Proteomics investigation of enhanced muscle development phenotypes

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Abstract

Protein expression in nuclear enriched, total cell membrane and cytosolic fractions of the *Semitendinosus* muscle was compared between lambs bred from control (C), high muscling (M) and high growth rate (G) sires. In total, 34 proteins were identified that were differentially expressed due to sire type. Differences in hind-limb muscle development between M lambs and C and G lambs were reflected in expression of proteins that regulate or function in cellular mechanisms of protein and energy metabolism. Despite no apparent difference in hind-limb muscle growth in G lambs compared to C, G lambs exhibited marked differences in regulation and function of energy metabolism. These results detail pathways than can be specifically targeted to enhance muscle accretion and growth in lambs. Developing mechanisms to specifically target these cellular mechanisms may yield greater gains in muscle accretion and growth rate than breeding on the basis for genetic capacity alone.

Executive Summary

The major objectives of this project were to develop a proteomics capability to provide new avenues for muscle development research in livestock species by applying new technology to investigate enhanced ovine muscle development and high growth phenotypes. Our purpose was to discover specific proteins and the critical cellular pathways that are involved in muscle development and growth that are genetically regulated. These findings are reported within the context of further understanding the cellular mechanisms driving muscle development. The proteins and genes reported here can be used to provide new tools for identification of superior muscling and growth genotypes, or they can be used as targets for treatments that may enhance muscle development and/or growth.

The *Semitendinosus* muscle samples used in this experiment were taken from lambs bred for enhanced genetic potential for muscling (M), growth rate (G) or neutral genetic potential for muscling and growth rate (C)[1]. Hegarty and co-workers[1] demonstrated in this lamb group, that breeding for enhanced genetic capacity for muscling resulted in increased hind-quarter weight and crude protein content at a high plane of nutrition compared to both control and growth lambs.

To elucidate changes in the underlying cellular mechanisms that support enhanced muscle development and growth rate, we enriched for populations of proteins of interest from within the complex tissue biopsies. Highly abundant contractile filament and extracellular matrix proteins were removed prior to protein expression analysis to enable enrichment of lower-abundance proteins. Further subcellular fractionation reduced the complexity of the cellular protein solution and yielded a nuclear enriched (NUC), total cell membrane (TCM) and clarified cytosolic fraction (CYT). The images presented here demonstrate that clear fractionation of cellular proteins into the NUC, CYT and TCM fraction with little duplication of proteins between fractions.

Proteins in the NUC, TCM and CYT fractions of the M, G and C genotypes were separated using two-dimensional electrophoresis and detected following staining with SYPRO Ruby fluorescent protein stain. Differential protein expression was determined using statistical analysis of relative concentration of matched proteins. Differentially expressed proteins were identified following peptide mass fingerprinting of proteins using Matrix Assisted Laser Desorption Ionisation (MALDI) – Time of Flight (TOF) mass spectrometry. Protein identities were determined using MS-Fit search software and mammalian protein and genome sequences. Proteome maps of the NUC, CYT and TCM fractions are presented, with differentially expressed proteins noted and proteins identified by peptide mass fingerprinting listed.

In total, 393 proteins were reproducibly detected in the NUC fraction. Of these proteins, 13 were differentially expressed between the M, G and C genotypes. Analysis using MALDI-TOF peptide mass fingerprinting identified 9 proteins. In the CYT fraction, 613 protein spots were reproducibly detected. Of these proteins, 24 were differentially expressed between the M, G and C genotypes. Analysis using MALDI-TOF peptide mass fingerprinting identified 11 proteins. In total, 639 proteins were reproducibly detected in the TCM fraction. Of these proteins, 24 were differentially expressed between the M, G and C genotypes. Analysis using MALDI-TOF peptide mass fingerprinting identified 14 proteins.

Differences in hind-limb muscle development between M lambs and C and G lambs were reflected in changes in protein expression related to shifts in protein and energy metabolism pathways. Changes in net protein accretion can occur through reduction in protein turnover, or increase in protein synthesis. Increased protein accretion in M lambs is suggested to be via increased protein synthetic capacity and muscle cell hypertrophy accompanied by elevated IGF-1 sensitivity and transition towards glycolytic fibre type. Changes in expression of ribosomal and translational regulatory proteins suggests that increased capacity for ribosomal biogenesis and elevated protein translational activity was the mechanism driving increased protein synthesis in the M genotype. Evidence was found for reduced recruitment of myonuclei in M lambs relative to C lambs, suggesting that elevated protein synthetic capacity was driven primarily through hypertrophy and not hyperplasia. This finding was supported by down regulation of some protein degradation pathway components, indicating that hypertrophy was supported by a reduction in energetic expenditure associated with protein turnover.

Hegarty and co-workers[1] demonstrated in these lambs, that breeding on the basis of high EBVs for growth produced no increase in lean muscle mass or crude protein in the hind-limb of lambs fed at a high plane of nutrition. Despite no apparent difference in hind-limb muscle growth in G lambs

compared to C, G lambs exhibited marked differences in regulation and function of energy metabolism pathways. Evidence presented here demonstrates a shift towards elevated glucose metabolism in G lambs. This is supported by changes in regulatory proteins that influence insulin sensitivity in concert with elevated expression of proteins involved in glucose metabolism pathways. The shift in energy balance in G lambs towards increased efficiency of glucose utilisation may be regulated by small GTPase proteins including those of the mitogen activated protein-kinase pathway and their interaction with the phosphatidylinositol second messenger system. Despite no apparent difference in muscle mass at the high plane of nutrition between G and C lambs, energetic efficiency associated with muscle maintenance processes and growth may be increased in G lambs. At low planes of nutrition, these inherent shifts in metabolism may provide a mechanism for greater efficiency of energy utilisation to support growth.

The research presented here details shifts in protein and energy metabolic and regulatory pathways associated with genetic capacity for muscling and growth in lambs. This information is of immediate use to the meat and livestock industry through furthering our understanding of the molecular mechanisms that underlie genetic selection for muscling and growth rate.

The pathways detailed within this report present opportunities to specifically target the mechanisms that enhance muscle accretion and growth in lambs. Developing a treatment means by which to alter the function of these pathways may provide a method for further improving muscle development or growth rate in animals that have been bred on the basis of enhanced genetic capacity for muscling and/or growth. Furthermore, development of mechanisms and treatments to specifically target these processes over coming years may yield greater gains in muscle accretion and growth rate than breeding on the basis for genetic capacity alone.

These findings provide candidate genes from which to scan the current industry flock for sequence variation. Defining the chromosomal location of this list of genes in light of current information on chromosomal location of quantitative trait loci (QTL) for muscling and growth rate may provide opportunities to seek single nucleotide polymorphism's within single genes that associate with these traits. Hence, this research has presented and opportunity to leverage further intellectual property from existing QTL projects.

Validation of differential protein expression of the proteins identified here through the use of antibodies specific for the individual proteins would provide more confidence in the findings relating to individual proteins. However, given the consistency of trends defined within each of the muscling and growth genotypes, a degree of confidence in the importance of the biological pathways presented here in influencing the traits of interest can be gained.

This project formed part of research within Project 2.2.1 of the Sheep CRC, Understanding muscle and fat biology to better meet consumer requirements. The work detailed in this report was funded through Meat and Livestock Australia, the Victorian Department of Primary Industries and the Sheep Co-operative Research Centre. As such, knowledge and information developed through this experimentation will be shared between DPI, MLA and the Sheep CRC.

Main Research Report

Background to Project and the Industry Context

The production of saleable muscle is the goal of sheep meat producers. This muscle production is driven by the deposition of structural proteins into muscle to increase its mass, a process that is in constant flux due to regulation of protein and energy metabolism in the animal as it responds to its environment. Changes in the cellular machinery of protein and energy metabolism are reflected in the proteins that undertake these functions. Hence, changes in muscle mass result from fluxes in key structural proteins in addition to regulatory proteins that control this process.

Traditional approaches to improving the production of muscle mass within the Australian flock have relied heavily on genetic improvement. Introduction and use of Estimated Breeding Values (EBV's) has enabled targeted improvement of specific traits within a flock[2]. Selected breeding of animals for improvement of a specific trait facilitates gains within that trait of interest, without any required understanding of the mechanism for trait improvement. Hence, genetic selection provides a coarse, but effective tool for providing improvement in capacity for overall trait performance. The expression of genetic capacity for traits such as growth rate and muscling are influenced heavily by environmental factors such as nutrition[3,4]. Understanding the cellular mechanisms that underlie genetic responses in trait performance is required to enable development of new strategies to further improve traits of commercial and industrial importance.

The genetic code (the genome) describes the proteins that cells can produce to perform the functions they are required to undertake. Mammalian genome sequences including human, bovine, mouse, domestic dog, and rat are now available within the public information domain. This availability of genetic information has enabled the prediction of protein sequence and structure on a whole genome basis. The characterisation of expression, structure, modification and function of these proteins is being addressed by a novel biotechnology discipline termed proteomics. In comparison to genomics, where the genetic code does not change over the life of an animal, the proteome is dynamic in character. Protein expression changes with development of an individual and as individuals respond to environmental factors. Proteomics, therefore, can provide much more detailed information on how individual proteins and genes are used to produce the outcomes of growth and interaction with the environment.

The control of muscle growth is exerted at many levels within the muscle cell. There are defined proteins that regulate gene transcription (transcription factors) through interaction with DNA in the nucleus to activate genes that are important for muscle growth[5]. Through these transcription factor-regulated pathways, changes in the expression of proteins that are required to alter cellular function to accommodate muscle growth are produced. These proteins can then effect the processes of muscle cell growth. For example, altering the levels or activity of protein synthesis machinery may influence the quantities of structural proteins of the cell cytoskeleton and contractile apparatus that are available for assembly. Other targets may include the energy metabolism pathways of the cell for redirecting energy expenditure towards support of protein accumulation, or the proteins of the signalling pathways within the cell that regulate these processes.

Skeletal muscle is a very complex tissue, comprised predominantly of skeletal muscle cells, but also containing satellite cells that sit on the surface of muscle cells, fibroblasts that produce connective tissue, adipocytes, smooth muscle tissue in the form of arterioles and veins and nerve tissue among others. Accurate interpretation of gene and protein expression data from skeletal muscle requires separation of the tissue into cell types of interest. Using fractionation techniques with proteomics enables targeted investigation of cell types of interest. Furthermore, separation of these cells into their organelles allows for more accurate interpretation of protein function and analysis of lower-abundance proteins. Utilising this approach to investigate differences in protein expression due to breeding for a trait of interest will allow the sensitivity to determine subtle changes in individual protein expression that may have large influence on cellular systems responsible for producing the animals phenotype.

Consumers place demands on the production of muscle, but their demands relate to the eating quality of this muscle when it is consumed as meat. A large proportion of eating quality is determined by the

structural integrity of muscle proteins. The ultimate nature of meat then, is a reflection of its protein deposition history and the changes that occur in these proteins during the animals' development up to slaughter. Understanding the processes that define muscle growth and meat quality requires investigation of how muscle proteins are expressed over development of the tissue and animal. Such studies will allow identification of the cellular systems involved in production of quality meat.

This project will utilise male progeny from the MS.002 research flock[6] that have been bred from sires with high EBV's for muscling (M), high EBV's for postweaning growth (G) and neutral EBV's for muscling and postweaning growth (C). Muscle tissue from these lambs will be prepared for proteomics to enable identification of proteins that are differentially expressed in response to genetic capacity for muscling and growth.

Project Objectives

Objectives of this project were to;

- Obtain muscle biopsies from the semitendinosus muscles of appropriate animals (of MLA Project No. MS.002)
- Adapt proteomics technologies at the University of Melbourne Dental School.
- Develop ovine bioinformatics know-how using public domain information and DPI bioinformatics capabilities.
- Identify and characterise proteins from ovine muscle that are differentially expressed in different genetic and nutritional environments.
- Provide information on potential roles of known and novel proteins/genes in cellular development that are related to muscle growth.

Methodology

Background Methodology Development

Scientists at Primary Industries Research Victoria (PIRVic) and researchers at the Korean National Livestock Research Institute collaborated to develop protocols for separation of proteins using 2DE from muscle biopsies. Protocols for muscle cell fractionation, subcellular separation, protein solubilisation and two dimensional electrophoresis were trialed to provide strategies for sub-cellular fractionation to reduce protein samples complexity for analysis of low-abundance proteins (Figure 1).

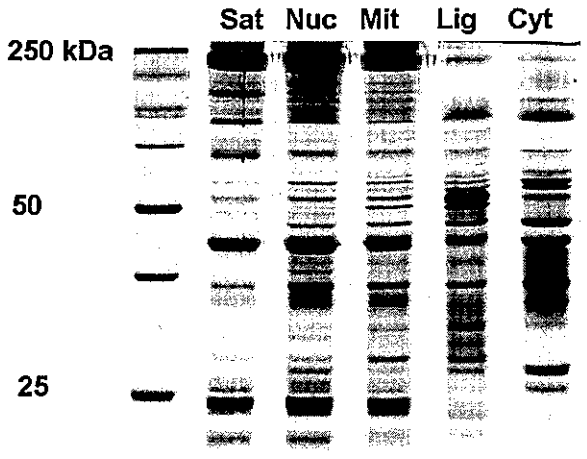


Figure 1. Small format SDS gel showing separation of muscle into single cell (sat) and muscle cell fractions followed by sub-cellular fractionation of muscle cells into nuclei (Nuc), mitochondrial (Mit), light vesicles (Lig) and cytosolic (Cyt) fractions.

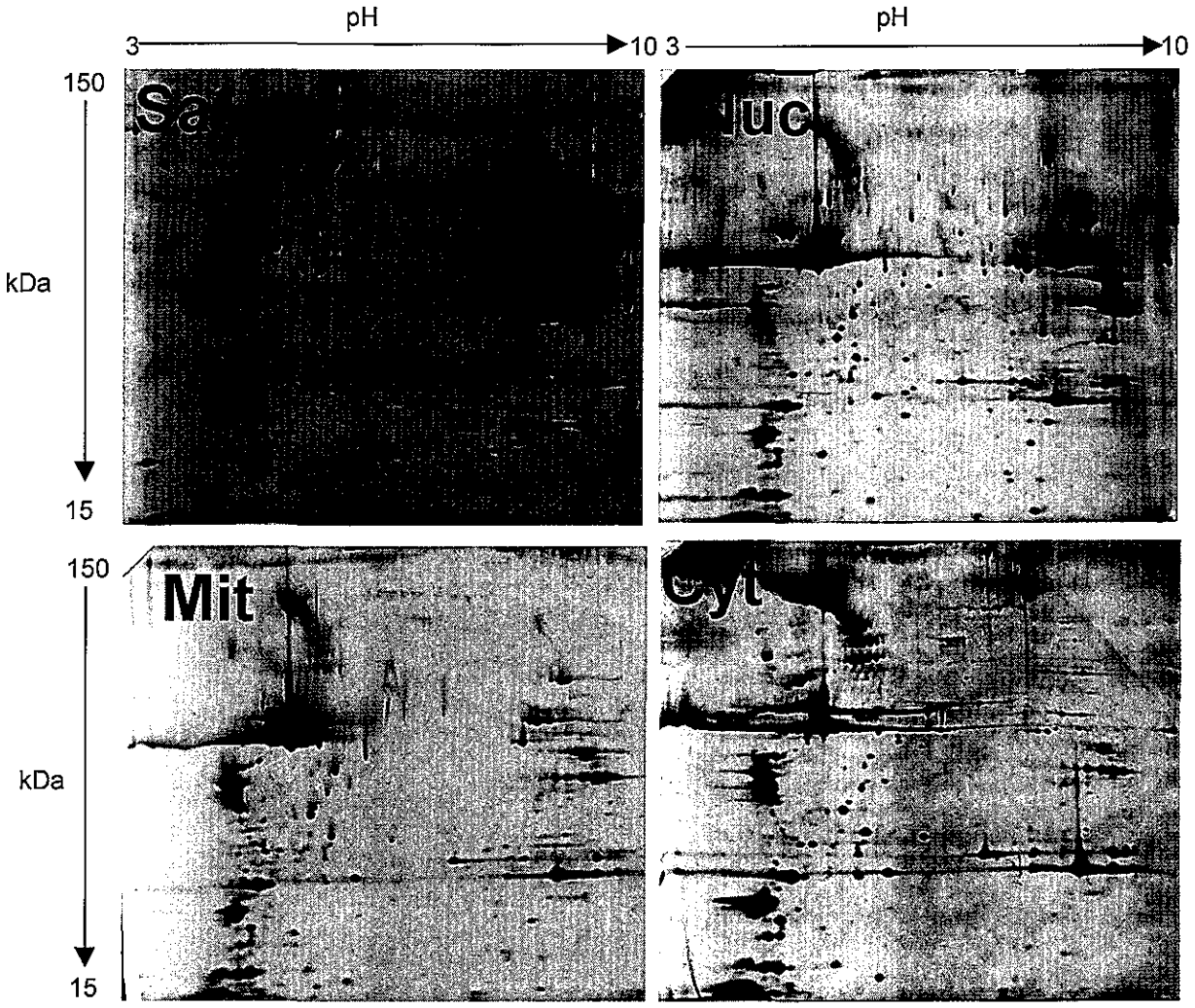
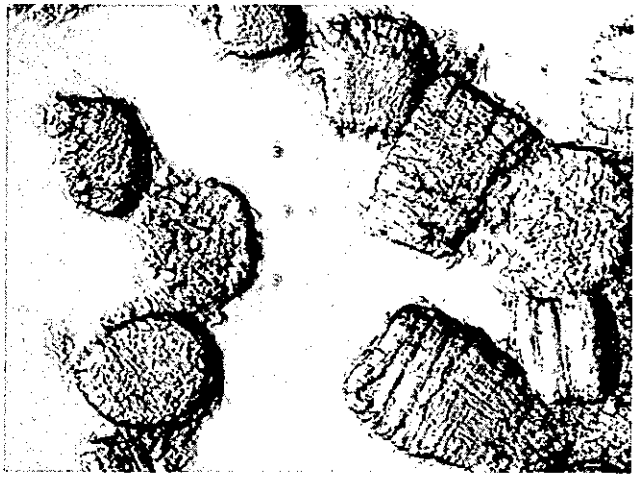


Figure 2. Large format 2DE gels using 18cm pH 3-10 non-linear isoelectric focusing strips in the first dimension, and 10% acrylamide SDS electrophoresis in the second dimension. 2DE protein profiles are of enriched single cells (Sat), muscle nuclei (Nuc), mitochondria (Mit) and cytosolic (Cyt) fractions.

Using frozen muscle tissue, homogenisation and fractionation protocols were trialed to determine the most appropriate conditions for reduction of proteome complexity in freeze/thaw compromised muscle samples. Results from test large-format gels demonstrated that some fractions of interest, showed considerable similarity in abundance of major protein groups (Figure 2. Nuc, Mit and Cyt fractions). These proteins resembled those identified by previous researchers as major structural components of the myofibrillar contractile apparatus[7].

In light of these findings, crude filtration and centrifugation was utilised to separate proteins from the muscle cell nuclear enriched fraction from the cellular contractile apparatus. Frozen muscle biopsies were homogenised briefly and muscle fibres were disrupted by trituration through a pipette prior to filtration through a 100μm cell strainer to remove non-disrupted tissue, this was discarded. Filtrates were then applied to 40μm filters to remove smaller particles including in-tact sarcomeric units (Figure 3. a), this was discarded. The filtrate (Figure 3. b) was then centrifuged to 1000g for 10 minutes at 4°C to sediment nuclei and heavy cell debris (Figure 3. c). This sample disruption and filtration protocol resulted in semi-purification of a heavy nuclear enriched and total cell membrane/cytosol fraction from crude muscle tissue that was depleted of myofibrillar proteins.

a.



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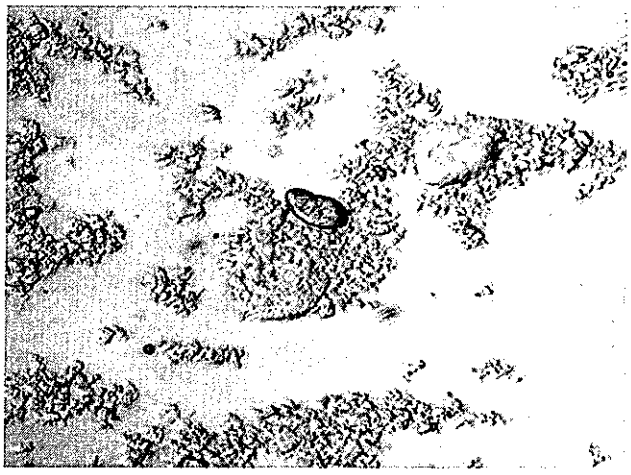


c.

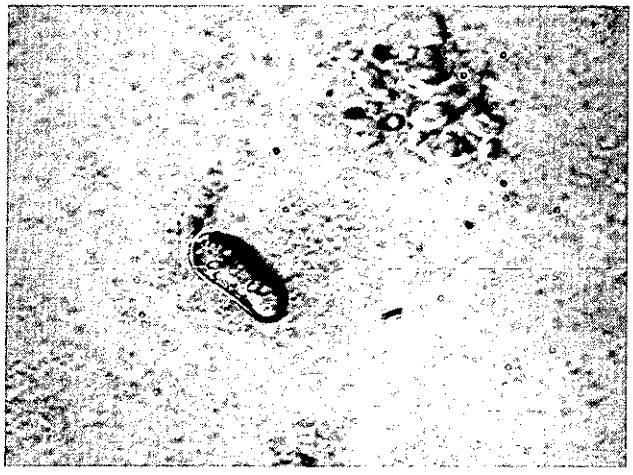
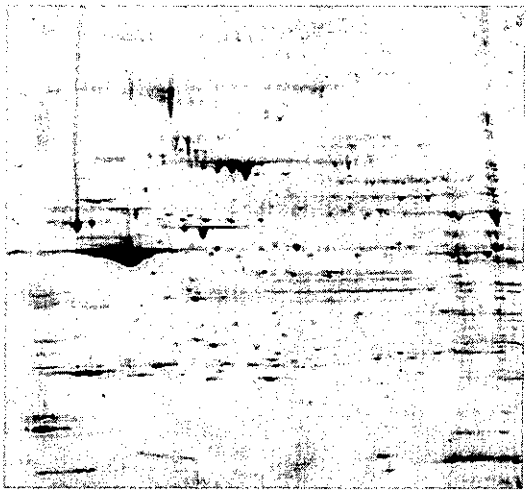


Figure 3. Fractionation of large myofibrillar particles (a. 40X magnification), small cellular debris (b. 100X magnification) and nuclear enriched fraction (c. 1000X magnification) using the experimental homogenisation and filtration protocol.

The total cell membrane and cytosol fractions were separated using ultra-centrifugation to 100,000g to sediment cellular organelles, vesicles and membranes (total cell membrane fraction). This produced three distinct fractions for proteomic analysis, a nuclear enriched fraction, a total cell membrane fraction and cytosolic fraction (Figure 4).

a.



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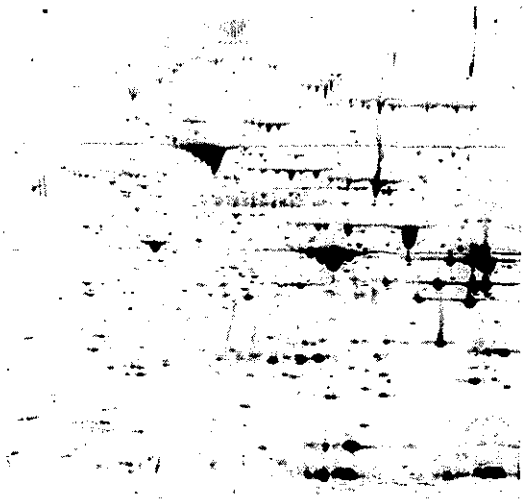

c.

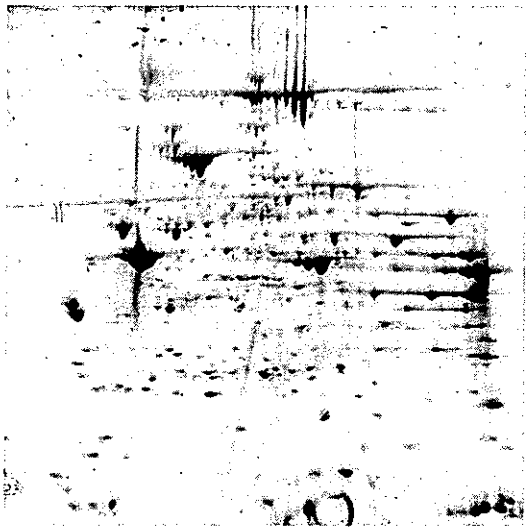

Figure 4. Large format 2DEs gel of the nuclear enriched fraction (a) total cell membrane fraction (b) and cytosolic fraction (c) using 8-18% acrylamide gels in the second dimension.

Animals, sample collection and pooling

Lambs from nine sires, representing 3 selected with high EBV for muscling (M), 3 selected with high EBV for post-weaning growth rate (G) and 3 with industry neutral EBV's for muscling and post-weaning growth rate (C) were used in this experiment[6]. Lambs for this experiment were maintained on a high plane of nutrition (*ad libitum* fresh pasture access). Lambs maintained on the low plane of nutrition were not analysed so that protein expression differences due to genotype alone could be determined. Biopsies (~200 mg) taken under local anaesthesia from the *Semitendinosus* muscle of male castrate lambs were snap frozen in liquid Nitrogen. Biopsies were transported under dry ice to PIRVic laboratories for preparation and analysis

To obtain adequate tissue for suitable technical replication of 2DE gels (triplicate is preferred), muscle biopsies from individual lambs were pooled together within sire (refer to Table 1). This provided 9 experimental units comprising of muscle tissue from between 2-4 animals per unit. Each experimental unit represents one sire with 3 sires for each of the control, growth and muscling lines.

Table 1. Sample pooling structure for analysis of MS002 *Semitendinosus* biopsies

MS002 Sample Biopsy Pools - ST Muscle			
Genotype	**Sire number**	***Pool number***	**Lamb ID.**
Muscling	5	***1***	**210**
Muscling	5		**215**
Muscling	5		**234**
Muscling	7	***2***	**303**
Muscling	7		**309**
Muscling	7		**320**
Muscling	7		**334**
Muscling	9	***3***	**401**
Muscling	9		**412**
Muscling	9		**437**
Growth	2	***4***	**65**
Growth	2		**68**
Growth	3	***5***	**111**
Growth	3		**128**
Growth	6	***6***	**290**
Growth	6		**298**
Growth	6		**464**
Control	1	***7***	**44**
Control	1		**49**
Control	1		**50**
Control	4	***8***	**457**
Control	4		**162**
Control	4		**176**
Control	4		**199**
Control	4		**164**
Control	8	***9***	**361**
Control	8		**362**
Control	8		**368**
Control	8		**383**

Sample preparation for two-dimensional electrophoresis

Pooled skeletal muscle tissue was diced finely and homogenised in 3 volumes of ice-cold homogenisation buffer (40mM Tris, 250mM sucrose, 1mM Magnesium acetate, 1mM EGTA, 0.1% Triton X-100, 1:50 complete enzyme inhibitor cocktail (Roche), pH 7.5 at 4°C) using a bead beater with two bursts of 20 seconds on high. Homogenates were cooled on ice for 60 seconds between homogenisation bursts. Muscle fibres were disrupted by trituration through a pipette prior to filtration through a 100μm cell strainer to remove non-disrupted tissue, this was discarded. Filtrates were then applied to 40μm filters to remove smaller particles including in-tact sarcomeric units, this was discarded. The filtrate was then centrifuged to 1000g for 10 minutes at 4°C to sediment nuclei and heavy cell debris. The supernatant that remained following sedimentation of nuclei was centrifuged again at 100,000g for 60 minutes at 4°C to sediment a total cell membrane fraction. The supernatant from centrifugation at 100,000g was frozen at -80°C and designated the cytosolic fraction. Both the nuclear enriched and total cell membrane pellets were resuspended in 10 volumes (w/v) of homogenisation buffer and re-centrifuged to dilute contaminating proteins prior to reconstitution in 1 volume (w/v) of homogenisation buffer and freezing at -80°C. The final fractions analysed by 2DE were the nuclear enriched fraction (NUC), total cell membrane fraction (TCM) and the cytosolic fraction (CYT).

Prior to solubilisation of proteins for 2DE, the protein concentration of each sample fraction was determined in triplicate using the 2D-Quant method (Amersham Biosciences). Aliquots of protein were frozen at-80°C for later solubilisation and use for 2DE.

Two-dimensional electrophoresis

Isoelectric focussing of nuclear enriched fraction

Proteins in the NUC fraction were solubilised for 1 hr in sample buffer (7M urea, 2M thiourea, 40mM Tris, 2% CHAPS, 2% ASB-14, 50mM DTT, 0.2% ampholytes) and centrifuged at 100,000g for 1hr at 20°C prior to IEF. One hundred μg of protein was loaded onto triplicate pH 5-8 IPG strips (Bio-Rad Laboratories) using passive in-gel rehydration for 16 hours. Focusing of pH 5-8 IPG strips was at 20°C for 85000 VhT using a step wise gradient of 100V for 1 hour, 300V for 1hr, 500V for 1 hour, 1000V for 1 hour, gradient to 8000V over 2 hours and 8 hours at 8000V. Following focusing, IPG strips were frozen immediately at -80°C until equilibrated prior to SDS PAGE.

Isoelectric focussing of cytosolic fraction

Proteins in the CYT fraction were solubilised for 1 hr in sample buffer and centrifuged at 100,000g for 1hr prior to IEF. One hundred μg of protein was loaded on triplicate pH 3-10 IPG strips (Amersham Lifesciences) using passive in-gel rehydration for 16 hours. Focussing was at 20°C for 69,000 VhT using a stepwise gradient 100V for 1 hour, 300V for 1hr, 500V for 1 hour, 1000V for 1 hour, gradient to 8000V over 2 hours and 6 hours at 8000V.

Isoelectric focussing of total cell membrane fraction

Proteins in the TCM fraction were solubilised for 1 hr in sample buffer and centrifuged at 100,000g for 1hr prior to IEF. One hundred μg of protein was loaded on triplicate pH 3-10 IPG strips (Amersham Lifesciences) using passive in-gel rehydration for 16 hours. Focussing was at 20°C for 69,000 VhT using a stepwise gradient 100V for 1 hour, 300V for 1hr, 500V for 1 hour, 1000V for 1 hour, gradient to 8000V over 2 hours and 6 hours at 8000V.

Equilibration and SDS PAGE

Equilibration of IEF strips was for 10 min each in equilibration buffer (6M urea, 2% SDS, 50mM Tris pH 8.8, 30% glycerol, 0.002% bromophenol blue) containing 100mg/10ml Dithiothreitol, then 250mg/10ml Iodoacetamide. Equilibrated IPG strips were applied to 6-18% duracryl gels (30T, 2.2B) and sealed with agarose solution (0.5% agarose in running buffer). Gels were run at 100volts for 1

hour followed by 500volts for 7 hours limited to 12 watts per gel. Gels were fixed for 1hr in 40% methanol, 10% acetic acid prior to staining overnight with SYPRO Ruby (Molecular Probes). The following day, gels were destained for 1hr in 10% methanol, 7% acetic acid prior to detection.

Imaging and statistical analysis

SYPRO Ruby stained images were captured using a cooled scanning CCD camera (PROExpress, Perkin Elmer Life Sciences) with excitation at 430nm, emission at 620nm and exposure for 4 seconds. Scanning used a dynamic range of 0 to 65,000 grey levels and a resolution of 100μm.
Image analysis used Phoretix 2D Evolution (Nonlinear Dynamics). Proteins were detected using Evolution Detection with background subtraction and normalisation for total protein volume. Protein patterns were warped to match individual proteins and synchronised protein data was exported to Excel (Microsoft). Differential protein expression was determined by analysis using ANOVA by grouping triplicate gels within sire, with sire nested within gene line as the fixed effect using Genstat 5.41 (VSN International Ltd, UK). Differences in protein expression were defined as significant where the normalised spot volumes between genetic groups differed by $P<.05$. Means presented are normalised spot volume ± standard error of the difference of means.

Protein picking and mass spectrometry

Following image analysis, gels were sealed into plastic bags and stored in destaining solution in the dark at 2°C until picking. Proteins were picked using automated protein excision (PROXcision, Perkin Elmer Life Sciences) followed by in-gel digestion with trypsin, peptide purification (ZipTip, Millipore) and MALDI spotting on a Multiprobe IIEX robot (Packard Biosciences).

Matrix Assisted Laser Desorption Ionisation –Time Of Flight (MALDI-TOF) analysis was performed on a Voyager-DE STR MALDI mass spectrometer (Applied Biosystems), equipped with a 337 nm N_2 laser. Parent ion masses were measured in reflector/delayed extraction mode, with accelerating voltage of 20kV, grid voltage of 65% and a 150 nsec delay. Positive polarity was used. Fifty scans were averaged per sample and spectra subject to 3 point external calibration using bradykinin, angiotensin I and adrenocorticotrophic hormone peptide (18-39) with M_r = 1060.5692, 1296.6853 and 2465.1989 respectively. The matrix used was α-cyano-4-hydroxy cinnamic acid saturated in a solution of 50% acetonitrile with 0.1% triflouro acetic acid.

Bioinformatics and protein identification

Database searching of peptide mass fingerprint results used Data Explorer™ software (ABI). Peptide Mass Fingerprints were compared in GenBank and SWISS-PROT mammalian protein sequence databases using MS-Fit™ (Protein Prospector, UCSF).

Results

This work focused on the separation of proteins from distinct fractions of the *Semitendinosus* muscle from control animals and animals bred for genetic capacity for muscling or growth. Differentially expressed proteins were identified by peptide mass fingerprinting. Two-dimensional protein maps are presented from each fraction with differentially expressed proteins identified. Significant differences in protein expression are relative to the control (C) lambs.

Nuclear enriched fraction

Good separation and resolution of nuclear enriched proteins based on isoelectric point was obtained between pH 5 – 8. Reproducible resolution of proteins based on molecular weight was gained between 10 – 120 kDa. In total, 393 proteins were reproducibly detected in the NUC fraction. Of these proteins, 13 were differentially expressed between the M, G and C genotypes (Figure 5). Analysis using MALDI-TOF peptide mass fingerprinting identified 9 proteins (Table 2).

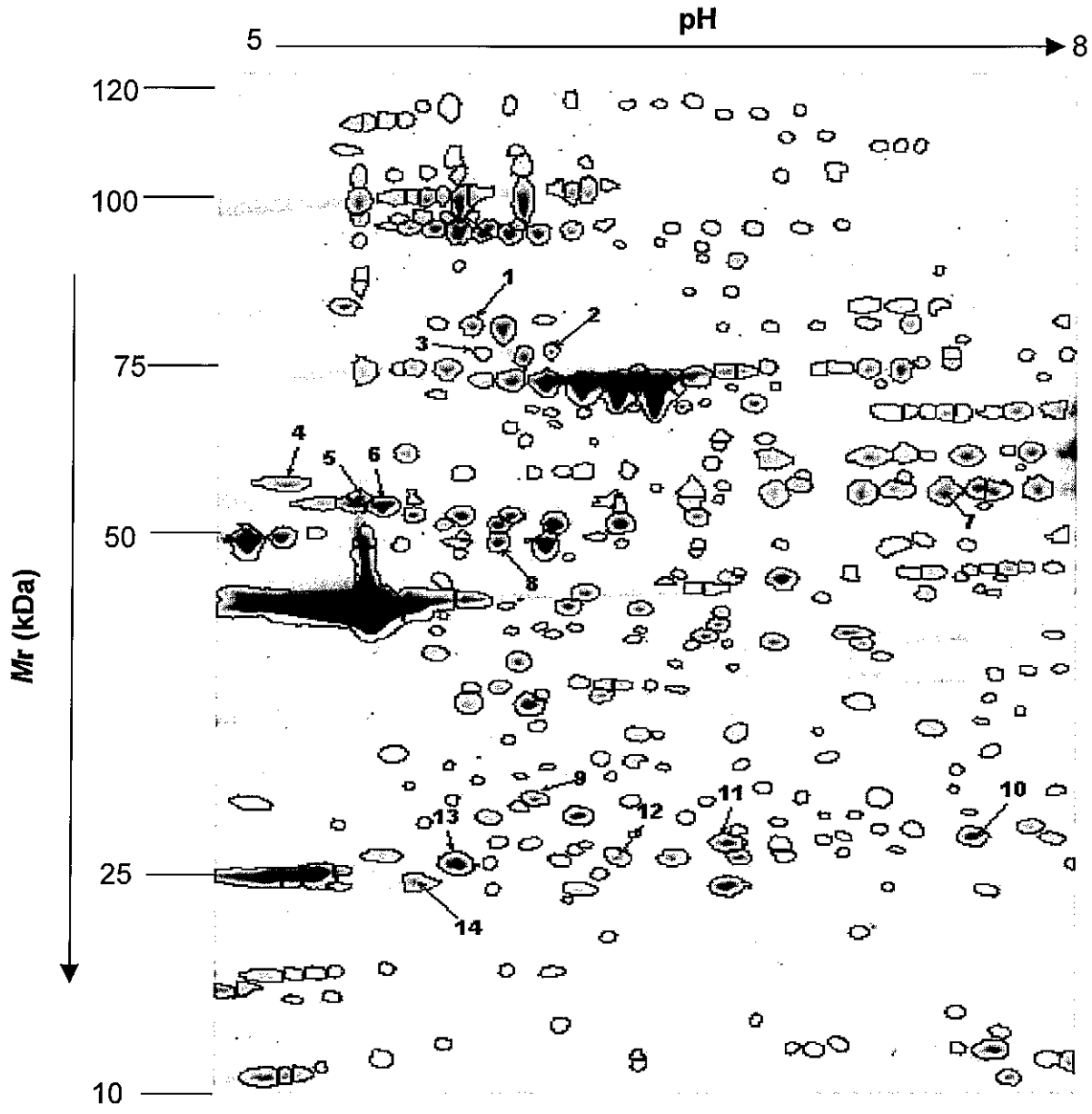

Figure 5. Two-dimensional reference gel of the nuclear enriched fraction of ovine *Semitendinosus* muscle. 2DE used a pH range of 5-8 in the first dimension and SDS PAGE (6-18% acrylamide) in the second dimension. Protein loading was 100 μg and the gel was stained using SYPRO Ruby. Spot numbers refer to proteins that were differentially expressed between muscling, growth and control.

Of the 9 differentially expressed proteins identified from the NUC fraction, 7 are involved in DNA binding or regulation of gene expression. Within the M lambs, down regulation of ribosome biogenesis protein BOP1, heat shock 70kDa precursor 9B, hypothetical protein DKFZp434H055 and C7orf31 protein and increased expression of NFkB essential modulator, prohibitin and DnaJ homolog subfamily C member 12 was observed. Down regulation of C7orf31 protein was also observed in G lambs.

The remaining 2 differentially expressed proteins were structural, with increased expression of vimentin seen in M and G lambs, whilst desmin expression was increased in M lambs only.

Table 2. Protein identification summary of differentially expressed proteins in the nuclear enriched fraction of *Semitendinosus* muscle from lambs bred from sires with EBV's for muscling, growth or sires with neutral EBV's for muscling and growth (control).

ID	Identification	Accession number	Means				P value	Experimental		Actual		Ref
			Muscling	Growth	Control	SED		mw	PI	mw	PI	
1	Ribosome biogenesis protein BOP1	P97452	4.22*	6.40	6.87	0.821	.023	82,000	5.7	82,545	5.86	8
2	Heat shock 70kDa precursor 9B	BC024034	0.32*	0.65	0.55	0.103	.032	76,000	6.0	73,728	6.03	9
3	Hypothetical protein DKFZp434H055	Q8NCX4	0.25*	0.25*	0.45	0.078	.054	75,000	5.8	65,715	-	10
4	Vimentin	L13263	0.92*	1.32*	0.53	0.171	.010	58,000	5.2	53,677	5.21	11
6	Desmin	AB011673	5.87*	3.49	2.17	1.265	.047	53,000	5.4	52,562	5.21	12
8	NFkB essential modulator	AF326207	1.50*	0.84	0.93	0.209	.024	49,000	5.8	47,973	5.61	13
9	Prohibitin	BT007411	1.89*	1.07	1.06	0.223	.002	30,000	5.9	29,804	5.57	14
11	C7orf31 protein	BC015397	0.75*	1.17*	3.00	0.601	.003	27,000	6.8	35,161	6.32	9
13	DnaJ homolog subfamily C member 12	Q9R022	8.56*	3.56	2.14	1.087	<.001	25,000	5.6	22,853	5.82	15

* Indicates significantly different to control.

Cytosolic fraction

Good separation and resolution of proteins in the CYT fraction based on isoelectric point was obtained between pH 4 – 9. Reproducible resolution of proteins based on molecular weight was gained between 10 – 150 kDa. In total, 613 protein spots were reproducibly detected in the CYT fraction. Of these proteins, 24 were differentially expressed between the M, G and C genotypes (Figure 6). Analysis using MALDI-TOF peptide mass fingerprinting identified 11 proteins (Table 3).

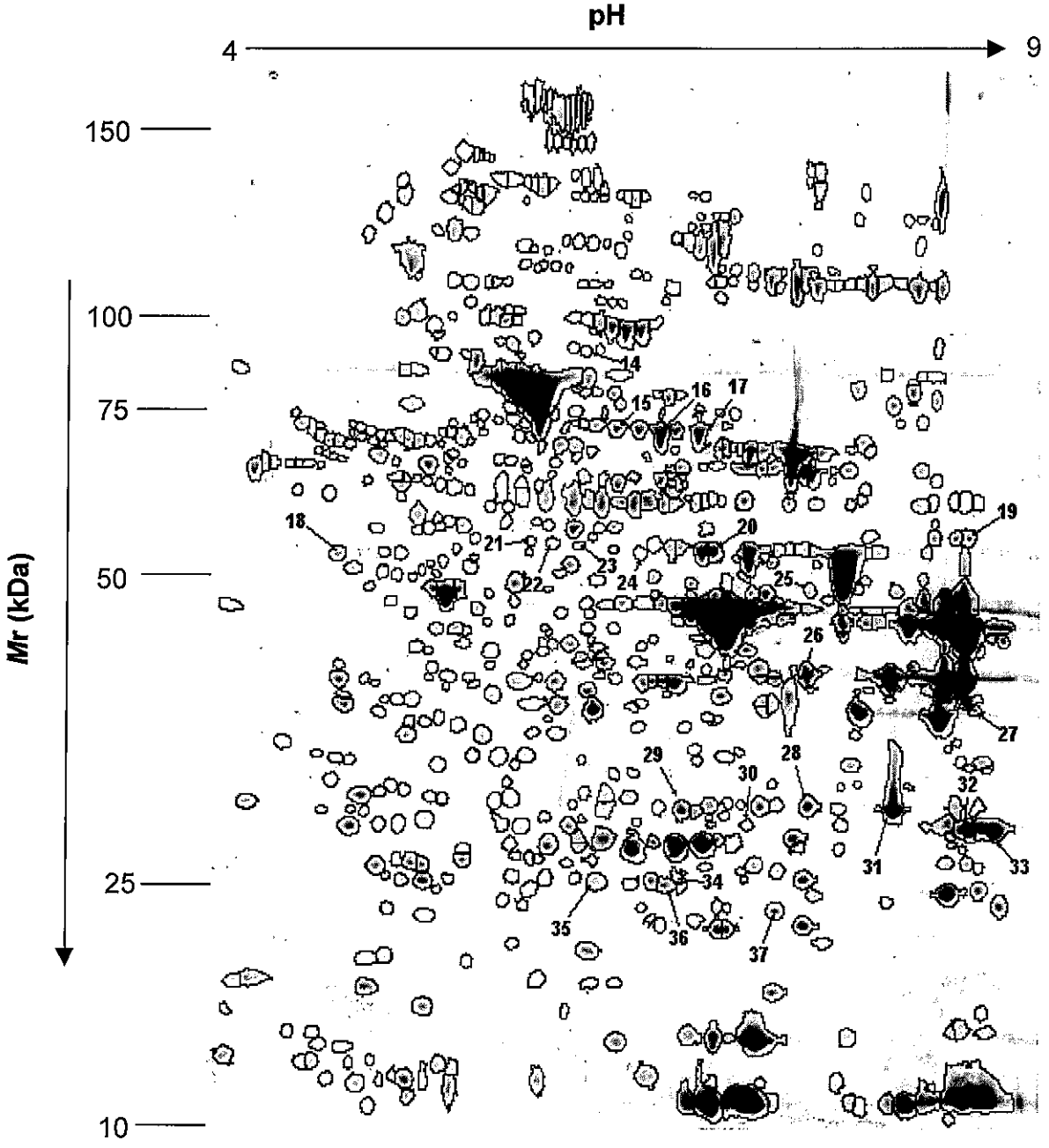

Figure 6. Two-dimensional reference gel of the cytosolic fraction of ovine *Semitendinosus* muscle. 2DE used a pH range of 3-10 in the first dimension and SDS PAGE (6-18% acrylamide) in the second dimension. Protein loading was 100 µg and the gel was stained using SYPRO Ruby. Spot numbers refer to proteins that were differentially expressed between muscling, growth and control.

Table 3. Protein identification summary of differentially expressed proteins in the cytosolic fraction of *Semitendinosus* muscle from lambs bred from sires with EBV's for muscling, growth or sires with neutral EBV's for muscling and growth (control).

ID	Identification	Accession number	Means			SED	P value	Estimated		Actual		Ref
			Muscling	Growth	Control			mw	PI	mw	PI	
14	Myh10 protein fragment	Q6PB65	0.85*	0.50	0.58	0.054	<.001	85,000	6.1	96,967	-	9
15	Phosphoglucomutase	L11694	3.29	2.66*	3.47	0.285	.046	70,000	6.2	61,599	6.31	16
16	Phosphoglucomutase 2	M97664	12.55	11.15*	15.06	1.279	.038	65,000	6.8	99,791	-	17
17	Phosphoglucomutase 1	BT006961	10.48*	9.89*	5.20	1.216	.005	65,000	7.2	61,370	6.20	14
19	Pleckstrin and Sec7 domain containing protein 3	Q9NYI0	0.60	0.19*	0.44	0.086	.021	58,000	8.8	60,193	8.83	18
22	Lamin B3 - S	D13455	0.26	0.46*	0.32	0.063	.045	54,000	6.0	53,269	5.74	19
23	Fgd2 protein	BC021845	0.26	0.40*	0.18	0.033	<.001	53,000	6.3	52,531	6.44	9
24	Uridine 5'-monophosphate synthase	P11172	0.40*	0.41*	0.30	0.040	.047	52,000	6.7	52,222	6.81	20
27	Skeletal muscle LIM-protein 1	P97447	0.50	0.98*	0.46	0.042	.011	34,000	8.9	31,899	8.76	21
29	Glyceraldehyde-3-phosphate dehydrogenase	Q28554	1.30*	0.93	0.88	0.165	.027	28,000	7.1	34,732	-	22
36	Thioredoxin domain containing protein 9	BC005968	2.48	4.57*	2.09	0.840	.017	23,000	6.4	26,534	5.61	9

* Indicates significantly different to control.

Of the 11 differentially expressed proteins in the CYT fraction, 5 are proteins with metabolic function. Three of these proteins were isoforms of phosphoglucomutase (PGM), with expression of PGM and PGM-2 reduced and expression of PGM-1 elevated in G lambs. Expression of PGM-1 was also elevated in the M lambs. Uridine 5'-monophosphate synthase expression was elevated both M and G lambs and glyceraldehyde-3-phosphate dehydrogenase expression was elevated in M lambs.

Three of the identified proteins were structural proteins, with expression of the myosin protein fragment of Myh10 protein being elevated in M lambs. Expression of Lamin B3 and skeletal muscle LIM-protein 1 were elevated in G lambs.

Two proteins associated with GTPase mediated signalling were identified, pleckstrin and Sec7 domain containing protein 3 and Fgd2 protein. Expression of these proteins was elevated in G lambs. The expression of thioredoxin domain containing protein 9 was also elevated in G lambs. This protein has no confirmed cellular function but is thought to be an ATP binding protein involved in regulation of cell differentiation.

Total cell membrane fraction

Good separation and resolution of TCM proteins based on isoelectric point was obtained between pH 4 – 9. Reproducible resolution of proteins based on molecular weight was gained between 10 – 150 kDa. In total, 639 proteins were reproducibly detected in the TCM fraction. Of these proteins, 24 were differentially expressed between the M, G and C genotypes (Figure 7). Analysis using MALDI-TOF peptide mass fingerprinting identified 14 proteins (Table 4).

Of the 14 differentially expressed proteins in the TCM fraction, 6 are proteins with metabolic function. Expression of N-acetylglucosaminyltransferase IVa, aldehyde dehydrogenase, ubiquitin ligase protein DZIP3 and creatine Kinase M chain was reduced in M lambs. Reduced expression of N-acetylglucosaminyltransferase IVa, Ubiquitin ligase protein DZIP3, ATPase, H+ transporting, as well as mitochondrial NADH ubiquinone oxidoreductase and lysosomal 42kD V1 subunit C isoform 1 was also observed in G lambs.

Four of the identified proteins are involved in cell signalling pathways. Mitogen-activated protein kinase kinase kinase 9 and inositol polyphosphate 1-phosphatase expression was reduced in G lambs. The expression of glandular kallikrein K9 was elevated and expression of insulin-like growth factor binding protein 4 was reduced in M lambs.

Two proteins associated with control of protein expression, Eukaryotic translation initiation factor 2 subunit 2 and prohibitin, showed increased expression in the M group. Prohibitin expression was also elevated in G lambs.

The expression of Flotillin-1 and Band 6 polypeptide, membrane associated structural proteins, was reduced in M and G lambs.

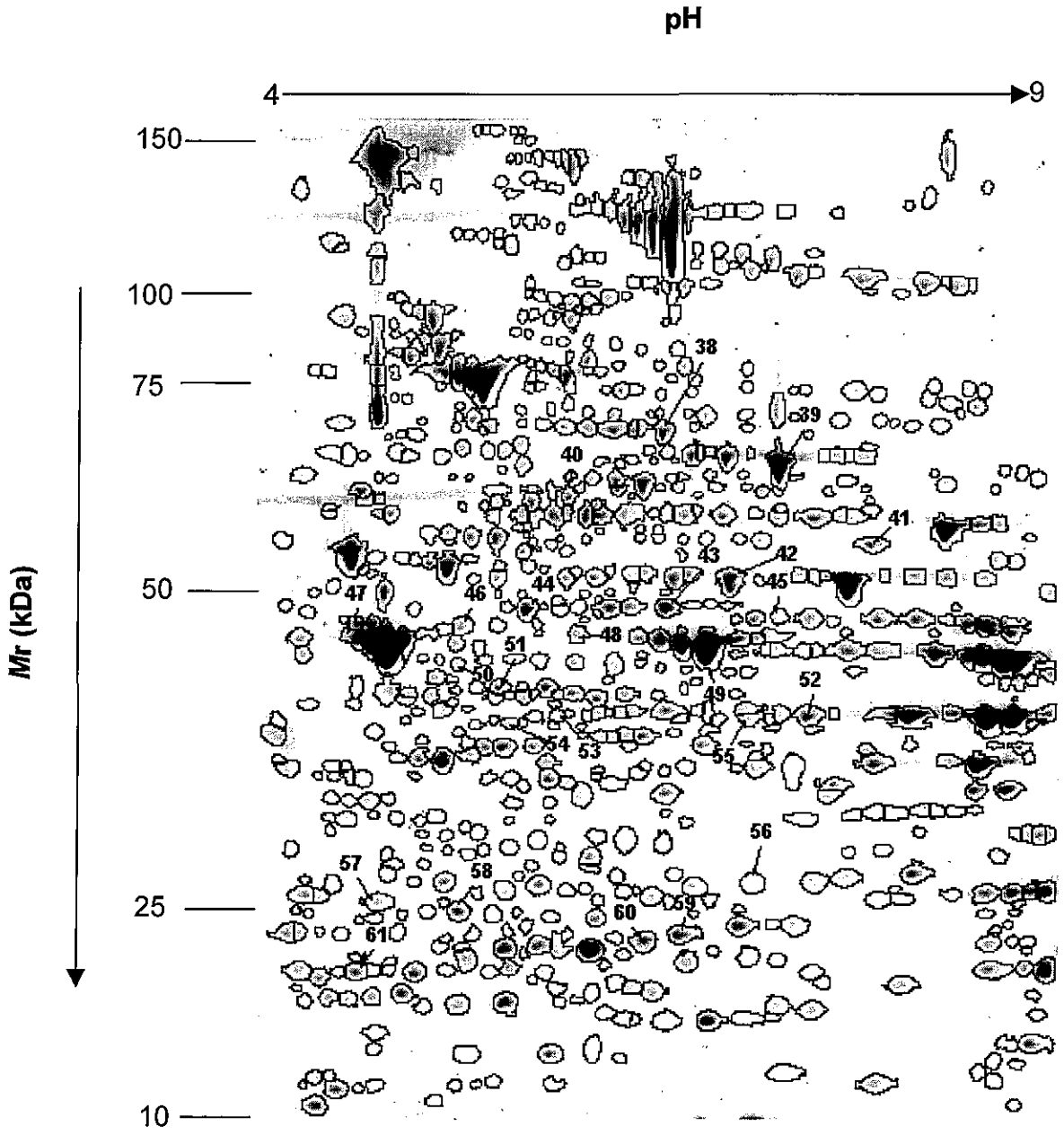

Figure 7. Two-dimensional reference gel of the total cell membrane fraction of ovine *Semitendinosus* muscle. 2DE used a pH range of 3-10 in the first dimension and SDS PAGE (6-18% acrylamide) in the second dimension. Protein loading was 100 μg and the gel was stained using SYPRO Ruby. Spot numbers refer to proteins that were differentially expressed between muscling, growth and control.

Table 4. Protein identification summary of differentially expressed proteins in the total cell membrane fraction of *Semitendinosus* muscle from lambs bred from sires with EBV's for muscling, growth or sires with neutral EBV's for muscling and growth (control).

ID	Identification	Accession number	Means			SED	P value	Estimated		Actual		Ref
			Muscling	Growth	Control			mw	PI	mw	PI	
39	N-acetylglucosaminyltransferase IVa	Q80UI0	9.22*	12.57*	16.27	1.508	0.004	64,000	7.7	61,151	7.68	23
40	Aldehyde dehydrogenase	U12761	0.429*	0.785	0.887	0.1358	0.023	58,000	6.2	54,825	6.37	24
43	Flotillin-1	O75955	1.69*	1.24*	2.91	0.277	.001	49,00	6.8	47,355	7.08	9
44	Mitochondrial NADH ubiquinone oxidoreductase	X14338	2.31	1.51*	2.31	0.289	.034	48,000	5.9	48,947	5.85	25
45	Mitogen-activated protein kinase kinase kinase 9	P80192	0.38	0.11*	0.32	0.055	.006	46,000	7.7	44,975	-	26
46	Ubiquitin ligase protein DZIP3	Q86Y13	0.59*	0.45*	0.84	0.109	.019	46,000	5.3	35,240	5.54	27
48	ATPase, H+ transporting, lysosomal 42kD, V1 subunit C, isoform 1	BC010960	0.33	0.23*	0.39	0.047	.053	43,500	6.3	43,942	7.02	9
49	Creatine Kinase M chain	AF120106	21.08*	25.56	27.95	2.133	.030	43,000	7.0	42,971	6.63	28
51	Eukaryotic translation initiation factor 2 subunit 2	P20042	0.83*	0.47	0.58	0.131	.014	40,000	5.7	38,388	5.60	29
53	Band 6 polypeptide B6P	S75710	0.12*	0.16*	0.29	0.045	.012	39,000	6.2	33,091	6.96	30
54	Inositol polyphosphate 1-phosphatase	M55916	0.19	0.09*	0.22	0.033	.011	38,000	5.8	43,931	5.25	31
56	Glandular kallikrein K9	P15949	0.70*	0.37	0.48	0.078	.003	26,000	7.5	26,323	7.18	32
58	Prohibitin	BT007411	1.14*	0.88*	0.18	0.075	<.001	25,000	5.5	29,804	5.57	14
59	Insulin-like growth factor binding protein 4	Q28893	2.27*	2.82	2.96	0.229	.044	23,000	6.9	25,869	6.66	33

* Indicates significantly different to control.

Discussion

The *Semitendinosus* muscle samples used in this experiment were taken from lambs bred for enhanced genetic potential for muscling, growth or neutral genetic potential for muscling or growth[6]. Hegarty and co-workers[6] demonstrated in this lamb group, that breeding for enhanced genetic capacity for muscling resulted in increased hind-quarter weight and crude protein content at a high plane of nutrition compared to both control and growth lambs.

Protein expression associated with muscling genotype

Changes in net protein accretion can occur through reduction in protein turnover, or increase in protein synthesis. Differences in hind-limb muscle development between M lambs and C and G lambs were reflected in protein expression in the NUC, CYT and TCM fractions. The ribosomal biogenesis protein BOP1 is a nuclear protein involved in blocking cell proliferation through inhibition of ribosomal RNA processing[34]. Reduced inhibition of ribosomal biogenesis through reduction in BOP1 levels in M lambs is consistent with increased capacity for protein synthesis and increased protein deposition in M lambs. The endoplasmic reticulum protein Eukaryotic translation initiation factor 2 subunit 2 (eIF-2; identified from the TCM fraction), functions in the early steps of protein synthesis by forming a ternary complex with GTP and initiator tRNA[29]. Increased expression of eIF-2, a biomarker of protein anabolism[35] is supportive of elevated protein synthetic capacity in M lambs.

Identification in the NUC fraction of vimentin and desmin, structural proteins associated with cytoskeletal intermediate filaments, indicates that the nuclear enriched fraction also contained some structural components of the muscle cell. Increased expression of vimentin and desmin has been linked to development of mature myofibres and to construction and reorganisation of skeletal muscle cells following injury[36]. Elevated expression of desmin and vimentin in M lambs, therefore, supports enhanced development of mature skeletal muscle fibres in M lambs. Detection of elevated levels of myosin heavy chain Myh10 protein fragment in the cytosolic fraction of M lambs is consistent with increased effort being directed towards muscle cell hypertrophy and protein deposition in M lambs.

Prohibitins are thought to inhibit DNA synthesis and repress cell proliferation[37]. Prohibitin 2 has been shown to specifically repress MyoD and MEF2 dependent gene transcription[38]. MEF2 is essential for muscle differentiation, with MyoD and MEF2 involved in myogenic transcription activation and myogenesis. Increased expression of prohibitin in M lambs, therefore, could be related to down regulation of myogenic cell proliferation in M lambs compared to C lambs. This would be consistent with muscle development in M lambs arising from increased hypertrophy in mature muscle cells and not from increased incorporation of activated myoblastic cells into skeletal muscle fibres. NF kappaB is thought to interfere with terminal myogenic differentiation of myoblastic cells[39]. Increased expression of NF kappaB essential modulator in M lambs, a transcriptional regulator that activates NF kappaB transcription factor following modification by ubiquination[13], also supports a shift in control of the myogenic process in M lambs. Reduced expression of the hypothetical protein DKFZp434H055DNA and C7orf31 protein was also observed in M lambs. Although these two proteins are predicted to be DNA binding proteins, their function in the nucleus is not known.

Ribosomal biogenesis can account for half of transcriptional and translational expenditure of cells [40], hence elevation in ribosomal activity to support increased protein synthetic capacity could also be associated with increased energy expenditure. However, down regulation of heat shock protein 70kDa precursor 9B (Hsp70-9B) was observed in M lambs. Heat shock protein expression in skeletal muscle has been associated with response to exercise and with change in fibre type[41]. Expression of Hsp70's in the developing diaphragm was related to adaptation towards a slower, more metabolically efficient muscle phenotype[42]. Hence, the metabolic profile of M lambs may be altered to counter increased energy expenditure associated with elevated ribosomal activity.

Insulin-like growth factor binding proteins (IGFBPs) prolong the half-life of the IGFs and have been shown to both inhibit and stimulate the growth promoting effects of the IGFs in cell culture[33]. IGFBPs alter the interaction of IGFs with their cell surface receptors. Specifically, IGFBP-4 has been shown to inhibit the interaction of IGF-1 with its receptor in skeletal muscle, with stabilisation of IGFBP-4 inhibiting IGF-1 mediated muscle growth[43]. IGF-1 increases muscle protein accretion in the ovine hind-limb[44] through inhibition of proteolysis and reduction in protein degradation rates[45]. Reduced

expression IGFBP-4 has also been also associated with shift in fibre type towards fast-twitch glycolytic muscle fibres (IIb) in rat masseter muscle in response to change in muscle use and exercise load[46]. Muscle hypertrophy in other ovine hypertrophy genotypes has been linked to shift towards enhanced growth of fast-twitch glycolytic fibres (type IIb). The most extreme of these muscling genotypes, the *callipyge* muscling genotype, is associated with increase in size and number of IIb fibres[47]. Reduced expression of IGFBP-4 in the TCM fraction of M lambs, therefore, is consistent with increased bioactivity of IGF-1 in M lambs and stimulation of muscle growth and protein accretion through IGF-1 mediated mechanisms.

Reduction of the E3 ubiquitin ligase protein DZIP3 in the TCM fraction indicates a down-regulation of ubiquitin-dependent protein degradation in M lambs. E3 ubiquitin ligase proteins mediate ubiquitination and subsequent proteasomal degradation of target proteins. E3 ubiquitin ligases accept ubiquitin from an E2 ubiquitin-conjugating enzyme in the form of a thioester and then directly transfer the ubiquitin to targeted substrates. Hence, down regulation of DZIP3 suggests that increased protein synthetic capacity in M lambs may be accompanied by reduced protein turnover, providing greater net gains in protein accretion and savings in energy expenditure in renewal of degraded proteins.

Elevation of the cytosolic glycolysis pathway enzymes, phosphoglucomutase 1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in M lambs, suggests an increase in glycolytic capacity in concert with enhanced protein deposition. This is consistent with down regulation of the membrane associated N-acetylglucosaminyltransferase IVa in the TCM fraction, suggesting sparing of amino acids from entry into the glycolytic pathway thereby providing substrate for protein synthesis. Increased expression of uridine 5'-monophosphate synthase, the enzyme responsible for undertaking the final step in pyrimidine biosynthesis, producing uridine 5'-monophosphate which can be shunted towards the urea or citric acid cycle, could indicate redirection of pyrimidines from nucleic acid biosynthesis towards provision of energy substrates in M lambs. Elevated creatine kinase M chain abundance, a mitochondrial enzyme that catalyses the ATP dependent phosphorylation of creatine, suggests increased capacity for provision of energy substrate for contraction in M lambs to support increased myofibrillar mass.

Down regulation of aldehyde dehydrogenase, a mitochondrial enzyme with high activity for oxidation of reactive aldehydes and oxidation of acetaldehyde to acetate as an end product of glycolysis was observed in M lambs. Under conditions of oxidative stress, modification of glycolytic enzymes including aldehyde dehydrogenase and GAPDH by S-thiolation (formation of mixed disulfides with low molecular weight thiols) may influence activity of these key glycolytic enzymes[48]. Oxidative stress is also a potent inhibitor of protein synthesis in skeletal muscle[49]. This inhibitory effect may be mediated through oxidation of cysteine residues via S-thiolation modification of translation factors or through reducing the expression of protein synthetic machinery[48]. With regard to M lambs, where evidence has been presented for enhanced glycolytic and protein synthetic capacity, the control of these processes may be influenced on a broader metabolic scale through a shift in cellular redox state.

Glandular kallikrein K9 is a member of the kallikrein gene family[50], a group of trypsin like proteases who's activity modifies other proteins such as kininogen to form bradykinin, a vasoactive peptide involved in local regulation of blood flow. Kallikrein expression has been demonstrated in kidney and pancreas and in cardiac and skeletal muscle, where they are thought to influence blood flow and contraction[50]. Increased expression in M lambs may provide capacity for enhanced perfusion of skeletal muscle to improve supply of substrates for enabling increased muscle growth and provide fuel for contraction.

Reduced expression of plasma membrane associated structural proteins band 6 polypeptide (B6P) and flotillin-1 was seen in the TCM fraction of M and G lambs. B6P is a constituent of desmosomal plaques, which order keratin in intermediate filaments and provide adhesion points for anchoring of the intermediate filaments with the cell membrane[51]. Flotillin-1 is a membrane scaffolding protein associated with caveolae, small indentations of the plasma membrane associated with signal transduction and vesicular transport, found abundantly in terminally differentiated cells such as skeletal muscle cells[52]. Reduction in both flotillin-1 and B6P in M and G lambs indicates reduced structural re-arrangement and stabilisation of the plasma membrane in muscle cells of these genotypes. This may reflect reduced remodelling of the plasma membrane associated with reduced

cell surface interaction with activated myoblastic cells in M lambs or reduced structural rearrangement of the cell surface of myofibres in M and G lambs.

Protein expression associated with growth genotype

Hegarty and co-workers[6] demonstrated in these lambs, that breeding on the basis of high EBVs for growth produced no increase in lean muscle mass or crude protein in the hind-limb of lambs fed at a high plane of nutrition. Few differences in protein expression in the NUC fraction were observed in response to breeding for high growth EBV's. Reduced expression of the DNA binding protein, the hypothetical protein DKFZp434H055DNA and C7orf31 protein was observed in G lambs, however, the function and activity of these proteins is unknown.

The majority of proteins identified in G lambs that were differentially expressed were involved in undertaking or controlling metabolic functions. Similar to M lambs, a shift in expression of phosphoglucomutase isoforms from PGM2 to PGM1 was observed in G lambs. Phosphoglucomutase performs conversion of glucose 1-phosphate to glucose 6-phosphate. PGM2 is known to be a substrate of the Ca^{2+}/calmodulin (CaM)-dependent protein kinase of junctional sarcoplasmic reticulum[53]. Interaction of PGM2 with sarcoplasmic reticulum membranes reduces PGM2 activity, with sarcoplasmic reticulum localised PGM2 thought to be involved in regulation of energy supply to Ca^{2+} ATPase for excitation-contraction coupling. Reduced cytosolic PGM2 could reflect localisation of PGM2 to sarcoplasmic reticulum, with increased abundance of PGM1 in the cytosolic fraction suggestive of increased cytosolic glycolysis potential as seen in M lambs. Increased expression of uridine 5'-monophosphate synthase and reduced expression of N-acetylglucosaminyltransferase IVa (as seen in M lambs), indicates greater capacity for shunting of pyrimidines from nucleic acid biosynthesis and sparing of amino acids from provision of energy substrates in G lambs. Hence, up-regulation of key glycolysis enzyme levels may provide increased capacity for energy provision for growth in G lambs through glucose dependent pathways.

Mitochondrial NADH ubiquinone oxidoreductase is component of the mitochondrial respiratory complex I, involved in electron transport. Studies in rats have demonstrated that levels of nutrition influence mitochondrial respiratory activity, specifically through complex I[54]. Under nutrition reduced levels of mitochondrial oxygen consumption and resulted in a shift in cellular energy balance. Reduction in NADH ubiquinone oxidoreductase, therefore, suggests a decreased mitochondrial capacity for oxidative phosphorylation in the mitochondria of G lambs in concert with increased capacity for glycolytic production of ATP. However, elevated passage of glucose derived substrates from glycolysis and pyrimidine derived substrates from pyrimidine biosynthesis to the Krebs cycle may generate excess NADH if oxidative phosphorylation is down-regulated in G lambs. Alterations in mitochondrial and glycolytic metabolism in under nutrition are also associated with transition in myosin heavy chain isoforms towards type I isoforms[54], which are more energetically efficient and have higher oxidative capacity[42]. These results indicate that energy generation and utilisation for growth in G lambs may be more efficient than in controls. This energetic advantage may be important during periods of low nutrition, where metabolism in G lambs may enable more rapid adaptation to environmental conditions and provide for more efficient use of energy for maintenance and growth.

The MAPK signalling pathways are a network of phosphorylation cascades that link transcriptional activity to cellular stress[55]. Mitogen-activated protein kinase kinase kinase 9 (MAP3K9), identified in the TCM fraction, is a serine/threonine kinase responsible for phosphorylation of a dual-specificity mitogen-activated protein kinase kinase (MAPKK). MAPKK in-turn regulates MAPK activity by specific phosphorylation. The MAPK signalling pathway has been linked to control of glucose uptake in skeletal muscle through regulation of insulin sensitivity. Specifically, the major negative control of insulin sensitivity results from phosphorylation of serine/threonine residues on the receptor and/or insulin receptor substrate proteins[56]. Hence, down regulation of MAP3K9 may reduce activity of the MAPK signalling pathway, thereby increasing insulin sensitivity and glucose uptake in skeletal muscle of G lambs. Increased glucose uptake in G lambs is consistent with the shift in metabolic profile of G lambs towards enhance glycolytic capacity.

Inositol polyphosphate 1-phosphatase (INPP1) is a component of the phosphatidylinositol second messenger-signaling pathway, catalysing the removal of a phosphate group from phosphatidylinositol. Glucose transport, the rate limiting step in glucose metabolism in skeletal muscle, is mediated by

insulin-sensitive glucose transporter 4 (GLUT4) and can be activated in skeletal muscle by insulin or by exercise[57]. Stimulation of phosphatidylinositol 3-kinase (PI3K) is a crucial step in stimulating glucose transport activity in response to insulin mediated increases in glucose uptake[58]. Reduced INPP1 expression may influence the phosphatidylinositol-signalling pathway such that PI3K dependent glucose transport regulation is elevated in G lambs.

Pleckstrin and Sec7 domain containing protein 3 (PS73) is a guanine exchange factor (GEF) for ADP-ribosylation factor guanine nucleotide factor 6 (ADRF6)[18]. FGD2 is a Rho protein GEF, also with a pleckstrin homology domain[9]. GEFs regulate the activity of the small GTP binding proteins, such as ADP-ribosylation factor and Rho-GTPase, by cycling them through active GTP bound states to inactive GDP bound states[59]. This process regulates many intracellular processes, mainly intracellular signalling and vesicular trafficking. Recently, a GEF for ADRF6 was demonstrated to play a critical role in regulating myoblast fusion[60]. Down-regulation of PS73 and FGD2, therefore, suggests that a GTP mediated cell signalling is down-regulated in G lambs. This may have implications for cell signalling, particularly with regard to myoblast fusion in the muscle growth process. Thioredoxin domain-containing protein (TXN9) is an ATP binding protein with predicted involvement in regulation of cell differentiation. Thioredoxins primarily function as general protein disulfide oxidoreductases, catalysing the oxidation of protein disulfides. In a non-mammalian system, thioredoxin-containing protein regulation of cell differentiation has been associated with secretory vesicle signalling, including interaction with GTPase mechanisms[61]. Flotillin-1, a membrane scaffolding protein associated with caveolae, small indentations of the plasma membrane involved in signal transduction and vesicular transport, is found abundantly in terminally differentiated cells such as skeletal muscle cells[52]. Considering the observed down-regulation of GEF proteins, increased expression of TXN9 and down-regulation of flotillin-1 in G lambs, considerable evidence is provided that a shift in cell signalling through the GTPase regulated vesicular mechanisms has occurred in response to increasing genetic capacity for growth.

Skeletal muscle LIM-protein 1 (SLIM1) is a structural protein with diffuse cytoplasmic expression and localised concentration around focal adhesions[62]. Elevated expression of SLIM1 in G lambs may be the result of membrane stabilisation through reduced secretory vesicle transport as indicated by a reduction in Flotillin-1 protein expression.

As seen in M lambs, prohibitin expression was elevated in G lambs, indicating repression of DNA synthesis and cell proliferation in muscle compared to C lambs[37]. This was thought to be consistent with greater muscle development in M lambs arising from increased hypertrophy in mature muscle cells and not from increased incorporation of activated myoblastic cells into skeletal muscle fibres[38]. In G lambs, although muscle mass in the hind-limb was not found to differ at high plane of nutrition[6], muscle mass may be maintained at a lower level of myoblastic cell proliferation and myonuclear turnover. Reasons for increased abundance of Lamin B3 in the cytosol in G lambs is unclear. Lamin B3 is a nuclear lamina protein, localised to the nucleoplasmic side of the nuclear envelope and is expressed during nuclear restructure associated with changes in nuclear morphology[19]. Detection of increased Lamin B3 in G lambs may be indicative of enhanced synthesis of Lamin B3 for maintenance of nuclear structure in myofibres.

Down-regulation of E3 ubiquitin ligase protein DZIP3 in the TCM fraction indicates a down-regulation of ubiquitin-dependent protein degradation in G lambs. Down regulation of ATPase, H+ transporting, lysosomal 42kD, V1 subunit C, isoform 1 in G lambs is also indicative of reduced lysosomal capacity for protein degradation in G lambs. In light of apparent shifts in energy metabolism, reduction in protein degradative pathways appears to offer G lambs an additional energetic efficiency advantage over C lambs.

Conclusions

Differences in hind-limb muscle development between M lambs and C and G lambs were reflected in changes in protein expression that were related to shifts in protein and energy metabolism. Increased protein accretion in M lambs is suggested to be via increased protein synthetic capacity and muscle cell hypertrophy accompanied by elevated IGF-1 sensitivity and transition towards glycolytic fibre type. Despite no apparent difference in hind-limb muscle growth in G lambs compared to C, G lambs exhibited marked differences in regulation and function of energy metabolism. Evidence presented

here demonstrates a shift towards improved efficiency of glucose metabolism in G lambs, supported by changes in insulin sensitivity signalled through GTPase and phosphatidylinositol related pathways concomitant with elevated expression of proteins involved in glucose metabolism.

Success in achieving objectives

All project objectives were completed successfully as described below.

Objective 1; Skeletal muscle samples were obtained from the semitendinosus muscles of male lambs from M, G and C lambs of MLA Project No. MS.002. Muscle samples were enriched for subcellular fractions of interest to enable detection of changes in protein expression in lower abundance proteins.

Objective 2; Proteomics technologies were developed at PIRVic for high-throughput analysis of muscle proteins using 2DE. Protein identification utilised MALDI MS facilities at the University of Melbourne Botany Department.

Objective 3; Ovine bioinformatics know-how was developed using public domain mammalian protein and genome sequence information and PIRVic and University of Melbourne bioinformatics software and computing facilities.

Objective 4; Differentially expressed proteins from the semitendinosus muscle of male lambs from the M, G and C genotypes were identified by MALDI MS peptide mass fingerprinting.

Objective 5; Information on the nature of the proteins identified from the semitendinosus muscle that are differentially expressed in response to breeding on the basis of genetic capacity for muscling and growth and their role in muscle development was detailed.

Impact on Meat and Livestock industry

The research presented here details shifts in metabolic and regulatory pathways associated with genetic capacity for muscling and growth in lambs. This information is of immediate use to the meat and livestock industry through furthering our understanding of the molecular mechanisms that underlie genetic selection for muscling and growth rate.

These results presented here detail pathways than can be specifically targeted to enhance muscle accretion and growth in lambs. Furthermore, development of mechanisms and treatments to specifically target these processes over coming years may yield greater gains in muscle accretion and growth rate than breeding on the basis for genetic capacity alone.

These findings provide candidate genes from which to scan the current industry flock for sequence variation. Defining the chromosomal location of this list of genes in light of current information on chromosomal location of quantitative trait loci (QTL) for muscling and growth rate may provide opportunities to seek single nucleotide polymorphism's within single genes that associate with these traits. Hence, this research has presented and opportunity to leverage further intellectual property from existing QTL projects.

Validation of differential protein expression of the proteins identified here through the use of antibodies specific for the individual proteins would provide more confidence in the findings relating to individual proteins. However, given the consistency of trends defined within each of the muscling and growth genotypes, a degree of confidence in the importance of the biological pathways presented here in influencing the traits of interest can be gained.

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