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

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

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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.

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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.

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"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: Immunogobulin 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

^{32}P: Phosphorous-32 isotope

^{31}P 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

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