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## Gene expression profiling of wool follicle growth cycles by cDNA microarray

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### ABSTRACT

Wool growth in Wiltshire sheep was synchronised by daylength manipulation to allow identification of genes that are regulated during transitions in the wool follicle cycle. Pair-wise comparisons between growing and resting phases were conducted using both direct and indirect cDNA microarray analysis. The two approaches were well correlated at three different levels: overall similar patterns of gene expression, detection of changes in keratin gene expression, and reduced differential expression between closer cycle stages. Using more slides, the indirect approach identified more expressed sequence tags (ESTs) (indirect 1132, direct 444), and had higher coverage of wool keratins (indirect 78%, direct 42%). This study demonstrates the usefulness of cDNA microarray for expression profiling in whole skin. Further characterisation of genes identified by this work, particularly the smaller sets of ESTs derived from comparisons at early stages of follicle regression and regrowth, would improve our understanding and provide leads for alternative wool harvesting technology or enhancing wool growth.

**Keywords:** expressed sequence tag (EST); hair cycle; sheep; New Zealand Wiltshire; wool harvesting.

### INTRODUCTION

The wool follicle cycle which underlies fleece shedding and wool growth is homologous to hair cycles in other mammals, and is distinct from metabolic-related wool breaks or fleece tenderness. This physiological cycle consists of four phases: prolonged steady-state growth (anagen), regression (catagen), quiescence (telogen), and regeneration of the follicle and re-establishment of wool growth (proanagen) (Parry *et al.*, 1995). The hormonal and environmental factors that regulate wool shedding in the New Zealand Wiltshire have been characterised and the resulting synchronised shedding and regrowth is established as a useful model for studying the mechanisms controlling wool growth (Nixon *et al.*, 2002). Identification of genes that control the wool follicle cycle is therefore potentially useful, either as a means to develop animal treatments that alter wool growth or to facilitate genetic selection.

Microarray analysis is a powerful tool to discover multiple differences in gene expression between cellular conditions (Duggan *et al.*, 1999). The relative levels of mRNA for thousands of genes can be simultaneously determined through competitive hybridisation. Although gene expression studies using microarrays over hair follicle growth have been reported in mouse (Ishimatsu-Tsuji *et al.*, 2005; Lin *et al.*, 2004), the contrasting results suggest that there is much more to be learned about follicle growth regulation.

In this study, a complete follicle growth cycle was induced in the New Zealand Wiltshire sheep and expression profiles compared over the time

course to identify genes involved in the key stages of wool follicle cycling. This report validates the approach using total RNA from whole skin samples with the ovine cDNA array.

### MATERIALS AND METHODS

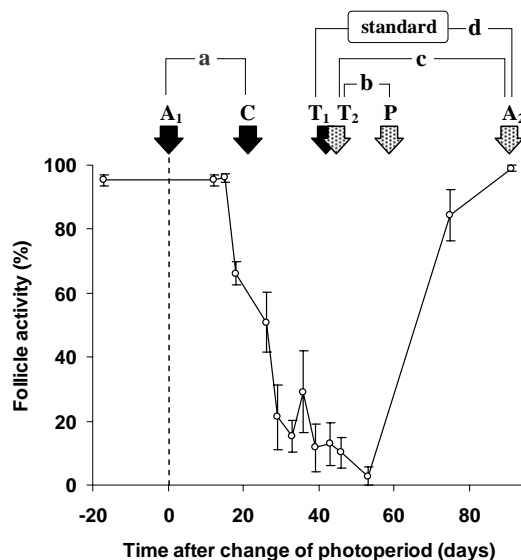
#### Animals, photoperiod manipulation and sample collection

Wool follicle cycles were synchronised in New Zealand Wiltshire sheep using artificial photoperiod to manipulate circulating prolactin as previously described (Parry *et al.*, 1995). Nineteen Wiltshire rams were maintained indoors on a constant diet of sheep pellets and hay from 20 September 2005. The animals were initially conditioned to short-days with eight hours of light and 16 hours of dark then exposed to long-days with 16 hours of light and eight hours of dark from 6 January 2006 (Day 0). The progression of wool follicle regression and reactivation was monitored histologically using skin biopsies from the lower mid-side (Nixon, 1993). Two sets of skin samples used in the microarray comparisons were collected from the flanks over the anagen to catagen (Group 1) and telogen to anagen (Group 2) transitions under anaesthesia, or at slaughter (T1 and A2) (Figure 1). Frozen skin samples from eight sheep, four from each cycle transition, were powdered and RNA extracted using TRIzol reagent (Invitrogen, Carlsbad, California, USA) and further purified (Qiagen GmbH, Hilden, Germany). A reference sample was constructed using equal amounts of total RNA from foetal skin, adult skin and adult liver of sheep.

#### Microarray and data analysis

Four experiments comparing gene expression at different stages of follicle regression and

**FIGURE 1:** Wool follicle cycle in eight Wiltshire sheep induced by change from short- to long-day photoperiod. The two sets of arrows indicate the sampling times of two groups of four sheep. Black arrows (Group 1) indicate samples from anagen ( $A_1$ ), catagen (C) and telogen ( $T_1$ ), used to profile regression (a). Stippled arrows (Group 2) indicate samples from telogen ( $T_2$ ), proanagen (P) and second anagen ( $A_2$ ) used to profile reactivation (b and c). Samples  $T_2$  and  $A_2$  were used for direct comparison (c), while samples  $T_1$  and  $A_2$  were each compared to reference standard RNA and thereby used in an indirect comparison (d). Error bars are SEM.



**TABLE 1:** Keratin ESTs identified from direct and indirect anagen-telogen comparisons. Percentages of differentially expressed ESTs are relative to the total number of ESTs.

| Type                           | Total EST | DE EST        |                 | Both | Total DE EST    |
|--------------------------------|-----------|---------------|-----------------|------|-----------------|
|                                |           | Direct        | Indirect        |      |                 |
| Overall                        | 20,736    | 444<br>(2.1%) | 1,140<br>(5.5%) | 372  | 1,212<br>(5.8%) |
| Keratin ESTs                   | 133       | 47<br>(35.3%) | 90<br>(67.7%)   | 47   | 90<br>(67.7%)   |
| Non follicle keratin ESTs      | 21        | 0             | 3 (14.3%)       | 0    | 3 (14.3%)       |
| Follicle-specific keratin ESTs | 112       | 47<br>(42.0%) | 87<br>(77.7%)   | 47   | 87<br>(77.7%)   |

reactivation were conducted with an overall balance of dye-labelling. Direct comparisons of samples within the same animals were made during the anagen-catagen phases (four comparisons and two repeated = six slides). The transition between the proanagen and telogen phases was also investigated with six slides (b in Figure 1). Four direct comparisons within each of four sheep in Group 2 were also performed between the telogen and anagen phases (c in Figure 1). Indirect comparisons of anagen (four slides) to telogen (four

slides, with one unsuccessful) were via the reference RNA (d in Figure 1). The ovine cDNA array contained 20,736 probes (spots or features) derived from mixed-tissue cDNA libraries of 140,000 expressed sequence tags (ESTs) (Diez-Tascon *et al.*, 2005). The identities of the ESTs were established by their top BLAST search hits against reference sequences (RefSeq) and proteins in GeneBank. Twenty-five  $\mu\text{g}$  of total RNA from each of the two compared skin samples was labelled (Invitrogen) with either Cy3 or Cy5 dye and hybridised to the microarray slides (Print128) using a commercial hybridisation buffer according to the manufacturer's protocols (Ambion, Foster City, California, USA). Slides were scanned and data generated from images using GenePix Pro 4.1 software (Axon Instrument, Foster City, California, USA). The data were normalised (Baird *et al.*, 2004) and analysed to identify differentially expressed (DE) ESTs between the two compared stages (1.5 fold difference in fluorescent signal,  $P < 0.05$ ). Wool follicle-specific keratin intermediate filament (KIF) and keratin associated protein (KAP) ESTs were used to further validate the outputs. DE ESTs were also classified into functional categories to assess their patterns of expression during the early transitional stages.

## RESULTS

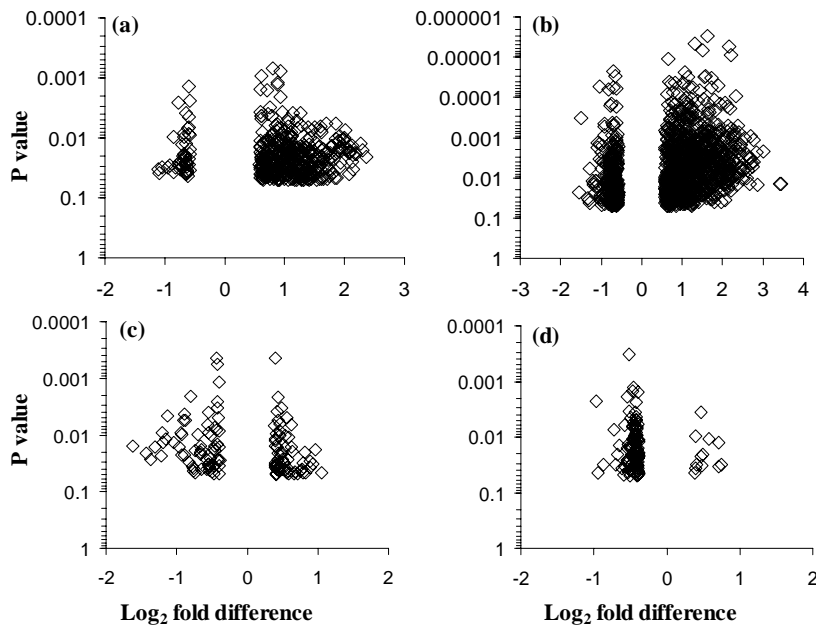
### Synchronisation of wool follicle growth

Wool follicles had entered the regression phase (catagen) by Day 18 and by Day 29 most follicles ceased growing (telogen) for approximately 20 days. Follicle growth (anagen) had resumed by Day 75 (Figure 1). More detailed analysis of follicle activity based on longitudinal sections confirmed the sequential regression and reactivation of wool follicles in each of the sheep selected for microarray. The samples used for anagen-telogen comparisons (c and d in Figure 1) were collected during the minimum and the new maximum of follicle activity. Samples used for early regression (a in Figure 1) and reactivation (b in Figure 1) studies were at the initial stages of follicle involution and regrowth.

### Identification of DE ESTs between anagen and telogen

Changes in expression were highly correlated amongst technical repeats of the indirect comparison. Linear relationships were found between anagen versus reference RNA and telogen versus reference RNA. When the data from direct and indirect profiling were overlaid, comparable expression patterns for all the probes were also seen. As a result, a total of 1,212 ESTs were identified (Table 1). Of these, 444 were revealed by direct comparison (using four slides; four sheep) and 1,140 ESTs were identified via a reference RNA sample

**FIGURE 2:** Differentially expressed ESTs at the compared follicle cycle phases. (a) Anagen versus telogen, direct. (b) Anagen versus telogen, indirect. (c) Proanagen versus telogen, direct. (d) Catagen versus anagen, direct. X axis is the fold difference in fluorescence signal ( $\log_2$ ) and Y axis is the probability (P) values of *t* tests.



**TABLE 2:** Classification of identified (1.3 fold difference,  $P < 0.05$ ) expressed sequence tags during early follicle reactivation and regression into functional categories.

| Classification                    | Proanagen vs Telogen |                 | Anagen vs Catagen |                 |
|-----------------------------------|----------------------|-----------------|-------------------|-----------------|
|                                   | Up regulation        | Down regulation | Up regulation     | Down regulation |
| Signalling and transcription      | 10                   | 8               | 4                 | 15              |
| Protein stability and degradation | 5                    | 2               | 0                 | 12              |
| Enzymes                           | 8                    | 9               | 0                 | 10              |
| Cell proliferation                | 2                    | 4               | 2                 | 4               |
| Structural proteins               | 5                    | 7               | 1                 | 17              |
| Other or unknown functions        | 8                    | 3               | 1                 | 17              |
| No identities                     | 27                   | 26              | 3                 | 28              |
| Total                             | 65                   | 69              | 11                | 103             |

(using seven slides; four same sheep for anagen and three different sheep for telogen) (Figures 2a and 2b, Table 1). In both approaches, the overwhelming majority of DE ESTs were up-regulated in anagen (Figures 2a and 2b). Of the 444 ESTs detected by direct comparison, 372 (83.8%) were also confirmed by the indirect method. Comparison between proanagen and telogen (Figure 2c), anagen and catagen (Figure 2d) revealed fewer DE ESTs.

**Identification of DE ESTs representing KIFs and KAPs**

A total of 133 ESTs included in the cDNA array represent KIF and KAP genes. Of these, 112 are expressed only in wool follicles. By direct

profiling, 47 (35%) of the represented KIF and KAP genes were differentially expressed between anagen and telogen (Table 1). In comparison, indirect profiling found that 90 (68%) were differentially expressed, including all of those detected by the direct approach. Three DE ESTs identified by the indirect method were keratins that are also expressed in skin epidermis, whereas all of those recognised by the direct approach were follicle-specific. Nearly 80% of the follicle-specific keratins were significantly different by the indirect approach, in contrast to 42% for the direct profiling (Table 1). Importantly, all but of one of these ESTs were expressed at a higher level in anagen.

**Differential expression during early reactivation and regression**

The smaller numbers of ESTs which showed 1.5-fold or greater difference in expression ( $P < 0.05$ ) in proanagen-telogen ( $n = 54$ ) and anagen-early catagen comparisons ( $n = 9$ ) were consistent with minimal structural reorganisation over early stages of reactivation and regression. When the threshold for ratio of fluorescence signal was relaxed to 1.3 fold, 134 and 114 DE ESTs were identified for the early regression and reactivation transitions respectively (Figure 2c and 2d). Classification of these ESTs into functional categories provided useful information on the cellular activities involved in follicle cycle progression (Table 2). Similar numbers of ESTs were up- and down-regulated through early proanagen (Figure 2c), whereas the majority of genes were downregulated during catagen (Figure 2d and Table 2).

**DISCUSSION**

The synchronisation of wool growth has enabled the discovery of genes that are regulated in association with follicle cycle transitions. Expression of KIF and KAP genes are required for the formation of follicles and fibre, thus their mRNA abundance is expected to be much higher in anagen than in telogen, as revealed by quantitative reverse transcriptase-polymerase chain reaction (qPCR) analyses (Z-D. Yu, Unpublished data). In contrast, the expression of non-follicle specific keratin remains relatively stable over the wool follicle cycle. Similar cycle-related differences in the expression of mouse KIF and KAP genes have also been previously detected by microarray (Ishimatsu-Tsuji *et al.*, 2005). Therefore, the

differential expression of these follicle-specific structural genes provides a useful means for assessing the reliability of microarray results.

The good agreement, including the overlap in DE KIF and KAP genes, between direct comparisons of samples from the same animals and indirect comparisons of different animals demonstrates the reliable performance of the cDNA microarray system. The higher discovery rate (78% versus 42%) of wool follicle-specific keratin and KAP genes by the indirect method was attributable to reduced variance through use of more slides. The generally smaller variance reported in the literature for direct comparison (Yang & Speed, 2002) and limited RNA from skin biopsies prompted us to adopt this approach for expression profiling between the closer transitional stages. Variation should be further reduced by hybridisation between samples from the same animal.

Significantly, 98% of the DE ESTs representing wool follicle-specific KIF and KAP genes were found to be expressed at a higher level in anagen than in telogen. This is consistent with the patterns detected in mouse skin samples with similar physiological conditions (Ishimatsu-Tsuji *et al.*, 2005; Lin *et al.*, 2004). The rare spots with higher affinity to one dye (Baird *et al.*, 2004) may explain why one keratin EST was found to be at a higher level in telogen by both approaches. The high level of reliability demonstrated is particularly important for a method which simultaneously detects the expression of many thousands of genes (Yu *et al.*, 2006). Nevertheless, confirmation by independent methods such as qPCR and/or *in situ* hybridisation is often required (Ishimatsu-Tsuji *et al.*, 2005; Lin *et al.*, 2004).

In comparison to the large number of genes that were differentially expressed between follicle dormancy and growth (Ishimatsu-Tsuji *et al.*, 2005; Lin *et al.*, 2004), the smaller sets of DE ESTs derived from comparisons of closer stages of anagen with early regression, and telogen with early reactivation, were consistent with the relatively small morphological changes in follicle growth status between these samples. The decreased level of expression for most genes in early catagen was also compatible with reduced follicle activities. An improved understanding of the differentially expressed genes has subsequently been achieved by functional classification and identification of the gene networks involved. Signalling and transcriptional control genes identified during the early stages of follicle transitions are particularly interesting. Knowledge of the nature of these genes, their polymorphisms and their associated signalling pathways will strengthen our understanding of the mechanisms controlling wool growth and specifying traits such as fibre length. More specifically, genes

regulated early in regression represent targets for interruption of wool growth, whereas those involved in early follicle regeneration provide leads to enhance wool growth. Further mining of the data derived will facilitate progress towards innovative technologies for wool harvesting and increasing production.

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