the controlling biochemical pathways are known. These new technologies could be integrated with more direct grower-manufacturer relationships where specified wool characteristics, reduced within-fleece variability, and efficient and ethical farm practices will add value.

Microarrays as a discovery tool in wool genomics

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ABSTRACT

Wool follicle development and growth is a highly ordered and complex process involving the sequential expression of numerous genes within diverse cell populations. The aim of present studies using microarray analysis is to identify genes and pathways involved in the growth of wool follicles which could be utilised to increase farm productivity and improve the characteristics of Australasian wools. An ovine cDNA array was developed including sequences derived from sheep skin containing wool follicles in different growth stages encompassing many structural and regulatory genes necessary for follicular growth. Initial comparison of stages of wool follicle regression show marked changes in the expression levels of numerous genes including wool keratins. Two parallel research projects in Australia and New Zealand supported by SheepGenomics are currently utilising microarray technology to profile gene expression during key stages of wool follicle development in the foetus and follicular cycling associated with fleece shedding.

Keywords: gene expression; microarrays; wool follicle development; wool follicle growth cycle.

INTRODUCTION

Innovative technologies are required to address issues of harvesting efficiency, fibre characteristics and animal welfare that are becoming critically important to the future of the wool industry. Such new technologies to control fibre growth will depend on advances in our understanding of the underlying genes and gene networks. For example, genes that encode signalling and adhesion molecules controlling wool follicle morphogenesis in the foetus profoundly affect adult fleece weight and fibre diameter. However, it is likely that the same or similar molecular mechanisms also govern organised interruption and reinitiation of post-natal fibre growth, with associated changes in fibre type and length. Discovery of the gene control networks will provide opportunities to accelerate the rate of genetic progress, and to develop bioactives to transform the costly farming operations of shearing, crutching and mulesing.

The pre-natal development of wool follicles progresses in waves across the surface of the foetus (Hardy & Lyne, 1956; Lyne & Brook, 1964). Ultimately, the nature and distribution of these follicle populations determine crucial aspects of the wool phenotype including fleece cover and variation in fibre attributes within the fleece (Scobie *et al*., 2006). Interactions of dermal and epithelial cells initially determine the patterning leading to primary wool follicle formation around day 60 of gestation in Merino sheep (Figure 1). This is followed by the development of original secondary follicles around day 85 of gestation. Branching of the original secondary follicles around day 100 generates the derived secondary follicles. The entire follicle complement is established by shortly after birth (Hardy & Lyne, 1956). Subsequently, and almost uniquely among organs of adult animals, hair follicles in the skin are regularly renewed in a process that, to some extent, recapitulates foetal development (Figure 2). This involves highly regulated inter-cellular communication, follicular stem cell activation, cell migration, proliferation and apoptosis (Stenn & Paus, 2001; Botchkarev & Kishimoto, 2003). These co-ordinated processes lead to the renewal of transient amplifying cells in the follicle bulb which divide and terminally differentiate to form the keratinised wool fibre.

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FIGURE 1: Stages in the development of primary wool follicles in the sheep foetus. Follicles form from two simple layers of skin: the epidermis and the underlying dermis. Dermal cells aggregate to form the dermal papilla (grey). In Merinos, the first generation of follicles initiate at about 60 days gestation. Adapted from Hardy (1992).

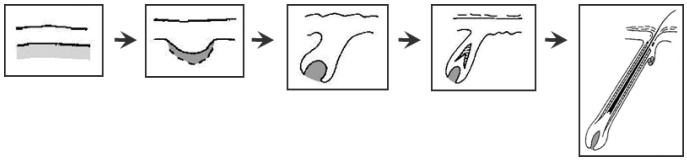
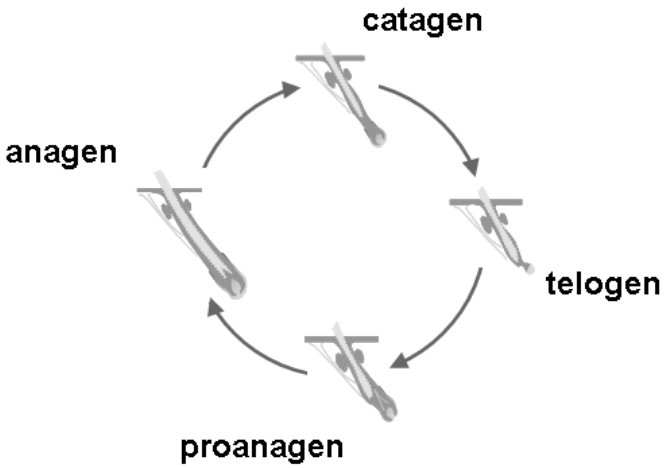


FIGURE 2: The wool follicle growth cycle. Active (anagen) and dormant (telogen) growth phases are separated by transitional phases of regression (catagen) and re-initiation of growth (proanagen). Exposure of Wiltshire sheep to long days in mid-summer causes wool follicles to transition from anagen, through catagen to telogen over a period of approximately 30 days.



If the cycles of individual follicles in a shedding sheep breed can be synchronised, skin can be collected for accessing specific cycle stages. In previous research we have established methods to artificially induce follicle cycling in Wiltshire sheep using abrupt changes in photoperiod or by the pharmacological manipulation of circulating prolactin (Pearson *et al*., 1996; Nixon *et al*., 2002). Skin samples from such experiments have already been used to identify genes associated with follicle growth cycles using differential display (Rufaut *et al*., 1999). However, differential display is an inefficient and unreliable methodology for identifying the large number of genes associated with wool follicle growth and development.

In contrast, microarray technologies developed over the last decade allow the relative expression levels of thousands of genes to be determined (Duggan *et al*., 1999; Lin *et al*., 2004). In a cDNA microarray, short selected sequences of cDNA (complementary DNA derived from RNA) are printed onto glass slides. Each spot of cDNA is approximately 100 μm in diameter so that many thousands of expressed sequences can be represented on a single slide. Microarray experiments typically involve the competitive hybridisation of two total RNA samples, each of which has been converted into cDNA and labelled with different fluorescent dyes. The differential expression of a particular gene is determined by the intensity ratio of the two signals emitted from the dyes when the hybridised spots are scanned by laser. Higher relative signal from either dye is a measure of difference in the specific RNA concentration, indicating up- or down-regulation of the corresponding gene. Hybridisations are performed in replicate using total RNA from different animals to determine variation and each RNA sample is alternately labelled with both dyes to control for dye-bias.

Hence, microarrays enable the simultaneous detection of changes in the expression of a large number of genes in a unified format. With appropriate experimental design and analysis software, the underlying gene networks operating in a tissue of interest can be identified. The aim of the studies described here was to test an ovine cDNA microarray by comparing sheep skin containing wool follicles in different stages of growth.

OVINE SKIN cDNA

The ovine cDNA arrays (Ovita Ltd., Dunedin, New Zealand) were fabricated by the AgResearch Molecular Biology Unit (Dunedin, New Zealand) utilising 20,736 annotated expressed sequence tags (ESTs). Approximately one half were selected from seven sheep skin libraries (Table 1) representing the principal wool follicle cycle stages (Figure 2). Other ESTs were derived from ovine muscle and ovarian tissues. Of the total, 10952 ESTs had high homology (e<1e-6) to entries in genomic (RefSeq) and protein (SwissProt) databases, while 9158 ESTs lacked any significant homologies by BLAST search. A small number of expressed sequences not represented in the EST libraries, but important in wool and hair growth processes, were cloned and added to augment the array. Each microarray slide included blank controls and repeats for quality control purposes.

TABLE 1: Sources of skin ESTs for the ovine cDNA microarray.

Library	Number of ESTs	Breed	Follicle growth status
OSAA	1081	Wiltshire	Anagen
OSAB	707	Romney	Anagen
OSCA	860	Wiltshire	Catagen
OSPA	1264	Wiltshire	Proanagen
OSTA	4467	Wiltshire	Telogen
OCS1	693	Romney	Anagen
OCS4	1509	Romney	Anagen

cDNA MICROARRAY ASSESSMENT

A wool follicle growth cycle (Figure 2) was induced in Wiltshire sheep that were housed indoors by way of an abrupt transition from short (8 h light : 16 h dark) to long day photoperiod (16 h light : 8 h dark) (Pearson *et al*., 1996). Mid-line skin samples were collected under local anaesthesia over 28 days following the photoperiod transition. Follicle growth status was determined by histological analysis (Nixon, 1993). The skin collections corresponded to a period in which growing wool follicles (100% anagen at day 0) began to regress. At day 4, 8% of mid-side follicles were classified as catagen, increasing to 16% at day 8. By day 28, 87% of the follicles were in telogen (Figure 2).

Frozen skin was ground to powder under liquid nitrogen. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and purified by DNase treatment and column chromatography (Qiagen, Hilden, Germany). Twenty five μg of total RNA was labelled with Cy3 and Cy5 dyes (Invitrogen, Carsbad, CA, USA) and hybridised to the microarray slides according to the manufacturer's instructions (Ambion, Austin, TX, USA). The expression data were generated from scanned images using GenePix Pro 4.1 (Axon Instruments, Foster City, CA, USA). Genes of interest were identified from the annotations of the EST database and classified by homology to genes of known function.

An initial experiment was conducted in three parts. The first involved a comparison of the same RNA sample against itself as a validation of experimental protocols and the microarray. As expected, such self-hybridisation slides yielded yellow spots arising from hybridisation of equal concentrations of the same mRNA species labelled with the two fluorophores (pseudocoloured red and green). The high intensity of the signals and the low background indicated fidelity of the microarray slides and appropriate hybridisation conditions.

In contrast, many spots on the slides hybridised with RNA from two different skin biopsies containing growing and dormant follicles were either red or green representing a significant degree of differential gene expression. The high correlation of relative signal intensities between the two dye-swap slides from this second part of the experiment (Figure 3) showed that the microarray generated consistent results when the test RNAs were labelled alternatively with either Cy3 or Cy5.

When the data were plotted as a volcano plot (Figure 4a), most genes had expression levels that were not dependant on follicle growth status as indicated by the concentration of spots in a central band comprising genes with less than 1.5 fold differences in expression level. Up- and down-regulation in anagen or telogen is represented by the spread of spots to the left or right of the central zone respectively. However, only those in the quadrants above the transverse line were up-regulated by 1.5-fold or more with a statistical significance of $P \leq 0.01$. With increased replication, the expression changes of more genes would likely reach statistical significance.

These results are supported by an examination of the expression of keratin intermediate filaments (KIFs) and keratin associated proteins (KAPs) (Figure 4b). In general, these genes were up-regulated in skin containing growing follicles, as would be expected. While some keratins of epithelial origin do not change over the growing and resting phases of the hair cycle, a higher proportion of wool keratins might have been expected to be significantly up-regulated as seen using quantitative real-time PCR (Yu *et al*., unpublished data). However, increased statistical power using additional microarray replicates will likely demonstrate that most, if not all, genes encoding for wool structural proteins are expressed at significantly higher levels in anagen.

FIGURE 3: Correlation of fold change in gene expression between anagen and telogen skin measured on two microarray slides with dye swap labelling. Each point represents an EST with measurable hybridisation signal. The axes represent log values of the intensity ratios measured on the two slides.

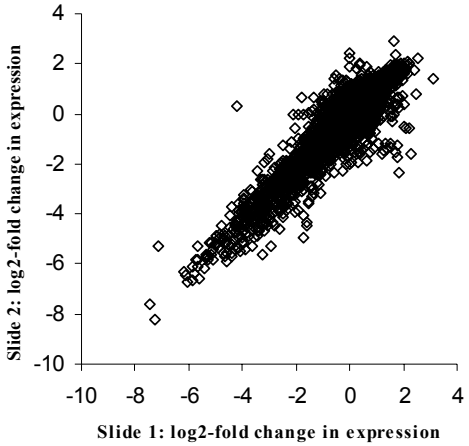


FIGURE 4: (a) A volcano plot of a microarray comparison between anagen and telogen wool follicles from a single Wiltshire sheep. This is based on two microarray slides with dye swap labelling (Figure 3). The X-axis shows the expression log fold change between the two growth stages. Up-regulation of gene expression in anagen is represented by negative values and down-regulation by positive values. The vertical dotted lines delineate 1.5-fold changes in expression. The Y axis represents the P-value for the differences between samples. The horizontal dotted line indicates P = 0.01. (b), as for (a) but including only those genes with the descriptor "keratin" included in the sequence annotation.

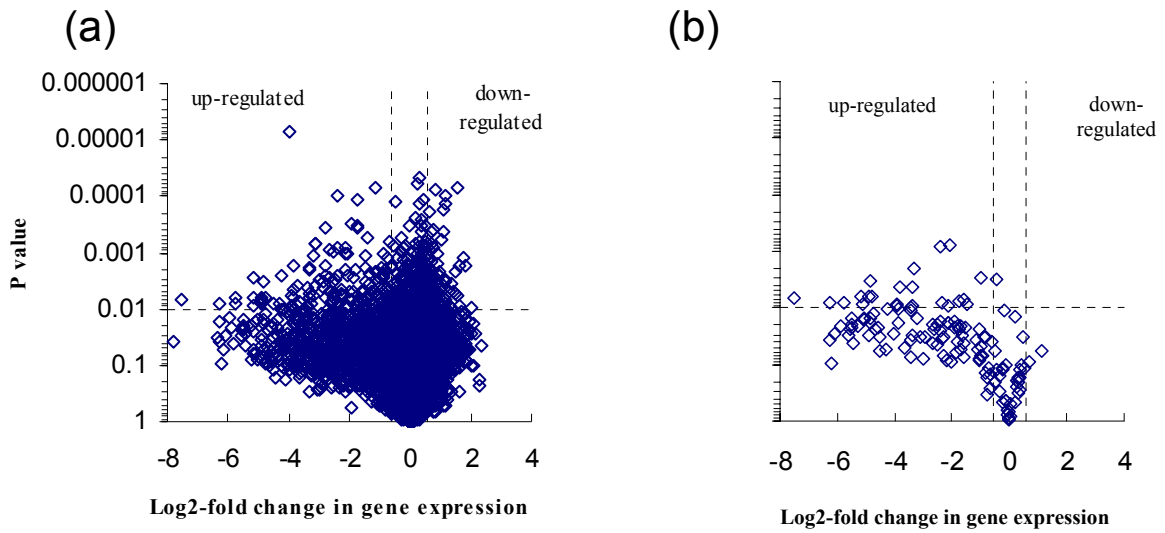
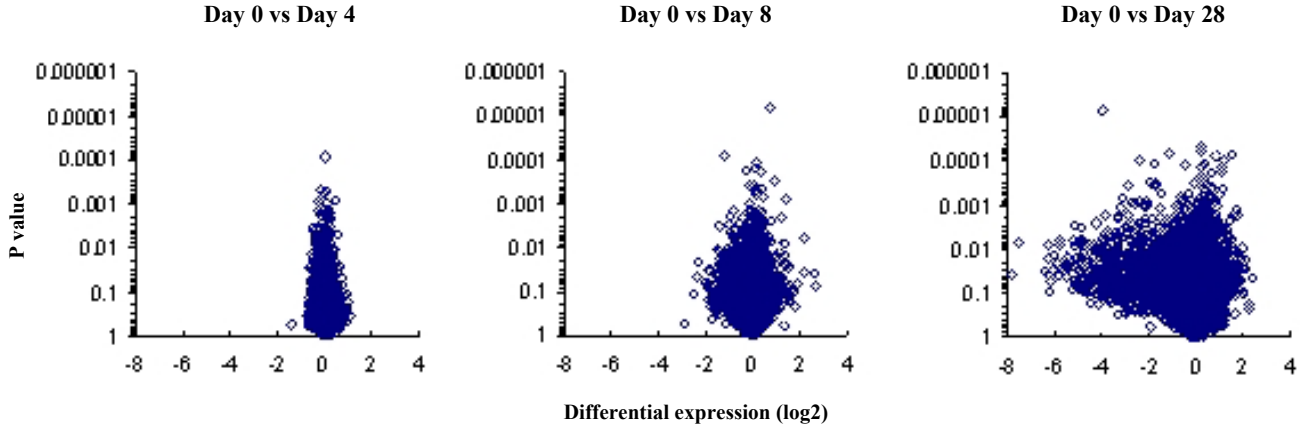


FIGURE 5: From left to right, volcano plots showing increasing changes in gene expression comparing anagen skin (day 0) with, respectively, early catagen (days 4 and 8) and telogen (day 28).



A third part of the experiment compared skin containing anagen follicles (day 0) with skin containing early regressing follicles at days 4 and 8 and with skin containing telogen follicles (day 28). Differential expression was markedly lower in early catagen (days 4 and 8) compared with the anagen-telogen comparison (Figure 5). The relative lack of differential expression over days 4 and 8 reflects, at least in part, the continuing expression of structural genes in anagen follicles over this period. Therefore, comparisons at these early stages of follicle regression facilitate the identification of regulatory genes involved in follicle regression. This is supported by the functional classification of the differentially expressed genes that were detected. Of the 42 genes that were differentially expressed between days 0 and 8, a third had functions associated with signal transduction and gene transcription, essential processes during the onset of follicle cycle transitions (Botchkarev & Kishimoto, 2003).

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.

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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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