AN INTEGRATED GENOMICS APPROACH TO IMPROVING WOOL PRODUCTIVITY AND QUALITY

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SUMMARY

This paper summarises the approach taken in a national research program designed to "identify and utilise genes of importance in the sheep industries". The sheepgenomics program as it was known, comprised meat, wool and parasite subprograms with an underpinning core technology subprogram. The wool subprogram used a combination of gene association and functional biology studies to identify genes and gene networks amenable to manipulation or selection to improve wool production and quality. Significant progress was made in identifying genes involved in wool follicle initiation, hair cycle regulation, recessive black pigmentation and fleece rot. Manipulation of key windows of foetal development resulted in lifetime positive changes in wool production, an important proof of concept in functional, developmental genomics.

INTRODUCTION

The Australian wool industry operates in a highly-competitive, global, textile fibre market in which it currently captures a small and diminishing share of the consumer's expenditure on apparel clothing. To remain competitive in this market, the industry must address a number of pressing issues which are limiting productivity, profitability and consumer acceptance of the products. These include mulesing, dark fibre contamination, fleece rot, flystrike, anthelminthic resistance, relatively coarse fibres, weak fibres, prickle in garments, poor easy-care attributes and high price relative to competitors. At the time of inception of the sheepGENOMICS program (2004), molecular genetics applied to animal breeding was in its infancy, with great expectations attached to the discovery of quantitative trait loci (QTL) for difficult-to-measure traits. Since then, high throughput single nucleotide polymorphism (SNP) genotyping has paved the way for whole genome selection and more targeted SNP marker identification. Developments in bioinformatics, and in particular, networked pathway analyses, now allow more functionally-relevant interpretation of gene expression studies. The wool subprogram of the sheepGENOMICS initiative developed an integrated, functional genomics approach to dissect the molecular and cellular events involved in the critical periods of development of the follicle population in the skin of developing sheep foetuses. A suite of techniques for gene detection, gene expression, gene localisation, gene transfection, *in vitro* cell functional assays, gene network analysis and biochemical manipulations were targeted at key windows of skin development and at the long-standing problems of fleece rot and recessive black fibre pigmentation. These techniques and a summary of progress to date are the subject of this paper.

THE EXPERIMENTAL APPROACHES TAKEN IN sheepGENOMICS.

Every aspect of lifetime wool production (clean fleece weight, wool growth efficiency), quality (mean diameter, variance in diameter, length growth rate of fibre, crimp characteristics, staple

strength, style, colour, fleece rot incidence), and even aspects of ectoparasite attraction (suint and wax contents), are established during the critical windows of development of skin and its appendages. The wool subprogram of SheepGENOMICS therefore concentrated on cellular and molecular events occurring during skin development, and particularly during critical windows of follicle initiation. The foetal development of wool follicles and their associated glands (sweat and sebaceous) is called neogenesis. The morphological changes that take place throughout the post-natal hair cycle are thought to be a recapitulation of these neogenic events (indicated in Fig 1 on the right side of the 'figure-of-six' diagram of Stenn and Paus, 2001).

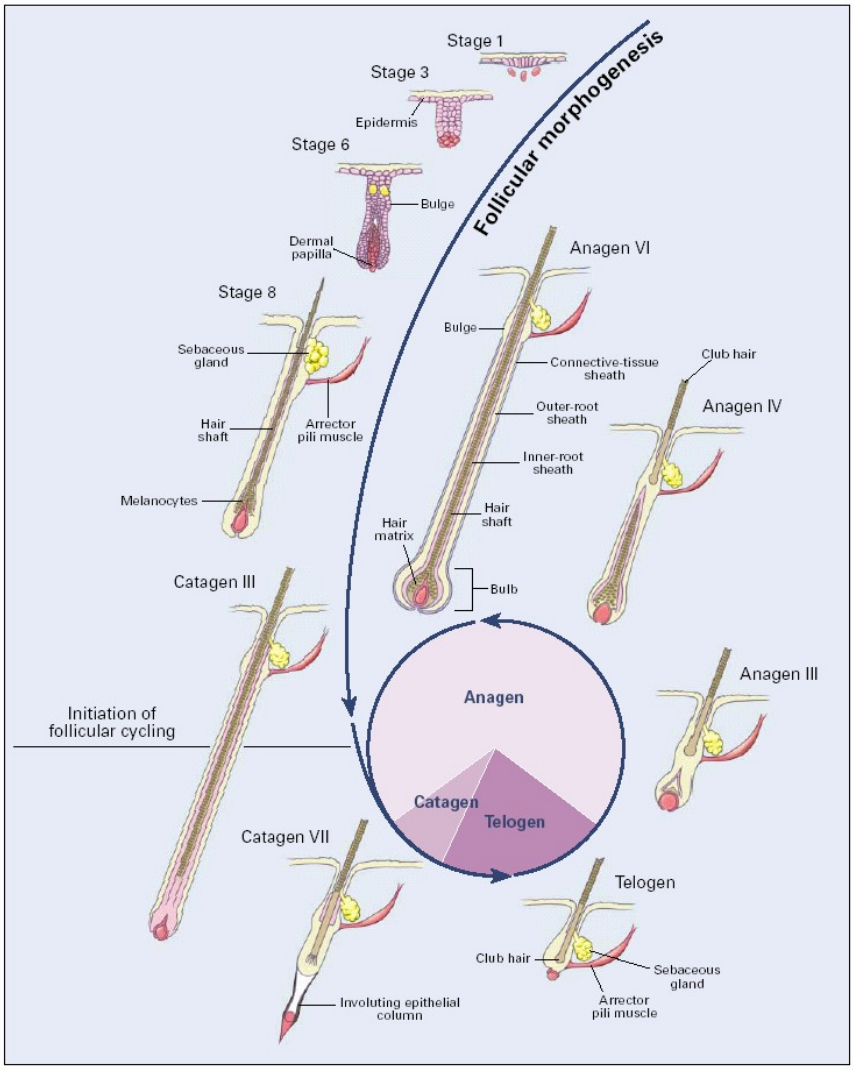

Figure 1. Targets of investigation of gene expression in the Wool subprogram. The left side of this figure-of-six' diagram represents the neogenesis of hair follicles and associated structures. The right side represents the on-going morphogenic events during the hair cycle in established follicles (after Stenn and Paus 2001).

In other words, the signalling events involved in the re-establishment of the follicle after the resting phase of the cycle (telogen) were hypothesised to be the same as those operating in neogenesis.

The strategy taken in the Wool subprogram of the SheepGENOMICS program was deliberately multi-pronged (from gene detection, gene sequencing, gene function and pathway modulations), because at that time, there were few examples of successful application of molecular genetics to applied livestock production. We took several approaches to identifying the key, and possibly unique, gene networks operating in follicle neogenesis and post-natal cycling: (1) expression studies based on candidate genes known to be involved in neogenesis in other epithelial tissues; (2) identification of genes and gene networks operating at critical phases of neogenesis and morphogenesis, using expression micorarrays; (3) targeting of pathways known to be involved in tissue neogenesis by direct biochemical/endocrinological intervention during critical periods of skin and appendage formation; and (4) identification of gene function by transfection of keratinocytes and assessment of their behaviour using *in vitro* assays. The candidate gene approach was also taken to identify the hitherto intractable problem of the agouti locus (see below), while the microarray global screen approach was taken to identify potential markers for fleece rot incidence. Our philosophy was that this multi-pronged candidate gene, targeted manipulation, and gene discovery approach would be a powerful model for dissecting functional genomics in its broadest sense. The results obtained using this approach are outlined below.

IDENTIFICATION OF TARGET GENES AND PATHWAYS IN FOLLICLE INITIATION

The major resource developed in SGP for the study of gene expression during foetal and early postpartum skin development was a tissue library of skin samples derived from lambs from artificially-inseminated, synchronised ewes. Samples were collected from cohorts of 3 foetalfoetal lambs sacrificed at 2-4-day intervals from days 35 to 143 post conception and from 8 lambs at 4-7 day intervals from days 2 to 100 postpartum. This resource, created by researchers from the South Australian Research and Development Institute and The University of Adelaide (CS Bawden, G. Nattrass, C.J. McLaughlan, H. McGrice, S.M. Dunn), allowed detailed identification of the histological stage of development of the skin and its appendages, localisation of mRNA expression (by *in situ* hybridisation) and quantification of mRNA expression by quantitative PCR.

A candidate gene approach was taken to characterise the expression profiles of approximately 100 genes, identified from literature related to the ontogeny of other organs and tissues which rely on similar ectodermal/mesenchymal interactions as skin. The genes included those involved in early epidermal/dermal signalling (eg members of the Wnt/βcatenin, FGF, EDA, BMP, Notch/Delta and Shh signalling pathways); mid- to late-signals in organ development (eg VEGF, VE-cadherin, Ephrin, NGF); structural proteins in the follicle, fibre, skin (eg trichohyalin, keratin and keratin-associated proteins); cell signalling molecules (eg β-Activin, Follistatin, SCF, HGF); transcription factors (eg Lef-1, Gli1, Prox1, HIF1); proteins involved in tissue remodelling (eg E-cadherin, matrix metalloproteinases); proteins involved in organ branching events (eg FGF10, BMP4, Sprouty, Netrin); and those involved in the formation and function of accessory glands (eg matrix metalloproteinases, cytokeratin 7 (CK7) and 1,5-alpha reductase (SRD5A1), Androgen receptor (AR) and Carcinoembryonic antigen cell adhesion molecule 1 / Biliary glycoprotein (CEACAM-1 / BGP)."

For each of these genes, spatiotemporal expression profiles were painstakingly quantified by qPCR and *in situ* hybridisation throughout the developmental time series. Expression profiles such as that depicted in Figure 2 were thereby obtained, allowing coincidence of changes in gene expression to developmental event to be determined. In this example the expression of the developmental protein, sonic hedgehog, begins expression at day 57 the time of primary follicle

initiation. Gene expression then peaks at day 83 when the large secondary population is initiated, with a secondary peak at day 101 when the secondary derived population is initiated.

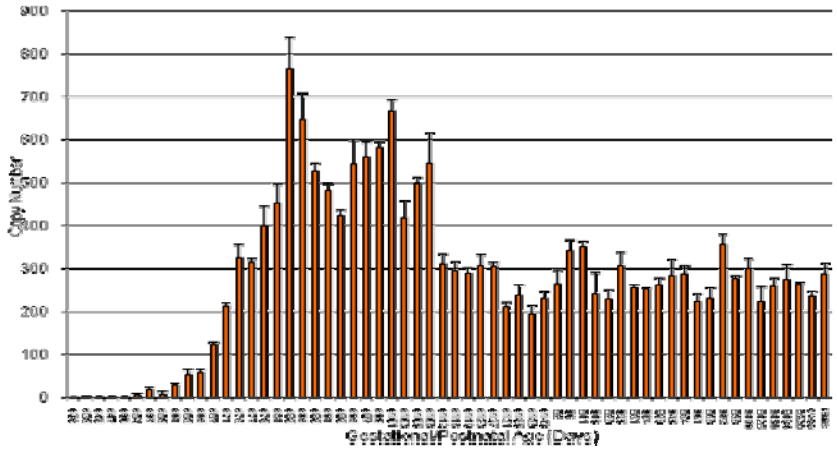

Figure 2 : qPCR assay data showing prevalence of transcripts encoding sonic hedgehog transcripts in midside skin during ovine development. Mean transcript prevalence for each developmental time point is plotted +/- the standard deviation (McLaughlan, Bawden, Nattrass, Dunn and McGrice unpubl.)

The challenge now facing the researchers is to coordinate this vast array of spatial and temporal information into an integrated pathway analysis which will focus targeted manipulations or identification of genes within which we might search for SNP mutations.. Ingenuity Pathway Analysis® is one means of integrating the data into a consolidated output, and is currently being employed to identify the major pathways related to developmental events.

One such pathway that appears to play a major role in tissue development in general and skin and appendage development in particular is the Delta/Notch signalling pathway. Philip Moore, Peter Wynn, Claire Gordon-Thompson and Stephanie Xavier found members of the Notch signalling pathway are indeed present in the cells that form the dermal condensate (the precursor to the dermal papilla of the follicle). Differences in the level of expression of Notch-1 and Delta-1 proteins were apparent between days 56 and 70 post-conception, and the ratio of Delta to Notch in dermal condensate cells was greater in Merino foetuses than Tukidale foetuses. A targeted biochemical approach was taken in a series of experiments initiated to determine the viability of manipulating the Delta/Notch pathway using an inhibitor of γ-secretase, DAPT ((N-[N-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester). The results of an *in vitro* trial of DAPT on cell aggregation in dermal papilla cells indicated that the inhibition of the Delta/Notch pathway resulted in complete inhibition of cellular aggregation (Figure 3).

It is well-established that properties of the dermal tissue exert a strong effect on the final structure of developing follicles *in vivo*. Thus the size of the dermal condensate and papilla seem to determine the size of the mature follicle and the diameter of the fibre it produces. The aggregation of papilla cells *in vitro* is a model for this morphogenetic process. The work of Nick Rufaut (AgResearch NZ), described below, encompasses the development of an *in vitro* bioassay which uses papilla cell aggregation to evaluating the impact of potential therapeutics on the specification of follicle size. Philip Moore's work suggests that the Delta/Notch signalling

pathway regulates the formation of dermal cell condensates. It is yet to be demonstrated that targeted manipulations of this pathway can result in increased follicle density and decreased fibre diameter *in vivo*, the desired outcome. Nevertheless, manipulation of this pathway has potential for *in utero* therapeutic manipulation of follicle formation and lifetime fibre growth. Moreover identification of SNPs in the Delta/Notch pathways may provide novel markers of wool growth.

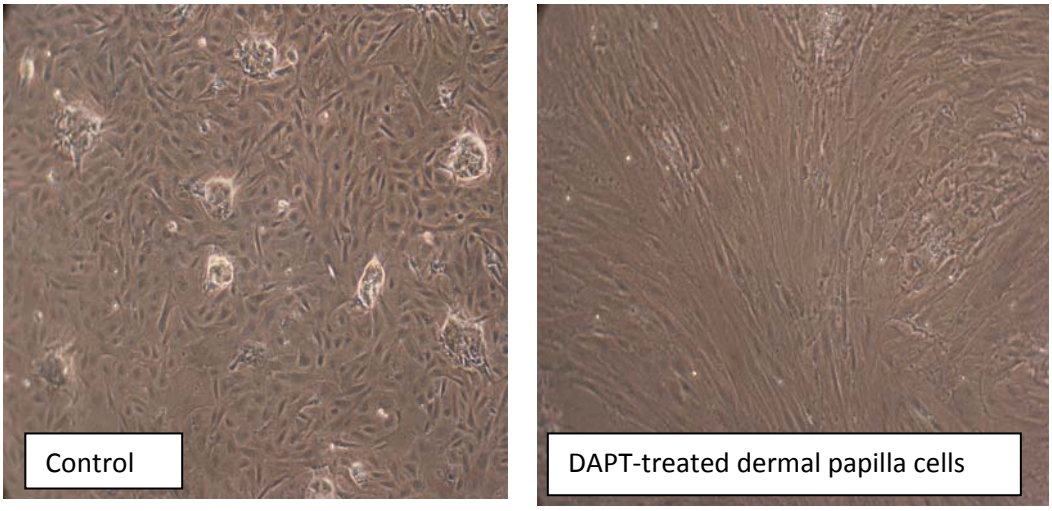

Figure 3. Blockage of Notch signalling by DAPT inhibits dermal papilla cell aggregation

DEVELOPMENT OF RAPID SCREENING METHODS FOR ASSESSING THE EFFECTS OF TREATMENTS ON SKIN DEVELOPMENT

As illustrated by the Delta/Notch work, it was clear from the outset of the sheepGENOMICS program that the ambitious goal of identifying gene pathways and therapeutic molecules with potential for beneficial manipulation of follicle and skin appendage formation would require test-bed systems which would allow screening of numerous candidates. We also needed a skin cell transfection system which would allow evaluation of the impact of gene changes on cell behaviour. Several models were developed in the wool subprogram to address these issues.

CELL CULTURE METHODS FOR SCREENING THERAPEUTICS WITH EFFECTS ON FOLLICLE INITIATION AND FIBRE GROWTH

Three cell culture methods were developed as screening tools: (a) a keratinocyte proliferation assay (Rufaut *et al.*, 2007); (b) a keratinocyte apoptosis assay (Rufaut *et al.*, 2007); and (c) the dermal papilla cell aggregation assay (Goldthorpe *et al.*, 2008). The latter relies on the unusual behaviour of papilla cells, which, on reaching confluence, begin 'clumping' in 3-dimensional aggregates spaced approximately equidistant from one another. As noted above, this behaviour is thought to recapitulate aspects of morphogenesis *in vivo*. Culture conditions were optimised to facilitate robust aggregation, and image analysis methods were developed for quantification of aggregate size and number. The value of this assay was evaluated by imposing a number of treatments which alter signalling pathways known to be important in morphogenesis *in vivo*, including the BMP, FGF and Wnt pathways. For example, lithium chloride is an inhibitor of GSK3B which mediates β-catenin degradation in the Wnt signalling pathway. Treatment with lithium diminished the size of DP cell aggregates in a dose-dependent fashion (Figure 4). This supports the gene expression results which indicated an important role for the Wnt signalling

pathway in follicle formation and implies a role for Wnt signalling in specifying follicle size and fibre diameter. Similarly, inhibition of BMP or FGF signalling by small molecules that target the receptors reduced aggregate size (Fig 5). Co-treatment with these inhibitors plus lithium chloride produced an additive effect.

Stratifin, a molecule involved in multiple cell signalling events, was identified in the microarray studies of hair cycle regulation. Apoptosis was induced in keratinocytes when stratifin expression was suppressed by RNAi, mediated by transfection with short interfering RNAs (Fig 6),. Stratifin and related molecules are potential candidates in wool growth regulation and should be the subject of further research in relation to regulation of wool growth (eg bioharvesting molecules).

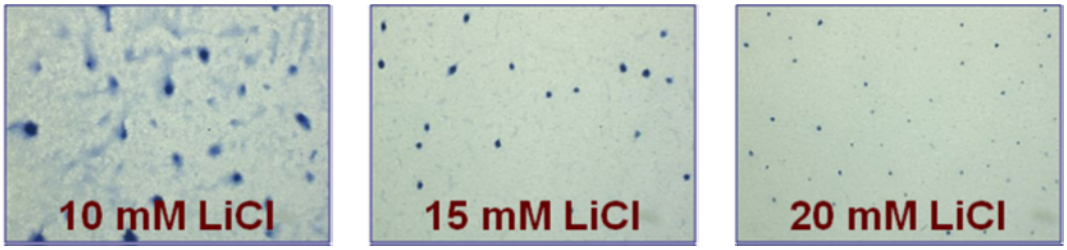

Figure 4. Lithium chloride, an agonist of the Wnt signalling pathway, affects dermal cell aggregate size in a dose-dependent manner. The aggregates of dermal papilla cells appear as dark blue clumps.

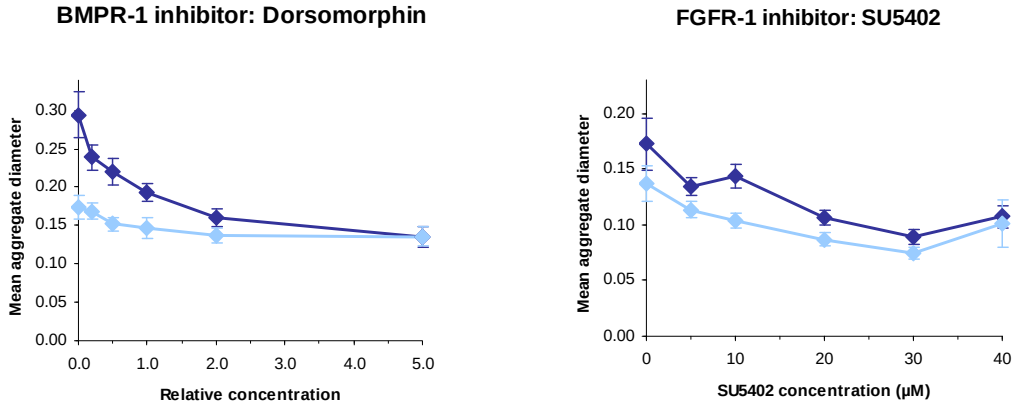

Figure 5. Inhibition of BMP and FGF receptors reduces aggregate size. Ovine dermal papilla cells were cultured in varying concentrations of dorsomorphin, an inhibitor of BMPR-1, and SU5402, an inhibitor of FGFR-1 (dark blue lines). Cells were also co-treated with the inhibitors and 10 mM lithium chloride (pale blue lines). Error bars show SEM for aggregate diameter. The cell culture system shows quantitative responses to changes in the signalling of developmental regulators that are likely to determine wool fibre diameter.

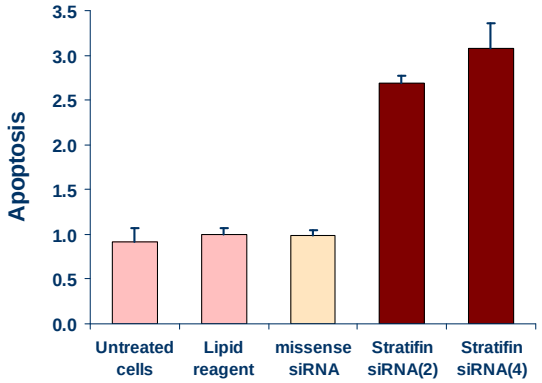

Figure 6. Treatment of ovine follicle keratinocytes with siRNAs targeting stratifin (columns 4-5) enhance apoptosis compared with control treatments (columns 1-3).

IN VITRO CULTURE OF EMBRYONIC MOUSE SKIN AS A TEST-BED FOR THERAPEUTIC MOLECULES TO ALTER WOOL FOLLICLE FORMATION

A mouse embryonic skin culture model was developed which allowed rapid screening of candidate molecules in a defined and quantifiable system. The advantages over the pregnant sheep model (see below) are that small quantities of therapeutic molecules can be tested, the gestation period is 20 days cf 147 days in sheep, the litter sizes are large (6-12), the strains of mice are inbred and therefore genetically-uniform, and the pattern of hair follicle formation is not unlike that of the developing foetal lamb. The method developed in the sheepGENOMICS program was based on that of Kashiwagi *et al.* (1997) with some modifications that allowed complete follicle formation to the point of hair growth. This model was used to determine the effects of manipulating the TGFβ superfamily signalling pathway. Two key components of that pathway are the activins and follistatins (see review by McDowall *et al.* 2008). Addition of exogenous follistatin stimulated follicle initiation *in vitro* by as much as 13%, while activinA induced a 20% reduction in follicle initiation. The culture of murine embryonic skin appears to be a responsive and robust system for testing the effects of signalling molecules on developmental events.

MANIPULATION OF FOETAL SKIN DEVELOPMENT USING A PREGNANT MOUSE MODEL

This model has many of the advantages of the murine embryonic skin culture system described above, but with the added advantage that the foetuses can be maintained into postnatal life to ascertain the long-term effects of treatment. Large litter sizes again provide a powerful experimental model. We used this model to investigate the effects of manipulation of the pentose phosphate pathway and retinoic acid signalling pathways. Manipulation of these pathways significantly influenced follicle initiation but a somewhat surprising result was that the vehicle control solution (which contained glucose), also significantly influenced follicle initiation. Further studies have validated this finding. The recent discovery that glucose availability can affect histone acetylation (Wellen *et al.* 2009), provides a potential mechanism whereby glucose could influence epigenetic events in developing foetal skin.

MANIPULATION OF FOETAL DEVELOPMENT IN PREGNANT SHEEP

Two methods of manipulating the development of foetal sheep skin *in vivo* were developed in the wool sheepGENOMICS program,: (1) by provision of therapeutics to the pregnant ewe (effective for manipulation of early stages of cutaneous development- days 0-50 pc). In this first

trimester most molecules supplied to the mother are delivered to the foetus via the foetal blood supply. Beyond day 50 the placental barrier forms and exposure of the foetus to the substances depends on the nature of the substance and its mode of delivery across placental tissues; (2) by provision of therapeutics directly to the foetus via intra-amniotic injections guided by ultrasound. This approach was used for pregnancy beyond day 50 and for substances known to be regulated by placental carrier systems.

An initial experiment designed to influence the cortisol axis in pregnant ewes at days 55-65pc (the time of primary follicle initiation) resulted in marked changes in the birthcoat scores of the resulting lambs. Metyrapone, which reduces cortisol biosynthesis, produced lambs which were hairier at birth ($P<0.003$) than control lambs, and those treated with betamethasone (Fig 7). The metyrapone lambs produced wool staples that were 10% longer than controls throughout life (up to 3 annual adult shearings) with no differences in staple strength or fibre diameter.

This is a major novel finding and one that has implications for all livestock species. It is the first 'proof of concept' that brief 'windows' of development exist in which therapeutic manipulations can induce lifelong changes in production traits. Further work has been conducted to identify the major gene network changes associated with this manipulation. Microarray data from the metyrapone experiment were analysed within a systems biology framework using Weighted Gene Co-expression Network Analysis (WGCNA). Four networks were created to determine those genes involved in metyrapone-mediated improvement of wool parameters. Using the WGCNA approach, we were able to detect co-expressed gene modules associated with metyrapone treatment. Gene ontology enrichment analysis of the genes comprising these modules identified networks associated with tube branching morphogenesis and with the BMP signalling pathway (Watson-Haigh, Kadarmideen, G. Nattrass, M. McDowall, N.M. Edwards and P.I. Hynd, unpubl. data). The known involvement of the BMPs and other members of the TGFβ-superfamily in skin development supports the notion that microarray analysis using this approach is a robust method. Interestingly the BMPs also featured strongly in the spatio-temporal expression studies referred to above. Furthermore, increased BMP signalling in association with a hairier birthcoat is consistent with the miniaturisation of papilla cell aggregates induced by BMP inhibition *in vitro*..

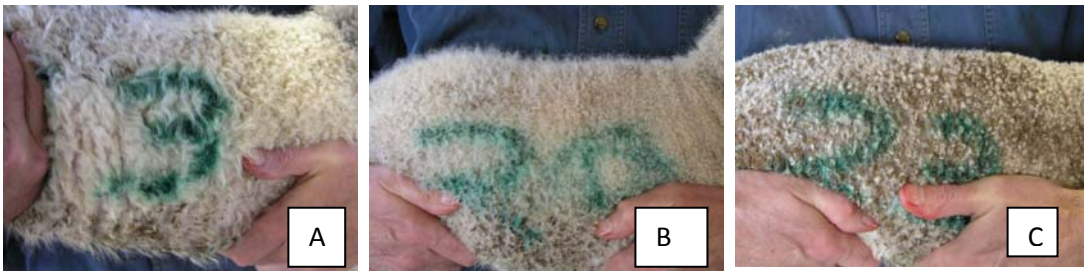

Figure 7. Lambs born after a 10-day treatment (days 55-65) with metyrapone (A), Vehicle (B) and betamethasone (C) differed in birthcoat score (BCS) and lifetime wool production. Lambs shown represent the mean BCS of each treatment (Mean± sem: A= 5.3±1.0; B= 3.7±1.3; C=2.6±1.9)

INVESTIGATIONS OF RECESSIVE BLACK (AGOUTI)

The white coat phenotype of domestic sheep breeds shows an autosomal dominant inheritance and has reached a high frequency in certain breeds as a result of selection for white fibres in an attempt to eliminate coloured sheep and coloured fibres. Self-colour black and badgerface are recessive pigmentation phenotypes of domestic sheep breeds caused by alleles at the agouti locus.

The dominant white or tan (A^{Wt}) agouti allele is responsible for the white wool phenotype in modern sheep breeds while the most recessive allele, non-agouti (A^a), results in black/brown wool (self-colour black). Another agouti allele, badgerface (A^b) is characterised by a pale dorsal, and darker ventral pattern; it is recessive to A^{Wt} and dominant to A^a. This project, lead by Dr Belinda Norris and colleagues at CSIRO Livestock Industries, has unravelled the mystery of the agouti locus, which has eluded researchers to date (Norris and Whan 2008). The principal findings are that the sheep dominant white allele (A^{Wt}) is characterised as having one or more extra copies of the gene at the agouti locus; that each point at which an additional agouti gene is inserted in the A^{Wt} allele (junction point) can be identified by a unique genomic sequence that spans the Junction Point; and finally that the recessive black alleles (A^a and A^b) each contain a single agouti gene with a dysfunctional promoter. The project team has developed an assay for counting Junction Points. However, the Junction Point assay is diagnostic of Carriers (ie heterozygotes A^{Wt}/A^a and A^{Wt}/A^b) only where the A^{Wt} allele contains a single junction point. Presently, for example, in our assays that detect two Junction Points, we cannot distinguish between a homozygous white animal in which each A^{Wt} allele has one junction point ($A^{Wt.1}/A^{Wt.1}$) and a Carrier with an allele with two Junction Points ($A^a/A^{Wt.2}$). Also for animals with higher numbers of junction points, there is a level of uncertainty in the junction point counts. Fortunately, we estimate that ~55% of Merino Carriers have only one junction point. We estimate that approximately 33.5% of Merino Carriers have 2 junction points (a triplicated agouti allele) and 11.5 % 3 junction points (a quadruplicated agouti allele) (Figure 1). These multiple junction point carriers cannot be classified as such by the junction point assay alone. Unfortunately a proposal to test for markers (SNP) adjacent to the duplicated region 3' breakpoints of recessive black and dominant white alleles to identify particular profiles (haplotypes) characteristic of different copy number carrier animals, failed to find an association between haplotype and agouti gene copy number or coat colour phenotype. Nevertheless the test has been developed to include a front-end, user friendly interface, which allows results to be readily interpreted based on parallel consideration of pedigree information.

GENE EXPRESSION THROUGHOUT THE HAIR GROWTH CYCLE

Drs Allan Nixon, Allan Pearson, Zhidong Yu and Nick Rufaut at AgResearch in New Zealand developed a wool shedding model based on the photoperiod-induced wool loss of Wiltshire Horn sheep (Nixon *et al.*, 2002). Gene expression profiling using an ovine cDNA microarray covering foetal initiation and postnatal hair cycling phases, revealed hundreds of differentially-expressed genes (Yu *et al.*, 2007). As hypothesised, many pathways are shared between follicle initiation in the foetus and hair cycling in the adult. Using Ingenuity Pathway Analysis® the differentially-expressed genes have been ordered into known gene networks. The opportunity now exists to identify inhibitors or stimulators of these pathways to control both foetal follicle formation and adult fleece growth.

IDENTIFICATION OF FUNCTIONAL GENES IN MUTANT SHEEP

Naturally-occurring or induced mutations, particularly in mice, have proven to be a valuable resource for studies of human disease. We reasoned that given the size of the Australian merino sheep population and a typical rate of spontaneous mutation, there should be a large number of phenotypes which would be valuable in dissecting functional genes involved in skin and follicle development. The media (including international outlets) were particularly interested in this project and it received widespread publicity, resulting in 31 unusual phenotypes being identified. These included a cohort of felting lustre mutants, sheep with very low follicle density, sheep with periodic shedding of fibres across the body, sheep with excessive skin wrinkle, sheep born with no wool follicles (so-called 'bald at birth'), sheep with unusual bare patches, and the previously described hypotrichosis phenotype. DNA, wool and skin samples have been taken from these

animals for analysis and cell lines or germplasm stored to enable rederivation of the phenotypes. To date, examination of gene expression and follicle and fibre structure in the felting lustre mutants has identified differences informative in defining the origin of fibre curvature and crimp (Li *et al.*., 2009).

GENES ASSOCIATED WITH RESISTANCE AND SUSCEPTIBILITY TO FLEECE ROT

During 2003-2004 genetically diverse resource flocks were measured for fleece rot resistance in artificial rain wetting shed trials at DPI NSW (Trangie) and CSIRO (Armidale). This provided fleece rot scores, blood and skin samples from two different genetic backgrounds (Merino and Merino x Romney) and genetic extremes (20 resistant and 20 susceptible animals from each genetic background). Samples of skin were collected to construct subtracted cDNA libraries and investigate gene expression patterns during the development of and recovery from fleece rot. Using skin samples from two resistant and two susceptible animals, six subtracted normalised cDNA libraries were made. From each library ~960 anonymous ESTs were printed together with CSIRO bovine and ovine skin ESTs in a 24K cDNA skin array. A microarray experiment of 31 array hybridisations was conducted. Analysis resulted in 297 differentially expressed (DE) array elements, the majority (72%) of which were anonymous ESTs from the six fleece rot subtracted cDNA libraries. SNPs within candidate genes amongst the group of differentially-expressed genes are currently being sought.

CONCLUSIONS

The Wool subprogram of the sheepGENOMICS program used a combination of molecular, biochemical and cellular approaches to dissect key spatio-temporal events in skin development. Identification of important genes, gene pathways and potential targets for therapeutic manipulation was attempted using cDNA micorarrays, qPCR of candidate genes, transfection of cells with genes of interest and assessment of their subsequent function, and 'best-guess' biochemical interventions. This approach has successfully identified potential targets for therapeutic intervention in skin development and has demonstrated that such intervention over brief 'windows' of foetal development can alter lifetime productivity.

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