UNIVERSITY OF LIVERPOOL

The role of microRNAs in the adaptive response of skeletal muscle to contractions during ageing

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By

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Abstract

As the age of the population keeps increasing, age-related disorders are becoming a bigger burden on health services and society as a whole. Sarcopenia is age-related loss of skeletal muscle mass and function that is associated with frailty and loss of independence of older people. There is currently no cure for sarcopenia with only dietary and exercise interventions available to some but not older people.

Skeletal muscle has the ability to adapt to intracellular and extracellular changes, such as exercise. Age-related loss of muscle mass and function is related to impaired adaptive response of muscle to exercise (contractions). However, the molecular mechanisms that regulate muscle adaptations to exercise are not well understood and it is therefore necessary to understand the molecular factors that control muscle adaptive response to design novel interventions against sarcopenia.

MicroRNAs are noncoding RNAs, approximately 20-24 nucleotides that regulate gene expression by binding to 3' untranslated region (UTR) of mRNAs, leading to mRNA degradation and /or inhibition of translation. microRNAs regulate approximately 30% of human genome, therefore they are likely to control muscle homeostasis during contraction. microRNA expression is regulated by extracellular and intracellular changes and microRNAs can regulate several signalling pathways simultaneously.

I hypothesize that microRNAs are a part of muscle adaptive response to contractions by regulating the expression of genes involved in this response and are a part of changes leading to blunted adaptive response of muscle during ageing.

I tested this hypothesis by selecting and validating microRNA candidates regulating the muscle adaptive response using qPCR. I characterised novel microRNA:-target interactions playing a role during muscle adaptive response to contractions *in vitro* (mouse H-2k^{b} myotubes) and validated these *in vivo* (C57BL6 wild type mice). Several microRNAs were validated and miR-181a and miR-181d were chosen for further functional studies due to their early response in muscle contraction and predicted target genes including Sirt-1, Jun and Fos.

I next used microRNA mimics and antagomiRs, to test whether manipulation of microRNA expression *in vivo* can positively affect more efficient adaptation of muscle to exercise. Sirt-1 was predicted as miR-181 target and Sirt-1 was shown to be regulated by miR-181 *in vitro* and *in vivo*. Moreover, treatment of mice with miR-181 regulated the size of the muscles and myofibres, as well as muscle function (strength). This suggests that miR-181 may be an attractive candidate for interventions aimed at ameliorating sarcopenia.

Dedication

To my beloved late sister, Chika Felicia Ugochukwu

Table of Contents

1

Acknowledgements....2

Abstract....3

Dedication....4

Table of Figures....9

List of Tables....13

Abbreviations....14

1. General Introduction....19

1.1. Skeletal muscle structure and function overview....20

1.1.1. Skeletal muscle structure: myofibre types....23

1.1.2. Skeletal muscle mechanical function: contraction....25

1.1.3. The mechanism of muscle contraction....26

1.1.4. Skeletal muscle mechanical function: energy metabolism....30

1.1.5. Types of contraction of skeletal muscle....32

1.1.6. Muscle contraction and reactive oxygen species (ROS)....34

1.1.7. Skeletal muscle adaptive response to contractions and exercise....35

1.1.8. Skeletal muscle metabolic function....37

1.2. Adult skeletal muscle homeostasis....38

1.2.1. Muscle homeostasis: muscle hypertrophy and atrophy....39

1.2.2. Skeletal muscle homeostasis: regeneration....41

1.2.3. Molecular pathways that regulate muscle homeostasis and response to contractions....43

1.3. Skeletal muscle ageing....50

1.3.1. Age-related changes in size and number of muscle fibres....52

1.3.2. Age-related changes in mechanical characteristics of skeletal muscle....53

1.3.3. The role of ROS in muscle ageing....54

1.3.4. Muscle adaptive response to exercise during ageing....55

1.3.5. Molecular changes in muscle during ageing 56
1.4. microRNAs as gene expression regulators in muscle 57
1.4.1. microRNA discovery 58
1.4.2. microRNA biogenesis 59
1.4.3. Prediction of microRNA target genes 61
1.4.4. microRNAs in skeletal muscle 61
1.4.5. microRNAs as regulators of muscle response to exercise 66
1.4.6. microRNAs in skeletal muscle ageing 72
1.4.7. microRNA in muscle disease 74
1.4.8. microRNAs as potential therapeutics against muscle ageing 74
1.5. Hypothesis 77
1.6. Aims and Objectives 77
2. Materials and Methods 79
2.1. Cell Culture 80
2.2. Isometric contraction protocol of H2kb myotubes *in vitro* 82
2.3. Animal maintenance 83
2.4. Isometric contraction protocol of skeletal muscle *in vivo* 83
2.5. Isolation of RNA from H2kb myotubes using Trizol. 84
2.6. Isolation of RNA from skeletal muscle 86
2.7. Reverse Transcription (microRNA). 88
2.8. Reverse Transcription (mRNA) 89
2.9. microRNA expression profiling using Real-Time quantitative polymerase chain reaction (qPCR) 93
2.10. Cryosectioning of skeletal muscle tissue 94
2.11. Preparation of tissue for cryostat sectioning 95
2.12. Preparation of tissue for cryostat sectioning 95
2.13. Tissue Embedding and Sectioning 96
2.14. Preparaton of chrome-alum gelatin coated slides (CAG) 96
2.15. Sirt-1 immunostaining 97
2.16. WGA staining: 98
2.17. MF20 immunostaining of *H-2k^b^* myotubes 99
2.18. Protein isolation from *H-2k^b^* myotubes 100
2.19. Protein content determination using Bichinchoninic Acid (BCA) 101
2.20. Preparation of Polyacrylamide gels. 102

2.21. Electrophoresis of proteins (SDS-PAGE). 103
2.22. Western blotting 104
2.23. Processing and development of nitrocellulose membrane and protein 105
2.24. microRNA and antagomiR treatment of mice 107
2.25. *In situ* force measurement of the EDL muscle. 108
2.26. Muscle fibre analysis 108
2.27. Statistical analysis of qPCR data. 109
3 Changes in microRNA expression in skeletal muscle during contractions and ageing 110
3.1 Introduction 112
3.2 Experimental Methods 116
3.2.1 Contraction protocol 116
3.2.2 RNA Analysis 116
3.2.3. cDNA synthesis and microRNA expression analysis. 116
3.2.4. Statistical analysis 117
3.3 Results 117
3.3.1 microRNA expression in the *tibialis anterior* muscle during ageing and following an isometric contraction protocol. 117
3.4 Discussion 125
4 Expression of microRNAs in contracted and non- contracted myotubes 128
4.1 Abstract 129
4.2 Introduction 129
4.3 Materials and methods 131
4.3.1 Contraction protocol of $H\text{-}2K^b$ myotubes 131
4.4 Results 132
4.5 Discussion 141
5. miR-181: target interactions in the regulation of $H\text{-}2k^b$ myotubes to contractions 145
5.1 Abstract 146
5.2 Introduction 147
5.2 Materials and methods 150
5.2.1. Cell culture 150
5.2.2 Isometric contraction protocol of $H\text{-}2k^b$ myotubes and RNA isolation 150

5.2.3 Reverse transcription (mRNA) and qPCR analysis of miR-181 target gene expression 151
5.2.4 Statistical analysis 151
5.3 Results 151
5.3.1. The effect of miR-181 on myotube size *in vitro* 151
5.3.2. The effects of miR-181 on putative target gene expression 155
5.4 Discussion 163
6. The role of miR-181 in muscle ageing and response to contractions: an in vivo study. 166
6.1 Abstract 167
6.2 Introduction 168
6.2 Materials & Methods 170
6.2.1. miR-181 overexpression and inhibition in the muscles of adult and old mice and isolation of RNA from skeletal muscle 170
6.2.2. Reverse Transcription and qPCR analysis (microRNA). 171
6.2.4. Sirt-1 and WGA immunostaining 171
6.2.5. *In situ* force measurement of the EDL muscle 171
6.2.6. Statistical analysis of qPCR and force data 172
6.3 Results 172
6.4 Discussion 194
7 Discussion 196
7.3 miR-181: target interactions in the regulation of *H-2k*^{b} myotubes to contractions 201
7.4 miR-181 role in muscle response to contractions and ageing *in vivo* 202
7.5 Future perspectives 205
7.5.1. Potential of microRNAs as future biomarkers 205
7.5.2 microRNA as Therapeutics 206
8 Appendix 208
8.1 microRNA-181a, b, c, d sequences 209
References 210

Table of Figures

Figure 1.1 Organisation of skeletal muscle……………………………………………………………...22

Figure 1.2. The mechanism of muscle contraction. ……………………………………….…………..28

Figure 1.3. An overview of energy metabolism in skeletal muscle. ……………………..31

Figure 1.4. Schematic diagram of skeletal muscle contraction…..…………………………..33

Figure 1.5. Schematic representation of the stages of muscle regeneration...........44

Figure 1.6. Schematic representation of molecular mechanisms involved in postnatal control of muscle growth and atrophy. …………………..…………………………50

Figure 1.7. The summary of cellular and molecular changes in muscle fibres contributing to skeletal muscle ageing. ……………..……………………………………………..52

Figure 1.8. The biogenesis of microRNAs. miRNA genes are transcribed by RNA polymerase II or III, in combination with specific transcription factors (TFs) as long primary transcripts (pri-miRNAs). …………………………………………………….…………61

Figure 2.1. Flow chart of RNA extraction using Trizol showing the separation of phases following the addition of chloroform………………………………….……………90

Figure 2.2 Schematic of western blotting setup………………………………………………………...107

Figure 3.1. Changes in miR-181a expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol...................……………………120

Figure 3.2. Changes in miR-181d expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol…………………...………………121

Figure 3.3. Changes in miR-133a expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. …………………..……………..…..121

Figure 3.4. Changes in miR-199a expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. ………………………………….….122

Figure 3.5. Changes in miR-378 expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. ……………………………………….122

Figure 3.6. Changes in miR-21 expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol……………………………………………123

Figure 3.7. Changes in miR-16 expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol…………………..…………….………123

Figure 3.8. Changes in miR-24 expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. ……………………………………….124

Figure 3.9. Changes in miR-130 expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. 124

Figure 3.10. Changes in miR-15 expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. 125

Figure 4.1. The expression of miR-181a following the isometric contraction protocol of *H-2k*^{b} myotubes. 136

Figure 4.2. The expression of miR-181d following the isometric contraction protocol of *H-2k*^{b} myotubes. 136

Figure 4.3. The expression of miR-133a following the isometric contraction protocol of *H-2k*^{b} myotubes. 137

Figure 4.4. The expression of miR-199a following the isometric contraction protocol of *H-2k*^{b} myotubes. 137

Figure 4.5. The expression of miR-378 following the isometric contraction protocol of *H-2k*^{b} myotubes. 138

Figure 4.6. The expression of miR-21 following the isometric contraction protocol of *H-2k*^{b} myotubes...138

Figure 4.7. The expression of miR-16following the isometric contraction protocol of *H-2k*^{b} myotubes. 143

Figure 4.8. The expression of miR-24 following the isometric contraction protocol of *H-2k*^{b} myotubes. 139

Figure 4.9. The expression of miR-130 following the isometric contraction protocol of *H-2k*^{b} myotubes...140

Figure 4.10 The expression of miR-5 following the isometric contraction protocol of *H-2k*^{b} myotubes...140

Figure 5.1. The effect of miR-181 upregulation or inhibition on C2C12 myotube size. 150

Figure 5.2. The effects of miR-181a overexpression and inhibition on *H-2kb* myotube size. 155

Figure 5.3. The effects of miR-181 overexpression and inhibition on the expression of atrogenes in *H-2k*^{b} myotubes................. 156

Figure 5.4. qPCR showing the expression of Jun in *H-2k*^{b} myotubes following an isometric contraction protocol. 159

Figure 5.5. qPCR showing the expression of JunB in *H-2kb* myotubes following isometric contraction protocol. 160

Figure 5.6. qPCR showing the expression of JunD in *H-2kb* myotubes following isometric contraction protocol. 160

Figure 5.7. qPCR showing the expression of Fos in *H-2kb* myotubes following isometric contraction protocol. ……………………………………………………………………...160

Figure 5.8. qPCR showing the expression of FosB in *H-2kb* myotubes following isometric contraction protocol. ………………………………………………………….………161

Figure 5.9. qPCR showing the expression of Pten in *H-2kb* myotubes following isometric contraction protocol. …………………………………………………….……..162

Figure 5.10. qPCR showing the expression of Sirt-1 in *H-2kb* myotubes following isometric contraction protocol. ………………………………………………….………162

Figure 5.11. qPCR showing the expression of p21 in *H-2kb* myotubes following isometric contraction protocol. ……………………………………………………..……..163

Figure 5.12. qPCR showing the expression of Igf1r in *H-2kb* myotubes following isometric contraction protocol. ………………………………………………………..…..163

Figure 5.13. Western blot showing the expression of SIRT-1 in *H-2kb* myotubes following isometric contraction protocol. ……………………………………..………164

Figure 6.1. The expression of miR-181a in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr). Expression relative to Rnu-6 is shown. N=3-4 per experimental group. ……………………………………………………………………………………………………175

Figure 6.2. The expression of miR-181d in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr). ……………………………………………………………………………………………………176

Figure 6.3. The effects of miR-181 overexpression and inhibition on muscle mass in adult and old mice. Adult (5 months old) and old (23 months old) mice were treated with antagomiR scrambled (Scr), miR-181a mimic (miR-181a) or antagomiR-181a (AM181) over 3 weeks…………………………………………………………………………177

Figure 6.4. The effects of miR-181 overexpression and inhibition on *gastrocnemius* myofibre diameter (cross-sectional area) of *gastrocnemius* muscle of adult and old mice. ……………………………………………………………………………………………………178

Figure 6.5. The expression of miR-181a and miR-181d in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr). ……………………………………………………………………………………………………180

Figure 6.6. The effects of miR-181 overexpression and inhibition on muscle mass in adult and old mice……………………………………………………………………………………181

Figure 6.7. The effects of miR-181 overexpression and inhibition on muscle mass in adult and old mice. ……………………………………………………………………………….……….182

Figure 6.8. The effects of miR-181 overexpression and inhibition on *tibialis anterior* myofibre size in adult and old mice. ………………………………………………….……….183

Figure 6.9. The effects of miR-181 overexpression and inhibition on EDL maximum force generation in adult and old mice. ……………….…………………………………….185

Figure 6.10. The expression of miR-181a in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. ……………………………………………….…..187

Figure 6.11. The expression of miR-181d in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. ………………………………………..…………188

Figure 6.12. The expression of Sirt-1 in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. ………………………………………….……….190

Figure 6.13. The expression of Pten in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. ……………………………………………………….…………191

Figure 6.14. The expression of JunD in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. ……………………………………..………………192

Figure 6.15. The expression of Fos in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. ………………………………………………………..……….193

Figure 6.16. The expression of Fosb in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. ………………………………………………………..……….194

Figure 6.17. The expression of SIRT-1 protein in the *tibialis anterior* muscle of adult (A - 6 months old) and old (B - 24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. ………………………………….………..195

List of Tables

Table 1.1 Classification of skeletal muscle fibre types based on the expression of myosin heavy chain type...24
Table 1.2 The role of miRNAs in maintaining processes associated with muscle homeostasis. 67
Table 1.3 Summary of microRNAs suggested to be involved in regulation of muscle homeostasis following exercise. 69
Table 1.4 Table summarising microRNA level changes in plasma/serum following exercise.. 71
Table 1.5 Summary of miRNA associated with muscle homeostasis and disorders. 76
Table 2.1 Primer sequences used in Real-Time quantitative PCR.............................91
Table 2.2 List of products used in qPCR experiments..92
Table 2.3 Lists of microRNA primers used in qPCR microRNA expression analysis ...94
Table 2.4 Antibodies used for western blotting and immunostaining analysis.......107
Table 3.1 Changes in muscle microRNA levels following exercise. 115
Table 4.1 Summary of changes in the expression of microRNAs following contraction in H-2kb myotubes and in muscle of adult and old mice.. 140

Abbreviations

Ach	-	Acetycholine
ANOVA	-	Analysis of Variance
AP	-	Activator Protein
APS	-	Ammonium Persulphate
ATP	-	Adenosine Triphosphate
AWERB	-	Animal Welfare and Ethical Review Body
BCA	-	Bicinchoninic Acid
BMD	-	Becker Muscular Dystrophy
BSA	-	Bovine Serum Albumin
CAG	-	Chrome-alum Gelatin
CAM	-	Calmodulin
CAT	-	Catalase
C.Elegans	-	Caenorhabditis elegans
cDNA	-	Complimentary deoxyribonucleic acid
CKD	-	Chronic Kidney Disease
CN	-	Contracted
CSA	-	Cross Sectional Area

CTX	-	Cardiotoxin
DAPI	-	4,’6-diamidino-2-phenylindole
DGCR8	-	DiGeorge Critical Region 8
DH2O	-	Distilled water
DM	-	Differentiation Media
DMD	-	Duchenne Muscular Dystrophy
DMEM	-	Dulbecco’s Modified Medium
DNA	-	Deoxyribonucleic acid
DTT	-	Dithiothreitol
EDL	-	Extensor Digitorum Longus
EPP	-	End Plate Potential
FBS	-	Fetal Bovine Serum
FGF	-	Fibroblast growth factor
FSHD	-	Facioscapulohumeral Muscular Dystrophy
Fstl	-	Follistatin-like
FOXO	**-**	Forkhead box protein 01
GAPDH	-	Glyceraldehyde 3-phosphate dehydrogenase
GH	-	Growth Hormone

GM	-	Growth Media
GPX1	**-**	Glutathione peroxidase 1
HGF	-	Hepatocyte Growth Factor
HPLC	-	High performance liquid chromatography
HS	-	Horse Serum
HSF	-	Heat Shock Factor
HSP	-	Heat Shock Protein
IGF	-	Insulin Growth Factor
IHC	-	Immunohistochemistry
K+	-	Potassium ion
miRNA	-	microRNA
miR	-	microRNA
MHC	-	Major Histocompatibility Complex
MyHC	-	Myosin Heavy Chain
mN	-	Millinewtons
MRF	-	Myogenic Regulatory Factor
MURF-1	-	Muscle ring finger protein 1
Na^+	-	Sodium ion

NF	-	Nuclear Factor
Pax	-	Paired-box Transcription Factor
PBS	-	Phosphate buffered saline
PGC1-α		-Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PRX3	-	Peroxiredoxin 3
PRX5	-	Peroxiredoxin 5
PTEN	-	Phosphate and tensin homolog
P13K	**-**	Phosphoinositide 3-kinase
qPCR	-	Quantitative polymerase reaction
RISC	-	RNA-induced Silencing Complex
ROS	-	Reactive Oxygen Species
SDS	-	Sodium Dodecyl Sulphate
SEM	-	Standard error of mean
SERCA	**-**	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SIRT1	-	Sirtuin 1
SOD	-	Superoxide Dismutase
SPF	-	Specific Pathogen Free
SRF	-	Serum Response Factor

TA - Tibialis Anterior

TCA - Tricarboxylic acid

TEMED - N N N' N'-Tetramethyl-ethylenediamine

TF - Transcription Factor

TRX2 **-** Thioredoxin2

TR2 **-** Thiol reductase 2

UCP2 **-** Uncoupled protein 2

UTR - Untranslated Region

Utrn - Utrophin

WGA - Wheat Germ Agglutinin

YFP - Yellow Fluorescent Protein

1. General Introduction

1.1. Skeletal muscle structure and function overview

Skeletal muscle makes up approximately 40% of total body weight and contains 50-75% of various type of protein found in the body. Muscle is associated with approximately 30-50% of body protein daily turnover. Skeletal muscle is composed of 75% of water and 20% of protein as well as fats, carbohydrates, inorganic salts and minerals making up the remaining 5%. Skeletal muscle homeostasis depends on the balance between muscle hypertrophy (growth), atrophy and muscle regeneration. Muscle regeneration is mainly dependent on satellite cells, adult skeletal muscle stem cells (Relaix & Zammit, 2012). Muscle growth and atrophy are largely dependent on protein synthesis and degradation. These mechanisms are controlled by hormonal balance, physical exercise and response of muscle to injury (Frontera & Ochala, 2015; Kraemer & Ratamess, 2005).

The structure of skeletal muscle is based on the arrangement of the muscle fibres, myofibres, associated with the connective tissue. A layer of connective tissue, the epimysium, is the fibrous tissue envelope that surrounds skeletal muscle and is continuous with the tendons. Skeletal muscle consists of bundles of myofibres, fascicles, separated by a connective tissue layer: the perimysium. Connective tissue separating individual myofibres is termed endomysium. Myofibres are multinucleated syncytia formed by fusion of undifferentiated myoblasts. The cytoplasm of myofibres is called sarcoplasm and is mainly occupied by contractile apparatus (Lieber, 2002).

The contractile apparatus of skeletal muscle is arranged into sarcomeres (Lieber, 2002). A sarcomere is the main functional unit of the muscle fibre and

responsible for the contractile properties of skeletal muscle. The sarcomere is composed of thick myosin and thin actin filaments (Figure 1.1). The myosin and actin filaments allow the contraction of the muscle fibres.

An intermyofibrillar network containing mitochondria, lipids, glycogen, T-tubules and sarcoplasmic reticulum is located between the myofibrils. T-tubules are responsible for conduction of electrical signals and the sarcoplasmic reticulum provides calcium storage, release of which is required for contraction to occur (Rayment et al., 1993).

The two most important functions of skeletal muscle are mechanical and metabolic (Komi et al., 1978). The mechanical function of the skeletal muscle involves converting chemical energy to mechanical energy for the production of force and power, the maintenance of health, posture and movement. The mechanical function is associated with muscle contraction. The mechanism of muscle contractions involves neuromuscular interactions and is described in section 1.1.3.

The metabolic function of skeletal muscle involves regulating whole-body protein metabolism, regulation of glucose and amino acid levels in the blood and energy storage (Larew et al., 2003; Spargo et al., 1979; Wolfe, 2006). This function of skeletal muscle is important for the heat production to maintain body temperature and for performance during exercise and physical activity (Frontera & Ochala, 2015).

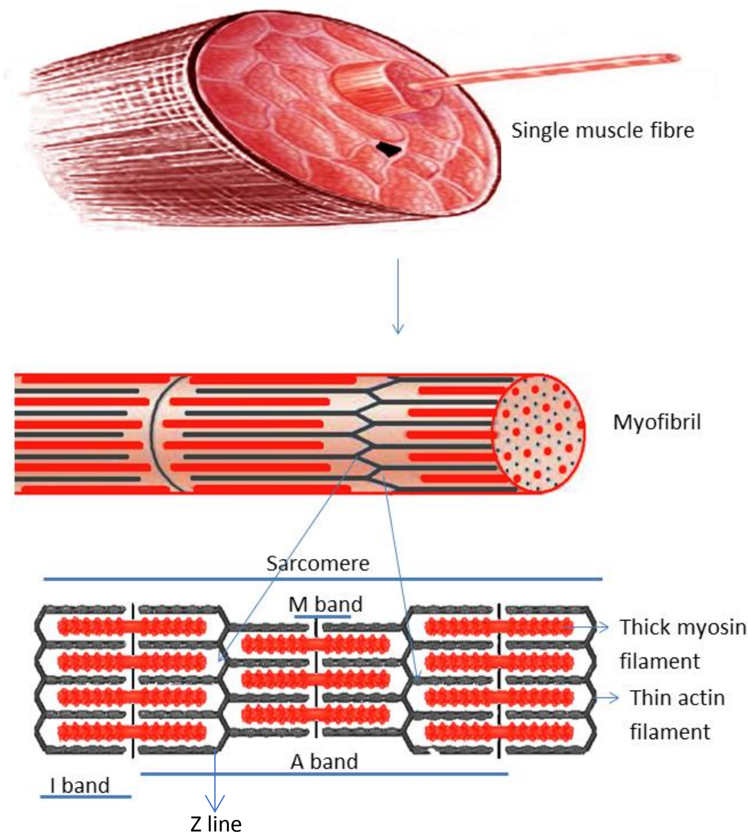


Figure 2.1 Organisation of skeletal muscle

Skeletal muscle generates adenosine triphosphate (ATP), an important molecule in the control of muscle tone at rest and during exercise and in maintaining glucose homeostasis (Stone, Evanson, & Kluess, 2014).

Skeletal muscle is one of the major tissues important for glucose homeostasis. Approximately 80% of glucose is utilised in the skeletal muscle (Thiebaud et al., 1982). Skeletal muscle also serves as storage for glycogen, the main storage of glucose in the body, which is associated with skeletal muscle adaptation to exercise (Kuo et al., 2013; Pilegaard et al., 2002).

Skeletal muscle provides amino acids required for organs such as the brain, heart and skin for the production of proteins which can be organ-specific. Amino acids released from the muscle also play a role in the maintenance of blood glucose levels during starvation or fasting (Brook et al., 2016; Frontera & Ochala, 2015).

1.1.1. Skeletal muscle structure: myofibre types

Skeletal muscle consists of fibres of different specialisation adapted to various roles. The contractile characteristics of these fibres are different, based on the Ca^{2+} kinetics activation, mitochondrial content, isoforms of myofibrillar proteins and energy metabolism. Myofibre types have been classified based on their contraction, morphological and metabolic properties. In recent years, the classification of fibre types has become based on the isoforms of myosin heavy chain (MyHC) that the myofibre types express (Spangenburg & Booth, 2003).

Based on the isoform of sarcomeric MyHC expressed, myofibres have been classified into four fibre types in most adult mammalian limb muscles: types IIB/X, and IIA fibres (Table 1.1) contain the isoforms of MyHC-IIb, MyHC-IIx and MyHC-IIa, respectively and type I fibres express MyHC type I. Unlike rodents, humans only express three MyHC isoforms, lacking the fast contracting type IIB MyHC isoform (Pellegrino et al., 2003; Smerdu et al., 1994), due to a genetic difference in the promoter of this gene (Brow et al., 2014) Based on the expression of these myosin types, the fibre types can be classified into slow and fast fibre type. Type I fibres are called “slow-twitch” fibres and type II are called “fast-twitch” fibres (Table 1.1; (Neunhauserer et al., 2011; Rivero et al., 1998).

1.1 Classification of skeletal muscle fibre types based on the expression of myosin heavy chain type. CSA – myofibre cross-sectional area.

Contractile property	Slow oxidative	Fast oxidative	Fast glycolytic
Myosin ATPase activity	Slow	Fast	Fast
Primary pathway for ATP synthesis	Aerobic	Aerobic, some anaerobic	Anaerobic glycolysis
Myosin heavy chain expressed	Type I	Type IIa	Type IIb/IIx
Fibre CSA	Small	Medium	Large
Glycogen storage	Low	Intermediate	High
Rate of fatigue	Slow	Intermediate	Fast
Mitochondrial content	High	High	Low
Capillaries	Many	Many	Few
Myoglobin content	High	High	Low
Colour	Red	Red to pink	White (pale)
Best suited for	Endurance type activities, eg. Running a marathon, maintaining a posture	Sprinting, walking	Short-term intense movements, such as hitting a baseball

Type I slow fibres, known as oxidative fibres, contain high levels of contractile proteins isoforms, high volumes of mitochondria, myoglobin and have

high oxidative enzyme capacity and high capillary densities (Table 1.1, (Spangenburg & Booth, 2003).

Type IIa oxidative fast fibres display a phenotype of fast contraction and high oxidative capacity and fatigue resistance (Table 1, (Spangenburg & Booth, 2003).

Type IIx and IIb glycolytic fast fibres have high ATPase activity, high glycolytic enzyme activity, and low volume density of mitochondria (Table 1. (Latorre et al., 1993). These myofibres have high level of expression of isoforms of contractile protein specific for fast fibres (for example fibre type IIx), high rate of contraction, but low fatigue resistance (Table 1.1, (Neunhauserer et al., 2011; Spangenburg & Booth, 2003).

It is noteworthy, that in rodents most muscle types contain a mix of different myofibre types, for example *tibialis anterior* muscle consists of mainly fast fibre types, whereas *soleus* muscle consists of slow and fast (up to 40%) of myofibre types (Soukup et al., 2002). Different types of fibres play distinct roles in muscle and are thought to be differentially affected by ageing, which may be associated with changing properties of skeletal muscle during ageing.

1.1.2. Skeletal muscle mechanical function: contraction

Skeletal muscle is estimated to have thousands of myofibrils which contain many myofilaments. Myofilaments arranged together form sarcomeres (Figures 1.1, 1.2). The sarcomeres are the main contractile units of the skeletal muscle. The thick and thin filaments between the Z disks are called a sarcomere. The thick

filaments constitute the dark A band. The thin filaments extend in each direction from the Z disk. The light band I is formed where the thin filaments do not overlap the thick filaments. The H zone is the part of the A band where the thick and thin filaments do not overlap. The M line runs through the center of the sarcomere. Molecules of titin extend from the M line to the Z disk and provide elasticity to the muscle and a scaffold for the assembly of myosin molecules in the thick filament (Figure 1.1). Skeletal muscle function depends on the regulatory proteins like calcium-dependent troponin and tropomyosin (Frontera & Ochala, 2015).

1.1.3. The mechanism of muscle contraction

Muscle contractions can be described or referred to as the activation of tension-generating sites within myofibres. Muscle contraction can be produced without changes in muscle length. The termination of muscle contraction is followed by muscle relaxation. In vertebrates, skeletal muscle contractions require synaptic input from motor neurons to produce muscle contractions. Following a stimulus of the motor neuron, the protein filaments within each skeletal muscle fibre slide past each other to produce a contraction, which is explained by the sliding filament theory (Yanagida, 2007). The contraction can be defined as a twitch, summation, or tetanus, depending on the frequency of action potentials.

Each muscle fibre is innervated by a motor neuron. The synapse between a motor neuron and a muscle fibre is known as the neuromuscular junction. Each neuromuscular junction contains the pre-synaptic terminal at the motor neuron end and the post-synaptic terminal, also known as the motor end plate, at the

muscle end. Muscle contraction starts when the action potential is initiated within the nervous system (Martyn et al., 1992).

Action potentials arrive at the pre-synaptic terminal of the neuromuscular junction causing voltage-gated Ca^{2+} channels open (Figure 1. 2). Ca^{2+} uptake into the terminal causes release of the neurotransmitter acetylcholine (Ach) into the synaptic cleft. Ach travels across the synaptic cleft to the motor end plate. Motor end plate contains nicotinic receptors for Ach (AchR), which are ligand-gated ion channels. Ach binds to the α subunits of the nicotinic receptors and causes conformational change. The central core of the ligand-gated channels opens and permeability of motor end plate to Na^{+} and K^{+} increases. When Ach triggers opening of these channels, more Na+ moves inwards than K+ outwards, depolarising the membrane. This potential change is called end plate potential (EPP). Once the EPP reaches a threshold value an action potential propagates along the sarcolemma.

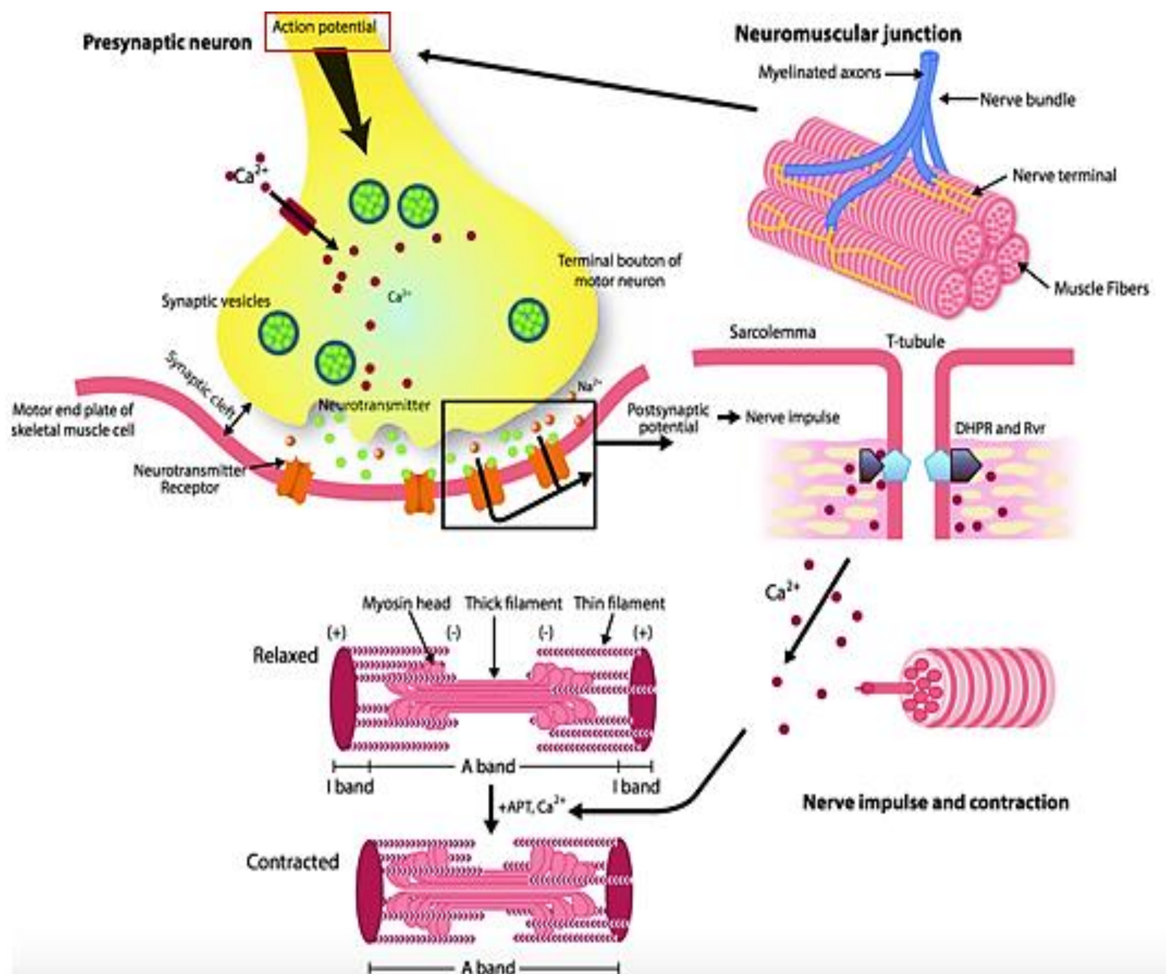


Figure 1.2. The mechanism of muscle contraction. The neuromuscular junction (NMJ) is composed of three elements: pre-synaptic: motor nerve terminal, intrasynaptic: synaptic basal lamina and post-synaptic: muscle fibre. The action potential reaching the motor nerve terminal is followed by the opening of calcium channels and calcium entering the neuron. ACh is delivered into the synaptic cleft. AChR activates events at the sarcolemma: calcium is released from the sarcoplasmic reticulum allowing cross-bridging cycling and force production. Figure taken from (Gonzalez-Freire et al., 2014).

When the nerve impulse arrives to the sarcolemma, it causes the release of calcium from the sarcoplasmic reticulum (Figure 1.2). Calcium then diffuses into the cytosol causing a calcium spark. Sarcoplasmic reticulum has a large calcium buffering capacity due to a calcium-binding calsequestrin. The activation of

thousands of calcium sparks by the action potential leads to a fibre-wide increase in calcium levels. The calcium released into the cytosol binds to Troponin C by the actin filaments and allows cross-bridge cycling (Berchtold et al., 2000). Troponin and tropomyosin are very important for physiological control of striated muscle contraction. Troponin is important for binding of calcium ions (Wakabayashi, 2015), (Geeves & Holmes, 2005).

Muscle contraction is a result of the sliding movement between the actin and myosin filament (Yanagida, 2007). Cross-bridge cycling underlies the sliding filament theory (Yanagida, 2007). A cross-bridge consists of two myosin heads extending from the thick filaments. Each myosin head has one binding site for ATP and one for actin. The binding of ATP to myosin leads to myosin release from actin and allows myosin to bind to another actin molecule. ATP hydrolysis by myosin positions the myosin in the "cocked position" weakly bound to actin binding site. The remainder of the actin binding site is blocked by tropomyosin. Following the action potential, the binding of calcium to troponin C on the actin filaments lead to tropomyosin sliding and unblocking the actin binding site. This allows the myosin to bind strongly to actin. Next, the myosin head then releases the inorganic phosphate resulting from previous ATP hydrolysis. This initiates a power stroke and generates a force. The actin filament move inwards and the sarcomere shortens (Geeves & Holmes, 2005). Next, ADP is released from myosin, however the myosin remains bound to actin until another ATP binds to myosin. Following ATP binding, the myosin head is detached from actin and another cross-bridge cycle can occur (Figure 1.3; (Berchtold et al., 2000)). Each round of cycle hydrolyses one ATP

molecule and each reciprocal action gives about 10-20nm of sliding movement (Yanagida & Ishii, 2012). As the sarcoendoplasmic reticulum calcium ATPase (SERCA) actively pumps calcium into the sarcoplasmic reticulum, calcium levels return to resting levels and the muscle relaxes.

1.1.4. Skeletal muscle mechanical function: energy metabolism

Skeletal muscle requires a steady supply of chemical energy to perform the mechanical function (movement). During exercise or muscle contraction chemical energy is converted to mechanical energy for body movement. Chemical energy is in the form of ATP in human and animal muscles. Four sources of this substance are available to muscle fibres: free ATP, phosphocreatine (provides phosphates to ADP molecules, producing high-energy ATP molecules), glycolysis and respiration (Boundless, 2016). A small amount of free ATP is available in the muscle for immediate use. ATP is also generated by two mechanisms: the anaerobic and aerobic mechanism (Chikani & Ho, 2014).

Anaerobic energy mechanism depends on ATP generation from anaerobic glycolysis. Glycolysis is a process in the absence of oxygen in which glucose is converted into pyruvate, water and NADH, resulting in production of two ATP molecules. The source of glucose for glycolysis can be from the circulation, but more often it is converted from glycogen stored in the myofibres. In case of glycogen stores in the muscle fibres being limited, glucose can be sourced from fats and proteins, however this process is not as efficient. The product of glycolysis, pyruvate, is continually processed into lactic acid. This leads to reduction of the pH

in the muscle and inhibits further anaerobic respiration. Excess pyruvate converted to lactic acid causes muscle fatigue (Figure 1.3).

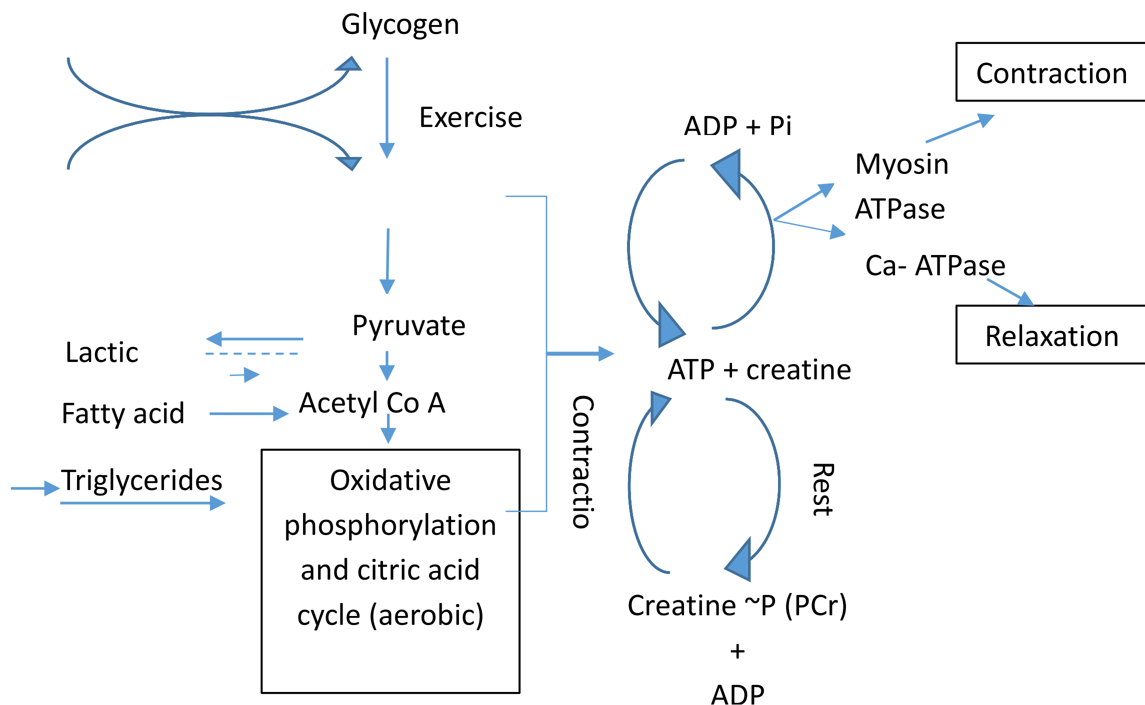


Figure 1.3. An overview of energy metabolism in skeletal muscle. Figure Taken from Slideplayer.com, slide 9349795.

Aerobic energy synthesis depends on production of ATP from oxidation of metabolic substrates, such as protein, lipids and carbohydrates. The pyruvate produced through glycolysis can be converted to lactic acid; however it can also be used to generate further ATP molecules in the presence of oxygen in the mitochondria via the Krebs cycle (Rooyackers et al., 1996). This cellular respiration is limited by oxygen availability and plays a key role in regenerating the stores of ATP, phosphocreatine, and glycogen in the muscle that are required for more rapid contractions (Figure 1.4).

1.1.5. Types of contraction of skeletal muscle

In skeletal muscle research, usually the following terms are used in studies associated with muscle contractions (David et al., 1985; Scott et al 1994):

- Muscular strength defined by the ability of muscle or a group of muscles to generate force;
- Force, measured in Newton (N), which can be calculated as muscle mass times acceleration due to gravity (g);
- Specific force is the maximum force generation per myofibre cross-sectional area; it is measured in kN/mm^2.
- Tension usually describes the resting force value (resting tension) and is measured in Newtons;
- Fatigue is defined as an inability to maintain a power output or force during repeated muscle contractions.

A single stimulus given to a muscle results in a simple twitch of the muscle (Figure 1.5a), divided into three phases: a latent period, a contraction period and a relaxation period. Depending on the type of the muscle, the duration and the maximum force generated by the twitch can vary. Type II myofibres contract in a shorter time period when stimulated than type I myofibres (Figure 1.5.a). With repeated stimulations (Figure 1.5b), individual responses fuse into one continuous contraction, tetanus, during which, maximum force is produced (Figure 1.5c). Muscular contractions that occur during normal body movements are primarily tetanic contractions (Ganong, 1993).

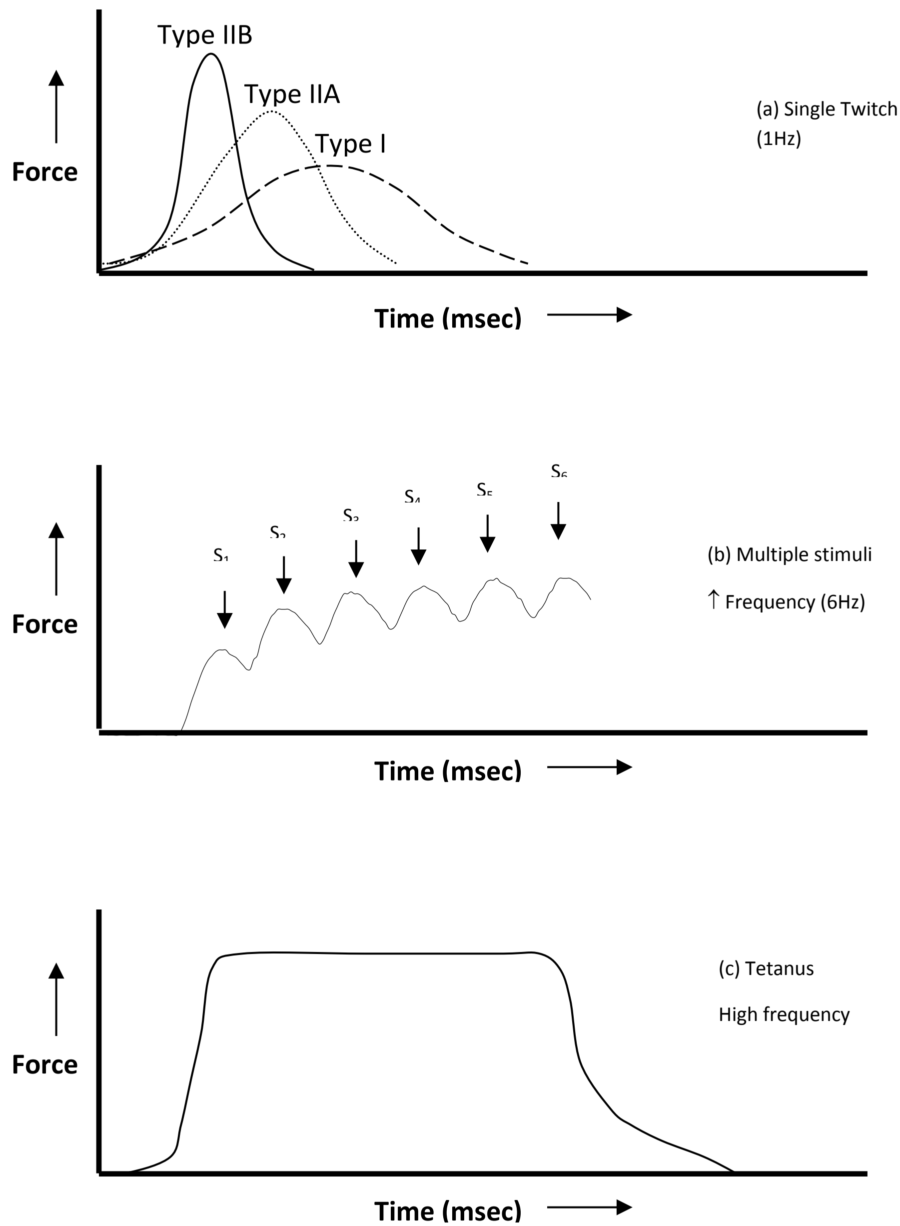


Figure 1.4. Schematic diagram of skeletal muscle contraction. (a) Maximum force produced by a single twitch in three different muscle fibres (b) Increasing the frequency of the stimulus results in a summation of twitches (c) High frequency results in tetanus. Source: Dr. Aphrodite Vasilaki, PhD thesis. (2003)

Muscle movement, not always associated with changes in the length of the muscle, involves three types of contraction:

1. Shortening (concentric or dynamic) contractions, during which muscle allows shortening of the contractile elements during activation. Shortening contractions are the most common in exercise;

2. Isometric (static) contractions, during which muscle contracts without an appreciable change in the length of the whole muscle, it involves pairs of contracting muscles work to oppose each other and thus cancel out any movement. Examples of this type of contractions include clenching of the fists or tensing of muscles (Lobish et al, 1996).

3. Lengthening (eccentric or plyometric) contractions, during which the muscle lengthens during activation. Lengthening contractions are thought to cause considerably more damage to skeletal muscle than shortening or isometric contractions (Faulkner et al, 1990). This may be due to the tension per fibre being generated during lengthening contractions due to the cross-sectional area of the fibre being reduced (Armstrong et al, 1991). However, the mechanisms underlying lengthening contraction-induced damage are not clearly understood.

1.1.6. Muscle contraction and reactive oxygen species (ROS)

Movement, and therefore exercise, is associated with muscle contractions described above. Exercise, therefore muscle contractions, has been shown to result in increased ROS *in vivo* (Davies et all, 1982; Jackson et all, 1985; McArdle et all, 2001a; Reid et al., 1992). Also an *in situ* model, (O'Neill et al., 1996) demonstrated that isometrically contracted cat skeletal muscle generated ROS detectable in the microvasculature of skeletal muscle. Moreover, it has shown that 15 minutes of

isometric contractile activity induced a rapid release of superoxide anion radicals from mouse skeletal muscle in *in vivo* and *in vitro* studies using contracting cultured primary skeletal muscle myotubes confirmed that this release was from muscle cells rather than other cell types present within the muscle (McArdle et al*,* 2001).

Ample evidence exists suggesting that the increased contractile activity of muscle results in increased production of ROS. It has been suggested that this increased production of ROS is associated with exercise-induced muscle damage. However, evidence exists that argues against this (Newham et al.,1983). It has been suggested that acute changes in ROS production do not result in skeletal muscle damage due to skeletal muscle cells having a complex antioxidant defence mechanism to provide protection against fluctuations in the production of ROS at resting conditions and during increased activity (Vasilaki et al., 2006). This is discussed below.

1.1.7. Skeletal muscle adaptive response to contractions and exercise

Skeletal muscle is a hugely adaptable tissue which undergoes a lot of metabolic and morphological adaptations during endurance exercise or stimuli (Safdar et al., 2009). During exercise, skeletal muscle adapts to protect the muscle from damage resulting from contraction (Close et al., 2005; Vasilaki et al.,2006).

Physical exercise is associated with a dramatic increase in oxygen consumption in the working muscles. Most of the oxygen is thought to be utilised in the mitochondrial electron transport chain, where it is reduced to water (Ji, 1995). However, there is now considerable data demonstrating that mitochondria are not

the only source of ROS production in skeletal muscle and that are other sources of ROS production (Powers & Jackson, 2008). In skeletal muscle, superoxide radicals are also found in the extracellular fluid and are produced at a relatively low rate with a significant increase during contractile activity (McArdle et al., 2001a; Reid et al., 1992).

Oxidative stress resulting from disrupted redox signalling has been shown as one of the causes of imbalance between protein synthesis and degradation causing muscle atrophy (Pellegrino et al., 2011). However, skeletal muscle has well developed mechanisms to respond to the increased ROS generation as an adaptation to protect against potential subsequent damaging insults. These mechanisms involve activation of redox-sensitive transcription factors such as Nuclear Factor kappa B (NF-κB) and activator protein-1 (AP-1) and the transcription of antioxidant defence enzymes, such as superoxide dismutase (SOD) and catalase (CAT), as well as the production of stress/heat shock proteins (HSPs) and glutathione peroxidase (GPX1) and catalase (CAT) enzymes (Vasilaki, Csete, et al., 2006). In addition, other proteins, such as sirtuins have been shown to play an important role in protection against a pathological increase in ROS production (Vasilaki & Jackson, 2013; Vinciguerra et al., 2010).

Interestingly, it has been demonstrated that a single bout of non-damaging isometric contractions results in significant increase in SOD and catalase activities and an increase in the HSP content of muscles of adult mice following contractile activity which was associated with increased activation of the transcription factors, NF-κB, AP-1 and heat shock factor 1 (HSF1) (Vasilaki et al., 2006).

In summary, specific transcription factors, antioxidant enzymes, HSPs and other proteins, such as sirtuins, are thought to protect the muscle against damage (Jackson, 2006).

1.1.8. Skeletal muscle metabolic function

Skeletal muscle metabolic function is crucial in maintaining energy homeostasis of the body. Muscle serves as source of storage organ in the body and maintains the levels of glucose and amino acid in circulation. The rapid protein turnover in muscle makes it possible for the muscle to adapt changes in its environment (Spargo et al., 1979).

The daily turnover of protein in skeletal muscle is about 12% (Spargo et al., 1979). This shows that the muscle and the amount of protein broken down in the muscle is at a very high rate daily and suggests skeletal muscle as a storage organ in the body. Muscle also takes part in maintaining the blood glucose and amino acid levels in the body (Spargo et al., 1979).

Mitochondria are the major site for metabolic activities in the muscle and play a vital role in energy metabolism of the body (Sakellariou et al.,1999). Cell function impairment and cell death can disrupt mitochondrial function resulting in disrupted energy metabolism. Mitochondria provide more than 90% of energy needed in the body (Bo et al., 2010). Mitochondria are key for respiration and oxidative energy production (Venditti et al., 2007). Abnormalities of mitochondria have been associated with the pathogenesis of musculoskeletal ageing (Cobley et al., 2015; Dimauro, Pearson et al., 2012; Jackson, 2013).

Skeletal muscle regulates glucose metabolism and plays a crucial role in insulin resistance development (Phielix & Mensink, 2008). Severe upregulation of plasma free fatty acids through intravenous (IV) lipid infusion can increase skeletal muscle insulin resistance both diabetic and non-diabetic proteins (Phielix & Mensink, 2008).

Skeletal muscle is also considered an endocrine organ; muscle has been shown to release myokines, muscle-originating cytokines, that affect the function of muscle and the surrounding tissues (Aoi,et al.,2011; Degens, 2010; Lightfoot et al., & McArdle, 2014). For example, IL-6 is a known myokine released from muscle during muscle contraction (Aoi et al., 2011). IL-6 has been shown to regulate muscle regeneration and hypertrophy (Aoi et al., 2011).

To summarise, skeletal muscle plays an important role in maintaining whole body metabolism and defective muscle function may be associated with whole body metabolic defects and disease.

1.2. Adult skeletal muscle homeostasis

Skeletal muscle is a very plastic tissue. Postnatal muscle growth and adaptation are mainly regulated by remodelling of the existing myofibres, whereas embryonic muscle growth is mainly regulated by proliferation of myogenic progenitors (Braun & Gautel, 2011). Adult skeletal muscle size is a balance between muscle growth (hypertrophy), atrophy and muscle regeneration (Moylan & Reid, 2007).

1.2.1. Muscle homeostasis: muscle hypertrophy and atrophy

Muscle mass increases by hypertrophy (increased protein content) and is controlled by anabolic and catabolic mechanisms controlling the increased synthesis or degradation of muscle proteins, respectively. Muscle hypertrophy can result from an adaptation to mechanical loading as a result of accumulated resistance training (Vigotsky et al., 2015).

Muscle atrophy is associated with decrease in myofibre size and number and can be triggered by disuse, catabolic steroids, cytokines, genetic factors, acidosis and catabolic nutritional states (Vigotsky et al., 2015).

Muscle hypertrophy and atrophy are associated with changes in sarcomeric protein composition, metabolic enzymes and contractile phenotype. The loss of balance between muscle growth and atrophy is also one of the main characteristics of ageing-associated loss of muscle mass and function – sarcopenia (Brown & Goljanek-Whysall, 2015).

Exercise and diet have been shown to regulate muscle mass and function, through controlling the balance between muscle hypertrophy and atrophy, which is largely associated with protein homeostasis. Exercise and diet are also important for metabolic control and skeletal muscle function (Fernandes et al., 2015).

Exercise is a key regulator of muscle homeostasis. Exercise regulates glucose uptake in skeletal muscle through the insulin dependent and insulin-independent signal transduction mechanisms (Aoi et al., 2011). Exercise has a

positive effect on muscle hypertrophy and has been suggested an intervention against muscle wasting (Aoi et al., 2011).

Several molecular pathways have been shown to be involved in regulating muscle mass and function, and these include growth hormone, insulin signalling (Schiaffino & Mammucari, 2011), TGF-beta signalling, including myostatin, (Amthor et al., 2007), as well as several cytokines (Buck et al.,1993; Haddad et al., 2005; Schaap et al.,2006).

Growth hormone (GH) is one of the factors that regulate muscle function through increasing muscle protein anabolism and therefore positively contributes to muscle mass (Chikani & Ho, 2014; Perrini et al., 2010).

Several cytokines, such as interleukin- 15 (IL-15) have also been shown to act as anabolic factors for skeletal muscle through preventing muscle wasting (Pistilli et al., 2007; et al., 2002).

Another cytokine, IL-6, has been reported to play a role in maintaining muscle homeostasis. The plasma concentration of interleukin-6 (IL-6) is increased following exercise (Fischer, 2006). IL-6 has been demonstrated to regulate muscle regeneration, as well as muscle mass (Carson et al., 2013). IL-6 levels are also increased during ageing (Ford et al., 2016) and it has been suggested that IL-6 may play a role in ageing-associated decline in muscle homeostasis (Soriano-Arroquia et al., 2016).

Insulin like growth factor 1 (IGF-1) is another very important regulator of muscle maintenance and repair, including regulating satellite cell function (Musaro

et al., 2001; O'Neill et al., 2010; Schiaffino & Mammucari, 2011; Song et al.,2013; Stitt et al., 2004). IGF-1 has also been shown to play a critical role in myogenesis during development, with mice lacking IGF-1 showing severe developmental phenotypes (Velloso, 2008).

Interestingly, the levels of IGF-1 have been shown to increase in muscle following exercise promoting muscle regeneration (Pelosi et al., 2007). Moreover, IGF-1 has been shown to ameliorate age-related skeletal muscle atrophy (Philippou et al., 2007).

1.2.2. Skeletal muscle homeostasis: regeneration

Satellite cells are adult muscle stem cells and play a key role in muscle regeneration. Satellite cells were discovered in 1961 by Mauro and were named by their anatomical location between the sarcolemma and the basal lamina of the muscle fibres (Mauro, 1961; Snijders et al., 2015).

Satellite cells remain dormant in quiescent adult muscles until a relevant cue. Quiescent satellite cells are characterised by expression of the paired-box transcription factor 7 (Pax7) and once activated in response to muscle injuries and other relevant cues, satellite cells down regulate the expression of Pax7 and enter cell cycle (Almeida et all., 2016).

Activated satellite cells proliferate; a proportion of satellite cells will undergo self-renewal and go back to dormant state where they remain until another response to stimuli or injury (Tian et al., 2016), whereas another group of

cells will proliferate and commit to myoblasts that will differentiate into myocytes and contribute to the repair of the muscle (Charge & Rudnicki, 2004). The mechanism by which satellite cells return to quiescent state or become myoblasts is not yet well understood (Figure 1.6).

The availability and functionality of satellite cells determine effective regeneration and changes in satellite cell number with ageing in human and rodents were shown (Brack & Rando, 2007; Dhahbi et al., 2012; Verdijk et al., 2007). Satellite cells from rodents and humans have also been shown to undergo senescence during ageing (Sousa-Victor et al., 2014). Human and rodent satellite cells from muscle of old individuals also show transcriptional profile switch, and reduced regenerative potential (Bortoli et al., 2003; Jejurikar et al., 2006; McArdle et al., 2003; Thalacker-Mercer et al., 2010). However, there is also evidence from mouse models that age-related loss of muscle mass and function can occur independently of defects in satellite cell function (Fry et al., 2014).

Circulating growth factors, such as interleukin 6 (IL-6), hepatocytes growth factor (HGF1) and insulin-like growth factor-1 (IGF-1), the abundance of which changes during ageing both in circulation and locally, have been shown to regulate the decline in function of satellite cells during ageing (Nederveen et al., 2016; Schiaffino & Mammucari, 2011; Tierney et al., 2014). Nevertheless, the relevance of the potential decline in number and function of satellite cells during ageing in sarcopenia development is not yet fully understood.

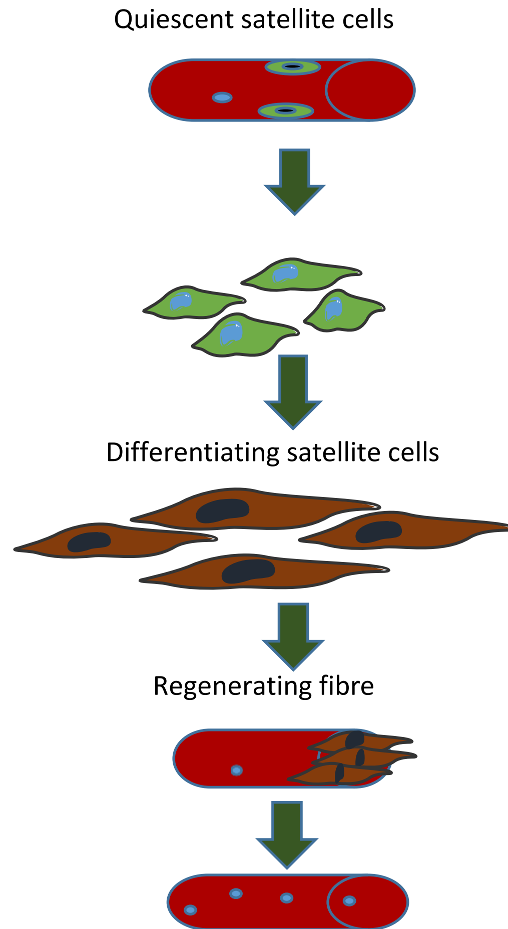


Figure 1.5. Schematic representation of the stages of muscle regeneration.

Upon relevant cues, satellite cells are activated; they proliferate and differentiate to repair the myofibres.

1.2.3. Molecular pathways that regulate muscle homeostasis and response to contractions

Muscle homeostasis, a balance between muscle hypertrophy, atrophy and regeneration, is regulated by distinct molecular mechanisms, including hormones, transcriptional and posttranscriptional mechanisms (Braun et all., 2011; Kinnunen et al., 2015).

Among the important hormonal signalling pathways regulating adult muscle growth are IGF or TGFβ signalling molecules, transcriptional regulators include serum response factor (SRF), JUN, FOS and FOXO families of transcription factors. Other important molecular regulators include regulators of protein homeostasis, such as SIRT1. Finally, microRNAs, epigenetic regulators of gene expression, have been shown to regulate adult muscle size and regeneration at the post-transcriptional levels (Brown et al., 2011).

Adult skeletal muscle mass is regulated by hormones. One of the most known muscle growth factors is IGF-1, which is secreted by myocytes in an autocrine manner in response to mechanical strain (Goldspink, 1999; Vinciguerra et al., 2010), or by the liver. IGF-1 binding to its receptors activates the PI3K–AKT signalling pathway and promotes muscle hypertrophy. The importance of insulin and IGF-1 signalling pathway in regulating glucose metabolism and protein homeostasis has been shown in mice lacking both IGF-1 receptors. These mice showed reduced muscle fibre size and muscle mass (O'Neill et al., 2015; O'Neill et al., 2010) validating the role of IGF signalling in muscle. The activity of IGF1 can be antagonized by myostatin (GDF8), a member of the TGFβ signalling molecules (Trendelenburg et al., 2009).

Another hormone that controls adult muscle mass is leptin, a major regulator of energy homeostasis. Leptin has been shown to positively regulate muscle mass by suppressing the activity of FOXO3A, further demonstrating how muscle and fat tissue metabolism are interlinked (Sáinz et al., 2009) (Fig. 1.6).

The AKT–FOXO, key molecular regulators of muscle growth, activity balance is also controlled by steroid hormones and their receptors: the anabolic androgen receptor and the catabolic glucocorticoid receptor (Braun & Gautel, 2011). The anabolic effects of androgens have long been known. However, using a muscle-specific androgen receptor knock-out mouse, it has also been shown that the muscle androgen receptor can regulate autocrine or paracrine activation of the muscle AKT signalling pathway (Fig. 1.6; (Braun et al., 2011; Chambon et al., 2010). Conversely, the glucocorticoid receptor is regulated in a feedback loop by the IGF1–AKT-activated mTOR, thus providing a feedback loop between anabolic and catabolic signals (Shimizu et al., 2011).

The balance between adult muscle hypertrophy and atrophy is also regulated on the transcriptional level. For example, the expression of catabolic genes is regulated by the transcription factor forkhead box O1 (FOXO1) and FOXO3 (Sandri et al., 2004). The activity of FOXO family proteins is regulated by phosphorylation: dephosphorylated FOXOs are located in the nucleus and are transcriptionally active. Phosphorylated FOXOs are sequestered in the cytoplasm.

The (PI3K)–AKT pathway, apart from its role in directly stimulating protein synthesis by activating mammalian target of rapamycin (mTOR) and its downstream targets (Sandri, 2008) (Fig. 1.6), it also blocks FOXO1 and FOXO3A nuclear import and transcriptional activity. Therefore, the AKT pathway indirectly regulates genes depending on FOXO proteins for their expression: atrogenes, such as atrogin 1 (FBXO 32, MAFBX1) and the muscle-upregulated RING finger (MURF) ubiquitin ligases (Sandri, 2008). Atrogin-1 and MURF1 target myofibrillar, metabolic and

transcriptional muscle proteins for degradation by the ubiquitin–proteasome system and autophagy (Willis et al., 2009). Apart from the FOXO proteins, NF-κB also regulates the expression of some atrogenes, thereby linking catabolic signalling and cytokine signalling (for example, through TNF) (Fig. 1.6).

Maintaining muscle homeostasis is important as skeletal muscle is one of the major metabolic tissues in the body. Another regulator of muscle metabolism is Sirtuin1 (SIRT-1) which controls mitochondria biogenesis, muscle hypertrophy and regeneration. SIRT-1 belongs to the family of Sirtuins, which are the class 3 protein deacetylases. SIRT-1 is a NAD^+-dependant deacetylase which regulates the peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α) that controls mitochondrial biogenesis and function (Gerhart-Hines et al., 2007; Mohamed et al., 2014).

One of the important characteristics of adult muscle is its ability to respond to changes in ROS levels, which occur during muscle contraction (Close et al., 2004). Muscle can respond to acute or chronic increases in ROS production by increased production of antioxidant defence enzymes and HSPs (Vasilaki, McArdle, et al., 2006). In recent years, much attention has been focused on the cellular mechanisms that initiate the adaptive response, specifically the induction of antioxidant defence enzymes and HSPs through the activation of transcription factors. Cells contain several redox-sensitive transcription factors; HSF1, NF-κB, AP-1 are three of the most well characterised transcription factors that play important roles in the response of cells to stress (Vasilaki, McArdle, et al., 2006).

There is also ample evidence that SIRT-1 is crucial in combating oxidative stress. SIRT-1 plays an important role in regulating cell survival and myogenesis, as well as adult muscle homeostasis (Pardo & Boriek, 2011). SIRT-1 has also been shown to regulate the recovery of cultured myoblasts from ROS-induced stress, and was therefore suggested to have an antioxidant role in muscle (Pardo & Boriek, 2011). Moreover, SIRT-1 regulates the expression of several antioxidant genes, including Mn superoxide dismutase (MnSOD), catalase, peroxiredoxins 3 and 5 (Prx3, Prx5), thioredoxin 2 (Trx2), thioredoxin reductase 2 (TRXR2), and uncoupling protein 2 (UCP-2) (Olmos et al., 2013). Moreover, the knockdown of FOXO3a or PGC-1α has been shown to prevent the induction of antioxidant genes by SIRT-1 overexpression, whereas SIRT-1 increased the formation of a FOXO3a/PGC-1α complex. Moreover, SIRT-1 inhibits NF-κB signalling, a major inducer of inflammatory response. Increased level of ROS has also been shown to directly and indirectly control the activity of SIRT-1 (Salminen et al., 2013).

In summary, There is clear evidence that SIRT-1 and ROS signalling are dependent on each other and this crosstalk controls hallmarks of ageing, such as disruption in proteostasis or low-grade chronic inflammation (Salminen et al., 2013).

Satellite cells play important role in adult muscle regeneration and therefore in homeostasis of muscle (Zammit et al., 2006). Satellite cells are also thought to take part in early postnatal muscle growth. However, their contribution in hypertrophic muscle growth is disputed, as hypertrophic growth of adult muscle has been shown to occur without satellite cell activation (Blaauw et al., 2009) .

Molecular pathways regulating embryonic and adult myogenesis, the latter requiring satellite cells, are largely common. These molecular players include Wnt and Notch signalling essential for proliferation of satellite cells (Buckingham & Montarras, 2008). The fibroblast growth factor (FGF) signalling pathway is also important in myogenesis through regulating the equilibrium between progenitor cell self-renewal and muscle differentiation (Armstrong & Esser, 2005). C-Fos and C-Jun transcription factors have also been shown to regulate myogenesis in adulthood. C-Fos and C-Jun are expressed in satellite cells within 3 hours after muscle trauma suggesting their role in early event during muscle regeneration (Kami et al., 1995).

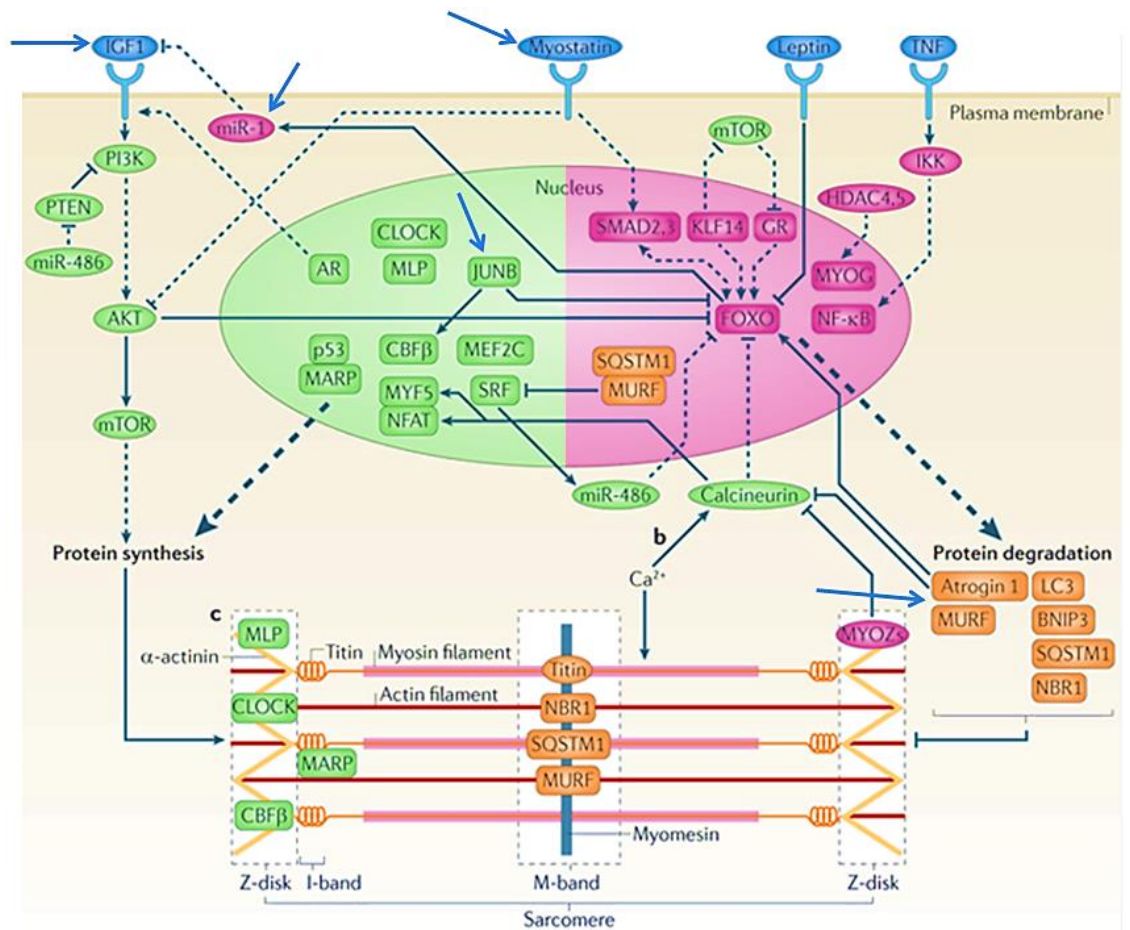


Figure 1.6. Schematic representation of molecular mechanisms involved in postnatal control of muscle growth and atrophy. Arrows indicate some of the key molecular players in muscle homeostasis. Taken from (Braun & Gautel, 2011).

Skeletal muscle has an ability to adjust to changes in physiological requirements, through changes in contractile properties, metabolism, and fibre size and mass (Braun & Gautel, 2011). These changes involve the control of transcription regulation, protein synthesis and degradation and metabolism. During adult muscle remodelling, early developmental programmes, such as SRF, NFATs, JunB or myogenin, can be reactivated to determine fibre-type characteristics (Bassel-Duby & Olson, 2006). Many of these responses are controlled by muscle activity, hormonal and cytokine signalling mechanisms that feed into the same pathways (Braun & Gautel, 2011).

In summary, the molecular control of adult muscle homeostasis is complex, regulated by hormonal influences, as well as changes within the muscle associated with its contractile properties. These mechanisms are interlinked, with many being regulated on the transcriptional level. Nevertheless, post-transcriptional regulation of gene expression, for example by micoRNAs has also been shown to play a crucial role in maintaining adult muscle homeostasis in the recent years (reviewed in (Brown & Goljanek-Whysall, 2015) and discussed in section 1.4).

1.3. Skeletal muscle ageing

One of the definitions of ageing is: “the progressive loss of function accompanied by decreasing fertility and increasing mortality with advancing age (Kirkwood & Austad, 2000). This complex physiological process involves morphologic and biochemical changes in cells, tissues and the whole organism.

The effect of ageing on skeletal muscle has been increasingly studied. Ageing in mammals leads to a decrease in skeletal muscle mass and function. The reduction in muscle mass attributed to muscle atrophy is the main cause of the age-related decrease in muscle strength and power (Porter et al., 1995). Moreover, the reduction of muscle mass is accompanied by a replacement with connective tissue and fat (Lexell, 1995).

Age-related loss of muscle mass and function is defined as sarcopenia (Brown & Goljanek-Whysall, 2015) . It results in the inability of older people to carry out daily activities (Arthur & Cooley, 2012).

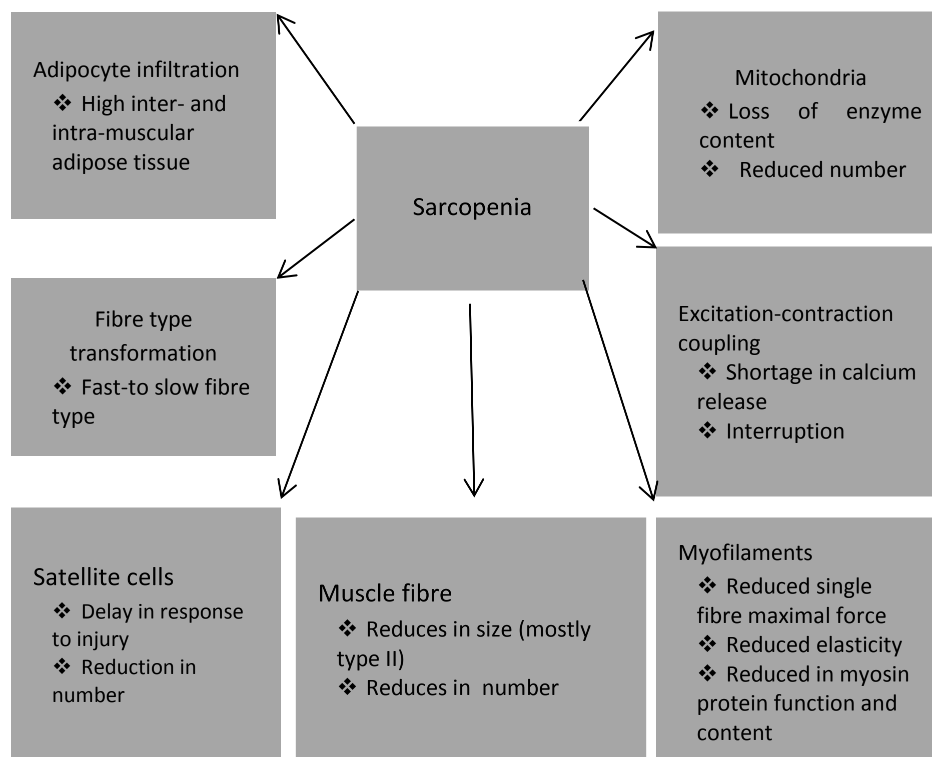


Figure 1.7. The summary of cellular and molecular changes in muscle fibres contributing to skeletal muscle ageing. Taken from **McCormick & McArdle (in press)**

Humans start losing muscle from the age of approximately 50 (Lexell et al., 1988). The loss of muscle mass and function in older humans is associated with a decline in the total number of muscle fibres and weakening of the remaining fibres (Brown & Goljanek-Whysall, 2015; Goljanek-Whysall et al., 2016); (Miljkovic et al., 2015). A difference in the loss of specific fibre types during ageing has been suggested between men and in women (Miljkovic et al., 2015). The mechanisms associated with age-related loss of muscle mass and function is described in Figure 1.8 and in chapters below.

1.3.1. Age-related changes in size and number of muscle fibres

Sarcopenia is accompanied by reduced fibre size and muscle cross sectional area (Lexell et al., 1988). The decline in muscle mass occurs mostly in the lower limbs, which reduce by 30%-40% in muscle fibre number (Miljkovic et al., 2015). In experiments investigating the size of muscle fibres during ageing, it was observed that 10%-40% of type II fibres were smaller in the older people when compared with the adult, however, type I fibre size was mostly unaffected (Holloszy et al., 1991; Miljkovic et al., 2015; Sato et al., 1984). Lexell *et al.* (1988) have characterised a decrease in the total number of fibres in human muscle during ageing, which was accompanied by a greater loss of contractile material of fast twitch type than of slow twitch type (Lexell et al., 1988). Studies using animal models have shown similar findings. For example, (Caccia et al., 1979) have reported an age-related decrease in the proportion of type II fibres in the rat soleus muscles and Holloszy et al (1991) have demonstrated a 37% decrease in the average cross-sectional area of the fast fibres in aged rats (Holloszy et al., 1991)

Interestingly, Grimby and Saltin (1983) found no age-related changes in type I fibre distribution in the vastus lateralis (VL) muscles of 66- to 100 years old individuals (Grimby & Saltin, 1983). Furthermore, Sato *et al* (1984) reported that the total volume of type I fibres in the minor pectoral muscle of women did not change with age (Sato et al., 1984). In addition, Lexell et al (1986) found that the type I distribution was 49% for adult men and 51-52% for older men (Lexell et al.,1986). These findings suggest that type I fibres are little affected by ageing. However, type I fibres have been shown to be more predisposed to loss of nerve

supply to the muscle due to lack of activity (Miljkovic et al., 2015). The differences in muscle fibre type response have been suggested to be a result of different responses to signalling pathways, such as Nf-κB and AP-1 pathways (Miljkovic et al., 2015; Rivero et al., 1998).

In summary, the reduction in muscle mass during ageing is associated with the decrease in fibre number, fibre size, or both, ultimately resulting in muscle of older individuals being weaker (Kirkendall et al., 1994).

1.3.2. Age-related changes in mechanical characteristics of skeletal muscle

Loss of muscle mass results in a loss of total force production (Kirkendall & Garrett, 1998). Young *et al* (1984) demonstrated that the isometric forces of the *quadriceps femoris* muscles from women were reduced by 35% reduction during ageing (Young, Stokes, & Crowe, 1984). (McDonagh et al., 1984) have also shown that the maximal isometric force of the *triceps surae* muscles from old men showed a 40% decrease during ageing (McDonagh et al., 1984). Also in small rodents, muscle has been shown to decrease in strength and power due to the loss in mass during ageing (Brooks et al., 1988; Faulkner et all., 1990).

Moreover, the muscles of old mice have been shown to be more susceptible to damage than those of young and adult mice (Young et al., 1984). The magnitude of the injury induced by stretches of muscle fibres from rats and mice has been shown to be increased with age and it has been suggested that the increased susceptibility of muscles from old rodents to contraction-induced injury resides in part within the myofibrils (Brooks & Faulkner, 1996).

During ageing, the ability of muscle to repair damage through activation and differentiation of stem cells from within the muscle bulk has also been shown to decline (Brooks & Faulkner, 1990; McBrideet al., 1995).

Interestingly, the ability of muscle to regenerate successfully has been shown to depend on the “environment” of that muscle. A muscle transplanted from an old rat into a young rat has been shown to regenerate at an equivalent rate to young muscle in contrast to the impaired regeneration in muscles of old rats. (Carlson & Faulkner, 1988).

1.3.3. The role of ROS in muscle ageing

The ageing process has been proposed to be the cumulative damage to lipids in cell membranes, DNA, and sub-cellular membranes and structures, by ROS. This theory, also known as the free radical theory of ageing, was first described by Harman in 1956 (Harman, 1956).

The increased ROS levels have been observed during ageing in mammals (Kowald & Kirkwood, 1994). In skeletal muscle, multiple studies on animals have suggested an ageing-related increased production of ROS and ROS-induced changes in protein homeostasis (McDonagh et al., 2014; Vasilaki & Jackson, 2013). Proteins containing sulphydryl groups, such as myosin and creatine kinase, can be oxidised and therefore non-functional. In addition, transcription factors containing redox-sensitive sites are also susceptible to damage.

Numerous studies have shown that cells from older organisms accumulate increased levels of oxidant-damaged mitochondrial DNA leading to abnormal

mitochondrial function and integrity (Goljanek-Whysall et al., 2016). In addition, an age-related decrease in the expression of genes involved in oxidative phosphorylation in old animals has been shown (Lee et al., 1999).

Perhaps as a response to age-associated ROS-induced cellular damage, antioxidant mechanisms have developed in mammalian cells. Evidence exists of an attempt in skeletal muscle to adapt to an age-related chronic increase in the production of ROS by adaptation of antioxidant defence enzymes.

Specifically, an increase in the activity of mitochondrial and cytosolic SOD in rat skeletal muscle during ageing has been shown (Ji et al., 1990) and CAT and GPX activities have also been shown to be significantly higher in muscle of old versus young rats (Leeuwenburgh et al., 1994). The activity levels of SOD and catalase have also been shown to be upregulated in quiescent TA muscle of old mice when compared to the muscles of adult mice (Vasilaki et al. 2006b). However, the response of the antioxidant defences during ageing appears to be highly tissue-specific (Leeuwenburgh et al., 1994).

In summary, ageing-related adaptation of cellular antioxidant defences is evident however it may be muscle fibre type-specific.

1.3.4. Muscle adaptive response to exercise during ageing

Skeletal muscles of adult mammals rapidly adapts to acute bouts of exercise by increasing their antioxidant activity (discussed in Chapter 1.2.3), muscles of old mammals show inability to adapt to exercise.

No apparent changes in most antioxidant defence enzymes in the skeletal muscles from old rats following exercise were demonstrated (Ji et al., 1990). Furthermore, Lawler et al., (1993) found no increase in the SOD and GPX activity in the muscles of old rats after treadmill running (Lawler et al., 1993). Moreover, skeletal muscles from older mice show higher baseline activity of antioxidant defence enzymes, however they seem unable to upregulate these activities following exercise (McArdle et al., 2006; Vasilaki, McArdle, et al., 2006).

The inability of muscle from aged mammals to adapt following exercise may be associated with the muscle weakness, increased susceptibility of myofibres to damage and their poor recovery from damage. The cellular and molecular mechanisms responsible for this age-related decline in skeletal muscle function remain unclear; however transcriptional and post-transcriptional mechanisms are undoubtedly involved and discussed in chapters below.

1.3.5. Molecular changes in muscle during ageing

The regulation of muscle wasting during ageing is multifactorial. Ageing affects transcription factor activation in several tissues associated with changes in gene expression. For example, HSF1 in the adrenals of old rats has a decreased ability to bind DNA following neuro-hormonal stress (Fawcett et al., 1994), Liu et al (1996) also reported a decrease in HSF1 DNA binding activity in diploid lung fibroblasts from older humans following heat shock (Liuet al., 1996). A reduction in HSF1 activation in hearts from old rats compared to hearts from young rats has also been demonstrated following thermal stress (Locke et al., 1995).

The activation of NF-κB and AP-1 has also been reported to be affected by ageing. An up-regulation of NF-κB binding activity during ageing in the quiescent mouse heart has been shown by (Helenius et al., 1996). The DNA binding of both NF-κB and AP-1 has also been shown to be significantly increased with age in the *TA* muscles of old mice compared with the DNA binding of NF-κB and AP-1 in the TA muscles of adult mice (Vasilaki et al., 2006b)

In addition to changes in the activity of transcription factors in muscle during ageing, an ageing-specific mRNA and protein signatures for human and rodent skeletal muscle revealed changes in gene expression specific for muscle from older people and rodents (Foulstone et al., 2003; Giresi et al., 2005; McArdle et al., 1999; McDonagh et al., 2014; Saini et al., 2008; Shi & Garry, 2006; Soriano-Arroquia et al., & Goljanek-Whysall, 2016; Stewart et all., 2004; Suetta et al., 2013). Among genes and proteins dysregulated in muscle during ageing, are hormones, such as IGF-1, members of TGFβ signalling pathway, components of the cellular stress response pathways, as well as some known molecular regulators of skeletal muscle homeostasis, such as SIRT-1 (Soriano-Arroquia et al., 2016). Recently, epigenetic regulators of muscle ageing have been described and these, specifically microRNAs, are described in Section 1.4.

1.4. microRNAs as gene expression regulators in muscle

microRNAs (miRNAs, miRs) are post-transcriptional gene regulators. microRNAs role has been described in most cell types and most biological processes. microRNAs are predicted to target two thirds of the human genome and are considered robust regulators of gene expression. In the recent years,

microRNAs have attracted interest as potential novel therapeutics against diseases, including muscle wasting. The role of microRNAs in muscle health and disease is discussed in the Chapters below.

1.4.1. microRNA discovery

The first two microRNAs discovered were *Lin*-4 and *Let*-7. *Lin*- 4 miRNA was first described in 1993, by Victor Ambros and Gary Ruvkun (Almeida et al., 2011).

Lin-4 was first characterised by Ambros and Ruvkun in nematode *Caenorhabditis elegans (C.elegans)*. They observed that the heterochronic genes in the nematode responsible for the development of the larval stages were regulated by Lin-4 Deletion of Lin-4 resulted in delayed development. (Almeida et al*.,* 2011). Ruvkun's laboratory also observed that Let-7, a miRNA first observed by Reinhart and Slack, controlled Lin-4 expression during adult stage of transition during larval development (Bhaskaran & Mohan, 2014). Let-7 was the second miRNA to be described by Reinhart and Slack et al in 2000. The absence of Let-7 during larval sages caused reoccurrence or delay of the larval stage and determined the outcome development till adult (Reinhart et al., 2000).

Interestingly, Let-7 is conserved and expressed in most species like humans and flies. Let-7 was characterised in arthropods, vertebrates in various tissues like kidney, lungs, brain and heart. The discovery of Let-7 led to more studies of microRNAs in other organisms.

To date, many microRNAs have been described in humans and other species and a curated miRNA online data base: miRBase (http://www.mirbase.org/), has been created.

1.4.2. microRNA biogenesis

miRNA genes are encoded intra-or intergenically and are transcribed by RNA polymerase II or III in combination with specific transcription factors (TFs) into long primary transcripts called pri-microRNAs (Gurtan & Sharp, 2013). These transcripts are processed in the nucleus by the RNase III enzyme Drosha, in complex with DiGeorge syndrome critical region regulator 8 (DGCR8), into pre-miRNA precursors, which are actively exported into the cytoplasm by Exportin-5 (Gurtan & Sharp, 2013). Pre-miRNAs are next processed in the cytoplasm by the RNase III enzyme Dicer, in complex with TRBP, into a duplex consisting of a traditionally named a guide strand (miRNA) and a passenger star strand (miRNA*). Usually only one strand is active and the other strand is degraded, however in some cases both strands may be active. The mature miRNA is loaded into the RNA-induced silent complex (RISC) and acts as a guide strand that recognises target sites within mRNAs, usually 3'UTR regions of transcripts, based on the sequence complementarity. The microRNA seed sequence (the first 2-8 nucleotides of the microRNA) is complementary to the binding site(s) with the mRNA(s). Some additional pairing between microRNA and the target mRNA may also happen, however in animals, microRNAs are only partially complementary to their target mRNAs.

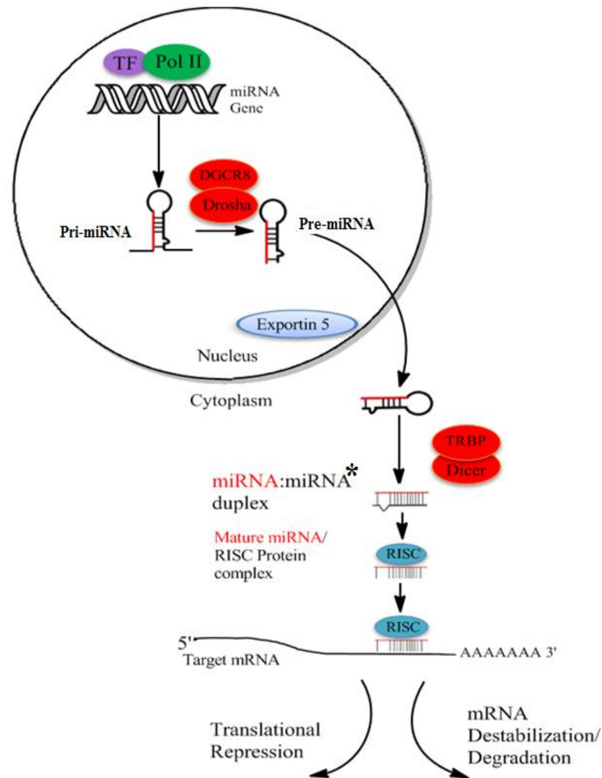


Figure 1.8. The biogenesis of microRNAs. miRNA genes are transcribed by RNA polymerase II or III, in combination with specific transcription factors (TFs) as long primary transcripts (pri-miRNAs). These are processed in the nucleus by the enzyme Drosha and DGCR8, into pre-miRNAs, which are exported into the cytoplasm by Exportin 5. Pre-miRNAs are processed by the enzyme Dicer, in complex with TRBP, into a duplex consisting of a guide strand (miRNA) and passenger star strand (miRNA*). The mature miRNA is loaded into the RISC and acts as a guide strand that recognizes target mRNAs based on sequence complementarity. The RISC subsequently represses target by inhibiting translation.

Binding of miRNA:RISC subsequently leads to target mRNA degradation and/or inhibition of translation, or both (Gurtan & Sharp, 2013). Currently, microRNA nomenclature takes into account the end of the pre-miRNA that the miRNA was generated from (-5p for miRs generated from the 5’ end of the pre-miRNA and -3p for miRs generated from the 3’end). microRNAs which are members of the same microRNA family usually have a, b, c, affix added to their name. In case of microRNAs encoded by multiple loci, additional number is added, for example

miR-133a-3p-1 and miR-133a-3p-2 are the names for miR-133a originating from the 3' end of the pre-miR-133a and from two different loci.

1.4.3. Prediction of microRNA target genes

The curated database containing information about validated microRNAs is called miRBase (http://www.mirbase.org). For the prediction of microRNA targets, several publically available target prediction algorithms can be used, for example;

Targetscan (www.targetscan.org)

D1ANA (http://diana.imis.athena-innovation.gr/DianaTools/index.php),

Miranda (http://cbio.mskcc.org/microrna_data/manual.html) and

Pictar (www.pictar.org)

However little overlap exists between different prediction algorithms, mainly due to partial rather than full complementarity between the microRNAs and their targets (Goljanek-Whysall et al., 2012). Target predictions use conservation between species, predicted RNA secondary structures and even expression patterns of microRNAs and their targets in addition to sequence complementarity.

1.4.4. microRNAs in skeletal muscle

The importance of miRNAs in tissue development and homeostasis has been shown by deleting the enzyme Dicer in muscle and other tissues. The deletion of Dicer in neural crest cells which is where the craniofacial skeleton is formed resulted in loss of morphogenesis in the tissue growth of the skull (Lian et al., 2012).

Muscle-specific loss of Dicer during development is associated with delayed myogenesis and lack of Dicer in satellite cells has a negative effect on muscle regeneration and results in mild atrophy of muscle (Cheung et al., 2012; Goljanek-Whysall et al., 2011; Sweetman et al., 2008; van Rooij et al., 2008).

Individual microRNAs have been overexpressed or downregulated *in vitro* and *in vivo* to demonstrate their role in muscle. For example, overexpression and knockdown of miR-206, miR-1 and -133 have demonstrated the role of these microRNAs in myogenic differentiation (McCarthy, 2008; Nakasa et al., 2010; Townley-Tilson et al., 2010).

Most miRNAs are universally expressed but some are expressed in tissues specific manner. Several miRNAs have been shown to be muscle specific: miRs-133, -208, -1, -499, -206 and -486. These are known as myomiRs (McCarthy, 2008).

A study using a genetic knockout of miR-208 and miR-499 demonstrated the significant role of microRNAs in skeletal muscle regeneration and function with the double knock-out displaying decreased proportion of type I myofibres in the *soleus* muscle (van Rooij et al., 2008).

Another study has shown that deletion or transient removal of miR-206 caused a delay in muscle regeneration after cardiotoxin (CTX)-induced muscle damage by preventing the fusion of new myofibres (Liu et al., 2012b). Deletion of another myomiR, miR-133, has been shown to contribute to the onset of centronuclear myopathy, as well as fast-to-slow myofibre switch, and mitochondrial dysfunction (N. Liu et al., 2011). miR-133 has also been shown to control myoblast

proliferation by regulating the expression of serum response factor (SRF). SRF is a transcript factor that is necessary for muscle differentiation (Sweetman et al., 2008).

miR-1 and miR-206, myomiRs, have been demonstrated to positively regulate myoblast differentiation through regulating the expression of SRF (Goljanek-Whysall, Pais, et al., 2012). miR-1 has also been shown to regulate muscle hypertrophy (McCarthy & Esser, 2007). miR-206 has been demonstrated to regulate *in-vitro* differentiation of myoblasts (Goljanek-Whysall et al., 2012; Nielsen et al., 2010) through the downregulation of Pola1, a fraction of DNA polymerase α, which is essential for cell proliferation (Horak et al., 2016).

Another microRNA, miR-486 does not show muscle-specific expression, however it has been shown to regulate skeletal muscle development through regulating Pax7 expression (Xu et al., 2015).

Downregulation of miR-486 expression in myoblasts has been shown to be associated with defective fusion into myotubes (Horak et al., 2016).

MyomiRs: miR-208a, -208b and -499 are encoded in myosin genes: MYH6, MYH7B and MYH7, respectively. These microRNAs have been shown to play a crucial role in regulating muscle myosin expression, and therefore muscle performance and myofibre type specification (van Rooij et al., 2009).

microRNA-181 plays an important role in regulating cellular growth, development and activation. During myogenesis, miR-181a has been shown to play a role in muscle differentiation by inhibiting the homeobox protein HOX-A11

(Naguibneva et al., 2006). Moreover, miR-181a has been shown to be upregulated immediately after exercise and the expression of miR-181a target genes suggests that it may play a role in muscle adaptation following exercise (Sharma et al., 2014). Our group has shown that miR-181a is downregulated in muscle during ageing and that it regulates myotube size *in vitro* (Soriano-Arroquia et al., 2016).

Recent studies have found that miR-378 controls myogenesis following muscle injury and in development through regulating Igf1r expression (Zeng et al., 2016). miR-378 is expressed in skeletal muscle and encoded in the first intron of gene encoding peroxisome proliferator-activated receptor γ coactivator 1β (Pgc-1β). miR-378 has been shown to regulate adipose tissue metabolism, however the *in vivo* target genes of miR-378 have not been confirmed in this study(van Rooij et al., 2009).

Another study used miR-182 gain- and loss-of-function to examine the role of miR-182 in regulating metabolic homeostasis. miR-182 has been shown to be preferentially expressed in fast twitch muscles and mice without functional miR-182 show fast-to-slow muscle fibre conversion and poor glucose metabolism (Zhang et al., 2016).

Among the transcription factors that regulate myomiR expression are MRFs (myogenic regulatory factors): Myogenin, Mrf4, MyoD and Myf5. MyomiRs are distinctively expressed and play a role during myogenesis, muscle atrophy, development, degeneration exercise and myopathies (Eisenberg et al., 2009; Goljanek-Whysall et al., 2012; Kirby & McCarthy, 2013; Sharma et al., 2014). For example, miR-1 and 206 accounts for 25% of most miRNAs and have key roles in

myoblast proliferation, differentiation and muscle hypertrophy (Nielsen et al., 2010).

The role of microRNAs in regulating muscle development and homeostasis is summarised in Table 1.2.

Table 1.2 The role of miRNAs in maintaining processes associated with muscle homeostasis. Taken from McCormick & Goljanek-Whysall, in press

miRNA	Process	Genes involved (if evidenced in study)	References
miR-133	Increases myoblasts proliferation *in vitro* and *in vivo* Increases myogenesis *in vitro* Inhibits myogenesis *in vivo* Regulates myogenesis *in vivo* Rhabdomyosarcoma *in vitro*	SRF IGF1 ERK 1/2 pathway, SRF SRF BAF chromatin remodelling complexes Micro-array with lots of different changes, see paper for details.	(Chen et al., 2006) (Huang et al.,2011) (Feng et al., 2013; Kim et al., 2014; Koutsoulidou et al.,2011) (Chen et al., 2006) (Katarzyna Goljanek-Whysall et al., 2014) (Rao et al., 2010)
miR-195	Inhibits myoblast proliferation *in vitro*	IGF1R, insulin receptor and cyclin genes	(Wei et al., 2016)
miR-1	Increases regeneration *in vitro* and *in vivo* Promotes myogenic differentiation *in vivo* Control cell fate of undifferentiated myoblasts *in vitro*	MyoD, myogenin and Pax7, GDF-8 Pax3, BAF chromatin remodelling complexes. CLCN3, Smarcd2, Smarcb1, MAP4K3, Fzd7, Meox2, RARB, NFAT5	(Kim et al., 2016; Tomoyuki Nakasa et al., 2010) (Katarzyna Goljanek-Whysall et al., 2014; Goljanek-Whysall et al., 2011) (Katarzyna Goljanek-Whysall et al., 2012)
miR-206	Promotes myogenesis *in vivo* Muscle cell fate *in vitro* Increases regeneration *in vitro* and *in vivo* Regulation of neuromuscular junction *in vivo*	Pax3, BAF chromatin remodelling complexes CLCN3, Smarcd2, Smarcb1, MAP4K3, Fzd7, Meox2, RARB, NFAT5 MyoD, myogenin and Pax7, GDF-8 HDAC4, FGF pathway	(Katarzyna Goljanek-Whysall et al., 2014; Goljanek-Whysall et al., 2011) (Katarzyna Goljanek-Whysall et al., 2012) (Kim et al., 2016; Liu et al., 2012a; Maciotta et al., 2012; Tomoyuki Nakasa et al., 2010) (McCarthy et al., 2007; Williams et al., 2009)
miR-486	Increases myoblast differentiation *in vitro*	Pax7	(Bijan et al., 2011)

miR-26a	Increases muscle regeneration *in vivo* Increase myogenesis *in vitro*	SMAD4 Ezh2	(Dey et al., 2012) (Wong & Tellam, 2008)
miR-181	Enhances myogenesis *in vivo* Regulates myofibres size *in vitro*	HOX-A11 Sirt-1	(Naguibneva et al., 2006) (Soriano-Arroquia et al., 2016)
miR-489	Controls satellite cell quiescence *in vivo*	DEK	(Tom Cheung et al., 2012)
miR-31	Controls satellite cell quiescence *ex vivo* and *in vivo*	Myf5	(Crist et al.,2012)
miR-155	Increase regeneration *in vivo*	SOCS1	(Nie et al., 2016)
miR-29a	Increase regeneration *in vitro* and *in vivo*	Fgf2, Fbn1, Lamc1, Nid2, Col4a1, Hspg2	(Galimov et al., 2016)
miR-675	Increase regeneration and myogenesis *in vitro*	BMP pathway, Cdc6	(Dey et al., 2014)
miR-431	Increase regeneration *in vivo*	Pax7	(Wu et al., 2015)
miR-208	Myofibre typing *in vivo*	Myosin genes	(van Rooij et al., 2009)
miR-378	Enhances myogenesis *in vitro*	MyoR	(Gagan et al., 2011)
miR-143	Regulation of satellite cell senescence *in vitro*	IFGBP5	(Soriano-Arroquia et al., 2016)

1.4.5. microRNAs as regulators of muscle response to exercise

microRNAs affect the whole protein content of the cell providing a high-throughput mechanism of responding to changes within the cell or the surrounding environment (Goljanek-Whysall, et al., 2012). miRNAs are predicted to target two

thirds of the human genome (Friedmanet al., 2009), therefore it is likely that microRNAs regulate muscle homeostasis following exercise.

The expression of myomiRs: miR-1/206 and miR-133has been shown to be acutely regulated in skeletal muscle during exercise (Drummond et al., 2008; Safdar et al., 2009). Moreover, Nielsen et al., (2010) have shown that microRNA expression can be modulated following acute and endurance exercise in humans and myomiR levels decrease to a new steady state level following inactivity period (Nielsen et al., 2010). miR-1 and miR-23 expression has also been shown to change following an acute bout of endurance exercise in mice (Safdar et al., 2009) and this correlated with changes in PGC1α expression, a mitochondrial biogenesis regulator. Interestingly overexpression of miR-499, which regulates the expression of slow myosin heavy chain, significantly has been shown to increase muscle endurance (van Rooij et al., 2009). The expression levels of several microRNAs, such as miR-181 and miR-378 have also been shown to correlate with muscle mass and strength (Davidsen et al., 2011), (Bloch et al., 2015).

Resistance exercise during which a mechanical strain is placed on the muscle, results in adaptations such as muscle hypertrophy. The hypertrophic response occurs mainly via accretion of contractile proteins and results in increased capacity to generate force (Kirby). In humans, an acute bout of resistance exercise has been shown to be associated with downregulation of miR-1 in skeletal muscle (McCarthy & Esser, 2007) suggesting that changes in miRNA expression following an acute bout of resistance exercise may be an integral part of the molecular changes related to muscle adaptive response.

Endurance exercise results in metabolic adaptations, such as increase in mitochondria, fibre type shift to a more oxidative profile and increase in capillary density, with little change in skeletal muscle. miRNAs have been suggested to play a role in metabolic adaptations following endurance exercise. For example, the expression of miRs: miR-1, miR-181 and miR-107 is downregulated following 90 minute bout of forced running (Safdar et al., 2009).

Despite microRNA expression having been shown to be regulated during exercise in humans and in animal models, the available data are correlative in nature and there is limited data on functional analyses of the role of microRNAs in muscle during exercise and muscle adaptation. As a single miRNA can target hundreds of genes and induce changes in multiple systems simultaneously, microRNAs are attractive candidates to regulate the adaptive response of skeletal muscle following contractions (Table 1.3).

Table 1.3 Summary of microRNAs suggested to be involved in regulation of muscle homeostasis following exercise.

miR	Exercise	Expression	Targets	Biological Function	Ref.
miR-181	Acute bout of endurance exercise (acute bout of treadmill)	Upregulated	Hoxa11 (MyoD repressor); Sirt-1	Inhibition of myogenic differentiation regulation of muscle hypertrophy/ atrophy	(Safdar et al., 2009)
miR-1	Acute bout of endurance exercise (acute bout of treadmill) Acute bout of endurance exercise and resistance exercise Endurance (6 weeks of cycling)	Upregulated immediately after exercise, downregulated after 12 weeks of training Downregulated	SRF	myomiR, muscle differentiation anti-hypertrophy, anti-proliferation	(Keller et al., 2011; McCarthy & Esser, 2007; Nielsen et al., 2010; Safdar et al., 2009)
miR-133	Acute bout of endurance exercise (acute bout of treadmill) Acute bout or 6 weeks of endurance exercise	No change Upregulated immediately after exercise, down after 12 weeks of training	SRF, Baf60, Igf1r	Control of muscle development and regeneration	(Keller et al., 2011; S. Nielsen et al., 2010; Safdar et al., 2009)
miR-23	Acute bout of endurance exercise (acute bout of treadmill)	Downregulated	PGC1alpha	Through PGC1α it might inhibit mitochondrial biogenesis	(Safdar et al., 2009)
miR-107	Acute bout of endurance exercise	Upregulated	PDK4	Maintenance of muscle homeostasis	(Safdar et al., 2009)
miR-126	Swimming (aerobic training, moderate and high volume)	Upregulated	Spred1	Pro-angiogenic through VEGF signalling through Raf-1/ERK1/2	(Silva ND et al., 2012)
miR-696	Treadmill 5 times/week for 4 weeks with increasing level of exercise	Downregulated	PGC1α	Inhibition of mitochondrial biogenesis and fatty acid oxidation in vitro	(Aoi et al., 2010)
miR-21	Treadmill	Upregulated			(Aoi et al., 2010)
miR-378	12 weeks of resistance training	Downregulated	MyoR PGC1β	Correlates with change in lean body mass; Promotes myogenic differentiationcontrols mitochondrial metabolism	(Davidsen et al., 2011)
miR-26a	12 weeks of resistance training	Downregulated	TGFβ	Muscle differentiation	(Davidsen et al., 2011; Dey et al., 2012)
miR-29a	12 weeks of resistance training	Downregulated	YY1	Enhances myogenesis	(Davidsen et al., 2011)
miR-451	12 weeks of resistance training	Upregulated	Unknown in muscle	Unknown in muscle	(Davidsen et al., 2011)
miR-101	Endurance (6 weeks of cycling)	Downregulated	Not validated in muscle	Regulates satellite cell proliferation	(Keller et al., 2011)
miR-455	Endurance (6 weeks of cycling)	Downregulated	PGC1α	Regulates brown adipogenesis	(Keller et al., 2011)
miR-494	Endurance (acute bout of swimming)	Downregulated	mTFA, Foxj3	Mitochondrial biogenesis	(Yamamoto et al., 2012)
miR-16	Endurance, swim training	Downregulated	Vegf, Vegf type 2 receptor	Angiogenesis	(Fernandes, et al., 2012)
miR-15a miR-15b	Endurance (6 weeks of cycling)	Downregulated	None validated in muscle	Protects against cardiac injury	(Keller et al., 2011)

To date there are no known microRNA biomarkers of muscle recovery, remodelling and adaptation to exercise training, which could facilitate monitoring of exercise intervention in older humans. The levels of several microRNAs however, have been reported to change in serum following exercise: miRNAs involved in angiogenesis (miRs: -20a, -210, -221, -222), inflammation (miRs: -21, -146a), skeletal and cardiac muscle contractility (miRs: -21, -133a) and hypoxia/ischaemia adaptation (miRs: -21, -146a, -210) (Baggish et al., 2014). Early muscle homeostasis biomarkers for mid-life screening and therefore interventions preventing healthy ageing remain to be established. Circulating microRNAs represent potential biomarkers of muscle remodelling and ageing (Table 1.4).

Table 1.4 Table summarising microRNA level changes in plasma/serum following exercise. Taken from (Safdar et al. 2009).

miRNA	Exercise	Expression	Targets	Biological Function	References
133a	Acute exhaustive exercise Sustained exercise training	no change no change	SRF, CCDN2	Anti-proliferation, anti-fibrosis, pro-differentiation	(Nielsen et al., 2010)
21	Acute exhaustive exercise Sustained exercise training	up up	PDCD4, RECK, PTEN, TIMP3, SPRY2, TPM1, RHOB	Anti-apoptosis, anti-proliferation, anti-inflammation, hypoxic adaptation	(Buscaglia & Li, 2011)
miR-146a	Acute exhaustive exercise Sustained exercise training	up up	IRAK1, TRAF6	Anti-inflammation, hypoxic adaptation	(Hung et al., 2013)
miR-210	Acute exhaustive exercise Sustained exercise training	no change no change	ISCU, EFNA3, E2F3	Mitochondrial metabolism, pro-angiogenesis, proliferation, survival, hypoxic adaptation	(Ullmann et al., 2016)
miR-20a	Acute exhaustive exercise Sustained exercise training	no change up	CDKN1a (p21/WAF1), E2F1, LRF	Anti-angiogenesis	(Baggish et al., 2011; Ramon et al., 2012)
miR-221	Acute exhaustive exercise Sustained exercise training	up up	KIT (c-kit), CDKN1B (p27/KIP)	Anti-angiogenesis	(Baggish et al., 2011)
miR-222	Acute exhaustive exercise Sustained exercise training	up up	KIT (c-kit), CDKN1B (p27/KIP)	Anti-angiogenesis	(Baggish et al., 2011; Gallach et al.,2014)
miR-328	Acute exhaustive exercise Sustained exercise training	no change no change	CD44	Anti-angiogenesis	(Jeyapalan et al., 2011)

1.4.6. microRNAs in skeletal muscle ageing

The mechanism that causes muscle ageing still remains unclear. Muscle-specific miRNAs, crucial regulators of skeletal muscle function, may contribute to age-dependent decline in muscle function. A satellite cell-specific Dicer knock-out, resulting in depletion of majority of microRNAs in satellite cells, has been shown to result in mild muscle fibre atrophy over time (Cheung et al., 2012)**.** miRNAs have been shown to regulate muscle development, function, hypertrophy and atrophy (Goljanek-Whysall, et al., 2012) and differential expression of miRNAs is associated with muscle disorders, such as muscular dystrophy (Eisenberg et al., 2007; Elia et al., 2009; Williams et al., 2009). Moreover, studies in *C.elegans* and humans suggest that miRNAs may act as longevity markers (Pincus et all., 2011; Serna et al., 2012). Recently, myomiRs were suggested to act as biomarkers of muscle turnover in mdx mice (Roberts et al., 2013). Interestingly, injection of miR-1/206 and miR-133 into injured muscle enhanced regeneration (Nakasa et al., 2010). Although differential expression of miRs in muscle has been described during ageing (Dhahbi et al., 2012; Drummond et al., 2008; McCarthy et all., 2009), the relevance of this is not yet understood. Nevertheless, among microRNAs dysregulated in muscle during ageing are miR-29, miR-378, miR-133, miR-143 and others (Drummond, 2010; Hu et al., 2014; Kim et al., 2014; Mercken et al., 2013; Rivas et al., 2014; Soriano-Arroquia et al., 2016; Soriano-Arroquia et al., 2016; Zacharewicz et al., 2014) .

miR-143 has been proposed to be a part of a failed compensatory mechanism aiming at improving defective skeletal muscle regeneration during ageing. miR-143 is downregulated in satellite cells of mice and humans during ageing and its downregulation leads to improved viability of satellite cells, however this is at cost of cellular senescence resulting from upregulation of mir-143 target genes: Igfbp5 (Soriano-Arroquia et al., 2016).

Let-7 miRNA has also been shown to be upregulated with age in skeletal muscle of humans. Let-7 regulates myoblast proliferation and the upregulation of Let-7 has been suggested to be associated of defective proliferation of satellite cells during ageing (McCarthy, 2014).

The cellular senescence is a process that causes permanent response to cells as a result of stress which contributes to ageing and age related disease (van Deursen, 2014). miR-29, regulator of senescence-associated genes, has been shown to be upregulated in the muscles of old mice when compared to the muscles of young rodents (Hu et al., 2014). Indeed, functional analyses of muscle following microRNA-29 gain- and loss-of-function approaches have confirmed that this microRNA may regulate senescence of the muscle. Therefore, upregulation of miR-29 expression in muscle during ageing has been suggested to drive some aspects of sarcopenia.

To summarise, limited data exists on functional aspects of microRNA dysregulation in muscle during ageing. It is not clear whether microRNAs drive loss of skeletal muscle or are a part of compensatory mechanism aiming at

improving muscle mass and function during ageing and further functional studies are need.

1.4.7. microRNA in muscle disease

Muscle loss during muscle and other tissues disorders is associated with disrupted molecular mechanisms regulating muscle homeostasis. Some of the muscular disorders include Becker muscular dystrophy (BMD), Facioscapulohumeral muscular dystrophy (FSHD), Duchenne muscular dystrophy (DMD) other muscular dystrophy are Limb-girdle muscular dystrophies which comprises of type 2A and 2B, inclusion body myositis, Miyoshi, Nemaline an polymyositis (Goljanek-Whysall et al., 2012). These disorders clearly have different mechanisms and are associated with a variety of pathophysiological changes in muscle. Nevertheless, common changes on the molecular level have been observed. Interestingly, the expression of microRNAs in muscle during these disorders changes and several microRNAs have been implicated in the pathogenesis of these disorders or even suggested to act as potential therapeutics (Table 1.5).

1.4.8. microRNAs as potential therapeutics against muscle ageing

The importance of miRNAs developed an interest towards the therapeutics aspect of these noncoding RNAs (Catalucci et al., 2008). Several microRNAs dysregulated in muscle during ageing and disease have important roles in muscle: miR-133 and miR-1 are necessary for skeletal muscle development, mIR-208a plays a role in skeletal muscle metabolic homeostasis,

miR-378, plays a role in inhibiting mitochondrial fatty metabolism (Wada et al., 2015) and miRs-23a, 499 and 208b control myosin heavy-chain expression (Wada et al., 2015).

Since microRNA mimics or inhibitors have the ability to alter the effects of overexpressed or underexpressed miRNAs in disease condition, microRNA-based therapeutics are currently under investigation (Reichel et al., 2015).

Some proof-of-principle studies have been performed: a study using rat skeletal muscle has demonstrated the positive effects of overexpressing miR-1, mir-206, and miR-133 mimics on muscle regeneration (Nakasa et al., 2010). microRNA-based therapeutics are currently in clinical trials therefore microRNAs may be emerging interventions for ageing- and disease-associated muscle loss.

Table 1.5 Summary of miRNA associated with muscle homeostasis and disorders. Taken from K. Goljanek-Whysall, et al 2012

Disease/adaptive response	**miRNA**	**References**
DMD	Expression of 37 miRNAs increased and 20 miRNAs decreased; increased expression of eight miRNAs including miR-206 , decreased expression of miR-1, miR-135a and miR-29c; increased expression of miR-31 targeting dystrophin; decreased expression of miR-486	(Alexander et al., 2011; Cacchiarelli et al., 2011; Eisenberg et al., 2007; Vasudevan et al., 2007)
BMD Limb-girdle muscular dystrophy type 2A Limb-girdle muscular dystrophy type 2B Miyoshi myopathy	Increased expression of miR-221 and miR-146b Increased expression of 80 miRNAs; decreased expression of miR-30a-3p and miR-197 Increased expression of 81 miRNAs; decreased expression of miR-30a-3p and miR-510 Increased expression of 64 miRNAs; decreased expression of miR-30a-3p, miR-30c and miR-302c	(Alexander et al., 2011; Cacchiarelli et al., 2011; Eisenberg et al., 2007; Vasudevan et al., 2007)
FSHD Polymyositis Inclusion body myositis Dermatomyositis	Increased expression of 57 miRNAs Increased expression of 35 miRNAs; decreased expression of miR-30a-3p Increased expression of 20 miRNAs; decreased expression of miR-197 Increased expression of 33 miRNAs; decreased expression of miR-30a-3p Increased expression of miR-206 in five out of seven patients; increased expression of miR-1 and miR-335; decreased expression of miR-29b , miR-29c and miR-33	(Alexander et al., 2011; Cacchiarelli et al., 2011; Eisenberg et al., 2007; Vasudevan et al., 2007)
DM1 RMS	Decreased levels of miR-1 and miR-133a; increased expression of miR-1, miR-133a, miR-133b and miR-206 in serum	(Alexander et al., 2011; Cacchiarelli et al., 2011; Eisenberg et al., 2007; Vasudevan et al., 2007)
ALS Atrophy	Increased expression of miR-206 miR-1, miR-206 , miR-133, miR-23a , miR-128 , miR-499 and miR-208b are potentially protective	[(Alexander et al., 2011; Cacchiarelli et al., 2011; Eisenberg et al., 2007; Vasudevan et al., 2007)
Hypertrophy	Expression of miR-206 , miR-1, miR-133a, miR-23, miR-499 , miR-378 , miR-29a , miR-26a and miR-451 was found to be modulated	(Alexander et al., 2011; Cacchiarelli et al., 2011; Eisenberg et al., 2007; Vasudevan et al., 2007)
Growth and regeneration	myomiRs	(Alexander et al., 2011; Cacchiarelli et al., 2011; Eisenberg et al., 2007; Vasudevan et al., 2007)

1.5. Hypothesis

As microRNA levels are responsive to environmental and intracellular changes and microRNAs can regulate several signalling pathways simultaneously (Alvarez et al., 2006), I hypothesise that microRNAs are a part of muscle adaptive response to contractions by regulating the expression of genes involved in this response and play a significant role in the blunted adaptive response of muscle to contractions during ageing.

I have tested this hypothesis by selecting and validating microRNA candidates regulating muscle adaptive response to contractions based on available literature and my preliminary data. I have characterised novel microRNA: target interactions playing a role during muscle adaptive response to contractions *in vitro* (mouse $H2K^b$ cells) and validated these *in vivo* (C57BL6 mice). Finally, using microRNA mimics and antagomiRs I have tested whether manipulation of microRNA expression *in vivo* drives a more efficient adaptation of muscle to exercise.

1.6. Aims and Objectives

The aims and objectives of this project were:

1. To select the most promising candidate microRNAs involved in regulation of the expression of target genes related to muscle adaptation following contractions, such as regulators of redox homeostasis.
2. To validate novel microRNA:target interactions during muscle contraction using an in vitro model: murine $H2K^b$ cells.

3. To characterise the role of disrupted microRNA:target interactions in quiescent and contracted skeletal muscle in order to establish whether changes in microRNA expression regulate muscle adaptive response following contractions in adult and old mice.

4. To test whether microRNA gain- or loss-of-function approaches may ameliorate muscle loss of mass and function (strength) during ageing using *in vivo* mouse model.

2. Materials and Methods

This study aimed to characterise the role of microRNAs in skeletal muscle adaptive response to contractions. Two model systems where used in this study:

- *In vitro* model: *H2k*^{b} mouse myoblast cell line,
- *In vivo* model: Adult (6 months) and old (24 months) C57BL6 mice.

2.1. Cell Culture

This study used differentiated H2k^{b} cells as an *in vitro* model of myofibres. Specifically, *H2k*^{b} myotubes were used to study changes in microRNA expression following an isometric contraction protocol and the effects of disrupted miR-181 expression on the myotube size.

Reagents:

- Dulbecco's modified medium (Sigma Aldrich, Dorset, UK)
- 15% v/v Foetal Bovine Serum (Life technology Warrington, UK)
- 2%v/v Horse serum (Life technology Warrington, UK)
- 1%v/v L-Glutamine (Sigma Aldrich, Dorset, UK,)
- 100µmol/ml and 0.1mg/ml streptomycin Penicillin/Streptomycin (Sigma Aldrich Dorset, UK)

Protocol:

H2k^b cells were originally cloned from the limb muscles of H2k^b-tsA58 transgenic mice (Beaucham et al., 1998) and were obtained from Prof. J. Morgan, University College London. The H2k^b cells were cultured in 6-well plates at 37°C, 5%CO2 in growth media (GM): Dulbecco's modified medium (DMEM) supplemented with 10% v/v Foetal Bovine Serum (FBS), 1%v/v L-glutamine and 1%v/v penicillin/streptomycin. Cells were cultured until they reached 90% confluency, then media was changed to differentiation media (DM): DMEM supplemented with 2%v/v Horse Serum (HS), 1%v/v/ L-glutamine and 1%v/v penicillin/streptomycin.

For miR-181 overexpression or inhibition in H2kb myotubes, cholesterol-conjugated microRNA mimic or antagomiR, (Dharmacon, Gelife sciences,UK) respectively, were added to differentiated H2K^b myotubes cultured in differentiation media, at 100nM final concentration. Transfection procedures using lipid vesicles were not required as miRNA mimics and antagomiRs used were conjugated with cholesterol moiety. This allowed for microRNA mimics and inhibitors (antagomiRs) to enter the cells efficiently: approximately 60-70% myotubes were successfully transfected and changes in micoRNA levels following miR-181 mimic or antagomiR-181 treatments were within physiological levels (2-4 times fold changes). Both miR-181 mimic and antagomiR were custom-made by (Dharmacon, Gelife sciences, UK). miR-181 mimic was predesigned (miRIDIAN miR mimic with 5'-cholesterol on the passive strand and ; 3'-Cy3 on the guide strand) AntagomiR-181 was designed as follows:

5’-FITC-mA*mC*mUmCmAmCmCmGmAmCmAmGmCmGmUmUmGmAmA*

mT*mG*mU*mU-Cholesterol 3' (m- 2'-hydroxy-methyl modified bases to reduce RNA digestion, *- phosphothioate bonds). Both miRNA mimic and antagomiR were synthesised using >70nmol synthesis scale and purified by *in vivo* HPLC. The control antagomiR “Scrambled” was used as a small RNA Control not predicted to target any known microRNA, as previously described (Goljanek-Whysall et al., 2011). Following the treatments, $H2K^b$ myotubes were incubated for 48h before RNA, protein isolation or contraction protocol.

2.2. Isometric contraction protocol of $H2k^b$ myotubes *in vitro*

Reagents:

- Phosphate buffer saline (PBS)
- Hugo Sachs Electronic Harvard Apparatus Type 223. (Stimulator CS, Germany)

Protocol:

Once differentiated, $H2k^b$ myotubes were stimulated with electrical stimulator (Hugo Sachs Electronic Harvard Apparatus Type 223 Stimulator CS, Germany) for 15 minutes using platinum electrodes made of 1mm platinum wire. In each well, two half- circle electrodes were aligned to the side of the well in other to obtain a maximal stimulation. Stimulation was performed with trains of alternating square wave pulses of 2 mines in duration for 0.5 s repeated every 5 s at 50 Hz and 30 V/well. Total stimulation time was 15 min.

Following the contraction protocol, cells were lysed immediately or incubated at 37^{0}C for a set period of time before lysis.

2.3. Animal maintenance

The study was performed using male wild type C57Bl/6 mice and Thy1-YFP mice which express Yellow Fluorescent Protein (YFP) in neuronal cells only as previously described. (McArdle et al., 2004).

Wild type C57BL6/J mice were obtained from Charles River (Margate). All mice were maintained under specific-pathogen free (SPF) conditions and fed ad libitum a standard chow and maintained under a 12 hour light-dark cycle. Experiments were performed in accordance with UK Home Office guidelines under the UK Animals (Scientific Procedures) Act 1986 and received ethical approval from the University of Liverpool Animal Welfare and Ethical Review Body (AWERB).

2.4. Isometric contraction protocol of skeletal muscle *in vivo*

Isometric contractions were performed as described previously. Mice that did not undergo the contraction protocol served as controls.

Reagents:

- 66mg/kg of body weight Ketaset/saline (Zoetis, USA)
- 55mg/kg of body weight Domitor/saline (Zoetis, USA)
- Hugo Sachs Electronic Harvard Apparatus Type 223(CS, Germany)

Protocol:

Wild type adult and old mice C57BL6/J were anaesthetised with 100µl of 1:5 ketaset/saline and 100ul of 1:6 domitor/saline (McArdle et al., 2004)Both hind limbs were subjected to a 15-minute period of isometric contractions through two electrodes, using a 100Hz pulse train at 60v for 0.5 seconds every 5 seconds with a pulse width of 0.1ms (Vasilaki et al., 2006). The mice were sacrificed immediately by cervical dislocation, and *tibialis anterior* and other muscles were removed, frozen in liquid nitrogen or mounted in OCT and stored in -80°C freezer until RNA analysis.

2.5. Isolation of RNA from H2kb myotubes using Trizol.

Reagents:

- RNAse-free water (Sigma-Aldrich, Dorset, UK)
- 100% Ethanol (Sigma-Aldrich, Dorset, UK)
- Chloroform (Sigma Aldrich, Dorset, UK)
- Trizol (Life technologies, Warrington, UK)
- Isopropanol:Isoamyl Alcohol 1:25 (Sigma-Aldrich Dorset, UK)
- Phosphate Buffered Saline (Sigma-Aldrich, Dorset UK)
- 6-well plates (Starlab Ltd, UK)
- ThermoScientific Nanodrop 2000c Spectrophotometer (Thermofisher Ltd UK).

Protocol:

All surfaces were cleaned with 70% ethanol; the pipettes were treated with RNaseZAP before commencement of the RNA extraction. RNA was isolated from all samples using the Trizol method according to the manufacturer's protocol. RNase-free filter tips (Star Lab) were used in all steps to avoid contamination with RNases. RNA extraction was performed in Class 2 fume cupboards (Clean Air Invent. UK)

Following isometric contractions of H-2k^b myotubes, the media was removed and cells were rinsed with PBS 500μl of Trizol was added into 6-well plates and sterile cell scraper was used to scrape the cells from the plates and to transfer them into Eppendorf tubes. Two hundred microliters of chloroform was added into each tube, samples were vortexed for homogenisation and centrifuged at 12,000rpm for 15 minutes at 4°C. Following centrifugation, 3 separate phases were observed, the upper phase was placed in a new Eppendorf tube 500μl of isopropanol. The samples were vortexed and incubated at room temperature for 10 minutes for RNA to precipitate. Next, samples were centrifuged at 12,000g for 15 minutes at 4°C and the isopropanol removed. Two hundred microliters of 80% ethanol was added, the samples were centrifuged at 12,000rpm for 2 minutes. Ethanol was removed and the RNA pellets were allowed to dry for 10minutes. Thirty microliters of RNAase-free water was added onto the pellet to dissolve the RNA. This protocol is pictured in Figure 2.1.

RNA concentration and quality were measured using Thermo Scientific Nanodrop 2000c Spectrophotometer. The RNA was stored at -80°C freezer for further analysis.

2.6. Isolation of RNA from skeletal muscle

Reagents:

- RNAse-free water (Sigma-Aldrich, Dorset, UK)
- 100% Ethanol (Sigma-Aldrich, Dorset, UK)
- Chloroform (Sigma Aldrich, Dorset, UK)
- Trizol (Life technologies, Warrington, UK)
- Isopropanol:Isoamyl Alcohol 1:25 (Sigma-Aldrich, Dorset UK)
- RNaseZAP™ AM9780 (Live Technologies Warrington, UK)
- ThermoScientific Nanodrop 2000c Spectrophotometer(Thermofisher Ltd, UK)

Protocol:

All surfaces were cleaned with 70% ethanol; the pipettes were treated with RNaseZAP (Life Technologies, Warrington, UK) on commencement of the RNA extraction.

RNA was isolated from all samples using the Trizol method according to the manufacturer's protocol. RNase-free filter tips Star Lab (Thermofisher Ltd, UK) were used in all steps to avoid contamination with RNases. RNA extraction was performed in Class 2 fume cupboards (Clean Air Invent, UK).

The *tibialis anterior* or other muscles from mice were ground to powder in liquid nitrogen and homogenized using 1ml of Trizol per muscle. Next, 200µl of chloroform per sample was added. The samples were mixed vigorously for 15 seconds by vortexing and incubated on ice for 5 minutes and centrifuged at 12,000g for 15 minutes at 4°C (Eppendorf centrifuge 5417R, Hamburg, Germany).

The upper aqueous phase was transferred to a new collection tube; five hundred microliters of 100% isopropanol was added into the tubes and mixed by vortexing. The remaining procedure was continued as described above in Chapter 2.2.

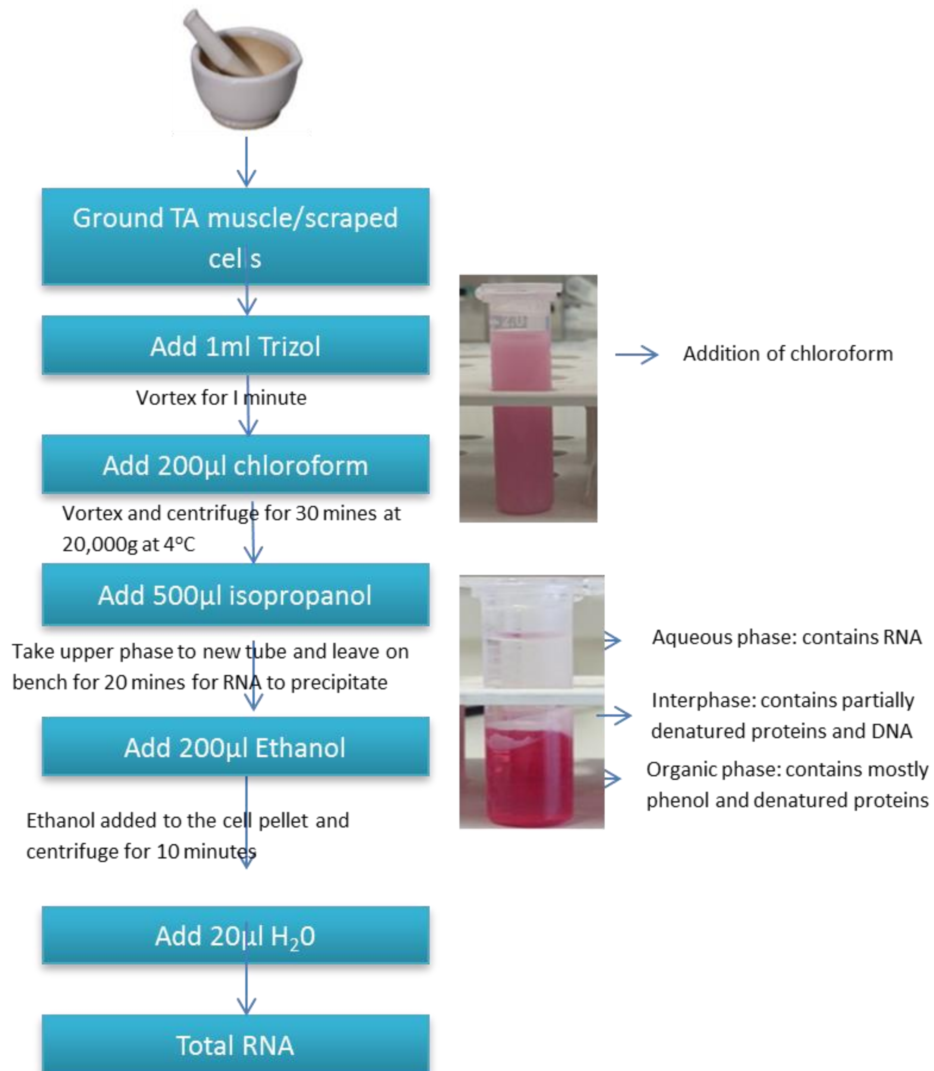


Figure 2.1: Flow chart of RNA extraction using Trizol showing the separation of phases following the addition of chloroform.

cDNA was synthesised from the RNA using miScript II Reverse Transcriptase kit (Qiagen).

Reagents:

- miScript HiSpec Buffer (Qiagen, UK)
- miScript Nucleics mix. (Qiagen, UK)
- miScript Reverse Transcriptase mix. (Qiagen, UK)

- RNAse-free water (Qiagen, UK)
- Total RNA (500ng)
- 96-wells, semi skirt straight Edges plates (Star Lab Ltd, UK)
- Bio-Rad CFX Connect ™ Real-Time System (Optics Model, Singapore)

Protocol:

Recommended amount of the Reverse Transcriptase mix was added into the PCR 96-wells plates. Appropriate volume of RNA (500ng) and water was added to obtain twenty microliters of total volume. The samples were incubated in thermal cycler as described below.

The reaction protocol:

60 minutes 37°C

5 minutes 95°C

5 minutes 4°C

cDNA was diluted 1:10 and was stored in -20°C for qPCR analyses.

2.8. Reverse Transcription (mRNA)

First-strand cDNA was synthesised from the RNA using Invitrogen reagents as described below.

Reagents:

- Random Hexamers (Sigma Aldrich, Dorset, UK)
- Reverse Transcription Buffer (5x) (Invitrogen, Paisley, UK)

- Dithiothreitol (DTT, Invitrogen, Paisley, UK)
- Deoxynucleotides (dNTPs),(Invitrogen, Paisley, UK)
- RNAse Inhibitor (Invitrogen, Paisley, UK)
- Superscript Reverse Transcriptase II (Sigma Aldrich, Dorset, UK)
- Bio-Rad CFX Connect ™ Real-Time System(Optics Model, Singapore)

Protocol:

Five hundred nanograms of RNA and 1µl of random hexamers were added into the PCR 96 wells plates to a total volume of 11µl. The samples were incubated at 65°C for 10 minutes. Next samples were placed on ice and 9µl of the following mix was added:

Reagent	**Volume (µl)**
Buffer (5X)	4
DTT	2
dNTP	1
RNAsa	1
Superscript Rev. Trans.	1

The reaction protocol:

60 minutes 65°C.

5 minutes 4°C

cDNA was diluted 10 times and stored at -20°C.

Table 2.1 Primer sequences used in Real-Time quantitative PCR.

Gene name	Forward primer sequence	Reverse primer sequence
Beta-2-microglobulin	GGAGAATGGGAAGCCGAACA	TCTCGATCCCAGTAGACGGT
Pten	TTGGCGGTGTCATAATGTCT	GCAGAAAGACTTGAAGGCGTA
mFOS	GGTGAAGACCGTGTCAGGAG	AGTTGATCTGTCTCCGCTTGG
mFOSb	GATCGACTTCAGGCGGAAAC	TTCGTAGGGGATCTTGCAGC
Sirt-1	GGCCGCGGATAGGTCCATA	CCACAGGAGACAGAAACCCC
mJunD	TACGCAGTTCCTCTACCCGA	AAACTGCTCAGGTTGGCGTA
mJunb	TCACGACGACTCTTACGCAG	TGTCTGATCCCTGACCCGAA
mPax7	CAGACAGGTGGCGACTCCG	CGGCTAATCGAACTCACTGAGGG
mPrdx3	TGAGTGTCAACGACCTTCCG	GGCTTGATCGTAGGGGACTC
mJun	GCACATCACCACTACACCGA	GGGAAGCGTGTTCTGGCTAT
mNfkb3	CGGATTCCGGGCAGTGAC	GAGGGGAAACAGATCGTCCA
mNFkb1	ACACGAGGCTACAACTCTGC	GGTACCCCCAGAGACCTCAT
mNfkb2	CGCCTCTCTTCACCTTAGGC	GATAGGGGCCATCAGCTGTC
Igfpb5	TACGGCGAGCAAACCAAGAT	CAGGGGCCTTGTTCGGATTC
Igf1r	CTGATGTCTGGTCCTTCGGG	TGATGCTGCCGATGATCTCC

Table 2.2 List of products used in qPCR experiments.

Product	Product number	Source
Real-Time qPCR		
miRScript RT II kit	218161	Qiagen
miRScript SybrGreen	218073	Qiagen
Sso-Advanced universal SybrGreen Super-Mix	L001893A	Biorad
Rnu-6 qPCR primer	MS00033740	Qiagen
Snord-61 qPCR primer	MS00033705	Qiagen
10x miScript universal primer	14802886	Qiagen
Sybergreen qPCR master mix	148023932	Qiagen
miR-181a primer	MS0008827	Qiagen
miR-181b primer	MS00006699	Qiagen
miR-181c primer	MS00008841	Qiagen
miR-181d primer	MS000031500	Qiagen
miR-378 primer	MS00002278	Qiagen
miR-15 primer	MS00001281	Qiagen
miR-24 primer	MS00005922	Qiagen
miR-143 primer	MS00001617	Qiagen
miR-34a primer	MS00001428	Qiagen
miR-130 primer	MS00001554	Qiagen

2.9. microRNA expression profiling using Real-Time quantitative polymerase chain reaction (qPCR)

Reagents:

- Sybergreen Master Mix (Qiagen, UK)
- Luminaris Higreen fluorescence Master Mix (Thermo Scientific UK)
- Universal primer (Qiagen, UK)
- microRNA Primer (Qiagen, UK)
- Rnu-6, snord-61 primers (Qiagen, UK)
- X-Clear Advanced polyolefin seal (Bio-Rad).

Protocol:

The qPCR reaction composition was as follows:

Reagents	Volume
Sso Advanced ™ universal SYBR Green Super mix	5µl
10x Universal primer	1µl
miRs Primer	2µl

The reaction Protocol:

3 minutes 95°C

30 seconds 95°C

30 seconds 55°C

30 seconds 72°C

Table 2.3 Lists of microRNA primers used in qPCR microRNA expression analysis

microRNA	miRcript primer ID	microRNA Sequence
miR-181a	mmu-miR-181a-5p MIMAT0000210	AACAUUCAACGCUGUCGGUGAGU
miR-378	mmu-miR-378a-5p MIMAT0000742	CUCCUGACUCCAGGUCCUGUGU
miR-16	mmu-miR-16-5p MIMAT0000527	UAGCAGCACGUAAAUAUUGGCG
miR-133	mmu-miR-133a-5p MIMAT0003473	GCUGGUAAAAUGGAACCAAAU
miR-199	mmu-miR-199a-5p MIMAT0000229	CCCAGUGUUCAGACUACCUGUUC
miR-15	mmu-miR-15b-5p MIMAT0000124	UAGCAGCACAUCAUGGUUUACA
miR-24	mmu-miR-24-1-5p MIMAT0000218	GUGCCUACUGAGCUGAUAUCAGU
miR-143	mmu-miR-143-5p MIMAT0017006	GGUGCAGUGCUGCAUCUCUGG
miR-34a	mmu-miR-34a-5p MIMAT0000542	UGGCAGUGUCUUAGCUGGUUGU
miR-34b	mmu-miR-34b-5p MIMAT0000382	AGGCAGUGUAAUUAGCUGAUUGU
miR-34c	mmu-miR-34c-5p MIMAT0000381	AGGCAGUGUAGUUAGCUGAUUGC
miR-130	mmu-miR-130b-5p MIMAT0004583	ACUCUUUCCCUGUUGCACUACU

2.10. Cryosectioning of skeletal muscle tissue

Muscles from mice intravenously injected with microRNA-181 mimic or inhitior (antagomiR) three times over 4-week time periodwere dissected following cervical dislocation and prepared for sectioning.

2.11. Preparation of tissue for cryostat sectioning

Reagents:

- 15% w/v Sucrose (Sigma Aldrich, Dorset, UK)
- 7.5%w/v Gelatin (Sigma Aldrich, Dorset, UK)
- 0.1%w/v Sodium azide (Sigma Aldrich, Dorset, UK)
- O.C.T (Cell path)
- 2-Methylbutane (Sigma Aldrich, Dorset, UK).

Protocol:

The gelatin powder was added to PBS, the mixture was heated to 45^{0}C and stirred until the gelatin has completely dissolved. After the mixture was homogenous, the gelatin solution was allowed to cool down for 1 min before sodium azide (for tissue preservation) and sucrose were added.

2.12. Preparation of tissue for cryostat sectioning

Reagents:

- Gelatin (Sigma Aldrich, Dorset, UK).

Protocol:

Muscles of mice were placed on a labelled weighing boats and covered with the gelatin embedding solution. The boats were covered with foil in to avoid evaporation. They were then placed in the oven at 37^{0}C for 3-4

hours and left at room temparature for 1 hour. The samples were covered with cling film and kept at $4^{o}C$ until ready to cut and snap freeze.

2.13. Tissue Embedding and Sectioning

Reagents:

- OCT (Cell Path).

Protocol:

Gelatin embedded muscles were mounted on a cork disk and further embedded with OCT. The block was then placed in isopentane pre-cooled with liquid nitrogen. All tissues were stored at $-80^{0}C$ until cryosectioning.

Muscles were cryosectioned (transverse oriented) at 8µm thickness using (leica Microsystem CM 1850 Germany). The serial section were collected using the gelatin-coated slides and allowed to air dry. On completion of sectioning, slides were frozen in $-20^{0}C$ until further staining.

2.14. Preparaton of chrome-alum gelatin coated slides (CAG)

Reagents:

- 1g gelatin (Sigma-Aldrich, Dorset, UK)
- 0.19g chrome alum (BDH Laboratory Supplies)
- 0.5 g sodium azide (Sigma-Aldrich, Dorset, UK)

Protocol:

All reagents were disolved in 200ml PBS, heated to 45^{0}C and filtered before use. The glass slides (Solmedia) were coated with gelatin to enable the tissue adherence to the slides.

2.15. Sirt-1 immunostaining

Reagents:

- 2% v/v Horse serum (Life technology, Warrington, UK)
- Monoclonal anti-rabbit Sirt-1 antibody (Abcam, UK)
- Goat anti rabbit-Alexa Fluor 488, A11008 (Invitrogen, Paisley, UK)
- Triton X (Sigma- Aldrich, Dorset, UK, Dorset UK)
- 4,'6-diamidino-2-phenylindole (DAPI; Sigma Dorset, UK)
- Phosphate buffer saline (Sigma Dorset, UK)
- 4% Paraformaldehyde (Sigma Dorset, UK).

Protocol:

For Sirt-1 immunostaining OCT-embedded muscle tissues were sectioned as described above, air-dryed for 10 minutes, washed twice with PBS for 5 minutes and fixed in 4% PFA for half an hour at room temperature. Slides were placed in 2% horse serum with 0.01% triton X for 1 hour and incubated overnight with Sirt-1 primary antibody diluted 1:150 in 2% horse serum with 0.01% triton X at 4°C. Next, three 5-minute PBS washes were performed and the secondary antiobody (Alexa-Fluor conjugated, Invitrogen) at 1:500 dilution in PBS added and incubated at room temperature for 1 hour followed by 3 5-minute PBS washes To allow visualisation of the nucleus, slides were incubated

in a solution containing DAPI (Sigma), diluted 1 in 10,000 in PBS for 10-minutes followed by a PBS wash at room temperature. Finally, sections were mounted in Hydromount (National diagnostics, USA) and placed at 4oC protected from light until visualisation with a fluorescent microscope (Eclipse Ti-Elnverted Microscope System Nikon. Eyepieces: CFI10x/22; Total magnification: 100x).

2.16. WGA staining:

Wheat Germ Agglutinin WGA staining was performed on muscle sections to determine myofibre cross sectional area.

Reagents:

- Phosphate buffer saline-Tween (Sigma Dorset, UK)
- Phosphate buffer saline (Sigma Dorset, UK)
- Wheat germ agglutinin (WGA; Vector Laboratory UK)
- DAPI (Sigma Dorset, UK)

Protocol:

The fixed sections were removed from -20^{0}C freezer and air dried for approximately 30 minutes. Next, the sections were washed with PBS-Tween 20 for 10 minutes and incubated with WGA (Vector Laboratory UK) diluted 1:1000 and DAPI diluted 1:1000 in PBS for 10 minutes. The sections were next washed with PBS for 10 minutes and mounted in Hydromount (National Diagnostics UK).

2.17. MF20 immunostaining of $H\text{-}2k^b$ myotubes

Reagents:

- 100% Ice-cold Methanol (ThermoFisher Ltd, UK)
- MF20 (Developmental studies hybridoma bank)
- Goat anti-mouse M488 (Invitrogen, Paisley, UK)
- DAPI (Sigma-Aldrich, Dorset UK, Dorset UK).

Blocking Solution 1

- 10% v/v Horse serum ((Sigma-Aldrich, Dorset UK)
- 0.1%v/v Triton-X (Sigma-Aldrich, Dorset UK, Dorset UK)
- Phosphate buffer saline (Sigma-Aldrich, Dorset UK).

Blocking Solution 2

- 10% v/v Horse serum (Sigma-Aldrich, Dorset UK)
- 0.05%w/v Triton-X ((Sigma-Aldrich, Dorset UK)
- Phosphate buffer saline (Sigma-Aldrich, Dorset UK).

Protocol:

At 90% confluence, cells were differentiated in 12 well plates with growth media. The media were taken off and cells were rinsed with PBS using 500µl per each well. The myotubes were fixed with cold Methanol for 5-10 minutes. Methanol was taken off and cells were rinsed with PBS. Next, blocking solution 1 was added and incubated on a rocker for 1 hour. Then primary antibody, MF20 diluted 1:1000 was added in blocking solution 2 and was kept overnight.

The following day, cells were rinsed 3 times for 5 minutes each and anti-mouse M488 (green) diluted 1:1000 in PBS was added for 1 hour. DAPI was added 1:1000 in PBS for 5-10 minutes and rinsed off with PBS. Next, 1 ml of fresh PBS was added to the cells and sealed with parafilm to avoid evaporation. Plates were wrapped with foil and stored at 4^oC until analysis.

2.18. Protein isolation from *H-2k^b* myotubes

Reagents:

- Phosphate buffer saline (Sigma-Aldrich Dorset, UK)
- 1%w/v sodium dodecyl sulphate (Sigma-Aldrich Dorset, UK)
- Protease inhibitors (Sigma Aldrich, Dorset, UK).

Protocol:

Cells in each well were harvested by scraping with 200µl of PBS into 1.5 eppendorf tube and centrifuged in eppendorf centrifuge 541R at 14,000rpm for 5 minutes at 4^0C. Supernatant was removed and each pellet was re-suspended in 50µl 1% sodium dodecyl sulphate (SDS) containing protease inhibitors.

Samples were sonicated on ice for 15 seconds (Soniprep 150) MSE and were centrifuge at 4^oC for 5 minutes at 14,000rpm. Supernatant was stored at -40^oC until analysis.

2.19. Protein content determination using Bichinchoninic Acid (BCA)

Reagents:

- Bovine serum albumin (BSA) 1mg/ml (Sigma-Aldrich, Dorset, UK)
- Reagent A: Bicinchoinic acid (BCA) solution, containg: 2mM BCA-Na, 160mM NaCO3.H_20, 7.0mM Na_2 tartrate, 0.1mM NaOH and 0.95% $NaHC0_3$, pH 11.2 (Sigma-Aldrich, Dorset, UK)
- Reagent B: 160mM $CuS0_4$.%5H_20 (Sigma-Aldrich, Dorset, U.K)
- Reagent C: 25ml of Reagent A and and 500µl of Reagent B
- Copper sulphate (Sigma Aldrich, Dorset, UK,)
- Distilled water
- Protein loading buffer (National diagnostics, Hull, UK)

Protocol:

Protein content of the samples was determined using a BCA (bichinchoninic acid) assay. Range of standards from 25-500µg/ml were prepared from a stock solution of 1mg/ml bovine serum albumin (BSA) and diluted with dH_20.

Twenty microlitres of each standard and samples that had been previously diluted with DH_2O was added to a well. Immediately before use, copper sulphate was diluted 50x with BCA solution and 200µl of this added to each well. The plate was then incubated at 37^oC for 30 minutes before measuring the absorbance at 570nm on the spectrophotometer (BMG laboratories, Buckinghamshire, UK). The protein content was calculated from the standard curve. Each standard and sample was run in duplicate. Fifty

micrograms of protein was diluted 1:1 with protein loading buffer in preparation for detection of protein content of specified antibodies.

2.20. Preparation of Polyacrylamide gels.

Reagents:

Stock acrylamide solution:

- 30% acrylamide, 0.8% bisacrylamide cross-link, in dH_20 (Protogel, National Diagnostics, USA)

Resolving buffer:

- 1.5M Tris/HCL, 0.384% SDS, pH 8.8 (Protogel buffer, National Diagnostics, USA)

Stacking buffer:

- 0.5M Tris/HCL, 0.4% SDS, pH 6.8 (Protogel stacking buffer, National Diagnostics, USA)

12% acrylamide gel:

- 8ml stock acrylamide solution (Protogel, National Diagnostics, USA)
- 5ml resolving buffer ((Protogel, National Diagnostics, USA)
- 6.8ml dH_20.

4% stacking gel solution:

- 1.3ml stock acrylamide solution, (Protogel, National Diagnostics, USA)
- 2.5ml stacking buffer, (Protogel, National Diagnostics, USA)
- 6.1ml dH_20.
- 10% (w/v) Ammonium persulphate (APS, Sigma Aldrich, Dorset, UK)

- NNN’N’-tetramethylene-diamine (TEMED, Sigma Aldrich, Dorset, UK).

Protocol:

Polyacrylamide gels were prepared as described above. To allow polymerisation of the resolving gel, 200µl of 10% Ammonium persulphate solution (APS) and 20µl of NNN’N’-tetramethylethylene-diamine (TEMED) were added to 12% acrylamide solution and immediately the gel solution was poured between glass plates (1.5mm spacers). Following the polymerisation of the 12% gel, 100µl of 10% APS and 25µl of TEMED were added to the 4% acrylamide solution. The 4% acrylamide solution was then immediately placed on top of the 12% acrylamide gel and a well comb placed in position to permit sample loading.

2.21. Electrophoresis of proteins (SDS-PAGE).

Reagents:

Electrophoresis buffer:

- Running buffer:

10x Tris/Glycine/SDS (0.025M Tris, 0.192M glycine, 0.1 %(w/V) SDS; National Diagnostics, Hull, UK)

- Distilled waterMolecular weight maker (17-225kDA) (Amersham, Buckinghamshire, UK).

Protocol:

Pre-prepared acrylamide gels were placed in running buffer in the electrophoresis tanks (Geneflow) using a power pack (Biorad, Hertfordshire, UK) and equal volume of proteins were loaded into the pre-prepared acrylamide

gels. To allow separation of the proteins a constant current of 12mA per gel was applied until the proteins ran through the 4% stacking gel. Next, current was increased to 20mA per gel until bromophenol blue dye had reached the bottom of the electrophoresis tank or bottom of the resolving gel. The proteins were then transferred onto a nitrocellulose membrane.

2.22. Western blotting

Reagents:

- Anode 1 buffer: 0.3M Tris (Sigma Aldrich, Dorset, UK) in 20% Methanol (Sigma Aldrich, Dorset, UK) in solution, pH 10.4
- Anode 2 buffer: 25mM Tris in 20% methanol solution, pH 10.4
- Cathode buffer: 40mM 6-amino n hexanoic acid (Sigma Aldrich, Dorset, UK) in 20% methanol solution, pH 7.6
- Ponceau S (Sigma Aldrich, Dorset, UK).

Protocol:

For the transfer of proteins onto a nitrocellulose membrane, Anode 1, Anode 2 and Cathode buffers were used. A ''sandwich'' was made from 6 filter papers soaked in Anode 1, 3 filter papers were soaked in Anode 2, the nitrocellulose membrane followed by the gel containing the proteins and a further 9 pieces of filter paper that had been soaked in Cathode Figure 2.2). Air bubbles were removed and a current of 45mA/ (per gel) for 1 hour was applied to allow transfer of the proteins from the gel onto the membrane. After 1 hour, the nitrocellulose membrane was removed and Ponceau S (Sigma, Dorset,

UK) solutions were added to the membrane to and ensure transfer of the proteins. Ponceau S solution was removed by washing 3-4 times with PBS/Tween20.

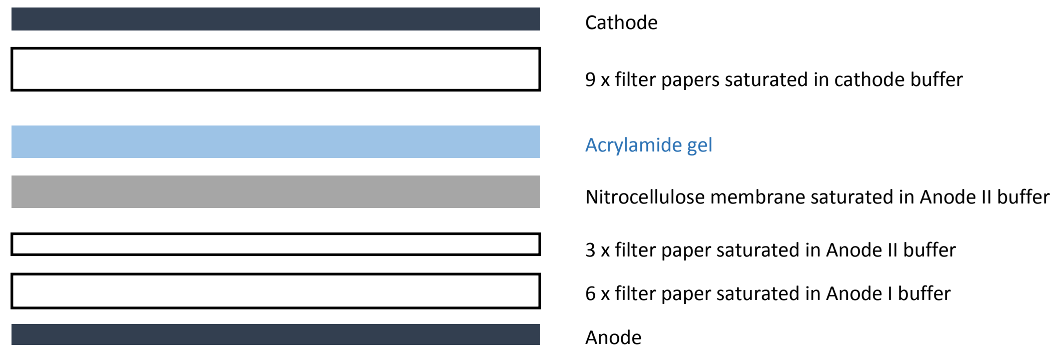


Figure 2.2. Schematic of western blotting setup.

2.23. Processing and development of nitrocellulose membrane and protein detection.

Reagents:

- PBS tween solution: 0.005% (v/v) polyoxyethylene-sorbitan monolaurate (Tween-20) (Sigma Aldrich, Dorset, UK) in PBS solution.
- Blocking solution: 5% w/v milk powder in 0.005% v/v PBS Tween
- Primary antibody solution: 1% w/v of milk powder in 0.005%v/v PBS Tween
- Secondary antibody solution: 3% w/v of milk powder in 0.005% v/v PBS Tween.
- List of antibodies (Table 2.4)
- Chemiluminescence reagent kit (ECL, Thermoscientific, Cheshire, UK).

Protocol:

To block unspecific interactions, 5% skimmed milk powder in 0.005% PBS/Tween was added onto the nitrocellose membrane for 1 hour. Primary antibody was diluted in 1% milk, and the membrane was incubated overnight at 4°C on a rocker. The following day, the antibody was removed and the membrane was washed 3 times for 5 minutes in PBS-Tween 20. Secondary antibody was added in 3% skimmed milk and the membrane was incubated for 1 hour at room temperature. After 1 hour, the membrane was washed 3 times (ECL, Thermoscientific, Cheshire, UK) and placed between acetate sheets and analysed using a chemiluminiescence imager (Biorad, Hertfordshire, UK).

Table 2.4 Antibodies used for western blotting and immunostaining analysis.

Antibody	**Cell Signalling Technologies**	**Dilution**	**Predicted molecular weight**
Anti-GAPDH	Mouse	1:1000	36kDa
Jun	Rabbit	1:1000	48kDa
Fos	Rabbit	1:1000	62kDa
Vinculin	Mouse	1:1000	124kDa
Sirt 1	Rabbit	1:1000	80kDa

2.24. microRNA and antagomiR treatment of mice

Wild type C57BL6/J adult (5 months old) and old (23 months old) mice were injected intravenously (via tail vein) with 100 µl of 2mg/kg body weight of microRNA mimic or inhibitor (See section 2.1 for sequences). The injections were done twice: at day 1 and day 15. Four weeks following the first injection, mice underwent *in situ* force measurements (See chapter 2.4 for details) or *in vivo* isometric contraction protocol). Mice were sacrificed using cervical dislocation and tissues were dissected for further analyses.

2.25. *In situ* force measurement of the EDL muscle.

The measurement of *Extensor digitorum longus* (EDL) contractile properties were carried out *in Situ* as described previously (Brooks & Faulkner, 1990). In order to measure the maximum isometric force, (P_0) of EDL, the distal tendon of anesthetised mouse was mounted on a lever arm of servomotor (Aurora Scientific Aurora, ON, Canada). The mouse hind limb was stationed while the peroneal nerve was exposed for stimulation with two platinum electrode wires attached to the nerve (Sakellariou et al., 2016) (McArdle et al., 2004) Stimulation voltage and muscle length were adjusted to produce maximum twitch force. With the muscle at optimum length, the maximum force was determined during 300 msec of voltage stimulation. The maximum force was identified by increasing the frequency of stimulation at 2-min intervals until the maximum force reached a plateau. Muscle fibre length (Lf) and cross-sectional area were calculated to determine specific force.

2.26. Muscle fibre analysis

Muscle fibre size and diameter were quantified using Fiji (Johannes Schindelin et al., 2012). ROIs were manually drawn or selected using *Tissue Cell Geometry Stats* macro (Advanced Digital Microscopy Core Facility, Institute for Research in Biomedicine, Barcelona, http://adm.irbbarcelona.org/image-j-fiji). Feret's diameter was used for the analysis. Statistical analysis was performed using Minitab (Minitab 17 Statistical Software (2010). [Computer software] State College, PA: Minitab, Inc. (www.minitab.com). Student's two tailed

unpaired *t*-test was used for comparison between two groups and one-way ANOVA followed by posthoc analysis (Bonferonni correction). Bonferroni corrects and adjust the *P* values in a statistical test and it also gives a more precise value. Results presented as ± S.E.M. n = 3 or 4 mice per group. P-value equal or less than 0.05 was considered as statistically significant (*, p≤0.05; **, p≤0.01; ***, p≤0.001), (Schindelin et al., 2012).

2.27. Statistical analysis of qPCR data.

Generated data were presented using mean ±SEM. Statistical analyses of different groups were determined by One-Way ANOVA followed by post hoc analysis. Each comparison between 2 experimental conditions was performed using unpaired Student's t test.

3 Changes in microRNA expression in skeletal muscle during contractions and ageing

3.1. Abstract

Ageing is associated with muscle loss and muscle losing its plasticity which may be related to a failure in adaptive responses. Skeletal muscle is known to be plastic in nature. In response to exercise, several transcriptional and signalling pathways become activated in order for the muscle to adapt to the contractions; this is called an adaptive response. These molecular factors are predicted to be regulated by non-coding RNAs, microRNAs.

microRNA expression has been shown to be dysregulated during ageing in *C. elegans*, animal models and human. However, the changes in several microRNA expression following contractions of muscle of older mice were different to those in muscle of younger adults.

The aim of this part of the study was to validate the changes in the expression of microRNAs that occur during contractions and ageing in the muscle of mice. qPCR analysis of microRNA expression was used to validate the expression of microRNAs in muscles of adult and old mice in response to contractions.

The results show that the expressions of microRNAs: miR-181a, miR-181d, was affected by ageing. The expression of these microRNAs was changed following the contraction protocol in the muscle of adult, but was blunted in the muscle of old mice.

These data shows that these microRNAs may play an important role in regulating muscle adaptive response and the disruption of their expression in

quiescent and contracted muscle of old mice may be associated with age-associated changes in response of the muscle to contractions.

3.1 Introduction

Skeletal muscle is important for maintaining posture, metabolic homeostasis and movement. Movement is possible due to muscle contraction. Exercise is important for the maintenance of muscle, as well as whole body health (Guller & Russell, 2010). Skeletal muscle is plastic in nature. In response to exercise, several transcriptional and signalling pathways become activated in order for the muscle to adapt to the contractions; this is called an adaptive response (McArdle et al., 2006). One of the mechanisms of adaptive response in muscle is through the upregulation of antioxidant enzymes, such as superoxide dismutase and catalase; this process is thought to be aimed at reducing muscle damage. Heat shock proteins, upregulated following contractions in rodents, also play a role in the adaptive response (McArdle et al.,2002). However, it has been shown that an isometric contraction protocol results in an increased production of antioxidant defence enzymes and HSPs in muscles of adult mice and theses effects were blunted in muscles of old mice, which show elevated basal levels of these factors (McArdle et al., 2006). Therefore, it has been suggested that the muscle adaptive response is disrupted during disease or ageing, in both humans and animal models. Understanding the mechanisms of muscle response to contractions during ageing is crucial since exercise, together with changes in diet, is currently the

only effective intervention against loss of muscle mass and function, even though this intervention may not be appropriate for all people.

Ageing-associated muscle loss in thought to be related to muscle losing its plasticity which may be related to a failure in adaptive responses. It has been shown that the increased production of free radicals, changes in protein content and disrupted transcriptional activity play an important role in the adaptive response in skeletal muscle (McArdle et al., 2002; McDonagh et al., 2014; Soriano-Arroquia et al., 2016; McArdle et al., 2006).

Despite a growing understanding of the roles of redox balance, transcriptional regulation and the antioxidant response in muscle adaptive response to contractions, little is understood about the mechanism regulating the blunted response of muscle to contractions during ageing. Changes in the expression of genes and proteins in muscle during ageing and following contractions have been characterised, however it is not well understood how these changes occur (reviewed in (Brown & Goljanek-Whysall, 2015)). Recently, epigenetic mechanisms, such as microRNAs, have also been shown to regulate muscle mass and function during ageing and disease (Davidsen et al., 2011) providing candidate mechanisms for regulating the differential responses of muscle from adult and old organisms to contractions.

MicroRNAs (miRNAs, miRs) are small non-coding RNA, approximately 20-22 nucleotides long. They function by binding to their complementary target sequences messenger RNA (mRNA), usually located within the 3' untranslated region (UTR). microRNA binding to its target usually results in mRNA

degradation and/or inhibition of translation (Ladomery et all., 2011).MicroRNAs have been shown to regulate almost all known biological processes (McGregor et al., 2014). They have been extensively studied and shown to regulate gene expression of approximately two thirds of the human genome (Friedman et al., 2009). Dysregulation of these small RNAs is also associated with many diseases, such as arthritis, cancer, myositis and muscular dystrophy (Bhaskaran & Mohan, 2014; Reis, 2016).

Muscle has been shown to express several muscle-specific and muscle-enriched microRNAs, called myomiRs (Ladomery et al., 2011). Muscle-specific-microRNAs include, but are not restricted to, miR-133, miR-1 and miR-206. These microRNAs play a significant role in regulation of muscle and development, regeneration, hypertrophy and atrophy and potentially ageing (Nakasa et al., 2010) ; Soriano-Arroquia et al., 2016)

microRNA expression has been shown to be dysregulated during ageing in *C. elegans*, animal models and humans (McGregor et al., 2014). microRNA expression has also been shown to specifically change in muscle during contractions and in ageing (Baggish et al., 2014; Drummond, 2010; McGregor et al., 2014; Safdar et al., 2009). Moreover, the changes in microRNA expression following contractions of muscle of older humans were different to those in muscle of younger adults. (McGregor et al., 2014). This is consistent with changes in gene expression in muscle following contraction in the old when compared to younger mammals. These changes in gene transcription could be

associated with differential expression of specific microRNAs (Kirby & McCarthy, 2013).

The aim of the studies reported in this chapter was to validate the changes in expression of microRNAs that occur during contractions and ageing in the muscle of mice. The candidate microRNAs were chosen based on available literature and data from our laboratory. Table 3.1 shows the candidate microRNAs chosen for this study and their potential role in regulating muscle adaptive response based on their validated target genes.

The table shows candidate miRNAs potentially involved in muscle adaptive response to contractions.

Table 3.1 Changes in muscle microRNA levels following exercise.

microRNA	**Expression during exercise**	**Validated targets**	**Validated Biological Function**	**References**
miR-181	Upregulated	HoxA11, Sirt-1	Regulates embryonic myogenesis and myotube size	(Naguibneva et al., 2006; Soriano-Arroquia et al., 2016)
miR-133	Upregulated	SRF, IGF, BAF60	Regulation of myoblasts proliferation and differentiation	(Goljanek-Whysall et al., 2012)
miR-21	Upregulated	Pten	Regulates Inflammation and Fibrosis	(Buscaglia & Li, 2011)
miR-378	Downregulated	MyoR, PGC1β	Promotes myogenesis, controls mitochondrial metabolism	(Zeng et al., 2016)
miR-16	Downregulated	Vegf, Vegfr	Angiogenesis	(Wahl et al., 2016)
miR-24	Unknown	P21	Regulation of cell senescence	(Bu &Baraldo 2016)
miR-15	Downregulated	None validated in muscle or cardiomyocytes	Protection of ischemia-induced cardiomyocyte injury; cross-talk between fat and muscle tissues	(Hullinger et al., 2012)
miR-130	Unknown	PGC-1α	Unknown	(Jiang et al., 2017)

3.2 Experimental Methods

3.2.1 Contraction protocol

This study was carried out using wild type adult male C57BL6/J mice (6 months old) and old male C57BL6/J mice (24 months old). The mice were maintained in a specific-pathogen free environment, were fed ad libitum with standard chow and maintained in a 12 hour light-dark cycle.

For this study, mice were anaesthetised and the hind limb was subjected to a 15-minute period of isometric contractions as described in Chapter 2.4 Following the contractions, mice were sacrificed immediately by cervical dislocation. Control mice were sacrificed without undergoing the isometric contraction protocol. The *tibialis anterior* muscle was removed, frozen in liquid nitrogen and stored at-80°C until analysed.

4 adult and 8 old mice were used for this experiment as indicated by power calculations. Old mice display greater variability in microRNA expression.

3.2.2 RNA Analysis

RNA isolation was performed using TRIZOL ™ (Ambion Life Technologies) as described in Chapter 2.6.

3.2.3. cDNA synthesis and microRNA expression analysis.

The cDNA synthesis was performed using miScript ll Reverse Transcriptase kit (Qiagen) (Section 2. 8) qPCR analyses of microRNA expression

in the *tibialis anterior* muscles were performed using micorRNA primers (Qiagen) as described in Section 2.7.

3.2.4. Statistical analysis

The qPCR data obtained are presented as mean ± SEM in all experiments. The statistical analyses of different groups were undertaken using One-Way Anova followed by post hoc analyses. Bonferroni is used due to it gives more tight values. Each comparison between two experimental conditions was done using the Students t-test.

3.3 Results

3.3.1 microRNA expression in the *tibialis anterior* muscle during ageing and following an isometric contraction protocol.

The effect of the isometric contractions on the expression of microRNAs in the *tibialis anterior* muscle of adult and old mice was studied in order to validate the changes in microRNA expression previously reported in rodents and humans**.**

The expression of microRNAs: miR-181a and miR-181d were downregulated in the *tibialis anterior* muscle of old mice compared with muscles from adult mice (Figures 3.1, 3.2). miR-181a and miR-181d expression

was upregulated in the *tibialis anterior* muscle of adult mice following isometric contraction when compared with muscles from mice which had not undergone the contraction protocol (Figures 3.1, 3.2). The expression of miR-181a and miR-181d in the *tibialis anterior* muscle of old mice showed no significant

changes in response to the contraction protocol (Figure 3.1). The expression of microRNAs: miR-181b and miR-181c was found to be very low in the *tibialis anterior* muscle of adult and old mice and was therefore not analysed in the muscle following the contraction protocol (Appendix 1).

The expression of miR-133a was downregulated in the *tibialis anterior* muscle of mice during ageing and in the *tibialis anterior* of adult mice following isometric contractions when compared with muscles from mice which had not undergone the contraction protocol (Figure 3.3), but the expression of miR-133a in the *tibialis anterior* muscle of old mice showed no significant changes in response to the contraction protocol (Figure 3.3).

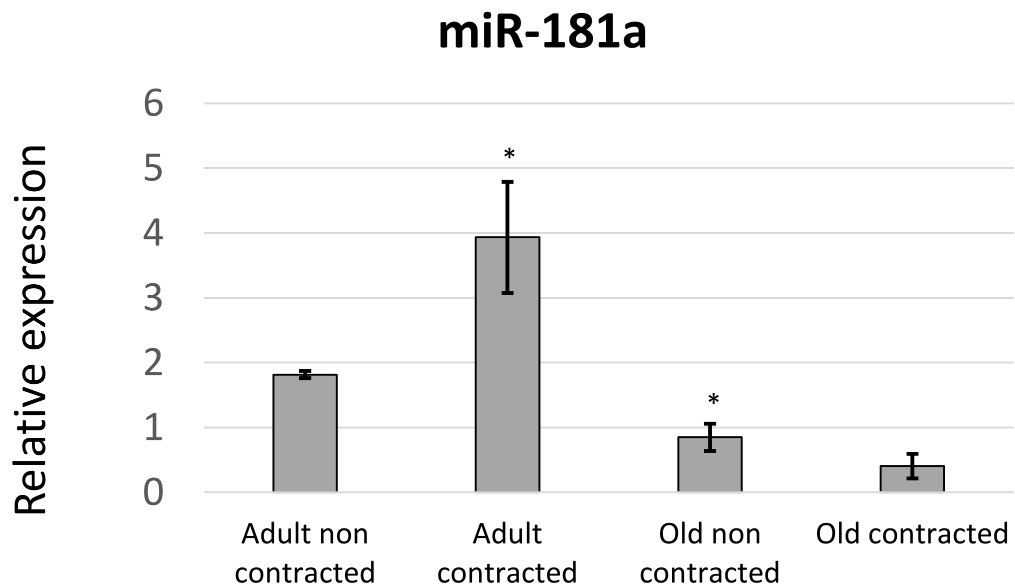


Figure 3.1. Changes in miR-181a expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. qPCR analysis of expression of miR-181a relative to Rnu-6, in quiescent (non-contracted) or after 15-minute contraction protocol (contracted) in *tibialis anterior* muscles from adult and old mice. Error bars show SEM; **P<0.05* (compared to Adult non-contracted) by One-Way Anova; n=4-8.

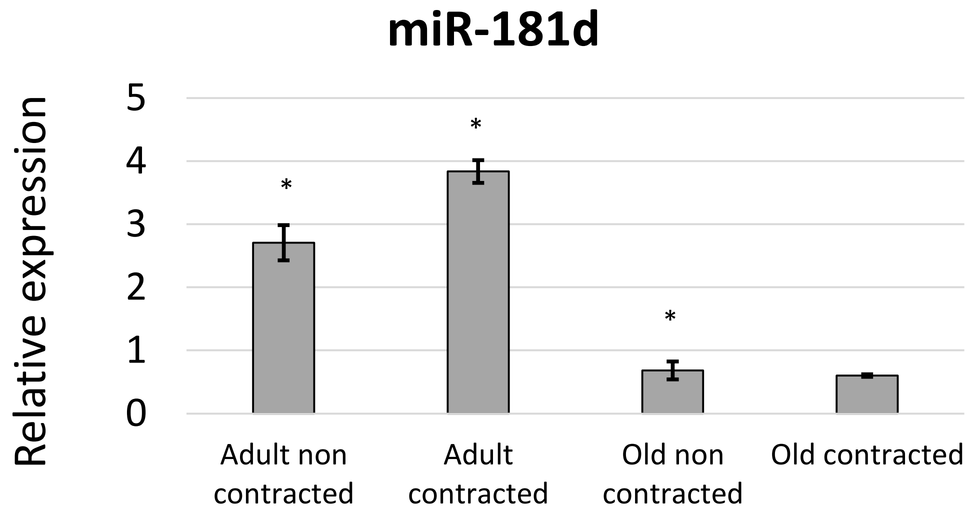


Figure 3.2. Changes in miR-181d expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. qPCR analysis of expression of miR-181d relative to Rnu-6, in quiescent (non-contracted) or after 15-minute contraction protocol (contracted) in *tibialis anterior* muscles from adult and old mice. Error bars show SEM; **P<0.05* (compared to Adult non-contracted) by One-Way Anova; n=4-8.

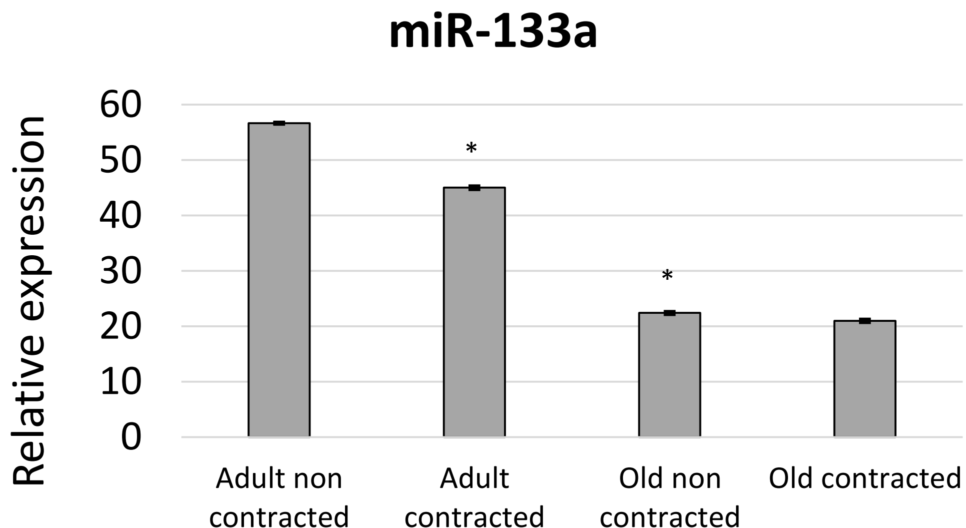


Figure 3.3. Changes in miR-133a expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. qPCR analysis of expression of miR-133a relative to Rnu-6, in quiescent (non-contracted) or after 15-minute contraction protocol (contracted) in *tibialis anterior* muscles from adult and old mice. Error bars show SEM; **P<0.05* (compared to Adult non-contracted) by One-Way Anova; n=4-8.

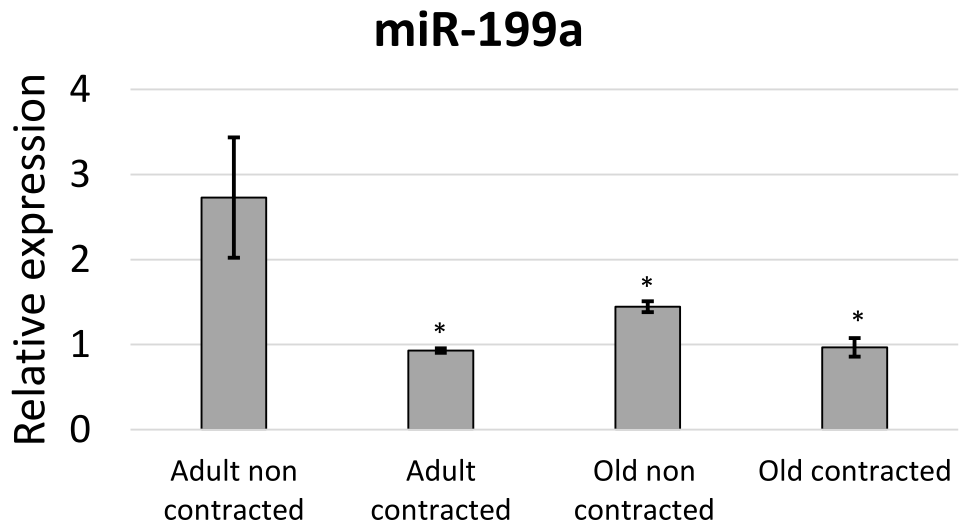


Figure 3.4. Changes in miR-199a expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. qPCR analysis of expression of miR-199a relative to Rnu-6, in quiescent (non-contracted) or after 15-minute contraction protocol (contracted) in *tibialis anterior* muscles from adult and old mice. Error bars show SEM; **P<0.05* (compared to Adult non-contracted) by One-Way Anova; n=4-8.

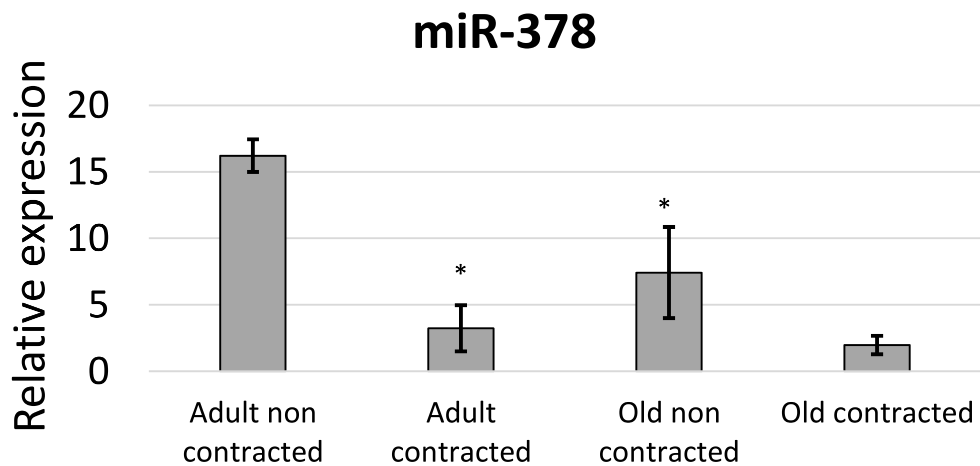


Figure 3.5. Changes in miR-378 expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. qPCR analysis of expression of miR-378 relative to Rnu-6, in quiescent (non-contracted) or after 15-minute contraction protocol (contracted) in *tibialis anterior* muscles from adult and old mice. Error bars show SEM; **P<0.05* (compared to Adult non-contracted) by One-Way Anova; n=4-8.

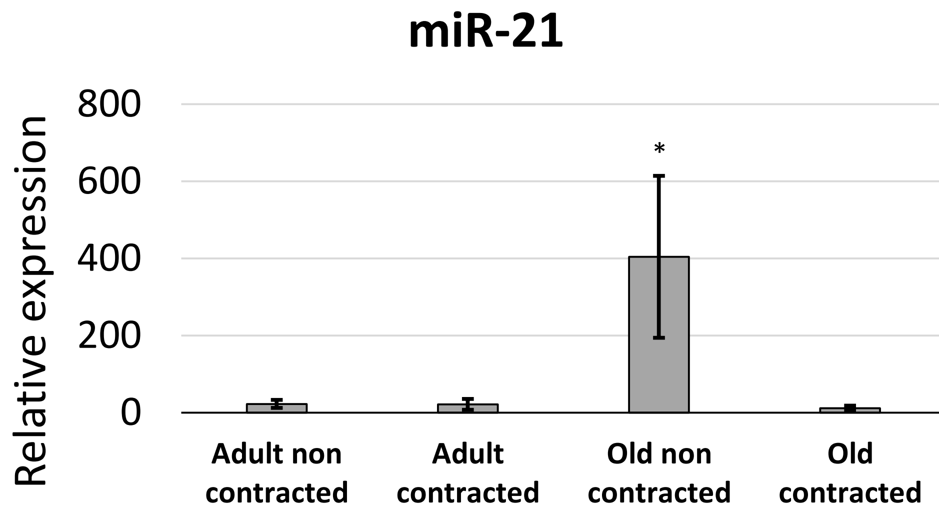


Figure 3.6. Changes in miR-21 expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. qPCR analysis of expression of miR-21 relative to Rnu-6, in quiescent (non-contracted) or after 15-minute contraction protocol (contracted) in *tibialis anterior* muscles from adult and old mice. Error bars shows SEM; **P<0.05* (compared to Adult non-contracted) by One-Way Anova; n=4-8.

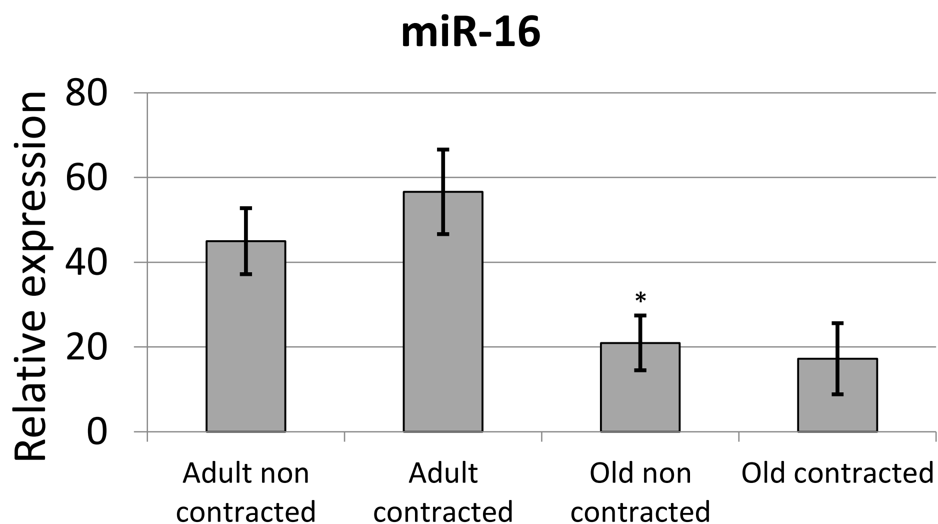


Figure 3.7. Changes in miR-16 expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. qPCR analysis of expression of miR-16 relative to Rnu-6, in quiescent (non-contracted) or after 15-minute contraction protocol (contracted) in *tibialis anterior* muscles from adult and old mice. Error bars show SEM; **P<0.05* (compared to Adult non-contracted) by One-Way Anova; n=4-8.

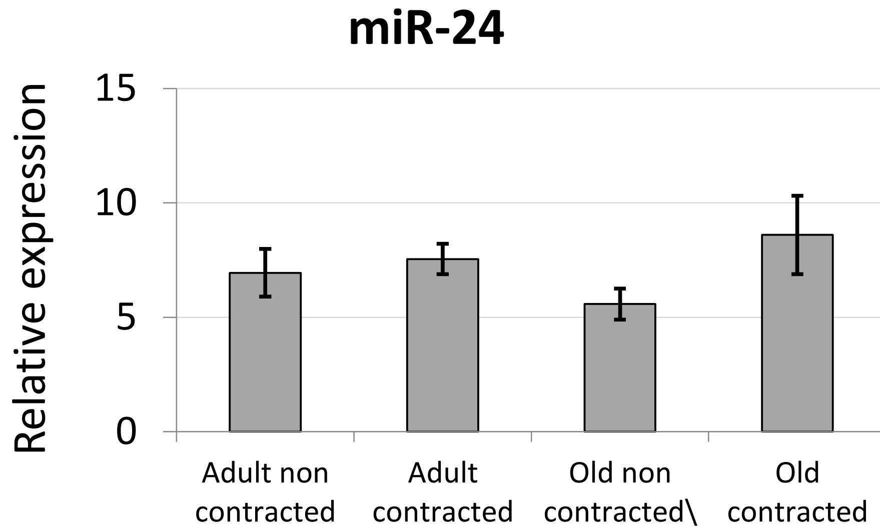


Figure 3.8. Changes in miR-24 expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. qPCR analysis of expression of miR-24 relative to Rnu-6, in quiescent (non-contracted) or after 15-minute contraction protocol (contracted) in *tibialis anterior* muscles from adult and old mice, n=4-8.

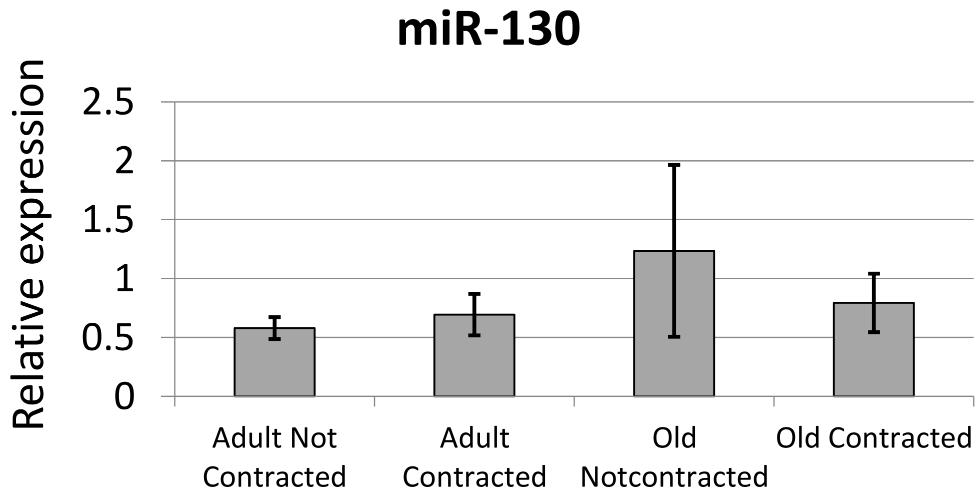


Figure 3.9. Changes in miR-130 expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. qPCR analysis of expression of miR-130 relative to Rnu-6, in quiescent (non-contracted) or after 15-minute contraction protocol (contracted) in *tibialis anterior* muscles from adult and old mice, n=4-8.

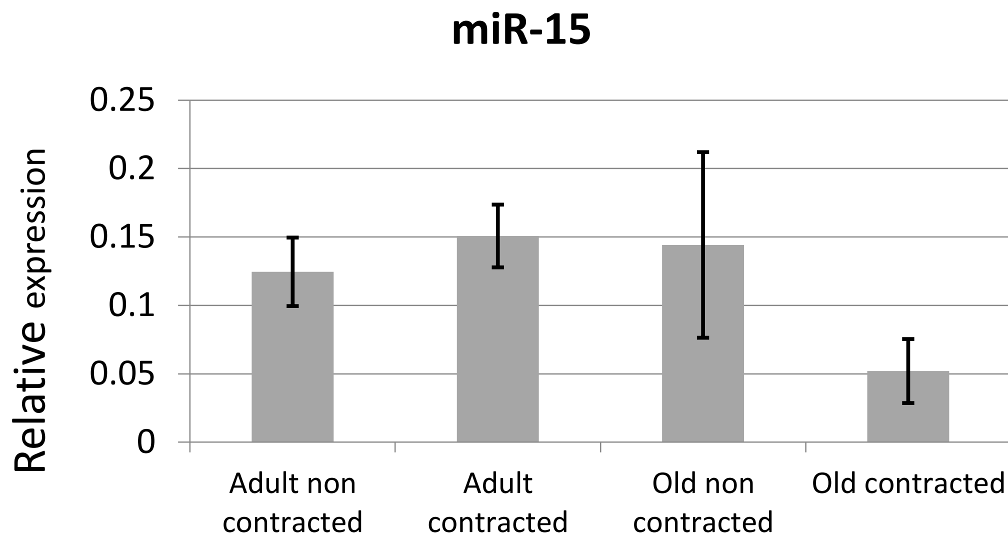


Figure 3.10. Changes in miR-15 expression in *tibialis anterior* of mice during ageing and following an isometric contraction protocol. qPCR analysis of expression of miR-15 relative to Rnu-6, in quiescent (non-contracted) or after 15-minute contraction protocol (contracted) in *tibialis anterior* muscles from adult and old mice, n=4-8.

The expression of miR-199a was significantly downregulated in the *tibialis anterior* muscle during ageing, as well as following the contraction protocol in the muscle of both adult and old mice (Figure 3.4).

miR-378 expression was significantly downregulated in the *tibialis anterior* muscle of mice during ageing and in the *tibialis anterior* muscle of adult mice following isometric contractions when compared with muscles from mice which had not undergone the contraction protocol (Figure 3.5). The expression of miR-378 in the *tibialis anterior* muscle of old mice appeared downregulated in response to the contraction protocol, but the changes were not statistically significant (Figure 3.5).

The expression of miR-21 was significantly upregulated in the *tibialis anterior* muscle from adult mice compared with muscles of old mice and it was downregulated in the muscle of old mice following the contraction protocol, however remained unchanged in the contracted muscle of adult mice (Figure 3.6).

miR-16 expression was downregulated in the *tibialis anterior* muscle of old mice as compared to the muscle of adult mice (Figure 3.7), but the expression of miR-16 in the *tibialis anterior* of adult and old mice was not affected by the isometric contraction protocol (Figure 3.7).

The expression of microRNAs: miR-24, miR-130 and miR-15 in the *tibialis anterior* muscle was not affected by ageing or the isometric contraction protocol (Figures 3.8-3.10).

To summarise, the expression of microRNAs: miR-181a, miR-181d, miR-133a, miR-378 and miR-199a was regulated by both ageing and the contraction protocol in the *tibialis anterior* muscle. Moreover, despite miR-181a, miR-181d, miR-378 and miR-133a expression being affected by the contraction protocol in the muscle of adult mice, it was not changed in the muscle of old mice following the contraction protocol, suggesting a potential role of these microRNAs in the blunted responses of muscle from old mice to contractions (Figures 3.1 – 3.5).

3.4 Discussion

Sarcopenia is the age-related loss of skeletal muscle mass and strength. During ageing, skeletal muscle responses to contractions are disrupted, and this is thought to be one of the reasons for the loss of muscle mass and function during ageing.

microRNAs have been shown to regulate up to 60% of coding genes in humans and are involved in most biological processes (Aoi, 2014; Friedman et al., 2009). The muscle-specific and muscle-enriched microRNA are called myomirs and include: miR-1, miR-133a, miR-133b, miR-499, miR-208 and miR-206. The abundance of these microRNAs is thought to be 25% of total microRNAs in skeletal muscles of human and mice (Aoi, 2014). Among other microRNAs reported to be highly expressed in skeletal muscle are: miR-378, miR-181, miR-24, miR-199 and others (Aoi, 2014; Brown & Goljanek-Whysall, 2015; Goljanek-Whysall et al., Pais, et al., 2012). Various studies have reported changes in microRNA expression in muscle following exercise in humans and rodents, however the results described are often not consistent due to different platforms and/or species used, and there is clear lack of data on the functional consequences of changes in microRNA expression in terms of muscle adaptation to exercise and ageing (Aoi et al., 2010; Baggish et al., 2014; Silva et al., 2012; Davidsen et al., 2011; Fernandes et al., 2012; Keller et al., 2011; McCarthy & Esser, 2007; Nielsen et al., 2010; Safdar et al., 2009; Yamamoto et al., 2012).

In this study, qPCR analysis of microRNA expression was used to profile the expression of microRNAs in muscles of adult and old mice in response to contractions in order to validate previously published data showing changes in microRNA expression in response to exercise. This approach led to a selection of the most promising microRNA candidates that might play a role in adaptive response of muscle of mice to contractions and the blunted adaptive response of muscle of old mice.

Among the microRNAs where the expression was disrupted during ageing, the expression of only five microRNAs was also significantly affected by the isometric contraction protocol: miR-181a, miR-181d, miR-133a, miR-378 and miR-199a (Figures 3.1-3.5).

The expression of microRNA-199a was downregulated during ageing and in response to contractions in the muscle of adult and old mice (Figure 3.5). This suggests that miR-199a is unlikely to control the disrupted mechanisms of muscle response to contraction during ageing but may regulate other aspects of muscle ageing.

The expressions of microRNAs: miR-181a, miR-181d, miR-378 and miR-133a were affected by ageing. Interestingly, the expression of these microRNAs was changed following the contraction protocol in the muscle of adult, but not old mice (Figures 3.1-3.4). These data suggest that these microRNAs may play a role in regulating muscle adaptive response and the disruption of their expression in quiescent and contracted muscle of old mice may be associated with age-associated changes in response of the muscle to contractions.

My data shows that miR-133a may be involved in response of muscle to contractions. miR-133 is one of the myomiRs and previous studies have demonstrated changes in the expression of this myomiR in the muscle of humans (Drummond, 2010). miR-133 has been demonstrated to play a role in embryonic myogenesis, muscle regeneration and hypertrophy, therefore miR-133a is an attractive candidate for further studies (Goljanek-Whysall et al., 2014; Goljanek-Whysall, Pais, et al., 2012; McCarthy & Esser, 2007).

miR-378 is a key regulator of adipose tissue metabolism (Carrer et al., 2012). Previous data from our laboratory suggest that miR-378 regulates mitochondrial homeostasis in muscle and its disrupted expression may contribute to sarcopenia onset (Whysall et al, manuscript in preparation).

miR-181 has been previously shown to regulate muscle development (Naguibneva et al., 2006) and our group has demonstrated that miR-181a expression is downregulated in the muscle of old mice when compared with muscle of adult mice (Soriano-Arroquia et al., 2016; Zacharewicz et al., 2013). Finally, our group has also shown that miR-181a targets the expression of Sirt-1 and regulates myotube size *in vitro* (Soriano-Arroquia et al., 2016). This makes miR-181 an attractive candidate for further studies.

4 Expression of microRNAs in contracted and non- contracted myotubes

4.1 Abstract

In this part of the study, I investigated the changes in miRNA expression following contraction in *H-2k*^{b} myotubes, *in vitro* model of muscle contractions, in order to compare microRNA changes of expression over time following contraction in the *in vitro* model**.**

The data presented here demonstrate the changes in the microRNA expression in *H-2k*^{b} myotubes following an isometric contraction protocol were studied and the *H-2k*^{b} myotubes underwent a 15-minute contraction protocol and non-contracted myotubes served as controls. The expression of microRNAs following the contraction protocol was studied over a 0-24 hour time course.

Data show that miR-181a and miR-181d expression was upregulated immediately after contraction protocol in *H-2k*^{b} myotubes as in the muscles of adult but not old, and mice returned to the basal levels after 4-8 hours in *H-2k*^{b} myotubes. This suggests that it is valid to study the role of microRNAs, and specifically miRNAs: miR-181a and miR-181d, in muscle response to contractions using *H-2k*^{b} myotubes.

4.2 Introduction

In Chapter 3.3, changes in the expression of selected microRNAs were validated in quiescent and contracted muscle of adult and old mice. The functional consequences associated with changes in the expression of the

selected microRNAs in muscle were further studied using *in vitro* and *in vivo* models (Chapters 5, 6, 7).

The use of cell lines is very important in molecular biological studies (Sunet al., 2014). Using cell lines reduces the use of animals in research, but the choice of cellular model is important (Nielsen et al., 2010). The C2C12 cell line, as well as primary myoblasts, are commonly used *in vitro* models in muscle biology studies (Goljanek-Whysall et al., 2012; Soriano-Arroquia et al., 2016), but there is limited data on the use of cell lines in studies of muscle responses to contractions (Pattwell et al., 2004).

In order to establish whether an *in vitro* cell line model can be used in studies of the role of microRNA(s) in muscle adaptation to contractions, the *H-2k^b^* cell line was used as previously described (McArdle et al., 2001b; Pattwell et al., 2004). Changes in the expression of selected microRNAs studied in Chapter 3.3 (Figures 3.1-3.10) following isometric contraction protocol were examined in *H-2k^b^* myotubes.

The aim of this part of the project was therefore to validate a previously used *in vitro* model: *H-2k^b^* mouse myoblast cell line for studying the role of microRNAs in muscle adaptive response to contractions.

4.3 Materials and methods

4.3.1 Contraction protocol of *H-2K^b* myotubes

H-2k^b cell were cultured in growth media until 80-85% confluent, at which point *H-2k^b* cells were switched to differentiation media and induced to form myotubes (please see Chapter 2.2) for detailed protocol). Myotubes were stimulated with electrical stimulator as described in Chapter 2.2 (McArdle et al., 2001b).

For the time course experiment, cells were maintained in an incubator for the duration of the study and RNA was extracted using the Trizol method as described in Chapter 2.5 for cDNA synthesis from myotubes and microRNA expression profiling.

Analysis of microRNA expression was performed using cDNA synthesised from RNA isolated from *H-2k^b* myotubes using miScript II Reverse Transcriptase kit (Qiagen). qPCR analysis was performed on Bio-Rad CFX Connect ™ Real-Time System as described in Chapter 2.9. First, expression of microRNAs was screened using technical replicates to allow for medium-throughput screening of many microRNA candidates. microRNAs, expression of which was significantly regulated in the first qPCR, was further analysed using biological replicates (n=3).

The qPCR data are presented as mean ± stdev when technical replicates were used and ± SEM when biological replicates were used in experiments (see figure legends). The statistical analyses of different groups

were determined by One-Way Anova followed by post hoc analyses Bonferroni Each comparison between two experimental conditions was done using the students T-test.

4.4 Results

The expression of microRNAs following the contraction protocol was studied over a 0-24 hour time course.

The expression of miR-181a and miR-181d in *H-2k*^{b} myotubes was significantly upregulated immediately after the contraction protocol compared with the non-contracted control cells and returned to basal levels within 4-8 hours (for miR-181a and miR-181d, respectively) following contractions. The expression of these microRNAs remained unchanged 8-24 hours after contractions compared with the non-contracted controls (Figures 4.1, 4.2).

miR-133a expression was significantly downregulated immediately following the isometric contractions of *H-2k*^{b} myotubes compared with the non-contracted control cells and returned to basal levels 4 hours following contractions. The expression of miR-133a remained unchanged between 4-24 hours after contractions compared with the non-contracted control cells (Figure 4.3).

The expression of microRNAs: miR-199a and miR-21 remained unchanged in *H-2k*^{b} myotubes immediately after the isometric contraction protocol (Figures 4.4, 4.6). The expression of miR-21 remained unchanged at all-time points after contractions compared with non-contracted control cells

(Figures 4.6). The expression of miR-199a was significantly upregulated in *H-2k^b^* myotubes 24 hours following the isometric contractions protocol, suggesting that this microRNA may play a role in later responses of muscle to contractions (Figure 4.4).

miR-16 and miR-378 expressions were significantly downregulated immediately following isometric contractions of $H\text{-}2k^b$ myotubes compared with the non-contracted control cells and returned to basal levels 4 hours following contractions. The expression of miR-16 remained unchanged between 4-24 hours after contractions compared with the non-contracted control cells (Figures 4.5, 4.7).

The expression of miR-24 was upregulated immediately and 12 hours following the isometric contractions of $H\text{-}2k^b$ myotubes compared with the non-contracted control cells and downregulated 4, 8 and 24 hours following the contractions (Figure 4.8). The variable expression of this microRNA appeared to cycle and resembled a circadian pattern of expression. miR-24 is predicted to target genes associated with circadian rhythms, suggesting it may be associated with circadian regulation of muscle gene expression.

The expression of miR-130 and miR-15 in $H\text{-}2k^b$ myotubes was not affected by the isometric contraction protocol (Figures 4.9, 4.10); this is consistent with no change in the expression of these microRNAs in muscle of adult mice in response to contractions (Figures 3.9, 3.10).

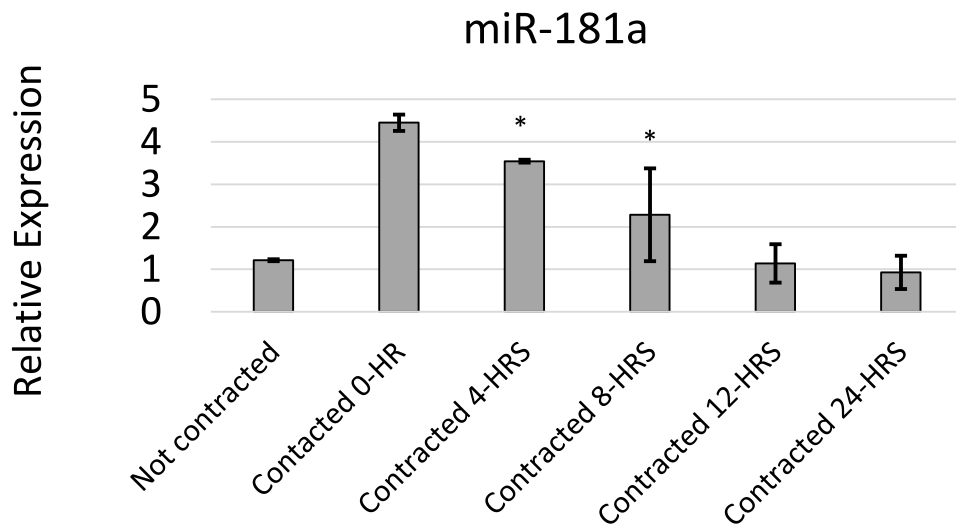


Figure 4.1. The expression of miR-181a following the isometric contraction protocol of *H-2k^b^* myotubes. qPCR showing the expression of miR-181a relative to Rnu-6 Not contracted (NC), Contracted (CN) at 0-4 hour time point after 15 minutes of isometric contractions of *H-2k^b^* myotubes. Error bars show SEM; * *P< 0.05 c*ompared to not contracted control; n=3.

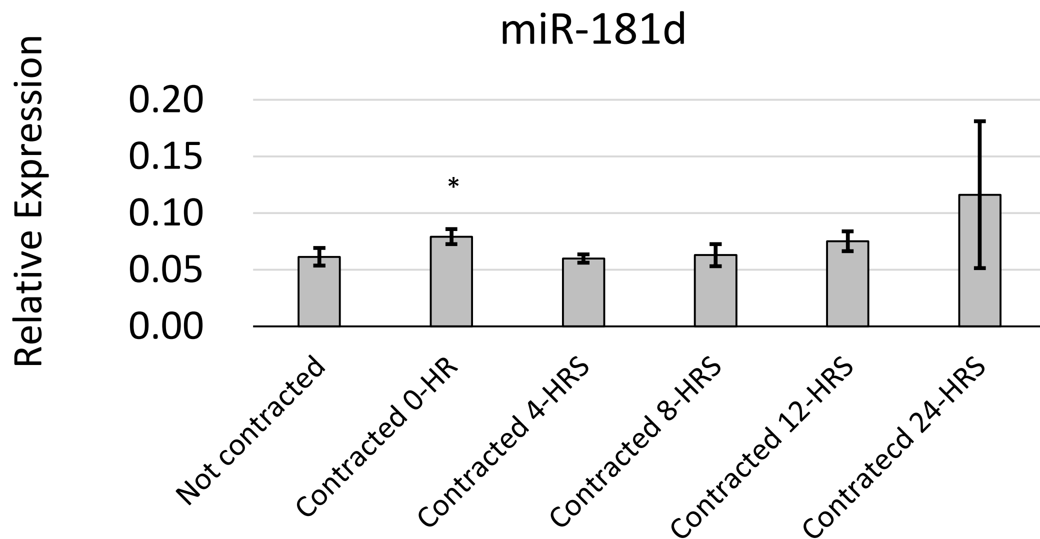


Figure 4.2. The expression of miR-181d following the isometric contraction protocol of *H-2k^b^* myotubes. qPCR showing the expression of miR-181d relative to Rnu-6. Not contracted (NC), Contracted (CN) at 0-4 hour time point after 15 minutes of isometric

contractions of *H-2k^b* myotubes. Error bars show SEM; * *P< 0.05* compared to not contracted control; n=3.

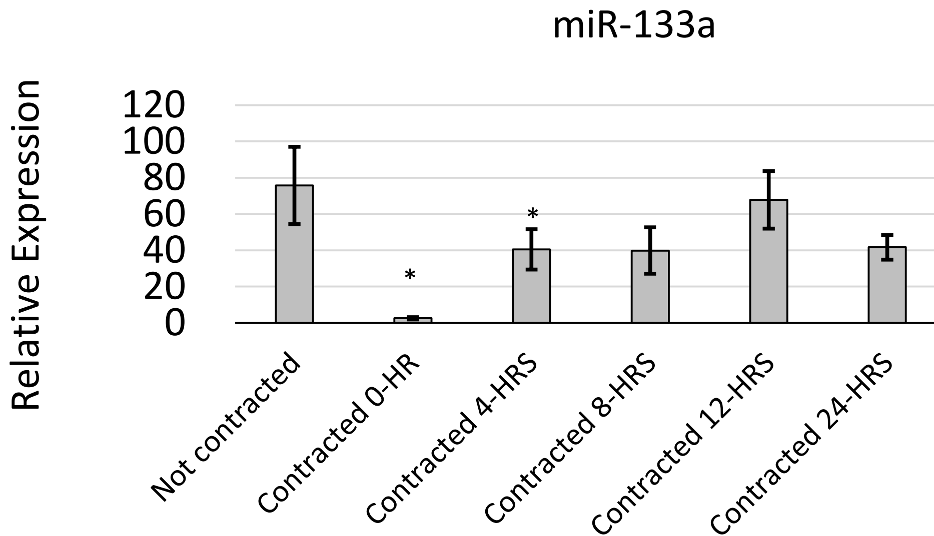


Figure 4.3. The expression of miR-133a following the isometric contraction protocol of *H-2k^b* myotubes. Expression of miR-133a relative to Rnu-6. Not contracted (NC), Contracted (CN) at 0-4 hour time point after 15 minutes of isometric contractions of *H-2k^b* myotubes. Error bars show SEM; * *P< 0.05* compared to not contracted control; n=3.

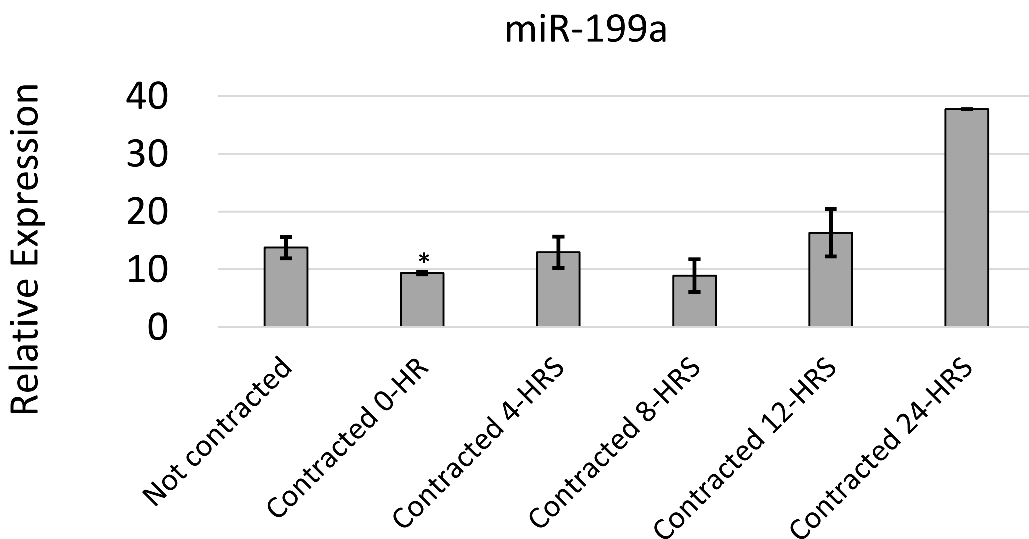


Figure 4.4. The expression of miR-199a following the isometric contraction protocol of *H-2k^b* myotubes. Expression of miR-199a relative to Rnu-6. Not contracted (NC), Contracted (CN) at 0-4 hour time point after 15 minutes of

isometric contractions of *H-2k*^{b} myotubes. Error bars show stdev; * *P< 0.05* *c*ompared to NC control; n=3.

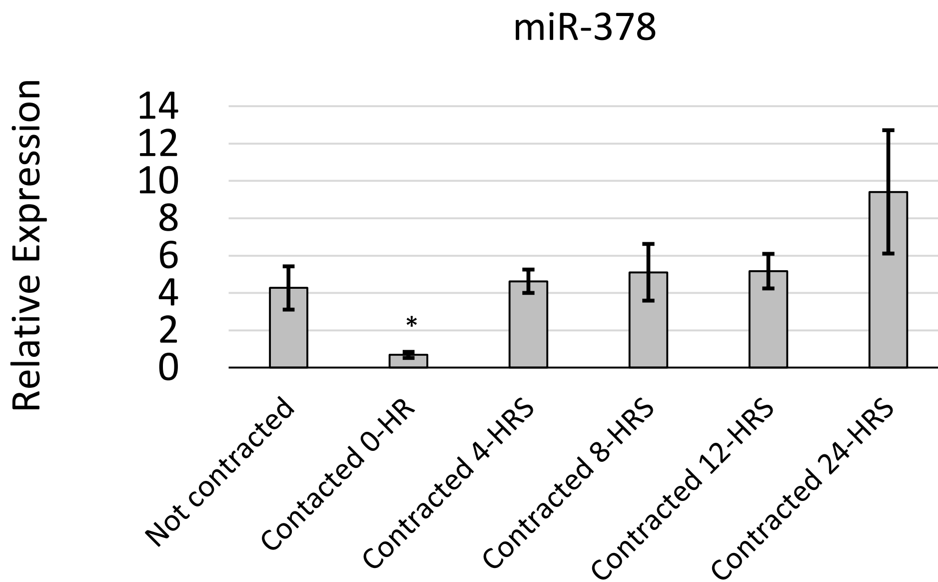


Figure 4.5. The expression of miR-378 following the isometric contraction protocol of *H-2k*^{b} myotubes. qPCR showing the expression of miR-378 relative to Rnu-6. Not contracted (NC), Contracted (CN) at 0-4 hour time point after 15 minutes of isometric contractions of *H-2k*^{b} myotubes. Error bars shows SEM; **P< 0.05 c*ompared to not contracted control; n=3.

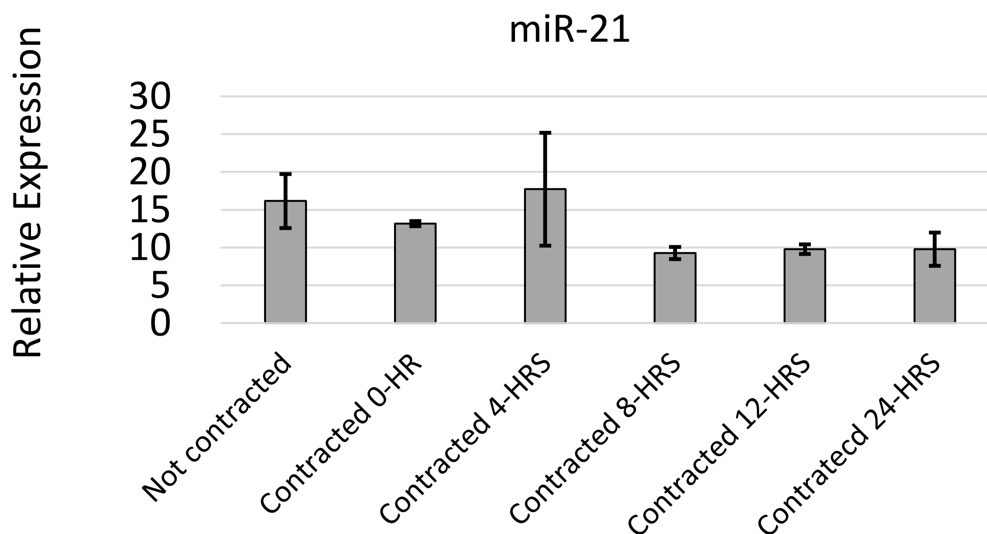


Figure 4.6. The expression of miR-21 following the isometric contraction protocol of *H-2k*^{b} myotubes. qPCR showing the expression of miR-21 relative to

Rnu-6. Not contracted (NC), Contracted (CN) at 0-4 hour time point after 15 minutes of isometric contractions of *H-2k^b^* myotubes n=3.

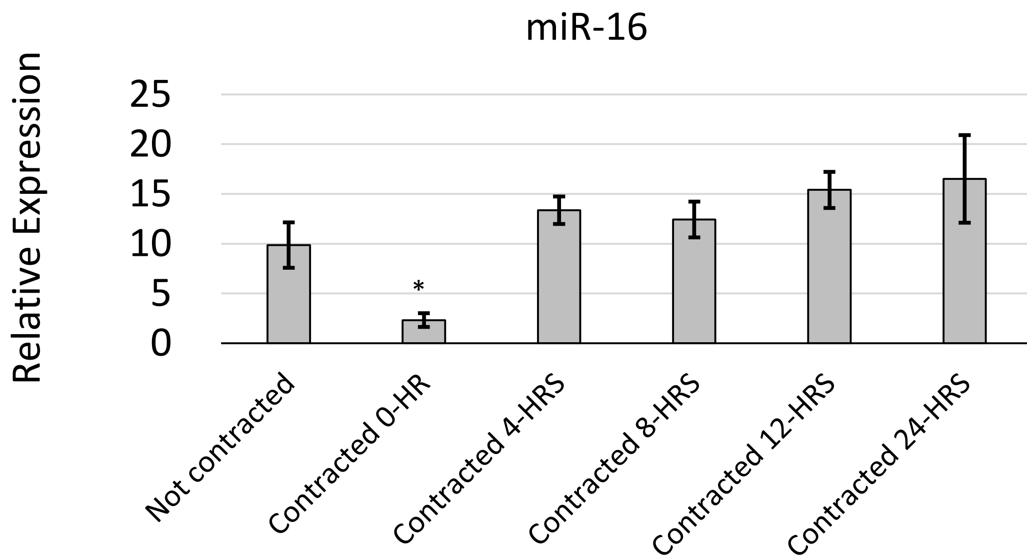


Figure 4.7. The expression of miR-16 following the isometric contraction protocol of *H-2k^b* myotubes. qPCR showing the expression of miR-16 relative to Rnu-6. Not contracted (NC), Contracted (CN) at 0-4 hour time point after 15 minutes of isometric contractions of *H-2k^b* myotubes. Error bars shows SEM; * *P< 0.05 c*ompared to not contracted control; n=3.

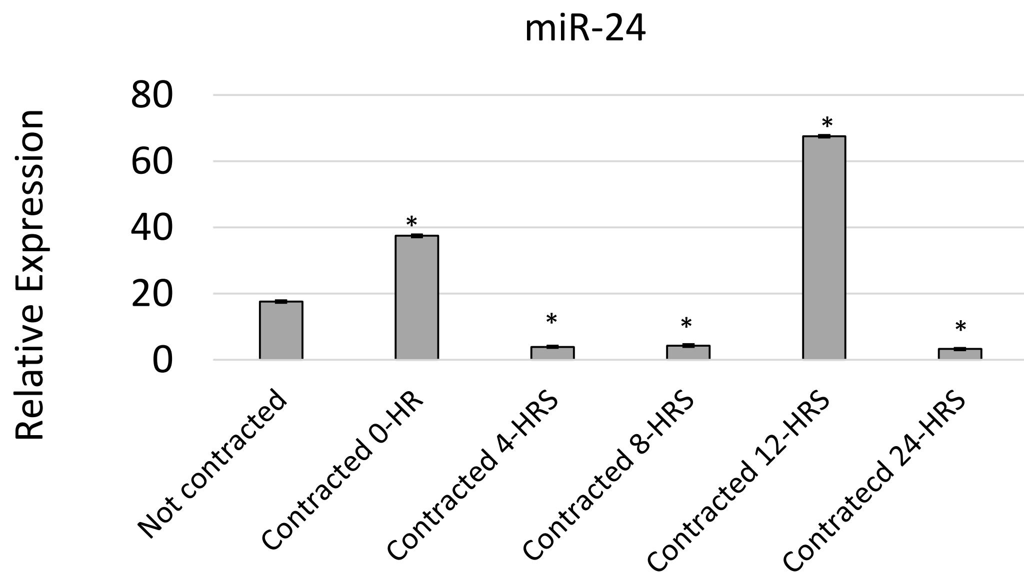
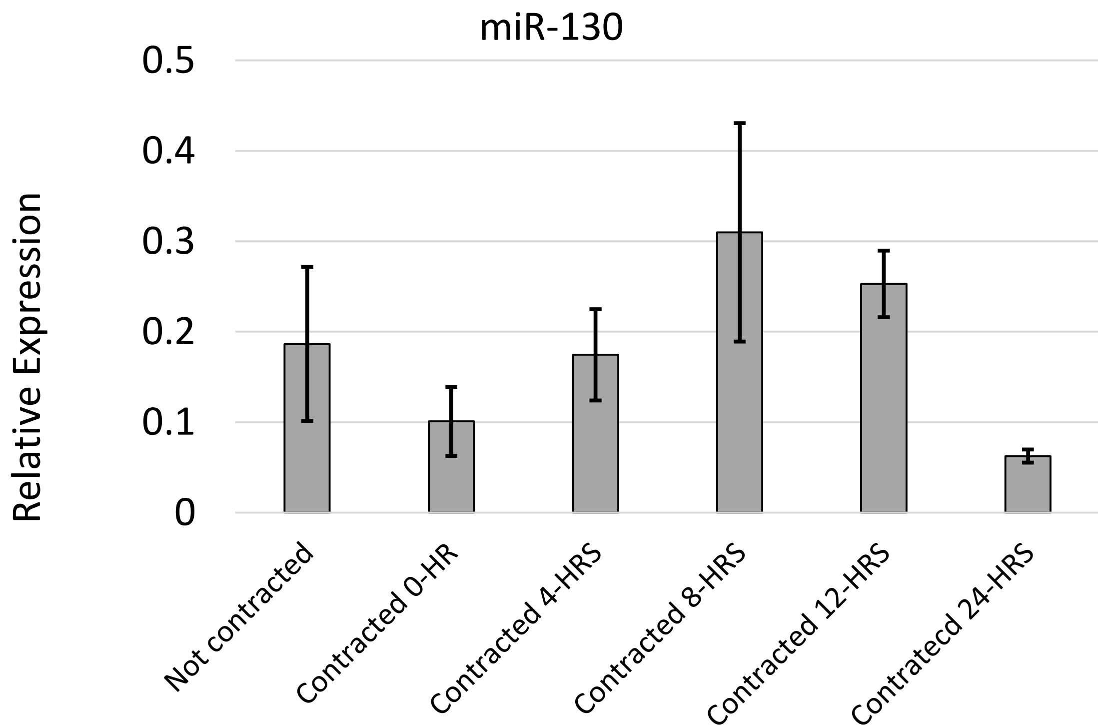


Figure 4.8. The expression of miR-24 following the isometric contraction protocol of *H-2k^b* myotubes. qPCR showing the expression of miR-24 relative to Rnu-6. Not contracted (NC), Contracted (CN) at 0-4 hour time point after 15

minutes of isometric contractions of *H-2k^b* myotubes. Error bars show stdev; *



*P< 0.05 c*ompared to not contracted control; n=3.

Figure 4.9. The expression of miR-130 following the isometric contraction protocol of *H-2k^b* myotubes. qPCR showing the expression of miR-130 relative to Rnu-6. Not contracted (NC), Contracted (CN) at 0-4 hour time point after 15 minutes of isometric contractions of *H-2k^b* myotubes. Error bars show stdev, n=3.

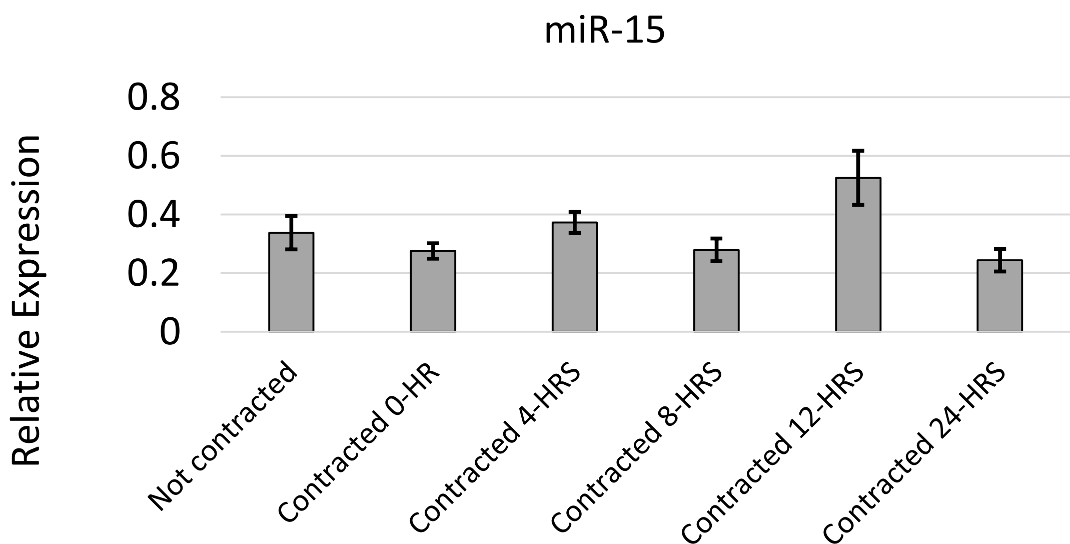


Figure 4.10 The expression of miR-5 following the isometric contraction protocol of *H-2k^b* myotubes. qPCR showing the expression of miR-15 relative to Rnu-6. Not contracted (NC), Contracted (CN) at 0-4 hour time point after 15 minutes of isometric contractions of *H-2k^b* myotubes. Error bars show stdev, n=3.

Table 4.1 Summary of changes in the expression of microRNAs following contraction in H-2kb myotubes and in muscle of adult and old mice. Up – upregulated, Down – downregulated; no significant changes in expression.

microRNA	Expression in H2kb following contraction protocol	Expression in muscle of adult mice following contraction protocol	Expression in muscle of old mice following contraction protocol	Expression in muscle of old mice compared to muscle of adult mice	Are changes in miRNA expression in muscle of adult mice after contractions recapitulated in H2kb cells?
miR-181	up	up	No change	Down	Yes
miR-181d	up	Up	No change	Down	Yes
miR-133a	Down	Down	No change	Down	Yes
miR-199a	No change	Down	Down	Down	No
miR-378	Down	No change	No change	Down	Yes
miR-21	No change	No change	No change	up	yes
miR-16	Down	No change	No change	Down	Yes
miR-24	Up	No change	No change		No
miR-130	Up	No change	No change	No change	No
miR-15	No change	No change	No change	No change	Yes

4.5 Discussion

During this part of the project, the changes in the microRNA expression in *H-2k*^{b} myotubes following an isometric contraction protocol were studied. This *in vitro* model has previously been used for studies of muscle contraction (Pattwell et al., 2004). The *H-2k*^{b} myotubes underwent a 15-minute contraction protocol and non-contracted myotubes served as controls.

The expression of six out of ten microRNAs: miR-181a, miR-181d, miR-133a, miR-24, miR-16 and miR-130 were changed in *H-2k*^{b} myotubes immediately after the isometric contraction protocol (summarised in Table 4.1).

The trends in microRNA expression in studies of *H-2k*^{b} myotubes and the muscles of adult mice (Chapters 3, 4) following contractions were largely comparable (approximately 70%). Specifically, the expression of seven out of ten microRNAs: miR-181a, miR-181d, miR-133a, miR-378, miR-21, miR-16 and miR-15, were affected by the isometric contraction protocol in the same direction in *H-2k*^{b} myotubes and in the muscles of adult mice (summary in Table 4.1). miR-181a, miR-181d expression was upregulated following contractions in the *H-2k*^{b} myotubes and the muscles of adult mice (Figures 3.1, 3.2, 4.1,4.2); the expression of miR-133a, miR-16 and miR-378 was downregulated in the *H-2k*^{b} myotubes and the muscles of adult mice (Figures 4.3, 4.7, 4.5, 3.3, 3.7, 3.5) and the expression of miR-21 and miR-15 remained unchanged in both the *H-2k*^{b} myotubes and the muscles of adult mice (Figures 4.6, 4.10, 3.6, 3.10). The difference in the response of three out of ten microRNA expression between *H-2k*^{b} myotubes and the muscles of adult mice may be due to several reasons: difference in the strength of response of *H-2k*^{b} myotubes

and the muscles of adult mice to isometric contractions protocol, lack of a muscle "environment" influence in the case of *H-2k^b^* myotubes (which may be important in case of microRNAs expressed in immune and fibroblast muscle resident cells, such as miR-21), or potentially the difference of statistical power resulting from a lower number (n=3) of biological replicates with the *H-2k^b^* myotubes than of the muscles of adult mice (n=5). Nevertheless, the microRNA candidates established in Chapter 3 as microRNAs potentially regulating the adaptive response of muscle to contractions during ageing: miR-181a, miR-181d, miR-378 and miR-133a, were also affected by the isometric contraction protocol in the same way in *H-2k^b^* myotubes and the muscles of adult mice (Figures 3.1, 3.2, 3.3, 3.5, 4.1, 4.2, 4.3, 4.5). The limitation of these data is however, that the expression of microRNAs was not studied in control cells 24 hours following the start of the protocol in which time it is possible that the differentiation/hypertrophy/atrophy events could have occurred in control cells. This has to be taken into account when interpreting the data.

Some of the advantages of the use of *H-2k^b^* myotubes in studying muscle response to contractions *in vivo* include reduction in mouse numbers used and the large number of time points following the contraction protocol that can be analysed to study the temporal changes in microRNA expression without the need of sacrificing large number of mice. This information can provide an indication on whether the microRNA may play a role in the early or later phase of muscle response to contractions.

Based on the data obtained in this chapter, miR-181a and miR-181d may be important in regulating the early response of the muscle to contractions: the expression of miR-181a was upregulated in *H-2k*^{b} myotubes immediately after the contraction protocol and returned to basal values (expression of miR-181a in non-contracted *H-2k*^{b} myotubes) after eight hours (Figure 4.1). Similarly, the expression of miR-181d was upregulated in *H-2k*^{b} myotubes immediately after the contraction protocol and returned to basal values (expression of miR-181a in non-contracted *H-2k*^{b} myotubes) after four hours (Figure 4.2).

The expression of miR-133a and miR-378 was downregulated in *H-2k*^{b} myotubes immediately after the contraction protocol and returned to the level of expression of miR-133a in non-contracted *H-2k*^{b} myotubes after four hours (Figures 4.3, 4.5).

miR-133 has been previously shown to be involved in embryonic myogenesis and muscle regeneration (Goljanek-Whysall et al., 2016; Goljanek-Whysall et al., 2012), therefore it is likely that changes in miR-133a expression following isometric contractions may be associated with satellite cells response to exercise and affect muscle regeneration. Moreover, miR-133a seems to be altered differently depending on the type of exercise (Drummond et al., 2008; McCarthy & Esser, 2007; Safdar et al., 2009) which may hold a clue to the precise role of miR-133a in muscle adaptive response to contractions.

miR-378 expression in *H-2k*^{b} was also downregulated immediately after the contractions and returned to the basal miR-378 expression after the contractions (Figure 4.5). miR-378 has been previously shown to regulate adipose tissue

metabolism and data from our lab suggests that miR-3378 may be involved in controlling mitochondrial homeostasis in muscle (manuscript in preparation). miR-378 has also been shown to regulate myogenesis and studies in heart tissue suggested that miR-378 may inhibit cardiomyocyte hypertrophy (Nagalingam et al., 2013). There is some lack of consistency in the role of miR-378 in controlling myocyte hypertrophy versus atrophy and functional studies in skeletal muscle are still missing.

miR-181a and miR-181d expression was upregulated immediately after contraction protocol in *H-2k*^{b} myotubes than of the muscles of adult, but not old, mice and returned to the basal levels after 4-8 hours in *H-2k*^{b} myotubes (Figures 3.1, 3.2, 4.1, 4.2). This is consistent with previously published data (Safdar et al., 2009). Our group has previously demonstrated that miR-181a expression is dysregulated during ageing in the muscle of mice and other groups have reported dysregulation of this microRNA expression in the muscles of humans during ageing (Drummond et al., 2008; Soriano-Arroquia et al., 2016). We have also shown that miR-181 regulates Sirt-1 expression and myotube size *in vitro* (Soriano-Arroquia et al., 2016).

Based on the above data, miR-181a and miR-181d, members of the miR-181 family which are predicted to target the expression of the same set of genes, were chosen for further functional validation of their role in muscle response to contractions during ageing using *in vitro* and *in vivo* models.

5. miR-181: target interactions in the regulation of *H-2k^b* myotubes to contractions

5.1 Abstract

This part of the project investigated microRNAs miR-181a and miR=181d predicted target genes and validated the previouly characterised role of miR-181a *in vitro*.

I used qPCR and western blotting to validate the expression of miR-181a/d predicted target genes previously reported to play an important role in maintaining adult muscle homeostasis, muscle aging and/or muscle response to contractions.

Data show that the expression of Sirt-1 mRNA and protein was downregulated, whereas the expression of miR-181a and miR-181d was upregulated immediately after the $H\text{-}2k^b$ myotubes contractions, suggesting Sirt1 as a real miR-181a and miR-181d target gene. Sirt-1 has been previously shown to regulate muscle differentiation and metabolism and redox balance of skeletal muscle, this makes Sirt-1 a good candidate for miR-181 target gene, potentially regulating the response of adult skeletal muscle to contractions

Other miR-181 predicted target genes were the genes encoding for AP-1 protein complex: Jun, JunB, JunD, Fos and FosB. AP-1 activity has been previously shown to be affected by contractions in the muscle from adult, but not old mice. However, the expression of these genes did not correlate with the expression of miR-181a and miR-181d in $H\text{-}2k^b$ myotubes following contractions and I was not able to examine the protein expression levels of these factors due to inability to detect them using western blot.

5.2 Introduction

Exercise and contraction of skeletal muscle are associated with changes in gene, protein and microRNA expression, as well as changes in transcriptional factor activation and upregulation of antioxidant enzymes activity (McDonagh et al., 2014; Safdar et al., 2009; Soriano-Arroquia et al., 2016; Vasilaki et al., 2010). Data from Chapters 3 and 4 shows that the expression of several microRNAs is changed in muscle and *H-2k^b* myotubes following an isometric contraction protocol. Moreover, the expressions of several microRNAs: miR-181a, miR-181d, miR-133a and miR-378, are dysregulated in both quiescent and contracted muscle from old mice. These microRNAs have been previously directly or indirectly shown to regulate myogenesis, muscle growth and/or muscle atrophy (Carrer et al., 2012; Goljanek-Whysall et al., 2012; Soriano-Arroquia et al., 2016). However, very few target genes have been validated for these microRNAs and the function of these microRNAs has not been yet studied in the context of ageing or muscle contraction.

H-2k^b myotubes were used in this project to study the role of microRNAs in muscle responses to contractions because of their ability to contract (Muses et al., 2011; Pattwell et al., 2004). Hypertrophy and atrophy of myotubes were also studied in *H-2k^b* myotubes, as these processes are significantly affected by ageing and exercise and are associated, at least to some degree, with the ability of skeletal muscle to respond to contractions: muscle of adult organisms typically responds to exercise/contractions via activation of hypertrophy-associated signalling resulting in muscle growth, whereas muscle of old organisms is characterised by increased rate of atrophy and disrupted response to exercise/contractions resulting in ineffective

hypertrophic response (Thomas Braun & Mathias Gautel, 2011; Brown & Goljanek-Whysall, 2015).

Our group has previously used C2C12 myotubes to investigate the role of miR-181 on myotube growth (Figure 5.1). miR-181 overexpression was shown to result in a decreased myotube size, whereas inhibition of miR-181 expression resulted in an increase in C2C12 myotube size (Figure 5.1).

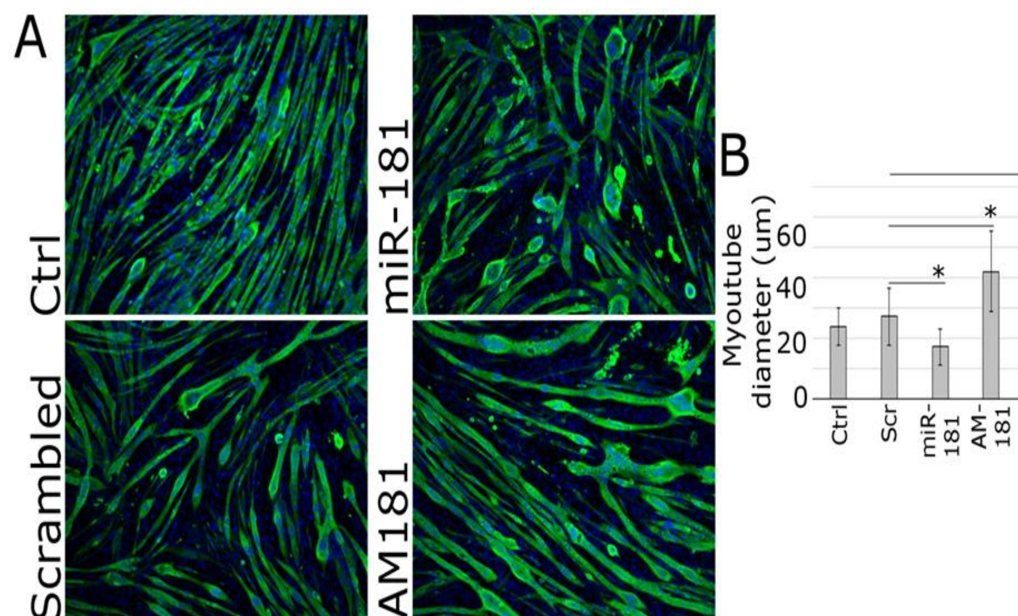


Figure 5.1. The effect of miR-181 upregulation or inhibition on C2C12 myotube size. C2C12 myotubes were transfected with miR-181 mimic or antimiR and the effects on myotube size were measured; green – MF20, blue – DAPI; from (Soriano-Arroquia, House, et al., 2016).

miR-181a and miR-181d, members of miR-181 family predicted to target the same set of target genes, were chosen for functional analyses based on the changes in their expression in the muscle of adult and old mice and in *H-2k^b^* myotubes following the isometric contraction protocol, changes in their expression in muscle of mice during ageing and data previously reported by us and others

showing the role of miR-181 in maintaining muscle size *in vitro* (Chapters 3 and 4; (Safdar et al., 2009; Soriano-Arroquia et al., 2016).

microRNAs exert their function through regulating the expression of target genes (Friedman et al., 2009). microRNA target predictions are difficult due to partial complementarity between the microRNA and its target genes. Nevertheless, publically available databases can be used to predict microRNA target genes and these predictions can be narrowed using gene ontology and network analyses. Since microRNAs are predicted to target several hundred genes, data previously published on genes and proteins differentially expressed in muscle of mice and humans during ageing were used to select most promising miR-181 target genes ((Giresi et al., 2005; McDonagh et al., 2014; Safdar et al., 2009; Soriano-Arroquia et al., 2016).

Targetscan v.7 (http://www.targetscan.org/) was used to predict miR-181a/d target genes. Moreover, gene ontology and network analyses were used to narrow the list of miR-181a/d putative targets.

Among miR-181a/d predicted target genes were: nuclear factor-kB (NF-κB) and activating protein-1 (AP-1) complex proteins: JUN and FOS. NF-kB has been shown to play an important role in pro-inflammatory response signalling in muscle (Hai el al., 2016). The activation protein-1 (AP-1) transcription factor plays an important role in the control of cellular responses to stimuli which regulate cell differentiation, proliferation, and immune response (Mechta-Grigoriou et al., 2001). The changes in activity of NF-κB and AP-1 in muscle of mice have been demonstrated in response to contractions and ageing (Vasilaki et al., 2010).

The aims of this chapter were to:

1. Examine the role of miR-181a/d in muscle hypertrophy and/or atrophy *in vitro*;

2. Investigate the function of miR-181a/d in regulating muscle responses to contractions *in vitro* through characterising its predicted and validated target genes.

5.2 Materials and methods

5.2.1. Cell culture

C2C12 cell culture and transfections were performed as describe by (Soriano-Arroquia et al., 2016). The *H-2k*^{b} cells were cultured, differentiated and transfected as described in Chapter 2.1. Briefly, the myotubes were treated with 100nM final concentration of miR-181a mimic or miR-181 antagomiR (AM181) for 48h prior to the contraction protocol and 10 days before immunostaining. AntagomiR scrambled, not predicted to target any known microRNA in the human or mouse genomes, was used as control transfection.

5.2.2 Isometric contraction protocol of *H-2k*^{b} myotubes and RNA isolation

The isometric contraction protocol of the *H-2k*^{b} myotubes was performed as described previously in Chapter 2 (section 2.2) (Pattwell et al., 2004)).

The isolation of RNA from the non-contracted (control) and contracted myotubes was undertaken immediately or at described time points following the isometric contraction protocol using Trizol reagent as described in Chapter 2.5.

5.2.3 Reverse transcription (mRNA) and qPCR analysis of miR-181 target gene expression

The first-strand cDNA was synthesised from the RNA isolated from treated *H-2k^b* myotubes using Invitrogen reagents as described previously in chapter 2.8. The qPCR was performed as described in Chapter 2.9.

5.2.4 Statistical analysis

Myotube diameter was measured and analysed using Image J as described previously (Chapter 2.15 (Goljanek-Whysall et al., 2012)) qPCR data are presented as the mean ± SEM in all experiments. The statistical analyses of different groups were determined by One-Way Anova followed by post hoc analyses. (Bonferroni)

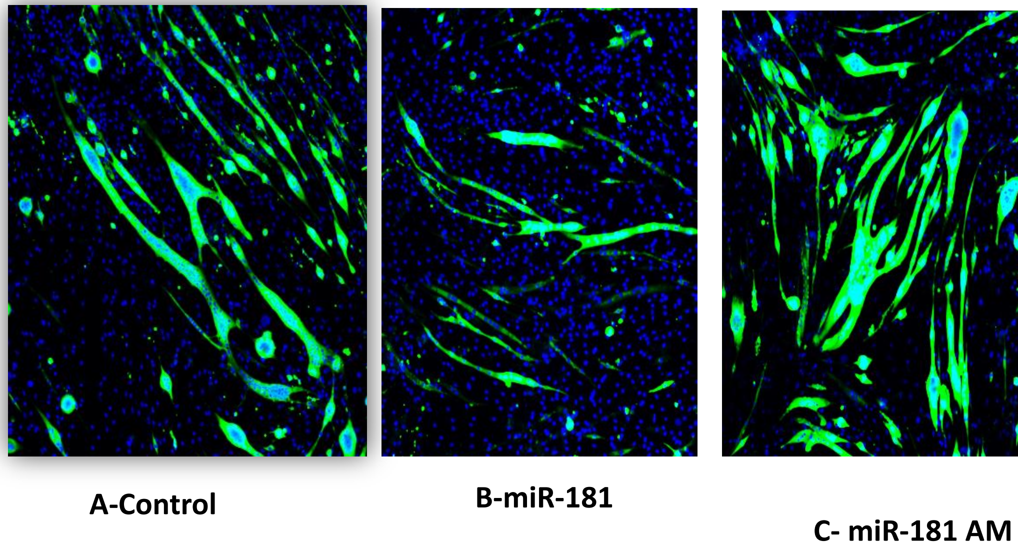
5.3 Results

5.3.1. The effect of miR-181 on myotube size *in vitro*

In order to validate the role of miR-181a/d on myotube size, miR-181a/d was overexpressed or inhibited in the differentiated *H-2k^b* myotubes and 10 days later, immunostaining for MF20 (myosin heavy chain – marker of differentiated myotubes) was undertaken. Myotube diameter was calculated to quantify the effects of miR-181a/d (from here called miR-181) on *H-2k^b* myotube size. miR-181 overexpression using miR-181a mimic was found to result in fewer and smaller myotubes (Figure 5.2), whereas inhibition of miR-181a expression using antagomiR led to the presence of a higher number and bigger myotubes (Figure 5.2). This is

consistent with data previously published by us using C2C12 cells (Soriano-Arroquia et al., 2016).

The changes in the size of the myotubes could be associated with hypertrophy or atrophy pathways being affected. Therefore, the expression of atrogenes, genes related to muscle atrophy, Atrogin-1 and MuRF-1, was analysed following miR-181a overexpression and inhibition in *H-2k^b* myotubes (Figure 5.3). The expression of neither Atrogin-1 nor MuRF-1 was not significantly affected by changes in microRNA-181a expression in *H-2k^b* myotubes suggesting that the effects of miR-181a on myotube size are not mediated by modification of this atrophy pathway (Figure 5.3). The effects of miR-181a on other signalling pathways associated with regulation of muscle growth were analysed below through investigating the expression of miR-181 target genes following miR-181a overexpression or inhibition in non-contracted and contracted *H-2k^b* myotubes.



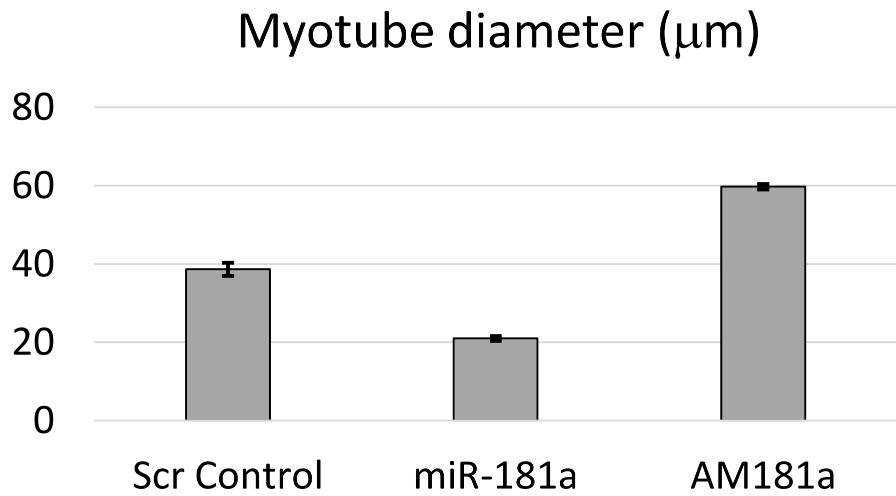
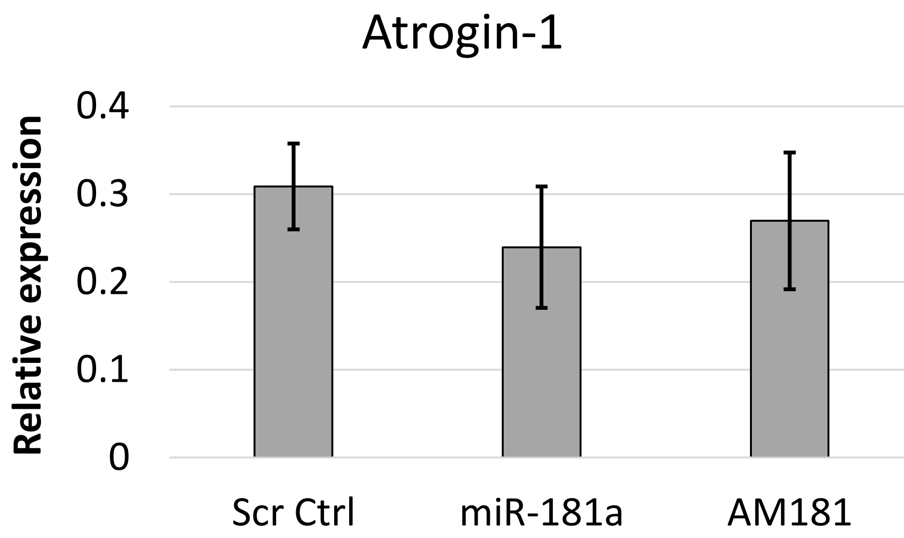


Figure 5.2. The effects of miR-181a overexpression and inhibition on *H-2k^b* myotube size. *H-2k^b* myotubes were transfected with miR-181a mimic or antagomiR-181 and immunostained for MF20 (myosin heavy chain). The effects of miR-181a overexpression and inhibition were established by quantification of myotube size. Scr Ctrl –myotubes treated with scrambled antagomiR; miR-181a – myotubes treated with miR-181a mimic; AM181a – myotubes treated with antagomiR181; Green- MF20; blue – DAPI. Error bars show SEM, *: $p<0.05$ (One-Way Anova); n=3.



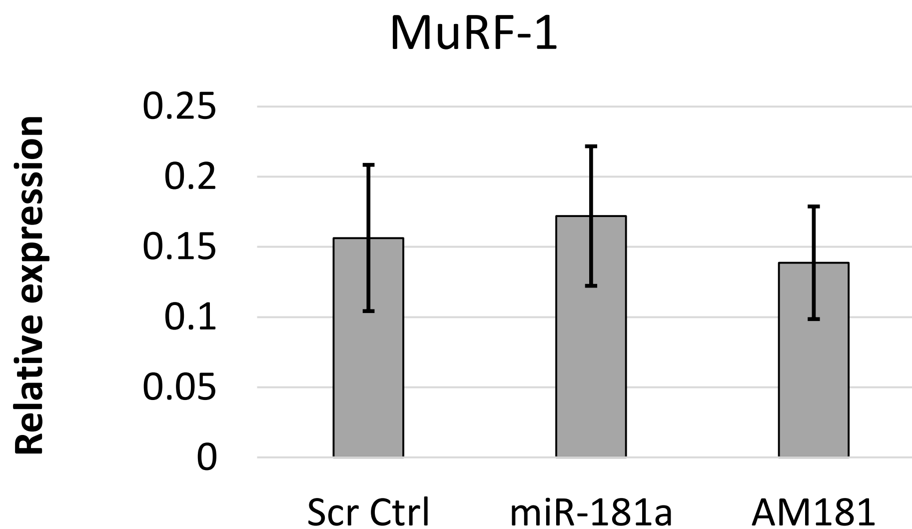


Figure 5.3. The effects of miR-181 overexpression and inhibition on the expression of atrogenes in *H-2k^b* myotubes. *H-2k*^{b} myotubes were transfected with miR-181a mimic or antagomiR-181 and immunostained for MF20 (myosin heavy chain). Scr Ctrl –myotubes treated with scrambled antagomiR; miR-181a – myotubes treated with miR-181a mimic; AM181a – myotubes treated with antagomiR 181. Error bars show SEM, *: $p<0.05$ (One-Way Anova); n=3.

5.3.2. The effects of miR-181 on putative target gene expression

In order to establish the mechanisms by which miR-181a/d affects mytoube size, the expression of miR-181a/d target genes was analysed. miR-181a/d has nearly 200 hundred putative target genes as predicted by TargetScan (http://www.targetscan.org/). In order to select miR-181 predicted targets relevant to muscle growth and response to contractions, network analyses and published data sets were used.

miR-181a/d predicted target genes chosen for further validation included: Jun, JunB, JunD, Fos and Fosb, encoding for AP-1 complex proteins previously shown to be altered during the contractions of adult, but not old mice (Vasilaki et al., 2010). Moreover, Pten, Igf1r and p21 were chosen for expression analyses due to their involvement in the regulation of muscle atrophy/hypertrophy and/or muscle loss during ageing (Glass, 2003; Grounds, 2002). Finally, Sirt-1 expression was also analysed as Sirt-1 has been shown to regulate redox balance and muscle hypertrophy and Sirt-1 has been validated as miR-181a target gene in C2C12 by us (Soriano-Arroquia, House, et al., 2016). The expression of miR-181 predicted target genes was analysed in $H\text{-}2k^b$ myotubes following isometric contractions protocol, similarly as miR-181a and miR-181d expression was analysed in Chapter 4 (Figures 4.1, 4.2). A predicted target gene was initially validated as miR-181 target and selected for further analyses if its expression was negatively correlated with the expression of miR-181a and/or miR-181d in $H\text{-}2k^b$ myotubes following the isometric contraction protocol.

The expression of Jun in *H-2k^b* myotubes was significantly downregulated immediately after the isometric contraction protocol and upregulated at 12 and 24 h after contractions in *H-2k^b* myotubes (Figure 5.4). The expression of JunB was significantly upregulated at 4, 12 and 24 hours following the contractions of *H-2k^b* myotubes, whereas the expression of JunD was significantly upregulated 4 and 8h after the contraction protocol of *H-2k^b* myotubes (Figures 5.5, 5.6).

The expressions of Jun, JunB and JunD in *H-2k^b* myotubes following the isometric contraction protocol were therefore not significantly negatively correlated with the expression of miR-181a and miR-181d in *H-2k^b* myotubes following the isometric contraction protocol (Figures 4.1, 4.2, 5.4, 5.5, 5.6). However, at 4h following *H-2k^b* myotubes contractions, the expression of JunB and JunD was correlated with that of miR-181d, therefore Jun genes were selected for further *in vivo* analyses (Chapter 6).

The expression of Fos and FosB in *H-2k^b* myotubes was significantly upregulated 4, 12 and 24 hours following the isometric contraction protocol, therefore the expression of Fos and FosB was not significantly correlated in the contracted *H-2k^b* myotubes (Figures 4.1, 4.2, 5.7, 5.8). However, as Fos and Fosb expression was affected at 4 hours after the contractions of *H-2k^b* myotubes, these genes were also selected for further *in vivo* validation (Chapter 6).

The expression of Pten in *H-2k^b* myotubes was significantly upregulated at 0, 12 and 24 hours following the myotube contractions, suggesting that Pten and miR-181a and miR-181d expressions were not negatively correlated in the

contracted *H-2k^b^* myotubes (Figures 4.1, 4.2, 5.9). However, Pten expression has been previously shown to be affected by miR-181 (Soriano-Arroquia et al., 2016).

Sirt-1 expression in $H\text{-}2k^b$ myotubes was significantly downregulated immediately after the contraction protocol and upregulated 4 and 12 hours following the contractions (Figure 5.10). This suggests that Sirt-1 expression may be negatively correlated to that of miR-181a and miR-181d in contracted $H\text{-}2k^b$ myotubes (Figures 4.1, 4.2, 5.10).

Both Pten and Sirt-1 expression were further analysed in *in vivo* studies following miR-181 gain- and loss-of-function approaches (Chapter 6).

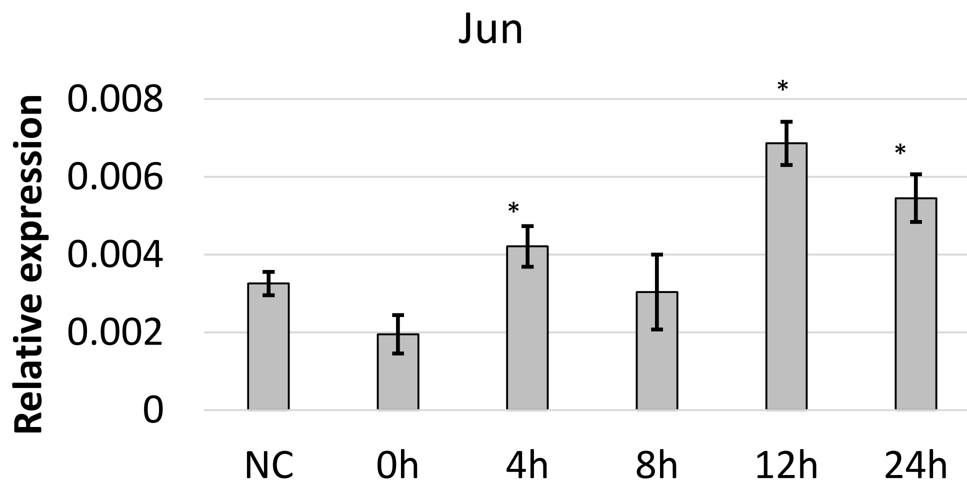


Figure 5.4. qPCR showing the expression of Jun in $H\text{-}2k^b$ myotubes following an isometric contraction protocol. Expression relative to beta-2-microglobulin and GAPDH and normalised to non-contracted control (100%) is shown. NC - not contracted myotubes, CN - contracted myotubes at 0-24 hours after 15-minute of isometric contractions. Error bars shows SEM * *P<0.05* compared to not contracted control, n=5.

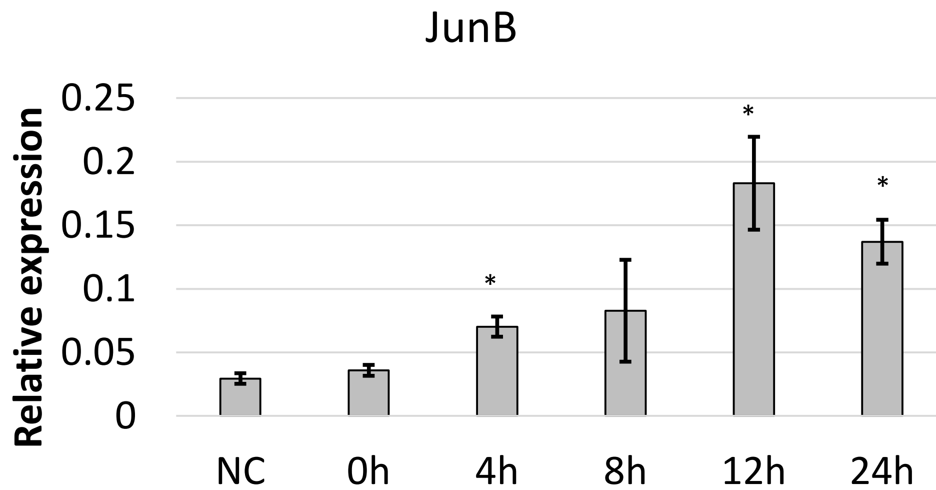


Figure 5.5. qPCR showing the expression of JunB in *H-2k^b* myotubes following an isometric contraction protocol. Expression relative to beta-2-microglobulin and Gapdh and normalised to non-contracted control (100%) is shown. NC - not contracted myotubes, CN - contracted myotubes at 0-24 hours after 15-minute of isometric contractions. Error bars shows SEM * $P<0.05$ compared to not contracted control, n=5.

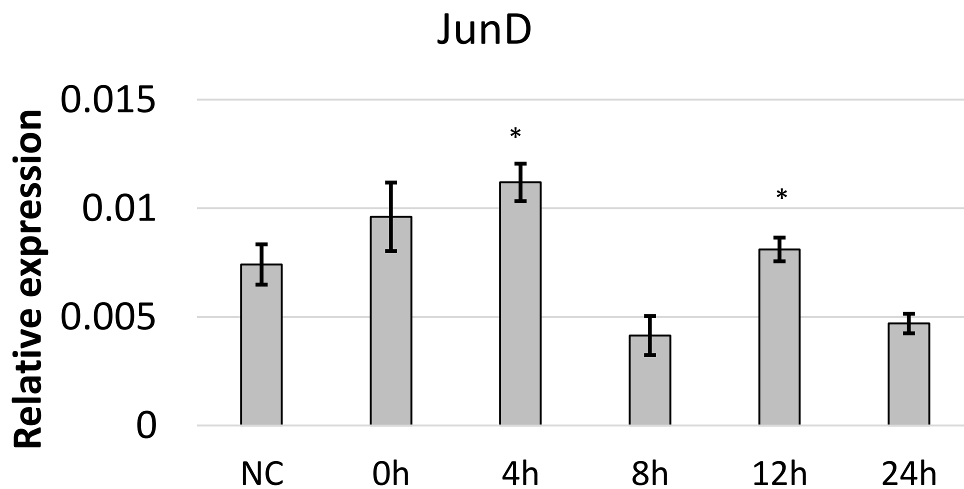


Figure 5.6. qPCR showing the expression of JunD in *H-2k^b* myotubes following an isometric contraction protocol. Expression relative to beta-2-microglobulin and Gapdh and normalised to non-contracted control (100%) is shown. NC - not contracted myotubes, CN - contracted myotubes at 0-24 hours after 15-minute of isometric contractions. Error bars shows SEM * $P<0.05$ compared to not contracted control, n=5.

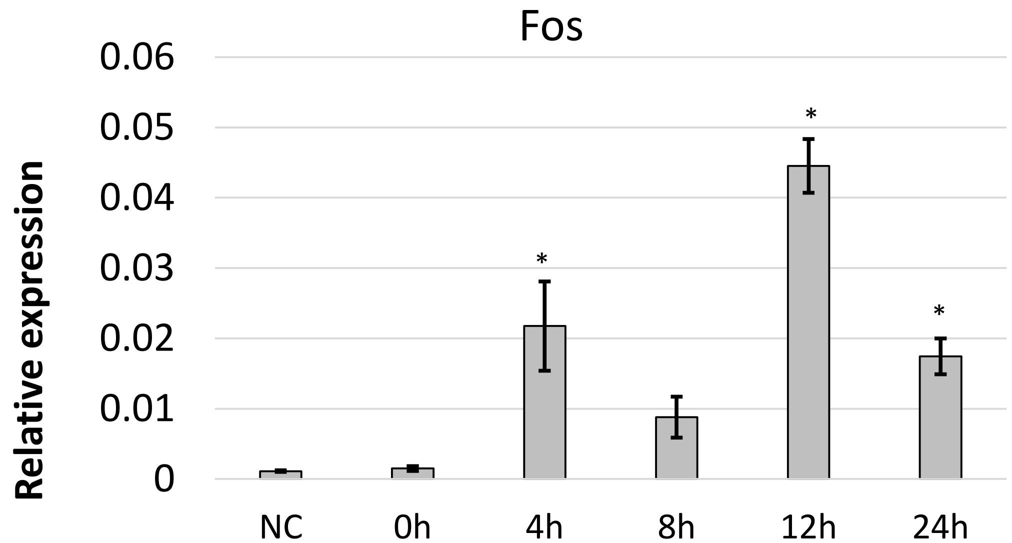


Figure 5.7. PCR showing the expression of Fos in *H-2kb* myotubes following an isometric contraction protocol. Expression relative to beta-2-microglobulin and GAPDH and normalised to non-contracted control (100%) is shown. NC - not contracted myotubes, CN - contracted myotubes at 0-24 hours after 15-minute of isometric contractions. Error bars shows SEM * *P<0.05* compared to not contracted control, n=5.

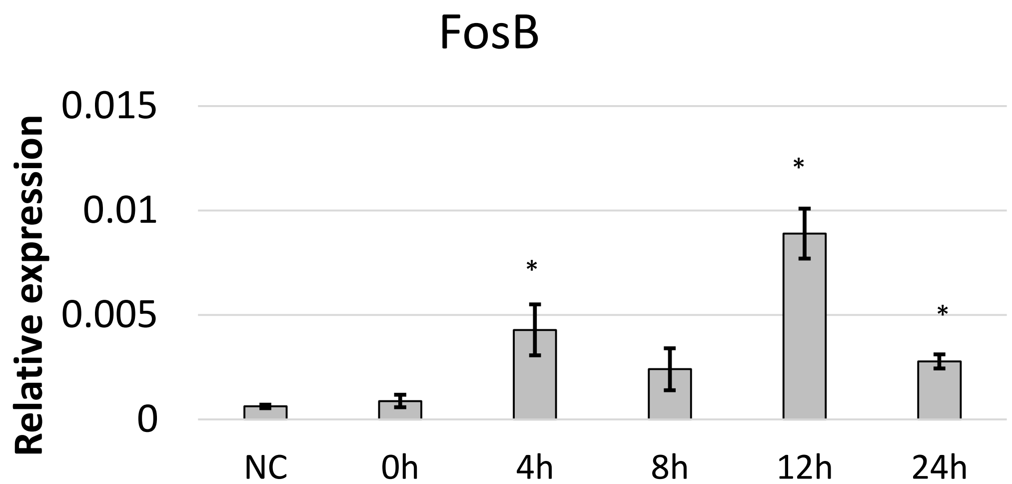


Figure 5.8. qPCR showing the expression of FosB in *H-2k^b^* myotubes following an isometric contraction protocol. Expression relative to beta-2-microglobulin and GAPDH and normalised to non-contracted control (100%) is shown. NC - not contracted myotubes, CN - contracted myotubes at 0-24 hours after 15-minute of isometric contractions. Error bars shows SEM * *P<0.05* compared to not contracted control, n=5

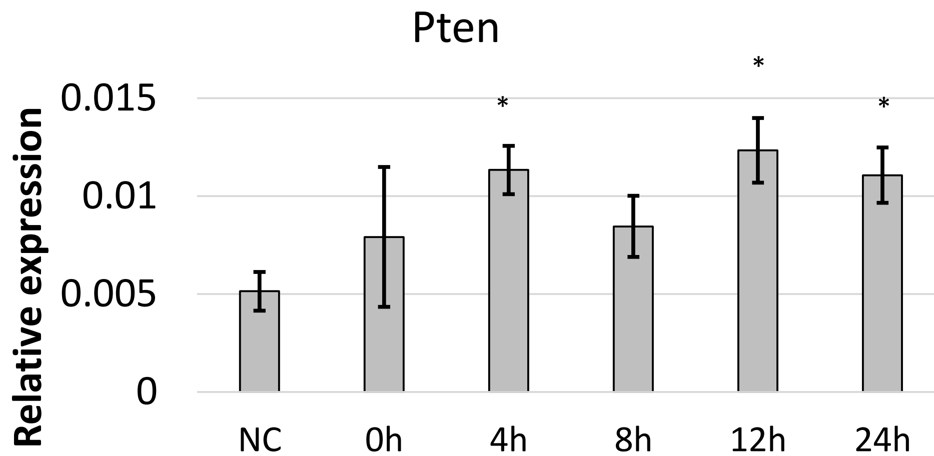


Figure 5.9. qPCR showing the expression of Pten in *H-2kb* myotubes following an isometric contraction protocol. Expression relative to beta-2-microglobulin and GAPDH and normalised to non-contracted control (100%) is shown. NC - not contracted myotubes, CN - contracted myotubes at 0-24 hours after 15-minute of isometric contractions. Error bars shows SEM * *P<0.05* compared to not contracted control, n=5.

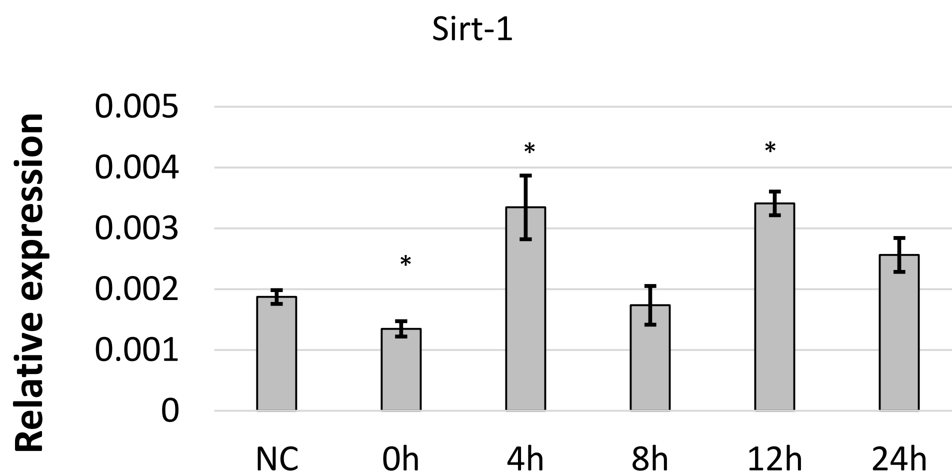


Figure 5.10. qPCR showing the expression of Sirt-1 in *H-2k^b^* myotubes following an isometric contraction protocol. Expression relative to beta-2-microglobulin and GAPDH and normalised to non-contracted control (100%) is shown. NC - not contracted myotubes, CN - contracted myotubes at 0-24 hours after 15-minute of isometric contraction -s. Error bars shows SEM * *P<0.05* compared to not contracted control, n=5.

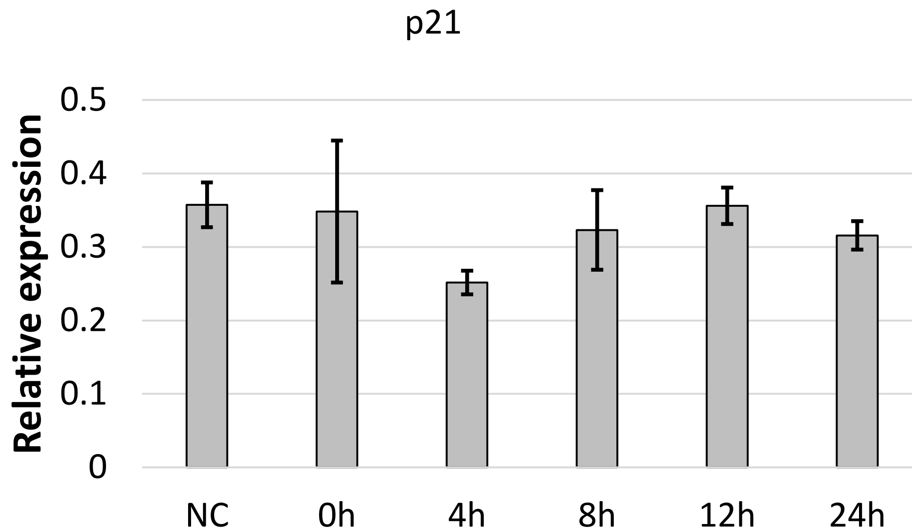


Figure 5.11. qPCR showing the expression of p21 in *H-2k^b* myotubes following an isometric contraction protocol. Expression relative to beta-2-microglobulin and GAPDH and normalised to non-contracted control (100%) is shown. NC - not contracted myotubes, CN - contracted myotubes at 0-24 hours after 15-minute of isometric contractions, n=5.

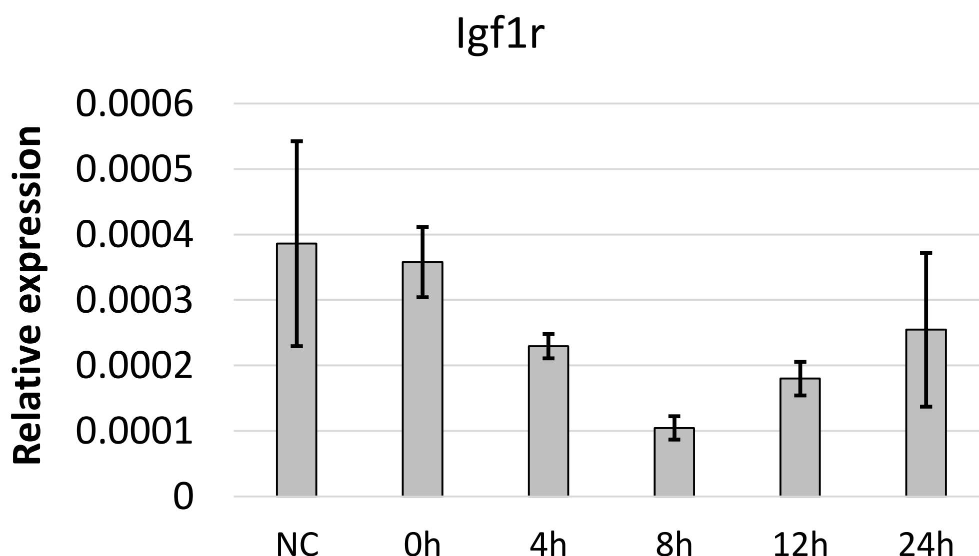


Figure 5.12. qPCR showing the expression of Igf1r in *H-2k^b* myotubes following an isometric contraction protocol. Expression relative to beta-2-microglobulin and GAPDH and normalised to non-contracted control (100%) is shown. NC - not contracted myotubes, CN - contracted myotubes at 0-24 hours after 15-minute of isometric contractions, n=5.

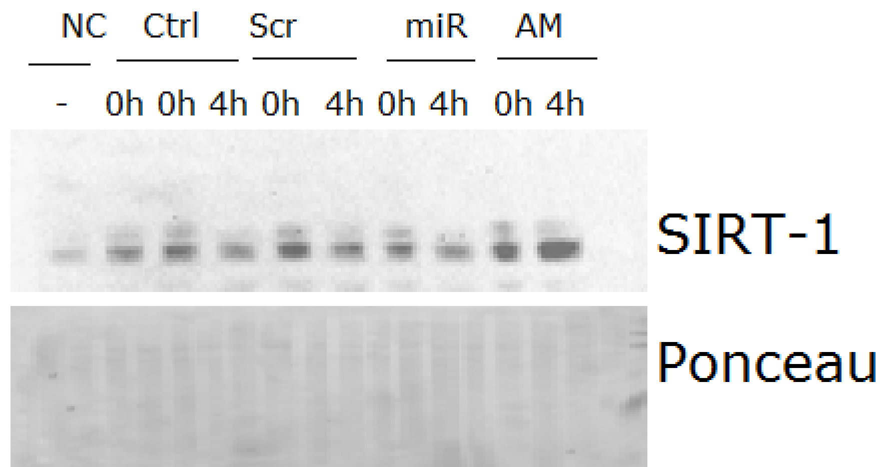


Figure 5.13. Western blot showing the expression of SIRT-1 in *H-2k^b^* myotubes following an isometric contraction protocol. NC - not contracted myotubes, CN - contracted myotubes at 0-24 hours after 15-minute of isometric contractions, n=5.

The expression of p21 and Igf1r was not significantly altered in *H-2k^b^* myotubes following the isometric contraction protocol (Figures 5.11, 5.12). This suggests that p21 and Igf1r are unlikely miR-181a/d target genes.

Based on the qPCR analyses of miR-181 target gene expression in *H-2k^b^* myotubes following the isometric contraction protocol, Sirt-1 was the most promising miR-181 target gene. Western blot analysis was therefore performed in *H-2k^b^* myotubes following the isometric contraction protocol to establish whether SIRT-1 protein levels were also affected by the isometric contractions of the in *H-2k^b^* myotubes. SIRT-1 protein expression was downregulated immediately after in *H-2k^b^* myotube contractions (Figure 5.13).

Our group has previously validated Sirt-1 as miR-181 target gene using C2C12 cells and GFP-reporter vectors (Soriano-Arroquia et al., 2016). Together,

these data indicate that Sirt-1 may be a relevant miR-181 target gene mediating its effects on myotube size and potentially regulating the response of muscle to contractions in adult mice and lack of thereof in the muscle of old mice. This was analysed in Chapter 6 using *in vivo* functional analyses of miR-181 overexpression and inhibition on muscle homeostasis.

5.4 Discussion

The data presented in this chapter validated the negative role of mir-181 on mytoube size *in vitro* (Figure 5.2), consistent with published data (Soriano-Arroquia et al., 2016).

qPCR and western blotting were used to validate the expression of miR-181a/d predicted target genes previously reported to play a role in maintaining adult muscle homeostasis, muscle aging and/or muscle response to contractions (Soriano-Arroquia et al., 2016).

Our data show that the expression of Sirt-1 mRNA and protein is downregulated, whereas the expression of miR-181a and miR-181d is upregulated immediately after the $H\text{-}2k^b$ myotubes contractions (Figures 4.1, 4.2, 5.10). Sirt-1 is a mammalian homologue of the yeast Sirt2 which has been shown to increase lifespan (Howitz et al., 2003). Sirt-1 has also been shown to regulate muscle differentiation and metabolism and redox balance of skeletal muscle (Cheng et al., 2016; Fulco et al., 2003; Pardo & Boriek, 2011). Moreover, Sirt-1 expression has been shown to be upregulated in the muscle of old mice (McDonagh et al., 2014) and our group has previously validated Sirt-1 as miR-181a target gene in vitro using

C2C12 cells (Soriano-Arroquia et al., 2016). Together, this makes Sirt-1 an attractive miR-181 target gene, potentially regulating the response of adult skeletal muscle to contractions and contributing to the defective response of muscle of old organisms to contractions. This hypothesis will be further investigated in Chapter 6 using an *in vivo* model and miR-181 gain- and loss-of-function approaches followed by physiological and molecular phenotype analyses.

Among other miR-181 predicted target genes were the genes encoding for AP-1 protein complex: Jun, JunB, JunD, Fos and FosB. AP-1 activity has been previously shown to be affected by contractions in the muscle from adult, but not old mice (Vasilaki et al., 2010). Moreover, JunB has been shown to play an important role in the maintenance of adult muscle mass and induce a rapid hypertrophy while preventing muscle atrophy (Raffaello et al., 2010). Moreover, FOS has been shown to be activated during exercise and be involved in the early regulation of cell growth and differentiation s (McGivney et al., 2009). This is consistent with expression data presented here (Figures 5.4-5.8).

The expression pattern of the Jun and Fos genes was not negatively correlated to the expression of miR-181a and miR-181d in $H\text{-}2k^b$ myotubes following isometric contraction protocol (Figures 4.1, 4.2, 5.1 – 5.5). It is possible that miR-181 regulated the expression of these genes through inhibition of translation rather than mRNA degradation, however we were not able to examine the protein expression levels of these factors due to inability to detect them using western blot. These factors are known to be notoriously difficult to be detected using western blot due to their sensitivity to protein isolation methods. Often, the

expression and/or phospohorylation of these proteins are affected immediately after changes within or outside the cells, rather than days after treamment. Despite the lack of evident correlation between the expression of the Jun and Fos factors with the expression of miR-181a and miR-181d in *H-2k*^{b} myotubes following isometric contractions, the expression of these genes was further studied in Chapter 6, using the *in vivo* model due to the importance of the AP-1 complex in muscle adaptive response to exercise and muscle ageing.

6. The role of miR-181 in muscle ageing and response to contractions: an in vivo study.

6.1 Abstract

The ability for muscles to maintain balance is impaired during ageing and the loss of this balance is associated with loss of muscle mass and function known as sarcopenia, which is associated with impaired balance and frailty.

The aims of this chapter were:

- To validate the role of miR-181 *in vivo,* in regulating muscle size and response to isometric contractions during ageing;

- To validate Sirt-1, Pten, JUN and FOS-encoding genes as miR-181a/d target genes *in vivo*.

The expression of miR-181a/d was downregulated in muscle during ageing and this was associated with the increased levels of Sirt-1 mRNA and protein, and decreased specific force of the EDL muscle. The expression of miR-181a and miR-181d was also negatively correlated with that of Sirt1 mRNA and protein in the muscles of adult, but not old mice suggesting that miR-181:Sirt-1 interactions may be disrupted in the muscle of old mice in response to contractions.

Finally, the effects of miR-181a and miR-181d on muscle mass in the adult and old mice using miR-181a/d gain and loss-of-function were studied. Data show no significant effects on muscle mass relative to whole body mass, both in adult and old mice treated with miR-181 and miR-181 antagomiR. However, miR-181a/d expression manipulation in the muscle of adult and old mice led to changes in myofibre size and specific force suggesting that changes in miR-181a/d expression may be a part of a mechanism associated with loss of muscle mass and function during ageing.

6.2 Introduction

Ageing is associated with decrease in muscle mass and function known as sarcopenia (McArdle et al., 2002). During ageing muscle also loses its plasticity and the response of muscle from older mammals to exercise is attenuated (McArdle et al., 2002). The molecular mechanisms of muscle ageing are not fully understood. Data presented in Chapters 4-6 shows that the expression of selected microRNAs, post-transcriptional regulators of gene expression, is changed in the *tibialis anterior* muscle of old mice (Figures 6.14, 6.15, 6.16, 6.17, 6.18). Moreover, our data show that the expression of several microRNAs: miR-181a, miR-181d and miR-133a, is differentially regulated in response to contractions in the muscle from adult and old mice (Figures 6.1, 6.2). As microRNAs can regulate the expression of multiple genes simultaneously and changes in gene and protein expression have been implicated in both muscle response to contractions and muscle ageing (Drummond et al., 2008; Giresi et al., 2005; McDonagh & Davies, 1984; Soriano-Arroquia et al., 2016), it is likely the dysregulated expression of microRNAs in muscle from older organisms may contribute to disrupted responses of older muscle to contractions. Data from our group (Soriano-Arroquia et al., 2016) and in Chapter 5 demonstrates that the changes in miR-181a and miR-181d may be crucial to regulating age-related changes in gene expression in muscle from mice and potentially humans. Moreover, changes in the expression of miR-181a and miR-181d result in changes in myotube diameter *in vitro*, in both C2C12 and *H2-k*^{b} cell lines (Figures 5.1, 5.2) (Soriano-Arroquia et al., 2016).

microRNAs play their function *via* regulation of gene expression, it is therefore necessary to characterise the target gene(s) of microRNA when deciphering its function. microRNA target gene predictions are difficult due to low complementarity between the microRNA and the target site within the 3'UTR of its target gene(s). Nevertheless, target prediction databases can be used to predict microRNA target genes. In Chapter 5, miR-181a/d target genes for *in vitro* validation were selected, based on their expression in muscle during ageing and/or contractions and their role in muscle. Genes previously described to play an important role in muscle ageing and/or response of muscle to contractions, such as Sirt-1 or Jun and Fos, were chosen. Analysis of miR-181a/d and target gene expression in quiescent or contracted muscle from adult and old mice and in *H2-k*^{b} myotubes following an isometric contraction protocol revealed Sirt-1 as most promising miR-181a/d target gene (Figure 5.10.). Genes encoding for JUN and FOS proteins, part of the AP-1 complex, previously shown to be a part of dysregulated response of muscle from old mice to contractions (Vasilaki et al., 2006), were also chosen for further *in vivo* analyses due to their important role in muscle contractions and the partial negative correlation of their expression with that of the expression of miR-181a/d in *H2-k*^{b} myotubes following an isometric contraction protocol (Vasilak et al., 2006) (Figures 4.1, 4.2).

SIRT-1 has been shown to play an important role in muscle metabolism, satellite cell function and myogenic differentiation, as well as in adaptation of muscle to changes in the environment (Cheng et al., 2016; Koltai et al., 2010; Olmos et al., 2013; Vinciguerra et al., 2010) (Pardo & Boriek, 2011). Moreover, SIRT-1 has

been shown to regulate redox balance in muscle – a key aspect of muscle response to contractions (Cheng et al., 2016; Soriano-Arroquia et al., 2016). Our group has previously shown that Sirt-1 is a miR-181 direct target *in vitro* using C2C12 cells (Soriano-Arroquia et al., 2016).

6.2 Materials & Methods

Adult (6 months old) and old C57BL6/J mice (24 months old) were used in this Chapter as described in section 2.4. The quiescent and contracted (15-minute isometric contraction protocol) muscle from mice was used for analyses as detailed in section 2.6. Mice were injected with miR-181 mimic, antagomiR-181 or anatgomiR-scrambled (control mice) twice in a three- or five-week period as described in section 2.24.

6.2.1. miR-181 overexpression and inhibition in the muscles of adult and old mice and isolation of RNA from skeletal muscle

Adult (5 months old) and old (23 months old) mice were treated with miR-181 mimic, antagomiR-181 or control antagomiR scrambled through two intravenous injections (at day 1 and day 10 or 14 of the experiment) as described in Chapter 2.24. For the contractions experiment, mice were treated for three weeks. For force measurements, mice were treated for five weeks. RNA isolation from the *tibialis anterior* muscle was performed using the Trizol method as described in Chapter 2.6. The n numbers were calculated based on previous data from our lab assuming greater variation in the expression of microRNAs in muscle of old mice as compared to muscle of adult mice.

6.2.2. Reverse Transcription and qPCR analysis (microRNA).

cDNA was synthesised using 500ng RNA using miScript II Reverse Transcriptase kit (Qiagen) or Superscript II, as described in sectons 2.7. and 2.8. The qPCR experiments were performed as described in section 2.9.

6.2.3. Western blot analysis of protein expression

Isolation of protein and western blot was performed as described in sections 2.18, 2.21 and 2.22. Briefly, the muscle was dissected in frozen in liquid nitrogen, the tissues was ground and protein isolated using SDS. BCA assay was used to determine the protein content. Antibodies used are described in Chapter 2.22, Table 6.

6.2.4. Sirt-1 and WGA immunostaining

SIRT-1 and WGA immunostaining was performed as described in Chapter 2.15 on PFA-fixed and cryosectioned *tibialis anterior* muscle.

6.2.5. *In situ* force measurement of the EDL muscle

The force measurement experiment was performed as described in Chapter 2.25. Briefly, the force of the *extensor digitorium longus* muscle was measured via peroneal nerve stimulation of anaesthetised mice. Forces were calculated as described in Chapter 2.25.

6.2.6. Statistical analysis of qPCR and force data

Generated data were presented using mean ±SEM. Statistical analyses of different groups were determined by T-test. Each comparison between 2 experimental conditions was performed using unpaired Student's t test.

6.3 Results

6.3.1. The effects of miR-181a/d on muscle mass and myofibre size *in vivo*.

The effects of miR-181a and miR-181d on muscle mass in adult and old mice were studied using miR-181 gain- and loss-of-function experiments. miR-181 mimic was designed based on the sequence of miR-181a and miR-181 antagomiR was designed to inhibit miR-181a and potentially mir-181d function. miR-181a is the member of miR-181 family expressed at the highest levels and most dynamically in the muscle of mice during ageing and following contractions (Figures 6.1.6.2). Adult (5 months old) and old (23 months old) mice were treated with miR-181 mimic or antagomiR-181 for three weeks *via* two intravenous injections. Mice treated with antagomiR scrambled (Scr), not predicted to target any known microRNA, served as controls.

The expression of miR-181a was efficiently regulated in the muscle from both adult and old mice by the intravenous injections of miR-181a mimic or antagomiR-181 (Figure 6.1). miR-181a was upregulated in the muscle from adult

and old mice following miR-181 mimic injections and downregulated following AM181 injections (Figure 6.1).

The expression of miR-181d was efficiently regulated in the muscle from adult mice by the intravenous injections of miR-181a mimic or antagomiR-181 (Figure 6.2). miR-181d expression was upregulated in the muscle from adult mice following miR-181 mimic injections and downregulated following AM181 injections (Figure 6.2). However, miR-181d expression in the muscle from old mice, even though significantly downregulated following AM181 treatment, was not significantly upregulated following miR-181a mimic injections (Figure 6.2). This is most likely due to the sequence of miR-181 mimic being designed to primarily mimic the endogenous miR-181a, the member of miR-181 family expressed at high levels in skeletal muscle and dynamically regulated in response to ageing and contractions of muscle.

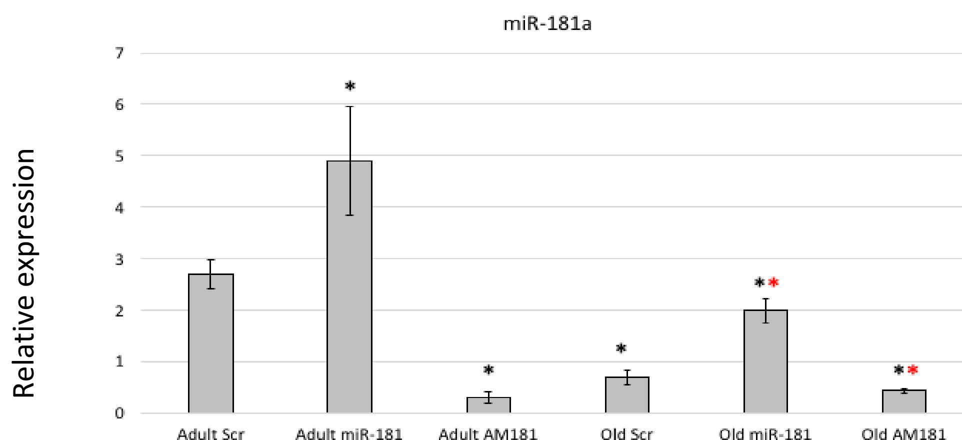


Figure 6.1. The expression of miR-181a in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr). Expression relative to Rnu-6. N=3-4 per experimental group. Error bars show SEM. * - $p<0.05$ as compared to Adult Scr NC; * - $p<0.05$ as compared Old Scr; One-Way Anova.

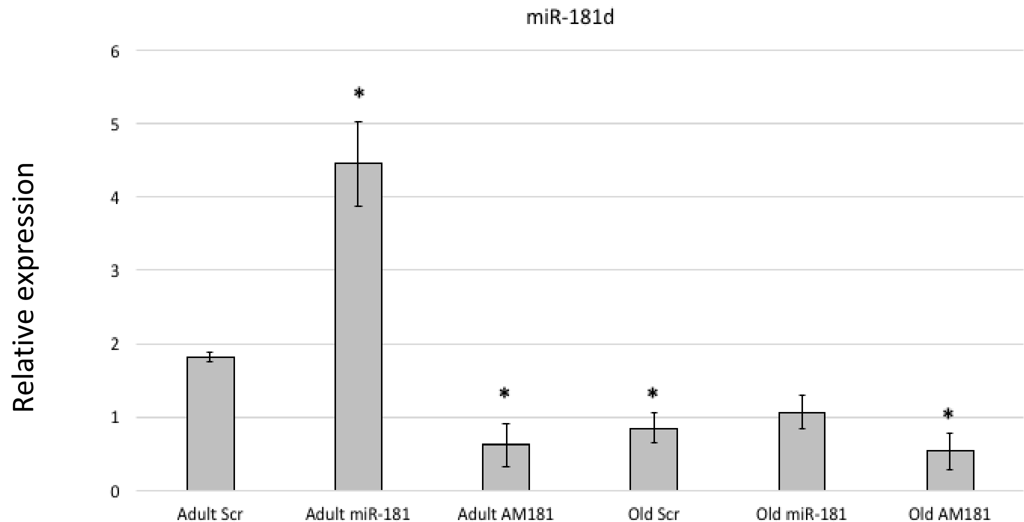


Figure 6.2. The expression of miR-181d in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr). Expression relative to Rnu-6. Error bars show SEM; n=3-4 per experimental group. * - $p<0.05$ as compared to Adult Scr NC; * - $p<0.05$ as compared Old Scr; One-Way Anova.

To establish the effects of miR-181 on muscle size, muscle mass from adult and old mice was measured three weeks after the first miR-181 mimic or antagomiR injection (Figure 6.3). The changes in the expression of miR-181a and miR-181d in the muscle from adult and old mice did not result in significant changes in the masses of *tibialis anterior*, *gastrocnemius*, *quadriceps* and *soleus* muscles (Figure 6.3). However, inhibition of miR-181a and miR-181d expression resulted in increased mass of the *extensor digitorium longus* (EDL) muscle from both adult and old mice (Figure 6.3). This could be due to differences in the expression levels of endogenous miR-181a/d in different types of mouse muscle or due to differences in the metabolism of different types of muscle depending or the fibre type

composition, mitochondria content or other endogenous molecular differences between the different muscle types. It is also possible that 3-week treatment of mice was not sufficient to observe significant changes in muscle mass.

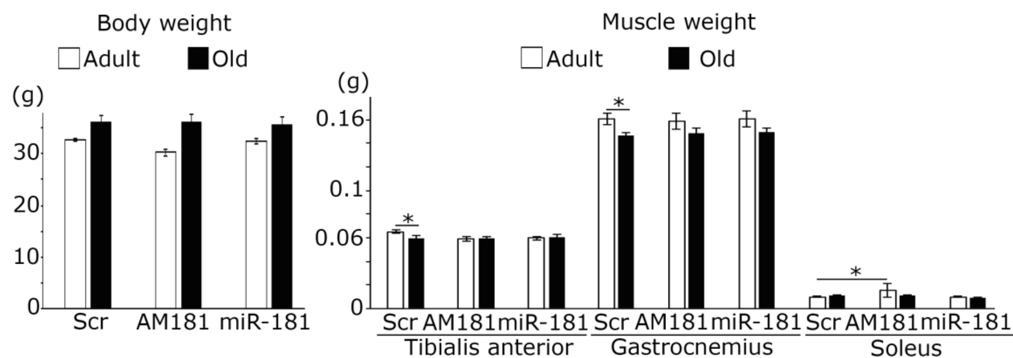


Figure 6.3. The effects of miR-181 overexpression and inhibition on body and muscle mass in adult and old mice. Adult (5 months old) and old (23 months old) mice were treated with antagomiR scrambled (Scr), miR-181a mimic (miR-181a) or antagomiR-181a (AM181) over 3 weeks. The weights of muscle are shown. Error bars show SEM; n=9-12; * - p<0.05 to Adult Scr; One-Way Anova.

In addition to muscle mass, myofibre size of the *tibialis anterior* muscle was also measured in the adult and old mice treated with miR-181 mimic or inhibitor. The cross-sectional area of the myofibres of the *tibialis anterior* muscle of adult and old mice was significantly affected by the treatment with miR-181 mimic or antagomiR-181 (Figure 6.4). Overexpression of miR-181a in the muscle of adult and old mice led to the presence of bigger myofibres, whereas inhibition of miR-181 expression led to the presence of smaller myofibres in the muscles of adult and old mice (Figure 6.4).

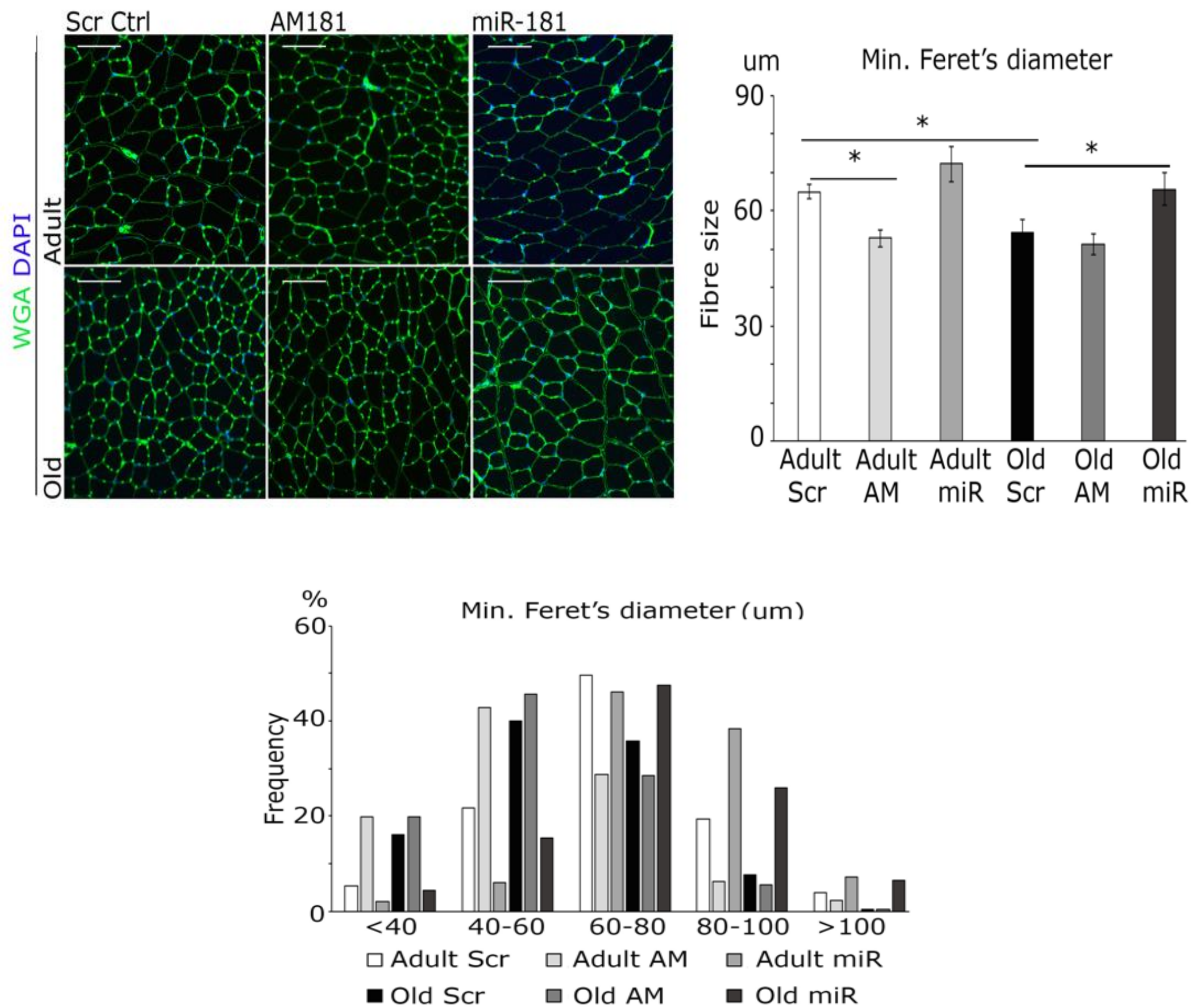


Figure 6.4. The effects of miR-181 overexpression and inhibition on *tibilais anterior* myofibre diameter (cross-sectional area) of *gastrocnemius* muscle of adult and old mice. Adult (5 months old) and old (23 months old) mice were treated with antagomiR scrambled (Scr), miR-181a mimic (miR-181a) or antagomiR-181a (AM181) over 3 weeks. The cross-sectional area of the myofibres is shown. Error bars show SEM; n=3.

6.3.2. The effects of miR-181 on muscle function *in vivo*.

Ageing is associated with loss of muscle mass and function. The data from the previous chapter show that 3-week treatment of mice does not have a significant effect on muscle mass in adult and old mice (Figure 6.3). However,

changes in miR-181 expression were associated with changes in myofibre size of adult and old mice (Figures 6.3., 6.4).

In order to establish the functional consequences of miR-181 expression levels, adult and old mice were treated with miR-181 mimic or antagomiR-181, respectively, for 5 weeks through two intravenous injections. Adult mice were treated with antagomiR-181a as miR-181 expression is downregulated druing ageing (Figure 5.1) Therefore, miR-181 overexpression in the muscle of adult mice was aimed at mimicking the changes occurring in the muscle of old mice. Old mice were treated with miR-181a mimic as overexpression of miR-181 has been demonstrated to increase myotube size *in vitro* (Figure 5.2). This treatment aimed at improving muscle function of old mice.

miR-181a and miR-181d expression in the *tibialis anterior* muscle was efficiently upregulated following the treatment of adult and old mice with miR-181 mimic and downregulated following the treatment of the mice with antagomiR-181 (Figure 6.5).

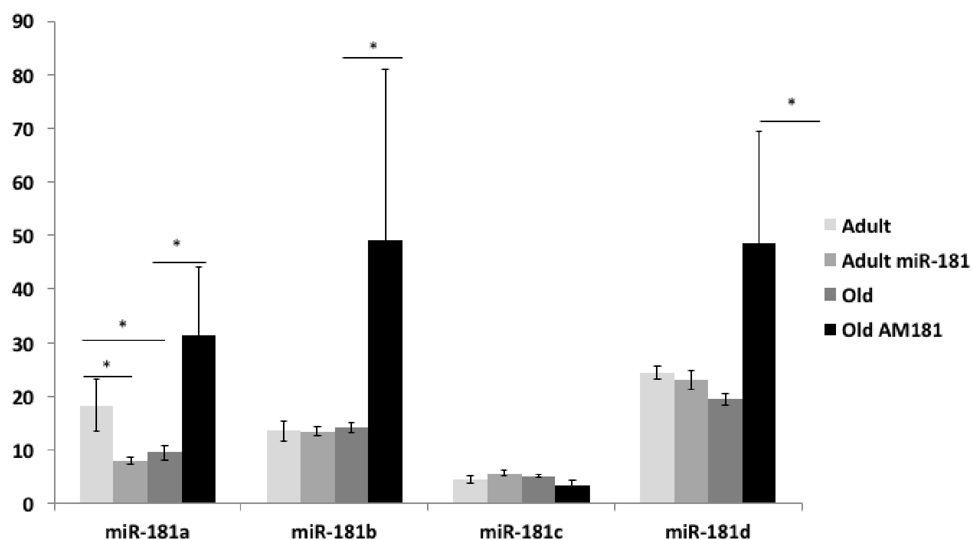


Figure 6.5. The expression of miR-181a and miR-181d in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr). Expression relative to Rnu-6. Error bars show SEM; n=4 (adult)-6 (old) per experimental group. * - p<0.05; One-Way Anova.

The upregulation of miR-181 expression in adult mice led to increased mass of *tibialis anterior*, *gastrocnemius*, *quadriceps*, *extensor digitorium longus* and *soleus* muscles of adult mice (Figure 6.6). The inhibition of miR-181 expression in the muscle of old mice had no significant effect on muscle mass (Figure 6.6). However, the adult mice treated with miR-181 mimic showed increased body weight, whereas old mice treated with AM181 inhibitor showed decreased body weight (Figure 6.6). The analysis of muscle mass normalised to body weight revealed no significant changes in the normalised muscle of adult and old mice following miR-181 mimic or antagomiR treatment, respectively (Figure 6.7).

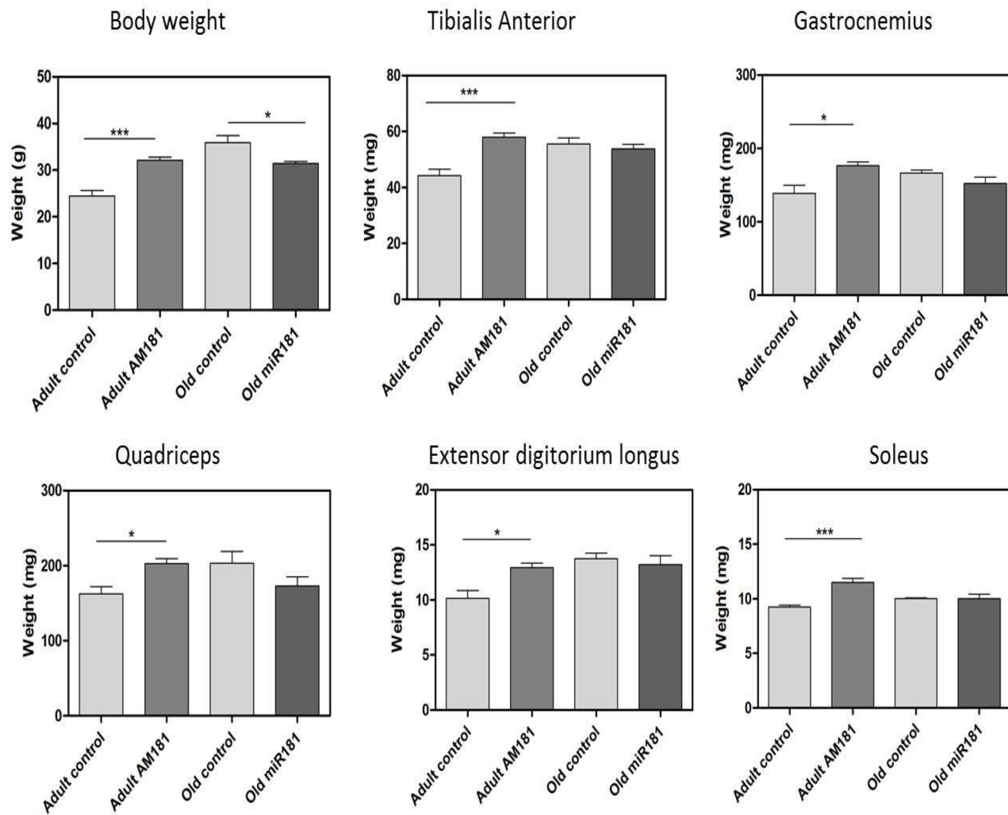


Figure 6.6. The effects of miR-181 overexpression and inhibition on muscle mass in adult and old mice. Adult (5 months old) and old (23 months old) mice were treated with antagomiR scrambled (Scr), miR-181a mimic (miR-181a) or antagomiR-181a (AM181) over 5 weeks. The weights of muscle are shown; n=4-6; error bars show SEM; * - $p<0.05$; One-Way Anova.

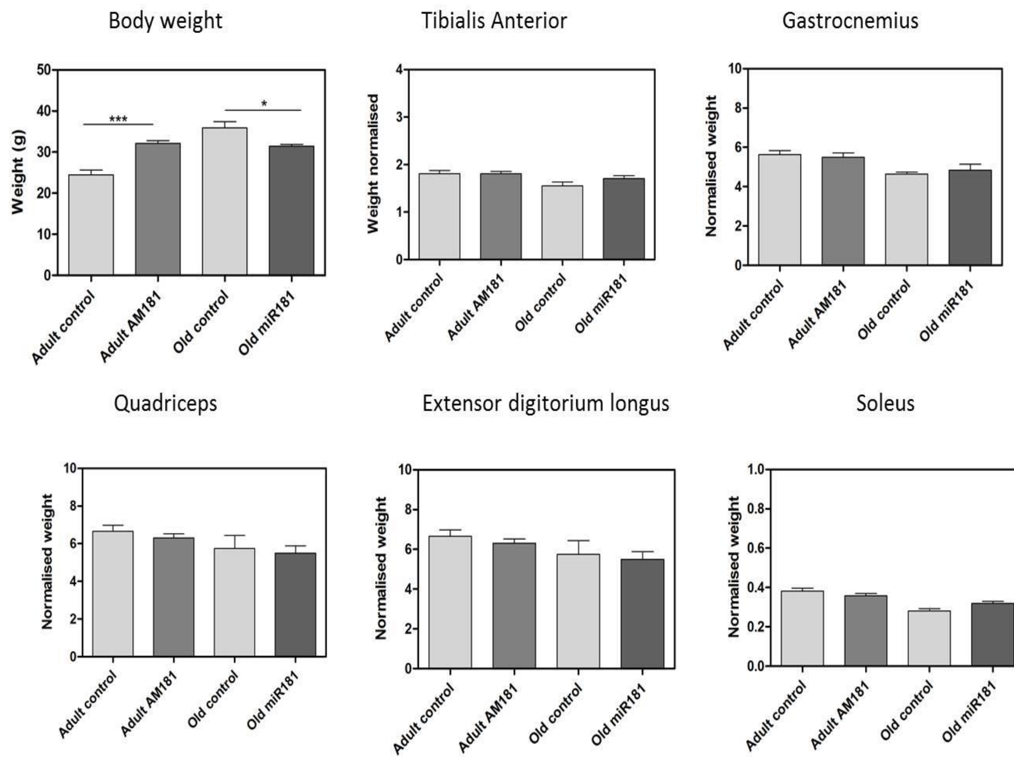


Figure 6.7. The effects of miR-181 overexpression and inhibition on muscle `mass in adult and old mice. Adult (5 months old) and old (23 months old) mice were treated with antagomiR scrambled (Scr), miR-181a mimic (miR-181a) or antagomiR-181a (AM181) over 5 weeks. The weights of muscle are shown; error bars show SEM; n=4-6; * - p<0.05; One-Way Anova.

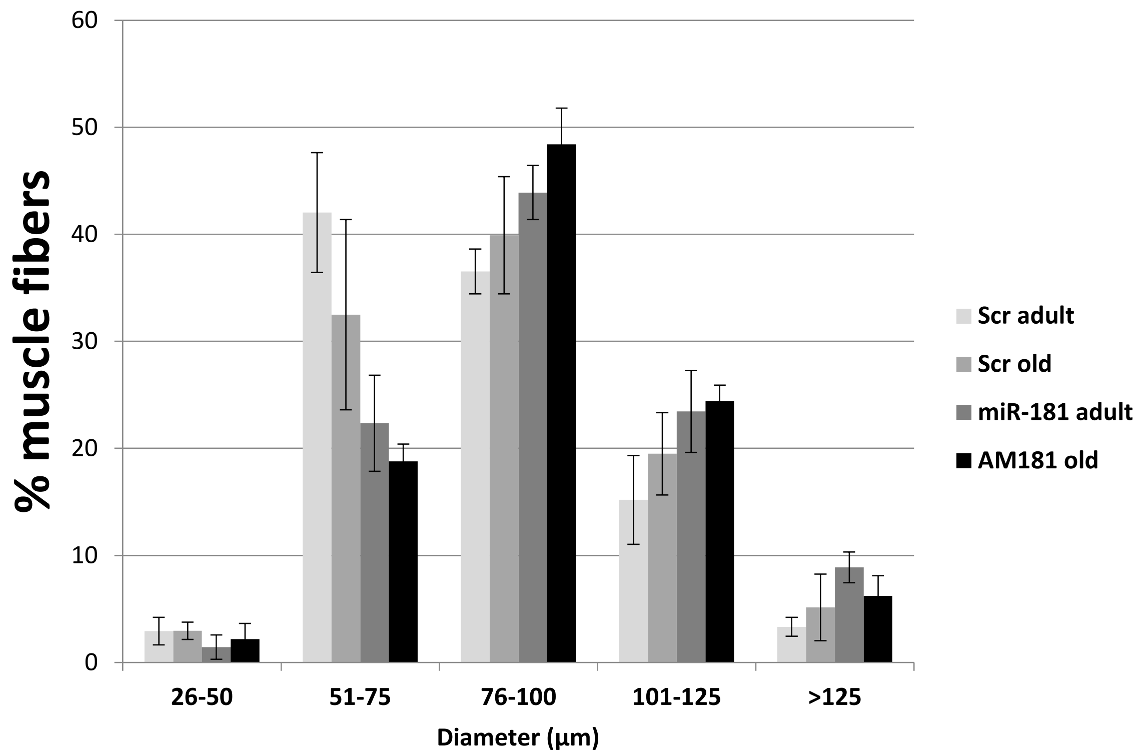


Figure 6.8. The effects of miR-181 overexpression and inhibition on *tibialis anterior* myofibre size distribution in adult and old mice. Adult (5 months old) and old (23 months old) mice were treated with antagomiR scrambled (Scr), miR-181a mimic (miR-181a) or antagomiR-181a (AM181) over 5 weeks. Minimum Feret's diameters of the tibialis anterior muscle are shown.

Changes in miR-181 expression in the *tibialis anterior* of adult and old mice led to changes in the distribution of the fibres according to their sizes (Figure 6.8). Overall, miR-181 inhibition in the *tibialis anterior* from adult mice led to the presence of fewer bigger myofibres as compared to control adult mice (Figure 6.8). The overexpression of mir-181 expression in the *tibialis anterior* of old muscle led to presence of higher number of bigger myofibres as compared to the muscle of control old mice (Figure 6.9).

In summary, data shown in Figures 6.1-6.8 demonstrate that miR-181 has limited effects on muscle mass; however it regulates the myofibre size of the muscle of adult and old mice (Figures 6.1-6.9). However, the effect of miR-181 on myofibre size is not as predicted from *in vitro* data (Figures 5.1, 5.2. and (Soriano-Arroquia, House, et al., 2016)) suggesting that miR-181 effects on muscle may be associated with surrounding tissues as well.

In order to establish the effects of miR-181 on muscle strength, the maximum and specific force of the EDL muscle of adult and old mice were measured following 5-week treatment of the mice with miR-181 mimic or inhibitor. miR-181 overexpression in the EDL muscle of adult mice led to decreased maximum force generation by the EDL as compared to the EDL muscle from adult mice treated with antagomiR scrambled, however this was not significant (Figure 6.9). Decreased levels of miR-181 in muscle of adult mice led to significant decrease in the maximum and specific force of the EDL muscle as compared to the muscle of adult mice treated with antagomiR scrambled (Figure 6.9). Both maximum and specific force of the EDL muscle of old mice treated with miR-181 were mimic were increased as compared to the EDL muscle of old mice treated with antagomiR scrambled (Figure 6.10, 6.11).

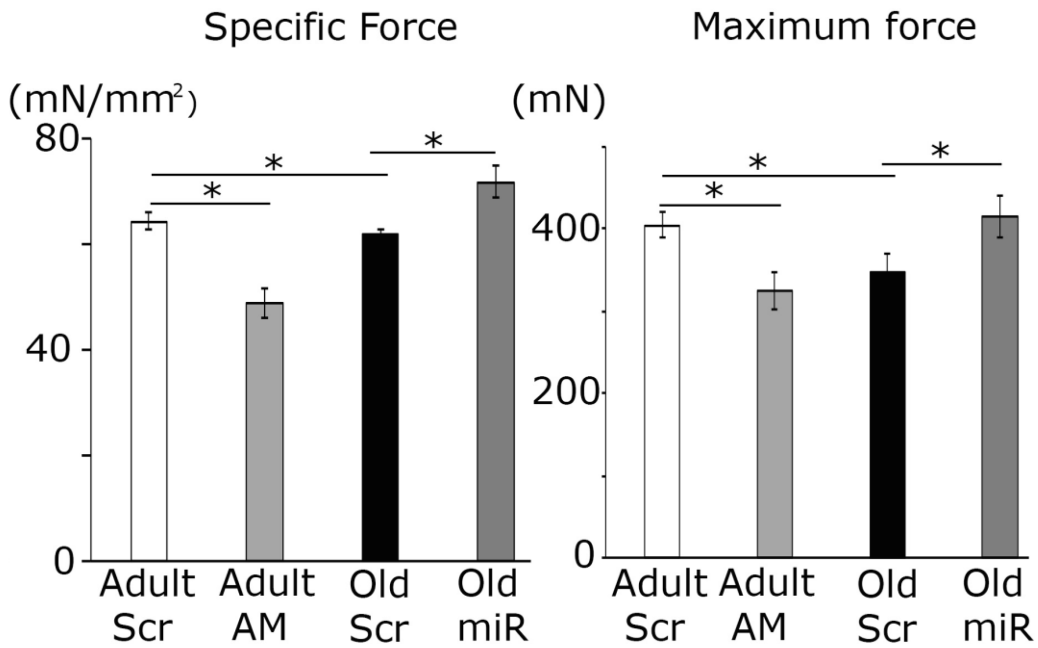


Figure 6.9. The effects of miR-181 overexpression and inhibition on EDL maximum and specific force generation in adult and old mice. Adult (5 months old) and old (23 months old) mice were treated with antagomiR scrambled (Scr), miR-181a mimic (miR-181a) or antagomiR-181a (AM181) over 5 weeks. The maximum force of the *extensor digitorium longus* muscle is shown, n=4 (adult)-6 (old). *<0.05 by Student T-test.

6.3.3. The effects of miR-181 on muscle response to contractions during ageing: miR-181 target genes validation *in vivo*

Data from previous chapters (5.3.1, 5.3.2) show that despite miR-181 having little to no effect on muscle mass *in vivo*, miR-181 may regulate myofibre size and miR-181 upregulation leads to decreased specific force of the EDL muscle of adult mice (Figure 6.11). In order to establish the mechanisms of miR-181 effects on muscle strength, miR-181 target genes were next validated *in vivo*. miR-181 levels were up- and downregulated in the quiescent and contracted muscle of adult and old mice. Briefly, adult and old mice were treated with miR-181 mimic or inhibitor. 3 weeks from the treatment, muscles of hind limbs of adult and old mice

were dissected or subjected to 15-minute isometric contractions protocol, as described in Chapter 2.4.

The expression of miR-181a and miR-181d in the muscle of adult mice was upregulated immediately after the 15-minute isometric contractions protocol and was restored to the levels of non-contracted muscle by 4 hours (Figures 6.12, 6.13). The expression of miR-181a and miR-181d in the muscle of old mice was not changed immediately after the contraction protocol, however it was significantly upregulated 4 hours after the contractions (Figures 6.12, 6.13). Moreover, the treatment of adult and old mice with miR-181 mimic or anatgomiR-181 led to increased or decreased levels of miR-181 in the quiescent and contracted *tibialis anterior* muscle, respectively, as compared to the control mice (Figures 6.12, 6.13).

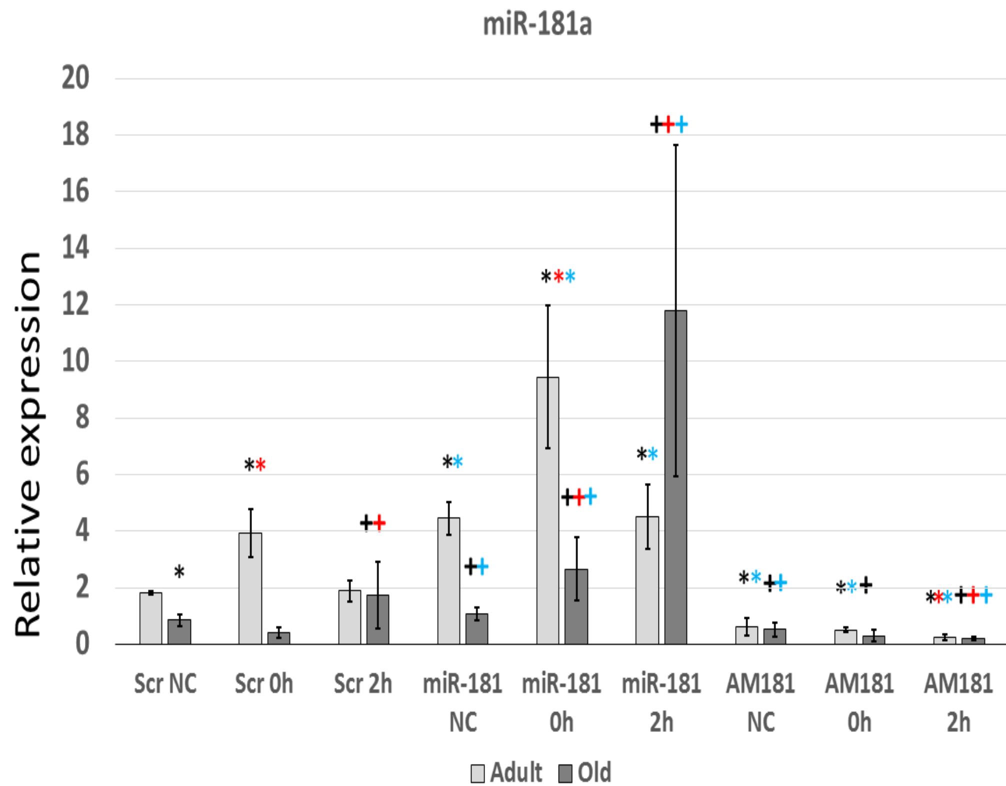


Figure 6.10. The expression of miR-181a in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. NC –not contracted, 0h – 15 minute contraction protocol followed by samples collection immediately after contractions, 2h- 15 minute contraction protocol followed by samples collection on average 2h after contractions. Expression relative to Rnu-6 is shown. N=3-4 per experimental group. * - $p<0.05$ as compared to Adult Scr NC; * - $p<0.05$ as compared the appropriate Adult treatment (Scr NC, miR NC or AM NC); * - $p<0.05$ as compared to the appropriate Old Scr time point (NC, 0h or 4h); + - $p<0.05$ as compared to Old Scr NC; + - $p<0.05$ as compared the appropriate Old treatment (Scr NC, miR NC or AM NC); + - $p<0.05$ as compared to the appropriate Old Scr time point (NC, 0h or 4h); One-Way Anova.

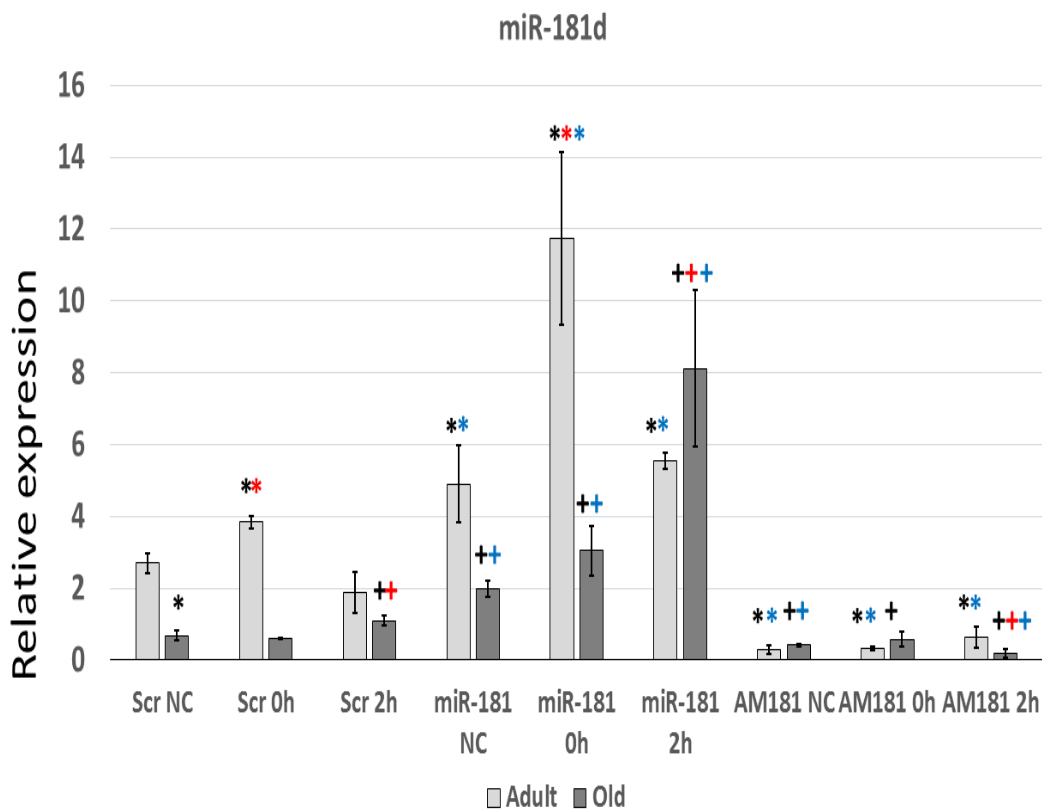


Figure 6.11. The expression of miR-181d in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. NC –not contracted, 0h – 15-minute contraction protocol followed by samples collection immediately after contractions, 2h- 15 minute contraction protocol followed by samples collection on average 2h after contractions. Expression relative to Rnu-6 is shown. N=3-4 per experimental group. * - p<0.05 as compared to Adult Scr NC; * - p<0.05 as compared the appropriate Adult treatment (Scr NC, miR NC or AM NC); * - p<0.05 as compared to the appropriate Old Scr time point (NC, 0h or 4h); + - p<0.05 as compared to Old Scr NC; + - p<0.05 as compared the appropriate Old treatment (Scr NC, miR NC or AM NC); + - p<0.05 as compared to the appropriate Old Scr time point (NC, 0h or 4h); One-Way Anova.

The expression of miR-181 putative target genes was first analysed by qPCR in the quiescent and contracted *tibialis anterior* muscle from adult and old mice following 3-week treatment with miR-181 mimic or antagomiR. The effects of the isometric contraction protocol and miR-181 mimic or antagomiR treatment on the expression of Pten, JunD, Fos and Fosb in the muscle of adult and old mice were

largely inconsistent with the relevant changes in miR-181a and miR-181d expression in the muscle of adult and old mice following the contraction protocol and miR-181 mimic and antagomiR treatment (Figures 6.15-6.18). Moreover, the expression of these genes was downregulated in the muscle of mice during ageing, similarly to the expression of miR-181a and miR-181d (Figures 6.11, 6,12, 6.15-6.19). These data is consistent with the data presented in the previous chapter (Figures 5.6, 5.7, 5.8, 5.9) and suggest that Pten, JunD, Fos and Fosb are not miR-181 target genes. Nevertheless, these genes are regulated by the contractions of the muscle of adult and/or old mice and may be a part of miR-181-independent mechanisms of muscle response to contractions, as described previously (Soriano-Arroquia et al., 2016).

The expression of Sirt-1 mRNA and SIRT-1 protein was regulated by the contraction protocol and changes in miR-181 levels in the *tibialis anterior* muscle of adult and old mice (Figures 6.14, 6.19). Both mRNA and protein expression of SIRT-1 were upregulated in quiescent muscle of old mice as compared to adult mice (Figures 6.14, 6.19). Moreover, overexpression of miR-181 expression led to downregulation of Sirt-1 mRNA and protein expression in quiescent and contracted muscle of adult and old mice (Figures 6.14, 6.19). Consistent with this, inhibition of miR-181 led to upregulation of Sirt-1 mRNA and protein expression in quiescent and contracted muscle of adult and old mice (Figures 6.14, 6.19).

The expression of Sirt-1 mRNA and protein was negatively correlated to that of miR-181a and miR-181d in quiescent and contracted tibialis anterior muscle of adult and old mice, suggesting that Sirt-1 is in fact a miR-181 target gene (Figures

6.14, 6.19). This is consistent with data presented in Chapter 5 Figure 5.10 and data previously published validating Sirt-1 as a direct target gene of miR-181 *in vitro* (Soriano-Arroquia et al., 2016)

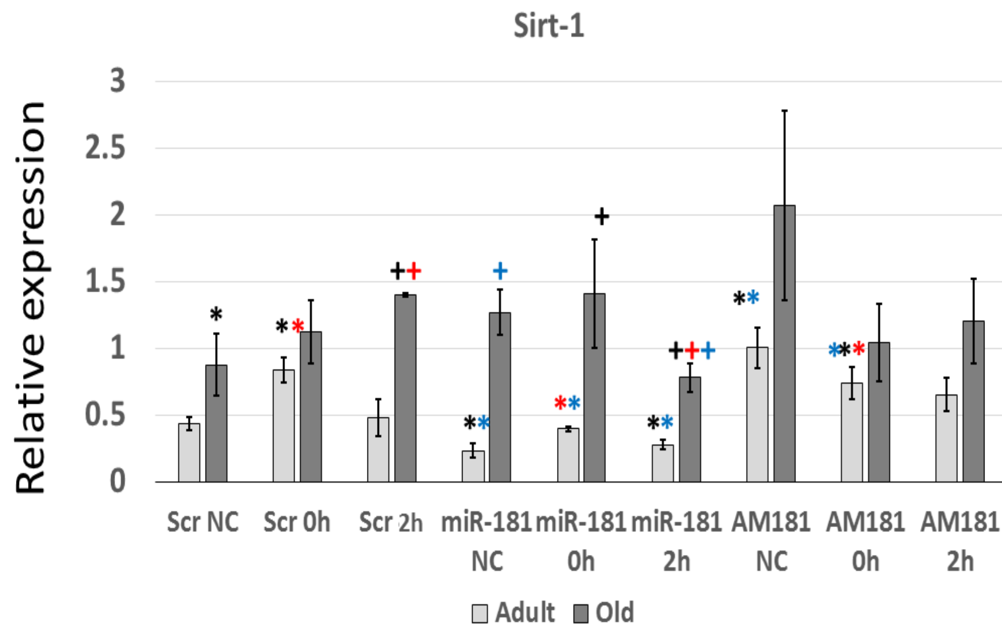


Figure 6.12. The expression of Sirt-1 in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. NC –not contracted, 0h – 15-minute contraction protocol followed by samples collection immediately after contractions, 2h- 15 minute contraction protocol followed by samples collection on average 2h after contractions. Expression relative to beta-2 microglobulin is shown. N=3-4 per experimental group. * - p<0.05 as compared to Adult Scr NC; * - p<0.05 as compared the appropriate Adult treatment (Scr NC, miR NC or AM NC); * - p<0.05 as compared to the appropriate Old Scr time point (NC, 0h or 4h); + - p<0.05 as compared to Old Scr NC; + - p<0.05 as compared the appropriate Old treatment (Scr NC, miR NC or AM NC); + - p<0.05 as compared to the appropriate Old Scr time point (NC, 0h or 4h); One-Way Anova.

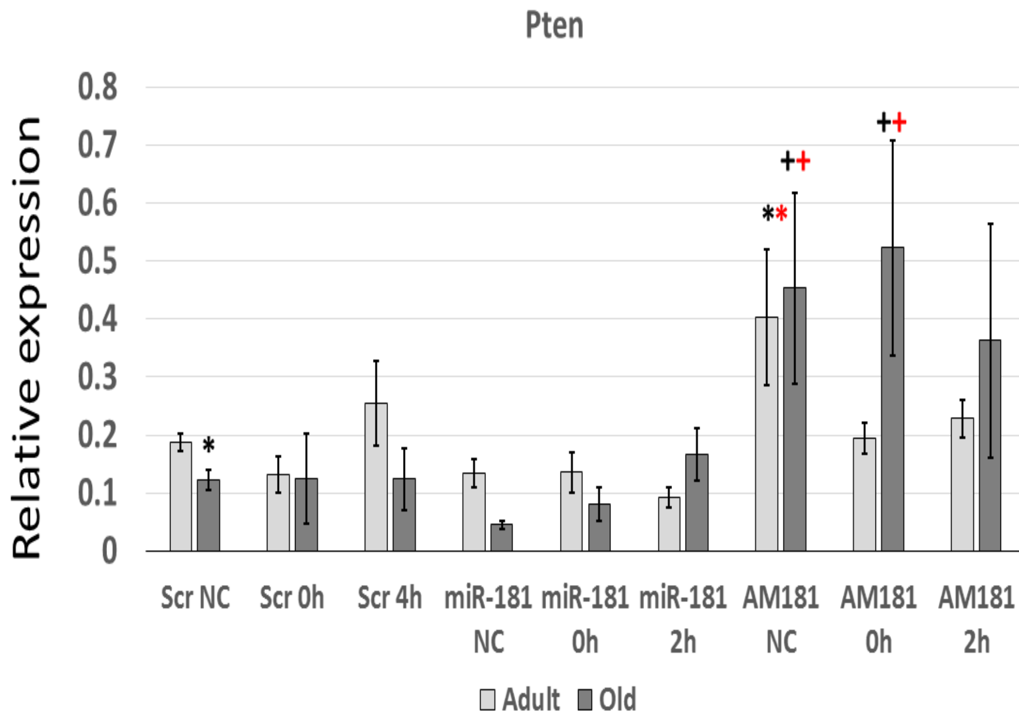


Figure 6.13. The expression of Pten in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. NC –not contracted, 0h – 15-minute contraction protocol followed by samples collection immediately after contractions, 2h- 15 minute contraction protocol followed by samples collection on average 2h after contractions. Expression relative to beta-2 microglobulin is shown. N=3-4 per experimental group. * - p<0.05 as compared to Adult Scr NC; * - p<0.05 as compared the appropriate Adult treatment (Scr NC, miR NC or AM NC); * - p<0.05 as compared to the appropriate Old Scr time point (NC, 0h or 4h); + - p<0.05 as compared to Old Scr NC; + - p<0.05 as compared the appropriate Old treatment (Scr NC, miR NC or AM NC); + - p<0.05 as compared to the appropriate Old Scr time point (NC, 0h or 4h); One-Way Anova.

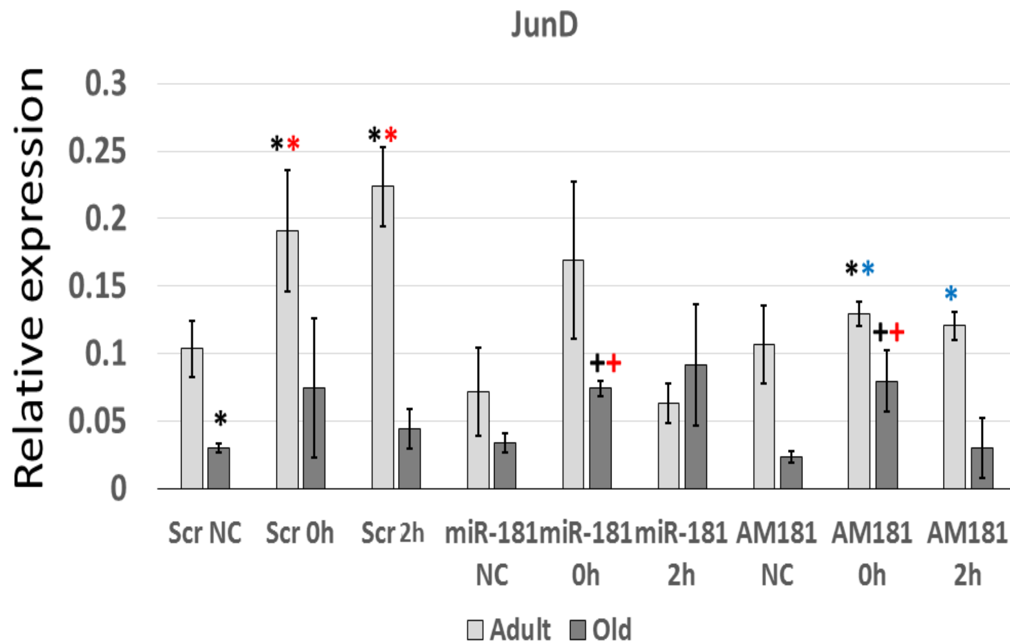


Figure 6.14. The expression of JunD in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. NC –not contracted, 0h – 15-minute contraction protocol followed by samples collection immediately after contractions, 2h- 15 minute contraction protocol followed by samples collection on average 2h after contractions. Expression relative to beta-2 microglobulin is shown. N=3-4 per experimental group. * - p<0.05 as compared to Adult Scr NC; * - p<0.05 as compared the appropriate Adult treatment (Scr NC, miR NC or AM NC); * - p<0.05 as compared to the appropriate Old Scr time point (NC, 0h or 4h); + - p<0.05 as compared to Old Scr NC; + - p<0.05 as compared the appropriate Old treatment (Scr NC, miR NC or AM NC); + - p<0.05 as compared to the appropriate Old Scr time point (NC, 0h or 4h); One-Way Anova.

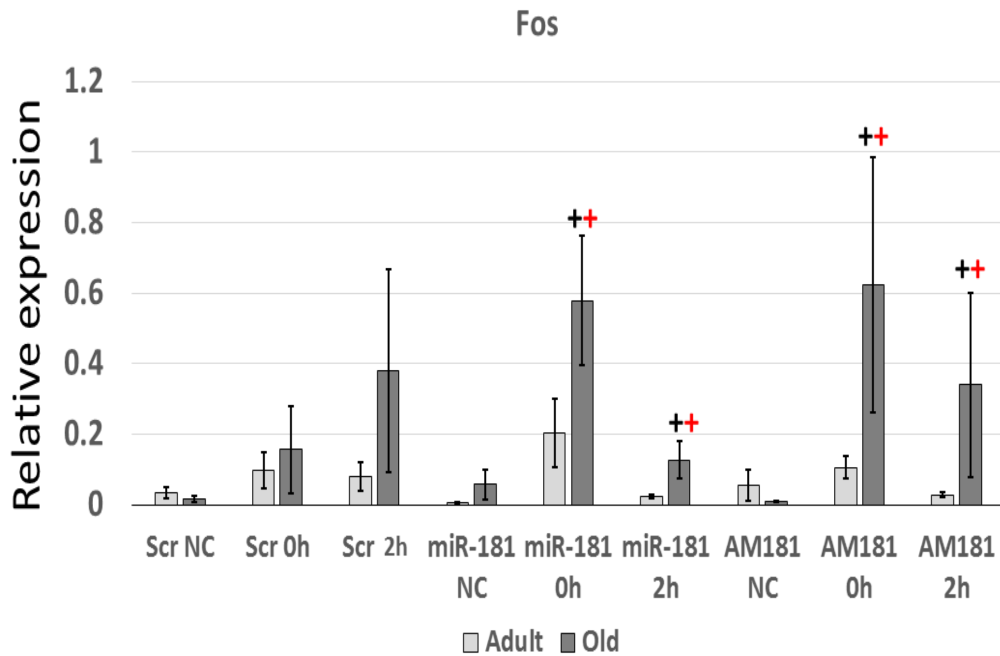


Figure 6.15. The expression of Fos in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. NC –not contracted, 0h – 15-minute contraction protocol followed by samples collection immediately after contractions, 2h- 15 minute contraction protocol followed by samples collection on average 2h after contractions. Expression relative to beta-2 microglobulin is shown. N=3-4 per experimental group. * - $p<0.05$ as compared to Adult Scr NC; * - $p<0.05$ as compared the appropriate Adult treatment (Scr NC, miR NC or AM NC); * - $p<0.05$ as compared to the appropriate Old Scr time point (NC, 0h or 4h); + - $p<0.05$ as compared to Old Scr NC; + - $p<0.05$ as compared the appropriate Old treatment (Scr NC, miR NC or AM NC); + - $p<0.05$ as compared to the appropriate Old Scr time point (NC, 0h or 4h); One-Way Anova.

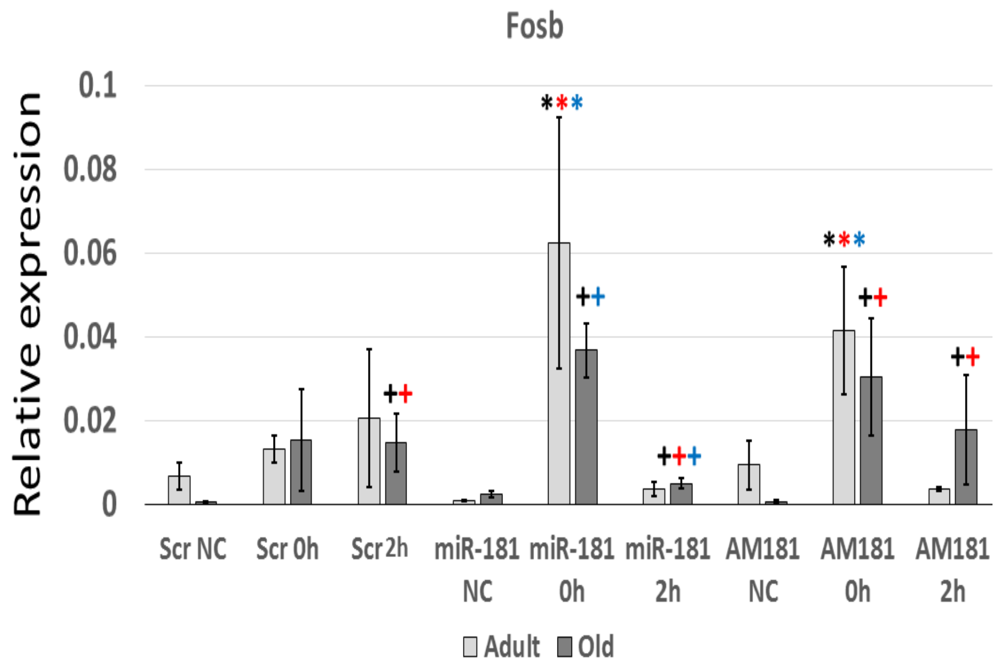


Figure 6.16. The expression of Fosb in the *tibialis anterior* muscle of adult (6 months old) and old (24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. NC –not contracted, 0h – 15-minute contraction protocol followed by samples collection immediately after contractions, 2h- 15 minute contraction protocol followed by samples collection on average 2h after contractions. Expression relative to beta-2 microglobulin is shown. N=3-4 per experimental group. * - p<0.05 as compared to Adult Scr NC; * - p<0.05 as compared the appropriate Adult treatment (Scr NC, miR NC or AM NC); * - p<0.05 as compared to the appropriate Old Scr time point (NC, 0h or 4h); + - p<0.05 as compared to Old Scr NC; + - p<0.05 as compared the appropriate Old treatment (Scr NC, miR NC or AM NC); + - p<0.05 as compared to the appropriate Old Scr time point (NC, 0h or 4h); One-Way Anova.

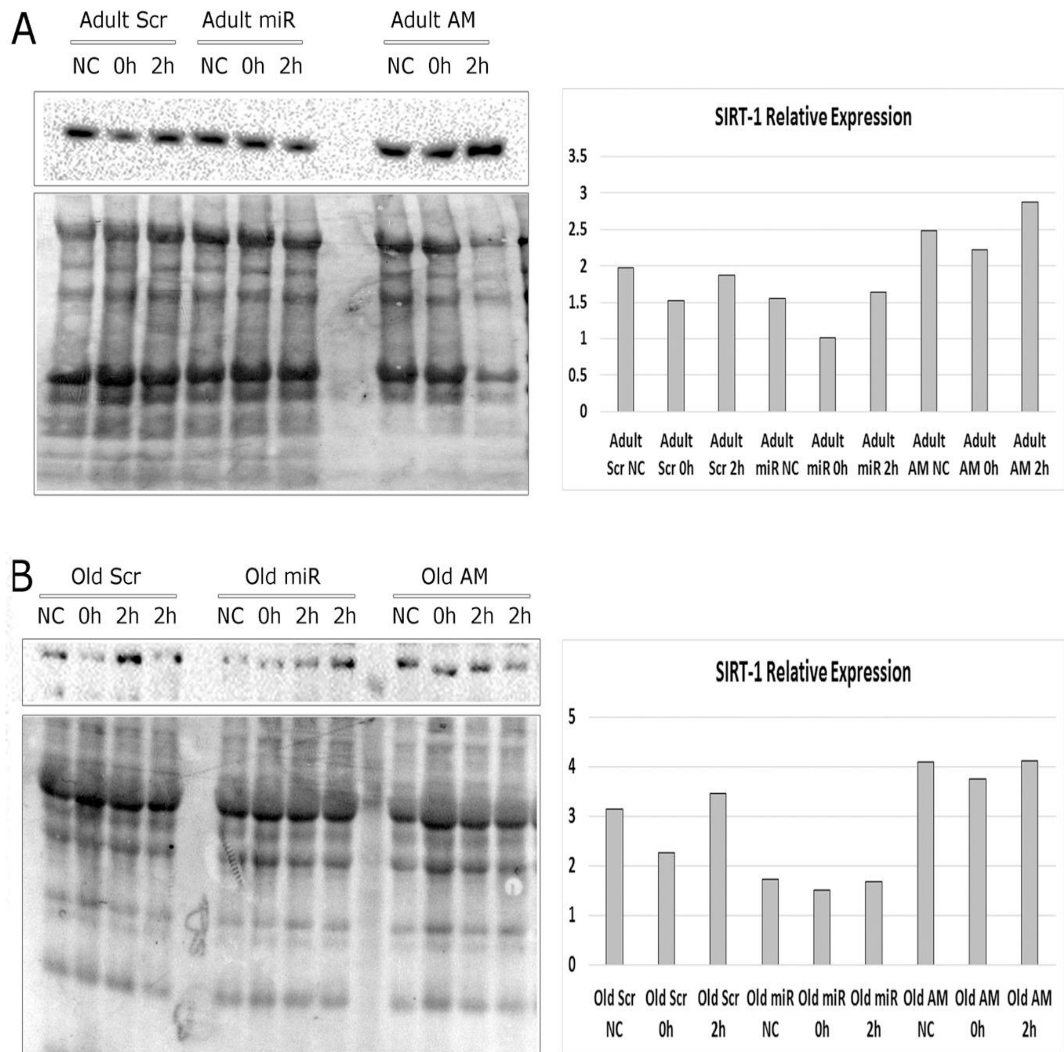


Figure 6.17. The expression of SIRT-1 protein in the *tibialis anterior* muscle of adult (A - 6 months old) and old (B - 24 months old) mice following a treatment with: miR-181 mimic (miR-181), antagomiR-181 (AM181) or antagomiR scrambled (Scr) and following an isometric contraction protocol. NC –not contracted, 0h – 15-minute contraction protocol followed by samples collection immediately after contractions, 2h- 15-minute contraction protocol followed by samples collection on average 2h after contractions. Quantification of the representative western blots is shown.

6.4 Discussion

Our group has previously shown that miR-181 negatively affects the myotube size in C2C12 cells in vitro (Soriano-Arroquia et al., 2016) The data presented in Chapter 5 is consistent with the data previously reported – miR-181 negatively regulates H-2k^b myotube size (Figure 5.2) Our published data and data presented in Chapter 5 also demonstrate Sirt-1 as key miR-181 target gene (Soriano-Arroquia, House, et al., 2016)(Figure 5.1). Among other miR-181 putative target genes investigated during this project in vitro and in vivo were Pten, a negative regulator of muscle homeostasis (Yue et al., 2016) genes encoding for JUN and FOS proteins, which form the AP-1 complex shown to regulate muscle response to contractions (Vasilaki et al., 2006). The validation of these genes as miR-181 targets was inconclusive based on the in vitro data presented in Chapter 5 (Figures 5.1, 5.2). Nevertheless, these genes were not validated as miR-181 targets in vivo using microRNA-181 gain- and loss-of-function approaches.

Next, the effects of miR-181 on muscle mass and function were validated *in vivo*. Data show limited to no effects of miR-181 on muscle mass however significant regulation of myofibre size in adult and old mice (Figures 6.1 – 6.9). Moreover, inhibition of miR-181 expression in the muscle of adult mice led to decreased maximum and specific force of the EDL muscle of adult mice and overexpression of miR-181 led to increase in the EDL maximum specific force of muscle of old mice (Figure 6.11). This may be due to the role of miR-181 in regulating the molecular mechanisms of muscle response to contraction, the

neuromuscular interactions, mitochondrial content or myofibre type composition of the muscle.

The data presented in Chapters 5 and 6 (Figures 5.9, 6.14) clearly validates Sirt-1 as a miR-181 target gene *in vitro* and *in vivo*. These data suggest that changes in miR-181 expression in the muscle of mice during ageing and contractions may regulate muscle response to contractions via regulating Sirt-1 expression (Figure 6.14). Interestingly, SIRT-1 has been shown to regulate redox balance of the muscle – a key aspect of muscle response to contractions that is disrupted during ageing (Prozorovski et al., 2008) SIRT-1 has been proposed to regulate PGC-1α activity and mitochondrial biogenesis during muscle contractions (Gurd, 2011). The activity of SIRT-1 has also been shown to be regulated by ROS and to regulate redox balance of skeletal muscle (Akkafa et al., 2015; Lim et al., 2010). Finally, SIRT-1 upregulation has been shown to result in changes in mitochondrial content, myofibre type switch and improved muscle performance in endurance treadmill exercise in the mouse model of Duchenne Muscular Dystrophy (Chalkiadaki et all.,2014) Together; these data suggest that miR-181 may regulate muscle function via regulating the expression of SIRT-1. It remains to be established whether miR-181 regulates fibre type composition and mitochondrial content of muscle via SIRT-1.

7 Discussion

The muscle of aged mammals does not respond to changes in its environment during exercise as efficiently as the muscle from adult mammals (Close et al., 2005). Ageing is associated with loss of muscle mass and eventually function (Florida-James et al., 2016) (Brown & Goljanek-Whysall, 2015; Vasilaki, McArdle, et al., 2006). This is known as sarcopenia (Brown & Goljanek-Whysall, 2015). Sarcopenia leads to reduced quality of life of older people and is associated with frailty (Goljanek-Whysall et al., 2016) . Exercise has been proposed to reduce age-related muscular atrophy; however exercise may not have as beneficial effects on the muscle of older people as of the younger people due to the blunted adaptive response of skeletal muscle to contractions in older people (Drummond et al., 2011; Goljanek-Whysall et al., 2016).

Among mechanisms proposed to be underlying the age-related loss of muscle mass and function are disrupted redox homoeostasis, mitochondrial dysfunction, changes in the activity of transcription factors, as well as epigenetic changes (Goljanek-Whysall et al., 2016; McCarthy & Esser 2007). Specifically, the expression of microRNAs has been proposed to regulate some aspects of muscle ageing and muscle response to contractions (Drummond, 2010; Goljanek-Whysall et al., 2016; McCarthy, 2008) have also shown that changes in the expression of myomiR in muscle of humans following exercise is not the same between adult and older people (Drummond, 2010).

Based on the above, the aims of this work were:

1. To select the most promising candidate microRNAs involved in regulation of target genes related to muscle adaptation following contractions;

2. To validate novel microRNA: target interactions regulating response of muscle to contraction;

3. To characterise the function of validated microRNA: target interactions during skeletal muscle adaptive response following contractions using two models: *in vitro* H-$2k^b$ cells- mouse myoblasts cell line and *in vivo:* adult (6 months old) and old mice (24 months old).

The expression of selected microRNAs was first validated in the quiescent and contracted muscle of adult and old mice. The expression of several microRNAs was altered in the muscle of adult but not old mice following the isometric contraction protocol (Figures 3.1, 3.2, 3.3). These data suggest that these microRNAs, also dysregulated in the muscle during ageing, may be regulators of disrupted muscle response to contractions during ageing.

The expression of the selected microRNAs was next validated in *H-$2k^b$* cells during contractions to establish the *H-$2k^b$* myotubes, a previously proposed *in vitro* model for studying microRNA: target interactions during muscle contractions. The expression of most microRNAs in *H-$2k^b$* myotubes following isometric contraction protocol mimicked the effects of contractions on muscle of adult mice and *H-$2k^b$* myotubes were validated as an appropriate in *vitro* model to study the effects of isometric contractions on miRNA:target interactions (Figures 4.1-4.410. Table 4.1).

Finally, the data presented in Chapter 4 show that miR-181 regulates myotube size *in vitro* and this may be through regulating Sirt-1 expression (Figures 5.4- 5.10).

Finally, the function of miR-181 and its targeting Sirt-1 were validated *in vivo* in quiescent and contracted muscle of adult and old mice (Figures 6.14, 6.15, 6.16, 6.17, 6.18).

7.1. Changes in microRNA expression in contracted skeletal muscle

The ability for muscles to maintain balance or homeostasis is impaired during ageing and the loss of this balance is associated with loss of muscle mass and function. This is known as sarcopenia, and is associated with impaired balance and frailty (Brown & Goljanek-Whysall, 2015).

The changes in microRNA expression during exercise have been described in the literature before. Based on the published data and own preliminary data, we validated the expression of selected microRNAs in the muscle of quiescent and contracted muscle from adult and old mice. Our findings largely agree with other literature (Drummond et al., 2008; McCarthy & Esser, 2007; Safdar et al., 2009; Soriano-Arroquia et al., 2016).

Our data show that the expression of miR-181a, miR-181d, miR-199, miR-378 and miR-133a in muscle changes during exercise. We observed that that miRs-181a, 181d, 133, and miR-378 were downregulated in the old muscle of mice following contraction protocol. The expression of these microRNAs (Figures 3.1, 3.2, 3.3, 3.4, 3.5) were upregulated in the muscle of adult mice following contraction but was blunted in the muscle of old mice.

Based on the above and on data from our group showing the role of miR-181 in regulation of myotube size *in vitro* (Soriano-Arroquia et al., 2016), we

selected miR-181a and miR-181d for further investigation. These microRNAs were downregulated in muscle during ageing and upregulated in the muscle of adult, but not old mice, following isometric contraction protocol (Figures 3.1, 3.2).

7.2. Expression of microRNA in *H-2k^b* myotubes following isometric contraction protocol

H-2k^b myotubes were used as *in vitro* model to study the mechanisms of response of muscle to isometric contraction (Pattwell et al., 2004) The *H-2k^b* myotubes underwent 15-minute isometric contraction in 6 well plates and the non-contracted ones were used as control as described previously

Data shows that miR-181a, miR-181d, miR-133a, and miR-24 were upregulated immediately after contraction, similarly to changes observed in muscle of adult and old mice. Specifically, we have observed that the expression of miR-181a was upregulated in *H-2k^b* myotubes after contractions and returned to basal levels after eight hours. miR-181d also was upregulated after contraction and returned to basal levels at four time-point. This shows that miR-181a and miR-181d may be involved in regulation of the early response in muscle contraction. Moreover, our studies have shown that *H-2k^b* myotubes can be used in microRNA:target interactions profiling in place of the use of animals.

7.3 miR-181: target interactions in the regulation of *H-2k^b* myotubes to contractions

The role of miR-181 in regulating mytoube size was first validated in *H-2k^b* myotubes using gain- and loss-of-function approaches (Figure 5.2). Data was consistent with our previously published data using C2C12 cells showing the negative role of miR-181 on myotube size *in vitro* (Soriano-Arroquia et al., 2016). Whether miR-181 affects myotube atrophy was analysed by studying the expression of atrogenes: Atrogin-1 and MuRF-1 (Figure 5.3). The expression of atrogenes remained unchanged following manipulation of miR-181 expression in *H-2k^b* myotubes, therefore miR-181 putative target genes were selected based on their role in muscle hypertrophy, including muscle hypertrophic response to contractions.

Following selection of candidate miRNAs with potential role in regulating the response of muscle to contractions, predicted targets of miR-181a and miR-181d were studied *in vitro*. Among the target studies were SIRT-1, a deacetylase with known functions in muscle (Vasilaki et al., 2006). Members of the AP-1 protein complex shown to be a part of blunted muscle response to contractions during ageing (Vasilaki et al., 2006) and targets associated with cell senescence and muscle hypertrophy: p21 and Igf1r (O'Neill et al., 2015; Soriano-Arroquia et al., 2016) Results show that the expression of Jun, JunD, Fos, FosB, Pten, and Sirt-1 were upregulated in *H-2k^b* myotubes following isometric contractions, Sirt-1 expression was also validated on the protein level by western blot analysis of *H-2k^b* myotubes following contraction and after contraction (Figures 5.4, -5.10). SIRT-1 was

downregulated in *H-2k^b* myotubes following contraction protocol and this is in agreement with the expression of miR-181a and miR-181d in *H-2k^b* myotubes following contraction protocol (Figures 4.1, 4.2).

7.4 miR-181 role in muscle response to contractions and ageing *in vivo*

The effects of miR-181a and miR-181d on muscle mass and function in the adult and old mice were studied using miR-181 gain and loss-of-function. The overall data shows no significant effects of miR-181 on muscle mass both in adult and old mice treated with miR-181 and miR-181 antagomiR (Figures 6.3). However, miR-181 regulates myofibre size and muscle function (strength) in the muscle of adult and old mice (Figures 6.1, 6.2).

Following selection of candidate miRNAs with potential role in regulating the response of muscle to contractions, predicted targets of miR-181a and miR-181d were studied *in vitro*. Among the target studies were SIRT-1, a deacetylase with known functions in muscle (Mohamedet al., 2014; Soriano-Arroquia et al., 2016) members of the AP-1 protein complex shown to be a part of blunted muscle response to contractions during ageing (Vasilaki et al., 2006) and targets associated with cell senescence and muscle hypertrophy: p21 and Igf1r (Soriano-Arroquia et al., 2016). Our results show that the expression of Jun, JunD, Fos, FosB, Pten, and Sirt-1 were upregulated in *H-2k^b* myotubes following isometric contraction (Figures 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 5.10.).

I studied the effects of miR-181a and miR-181d on muscle mass in the adult and old mice using miR-181 gain and loss-of-function. The overall data shows

no significant effects on muscle mass both in adult and old mice treated with miR-181 and miR-181 antagomiR (Figures 6.1, 6.2).

The reduction in muscle mass and function in older people is due to loss of muscle fibres; loss of motor neurons is also associated with the loss of muscle fibres and other mechanisms, such as defective regeneration following injury (Goljanek-Whysall et al., 2016). Muscle atrophy has been proposed to be associated with reduction in number of myofibres (Deschenes, 2004; Koltai et al., 2010).

Data show that despite the overall lack of significance on muscle mass, a significant effect of miR-181 on the average myofibre size was observed (Figures 6.6, 6.7). Moreover, the distribution of the myofibre sizes was affected by overexpression or inhibition of miR-181 in the *tibialis anterior* muscle of adult and old mice (Figure 6.8).

Data shows the effects of miR-181 on maximum and specific force of the EDL muscle (Figure 6.10). Specifically, downregulation of miR-181 expression in the EDL muscle of adult mice led to reduction in the specific force as compared to control mice and upregulation of miR-181 expression in the muscle from old mice resulted in improved specific force (Figure 6.11).

The changes in the specific force of the EDL muscle may be explained by changes in myofibre size, fibre type and increase in muscle protein like the myosin and actin changes in the mitochondria. Sirt-1, miR-181 validated target gene, was shown to regulate the above. (Brown, 1987; Chalkiadaki et al., 2014; Cheng et al., 2016; Deschenes, 2004)

In order to decipher the mechanism of miR-181 effects on muscle force *in vivo*, microRNA target genes were validated. Sirt-1 was validated as miR-181 target gene (Figure 6.14). Sirt-1 is a member of the Sirtuin family and a NAD+-dependent protein deacetylase known to prevent anti-ageing and promote muscle hypertrophy and regulate autophagy. (Gurd 2011; Lee et al., 2013). Sirt-1 regulates myotube size (Figure 5.10) and muscle size and strength (Chalkiadaki et al., 2014; Koltai et al., 2010).

The expression of miR-181 is downregulated in muscle during ageing. Our data show that miR-181 downregulation in the muscle of old mice leads to increased levels of Sirt-1 mRNA and protein, as well as increased specific and maximum, force of the EDL muscle (Figures 6.6, 6.7). This suggests that changes in miR-181 expression may be a part of a mechanism underlying loss of muscle function during ageing. Further analyses are however required in order to establish whether earlier or longer interventions using miR-181 loss-of-function approaches may lead to more effective regulation of muscle mass and function during ageing. Moreover, it is essential to establish how miR-181:Sirt-1 interactions regulate myofibre size and muscle function – whether by hypertrophy or autophagy pathway, both processes previously shown to be regulated by Sirt-1.(Pardo & Boriek, 2011)

7.5 Future perspectives

7.5.1. Potential of microRNAs as future biomarkers

microRNAs are considered novel post-transcriptional regulators of gene expression. Due to their small size and straight-forward detection, as well as presence in bodily fluids (blood, urine) and their levels changing in the tissues and bodily fluids during various disease cinditions, microRNAs are thought as promising biomarkers (Goljanek-Whysall et al., 2012)

Several microRNAs have been identified as potential serum biomarkers of ten muscle diseases, for example Duchenne muscular dystrophy (DMD) (Eisenberg et al., 2009). The common test, creatine kinase blood levels, is often used to detect muscle damage. Eisenberg and colleagues have shown that the levels of miR-133, miR-1 and miR-206, which are muscle specific microRNAs, were increased in patients with DMD (Goljanek-Whysall et al., 2012). Other circulating (present in the serum or plasma) microRNAs, such as miR-378 and miR-31, have also been found to be associated with muscular dystrophy in humans (Aoi, 2014). However, as much microRNA, such as miR-378 or miR-31, are expressed in many different tissue types, not only muscle, attention must be paid to the potential use of microRNAs as biomarkers. Therefore, muscle-specific microRNAs are attractive in this field of research and may be used as potential biomarkers for the diagnosis of muscular dystrophy.

7.5.2 microRNA as Therapeutics

MicroRNAs have recently been considered potential therapeutics for various disorders, including muscle disorders, due to their small size and effective delivery. Indeed, several microRNA-based therapeutics has been patented and clinical trials are ongoing (Goljanek-Whysall et al., 2012).

microRNA expression can be upregulated using microRNA mimics or downregulated using antagomiRs (Krutzfeldt et al., 2005). For muscle diseases, microRNA mimics or antagomiRs could be delivered into muscle via intramuscular(not practical) or intravenous injection. Intravenous injection, even though more efficient, could potentially result in delivery of microRNA mimics/antagomiRs into other than muscle tissues. Moreover, as microRNAs regulate the expression of many genes, off-target effects have to be defined before microRNA mimics/antagomoR can be considered a potential therapeutic. Nevertheless, several microRNa has been shown a potential to aid muscle repair or maintain muscle mass.

For example, in a rat skeletal muscle injury model, it was found that a local injection of miR-1, miR-133, miR-206 mimics accelerated muscle regeneration (Zhang et al., 2016).

Moreover, in DMD patients, an intra-muscular injection of stabilised miR-486 together with exon-skipping morpholinos was proposed to restore muscle function and prevent the loss of m8uscle in a DMD patient (Goljanek-Whysall et al., 2012)

To summarise, even though in early days, microRNAs may hold a potential as non-invasive biomarkers and/or potential therapeutics against various disorders, including loss of muscle mass and function during ageing. However, more research is needed to functionally characterise microRNAs in muscle and to define the effects of microRNA level manipulation on the whole body level.

8 Appendix

8.1 microRNA-181a, b, c, d sequences

hsa-mir-181a-2 MI0000269
AGAAGGGCUAUCAGGCCAGCCUUCAGAGGACUCCAAGGAACAUUCAA
CGCUGUCGGUGAGUUUGGGAUUUGAAAAAACCACUGACCGUUGACU
GUACCUUGGGGUCCUUA

mmu-mir-181a-2 MI0000223
CCAUGGAACAUUCAACGCUGUCGGUGAGUUUGGGAUUCAAAAACAAA
AAAACCACCGACCGUUGACUGUACCUUGG

>hsa-mir-181b-1 MI0000270
CCUGUGCAGAGAUUAUUUUUUAAAAGGUCACAAUCAACAUUCAUUG
CUGUCGGUGGGUUGAACUGUGUGGACAAGCUCACUGAACAAUGAAU
GCAACUGUGGCCCCGCUU

>mmu-mir-181b-1 MI0000723
AGGUCACAAUCAACAUUCAUUGCUGUCGGUGGGUUGAACUGUGUAG
AAAAGCUCACUGAACAAUGAAUGCAACUGUGGCC

hsa-mir-181c MI0000271
CGGAAAAUUUGCCAAGGGUUUGGGGGAACAUUCAACCUGUCGGUGA
GUUUGGGCAGCUCAGGCAAACCAUCGACCGUUGAGUGGACCCUGAGG
CCUGGAAUUGCCAUCCU

mmu-mir-181c MI0000724
GCCAAGGGUUUGGGGGAACAUUCAACCUGUCGGUGAGUUUGGGCAG
CUCAGACAAACCAUCGACCGUUGAGUGGACCCCGAGGCCUGGA

>hsa-mir-181d MI0003139
GUCCCCUCCCCUAGGCCACAGCCGAGGUCACAAUCAACAUUCAUUGUU
GUCGGUGGGUUGUGAGGACUGAGGCCAGACCCACCGGGGGAUGAAU
GUCACUGUGGCUGGGCCAGACACGGCUUAAGGGGAAUGGGGAC

mmu-mir-181d MI0005450
ACAAUUAACAUUCAUUGUUGUCGGUGGGUUGUGAGGAGGCAGCCAG
ACCCACCGGGGGAUGAAUGUCACUGU

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