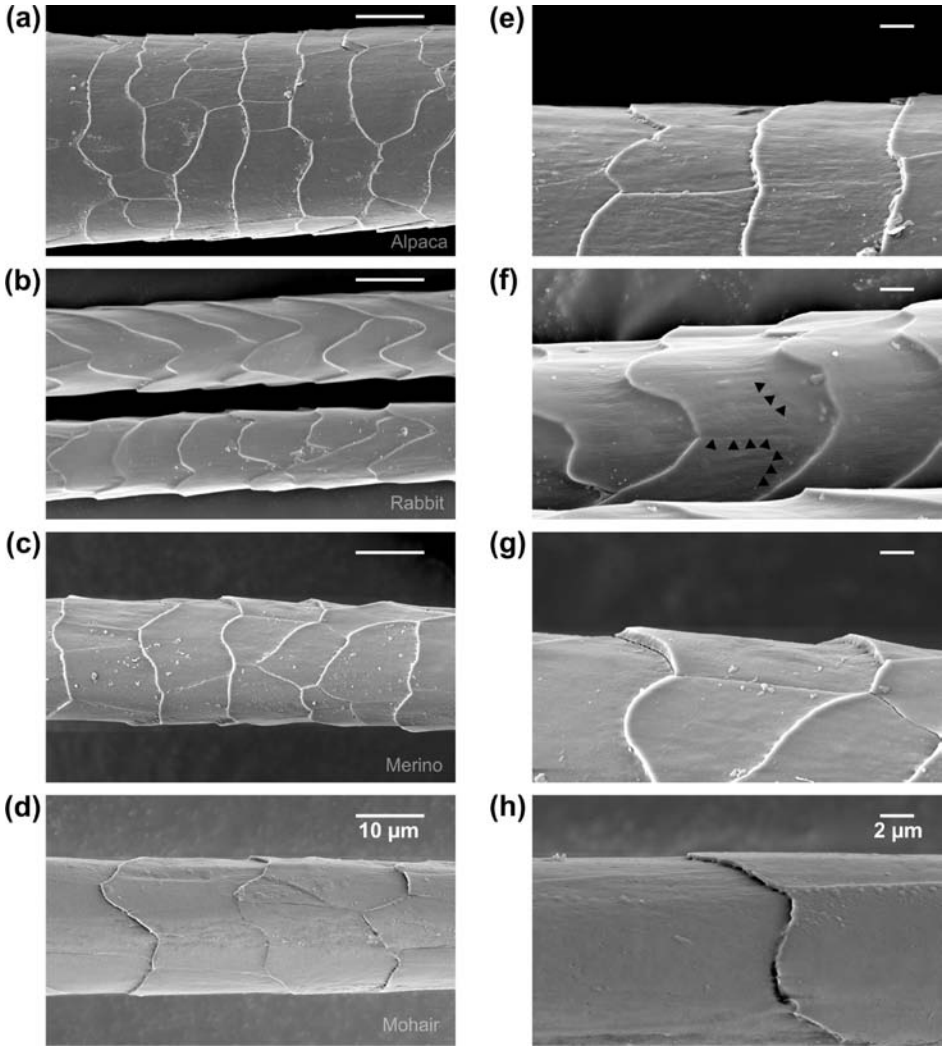

Figure 3. Scanning electron micrographs of (a−d) of typical fine fibers and (e−h) details of cuticle scale pattern of each species. (a,e) alpaca, (b,f) rabbit, (c,g) Merino, and (d,h) mohair. Scale bar for a−d: 10 μm. Scale bar for e−h: 2 μm. In f, some cuticle boundaries that do not correspond to scale edges are indicated (arrow heads). See text for scale pattern types for each species.

Morphology and Ultrastructure of the Fiber Interior. In transverse TEM sections, all fibers exhibited the general morphology expected for mammalian hairs: an outermost layer of one or more overlapping cuticle cells surrounding the cortex and, in many hairs, a centrally located medulla (Figure 4). Hair cross-section shape appeared to be species dependent, with mohair being typically highly circular, rabbit being often oblong and irregular, and the rest ranging from circular to roughly elliptical.

Merino Wool. Sheep wool has been extensively described at the micro and nanostructural level.[12,13,24−26] The Merino wool fibers in this study were typical specimens, with a single cuticle cell wrapped around the cortex and overlapping adjacent to the paracortex (Figure 4c). The cortex was composed of three cell types (Figure 5a); orthocortex, mesocortex, and paracortex. The general organization of wool cortex cells was identical to that of cells from other species in this study, having a trilaminar cell membrane complex (CMC) delineating each cell, and various amounts of cytoplasmic remnant material (CR) and intermacrofibrillar material (IMM) dispersed between macrofibril bundles composed of intermediate filament (IF) and matrix. Orthocortex cells were easily distinguished from the paracortex at moderate magnifications by the presence of circular macrofibrils (Figure 5a), but mesocortex cells required a magnification high enough to visualize IFs (Figure 5b). Mesocortex cells are typically a feature of high-diameter (coarse) wools[27] and were rare or absent in Merino fibers examined. At high magnification, orthocortex macrofibrils had a clear patch of transversely cut IFs at their center, which was surrounded by circular impressions of obliquely oriented IFs, this being characteristic of a double-twist arrangement in which concentric shells of IFs are helically wound about a central axis and the IFs of each successive shell increase in pitch.[13,28]

Alpaca. Alpaca fibers were of higher diameter than Merino and all medullated. The cuticle on alpaca fibers was up to four cells thick. There was separation between some cuticle cells on all fibers, indicating damage to the CMC. However, the intracellular layers appeared undamaged and closely resembled those of Merino and all other fibers examined. The cortex of all alpaca fibers appeared to be undamaged, and at magnifications in which the entire cross-section was visible (Figure 4a), melanin granules, often clumped, were evident across the cortex as small electron dense dots.

Different types of cells were evident, even at low magnifications, with the different types arranged bilaterally across the cortex (Figure 4a-dashed line). Medullae were slightly

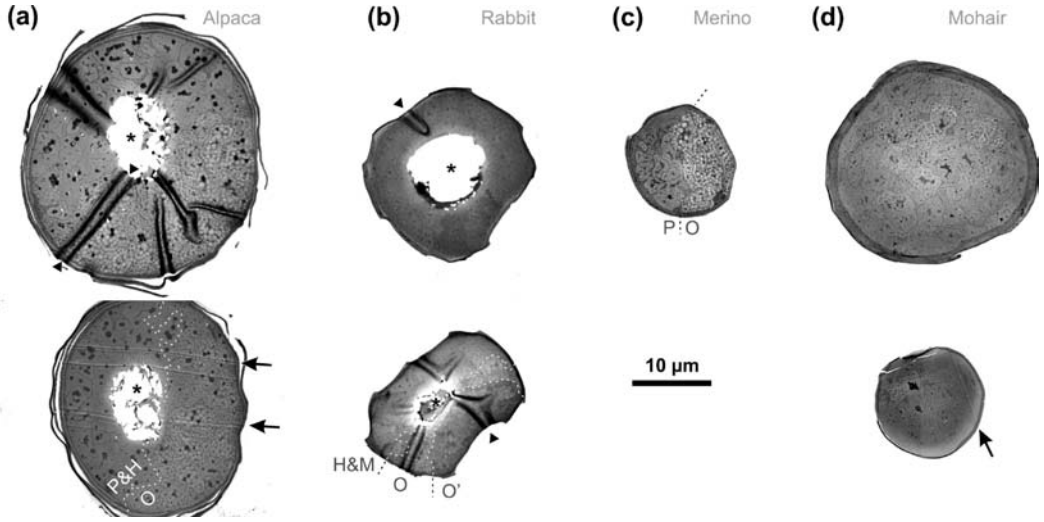

Figure 4. Transmission electron micrographs of mean-diameter fine-fibers (top row) and fibers from each species with diameters most similar to the mean Merino wool diameter (bottom row): (a) alpaca, (b) rabbit, (c) Merino sheep, and (d) mohair. Examples of artifacts, folds, and microtome score marks are indicated with arrowheads and arrows, respectively. The line surrounding the cortex and just inside the cuticle for mohair (top) is an artifact, as is the diffuse pale ring just inside the cuticle in the bottom image. Medullas (*) are visible in the centers of alpaca (a) and rabbit (b) fibers. Examples of bilateral distribution of cortical cell types is indicated in some fibers (a–c) by dashed lines, and the letters refer to cell types identified in later figures for each species. Bilateral cell distribution and clearly defined cell types were not found in mohair samples.

off-center toward the side with fused cells, and the cuticle in a number of cells tended to be thicker on the same side. In comparison with wool, there were notably fewer cytoplasmic remnants.

At higher magnification (Figure 6a), three cell types were observed. One cell type appeared very similar to the orthocortex of wool, and we referred to this as "ortho-like" (O). IFs were arranged within macrofibrils of ortho-like cells (Figure 6c) in a double-twist architecture similar to that of wool orthocortex macrofibrils (Figure 5b). The second cell type was somewhat less like the orthocortex because it stained more densely than the ortho-like cells and because the macrofibrils were slightly larger and less circular (Figure 6a). Many macrofibrils in these cells had a large core of pseudohexagonally packed IFs surrounded by a thick coat of obliquely viewed filaments (Figure 6c), a pattern familiar from human scalp hair[5] and which corresponds to a double-twist architecture with a lower intensity than that typical of orthocortex macrofibrils.[13] We refer to cells composed of macrofibrils of this type as "human-hair-like" (H).

A third type of alpaca cortex cell resembled wool paracortex cells because many macrofibrils appeared to be large and had irregular edges (Figure 6b). Despite their general "para-like" (P) appearance, these cells had fewer cytoplasmic remnants and more intermacrofibrillar material than typical for wool paracortex. IFs were pseudohexagonally packed into macrofibrils that had a distinctly polygonal shape (Figure 6d) and were separated from neighboring macrofibrils by a thin layer of intermacrofibrillar material.

In addition, each section had one or two cortex cells of unusual appearance, occurring close to the cuticle (Figure 6e) in which macrofibrils were often large and elongated, surrounded by considerable amounts of intermacrofibrillar material or cytoplasmic remnant, and in which many macrofibrils contained darkly stained centers. At high magnification (Figure 6f), these dark centers appeared to be formed of large inclusions of staining density similar to that of the material surrounding the macrofibrils. Arrangement of IFs in these macrofibrils suggest they had double-twist architecture.

Angora Rabbit. Like alpaca, rabbit fibers were medullated. In some cases, the medulla was partially filled with an amorphous material. The one-cell-thick cuticle differed considerably from those of alpaca and wool because it varied in thickness considerably. These variations in cuticle thickness and shape were mostly responsible for the jutting and irregular shape of fiber transverse sections (Figure 4b). Typically, a single cuticle cell was wrapped around the entire cortex with minimal overlap (Figure 7a).

Four cell types were evident, and some of the dark and light staining cells could be seen easily at low magnification (Figure 4b). Two cell types had a double-twist macrofibril architecture that was similar to the wool orthocortex, but one stained more densely than the other (Figure 7a,b). Two other cells types had macrofibrils that had a more fused appearance, but both had cores surrounded by obliquely tilted IFs, indicative of double-twist architecture. One type of cell was very similar to the wool mesocortex, being characterized by sizable patches of highly aligned IFs that, when viewed end-on, formed patches of hexagonal mosaic (Figure 7c). We termed cells with this pattern of IF arrangement "meso-like". The remaining cell type was indistinguishable from that described for alpaca as "human-hair-like" (Figure 7d).

Across the cortex of most rabbit fibers the cell types were arranged such that dark ortho-like cells were clustered at one side, light ortho-like cells clustered or scattered adjacent to them, and the more fused-looking meso-like and human-hair-like cells predominated on the side furthest from the dark ortho-like cells. Overall, there was a bilateral appearance, with the two ortho-like cell populations on one side of the cortex (Figure 4b, dashed line).

Mohair. All mohair fibers had notably circular cortices (Figure 4d) enveloped by 1–3 layers of uniformly narrow and heavily overlapping cuticle cells. The cuticle cells had a layered structure similar to that of wool. None of the fibers examined with TEM had medullas.

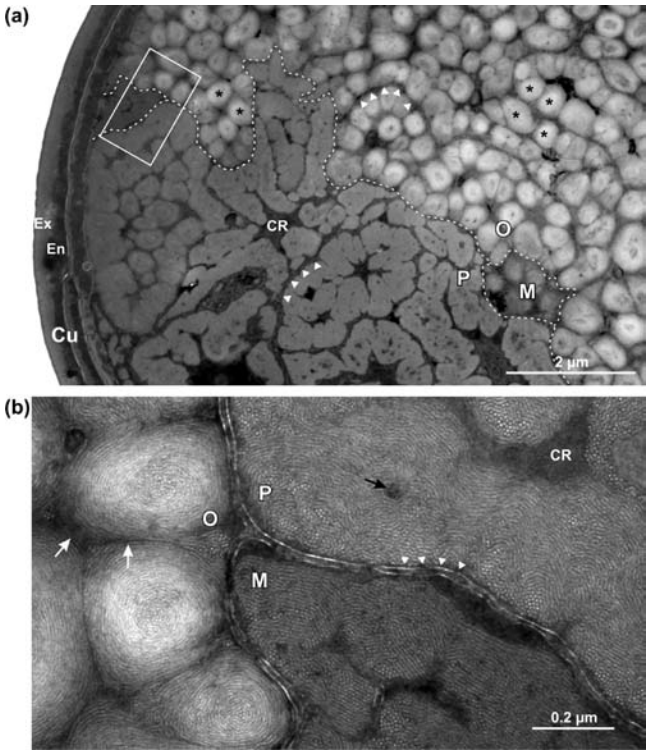

Figure 5. TEM of Merino wool fiber. (a) Cortex and cuticle (Cu). The two main layers of each cuticle cell are exocuticle (Ex) and endocuticle (En). Cortex cells vary in appearance (delineated by a white dash line) with some composed primarily of fused material (P), frequently with centrally located electron-dense stellate cytoplasmic remnants (CR). A narrow band of densely staining material and the cell membrane complex delineate cell edges (arrow heads). Orthocortex (O) and uncommon mesocortex (M) cells have macrofibrils (asterisks) that are rounded and separated from neighbors. (b) Macrofibrils are made up of intermediate filaments (IFs), appearing as electron-lucent circles when transverse and wavy or circular lines when oblique. Orthocortex macrofibrils have a ring-core appearance diagnostic of a double-twist architecture and are surrounded by a thin layer of intermacrofibrillar material (IMM) (white arrows). Paracortex cell macrofibrils have no IMM, but frequently, various sized inclusions occur (black arrow).

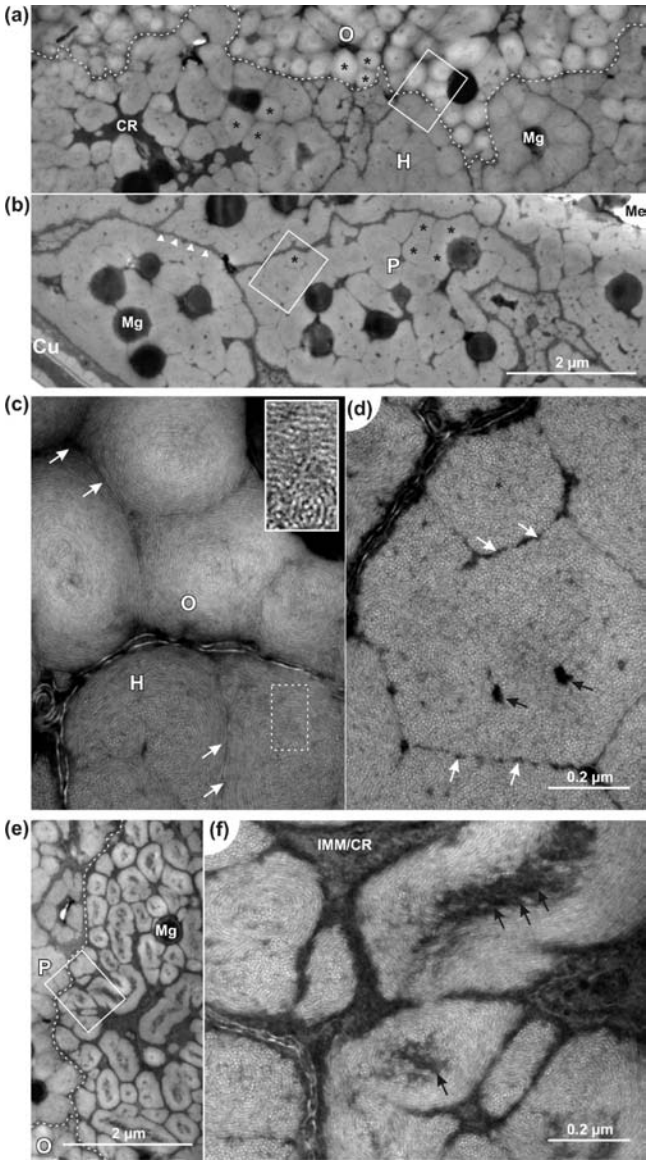

Figure 6. TEM of alpaca fiber. (a) Part of cortex showing ortho-like (O) and human-hair-like (H) cells. Melanin granules (Mg) occur inside cells as electron-dense ovals. Other labels are as for Figure 5. (b) Another cortex region composed of para-like (P) cells. Micrograph extends from the cuticle (Cu) to the central medulla (Me). (c) Ortho-like macrofibrils and the neighboring cell with larger human-hair-like macrofibrils. Inset: enlargement of the dashed box showing transition from pseudohexagonal to the oblique appearance of filaments. (d) Details of the para-like cell showing the polygonal shape of the macrofibril and intermacrofibrillar material. (e and f) Low and high magnification micrographs of an uncommon cell consisting of often-distorted macrofibrils with large central inclusions.

At low magnifications (Figure 4d) and at magnifications where cell-level details were visible (Figure 8a), variation in the size and shape of macrofibrils was evident. However, discrete natural classification of cell types was less clear than in Merino or other fibers in this study because, irrespective of size and architecture, macrofibrils were often fused to neighbors, and separation by IMM was sporadic, leading to a general impression of disorganization. Staining was similar in all cells. At high magnification, all cells contained macrofibrils that had patches of IFs packed into a regular hexagonal mosaic (Figure 8b–e), reminiscent of the wool mesocortex. Following Orwin, Woods, and Ranford's[12] naming convention for describing macrofibril variation in the mesocortex, we might describe two of the cell types as similar to the ortho-like mesocortex (Figure 8b) and para-like mesocortex (Figure 8e). A third cell type, containing large rounded macrofibrils that had low-intensity double-twist architectures (Figure 8c,d), could be described as the "human-hair-like mesocortex".

Within the cortex cross-section, there was a tendency for ortho-like cells with smaller macrofibrils to occur centrally and those with a larger, more fused appearance to occur toward the edge. In no fiber did we see a bilateral organization.

2-Dimensional Gel Electrophoretic Protein Mapping of the Fibers. Proteins that focused between pH 3 and 11 from fine mohair, alpaca, and rabbit fibers resolved into 2-DE maps that all showed a general pattern of long strings of type II intermediate filament forming keratins, and a tight cluster of type I keratins. In the lower molecular mass region, marked and specific differences were observed between the samples. Whereas alpaca (Figure 9a) and Merino (Figure 9c) showed a limited number of spots in the 10–25 kDa region, in the

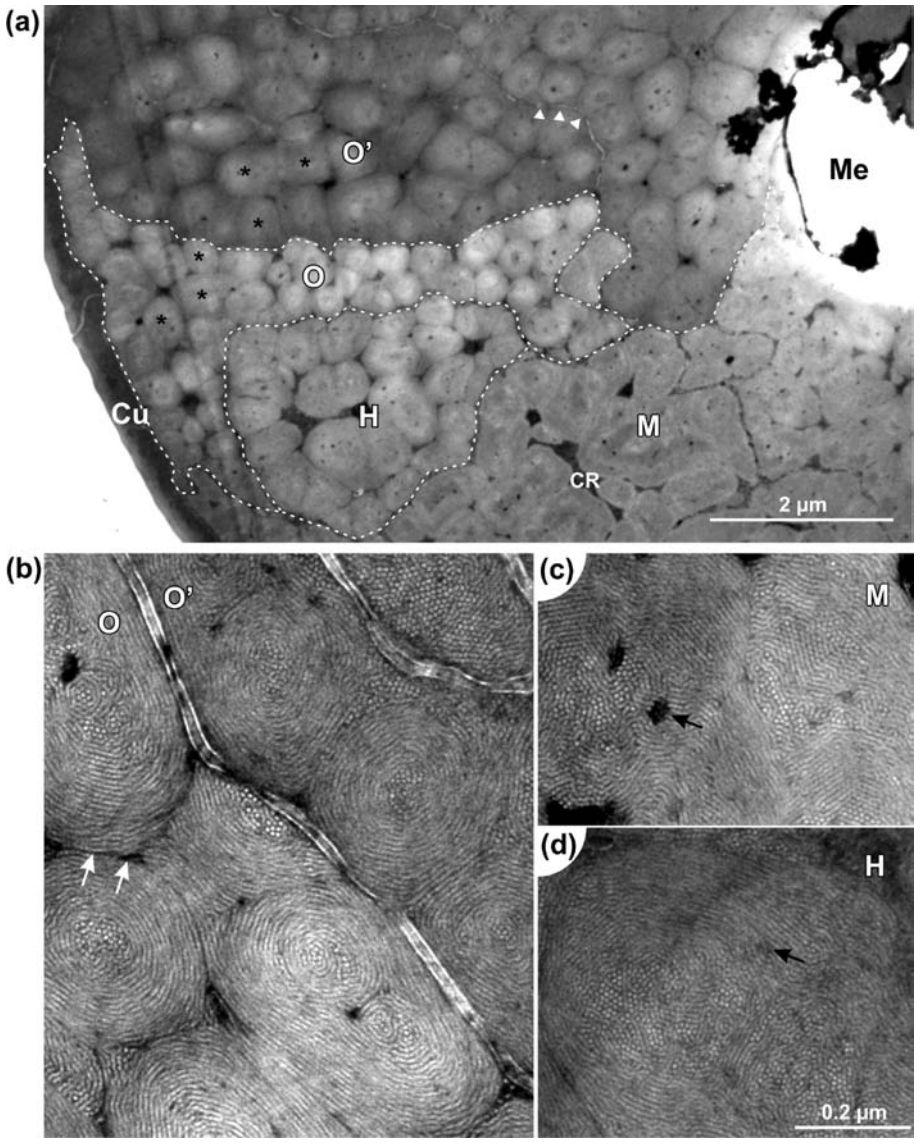

Figure 7. TEM of rabbit fiber. (a) Part of cortex showing the two ortho-like (O and O'), meso-like (M), and human-hair-like (H) cell types. Other labels are as for Figure 5. (b) Ortho-like macrofibrils from dark- and light-staining cells. (c) Details of the meso-like cell. (d) A large low-intensity double-twist macrofibril in a human-hair-like cell.

2-DE map of rabbit fiber (Figure 9b) a large number of distinct spots appeared in this same area of the gel, in addition to some new strings between 25 and 37 kDa. In mohair (Figure 9d), a number of unique strong spots appeared between 10 and 37 kDa.

In order to examine the keratin region more closely, fiber protein extracts were focused between pH 4 and 7. The resulting spot patterns (Figure 10) could be directly compared with that from Merino wool (Figure 10c); with, as expected, their highly abundant type II keratins forming a single long string in the basic region, and type I keratins forming a tight cluster, which, in accordance with previous studies, was found to consist of four strong strings.[17] Comparison of the 2-DE protein patterns of alpaca, rabbit fiber, and mohair with Merino revealed distinct and reproducible differences in type I and type II keratin spots.

The intensity of the spots at the basic end of the type II keratin string in the alpaca (Figure 10a), rabbit (Figure 10b), and mohair (Figure 10d) 2-DE maps was weaker compared to those for Merino. Type II keratins appeared in two distinct rows for alpaca and in two well separated rows for mohair. Only a single line of spots was apparent in the rabbit sample, though the shape of some suggested there were several overlapping strings of spots present in the acidic region of the type II keratin string.

The 2-DE gels also revealed distinct differences in the type I keratin pattern. While Merino type I keratins (Figure 10c) separated into four strings of proteins, alpaca type I keratins (Figure 10a) formed a very compact cluster of three strings, and rabbit (Figure 10b) type I keratins were split into six or seven strings of spots. In contrast, the distribution of mohair type I keratins (Figure 10d) appeared to be similar to that of Merino wool with a cluster of three or four strings, the difference being that in mohair the lowest molecular mass string extended to a higher pH compared to the rest of the cluster.

Protein Composition of the Fibers. To further characterize protein profile differences between the species as evidenced by 2-DE, mass spectrometric analysis was performed on selected protein spots and compared to the extensive body of

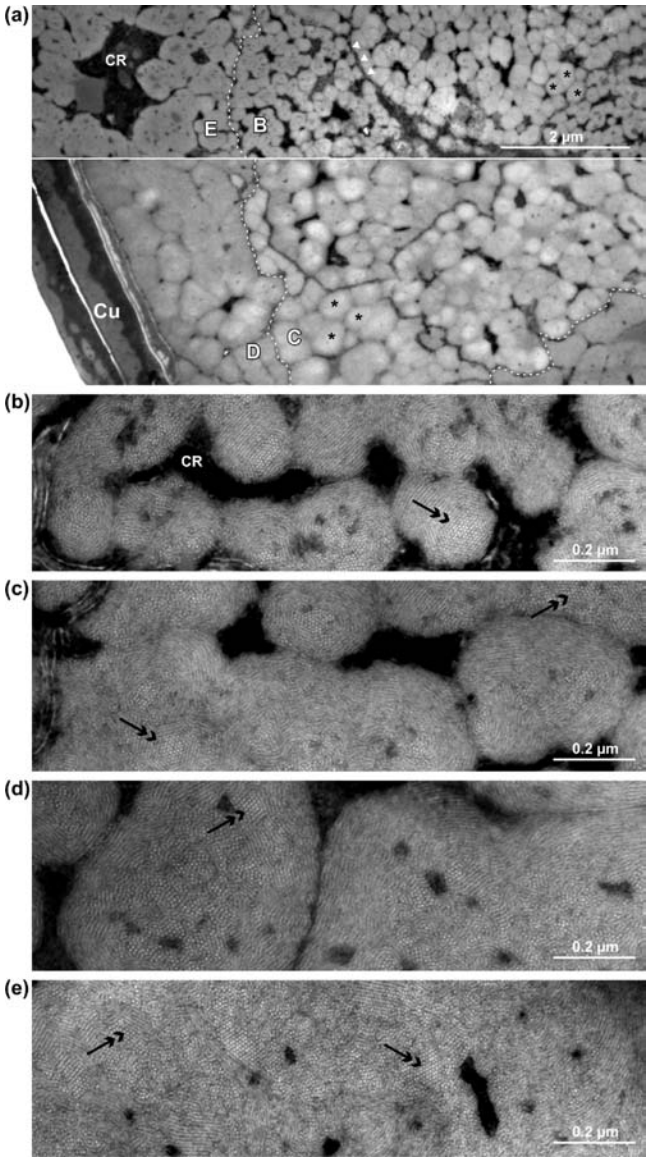

Figure 8. TEM of mohair fiber. (a) Two parts of the cortex showing variation in macrofibril appearance between cells. Letters refer to cells with macrofibril structure represented by micrographs in corresponding figure parts. Other labels are as for Figure 5. (b) Cell with small macrofibrils separated by cytoplasmic remnant material. (c) Cell with larger fused macrofibrils. (d) Cell with large human-hair-like macrofibrils. (e) Cell with large fused macrofibrils. In each case, an example of hexagonally packed IFs (double-head arrow) is shown, and note that some pseudohexagonal packing occurs in each cell.

knowledge on the Merino protein map.[15,17] Generally, protein identification was hampered by a lack of database representation; this was especially the case for alpaca. For this reason, we selected proteins from mohair, a species closely related to sheep and proteins from one distinctly different species (rabbit).

Rabbit. Nine spots from the keratin region of the rabbit 2-DE were analyzed by mass spectrometry after excision, destaining, digestion with trypsin, and extraction from the gel. The proteins identified from searches of the rabbit protein database are listed in Table 3 along with matches obtained by searching the wool protein database. When the taxonomy was restricted to *Oryctolagus cuniculus*, spots 1 and 2 in the type I keratin region were matched to a single predicted rabbit sequence, in this case K38-like. Analysis of the other type I keratins was less definitive, two type I keratins being candidates for the protein present in spot 3, while it was not possible to uniquely identify the keratins in spots 4, 5, and 6, which may not be surprising given the strong overlap between the two strings in which these spots were found.

In an effort to look for relationships between these proteins and those of sheep, the same searches were performed with the taxonomy restricted to *Ovis aries*. No match was obtained for spots 1 and 2, while spot 3 was found to match with sheep K33b, and there were indications of up to four sheep type I keratins present in the strings in which spots 4−6 were located. In the type II region, spots 7−9 appeared to be either K83-like or K86-like, or a mixture of both.

A number of spots from the gel region where keratin associated proteins (KAPs) are expected were analyzed by LC-MS/MS and MALDI MS. In each case, the only matches found were for keratin proteins when the taxonomy was restricted to either *Oryctolagus cuniculus* or *Ovis aries*. Curiously, no matches were obtained for members of the KAP1 family, despite the fact that spots 1−3 (Figure 9b) all had the peptide of m/z 1077.50, which is the mass of the peptide for the propionamide-modified sequence, WCRPDCR, and diagnostic for the sheep KAP1 family and two KAP1-like protein sequences that are known to be in the NCBInr database for rabbit.

Mohair. A further seven spots from mohair fiber were analyzed by mass spectrometry; five from the type I keratin region and two from the type II keratin region (Table 4). Spots 1−5 were matched to what was described in the NCBI nr database as goat "Hair Acidic Keratin I Protein" (multiple sequence alignment showing it having the strongest homology to sheep K31) when searches were conducted against *Capra* spp., possibly because only one complete and one partially complete goat fiber keratin sequence, and one inner root sheath keratin sequence are known. When searches were conducted against *Ovis aries*, it was not possible to distinguish between the four major type I keratins in sheep, specifically K31, K33a, K33b, and K34. In the case of spots 6 and 7 in the type II keratin region, no matches were obtained for any goat keratins when searched against *Capra* spp, but matches to either K81 or K86, were obtained when searched against *Ovis aries*.

A number of spots from the KAP gel region were also analyzed (Table 5). A match was obtained for either hair acidic keratin or a KAP from *Capra aegagrus*, with the latter seeming more likely as this spot was in the KAP region of the 2-DE gel. This particular KAP is a member of the KAP1 family and showed strongest homology to KAP1.3 from sheep. This is, however, the only known goat KAP1 sequence, and as there may be more members of this family, the identification was provisional. Spot 2 was not identified but, like spot 1, it had the m/z 1077.50 peptide, characteristic of the sequence WCRPDCR, suggesting that it was also from the KAP1 family. Matches were found for KAP13.1 in spot 3 when the search was restricted to *Capra* spp., and further confirmation of this was obtained when a match was found for sheep KAP13.1 when the search was restricted to *Ovis aries*.[15,17]

DISCUSSION

Keeping in mind that morphological variation is often greater between fibers of different diameters from the same animal than between similar diameter fibers from distantly related species,[12] variations in fiber diameter were kept to a minimum for this study. As an example, a 20 μm diameter Merino wool

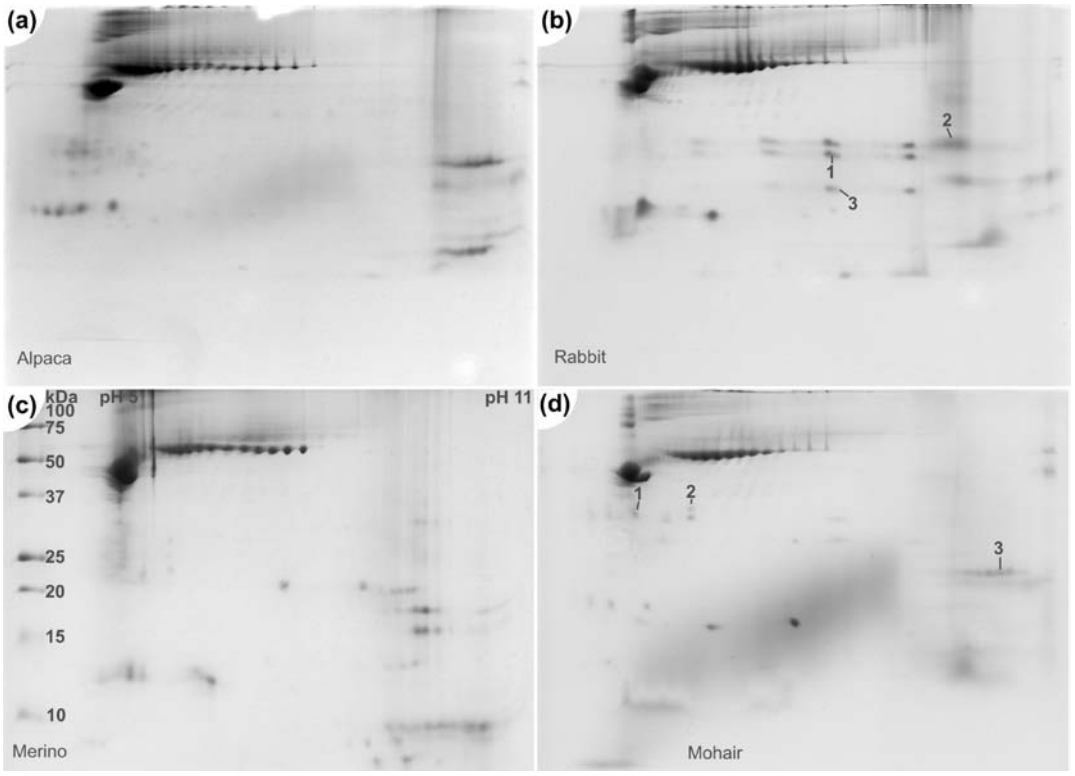

Figure 9. 2-DE gels over the pH range 3−11 of protein extracts highlighting the keratin and KAP regions of (a) alpaca, (b) rabbit, (c) Merino wool, and (d) mohair. The keratins appear between 37 and 73 kDa, while the KAPs are between 10 and 37 kDa. The numbers in panels b and d refer to spots excised and analyzed by mass spectrometry.

fiber can be more similar to a 20 μm diameter deer under hair[2] than to a 40 μm diameter Romney wool fiber.[12]

Macrofibril Architecture. While cuticle cells shared a very similar structure and staining pattern in TEM micrographs across all species, suggesting that many parameters of these cells are closely linked, cortical cell types showed considerable interspecies variation. A common feature of alpaca, rabbit, and mohair fibers was the presence of cortex cells that contained large macrofibrils with circular/elliptical transverse profiles. Within these macrofibrils, IFs were pseudohexagonally packed (similar to that seen in wool paracortex macrofibrils), but the macrofibrils also had an overall double-twist arrangement similar to, but less intense than, that of an orthocortex macrofibril.[28,29] Such low-intensity double-twist macrofibrils have been observed very rarely in the wool paracortex[13] but are common in cell types of deer antler fiber cortex[2] and in human scalp hair cortex cells.[5]

Cell types containing low-intensity double-twist macrofibrils appear to be intermediate in structure between the familiar wool cell types of orthocortex and paracortex, yet they are not mesocortex (the traditionally viewed intermediate). It is not clear whether the situation found in human scalp hair, in which there is a negatively correlated linear relationship between macrofibril size and double-twist intensity (Harland, D. P., Walls, R. J., Vernon, J. L., Dyer, J. M., Woods, J. L., Bell, F., unpublished data), occurs in any of the hairs in this study. Therefore, general macrofibril architecture and a tendency for strict hexagonal packing may be explained by different keratin and KAP combinations, which would help explain the diversity found in species such as rabbit. Therefore, any cell could have a "meso-like" IF packing component, and this perhaps goes some way to explaining the relatively large variation in macrofibril structure observed in wool mesocortex cells, and that gave rise to the terms ortho-like mesocortex and para-like mesocortex.[12]

The macrofibrils from alpaca that had distorted profiles and frequently contained central darkly staining inclusions (Figure 6e,f) are more difficult to explain. To our knowledge, macrofibrils of this type have not been described previously for any species. The rarity of these cells, the distorted macrofibril profiles, and excess of intermacrofibrillar material or cytoplasmic remnant material are reminiscent of so-called type D cells, which were uncommonly found in Japanese human hair cortex in an earlier study[5] and which were speculated to be from a region close to one end of the spicule-like cell. If this is the case, further investigation may clarify some of the events occurring during the later stages of hair fiber assembly during which the cytoplasm condenses and distortion of macrofibrils may occur.

Staining Intensity Differences. In wool, the TEM methods used here (reduction followed by osmication) show stain intensity differences between orthocortex and paracortex cells in terms of matrix density, with the paracortex matrix appearing darker in micrographs (see Figure 5). This darker staining is one specific characteristic of the paracortex and sometimes the mesocortex, and correlates with increased sulfur content, diagnostic of increased abundance of sulfur-rich KAPs. In rabbit fibers, we found evidence that an increased stain density is not necessarily tied to a paracortex-like macrofibril architecture or loose local IF packing because there were two cell types present that both contained double-twist macrofibrils with hexagonal local IF packing reminiscent of that found in the wool orthocortex, but one of these cell types contained much more intensely stained matrix (Figure 7). Further investigation

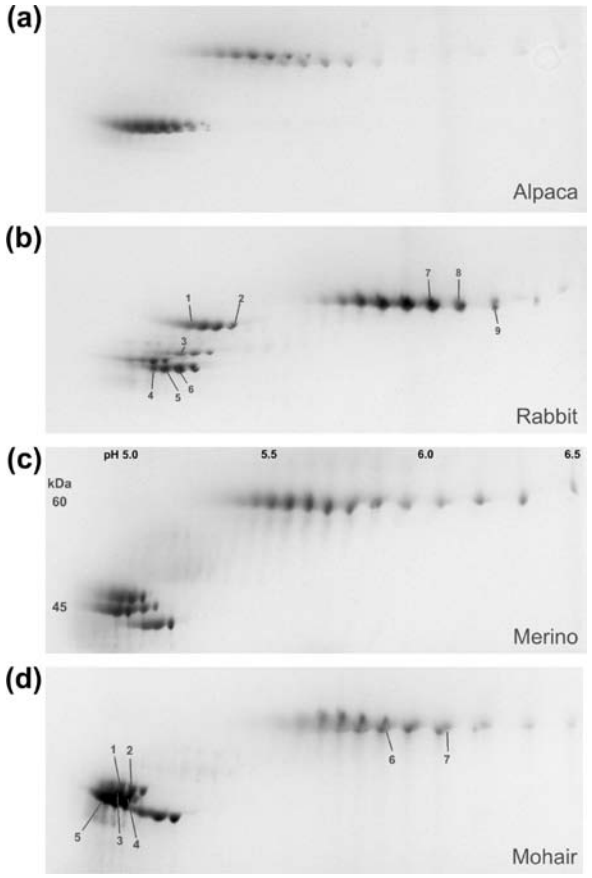

Figure 10. 2-DE gels over the pH range 4–7 of protein extracts showing the keratin region of (a) alpaca, (b) rabbit, (c) Merino wool, and (d) mohair. The type I keratins form a compact region at low pH, while the type IIs appear as a long string at higher pH and molecular weight. The numbers in b and d refer to spots excised and analyzed by mass spectrometry.

Table 3. Identification of Rabbit (*Orcytolagus cuniculus*) Proteins in the 2-DE Map of Rabbit Wool and the Equivalent Proteins in Sheep (*Ovis aries*)[a]

spot no.	rabbit	sheep equivalent(s)
1 and 2	predicted K38-like	
3	predicted K31-like	K33b
	predicted K33a-like	
4	predicted K31-like	K31
	predicted K33a-like	K33a
	predicted K3B-like	K33b
		K34
5	predicted K33a-like	K31
		K33b
6	predicted K31-like	K31
	predicted K33a-like	K33b
7	predicted K83-like	K83
	predicted K86-like	
8 and 9	predicted K83-like	
	predicted K86-like	

[a]The spot numbers relate to the gel in Figure 10b.

will be required to clearly tie this finding to increased sulfur content.

Cell Distribution Across the Fiber. Angora rabbit might be considered an out-group in our comparison, and differences

Table 4. Identification of Goat (*Capra hircus*) Proteins in the 2-DE Map of Mohair Wool and the Equivalent Proteins in Sheep (*Ovis aries*)[a]

spot no.	mohair	sheep equivalent(s)
1, 3, and 4	hair acidic keratin 1	K31
		K33a
		K33b
		K34
2	hair acidic keratin 1	K31
		K33a
		K33b
5	hair acidic keratin 1	K31
		K33b
		K34
6 and 7		K81
		K86

[a]The spot numbers relate to the gel in Figure 10d.

Table 5. Identification of Goat (*Capra hircus*) proteins in the 2-DE Map of Mohair Wool and the Equivalent Proteins in Sheep (*Ovis aries*) from the KAP Region[a]

spot no.	mohair	sheep equivalent(s)
1	hair acidic keratin 1	K10, K33b, K75, K33a, K1, K6A, K81, K83
	KAP [*Capra aegagrus*]	KAP1.3 KAP1.1, KAP1.4
3	KAP13.1	KAP13.1
		K1, K10, K75, K6A, K18

[a]The spot numbers relate to the gel in Figure 9d.

in protein composition, morphology, and single-fiber properties might be less surprising than for more related species, yet it was the species most related to Merino that provided some of the most unexpected results. Mohair was notably different from the others because there was no detectable relationship in these very straight fibers between curvature and diameter (Figure 2). Morphologically, mohair had more cuticle layers than did wool, and in addition to the "meso-like" hexagonal packing motif seen in all cells, was the only fiber in which cell types were not distributed asymmetrically across the fiber, instead being semirandomly arranged in a roughly annular pattern.

Protein Content. Apart from the obvious differences in the 2-DE patterns of the KAPs, there were distinctive differences in the keratin proteins. While mohair and Merino fibers appear to be very similar in the type I keratin region, differing only in the relative position of the lowest molecular weight string, the others differ considerably. In the case of the alpaca fiber, these formed a very compact cluster of three strings, whereas in rabbit fiber, they were split into six or seven strings of proteins. In many cases, we were able to identify the protein class to which 2-DE spots belonged, and definitive identification was possible for some spots, but in general, this was hampered by a lack of species-specific sequence database coverage.

In contrast, there were strong similarities between the 2-DE pattern of all four fiber types in the type II keratin region. Two distinct strings were evident in both the alpaca and mohair 2-DE gel at the acidic end. Mass spectrometric analysis revealed the presence of proteins like K81, K83, and K86 in this region, proteins that have also been found in this region in Merino fibers.[17] Notable among the type II keratins for the alpaca, rabbit, and mohair fibers was the lower abundance of spots at the basic end of the string compared to the acidic end, in

relation to Merino wool, and in the case of mohair and rabbit, no peptides consistent with K85-like proteins were identified by mass spectrometric analysis. This is interesting because the sequence homology (based on % sequence identity) between K85 and the other type IIs is lower, ranging between 80 and 87%, whereas the homology between K81 and the other two proteins, K83 and K86, is between 90 and 93%. K85 is distinct from other keratins for another reason; while its role/function in fiber structure is still not clear, it is interesting that mutations in this protein are also known in humans to correlate to pure hair and nail ectodermal dysplasia, a disorder characterized in part by abnormal hair morphology, including high-levels of single-fiber curvature.[30]

Conclusions. The traditional definitions of wool cell type names (e.g., orthocortex) are collective terms based on a set of parameters that include macrofibril architecture, IF packing and spacing, and protein composition. However, this work challenges that view in a number of ways, one being that an increase in sulfur content of the cell does not correlate specifically with paracortical-like macrofibril architecture. In addition, a major morphological conclusion from our study is that the low-intensity double-twist macrofibrils, which if they occur at all in wool are uncommon and associated with paracortex cells, are common in other mammalian fibers. High-intensity double-twist macrofibrils, as found in the wool orthocortex, are also common in other mammalian fibers. The relative lack of variation in double-twist intensity in wool is likely the exception to the rule.

Likewise, from past studies relating relative protein composition to wool fiber morphology a view has developed of how specific classes of wool proteins might influence the macrofibril architecture of the major cell types. So, in looking beyond the wool fiber, we have found that fibers whose cell architecture departs from the so-called norm also differ considerably in their keratin and KAP composition. For instance mohair, the fiber most closely related to wool in our study, is notable for a lack of any relationship between diameter and curvature as well as any asymmetric distribution of cell types. Therefore, it is of interest that there are considerable differences in its KAP component and even some noticeable differences in its keratins. In particular, in a 2-DE map, the type I keratins are marked by having their lowest molecular weight string separating at a higher pH to the rest of the cluster, while in the type II string, there appears to be evidence for decreased amounts of a K85-like protein at the basic end of the type II region. This K85 decrease was also evident in the 2-DE gels of alpaca and rabbit. The rabbit 2-DE was also notable for a pattern of KAPs that differed considerably from Merino and mohair, as well as the appearance of extra strings of spots in the type I keratin region, all this in a fiber whose cells exhibit similar architectures but differ in stain density. It certainly looks like the protein−structure relationships in fibers are considerably more flexible than can be accounted for using the wool cell type framework. What is more, low-intensity double-twist macrofibrils appear to occur in the hairs of most species other than wool, and this raises the question of whether wool itself is something of a special case rather than being typical of fibers in general.

In a postwoolcentric world, how do we approach fiber protein−structure relationships? We saw noticeable differences in the types of proteins found in the fibers and their relative of composition, in both the intermediate filament and keratin associated proteins. Such differences in protein profiles may be linked to the changes in morphology that we have been seeing. In addition, the mature fiber is not just defined by its constituent proteins but is also the product of processes occurring in the follicle and the keratinization zone above it. As these processes may include self-assembly driven by the very complement of proteins present,[28] the systems under study are of an extremely complex nature, and it is anticipated that mono-disciplinary studies will only ever succeed in revealing fragmentary information. We see interspecies comparison as a useful approach for teasing out general relationships between the genetic, proteomic, structural, and fiber mechanical properties.

AUTHOR INFORMATION

Corresponding Author

*AgResearch Ltd., Private Bag 4749, Christchurch 8140, New Zealand. Tel: +64-3-321-8800. Fax: +64 3 321 8811. E-mail: ancy.thomas@agresearch.co.nz.

Author Contributions

†These authors contributed equally to this article.

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