

Characterisation of the Ovine Model of McArdle's Disease: Development of Therapeutic Strategies.

Kendall Rae Walker BSc (Hons.)

**Department of Veterinary Biology and Biomedical Sciences
Murdoch University**

**"This thesis is presented for the degree of Doctor of
Philosophy of Murdoch University."**

2006

Declaration

I declare that this thesis is my own account of my research and contains as its main content work that has not previously been submitted for a degree at any tertiary education institution.

Kendall Rae Walker

Abstract**Characterisation of the Ovine Model of McArdle's Disease: Development of Therapeutic Strategies.**

McArdle's disease (OMIM 232600) is one of the most common glycogen storage diseases affecting skeletal muscle. It is inherited in an autosomal recessive manner and is caused by a defect in the muscle glycogen phosphorylase gene (PYGM) (Ch 11q13), a key metabolic enzyme. As a result, patients are unable to mobilise muscle glucose stores to provide the energy for muscle contraction; and suffer from exercise intolerance (myalgia, tachycardia, breathlessness and early fatigue) as well as cramps / contractures and occasionally rhabdomyolysis and myoglobinuria after bouts of strenuous exercise. Histopathologically, McArdle's disease is characterised by an increase in subsarcolemmal storage of glycogen and reduced or absent glycogen phosphorylase activity. McArdle's disease displays significant genetic heterogeneity, with 47 different disease-causing mutations in PYGM having been identified in human sufferers. In keeping with this genetic heterogeneity, significant molecular heterogeneity is observed in patients with regard to the effect of the disease-causing mutation on the PYGM mRNA transcript. In contrast, little biochemical heterogeneity is associated with the disease, with approximately 90% of human patients having no residual pygm protein and therefore no enzyme activity.

A naturally occurring ovine model of McArdle's disease was identified a number of years ago in a flock of merino sheep in Western Australia. The disease causing mutation responsible for ovine McArdle's disease was identified and published in 1997 by Tan *et al.*, (1997) [1]. The mutation occurs in the 3' acceptor splice-site of intron 19 of the ovine PYGM gene, resulting in the activation of a cryptic splice-site site in exon 20 and the premature termination of the transcript. Hypothetically this mutant pygm protein should be 31 amino acids smaller than the wild-type. When I began my PhD, it was known that the ovine sufferers displayed exercise intolerance and histological

examination of affected muscle revealed an excess subsarcolemmal storage of glycogen and absent phosphorylase, as occurs in their human counterparts. Due to their similarities in muscle mass to humans and the relative ease and low cost of maintenance, the ovine model of McArdle's disease is an important and highly relevant test-bed for therapeutic strategies.

This thesis can essentially be divided into two parts:

1) The characterisation of the ovine model of McArdle's disease-

In particular, determining the effect of the disease-causing mutation on both the PYGM mRNA and protein, characterising the sequences of the ovine glycogen phosphorylase isoforms and their developmental and tissue specific expression patterns, as well as determining the identity and the activity of the glycogen phosphorylase isoforms expressed in certain muscle groups (cardiac, extraocular and smooth muscles) which appear to be protected from the disease.

Based upon this data and making use of the ovine model;

2) Development of therapeutic strategies-

A number of potential therapeutic strategies were investigated including:

- The upregulation of a functionally related gene, through the re-expression of non-muscle isoforms of glycogen phosphorylase via notexin induced muscle regeneration.
- As well as the replacement of the PYGM gene using modified adenovirus 5 (AdV5) and adeno-associated virus 2 (AAV-2) vectors.

Based upon the ability of the non-muscle isoforms of glycogen phosphorylase to function in-vivo in McArdle's muscle in the absence of the muscle isoform, the ability of tributyrin (a butyrate prodrug and potent histone deacetylase inhibitor) to re-express brain glycogen phosphorylase in mature mouse muscle was also investigated.

Acknowledgements

I would like to thank my principal PhD supervisor, Prof. Nigel Laing, for his supervision throughout this PhD and for giving me the freedom to follow my ideas and to “suck it and see”. I would also like to thank him for his financial support, without which I could not have completed this PhD thesis.

I would like to thank my co-supervisor Prof. John Howell for allowing me to be a part of the viral gene therapy and muscle regeneration studies.

I would like to thank Dr Kristen Nowak for her unerringly positive and cheery disposition, even in the face of computer crises! My fellow PhD student, Ms Danielle Dye, thanks for sharing the “PhD experience” with me! Your friendship and support made all the difference in the retention of my sanity!

I would like to thank Dr. Anthony Akkari for all his support and guidance throughout my PhD, and for having the ability to make me laugh on a regular basis.

I would also like to thank my parents for their support, which allowed me to pursue a postgraduate degree. Thanks for all the bills you paid, the loans and the numerous car repairs and for not despairing (overmuch!) at the prospect of your daughter becoming a “professional student”!

*"The very powerful and the very stupid have one thing in common. Instead of altering their views to fit the facts, they alter the facts to fit their views... which can be very uncomfortable if you happen to be one of the facts that needs altering."
- Dr Who*

Animal Experimentation

All animal experimentation carried out for this thesis work, was performed with the approval of Murdoch University and the University of Western Australia's animal ethics committees. Animal care and experimentation were performed according to the relevant guidelines set out by Murdoch University, University of Western Australia and the National Health and Medical Research Council of Australia. The sheep used in this PhD work were housed at the Murdoch University Farm, and all experimentation was carried out at Murdoch University. The rodents used in this PhD work were housed at the University of Western Australia's animal house, and all experimentation was carried out at the University of Western Australia.

Ethics Approval Numbers

Murdoch University:

740R/99

873R/01

1097R/04

1021R/03.

University of Western Australia:

00/100/111

01/100/137

List of Abbreviations

- AMP:** Adenosine monophosphate
- BAC:** Bacterial artificial chromosome
- bp:** Base pairs
- cDNA:** Complementary DNA
- CFII:** Cystic fibrosis type II
- CFTR:** Cystic fibrosis transmembrane regulator
- cfu:** Colony forming units
- CnBr:** Cyanogen Bromide
- cpm:** Counts per minute
- cRNA:** Complementary RNA
- DAPI:** 4',6-diamidino-2-phenylindole
- DMSO:** Dimethylsulfoxide
- dATP:** 2'-deoxyadenosine 5'-triphosphate
- dE:** Embryonic gestation (days)
- dNTP's:** deoxynucleotide-triphosphate
- dPN:** Post-natal age (days)
- DTT:** Dithiothreitol
- EDL:** Extensor digitorum longus
- EI:** Exercise intolerance
- FDA:** Food and drug administration (USA)
- G-1-P:** Glucose-1-phosphate
- GC:** Gas chromatography
- GC/MS:** Gas chromatography / Mass spectrometry
- GCOS:** Genechip® operating software
- HDAC:** Histone deacetylase
- h.p.i.:** Hours post infection
- HPRT:** Hypoxanthine-guanine phosphoribosyl-transferase

HRP: Horse-radish peroxidase

IEF: Isoelectric focusing

IgG: Immunoglobulin G

IMP: Inosine monophosphate

IVT: *In-vitro* transcription

kDa: Kilodaltons

K_m: Michelis-Menten constant

MMLV: Moloney murine Leukaemia virus

MOI: multiplicity of infection

MOPS: 3-(N-morpholino) propanesulfonic acid

MPL-TDM: Monophosphoryl-lipid A + Trehalose dicorynomycolate

mRNA: messenger RNA

NADP: Nicotinamide adenine dinucleotide phosphate

NPA: Nuclease protection assay

nt: Nucleotide

³²P: Phosphorous-32 isotope

³¹P MRS: Magnetic resonance spectroscopy

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffered saline

PBS-T: Phosphate buffered saline/0.1% Tween-20

PCR: Polymerase chain reaction

Pfu: Plaque forming units

P_i: Inorganic phosphate

PYGB: Brain glycogen phosphorylase DNA

pygb: Brain glycogen phosphorylase protein

Pygb: Murine brain glycogen phosphorylase gene

PYGM: Muscle glycogen phosphorylase DNA

pygm: Muscle glycogen phosphorylase protein

PYGL: Liver glycogen phosphorylase DNA

pygl: Liver glycogen phosphorylase protein

rAAVCMVPYGM: adeno-associated viral vector with PYGM insert & a cytomegalovirus promoter.

RACE: Rapid amplification of cDNA ends

rAdVCMVPYGM: adeno viral vector with PYGM & a cytomegalovirus promoter

RT: Room temperature

RT-PCR: Reverse transcriptase polymerase chain reaction

S.D.: Standard deviation

SDS: Sodium dodecyl sulfate

Sf9: Spodoptera frugiperda insect cells (serum free adapted)

SMA: Spinal muscular atrophy

SSCP: Single-strand conformational polymorphism.

TAE: Tris acetate EDTA buffer

TBE: Tris borate EDTA buffer

TBS: Tris buffered saline

U: Units of enzyme

UTR: Untranslated region

VLCFA: Very long chain fatty acids

V_{max}: Maximal velocity

X-ALD: X-linked adrenoleukodystrophy

Table of Contents

Declaration	i
Abstract	iii
Acknowledgements	v
Animal Experimentation	vii
List of Abbreviations	ix
Chapter 1.0: An Overview of McArdle's Disease and Glycogen Phosphorylase	3
1.1: Inborn Errors of Metabolism	3
1.1.1: Metabolic Myopathies	3
1.2: McArdle's Disease (Myophosphorylase Deficiency)	11
1.2.1: Unusual Patient Presentations	12
1.2.2: Genetic heterogeneity of McArdle's disease	15
1.2.3: Genotype-Phenotype correlations	18
1.2.4: Exercise Intolerance (EI)	19
1.2.5: Therapy	22
1.2.6: Animal Models	25
1.3: Glycogen Phosphorylase	25
1.3.1: Regulatory mechanisms of glycogen phosphorylase	26
1.3.2: Tissue specific and developmental regulation of the mammalian glycogen phosphorylases	30
1.4: Aims of this PhD Thesis	32
Chapter 2.0: Characterisation of the Ovine Brain (PYGB) and Liver (PYGL) Sequences	37
2.1: Summary	37
2.2: Introduction	37
2.2.1: Glycogen phosphorylase: sequences, homology and evolutionary relationships	37
2.2.2: Aims of Chapter 2	39
2.3: Materials and Methods	40
2.3.1: Determination of the ovine brain glycogen phosphorylase (PYGB) mRNA sequence	40
2.3.1.1: Extraction of total RNA from ovine heart tissue and cDNA synthesis	40
2.3.1.3: Amplification of the 5' region of ovine PYGB cDNA	41
2.3.1.4: Amplification of the 3' region of ovine PYGB cDNA	42
2.3.1.5: Determination of the prevalence of the A/G substitution (at nucleotide position c1249) of the ovine PYGB coding region	45
2.3.2: Determination of the ovine liver glycogen phosphorylase (PYGL) mRNA sequence	46
2.3.2.1: Extraction of total RNA from ovine liver tissue and cDNA synthesis	46
2.3.2.2: Amplification and sequencing of the ovine PYGL coding region	46
2.3.2.3: Amplification of the 5' region of ovine PYGL cDNA	47
2.3.2.4: Amplification of the 3' region of ovine PYGL cDNA	48
2.3.3: Characterisation of multiple 3'UTR transcripts of ovine PYGB and PYGL	51
2.3.3.1: PCR characterisation of multiple 3'UTR transcripts isolated from ovine PYGB and PYGL	51
2.3.3.2: Northern blot analysis of multiple 3'UTR transcripts of ovine PYGB and PYGL	57
2.3.4: Analysis of ovine glycogen phosphorylase cDNA and protein sequences	57
2.3.5: Phylogenetic analysis of glycogen phosphorylases	58
2.3.6: Semi-quantitative PCR analysis of glycogen phosphorylase isoforms in ovine tissues	58
2.4: Results and Discussion	60
2.4.1: PCR amplification of the ovine PYGB mRNA sequence	60
2.4.1.1: PCR analysis of ovine PYGB 3'UTR transcripts	61

2.4.1.2: Northern blot analysis of ovine PYGB 3'UTR transcripts.....	61
2.4.1.3 Prevalence of G/A polymorphism at nucleotide c1249 of ovine PYGB coding region.....	62
2.4.1.4 Conservative polymorphisms in ovine PYGB coding region:.....	63
2.4.2: PCR amplification of the ovine PYGL mRNA sequence.....	63
2.4.2.1 PCR analysis of ovine PYGL 3'UTR transcripts.....	64
2.4.2.2 Northern blot analysis of ovine PYGL 3'UTR transcripts.....	64
2.4.2.3 Conservative polymorphisms in ovine PYGL coding region:.....	67
2.4.3: Characterisation of the ovine glycogen phosphorylase cDNA sequences and predicted protein sequences.....	67
2.4.3.1: Conservation of ligand binding residues in ovine pygb and pygl.....	70
2.4.4: Analysis of non-mammalian and mammalian phosphorylases.....	72
2.4.5: Semi-quantitative PCR analysis of glycogen phosphorylase isoforms in ovine tissues.....	74
2.5: Conclusions and Future Work	91

Chapter 3.0: Expression of the Two Polymorphic Variants of Ovine pygb (Ala416pygb and Thr416pygb) using the Baculovirus Expression System (BEVS).	97
3.1: Summary	97
3.2: Introduction	97
3.2.1: Brain Glycogen Phosphorylase (pygb).....	97
3.2.1.1: Kinetics of pygb.....	98
3.2.2: Recombinant expression of glycogen phosphorylase.....	98
3.2.3: Aims for chapter 3.....	100
3.3: Materials and Methods	101
3.3.1: Insect Cells.....	101
3.3.2: Cloning of ovine 1249A-PYGB (Threonine @ a.a. 416) and 1249G-PYGB (Alanine @ a.a. 416) cDNA:.....	101
3.3.3: Construction of BacPAK8 transfer vector.....	102
3.3.4: Generation of recombinant baculoviruses Ala416PYGB and Thr416PYGB.....	103
3.3.4.1: Confirmation of the identity of the recombinant baculoviruses Ala416PYGB and Thr416PYGB, by immunoblotting with a pygb specific antibody.....	104
3.3.5: Determination of the optimal incubation period for recombinant protein production.....	105
3.3.5.1: SDS-PAGE.....	105
3.3.5.2: Native gel activity assays for glycogen phosphorylase.....	106
3.3.6: Recombinant protein production.....	106
3.3.8: Glycogen phosphorylase assay of purified recombinant Ala416pygb and Thr416pygb.....	107
3.3.9: Statistical analysis of kinetic data.....	108
3.4: Results and Discussion	109
3.4.1: Expression and purification of recombinant Ala416pygb and Thr416pygb proteins using the baculovirus expression system (BEVS).....	109
3.4.1.1: Generation of recombinant Ala416PYGB and Thr416PYGB baculoviruses.....	109
3.4.1.2: Pilot-scale expression and purification of recombinant Ala416pygb and Thr416pygb proteins.....	110
3.4.1.2.1: Characterisation of the optimal incubation period for expression of recombinant Ala416pygb and Thr416pygb proteins.....	110
3.4.1.2.2: Purification of recombinant Ala416pygb and Thr416pygb proteins.....	110
3.4.1.3: Expression and purification of recombinant Ala416pygb and Thr416pygb proteins.....	111

3.4.2: Kinetic analysis of the polymorphic variants Ala416pygb and Thr416pygb.	112
3.5: Conclusions and Future Work	125
Chapter 4.0: Purification of Ovine Muscle (pygm) and Liver (pygl) Glycogen Phosphorylase: Generation of Isoform Specific Antibodies	129
4.1: Summary	129
4.2: Introduction	129
4.2.1: Glycogen phosphorylase antibodies.	129
4.2.2: Purification of muscle (pygm) and liver (pygl) glycogen phosphorylase. ..	130
4.2.3: Aims of chapter 4	131
4.3: Materials and Methods	133
4.3.1: Animal tissues	133
4.3.2: Purification of muscle glycogen phosphorylase (pygm) from ovine skeletal muscle.	133
4.3.3: Purification of liver glycogen phosphorylase (pygl) from ovine liver.	134
4.3.4: Generation of polyclonal antibodies against ovine pygm and pygl.	135
4.3.5: Purification of polyclonal antibodies against ovine pygm and pygl.	136
4.3.5.1: pygb, pygm and pygl coupled sepharose 4B columns	136
4.3.5.2: IgG fractionation	136
4.3.5.4: Cross-adsorption and affinity purification to generate a polyclonal antibody specific for pygl.	137
4.3.6: Production of a pygb specific polyclonal antibody.	137
4.3.7: Dot-blot analysis to determine optimal 1° antibody concentration for western blot analysis.	138
4.3.8: Confirmation of specificity of polyclonal antibodies against pygb, pygm and pygl.	138
4.3.8.1: Native gel activity assay	138
4.3.8.2: Western blot analysis	139
4.3.9: Characterisation of multi-species interaction of polyclonal antibodies	139
4.4: Results and Discussion	140
4.4.1: Purification of pygm.	140
4.4.2: Purification of pygl.	141
4.4.3: Generation of polyclonal antibodies against ovine pygm and pygl	141
4.4.4: Production of a polyclonal antibody against pygb	143
4.4.5: Characterisation of glycogen phosphorylase isoforms by native gel activity assays	143
4.4.6: Confirmation of specificity of polyclonal antibodies against pygb, pygm and pygl.	144
4.4.7: Cross-reaction of glycogen phosphorylase antibodies with other mammalian glycogen phosphorylases.	145
4.5: Conclusions and Future Work	153
Acknowledgements:	154
Chapter 5.0: Characterisation of the Ovine Model of McArdle's Disease	157
5.1: Summary	157
5.2: Introduction	157
5.2.1: McArdle's Disease.	157
5.2.1.1: Molecular genetic heterogeneity of McArdle's disease.	158
5.2.1.2: In-vitro and in-vivo models of McArdle's disease.	158
5.2.2: Aims of Chapter 5.	160
5.3: Materials and Methods	161
5.3.1: Tissue collection.	161
5.3.2: Genotyping of sheep for McArdle's status	161
5.3.3: Northern blot analysis of PYGM mRNA transcript in McArdle's affected semitendinosus muscle.	162

5.3.4: Characterisation of glycogen phosphorylase isoenzyme composition in tissues of normal, carrier and McArdle's affected sheep	162
5.3.4.1: SDS-PAGE and immunoblotting.....	163
5.3.4.2: Native gel activity assay	163
5.3.5: Glycogen Phosphorylase Assays	163
5.3.6: Immunohistochemistry of ovine tissues	164
5.4: Results and Discussion	165
5.4.1: Molecular and biochemical characterisation of the ovine model of McArdle's disease.....	165
5.4.1.1: Molecular and Biochemical consequences of ovine McArdle's disease	165
5.4.1.2: Phosphorylase activity in ovine carrier semitendinosus	167
5.4.2: Glycogen phosphorylase isoform composition in tissues from normal and McArdle's affected sheep.....	167
5.4.2.1: Glycogen phosphorylase isoforms expressed in muscles selectively spared in McArdle's disease.....	169
5.4.2.2: Western blot analysis of normal and McArdle's affected tissues with isoform specific glycogen phosphorylase antibodies.....	172
5.4.2.3: Native gel activity assays of normal and McArdle's affected tissues ..	173
5.4.2.4: Summary of isoforms identified in normal and McArdle's affected tissues:	174
5.5: Conclusions and Future Work	194
Acknowledgements:	195

Chapter 6.0: Time-course of Expression of Glycogen Phosphorylase in Ovine Fetal Skeletal Muscle	199
6.1: Summary	199
6.2: Introduction	199
6.2.1: Growth and Development of the sheep fetus	199
6.2.1.1: Ovine fetal skeletal muscle development	200
6.2.2: Muscle maturation and differentiation.....	201
6.2.2.1: Regulation of muscle glycogen phosphorylase (PYGM) during myogenesis.....	202
6.2.3: Developmental expression of glycogen phosphorylase	203
6.2.4: Aims of Chapter 6	205
6.3: Materials and Methods	207
6.3.1: Mating of ewes to obtain normal fetuses	207
6.3.1.1: Determination of fetal age.....	207
6.3.1.2: Collection of tissue samples from normal fetuses / lambs.....	208
6.3.2: Mating of ewes to obtain McArdle's affected fetuses / lambs	208
6.3.2.1 Collection of tissue samples from McArdle's fetuses / lambs	208
6.3.2.2 Genotyping of fetuses for McArdle's status	209
6.3.3: Northern Blot Analysis of PYGB mRNA in the hind-limb skeletal muscle of normal sheep during ovine fetal development.....	209
6.3.4: Analysis of PYGB and PYGM mRNA transcripts in the hind-limb skeletal muscle of normal sheep during ovine fetal development using nuclease protection assays (NPA).....	210
6.3.4.1: Generation of RNA probes specific for PYGB and PYGM.....	210
6.3.4.2: RPA of fetal ovine hind-limb skeletal muscle using RPA III kit (Ambion)	210
6.3.5: Semi-quantitative RT-PCR analysis of PYGB and PYGM mRNA transcripts in ovine fetal skeletal muscle.....	211
6.3.6: Western Blot Analysis of glycogen phosphorylase isoforms in normal ovine fetuses.....	212
6.3.6.1: Preparation of tissue lysates.....	212
6.3.6.2: SDS-PAGE and immunoblotting.....	212

6.3.7: Native gel activity assays of glycogen phosphorylase isoforms in normal ovine fetuses and lambs.	213
6.3.8: Analysis of glycogen phosphorylase isoforms in the hind-limb skeletal muscle of McArdle's affected fetuses.....	213
6.4: Results	214
6.4.1: Determination of fetal age.....	214
6.4.2: Determination of McArdle's status of 130-day gestation.....	214
6.4.3 Characterisation of the expression of PYGB and PYGM mRNA transcripts in ovine hind-limb skeletal muscle during fetal development.	215
6.4.3.1: Northern blot analysis of ovine fetal skeletal muscle with a PYGB specific riboprobe.	215
6.4.3.2: Nuclease protection assays of ovine fetal skeletal muscle with PYGB and PYGM specific riboprobes.....	215
6.4.3.3: Semi-quantitative RT-PCR analysis of PYGB and PYGM mRNA transcripts in ovine fetal skeletal muscle.....	216
6.4.4: Characterisation of glycogen phosphorylase isoforms expressed in ovine fetal hind-limb skeletal muscle by native gel activity assays.....	216
6.4.5: Western blot analysis of glycogen phosphorylase isoforms in ovine fetal skeletal muscle using isoform specific antibodies.....	217
6.4.6: Analysis of pygb protein levels and enzyme activity in ovine liver and heart throughout fetal gestation.....	217
6.4.7: Analysis of glycogen phosphorylase isoenzymes in McArdle's affected skeletal muscle from fetuses of 130-days gestation, day of birth (0 days post-natal) lambs and 15 day post-natal lambs.....	218
6.5: Discussion	229
6.5.1: Developmental expression of muscle glycogen phosphorylase in ovine fetal skeletal muscle.....	229
6.5.2: Developmental expression of brain glycogen phosphorylase.....	230
6.5.2.1: Developmental expression of brain glycogen phosphorylase in ovine fetal skeletal muscle.....	230
6.5.2.2: Expression of brain glycogen phosphorylase in ovine fetal liver.....	234
6.5.2.3: Expression of brain glycogen phosphorylase in ovine fetal heart.....	234
6.6: Conclusions and Future Work	236
Acknowledgements:.....	238
Chapter 7.0: Investigation of Therapeutic Strategies: Viral Gene Therapy and the Confounding Effect of Regeneration Explored Using Notexin.....	241
7.1: Summary	241
7.2: Introduction	241
7.2.1: Gene therapy and myopathies.....	242
7.2.1.1: Viral based gene therapy for muscle disease.....	243
7.2.1.2: Gene therapy and McArdle's disease.....	245
7.2.2: Muscle regeneration and McArdle's disease.....	246
7.2.2.1: Muscle regeneration.....	247
7.2.3: Aims for chapter 7.....	251
7.3 Materials and Methods	252
7.3.1: Surgical procedures:.....	252
7.3.2: Notexin induced muscle regeneration in McArdle's affected sheep.....	253
7.3.2.1: Notexin administration:.....	253
7.3.2.2: Biopsy collection.....	253
7.3.2.3: Histochemical and immunohistochemical analysis of muscle blocks.....	253
7.3.2.4: Analysis of glycogen phosphorylase isoforms in regenerating muscle fibres.....	254
7.3.3: Expression of human myophosphorylase in the semitendinosus muscle of McArdle's affected sheep using modified adenovirus 5 and adeno associated virus 2.....	254

7.3.3.1: Production of recombinant modified human myophosphorylase adenovirus 5 (rAdVCMVPYGM) and recombinant human myophosphorylase adeno-associated virus 2 (rAAVCMVPYGM).....	254
7.3.3.2: Trials using rAdVCMVPYGM serotype 5.....	255
7.3.3.3: Trials using rAAVCMVPYGM serotype 2.....	255
7.3.3.4: Analysis of Biopsies collected from rAdVCMVPYGM and rAAVCMVPYGM trials.....	256
7.4: Results and Discussion	257
7.4.1: Muscle regeneration in the hind-limb semitendinosus muscle of McArdle's affected sheep after notexin administration.....	257
7.4.1.1: Glycogen phosphorylase expression in notexin induced regenerating muscle.....	258
7.4.2: Intramuscular injection of recombinant modified human myophosphorylase adenovirus 5 (rAdVCMVPYGM) and adeno-associated virus 2 (rAAVCMVPYGM) into McArdle's affected ovine muscle.....	259
7.4.2.1: Trials using rAdVCMVPYGM.....	259
7.4.2.2: Trials using rAAVCMVPYGM.....	260
7.4.2.3: Summary of histochemical analysis of phosphorylase positive fibres in rAdVCMVPYGM and rAAVCMVPYGM trials.....	261
7.4.2.4: β -galactosidase positive controls rAdVCMVLacZ and rAAVCMVLacZ.....	261
7.4.2.5: Cellular response associated with intramuscular injection.....	261
7.4.2.6: Muscle regeneration associated with intramuscular injection.....	262
7.4.2.7: Summary of histochemical analysis.....	262
7.4.2.8: Isoform identification in muscle biopsies from rAdVCMVPYGM and rAAVCMVPYGM trials.....	263
7.5: Conclusions and Future Work	277
Acknowledgements:.....	281
Chapter 8.0: Investigation of Therapeutic Strategies: Attempts to Re-express Brain Glycogen Phosphorylase in Skeletal Muscle using Tributyrin.....	285
8.1: Summary	285
8.2: Introduction	285
8.2.1: Exploitation of gene redundancy as a therapeutic strategy.....	285
8.2.1.1: Pharmacological re-expression of pygb as a therapy for McArdle's disease.....	286
8.2.1.2: Butyrates and gene redundancy.....	288
8.2.2: Aims of Chapter 8.....	291
8.3: Materials and Methods	292
8.3.1: Animal Care.....	292
8.3.2: Experimental Protocol.....	292
8.3.3: Determination of intestinal butyric acid levels by gas chromatography.....	293
8.3.4: Western blot analysis with polyclonal antibodies against brain glycogen phosphorylase (pygb).....	293
8.3.4.1: Tissue lysates.....	294
8.3.5: Microarray analysis of mouse EDL muscle with the Genechip® Mouse Genome 430 2.0 Affymetrix array.....	294
8.3.5.1: RNA isolation and cDNA preparation for array analysis.....	294
8.3.5.2: Biotinylated cRNA hybridisation and analysis.....	294
8.4: Results and Discussion	296
8.4.1: Tolerance of 5% Tributyrin diet by mice.....	296
8.4.2: Analysis of faecal butyrate levels by GC-Mass spec.....	296
8.4.3: Microarray analysis of EDL muscle from mice fed a 5% tributyrin diet.....	299
8.4.4: Effect of a 5% tributyrin diet on the expression of brain glycogen phosphorylase protein in the EDL muscle of mice.....	301
8.5: Conclusions and Future Work	307
8.6: Acknowledgements.....	309

Chapter 9.0: Conclusions and Future Work	313
References:.....	318
Appendix A1: Genbank sequences	355
Appendix A2: ClustalW alignment of ovine glycogen phosphorylase protein sequences.....	359
Appendix A3: Composition of Mouse Feeds.	360
Appendix A4: List of Suppliers	361
Appendix A5: Buffers and Solutions.....	362

List of Figures

Figure 1.1: Schematic representation of the Glycogen Storage Diseases (GSD's). ...	10
Figure 2.3.1: Schematic representation of amplification strategy for ovine PYGB cDNA.....	43
Figure 2.3.2: Sequencing strategy for ovine PYGB coding region.....	43
Figure 2.3.3: Schematic representation of amplification strategy for ovine PYGL cDNA.	49
Figure 2.3.4: Sequencing strategy for ovine PYGL coding region	49
Figure 2.3.5: PCR amplification strategy and probes for northern blot analysis to confirm 3'UTR splicing of ovine PYGB.....	53
Figure 2.3.6: PCR amplification strategy and probes for northern blot analysis to confirm 3'UTR splicing of ovine PYGL.	55
Figure 2.4.1: PYGB 3'RACE products analysed on a 1.5% agarose gel.....	77
Figure 2.4.2: Sequence of ovine PYGB 3'UTR transcript A and transcript B.	77
Figure 2.4.3: PCR analysis of ovine PYGB 3'UTR transcripts on a 1% agarose gel. ...	79
Figure 2.4.4: Illustration of Fragment 3 amplified from ovine cDNA and genomic DNA	79
Figure 2.4.5: Northern blot analysis of 10µg of total RNA with ovine PYGB probe A.	81
Figure 2.4.6: MscI digestion of ovine genomic DNA analysed on a 3% agarose/TBE gel.	81
Figure 2.4.7: Schematic representation of the conservation of Ala416 in mammalian and non-mammalian glycogen phosphorylases.....	81
Figure 2.4.8: PYGL 3'RACE products analysed on a 1.5% agarose gel.	83
Figure 2.4.9: Sequence of ovine PYGL 3'UTR transcript A and transcript B.	83
Figure 2.4.10: PCR analysis of ovine PYGL 3'UTR transcripts on a 1% agarose gel.	85
Figure 2.4.11: Northern blot analysis of 10µg of total RNA with ovine PYGL probe C.	85
Figure 2.4.12: Northern blot analysis of 10µg of total RNA with ovine PYGL probe C.	85
Figure 2.4.13: Phylogenetic analysis of mammalian and non-mammalian glycogen phosphorylase protein sequences.	87
Figure 2.4.14: Analysis of PCR products from semi-quantitative RT-PCR of ovine tissues.....	89
Figure 3.4.1: SDS-PAGE of insect cell lysates used for the double homologous recombination to generate recombinant baculoviruses Ala416PYGB and Thr416PYGB.....	115
Figure 3.4.2: Western Blot using pygb specific polyclonal antibody (1:3,500 dilution).	115
Figure 3.4.3: Native gel activity assay of insect cell lysates used for the double homologous recombination to generate recombinant baculoviruses Ala416PYGB and Thr416PYGB.....	115
Figure 3.4.4: SDS-PAGE of 10µg of insect cell lysates from the time-course experiments.....	117
Figure 3.4.5: Native gel activity assay of insect cell lysates from the time-course experiments.....	117
Figure 3.4.6: Elution profile of crude insect cell lysate on a High-Q anion exchange column.....	119

Figure 3.4.7: Native gel activity assay of recombinant Ala416pygb containing fractions from figure 3.4.6.	119
Figure 3.4.8: Elution profiles of recombinant pygb's on a High-Q (BioRad) anion exchange column.	121
Figure 3.4.9: Elution profiles of recombinant pygb's on a High-Q (BioRad) anion exchange column.	121
Figure 3.4.10: SDS-PAGE of purified recombinant Ala416pygb and Thr416pygb proteins.	121
Figure 3.4.11: Graphical Representation of V/K vs. P_i Concentration ($v = V/K / (1/[S] + 1/K$, with a weighting of $1/S.D.^2$).	123
Figure 3.4.12: Graphical Representation of the effect of P_i on activity of recombinant Ala416PYG and Thr416PYGB (Global fits weighted by $1/S.D.^2$).	123
Figure 4.4.1: Concanavalin-A (Con A) purification of muscle glycogen phosphorylase from ovine semitendinosus muscle.	147
Figure 4.4.2: Elution profile of pygm on High-Q (BioRad) anion exchange column. .	147
Figure 4.4.3: Elution profile of pygm on a Methyl-HIC (BioRad) column.	147
Figure 4.4.4: Concanavalin-A (Con A) purification of liver glycogen phosphorylase from ovine liver.	147
Figure 4.4.5: Elution profile of pygl on High-Q (BioRad) anion exchange column. ...	149
Figure 4.4.6: Elution profile of pygl on High-Q (BioRad) anion exchange column. ...	149
Figure 4.4.7: Native analysis of purified ovine pygm and pygl.	149
Figure 4.4.8: Native gel activity assay of purified ovine pygm and pygl.	149
Figure 4.4.9: Native gel activity assay of 20 μ g of ovine tissue lysates.	149
Figure 4.4.10: Native gel activity assay of 50 μ g of ovine tissue lysates.	151
Figure 4.4.11: Native gel activity assay of 50 μ g of ovine tissue lysates.	151
Figure 4.4.12: Western blot analysis of 20 μ g of ovine tissue lysates to confirm antibody specificity.	151
Figure 4.4.13: Western blot analysis of mammalian tissues with polyclonal antibodies against A) pygb, B) pygm and C) pygl.	151
Figure 5.4.1: Northern Blot Analysis of Total RNA extracted from ovine semitendinosus muscle.	177
Figure 5.4.2: Western Blot Analysis of total protein lysates from ovine semitendinosus muscle.	177
Figure 5.4.3: Native Gel Activity Assay of total protein lysates from ovine semitendinosus muscle.	177
Figure 5.4.4: Kinetic analysis of glycogen phosphorylase activity in normal and carrier semitendinosus muscle.	177
Figure 5.4.5: Native gel activity assay of control tissue lysates.	179
Figure 5.4.6: Kinetic analysis of glycogen phosphorylase activity in normal and McArdle's affected heart.	179
Figure 5.4.7: Native gel activity assay of heart from normal, carrier and McArdle's affected sheep.	179
Figure 5.4.8: Western blot analysis of heart from normal, McArdle's affected and carrier sheep (n=3) with polyclonal antibodies against A) pygb, B) pygm and C) pygl.	181
Figure 5.4.9: 6 μ m frozen section from McArdle's affected extraocular muscle stained for glycogen phosphorylase.	181
Figure 5.4.10: Immunohistochemical analysis of 8 μ m frozen sections of McArdle's affected extraocular muscle with a polyclonal antibody specific for pygb.	181
Figure 5.4.11: Kinetic analysis of glycogen phosphorylase activity in normal and McArdle's affected extraocular muscle.	183
Figure 5.4.12: Native gel activity assay of extraocular muscle from normal, carrier and McArdle's affected sheep.	183
Figure 5.4.13: Western blot analysis of extraocular muscle from normal, McArdle's affected and carrier sheep (n=3) with polyclonal antibodies against A) pygb, B) pygm and C) pygl.	183

Figure 5.4.14: Native gel activity assay of bladder from normal, carrier and McArdle's affected sheep.....	185
Figure 5.4.15: Western blot analysis of bladder from normal, McArdle's affected and carrier sheep (n=1) with polyclonal antibodies against A) pygb, B) pygm and C) pygl.....	185
Figure 5.4.16: Native gel activity assay of intestine from normal, carrier and McArdle's affected sheep.....	185
Figure 5.4.17: Western blot analysis of intestine from normal, McArdle's affected and carrier sheep (n=1) with polyclonal antibodies against A) pygb, B) pygm and C) pygl.....	187
Figure 5.4.18: Western blot analysis of tissues from normal and McArdle's affected sheep using polyclonal antibodies against A) pygb, B) pygm and C) pygl.....	187
Figure 5.4.19: Native gel activity assay of normal and McArdle's affected ovine tissues.....	191
Figure 6.4.1: Crown-rump measurements of ovine fetuses.....	219
Figure 6.4.2: Total RNA extracted from ovine fetal muscle analysed on a 1% agarose/TBE gel.....	219
Figure 6.4.3: Northern blot analysis of ovine fetal skeletal muscle using a P ³² labelled PYGB specific riboprobe.....	219
Figure 6.4.4: Nuclease protection assay of ovine fetal skeletal muscle with riboprobes specific for PYGB (A&B) and PYGM (C&D).....	221
Figure 6.4.5: Analysis of PCR products from semi-quantitative RT-PCR of ovine fetal skeletal muscle.....	221
Figure 6.4.6: Native gel activity assay of 50 µg of ovine fetal skeletal muscle.....	223
Figure 6.4.7: Western blot analysis of ovine fetal skeletal muscle with polyclonal antibodies against A) pygb, B) pygm and C) pygl.....	225
Figure 6.4.8: Western blot analysis of ovine fetal liver with a polyclonal antibody against pygb.....	225
Figure 6.4.9: Native gel activity assay of ovine fetal liver.....	225
Figure 6.4.10: Western blot analysis of ovine fetal heart with a polyclonal antibody against pygb.....	227
Figure 6.4.11: Western blot analysis of ovine fetal skeletal muscle from normal and McArdle's affected sheep with polyclonal antibodies against.....	227
Figure 6.4.12: Native gel activity assay of ovine fetal skeletal muscle from normal and McArdle's affected sheep.....	227
Figure 7.4.1: Native gel activity assay of 50µg of muscle biopsy lysate from notexin injected McArdle's lambs. IEF gel pH 3-10 (BioRad), incubated in 5mM AMP and 0.7M Na ₂ SO ₄ , stained with KI/I ₂	265
Figure 7.4.2: Western blot analysis of 20µg of muscle biopsy lysate from notexin injected McArdle's lambs.....	267
Figure 7.4.3: Serial frozen sections (6µm) of a 10-day semitendinosus muscle biopsy from a notexin injected McArdle's lamb. Stained for glycogen phosphorylase (A) and glycogen (B).....	267
Figure 7.4.4: Graphical representation of the phosphorylase positive fibre counts from the rAdVCMVPYGM trial.....	273
Figure 7.4.5: Graphical representation of the phosphorylase positive fibre counts from the rAAVCMVPYGM trial.....	273
Figure 7.4.6: Native gel activity assay of 50µg of muscle lysate from McArdle's affected lambs injected with AdVCMVPYGM or AAVCMVPYGM vectors.....	275
Figure 7.4.7: Western blot analysis with pygm antibody, of 20µg of muscle lysate from McArdle's affected lambs injected with AdVCMVPYGM or AAVCMVPYGM vectors.....	275
Figure 8.4.1: A comparison of the average weights of the mice fed the control and the test diet.....	296
Figure 8.4.2: Western blot analysis of 20µg of total protein extracted from mouse extensor digitorum longus (EDL) muscle with a pygb specific antibody (1:10,000diln).....	301

List of Tables

Table 1.1: Summary of 15 types of Glycogen Storage Disease (GSD).....	6
Table 1.2: A summary of the mutations identified in the PYGM gene in human McArdle's patients.	17
Table 2.3.1: PCR conditions for the amplification of ovine PYGB cDNA fragment.....	41
Table 2.3.2: Ovine PYGB sequencing primers.....	41
Table 2.3.3: PCR conditions for 5' Rapid Amplification of cDNA ends (RACE) of ovine PYGB 5'UTR.	42
Table 2.3.4: PCR conditions for 3' Rapid Amplification of cDNA ends (RACE) of ovine PYGB 3'UTR.	42
Table 2.3.5: PCR conditions for the amplification of ovine PYGL cDNA fragment.	47
Table 2.3.6: Ovine PYGL sequencing primers.....	47
Table 2.3.7: PCR conditions for 5' Rapid Amplification of cDNA ends (RACE) of ovine PYGL 5'UTR.....	48
Table 2.3.8: PCR conditions for 3' Rapid Amplification of cDNA ends (RACE) of ovine PYGL 3'UTR.....	48
Table 2.3.9: PCR primers used to characterise the ovine PYGB 3'UTR transcripts....	53
Table 2.3.10: PCR primers used to characterise the ovine PYGL 3'UTR.....	55
Table 2.3.11: Probes used in northern blot analysis to characterise multiple 3'UTR transcripts generated with 3'RACE.....	57
Table 2.3.12: Conditions employed in Semi-quantitative PCR of PYGB, PYGM, PYGL, HPRT and 18s rRNA.	59
Table 2.4.1: Conservative polymorphisms detected in ovine PYGB coding region.....	63
Table 2.4.2: Polymorphisms detected in ovine PYGL coding region.	67
Table 2.4.3: Homology of published mammalian and non-mammalian glycogen phosphorylases to the ovine phosphorylases.....	69
Table 2.4.4: Glucose-6-Phosphate binding residues.	70
Table 2.4.5: AMP binding residues.	70
Table 2.4.6: Results of semi-quantitative RT-PCR analysis of ovine tissues.	89
Table 3.3.1: Sequences of primers used to sequence PYGB clones.	102
Table 3.4.1: Summary of data generated from the kinetic analysis of the polymorphic variants Ala416pygb and Thr416pygb.....	113
Table 4.3.1: 1° and 2° antibody concentrations used in western blot analysis.	139
Table 5.3.1: 1° and 2° antibody concentrations used in western blot analysis.	163
Table 5.4.1: Glycogen phosphorylase assay of normal and carrier semitendinosus muscle.	177
Table 5.4.2: Glycogen phosphorylase assay of normal and McArdle's affected heart.	179
Table 5.4.3: Glycogen phosphorylase assay of normal and McArdle's affected extraocular muscle.	183
Table 5.4.4: Summary of glycogen phosphorylase isoforms detected in normal and McArdle's affected tissues determined by western blot analysis with isoform specific antibodies.	189
Table 5.4.5: Summary of glycogen phosphorylase isoform activity in normal and McArdle's affected tissues as determined by native gel activity assay in the presence of 5mM AMP and 0.7M Na ₂ SO ₄	193
Table 6.3.1: Conditions employed in semi-quantitative PCR of PYGB, PYGM, HPRT and 18s rRNA.	212
Table 7.4.1: Summary of results of phosphorylase positive fibres following notexin injection into the hind-limb semitendinosus muscle of 16 McArdle's affected lambs and 4 mature McArdle's affected sheep.	265
Table 7.4.2: Results of histochemical analysis of McArdle's lambs (2-7 days old) injected with AdVCMVPYGM.	269

Table 7.4.3: Histochemical analysis of McArdle's lambs injected with AdVCMVLacZ positive control.	269
Table 7.4.4: Results of histochemical analysis of McArdle's sheep injected with AAVCMVPYGM.	271
Table 7.4.5: Histochemical analysis of carrier lambs injected with AAVCMVLacZ positive control.	271
Table 8.3.1: Antibodies and antibody dilutions used in western blot analysis.	294
Table 8.4.1: Analysis of intestinal butyrate levels in mice as determined by gas chromatography/mass spectrometry.	298
Table 8.4.2: Genes up-regulated >2 fold by microarray analysis with GeneChip® Mouse Genome 430 2.0 Affymetrix array, in mice fed 5% tributyrin diet.	303
Table 8.4.3: Genes down-regulated >2 fold by microarray analysis with GeneChip® Mouse Genome 430 2.0 Affymetrix array, in mice fed 5% tributyrin diet.	304