final report

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SheepGenomics Wool Subprogram

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Executive summary

The wool Subprogram brought together a group of internationally-recognised wool and skin biologists from The University of Adelaide, AgResearch New Zealand, University of Western Sydney, CSIRO Livestock Industries, and the South Australia Research and Development Institute. The Subprogram focussed on 5 major activities:

1. gene expression during critical periods of development of the primary, secondary original and secondary derived follicles population in the skin during fetal life
2. gene expression at critical periods of change in follicle activity throughout the hair growth cycle
3. therapeutic manipulation of targeted pathways during critical periods of fetal skin development
4. identification of gene markers for difficult-to-measure and novel wool traits
5. identification and sequencing of genes involved in resistance to fleece rot, and production of black wool associated with the agouti locus

Outcomes summary

1. first proof of concept that lifetime wool production can be altered by brief, targeted periods of treatment of the pregnant ewe

2. identified the genetic basis of the recessive black trait

3. identified gene networks involved in wool follicle initiation and branching

4. identified gene networks involved in hair cycle regulation

5. developed an in vitro rapid assay for assessing the impact of therapeutic agents on wool growth

6. developed a suite of wool traits for association mapping in the gene mapping flock

7. in partnership with wool producers across Australia, identified a range of sheep with unusual fleece and skin characteristics, which may assist us to identify mechanisms controlling wool growth

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1. Background

1.1 Industry context at the outset of SheepGENOMICS

The Australian wool industry operates in a global textile fibre market in competition with synthetic fibres (polyester, acrylic, microfibre, nylon, polyamide), and other natural fibres (cotton, silk, cashmere, alpaca, mohair). Wool occupies a small niche (4% of total apparel fibres) in the market but is at the high-quality and high-value end of the chain, reflecting the unique performance of the fibre as a textile in terms of drape, breathability, softness, and elasticity. Consumers demand fabrics that are casual, comfortable, easy-care, versatile, value-for-money, and 'branded'. While wool meets many of these demands, it is relatively expensive (approx. 3-4 times the price of competitors), is considered old-fashioned, prickly, heavy, winter-only wear, not easy-care (shrinks, pills, loses shape) and is often contaminated by dark fibres. The fibres are often weak, resulting in reduced fibre length in the top leading to increased yarn thickness after spinning, and increased fabric weight at garment manufacture. From an on-farm viewpoint the sheep wool industry is under increasing pressure from animal welfare activists particularly in relation to mulesing and to blowfly strike. A major predisposing cause of body blowflystrike is fleece rot, a condition in which fleece architecture and the immune responsiveness of the sheep, contribute to a bloom of bacteria on the skin (largely *Pseudomonas aeruginosa*) under conditions of high rainfall. The solution to this problem and associated fly strike, is to identify animals resistant to the development of fleece rot. Obviously genetic selection for fleece rot resistance depends on the climatic conditions predisposing animals to the condition being present. Such a situation lends itself to the use of DNA markers. Similarly the occurrence of black sheep is dependent on the presence of carrier animals in the flock at the time that an unidentified carrier ram is used. Gene methods for identifying carrier animals would eliminate this problem.

1.2 Wool science research and development capability at the outset of the SheepGENOMICS program

The Australian wool industry through its variously-named R&D funding bodies (Wool Trust Fund, AWRAPO, IWS, AWI) had a history of funding strategic, applied and commercial research and of supporting postgraduate students in wool research in the 1970's, and 1980's. However in the 1990's funding of postgraduate students was discontinued on the grounds that few of the students ended up in the wool industry (the fact that the wool industry benefitted in attracting scientists trained by other industries was ignored). This sent a very loud and clear signal to bright young students that the wool industry saw no future for them in research and development. At the same time the University of NSW closed its School of Wool and Pastoral Sciences, which had a history of training wool scientists. At the same time the CSIRO closed its wool research laboratories at Prospect NSW. Together these events culminated in an exodus of scientists from wool research to the point that by the year 2000 the only 'wool research' groups left in the country were at Geelong (CSIRO Textiles), a small wool biology group at The University of Adelaide (Prof Phil Hynd's group), and a scattering of scientists with on-farm wool interests in various state departments of agriculture. There was a complete absence of young scientists trained in aspects of wool chemistry, processing, biology and production. At the commencement of sheepgenomics there were effectively 2 wool biology researchers; this was built to a team of 10 senior scientists, 3 postdoctoral fellows, 6 support staff and 3 postgraduate students. Following the decision not to continue funding sheepgenomics there are now 2 dedicated wool scientists considering their futures. Having built a significant and capable team of dedicated wool scientists we now have lost that capability. It is unlikely that it will ever be rebuilt.

2. Planned outputs from the Wool Subprogram

2.1 Scientific outputs

- Development of markers for difficult-to-measure traits (staple strength, pigmented fibres, shrinkproof wool, fleece rot resistance)
- Development of therapeutic approaches to manipulate critical 'windows' of fetal development with the aim of altering the lifetime fibre characteristics (fibre diameter, fibre length growth, fibre crimp, clean fleece weight)
- In the course of the program it became apparent that the mulesing phase-out had major implications for sheep breeding so added emphasis was placed on easy-care characteristics such as the barebreech trait.

2.2 Capability building for the wool sheep industry

As indicated above the R&D capability to achieve these scientific outcomes and to deliver them to industry did not exist at the outset of Sheepgenomics. The first task was to:

- develop a team of researchers with the skills in molecular biology, understanding of gene function, fetal physiology, cell biology, and follicle science
- develop an integrated program with a pipeline of activities from gene discovery, gene function, gene localisation, gene network analysis, and biochemical interference, using in vitro and in vivo models
- attract and train young scientists into wool biology research as a capability for the future.

3. Structure and focus of the Wool Subprogram

The subprogram was focussed on:

(a) identifying markers for key traits dictating the profitability of wool enterprises;

(b) identifying key genes and their functional pathways involved in the 'initiation' of wool follicles and their appendages in the fetal sheep;

(c) identifying the 'start' and 'stop' switches operating in the hair growth cycle and

(d) identifying therapeutic means of manipulating the 'initiation', 'start' and 'stop' signalling.

These activities are summarised in Figures 1 and 2.

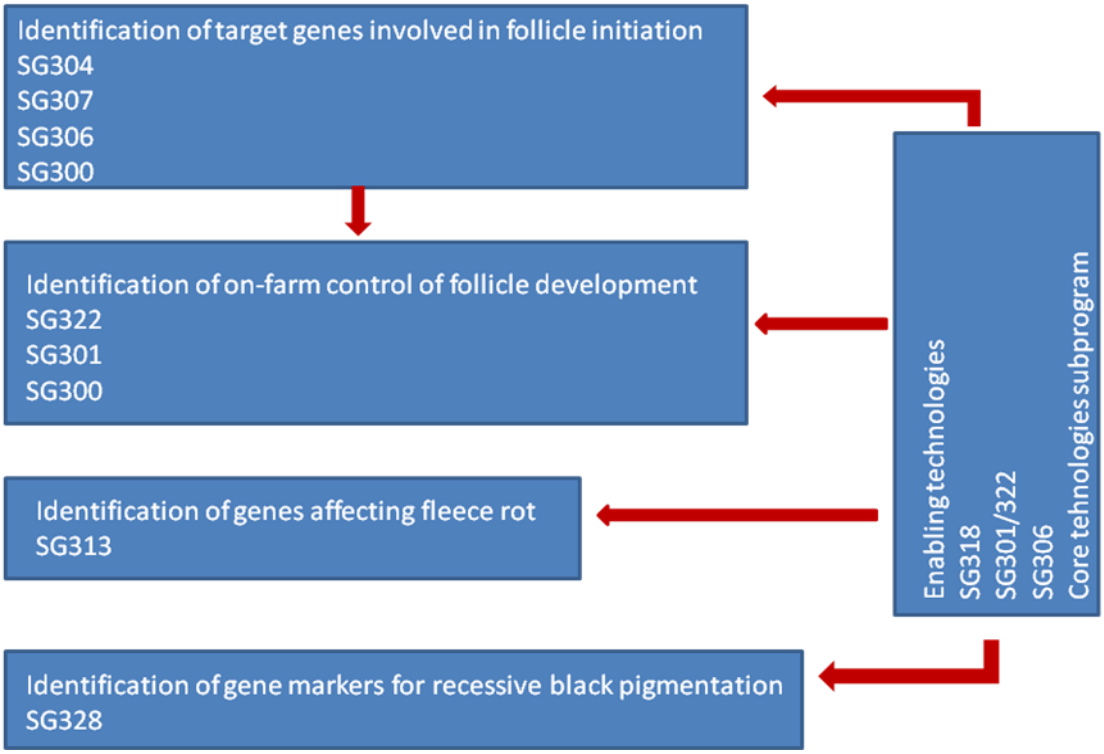

Figure 1. Wool Subprogram project map

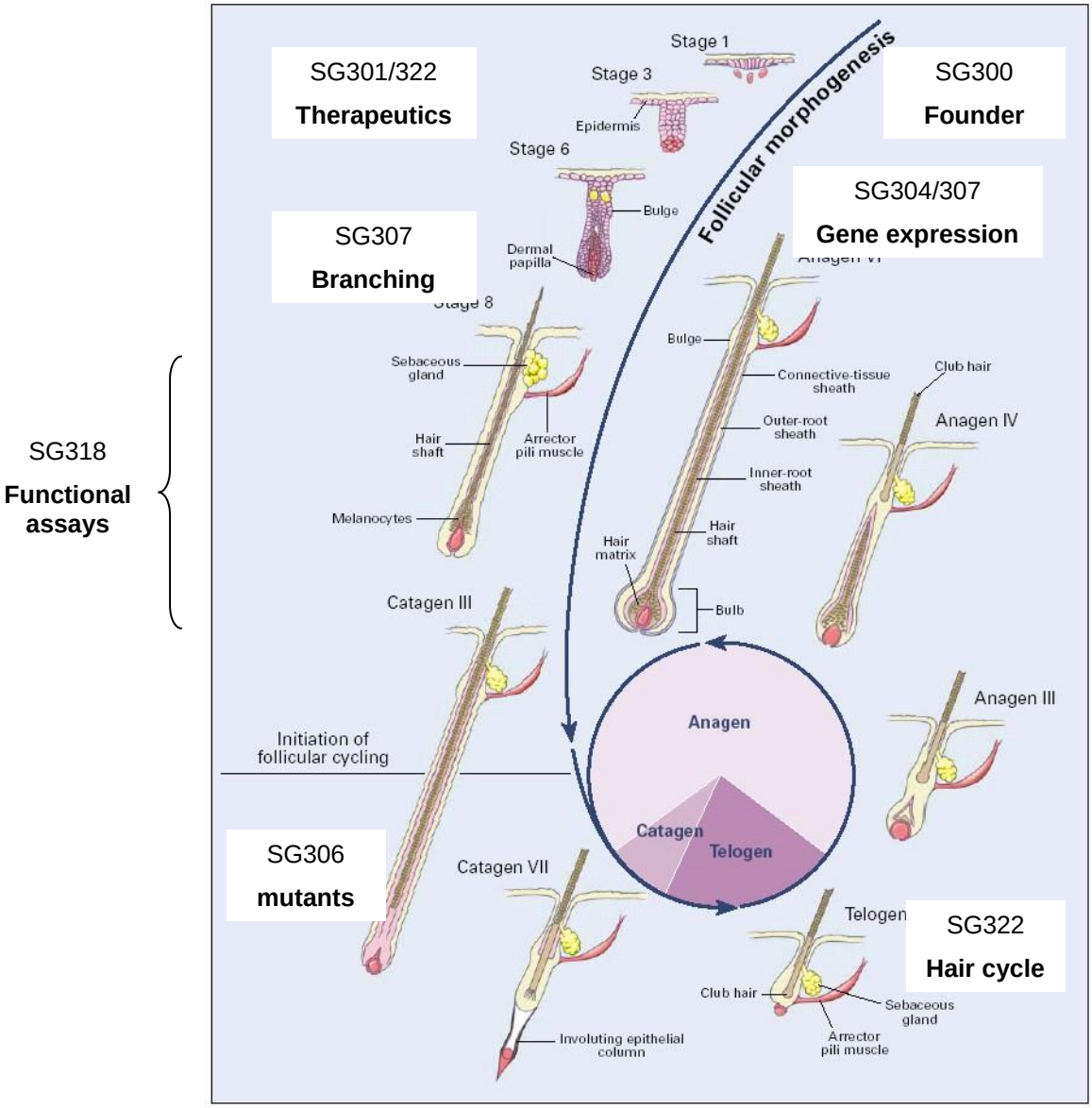

Figure 2. Targets of investigation of gene expression in the Wool Subprogram. Genes involved in follicle 'neogenesis' (formation) during fetal life, and then in follicle morphogenesis in cycling sheep, were studied as a precursor to identification of pathways with potential for therapeutic manipulation. The 'start' signals in the hair cycle were considered relevant to those involved in neogenesis, and the 'stop' signals in the hair cycle were considered relevant to applications such as shearing and mulesing alternatives.

It was important at the outset of the wool genomics program that a system of gene discovery, analysis of gene expression (quantitative, spatial, temporal), and assessment of gene function in *in vitro* and *in vivo* test beds, be established. To do so required the identification of leading researchers with the skills and tools available to rapidly integrate functional genomics activities in a coordinated and collaborative manner. This was achieved as follows:

- a team of Australian and New Zealand researchers with high credibility in wool and hair genomics research was established

- a tissue bank of ovine fetal skin samples was established at frequent and regular intervals throughout pregnancy, to allow gene expression at critical phases of development to be quantified
- Gene expression systems were established including robust methods for quantification of candidate genes likely to be involved in fetal skin development, and the inclusion of a 20,000 EST microarray based on ovine sequences derived from skin at various stages of the hair cycle.
- A cell culture-based gene transfection system was established using a Lentiviral transfection system and subsequent cell function assays based on *in vitro* cell behaviour (motility and aggregation) and *in vivo* performance in tissue grafts.
- A national search program for sheep exhibiting extreme wool and skin phenotypes was instigated.

4. The participants in the Wool Subprogram

At various (and not always contiguous) times, the following research organisations and personnel were involved in the subprogram.

Research Organisation	**Researcher**	**Technical staff**	**Postdoctoral Fellows**	**PhD students**
SARDI	Dr Simon Bawden Dr Greg Nattrass	Mr Clive McLaughlin	Dr Stephanie Dunn	
University of Adelaide	Prof Phil Hynd,	Ms Rachel Collett Ms Nerida Sweet Ms Tamara Smith Ms Natasha Penno	Dr Melanie McDowall	Ms Hayley McGrice
AgResearch NZ	Dr Allan Nixon Dr Nick Rufaut Dr Allan Pearson			
University of Western Sydney	Assoc Prof Philip Moore		Dr Claire Gordon-Thompson	Ms Stephanie Xavier
CSIRO LI	Dr Aaron Ingham Dr Belinda Norris Dr Graham Cam	Ms Vicki Whan		

5. Research activities and outcomes from the Wool subprogram

5.1 Identification of target genes and pathways involved in follicle initiation

(Projects addressing this goal: SG300, SG304, SG307)

The major resource developed in SGP for the study of gene expression during fetal skin development was a tissue library of skin samples derived from fetal lambs from AI'd, synchronised ewes. Cohorts of 3 fetal lambs were slaughtered at 3-day intervals from days 35 post conception to day 100 postpartum. This resource, developed by SARDI researchers, 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 qPCR. A similar series was developed at CSIRO Livestock Industries but with greater emphasis on the period of secondary follicle initiation and development.

100 candidate genes were selected for analysis of changes in their expression during fetal development based on their likely involvement in early epidermal/dermal signalling, mid-to-late signals involved in development, structural proteins in the follicle/fibre/skin, cell signalling molecules, transcription factors, proteins involved in tissue remodelling, proteins involved in branching events, and those involved in the formation and function of accessory glands. Transcripts of interest include Frizzled10, beta catenin, Wnt5a, Shh, BMPR1A, Notch1, FGF10, ectodysplasin, MMP7, Androgen receptor, and E-cadherin. All showed temporal changes in expression throughout fetal life, often consistent with potential roles in follicle initiation and development.

qPCR profiling of gene expression throughout the fetal series was conducted for a suite of molecules involved in epithelial/mesenchymal interactions. These included: PDGF and its receptor, HGF/SF and its receptor (c-met), Activin and follistatin, and E-cadherin. Molecules thought to play a role in development of the accessory glands of skin were also profiled; these included cytokeratin7, MMP7, 1,5 alphareductase, Ihh, androgen receptor, and CEACAM1. Again, all these molecules displayed expression changes consistent with functional roles in development of the follicles and their appendages.

The temporal expression of nuclear receptors for 2 ligands, namely thyroid hormone and cortisol coincided temporally with primary and secondary follicle initiation, and spatially with components of the dermis and epidermis that are involved in follicle formation. This result is of particular interest given the demonstrated effects of cortisol status on follicle initiation shown in Project 301 (above), and the effects of thyroid status on follicle maturation (Project 301 but data not shown). Further development of the activation/inactivation of the ligands for thyroid and cortisol hormones is warranted (see below).

A series of expression profiles was derived relating to the development of the neural, immune and circulatory support systems in developing skin (HIF-1, VEGFA, EphrinB1, VE-cadherin, Sox18, NGF, NTF3, NGRF3, N-cadherin, neuralin-1 and SCF). The changes in expression reflect expected changes in development of the support networks, but it is difficult to see how these might be used to manipulate follicle and accessory gland development.

To coordinate the vast array of spatial and temporal information arising from the expression studies, Ingenuity Pathway Analysis is being employed to identify the major pathways related to developmental events. IPA is a powerful tool for integrating gene expression data

into a consolidated output which might identify potential targets for manipulation or SNP discovery.

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. Project SG300 found that members of the Notch signalling pathway are indeed present in the cells that form the dermal aggregate (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 postconception, and the ratio of Delta to Notch in dermal condensate cells was greater in Merino foetuses than Tukidale foetuses. The role of cell aggregation in follicle formation is a common theme arising from the sheepgenomics program. The work of Nick Rufaut (AgResearch NZ), described later, highlights the importance of dermal cell aggregation in follicle formation *in vivo*, and describes an *in vitro* bioassay which uses dermal papilla cell aggregation as a screening method for evaluating the impact of potential therapeutics on follicle-forming capacity. If, as appears the case, the Delta/Notch signalling pathway regulates dermal cell condensate formation, 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.

A series of experiments was 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 inhibitor of the Delta/Notch pathway resulted in complete inhibition of cellular aggregation. For the first time a pathway has now been identified which appears to control fibre diameter directly by influencing follicle density. However it is yet to be demonstrated that targeted manipulations of the Notch/Delta signalling pathways can result in increased density and decreased fibre diameter, the desired outcome.

Publications

Yu Z, Gordon SW, Nixon AJ, Bawden CS, Rogers MA, Wildermoth JE, Maqbool NJ, Pearson AJ. (2009) Expression patterns of keratin intermediate filament and keratin associated protein genes in wool follicles. Differentiation 77:307-16.

Potential commercial outcomes

1. a gene marker test for follicle branching/density/diameter

By identifying polymorphisms (SNPs) within the genes encoding proteins active within the Notch/Delta pathway, it may be possible to develop a gene marker for fibre diameter. Such a marker may be usefully applied to the industry SNP-chip being developed.

2. a therapeutic to increase follicle density and reduce fibre diameter

Manipulation of the Notch signalling pathway or associated signalling pathways may result in increased dermal aggregation hence increased density and reduced diameter.

Work to realise commercial outcomes

1. marker development:

i. sequence through the Notch family of ligands/receptors in high density, low diameter Merinos and low density, high diameter Merinos, looking for polymorphisms

ii. apply the SNPs to the SNP chip

2. therapeutics

iii. identify candidate targets and inhibitors, in the Notch signalling pathways

iv. screen the effects of these compounds on dermal cell behaviour, using the dermal cell aggregation assay developed in Project SG318

v. test successful therapeutics in the embryonic mouse skin model, pregnant mouse model, and pregnant sheep model developed in SG301 (effects on lifelong wool production)

5.2 Development of functional assays for testing the impact of therapeutic molecules on follicle initiation and appendage formation

(Projects addressing this goal: SG 318, SG301/322)

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 a testbed system which would allow high-throughput screening of candidates. Several models were developed in the Wool Subprogram to address this issue:

5.2.1 In vitro assays for cell behaviour

Three methods were developed as screening tools for therapeutics; (a) keratinocyte proliferation and migration assay; (b) keratinocyte apoptosis assay and (c) a dermal papilla cell aggregation assay. The latter relies on the unusual behaviour of dermal cells, which, on reaching confluence, begin ‘clumping’ in 3-dimensional aggregates spaced approximately equidistant from one another. The robustness and value of these assays was evaluated by imposing a number of treatments likely to influence cell behaviour. These included stratifin, a growth regulator, PD173074, PDGFR inhibitor V, A Wnt agonist and activin A. LiCl an inhibitor of the Wnt signalling pathway, inhibited cell aggregation in a dose-dependent fashion. This supports the gene expression results which indicated an important role for the Wnt signalling pathway in follicle formation. Stratifin, a molecule involved in multiple cell signalling events proved to be important in the microarray results of SG322 (Hair cycle regulation), and in apoptosis in keratinocytes in vitro. 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).

5.2.2 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 allows 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 uniform genetically, and the pattern of hair follicle formation is not ulike that of the developing fetal 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.

5.2.3 Manipulation of fetal 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 provides a powerful experimental model.

5.2.4 Manipulation of fetal development pregnant sheep

Methods for manipulating the development of fetal sheep skin in vivo were developed in the wool sheepgenomics program, by two methods:

(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 fetus via the fetal blood supply. Beyond day 50 the placental barrier forms and exposure of the fetus 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 fetus 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.

These methods were successfully used to manipulate the cortisol, and thyroxine status of developing fetal sheep (see below).

Publications

1. McDowall, M., Penno, N.M., Jahoda, C.A.B. and Hynd, P.I. (2006). Optimising a fetal skin organ culture system to study pelage hair folliculogenesis in mice. **The 2nd Stradie Meeting on Skin and Hair Biology. 22-26 Sept 2006.**

2. The role of activins and follistatins in skin and hair follicle development and function.

McDowall M, Edwards NM, Jahoda CA, Hynd PI.

Cytokine Growth Factor Rev. 2008 Oct-Dec;19(5-6):415-26. Epub 2008 Oct 14. (Impact Factor 11.6)

3. ActivinA and Follistatin influence hair follicle development in mice. McDowall, M., McGrice, H., Penno, N.M., Nattrass, G., Hebart, M. and Hynd, P.I. (2007). Proc. 5th Int. Congress on Hair Research. Vancouver Canada.

Potential commercial outcomes

1. Dermal papilla aggregation assay

This assay has potential as a screening mechanism for therapeutics with potential as hair-modifying agents. In particular as treatments for androgenetic alopecia, alopecia areata, alopecia universalis, alopecia totalis, and hirsutism. The size and density of the papilla aggregations reflects the impact of the compounds on biochemical networks involved in dermal cell activity and behaviour. The in vitro behaviour seems to reflect the behaviour of the dermal cells in vivo. This assay would be valuable for pharmaceutical companies with interests in human hair growth (Johnson & Johnson, Unilever, Proctor & Gamble and many others).

2. Identification of agents with potential as defleecing or mulesing alternatives

The assays have identified some compounds which significantly alter keratinocyte proliferation and dermal cell behaviour, and therefore may have potential as wool-modifying compounds. Further work is required to develop these compounds.

Further work required to realise commercial outcomes

1. The dermal cell aggregation assay has been patented and is available for presentation to potential customers. It is yet to be determined what the size of the market for the assay might be but AgResearch is pursuing the commercialisation of the product.

2. Mass screening of a wide range of potential wool-regulatory compounds through the aggregation, proliferation and apoptosis assays is required to identify promising candidates for wool growth applications. In particular compounds that might induce miniaturisation of follicles (mulesing alternatives) or induce a break in the fleece (biodefleecing) should be sought. Promising candidates must then be tested *in vivo* (intradermal, intravenous administration to sheep), a system currently used by the Wool Research group at Adelaide University. Successful compounds then need to be developed in a form that allows large-scale testing, to evaluate effects on different genotypes, environmental conditions, dietary regimes and so on.

5.3 Manipulation of initiation of wool follicles and follicle appendages during fetal development

(Projects addressing this goal: SG301/332, SG322)

5.3.1 Application of therapeutic treatments using the pregnant sheep model

Ultimately the application of the genomics research in sheepgenomics wool subprogram will be via delivery of gene markers and use of therapeutic treatments which can be appl;ied to pregnant ewes at an appropriate time to alter follicle and follicle appendage formation. An initial experiment designed to influence the cortisol axis in pregnant ewes at days 55-65pc 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 (to increase the circulating levels of corticosteroids), 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 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 a Bone Morphogenetic Protein (BMP4), a ligand known to be involved in hair follicle development and expressed at the time of branching of secondary-derived follicles in Merino sheep. This finding has been submitted for publication (see below). The implication of BMP-4 is exciting given the known involvement of the BMPs and other members of the TGFβ-superfamily in

skin development. This ligand also featured strongly in the spatio-temporal studies referred to earlier (Section 7.1).

A simlar approach was taken to manipulation of the thyroid status of pregnant sheep during windows of development of Primary and Secondary follicles. This was based on previous studies which had shown strong effects of thyroxine on initiation of wool follicles (Hopkins and Thorburn 1972), and effects of thyroxine on development of other tissues, including branching morphogenesis in lung tissue. No effects of thyroxine manipulation (deletion or enhancement) were found.

Cysteine status was manipulated during periods of secondary follicle initiation had no significant effects, although there was a trend towards increased follicle density in sheep treated dwith additional cysteine during secondary-derived follicle formation. There was also a trend towards reduced sebaceous gland density in these animals at birth.

5.3.2 Application of therapeutic treatments using the pregnant mouse model

- Manipulation of the polyamine pathways produced dose-dependent decreases in follicle development in cultured murine embryonic skin
- Manipulation of activin/follistatin signalling in murine embryonic skin significantly altered follicle initiation. Follistatin increased the density of 'secondary' follicles while activin depressed follicle density. These findings are in accord with literature effects of these molecules on epithelial/mesenchymal signalling pathways.
- Manipulation of thiamine status of pregnant mice had no consistent effect on follicle initiation or density
- Manipulation of retinoic acid status during critical periods of follicle initiation significantly increased follicle density in mouse pups, but had adverse effects on litter size.
- Glucose supplied to pregnant mice during critical periods of follicle development significantly increased follicle density and total follicle number/animal. This result was surprising and potentially very important. The increase was of the order of 25%.

Project SG322. Discovery of genes for on-farm control of wool follicle growth

Scientific outcomes of the research

The objective of this work was to identify genes controlling the hair growth cycle. The propensity for Wiltshire Horn sheep to undergo seasonal fleece shedding was used as the experimental model with samples taken to represent the 'stop' and 'start' points. cDNA microarrays were used to identify genes that were upregulated or downregulated and Ingenuity Pathway Analysis used to order the many hundreds of differentially-expressed genes into ordered gene networks. 26 canonical pathways were identified during primary follicle formation and 43 pathways associated with hair growth cycling. Some of the gene pathways common to follicle initiation and cycling are well-known, but 6 common networks have not been previously described in relation to wool or hair growth. These 6 high-priority candidate genes were selected and full-length sequences obtained. This represents an exciting novel finding and one with significant commercial potential.

Publications

Potential commercial outcomes of the research

The complete list of differentially-expressed genes operating at different stages of the hair cycle, and the 6-pathway shortlist in particular, is a very significant resource with potential

application to human hair (diseases and cosmetic) and wool industry (defleecing agents and mulesing alternatives). Biochemicals within those pathways can now be targeted to induce follicle shutdown and miniaturisation, which might provide a means of selectively causing bare areas on the sheep (mulesing alternative) or systemic follicle shutdown (novel biodefleecing agents). Knowledge of these pathways also has potential application to human hair. By activating components of the 'stop' pathways, hair growth in unwanted areas could be reduced, while activation of 'start' signals could induce hair growth in 'hairless' skin. These human hair applications are worth many billions of dollars/annum.

Further work required to realise commercial outcomes

- complete thorough investigations of the roles of the 6 pathways using siRNA, and antibody approaches to protein function knockout, in dermal cells and keratinocytes in vitro
- identify candidate therapeutic targets within the pathways and develop small molecule inhibitors of key candidates. Test effects of inhibitors on hair growth in vitro and in vivo.
- Develop commercial products for large-scale testing of therapeutics on sheep and humans

Potential commercial outcomes of therapeutic manipulations

This project has clearly demonstrated the proof of concept that therapeutic manipulation of brief critical windows of fetal development can produce positive effects on hair follicle development. Further, the effects are permanent and not associated with detrimental side effects. Of all the treatments trialled, the 2 most promising were cortisol and glucose. It is unlikely that steroid hormone manipulation is ever likely to have commercial application in food animals. However identification of 'downstream' pathways and signalling molecules may identify potential therapeutic targets. A microarray experiment has been conducted to commence this process but further work is required.

The glucose finding was surprising and exciting as it has potential commercial application. One can envisage a simple, inexpensive treatment that could be applied to pregnant ewes for critical periods of development post conception. Such a treatment may consist of rumen-protected glucose capsules (slow-release) with say a 21 day life. If the results from the mouse experiments are verified in sheep (ie a 25% increase in follicle density), this would project to a lifetime decrease in fibre diameter of approximately 2microns. The benefit/cost of such a change would be very high indeed.

Further work to realise commercial outcomes

1. Cortisol result:
    - analyse the microarray data from the metyrapone experiment to identify potential target pathways for manipulation, using ingenuity Pathway Analysis
    - conduct experiments on small molecule therapeutics directed towards target biochemical pathways
    - patent approach and therapeutic molecule
    - conduct large-scale trials with sheep of different genotype to validate concept and provide real-world data on costings, benefit/cost ratios, problems etc

2. Glucose result:
    - Patent the idea and preliminary results
    - Repeat the experiment in pregnant sheep to validate result
    - Identify downstream effects and mode of action of glucose on skin development (hexokinase, glucose-6-phosphate, transketolase and transaldolase) and cellular homeostasis
    - Develop a commercial delivery system for postruminal glucose provision to pregnant ewes
    - Conduct large-scale trial in different genotypes to provide real-world data on costings, problems, benefit/cost ratios

Project SG306. Mutants and extremes for gene discovery

Scientific outcomes of the research

The objective of this project was a novel attempt to utilise naturally-occurring genetic mutations that appear in all populations spontaneously. Such mutations in mice have been very successfully used to determine the underlying causes of disease in humans. We aimed to use this untapped resource based on identification of phenotypic extremes by Australian sheep farmers. Most if not all of the extreme phenotypes fell into one of the following categories:

- non-genetic (facial eczema, photosensitisation) and therefore of little value in a genomics program
- Merino Felting Lustre mutant. This mutation has been identified previously. It has relevance to the mechanisms operating in crimp formation and cell fate within the follicle (differentiation into ortho-or paracortical cell type), but is of limited value to commercial outcomes for the Merino industry
- Delayed development (eg 'bald-at-birth') mutants. These are of value in identifying developmental signals as targeted in Projects SG304 and SG307 but would require matings to fix the genotype followed by subsequent examination of fetal gene expression in parallel to 'normal' development genotypes.
- Hypotrichosis (hairless) mutations. These have been well characterised in other species and the gene identified.

Publications

While no scientific publications arose from this project, the PR value for wool research was enormous. Print, television and radio media coverage was extensive and spread as far afield as the UK and USA. 28,000 wool producers were sent a copy of Beyond the Bale, which detailed the search for extreme phenotypes.

Potential commercial outcomes

1. a SNP genotype test for hypotrichosis.

Hypotrichosis is a recessive, so-called 'hairless' gene present in Dorset and White Suffolks. A test for carriers of hypotrichosis could be developed, depending on the frequency of the problem in these breeds.

2. use of hypotrichosis in sheep as a model for congenital atrichia with popular lesions in humans

The sheep may be a useful model for this debilitating disease of humans given their size, availablility of large amounts of tissue, knowledge of wool growth, availability of gene primers and so on.

Further work required to realise commercial outcomes

1. SNP genotype test for hypotrichosis carriers in Dorset and White Suffolk sheep

Cloning of cDNA and genomic DNA from unaffected carriers and affected carriers and homozygotes that contains the likely defective region of the hairless gene would allow differences in the nucleotide sequences to be identified.

2. other commercial opportunities

Given the following, the value of continuing to use sheep mutants for discovery of genes associated with fleece development is questionable:

- the existence of hundreds if not thousands of skin and hair mutants being generated naturally and chemically (Phenomics Centre) in mice
- the existence of wide genetic variance already in fleece and skin traits within the Ovine genus (breeds, strains, bloodlines, individuals)

Other remarks

This project played an important role in involving sheep producers in the research program. Future research for the wool industry should learn from this. For a relatively low cost (approx $50k per annum) an enormous amount of media involvement was achieved (national and international), and the opportunity to involve, inform and educate producers was presented. Whilst the direct research outcomes were modest, many producers were introduced to genetic and genomic concepts and became more involved in the entire program.

Project SG328 Gene markers for pigmentation

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 has unravelled the mystery of the agouti locus, which has eluded researchers to date. 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

- That the recessive black alleles (A^a and A^b) each contain a single agouti gene with a dysfunctional promoter

The project team have developed an assay for counting Junction Points referred to as the ABI3130xl copy number assay. 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. The assay result provides an estimate of the number of junction points from PCR product peak area ratio data that are not discrete junction point groupings. Thus, some animals with two or more junction points will be assigned a unique junction point number (count), based on a probability of having one of two possible 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.

Project SG313. Identification of genes affecting fleece rot resistance/susceptibility

Scientific outcomes of the project

The objective of this project was to identify genes associated with resistance or susceptibility to fleece rot using a microarray approach to identify differentially-expressed genes in resistant and susceptible lines of sheep. A subset of 28 differentially-expressed genes was selected on the basis of known functions, expression patterns and gene connections from an initial identification of 297 differentially expressed array elements between the susceptible and resistant lines. A further selection was then made down to 4 candidate genes for SNP detection. A SNPlex assay has been designed that successfully incorporates 24 of the SNP within 10 of the target genes into a single reaction assay.

Potential commercial outcomes of this work

A SNP marker for early detection of fleece rot susceptibility/resistance would be a valuable development for the Merino sheep industry. Fleece rot is an ideal candidate for marker-assisted selection in that the environmental conditions must be conducive to the development of the condition before susceptible/resistant individuals can be identified. A SNP marker (s) would enable identification of superior individuals in this respect, regardless of the prevailing conditions.

Further work required to realise the commercial outcomes

If SNPs can be found within the candidate gene regions, they could be added to a commercial SNP chip. If no SNPs can be identified within the candidate gene regions, further work will be required to identify other potential candidates. The fleece rot selection

lines at Trangie are an ideal resource for this purpose. There is the possibility that this resource will be lost if no funding is forthcoming for fleece rot research.

6. Capability and capacity building aspects of the wool subprogram

At the outset of sheepGENOMICS the capacity for high-quality strategic wool research in Australia had been decimated over the previous decade. Remnants of CSIRO's wool biology research capability remained scattered and unconnected, and the only other group concentrating on wool biology was at The University of Adelaide. A team of scientists was built around these remnants and supplemented by a strong group in AgResearch with complementary skills. Four postdoctoral fellows with no previous involvement in wool research were employed in the wool subprogram (McDowall, Dunn, Nattrass, Gordon-Thompson) and 2 postgraduate students (Xavier and MGrice) completed their PhDs in the program. A Science Advisory Committee was established (see table) comprising 2 external members (Franklin and Nash). This committee was frequently consulted for advice, and to make recommendations on project directions, terminations, invitations to join the program and all matters related to the on-going development of the subprogram.

Prof. Frank Nicholas	Sydney University	Quantitative and Molecular genetics
Dr Ian Franklin	Ex CSIRO PRS	Molecular genetics, gene mapping
Dr Andrew Nash	Amrad Corporation	Biotechnology and commercialisation
Dr Paul Swan	AWI	Manager Genetics Research
Dr Troy Fischer	AWI	Manager Molecular Genetics
Dr Terry Longhurst	MLA	Molecular genetics
Dr Rob Forage	SGP	Program Director Sheep Genomics

Unfortunately having developed this increased capability for a high-quality science base to address ongoing issues of strategic importance to the wool industry, the base has disintegrated rapidly. McDowall has returned to human medical research, Gordon-Thompson has retired, Dunn is continuing on wool research with SARDI funds but this is unlikely to continue, and Nattrass is no longer working on wool research. The AgResearch group has also disintegrated with the move of Rufaut to St Vincent's Hospital and Nixon under threat of closure of their program. The 2 PhD students have not found opportunities in wool research. Key research personnel such as Norris, who cracked for the first time the complex agouti multi-copy locus (which paves the way for other multicopy traits), have been made redundant by strategic decisions based on the likelihood of continued funding from the wool industry.

7. Conclusions and recommendations

The merino that will carry the wool industry forward will have the following characteristics:

- Plain-bodied, wrinkle-free, barebreech (no mulesing required)
- Guaranteed free of pigmented fibres
- High staple strength, low fibre diameter, high clean fleece weight
- Resistant to fleece rot and flystrike

The Wool subprogram of sheepGENOMICS has developed tools and opportunities to contribute to the development of this animal:

1. identified the causative genes involved in development of recessive black pigment and developed a copy number assay with user-friendly front end to identify carriers with reasonable and quantified certainty.
2. identified that it is possible to favourably manipulate fetal development over short, targeted periods of gestation to produce enhanced wool growth for the animal's lifetime
3. developed tools that allow rapid screening of bioactive compounds with potential to alter fetal follicle initiation, and fibre growth in adults (as defleecing and mules-crutching agents)
4. tested candidates for alteration of fetal follicle initiation (DAPT1, glucose, benfotiamine, polyamine enzymes, thiamine antagonists, retinoic acid, activin, follistatin, T4, cysteine, cortisol antagonists and analogues)
5. developed an extensive library of fetal tissue, expression profiles and in situ localisation for 100 candidate genes