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Regulation of Skeletal Muscle Metabolism and Development by Small, Non-Coding RNAs:
Implications for Insulin Resistance and Type 2 Diabetes Mellitus

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Implications for Insulin Resistance and Type 2 Diabetes Mellitus

THESIS FOR DOCTORAL DEGREE (Ph.D.)

by

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ABSTRACT

microRNAs (miRNAs) are a class of epigenetic post-transcriptional regulators. These short (~22 nucleotides) non-coding RNAs can potently reduce protein abundance through direction of the RNA-induced silencing complex to targeted genes. Ultimately, miRNAs will reduce protein levels of target genes through mechanisms involving either inhibition of protein translation or destabilization and cleavage of target gene mRNA molecules.

miRNAs are implicated in the regulation of several cellular processes, including growth, differentiation, and metabolism. The expression of miRNAs is altered in several clinical conditions including cancer, obesity, and diabetes. In this thesis, the aim was to elucidate how miRNAs are regulated in human skeletal muscle during three conditions, including *in vitro* skeletal muscle development, in type 2 diabetic patients, and following endurance exercise training. After identifying miRNAs that have altered expression in the aforementioned conditions, an additional aim was to characterize the functional effects of these miRNAs, including effects on target gene abundance and regulation of cellular metabolism.

In **Study I**, miRNA expression was determined during human skeletal muscle cell differentiation with 48-hour resolution. Transcriptomic miRNA expression profiles of proliferative and differentiated cells were overlapped with gene expression alterations to identify reciprocal miRNA-mRNA expression patterns. Using this approach, miRNA-centered regulatory networks involved in *in vitro* human skeletal muscle development were predicted. miR-30b and miR-30c were among those differentially regulated miRNAs for which regulatory networks were modelled.

In **Study II**, increased expression of miR-29a and miR-29c was identified in skeletal muscle from type 2 diabetic patients. Specifically, these miRNAs were expressed to a greater extent in type 2 diabetes. Thereafter these miRNAs were identified to induce insulin resistance and disturbances in glucose metabolism in human and mouse skeletal muscle. Several genes implicated in regulation of glucose and lipid metabolism were identified to be sensitive to altered miR-29 expression, including hexokinase 2.

In **Study III**, miR-19b-3p and miR-107 were found to be induced in human skeletal muscle following 14 consecutive days of endurance exercise. The roles of these two miRNAs in the regulation of mRNA abundance and metabolic traits associated with skeletal muscle adaptation to endurance exercise training were determined. miR-19b-3p and miR-107 were identified to potently increase insulin sensitivity and glucose metabolism in human skeletal muscle cells, whereas functions of miR-19b-3p were also conserved *in vivo* and in *in vitro* models of mouse skeletal muscle.

Together, these studies highlight roles of skeletal muscle miRNAs in post-transcriptional regulation of gene expression and modifications of insulin sensitivity in conditions such as type 2 diabetes and following endurance training.

LIST OF SCIENTIFIC PAPERS

I. **Sjögren RJ**, Egan B, Katayama M, Zierath JR, Krook A. *Temporal analysis of reciprocal miRNA-mRNA expression patterns predicts regulatory networks during differentiation in human skeletal muscle cells.* Physiol Genomics. 2015; 47(3):45-57.

II. Massart J, **Sjögren RJ**, Lundell LS, Mudry JM, Franck N, O'Gorman DJ, Egan B, Zierath JR, Krook A. *Altered miR-29 Expression in Type 2 Diabetes Influences Glucose and Lipid Metabolism in Skeletal Muscle.* Diabetes. 2017; 66(7):1807-1818.

III. **Sjögren RJ**, Massart J, Egan B, Garde C, Gu W, Lindgren M, Barrés R, O'Gorman DJ, Zierath JR, Krook A. *Endurance exercise training-responsive miR-19b-3p and miR-107 improve glucose metabolism and insulin signaling in human skeletal muscle*. Unpublished, in manuscript.

CONTENTS

LIST OF ABBREVIATIONS

AGO	Argonaut
AKT2	AKT Serine/Threonine Kinase 2
BMI	Body mass index
CAV3	Caveolin 3
CLIP	Crosslinking and immunoprecipitation
CLIP4	CAP-Gly Domain Containing Linker Protein Family Member 4
E2F7	E2F Transcription Factor 7
EDL	*Extensor digitorum longus*
FAM98A	FAM98A
FDB	*Flexor digitorum brevis*
GIP	Gastric inhibitory protein
GLP1	Glucagon-like peptide 1
GLUT1	Glucose Transporter Type 1
GLUT4	Glucose Transporter Type 4
GSK3	Glycogen synthase kinase 3
HbA1c	Glycated hemoglobin
HBP1	HMG-Box Transcription Factor 1
HDL	High-density lipoprotein
HEK293	Human embryonic kidney cells
HEYL	Hes Related Family BHLH Transcription Factor With YRPW Motif-Like
HGP	Hepatic glucose production
HITS	High-throughput sequencing
HK2	Hexokinase 2
IRS	Insulin receptor substrate
KIF13A	Kinesin Family Member 13A
LDL	Low-density lipoprotein
MAPK6	Mitogen-Activated Protein Kinase 6
miRNA	microRNA
MRF	Myogenic regulatory factor
MYF5	Myogenic factor 5
MYH	Myosin heavy chain
MYOD	Myogenic differentiation

MYOG	Myogenin
ncRNA	Non-coding RNA
NGT	Normal glucose tolerance
PAX3, PAX7	Paired Box 3, Paired box 7
PFKFB3	6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PHLDA1	Pleckstrin Homology Like Domain Family A Member 1
PI3K	Phosphoinositide 3-kinase
PIK3R3	Phosphoinositide-3-Kinase Regulatory Subunit 3
PKB	Protein kinase B; Akt
PRDM1	PR/SET Domain 1
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
PTEN	Phosphatase And Tensin Homolog
RASAL2	RAS Protein Activator Like 2
RISC	RNA-induced silencing complex
RNA-seq	RNA sequencing
RNF11	Ring Finger Protein 11
RT-qPCR	Reverse transcription-quantitative PCR
siRNA	Small interfering RNA
SLAIN2	SLAIN Motif Family Member 2
SNAI1	Snail Family Transcriptional Repressor 1
T2D	Type 2 diabetes
TA	*Tibialis anterior*
TBC1D1	TBC1 domain family member 1
TBC1D4	TBC1 domain family member 4; Akt substrate of 160 kDa (AS160)
UTR	Untranslated region
VO_{2peak}	Peak oxygen uptake
VPS37A	VPS37A, ESCRT-I Subunit
WNT5A	Wnt Family Member 5A
ZBTB4	Zinc Finger And BTB Domain Containing 4
ZDHHC7	Zinc Finger DHHC-Type Containing 7

1 INTRODUCTION

1.1 REGULATION OF POST-TRANSCRIPTIONAL EVENTS BY NON-CODING RNAS

1.1.1 Transcriptional homeostasis and epigenetics

Homeostasis is the regulation and control of an organism's internal state in response to alterations in the external environment. Evolution has led to numerous systems that keep physiological processes under tight control to ensure survival of the organism. Homeostatic regulation is dynamic, keeping a system within a close range of a biological set point. For example, blood glucose fluctuates over the course of the day, but returns in the fasted state to ~5 mmol/L in healthy humans. Homeostatic mechanisms are observed at various scales in biology. There is complex crosstalk between different tissues and cell types in multicellular organisms. Within individual cells, examples of homeostatic control include maintenance of membrane lipid composition, progression through the cell cycle, substrate metabolism, RNA transcription, and protein translation.

The central dogma of molecular biology was postulated in the 1950s and describes the flow of information in biological systems [1]. The flow can simply be summarized as DNA is transcribed into RNA and then RNA is translated into protein. The tight control of this stepwise progression in the cell maintains the informational integrity in DNA while producing necessary proteins in response to homeostatic challenges. Transcription and translation of a gene into its functional unit is regulated at multiple levels. All diploid cells in an organism share a nearly identical DNA sequence. Nevertheless, there is a myriad of different cell types in the human body. This diversity in cell types arises due to differential expression of functional units: proteins and non-coding RNAs (ncRNAs). Also, the normal function of cells requires proper transcriptional responses to changes in the external environment. Several regulatory mechanisms function to sustain this diversity including epigenetic modifications, transcription factor activation, post-transcriptional processes, and various post-translational modifications.

Epigenetics (*epi-*, Greek prefix meaning "over, around") describes changes in gene function that do not involve alterations of the genetic sequence. Epigenetic control mechanisms include histone modifications, DNA methylation, and ncRNA. Histones are proteins which the DNA is coiled around to form nucleosomes, facilitating the packing of large DNA molecules into chromosomes [2]. Histone proteins ensure selective accessibility of functional units within DNA (e.g., enhancers, promoters, and open reading frames) for the transcriptional machinery. Histones can be modified by different post-translational modifications, including methylation, acetylation, and phosphorylation. These alterations regulate accessibility to the genome and create a so-called 'histone code' [3]. The DNA of a cell can also be methylated on cytosine nucleotides, specifically in CpG sites, creating 5-methylcytosine [2]. The location being methylated within the genome ultimately determines the functional effects of such modifications. For example, methylation of cytosine in gene

promoters causes recruitment of proteins, including histone modifying-proteins, which repress transcription [2]. Another epigenetic mechanism to regulate gene expression and translation is through ncRNAs. ncRNAs are a diverse family of RNA species that regulate gene expression, protein translation and genome stability.

1.1.2 Overview of non-coding RNAs

The human genome consists of ~3 billion base pairs and 62% of the DNA is transcribed into RNA molecules [4]. While only 1.5% of the DNA gives rise to protein coding exons, a large part of the transcribed RNA is of a non-coding nature. Whether all of these ncRNA molecules have functional potential remains undetermined. Nevertheless, several of the transcribed ncRNA are well characterized and known to be functional, including structural ncRNAs such as ribosomal RNA (rRNA) and transfer RNA (tRNA) that are necessary for protein synthesis. Thus, besides coding for proteins, a large part of the human DNA gives rise to functional ncRNA molecules.

Several different classes of ncRNAs exist and these can be divided into two groups based on their size: short (<200 nt) and long (≥200 nt) ncRNAs. Short ncRNAs can further be divided into multiple different subclasses, some of which are listed in **Table 1**. One such class of short ncRNAs is the focus of this thesis: microRNAs (miRNAs).

Table 1. Examples of short non-coding RNAs

Type of short ncRNA	Abbreviation	Length (nt)	Function
Small nuclear RNA	snRNA	~150	Splicing and maturation of pre-mRNAs [5]
Small nucleolar RNA	snoRNA	60–300*	Directs chemical modifications of RNA molecules [5]
Short hairpin RNA	shRNA	20–25	Artificial RNA molecules used to silence genes [6]
Small interfering RNA	siRNA	20–25	Post-transcriptional regulation and antiviral defense mechanism [5]
microRNA	miRNA	~22	Post-transcriptional regulation of gene expression [7]
Piwi-interacting RNA	piRNA	~30	Silencing of transposable elements and important for genome stability [5]

*Some snoRNAs are classified as long non-coding RNAs.

1.1.3 miRNAs as post-transcriptional regulators

miRNAs are about 22 nucleotides long and act as post-transcriptional regulators of gene expression [7]. This ncRNA family was identified in the early 1990s when two groups of researchers showed that *C. elegans* development was controlled by the non-coding *lin-4* gene that regulates Lin-14 protein abundance dependent on the *lin-14* 3'-untranslated region

(UTR) [8, 9]. During the following years, additional short ncRNAs were discovered, which have conserved sequences in the human genome [10, 11]. These short ncRNAs were named miRNAs. In the end of the 1990s and beginning of the 2000s, the functional aspects of post-transcriptional regulation by miRNAs were elucidated [12].

miRNAs ultimately act to reduce protein abundance of targeted transcripts. miRNAs are transcribed from the nuclear DNA into primary miRNAs (pri-miRNAs) that can be 100s of base-pairs long and have a hairpin structure [13]. See **Figure 1** for an overview of miRNA biogenesis and mode of action. In the nucleus, the pri-miRNA is cleaved at the base of the stem-loop into a precursor miRNA (pre-miRNA) by the RNase III protein Drosha, which is part of the Microprocessor complex [14, 15]. The pre-miRNA still contains its characteristic hairpin structure, which is important for later stages of miRNA maturation and function. Following cleavage, the pre-miRNA is exported from the nucleus by Exportin-5 [16]. In the cytosol, the pre-miRNA is further cleaved by the RNase III enzyme Dicer, resulting in a miRNA:miRNA duplex [17]. One of the strands, the guide strand, is introduced into the RNA-induced silencing complex (RISC) [18]. The selection of the guide strand is not random. Factors influencing this selection include strand stability at the 5'end of the miRNA:miRNA duplex, as well as regulation by proteins involved in miRNA maturation including Dicer and RISC-associated proteins [19]. The miRNA strand in RISC will guide the complex to target genes through direct Watson-Crick base pairing between the miRNA and 3'UTRs of targeted mRNAs. Nucleotides 2–8 in the 5' end of the miRNA, known as the seed region, is under higher selective pressure compared to other nucleotides of miRNAs [20]. The seed region of the miRNA is the most important component for guidance of the RISC to target genes. Within RISC, a large multiprotein complex, the protein Argonaut (AGO) binds the miRNA and guides RISC to target mRNAs [21]. This guidance will result in decreased translation of protein from the target gene due to either destabilization and cleavage of the mRNA or inhibition of protein translation [13]. Cleavage of targeted mRNA requires a catalytically active AGO protein (i.e., AGO2 in humans) and also a high sequence complementary between the miRNA and mRNA target [13, 22]. Inhibition of protein translation of target genes by miRNA-loaded RISC can be achieved by prevention of initiation or by termination of ongoing protein translation [23, 24]. In contrast to earlier beliefs that human miRNAs mainly worked through inhibition of translation, miRNAs in mammals predominantly act to reduce mRNA abundance [25]. In humans, approximately 60% of protein-coding genes have conserved sites for miRNA binding, implicating that miRNAs can alter the expression of a majority of human coding transcripts [26]. Thus, miRNAs are potent post-transcriptional regulators that cause decreased abundance of target genes.

Conservation of miRNA sequences between species also suggests conservation of regulated targets and functions. The machinery used for miRNA maturation and target recognition is well conserved in eukaryotes and believed to have evolved initially as a defence mechanism against viruses [27]. The evolution of RNA interference-mechanisms

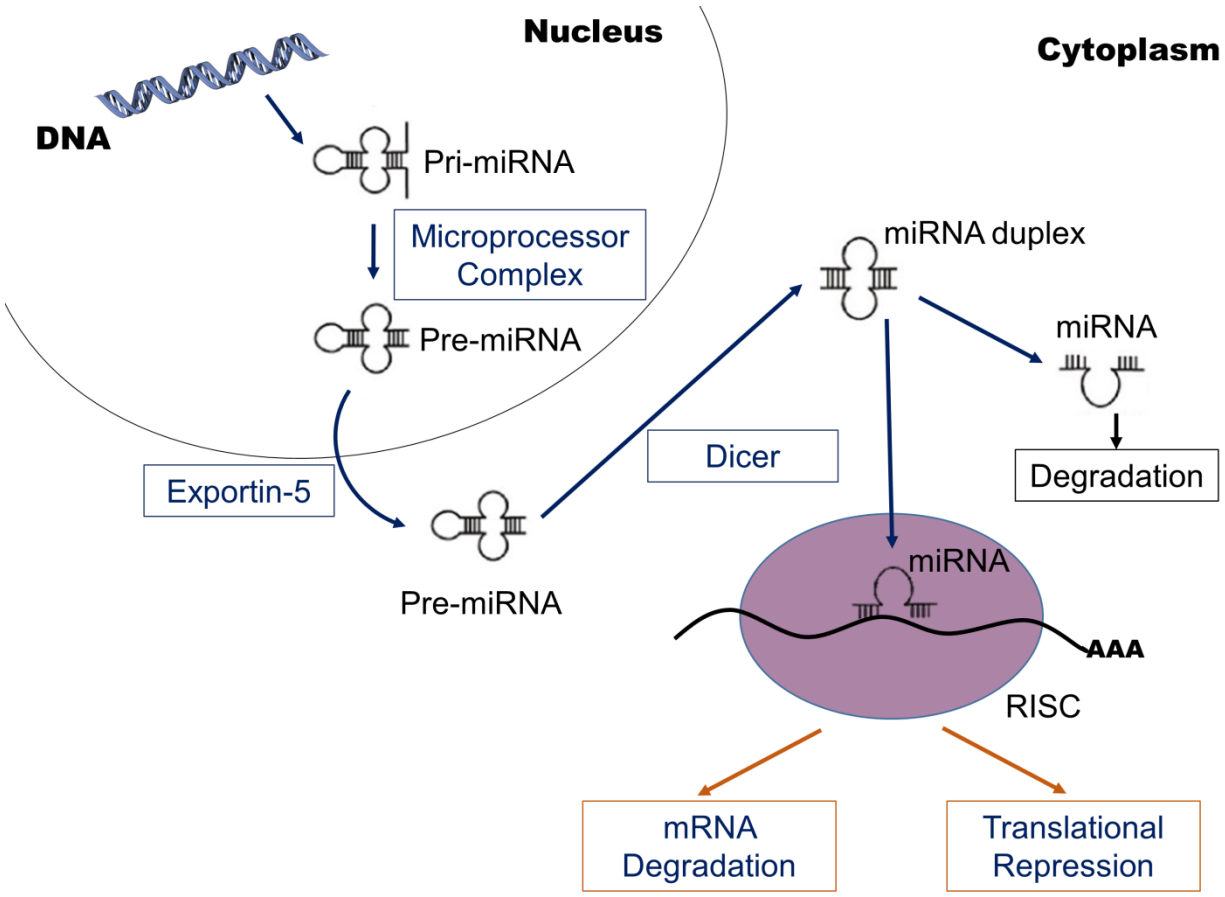

Figure 1. Overview of miRNA maturation, function and target recognition. miRNAs are transcribed as primary miRNAs (pri-miRNA) and processed within the nucleus to a precursor miRNA (pre-miRNA) by the Microprocessor complex. Thereafter, the pre-miRNA is exported from the nucleus by Exportin-5 into the cytoplasm where Dicer cleaves the pre-miRNA to a miRNA duplex. The guide strand will get incorporated into the RNA-induced silencing complex (RISC), while the other strand is degraded. The miRNA will guide RISC to targeted mRNAs and bind through Watson-Crick complementary binding, which results in either mRNA degradation or translational repression, ultimately decreasing translation of protein of miRNA target genes.

also permitted improved possibilities for fine-tuning gene expression and regulation of cellular functions. Whereas the machinery for RNA interference is conserved in eukaryotes, miRNAs are not conserved between the animal and plant phyla, indicating that they have evolved independently. Whereas animal miRNAs bind targets through incomplete complementarity, plant miRNAs often show full complementarity to their targets resulting in mRNA decay [13]. In animals, several miRNA families are conserved from worms to humans [28]. Furthermore, some miRNAs have arisen within distinct branches of evolution; for example, there are primate- and human-specific miRNAs [29]. Evolutionary young miRNAs were found to show predominant expression in neural tissues, implicating that young miRNAs might have contributed to the evolution of more complex mammalian brains [29]. Additionally, conserved miRNA target sites within target gene 3'UTRs are more likely to be functional and physiologically relevant [30]. In total, 2,693 mature miRNA sequences have been identified in humans (miRbase, release 22 (March 2018)). The number of miRNAs in animal genomes correlates to DNA size, and thus with genomic complexity [29]. With the

knowledge that ~60% of protein-coding genes in humans have conserved miRNA target sites [26], miRNAs are conserved post-transcriptional regulators of target abundance that have co-evolved together with an increased complexity in the regulation of the mammalian genome.

1.2 SKELETAL MUSCLE

1.2.1 Overview of skeletal muscle function and morphology

Skeletal muscle is needed for posture, locomotion, and ultimately for survival. The tissue accounts for approximately 40-45% of body mass in healthy humans and play a vital role in energy metabolism [31]. Skeletal muscle is composed of multinucleated fibers that contain myofibrillar proteins including actin and myosin organized into filaments. Together, repeated units of thin actin and thick myosin filaments create the functional contractile apparatus of skeletal muscle: the sarcomere. In response to motor neuron activation, Ca^{2+} is released from intracellular stores and will through regulatory proteins trigger muscle contraction due to sliding filaments and cross-bridging [32, 33]. In response to a spike in Ca^{2+}, myosin motor proteins will hydrolyse ATP leading to conformational changes of the protein that ultimately shortens the sarcomere. The conformational changes of myosin and reshuffling of intercellular Ca^{2+} are energy demanding, and, thus, skeletal muscle is a main contributor to resting metabolic rate in mammals [34, 35].

Skeletal muscle tissue consists of different fiber types that can be characterized by specific contractile or metabolic properties [31]. Basically, there are three fiber types: type I fibers (slow-twitch; oxidative), type IIa fibers (fast-twitch; oxidative), and type IIx fibers (fast-twitch; glycolytic). Type I fibers are oxidative and used during low-intensity exercise, while type II fibers are more glycolytic and active for short bursts of intense activity. Skeletal muscle fiber type composition is sensitive to exercise interventions and disease states. Athletes in sports that demand endurance (aerobic exercise) have higher relative amounts of type I fibers, whereas athletes in sports that require strength and power (resistance) have more type II fibers [31, 36]. Additionally, changes in fiber type composition can occur in response to endurance and resistance exercise [36]. For example, endurance training increases the relative amount of type I fibers while decreasing type IIx fibers [37, 38]. Insulin resistance is associated with reduced capillary density and abundance of type I fibers in humans [39]. For example, in type 2 diabetic patients there is a reduction in slow oxidative fibers and a concomitant increase in fast glycolytic fibers [40]. These findings are of clinical importance since larger relative amounts of type I fiber in skeletal muscle are associated with increased expression of the contraction and insulin-responsive glucose transporter GLUT4 and capacity for insulin-stimulated glucose transport [41, 42].

miRNAs have been implicated in fiber type specification. Three genes encoding for myosin heavy chain (MYH) isoforms, *MYH6*, *MYH7*, and *MYH7B*, encode intronic miRNAs, miR-208a, miR-208b, and miR-499, respectively [43]. MYH6/miR-208a are expressed in cardiac muscle, MYH7/miR-208b in skeletal muscle type I fibers, and MYH7B/miR-499 in both cardiac muscle and skeletal muscle type I fibers. These miRNAs are potent regulators of

skeletal muscle fiber type specification in mice, as double knockout of miR-208b/miR-499 in skeletal muscle reduce the number of type I fibers [43]. These miRNAs are required for part of the transcriptional networks that specify skeletal muscle fiber type [44]. Thus, skeletal muscle characteristics are linked to energy metabolism in conditions such as exercise and insulin resistance, and are influenced by miRNA-dependent regulatory mechanisms.

1.2.2 Skeletal muscle development

Skeletal muscle consists of myofibers responsible for contraction, as previously mentioned. Several other cell types are also present in skeletal muscle tissue, including progenitor cells (satellite cells) [45]. In adult skeletal muscle these progenitors are quiescent but can respond to stressors, such as contractile activity or injury to myofibers, to re-enter the cell cycle and regenerate muscle tissue. For example, hypertrophy of skeletal muscle in response to resistance exercise is linked to the ability of satellite cell activation [46, 47]. Furthermore, satellite cells are required for regeneration of skeletal muscle following toxin-induced injury [48]. Satellite cells represent an important cell type in skeletal muscle and are needed for its regenerative and plastic potential.

An extensively used tool to study skeletal muscle development and regeneration is the isolation of satellite cells from either human skeletal muscle biopsies or intact muscle from mice. These cells can proliferate and differentiate into mature multinucleated myotubes *in vitro*. Several different transcription factors, including Paired Box 3 (PAX3) and Paired Box 7 (PAX7), are determinants for the myogenic lineage and the proliferation of satellite cells [49]. PAX3 is essential for embryogenic myogenesis [50], whereas PAX7 is required for satellite cell specification and adult skeletal muscle regeneration [48, 51]. Activation of myogenic precursors stimulates their re-entering of the cell cycle to start proliferating. The commitment of satellite cells to differentiation into multinucleated myotubes is under the control of other transcription factors known as myogenic regulatory factors (MRFs), including myogenic differentiation (MYOD), myogenin (MYOG), and myogenic factor 5 (MYF5) [49]. These regulators promote differentiation of satellite cells by controlling expression of multiple myogenic genes. Since loss-of-function studies of MYF5 and MYOD in mice result in ablated skeletal muscle formation, these genes are considered determination factors that are required for myogenic identity [52]. MYF5 and MYOD are also upstream regulators of later MRFs, such as MYOG, that are important for terminal myotube differentiation and formation as MYOG-deficiency is postnatally lethal in mice due to muscle deficiency [53]. Nevertheless, other functional regulators of skeletal muscle formation exist and include miRNAs.

1.2.3 miRNA-regulation of skeletal muscle development

Evidence for regulatory roles of miRNAs in skeletal muscle development and regeneration comes from models where miRNA maturation is disturbed. Skeletal muscle-specific Dicer ablation in mice reduces the expression of several miRNAs and causes perinatal death, which associates with reduced skeletal muscle mass [54]. Additionally, conditional knockout of

Dicer in satellite cells of mice results in spontaneous re-entering of the cell cycle causing impaired injury-induced skeletal muscle regeneration [55]. Thus, normal biogenesis of miRNAs in satellite cells and in mature muscle is required for skeletal muscle plasticity and development.

Several miRNAs show enriched or specific expression in muscle tissue (skeletal and cardiac muscle) and are therefore termed 'myomiRs' [56]. Examples of myomiRs include miR-1, miR-133a/b, miR-206, miR-208a/b, and miR-499. Duplication of some of these miRNAs during evolution has endowed them with similar sequences, and are located in duplicate locations of the genome. For example, miR-1 and miR-206 differ in only four nucleotides, but share identical seed sequences, and thus also similar, but not identical, targets [57]. Several of the myomiRs are induced upon skeletal muscle differentiation by regulation of different MRFs [58-60]. miR-1 and miR-206 are induced during differentiation, and target repressors of skeletal muscle differentiation, including histone deacetylase 4, PAX3, and PAX7 [59, 61, 62]. Nevertheless, while *in vitro* effects of these miRNAs on skeletal muscle cell differentiation are clear, ablation of miR-1-2 or miR-206 in mice does not disturb embryonic development of skeletal muscle [63, 64]. The lack of functional effects on skeletal muscle development in these models could indicate methodological differences of studying this process when using *in vitro* cell cultures or *in vivo* models of embryonic muscle development. Another plausible explanation for the lack of effects are that miR-1 and miR-206 share the same seed sequence and could thus compensate for ablation of one of the myomiR members. Evidence pointing towards the latter explanation comes from experiments performed on *D. melanogaster* which only have a single copy of miR-1 and lack miR-206. Ablation of miR-1 in flies cause deformed musculature in larvae [65]. Furthermore, deletion of miR-206 disturbed post-embryonic regeneration of muscle in response to injury and exacerbate dystrophic symptoms in mice [64, 66]. Additionally, miR-1 and miR-133a also modulate differentiation-associated increases in mitochondrial function and abundance [67]. Thus, members of the myomiRs, including miR-1 and miR-206, are critical regulators of skeletal muscle development and regeneration.

Non-skeletal muscle-enriched miRNAs are also involved in processes related to skeletal muscle development and differentiation. Expression of several miRNAs is altered upon skeletal muscle cell proliferation and differentiation as determined by transcriptomic approaches [55, 68-72]. Whereas myomiRs are among the miRNAs with the largest relative expressional changes, numerous other miRNAs are altered during skeletal muscle cell proliferation and differentiation. Examples of such miRNAs that have functional effects during myogenesis *in vitro* include miR-20a [73], miR-26a [70], and miR-30 [74]. Regulation of skeletal muscle cell differentiation is not only restricted to miRNAs among the ncRNA family. For example, numerous long ncRNAs are implicated in the regulation of skeletal muscle differentiation and myogenesis [75, 76]. Interestingly, some long ncRNAs regulate skeletal muscle differentiation acting as 'sponges', endogenous decoys, for myogenic miRNAs [77-79]. These findings further support an important role for miRNAs in regulation of myogenesis.

1.3 GLUCOSE HOMEOSTASIS AND TYPE 2 DIABETES

1.3.1 Regulation of whole-body glucose homeostasis and skeletal muscle glucose metabolism

Blood glucose levels are tightly regulated and under homeostatic control in fed and fasted conditions. This regulation is complex and involves several tissues, including the brain, gastrointestinal tract, pancreas, liver, adipose, and skeletal muscle. The brain regulates energy metabolism and feeding behaviors through regulatory centers in the hypothalamus and brain stem [80]. These centers receive information, often in the form of hormonal signals, from the peripheral tissues about energy availability. One such example is the hormone leptin, which is released from adipose tissue when energy supply is high and signals to satiety centers in the brain. The brain can thereby regulate feeding behaviors and stimulate food intake. Ingested carbohydrates (e.g., starch and sucrose) are broken down into monosaccharides, the most common in our diet being glucose. The presence of monosaccharides will stimulate release of several gastrointestinal hormones such as gastric inhibitory protein (GIP) and glucagon-like peptide 1 (GLP1) [81]. An increase in circulating blood glucose following a meal is sensed by the pancreas [82]. Endocrine cells in the pancreas, namely α- and β-cells, produce glucagon and insulin, respectively. These hormones are major regulators of whole-body glucose homeostasis. With increased blood glucose, β-cells release insulin into the blood stream to reduce levels of circulating glucose. Primary target tissues for insulin action are liver, adipose, and skeletal muscle, as insulin stimulation will increase glucose uptake, glycolysis, glycogen synthesis, and lipogenesis in these tissues [83]. As previously mentioned, GIP and GLP1 are released into the blood in response to monosaccharides in the gastrointestinal tract, and these hormones stimulate insulin-release from β-cells. The actions of GLP1 and GIP are thought to account for about 50% of the insulin released after a meal, and thus a large part of the insulin-stimulated glucose disposal [81]. Under fasting conditions, where glucose levels are low, glucagon is released to activate hepatic glucose production (HGP) by promoting gluconeogenesis and glycogenolysis in the liver, leading to an elevation in blood glucose levels [82]. Thus, glucose homeostasis is tightly controlled by numerous tissues and endocrine regulatory nodes.

Skeletal muscle has an important role in the regulation of whole-body energy metabolism and glucose homeostasis. Skeletal muscle tissue is a main contributor to resting metabolic rate [35]. Skeletal muscle also serves as a pool of energy reserves in states of starvation when amino acids can be released to sustain HGP. Skeletal muscle production of lactate through anaerobic glucose metabolism can also provide substrates for HGP [84]. Additionally, skeletal muscle absorbs ~25% of ingested glucose in the postprandial state and is estimated to be responsible for ~85% of insulin-stimulated glucose uptake [85, 86]. This effect is mediated by insulin binding to its receptor on the skeletal muscle plasma membrane, which activates a signaling cascade involving insulin receptor substrate (IRS), phosphoinositide 3-kinase (PI3K), and protein kinase B (PKB/Akt) activation [83]. Downstream of Akt, several different branches of insulin-signaling are responsible for insulin's functional effect in skeletal muscle. For example, Akt inhibits glycogen synthase

kinase 3 (GSK3) and activates mammalian target of rapamycin (mTOR) resulting in increased glycogenesis and protein synthesis, respectively. Insulin-stimulated glucose uptake in skeletal muscle is regulated by Akt-dependent inhibition of TBC1 domain family member 4 (TBC1D4/AS160), thus permitting GLUT4-containing vesicles to fuse with the plasma membrane, which results in increased glucose transport. Thus, skeletal muscle contributes to glucose homeostasis due to its great capacity to clear glucose from the circulation in response to insulin-stimulation (see **Figure 2** for an overview of skeletal muscle insulin-signaling).

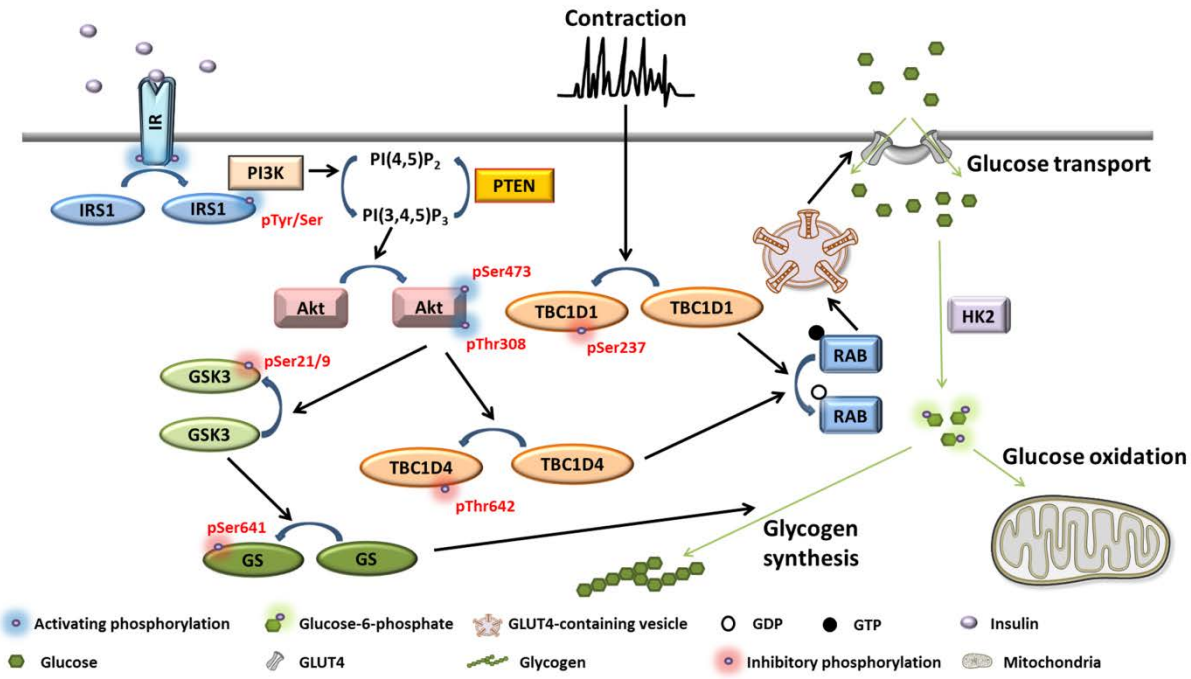

Figure 2. Schematic of insulin and contraction-induced glucose transport. Insulin and contraction induce skeletal muscle glucose uptake through different mechanisms. Insulin will bind to the insulin receptor (IR) causing autophosphorylation of intracellular domains of the receptor. Insulin receptor substrate 1 (IRS1) will bind and become activated through phosphorylation at serine and tyrosine residues. This will cause phosphoinositide 3-kinase (PI3K) to interact with IRS1 resulting in PI3K activation and phosphorylation of the phospholipid phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) generating phospholipid phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$). $PI(3,4,5)P_3$ will recruit Akt/PKB (Akt) to the plasma membrane resulting in phosphorylation of Thr^{308} and Ser^{473}, thus activating Akt. Insulin-stimulated Akt activation is responsible for several of insulin's functional effects in skeletal muscle. Akt will phosphorylate glycogen synthase kinase 3 (GSK3) at $Ser^{21/9}$ ($GSK3\alpha^{Ser21}$ and $GSK3\beta^{Ser9}$), and this phosphorylation will relieve GSK3-dependent inhibitory phosphorylation of glycogen synthase (GS) at Ser^{641} ultimately increasing glycogen synthesis. Akt will also phosphorylate and inhibit TBC1 domain family member 4 (TBC1D4/AS160) through phosphorylation at Thr^{642}, resulting in inhibition of TBC1D4's GTPase activating domain that, when activated, keeps several different Ras-related proteins (RAB) inhibited. Thus, when Akt inhibits TBC1D4, RAB proteins will get activated, ultimately causing intracellular pools of GLUT4-containing vesicles to fuse with the plasma membrane. GLUT4 will then be able to transport glucose across the plasma membrane. During contraction, several different contraction-induced mechanisms will cause phosphorylation at different residues of TBC1D1, including Ser^{237}, resulting in inhibition of TBC1D1 GTPase activity. Similarly to TBC1D4, inhibition of TBC1D1 will cause activation of RAB proteins resulting in GLUT4 translocation. Following transport, glucose gets phosphorylated by hexokinase 2 (HK2) to generate glucose-6-phosphate (G6P). G6P is trapped intracellularly through phosphorylation, and can be metabolized by mitochondria or stored as glycogen.

1.3.2 Type 2 diabetes and insulin resistance

Disturbed homeostasis of glucose is observed in states of insulin resistance, such as in patients with type 2 diabetes (T2D). Prevalence of obesity and T2D has dramatically increased during the last decades. In 2016, 650 million adults were obese and over 380 million children and adolescents were overweight or obese [87]. Thus, worldwide obesity has close to tripled in the last 40 years. As obesity is a well characterized risk factor for the development of T2D [88], the prevalence of diabetes has also increased to 422 million adults [89]. Furthermore, by 2045 the number of individuals with diabetes is estimated to increase by 50% [90]. The rise in obesity and T2D coincides with increases in a sedentary life style, reduced physical activity, and increased caloric intake. Complications arising due to diabetes include a higher risk for cardiovascular disease [91]. Furthermore, diabetes is the sixth leading cause of death [92], and thus shortens life expectancy.

The etiology of T2D is complex and involves multiple organs in the body. T2D is defined as having fasting blood glucose ≥7.0 mmol/L or blood glucose ≥11.1 mmol/L two hours after an oral glucose tolerance test [93]. Major determinants of disease progression are disturbed release of insulin from the insulin producing cells in the pancreases and insulin resistance in peripheral tissues. Disturbed β-cell function and apoptosis is observed before clinical development of T2D, resulting in decreased insulin release in response to elevated blood glucose [94]. Furthermore, the gastrointestinal-induced glucose disposal that is mediated through GIP and GLP1 is disturbed in T2D due to a defective β-cell response [95]. Peripheral insulin resistance in liver, adipose, and skeletal muscle disturbs whole-body glucose and lipid metabolism. Increased adiposity with obesity interferes with insulin's anti-lipolytic effect on adipose tissue, which results in excessive amount of circulating free fatty acids and ectopic lipid storage in liver and skeletal muscle, that negatively regulate insulin sensitivity through different mechanisms [96]. In addition, insulin resistance in adipose tissue is associated with increased tissue inflammation, further disturbing adipose tissue glucose and lipid metabolism [97]. In liver, insulin fails to inhibit gluconeogenesis and thus HGP in T2D, resulting in disproportionate glucose release from hepatocytes even in the fed state [98]. Hence, the pathophysiology of T2D is complex and involves dysfunctional regulation of metabolism in numerous tissues.

Skeletal muscle regulation of glucose metabolism is disturbed in T2D due to insulin resistance. Insulin resistance in skeletal muscle is a primary defect in T2D disease progression [99], and is characterized by reduced insulin-stimulated glucose uptake and glycogen synthesis. Factors mediating insulin resistance in skeletal muscle include ectopic lipid deposition, increased local and systemic inflammation, glucotoxicity, and mitochondrial dysfunction [83]. Skeletal muscle insulin resistance is linked to reduced phosphorylation and activation of proteins involved in insulin signaling such as IRS1, PI3K [100], and AS160 [101], defective insulin-stimulated GLUT4 translocation to the plasma membrane [102, 103], and reduced insulin-stimulated glucose transport [102, 104]. Although, identifying the molecular regulators responsible for these dysfunctions in skeletal muscle is still needed,

there is considerable evidence that skeletal muscle insulin resistance plays an important role in the etiology of disturbed glucose homeostasis in T2D.

1.3.3 miRNA-regulation of glucose metabolism

miRNAs have been implicated as regulators of T2D-related traits in several tissues including adipose, liver, pancreas, and skeletal muscle. In adipose tissue, miRNAs regulate inflammation in humans [105], and a recent publication indicates that adipose-derived circulating miRNAs in exosomes of mice can regulate gene expression in other tissues such as the liver indicating that dysregulated adipose tissue miRNA expression directly could impact whole-body metabolism [106]. A miRNA enriched in hepatocytes, miR-122, regulates cholesterol metabolism and is important for anti-inflammatory functions within the liver [107, 108]. Inhibition of the miR-103/107-family in adipose and liver improves insulin sensitivity and glucose homeostasis in obese animal models [109]. Pancreatic β-cells are also affected by miRNA-dependent regulation as evidenced by the involvement of miR-375, a miRNA enriched in β-cells, in β-cell proliferation and, thus, regulation of insulin secretion [110]. The examples reported herein simply highlight the important regulatory roles of miRNAs in glucose homeostasis.

In skeletal muscle, the expression profile of individual genes in T2D subjects is not markedly different than non-diabetic subjects [111, 112]. However, analysis of functionally related networks reveals that genes related to mitochondrial function are dysregulated in diabetic skeletal muscle [112]. Thus, T2D skeletal muscle dysfunction is characterized by alterations in transcription, indicating a potential role for miRNA-dependent regulation. Several miRNAs have altered expression in skeletal muscle of insulin resistant mouse models [113-115] and in humans with T2D [111]. Expression of miRNAs is also altered in skeletal muscle following a hyperinsulinemic-euglycemic clamp [116]. Accordingly, skeletal muscle miRNA expression is sensitive to endocrine and metabolic regulation. The myomiRs miR-133a and miR-206 are decreased in skeletal muscle from T2D patients [111]. Interestingly, a recent report highlights the role of miR-133a, in combination with miR-1, in the regulation of genes involved in mitochondrial function [67]. Although there is a clear role for miRNA-mediated regulation of metabolism, only a few miRNAs have been studied *in vitro* or *in vivo* in relation to insulin resistance and diabetes in skeletal muscle.

1.4 EXERCISE

1.4.1 Health benefits of exercise training

The health benefits of physical activity and regular exercise training include improved bone strength, reduced adiposity, improved glucose homeostasis, decreased blood pressure, increased cardiac output, as well as a reduced risk to develop depression and T2D [117]. Overall, a lack of physical activity is strongly associated with reduced health-related quality of life and shortens life expectancy by 0.4–4.2 years [118, 119]. These findings are not unexpected with regard to theories of human evolution indicating that the genus *Homo* is well adapted for endurance-related activities [120]. Thus, the reduced physical activity and increased sedentary time observed in modern societies could be considered inconsistent with how humans have evolved. Nevertheless, evolutionary theories also predict that while humans are adapted to perform endurance-related activities, we are also prone to avoid unnecessary exertion [120], possibly explaining why so few individuals meet exercise guidelines and comply with recommendations of increased physical activity [121].

Direct or indirect effects of endurance exercise training are observed in several tissues including adipose [122], liver [123], pancreas [124], and the contracting skeletal muscle. Physical activity reduces adiposity in obese individuals [125]. Since obesity is a risk factor for developing insulin resistance and T2D [88], physical activity and endurance training also reduce the risk of acquiring these conditions [126]. Nevertheless, not all of the positive effects of endurance exercise training in obese individuals are connected to weight loss, as glycemic control is improved before changes in body weight are achieved [127]. Physical activity, defined as at least 150 min per week of combined resistance and endurance exercise, improves glycemic control in individuals with T2D [128], and is therefore a recommended intervention for patients [129]. Exercise in combination with diet interventions can be as effective as pharmacological interventions in the prevention of T2D development [130]. Additionally, endurance exercise affects glucose homeostasis in healthy individuals. Healthy young volunteers exhibit exercise-induced improvements in glucose tolerance and fasting glucose levels [131, 132], while only ten days of exercise abstention worsens glucose tolerance and insulin sensitivity in master athletes [133]. Thus, exercise training is a potent tool to maintain normal glucose homeostasis and prevent the development of insulin resistance and T2D.

1.4.2 Exercise-induced alteration in skeletal muscle function

Skeletal muscle is a plastic tissue that is responsive to environmental cues and can readily remodel to adapt to increased demands of repeated skeletal muscle activity. With repeated bouts of contraction with exercise, alterations in substrate metabolism, mechanical stress, calcium flux, and redox balance will change the activities of multiple signaling pathways [134]. These signaling pathways ultimately alter gene expression via transcription factors and epigenetic mechanisms. Changes in the skeletal muscle transcriptome occurs in response to a single bout of endurance exercise [135], as well as training [136, 137]. Specifically, genes

related to mitochondrial biogenesis and oxidative metabolism are altered [137], as well as genes regulating the extracellular matrix composition [138, 139], vascularization [140], and glucose metabolism [136]. Thus, several of the defects associated with skeletal muscle function in T2D are reversed by exercise and exercise training.

Improved glucose uptake and insulin sensitivity are observed in skeletal muscle following exercise. Contraction of the working skeletal muscle promotes glucose uptake through an insulin-independent mechanism [141-143]. This uptake is mediated by phosphorylation of TBC1 Domain Family Member 1 (TBC1D1) [144], a functionally related protein to TBC1D4 (see **Figure 2** for an overview of contraction-induced glucose uptake). Nevertheless, exercise improves skeletal muscle insulin sensitivity, as evidenced by the fact that endurance exercise training improves insulin-stimulated glucose uptake [145]. Improved insulin-stimulated glucose uptake following exercise is associated with increased insulin-mediated phosphorylation of AS160 [146], and increased protein abundance of GLUT4 following endurance exercise and training [136, 147]. These modifications are also observed in skeletal muscle of T2D patients following exercise training [148, 149]. Thereby, endurance training is a potent inducer of insulin-stimulated signaling and glucose transport in skeletal muscle of people with T2D.

Endurance exercise training-associated improvements in skeletal muscle insulin sensitivity have several mechanistic explanations. For example, the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) is a transcriptional coactivator with increased expression upon endurance training [150]. PGC1α is considered a master regulator orchestrating transcriptional networks that alter mitochondrial function and lipid oxidation [151]. Metabolic flexibility, defined as the ability of an organism or tissue to adapt fuel oxidation to fuel availability, is disturbed in T2D [152] and is associated with mitochondrial dysfunction, as well as a reduced capacity to oxidize lipids in the fasted state [153, 154]. Improvements in fasting lipid oxidation is a strong predictor for enhanced insulin sensitivity [155]. Furthermore, skeletal muscle oxidative capacity is a predictor of whole-body insulin sensitivity [156]. Skeletal muscle has an increased reliance on lipids as an energy source following endurance training [157]. This increased skeletal muscle oxidative capacity is associated with improvements in insulin sensitivity in T2D after endurance exercise training. PGC1α is a candidate regulating these alterations, although further mechanistic insights into the specific molecular and cellular events by which exercise enhances insulin sensitivity may uncover novel therapeutic entry points for insulin resistance and diabetes.

1.4.3 miRNA-regulation during exercise training

Acute endurance exercise and regular exercise training alter the expression of miRNAs and genes involved in miRNA processing. One bout of endurance exercise increases the mRNA abundance of Drosha, Exportin-5, and Dicer transcripts in human skeletal muscle [158]. Endurance exercise also increases the expression of several myomiR members, including miR-1 and miR-133a [158, 159]. Conversely, endurance training has the opposite effect on myomiR expression, as miR-1, miR-133a/b, and miR-206 are decreased following 12 weeks

of endurance training [159]. Several of these miRNA species, including miR-1 and miR-133a, are also downregulated following six weeks of endurance training [137]. Resistance training has not been reported to be associated with altered myomiR expression [160]. Furthermore, skeletal muscle disuse induced by bed rest is associated with decreases in myomiR expression [161, 162]. Thus, the divergent modulation of myomiRs expression in response to muscle activity may be explained by remodeling processes that differ depending on the type and duration of stimuli.

Most studies that have determined miRNA expression following endurance exercise have focused on either myomiR expression or pre-selected candidates. Several non-myomiR miRNAs are altered following six weeks of endurance training [137], where more miRNAs are downregulated compared to upregulated (14 and 7, respectively). As there was enrichment for miRNA target genes in gene ontologies related to metabolism, miRNAs are suggested to regulate part of the exercise-induced alterations in skeletal muscle metabolism [137]. Future mechanistic studies are required to provide direct evidence for miRNA-mediated regulation of the endurance exercise-induced adaptive response of human skeletal muscle.

2 AIMS

Proper post-transcriptional control is essential for the regulation of multiple cellular functions including proliferation, differentiation, and energy metabolism. How miRNAs regulate human skeletal muscle function is incompletely understood. To address this, the overarching aim of this thesis was to determine roles for miRNAs in the regulation of skeletal muscle development and function. Specifically, the sub aims were:

- To determine the expression of miRNAs during the differentiation of primary human skeletal muscle cells. In conjunction with this, the functional effects of differentially expressed miRNAs on target gene expression were characterized during the differentiation process.

- To determine the expression of the miR-29 family members in skeletal muscle from glucose tolerant individuals as compared to type 2 diabetic patients. The thesis also aimed to charactherize the roles of these miRNAs in regulation of human skeletal muscle metabolism and insulin sensitivity.

- To characterize the miRNA expression in human skeletal muscle in response to endurance exercise training. Aims of the thesis also included the investigation of the roles played by differentially expressed miRNAs on functions associated with training-induced alterations, including insulin sensitivity, as well as glucose and lipid metabolism.

3 EXPERIMENTAL PROCEDURES AND METHODOLOGICAL CONSIDERATIONS

Reported here are selected methods utilized for data acquisition in studies presented in this thesis. The order in which the methods are presented provides an overview of the workflow employed in these studies. Biological material was required in order to answer the scientific questions that this thesis aimed to address. The cohorts representing different conditions (i.e., skeletal muscle differentiation, diabetes, or endurance exercise training) are presented first. Thereafter, a description of techniques to determine expression of miRNAs is provided. Differentially regulated miRNAs were studied and the effects of these miRNAs on post-transcriptional regulation and/or metabolism were assessed in skeletal muscle cells or in mouse skeletal muscle tissue. Lastly, the effects of the specific miRNAs studied were linked to predicted or validated target genes using different approaches.

A detailed description of methods used in the thesis is provided in the individual papers. The goal of this section, however, is to discuss advantages and disadvantages of the techniques and why certain methods were chosen for data acquisition.

3.1 SKELETAL MUSCLE CELL CULTURES

Satellite cells were extracted by enzymatic dissociation from human skeletal muscle biopsies or whole mouse skeletal muscle [163, 164]. Isolated cells, growing as proliferating myoblasts, were cultured in CO_2-ventilated, humidified incubators at 37°C with liquid media containing all necessary nutrients for growth. The cells originated from skeletal muscle biopsies taken from healthy volunteers and all subjects provided written informed consent. The ethical committee at Karolinska Institutet approved the study protocols and all protocols followed the principles of the Declaration of Helsinki.

Skeletal muscle myoblasts differentiate into multinucleated myotubes that resemble mature skeletal muscle in expression of muscle-enriched miRNAs and genes. Nevertheless, results obtained from *in vitro* models cannot simply be used to draw direct conclusion to clinical practice. Using primary human skeletal muscle cells from several donors improves the ability to translate results from *in vitro* studies to human physiology since studies are performed with a diversity of genetic backgrounds. Thus, primary human skeletal muscle cells provide an invaluable research tool in the study of skeletal muscle development, differentiation, and metabolism. In **Study I**, primary human skeletal muscle cells were utilized for determination of miRNA and gene expression during the differentiation process. RNA was collected at 48-hour intervals and expression of miRNAs and genes was assessed.

Immortalized skeletal muscle cell lines of human (LHCN-M2), rat (L6), and mouse (C2C12) divide rapidly. Thus, they can be used to quickly collect large amounts of data. Furthermore, these cells are easily accessible for most laboratories and thus provide opportunities for investigators to validate results published from other groups. Nevertheless, compared to primary human skeletal muscle cells, translatability of results from these models is limited since these cells are genetically homogeneous and some of these models have

perturbed insulin signaling and glucose metabolism. Representative images of proliferative and fully differentiated skeletal muscle cell models used in this thesis are shown in **Figure 3**.

3.1.1 Functional experiments in cells

Many of the results in this thesis were produced by studying the effects of selected miRNAs in skeletal muscle cells cultured *in vitro*. Modulation of expression of miRNAs, genes, and proteins can be achieved by several methodologies. Short RNA molecules such as miRNA precursors or inhibitors or short interfering RNA (siRNA) can be introduced into skeletal muscle cells by transfection, including liposome-based transfection. Other methods of altering expression can be through DNA-based delivery methods (e.g., transfection, electroporation, or transduction), although large DNA plasmids are not easily introduced into primary skeletal muscle cells.

For the studies reported in this thesis, cells of different tissue and species origin were transfected with miRNA precursors or inhibitors. In **Study I**, miRNA precursors for miR-30b and -30c were transfected into human skeletal muscle cells to determine the effects on gene expression of miR-30b/c predicted targets. Additionally, luciferase assays were used in human embryonic kidney cells (HEK293) transfected with miR-30b or -30c precursors to determine if the miRNAs could bind directly to the 3'UTRs of predicted target genes *RASAL2* and *WNT5A*. In **Study II**, miR-29a and -29c precursors or inhibitors were transfected into human skeletal muscle cells to determine the effects on glucose and lipid metabolism, expression of genes, and protein signaling. In **Study III**, endurance exercise training-regulated miR-19b-3p and miR-107 were overexpressed in primary skeletal muscle cells from humans and mice, as well as in C2C12 cells, through transfection of miRNA precursors. Inhibitors of miR-19b-3p and miR-107 were also transfected into human skeletal muscle cells to determine endogenous effects of these miRNAs. Additionally, siRNA targeting either *MAPK6* or *RNF11* were transfected into human skeletal muscle cells. Following transfection of miRNA precursors or inhibitions, or siRNAs, substrate metabolism and insulin sensitivity were studied in addition to gene expression and protein signaling. Primary mouse skeletal muscle cells and C2C12 cells were used to elucidate conservation of miRNA functionality and target recognition between species.

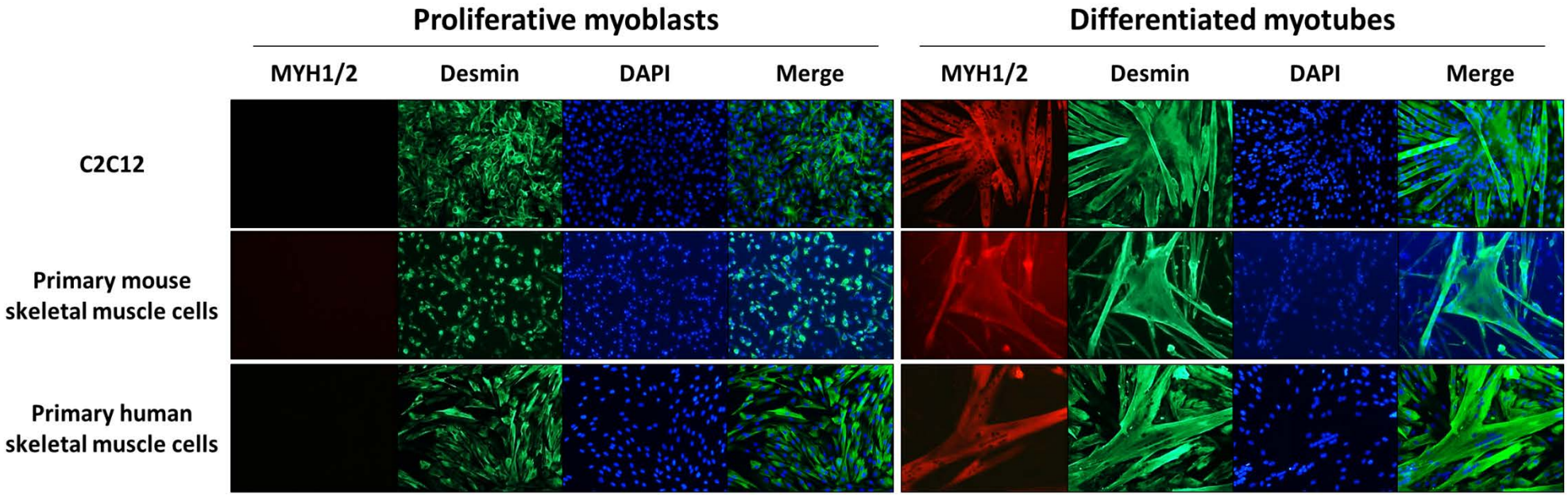

Figure 3. Overview of cell culture models utilized in this thesis. Representative immunocytochemistry images of C2C12 skeletal muscle cells and primary mouse and human skeletal muscle cells before induction of differentiation (Proliferative myoblasts) and following differentiation (Differentiated myotubes). Fixated cells were incubated with primary antibodies targeting myosin heavy chain 1 and 2 (MYH1/2) and Desmin prior to being incubated with fluorophore-conjugated secondary antibodies directed against the primary antibodies. Nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI). Desmin and DAPI staining is merged (Merge).

3.2 COHORT FOR STUDIES OF miRNA EXPRESSION IN TYPE 2 DIABETIC PATIENTS

T2D is characterized by disturbed glucose homeostasis resulting in increased fed and fasted blood glucose levels. In **Study II**, a cohort of individuals with normal glucose tolerance (NGT) or T2D was used to determining skeletal muscle expression of miR-29 family members. The clinical characteristics of these individuals are reported in **Table 2**. The cohort consisted of male volunteers who were not insulin treated. The cohort was matched for age, weight, and BMI. Skeletal muscle biopsies from *vastus lateralis* was obtained under local anesthesia following an overnight fast. The ethical committee at Karolinska Institutet approved the study protocols that adhered to the principles of the Declaration of Helsinki. All subjects provided written informed consent.

3.3 COHORT FOR STUDIES OF miRNA EXPRESSION FOLLOWING ENDURANCE EXERCISE TRAINING

In **Study III**, miRNA expression was assessed in skeletal muscle biopsies from young and sedentary, but otherwise healthy, men. Four biopsies were collected: before the start of the program, and after one day, ten days and fourteen days of an endurance exercise-training program. This program consisted of supervised exercise for one hour per day on an electronically braked cycle ergometer at 80% of pre-determined peak oxygen uptake (VO_{2peak}) for 14 consecutive days. Biopsies from the *vastus lateralis* skeletal muscle were taken under local anesthesia in the morning following an overnight fast. All biopsies were taken at least 16 hours after completion of the last exercise bout. All participants provided written informed consent and the research ethical committee at Dublin City University approved the study protocols that were conducted according to the Declaration of Helsinki. The anthropometric characteristics are given in **Table 2**.

3.4 METHODS TO DETERMINE miRNA AND GENE EXPRESSION

Although the DNA sequence is nearly identical across cell types in an organism, the expression of RNA molecules is highly variable between cell populations. There are many different methods to assess the expression of RNA molecules in biological material. When determining the expression of single miRNAs or mRNAs, reverse transcription-quantitative PCR (RT-qPCR) with specific DNA primers is often the preferred method. There are also methods available to determine expression of all RNAs in a biological sample at once. This is achieved with gene arrays or by RNA sequencing (RNA-seq), which generate transcriptomic expression profiles of multiple RNAs simultaneously. Gene arrays are relatively inexpensive and easy to process, although RNA-seq is becoming less expensive and easier to analyze. One advantage of RNA-seq over gene arrays is that detection is limited to transcripts existing in the sample, whereas gene arrays give signal intensities for all probes on the chip (even though a transcript might not be expressed). Another advantage of RNA-seq is that it does not require prior knowledge about what RNA sequences that can be detected. Transcriptomic methods often suffer from lower sensitivity and specificity compared to univariate analysis

methods, making validation of data generated by transcriptomic approaches critical. Detection and determination of miRNA abundance is complicated by their lack of a polyA-tail and their short length, complicating sequencing-library preparation and amplification by standard RT-qPCR methods.

Table 2. Anthropometric data of human cohorts studied in this thesis.

	STUDY II		**STUDY III**	
	NGT	***T2D***	***Pre-training***	***Post-training***
n *(male/female)*	10 (10/0)	12 (12/0)	8 (8/0)	-
Age *(years)*	59.0 ± 5.3	62.1 ± 4.4	22.9 ± 6.5	-
Height *(cm)*	178.7 ± 7.8	175.4 ± 4.1	178.8 ± 7.6	-
Weight *(kg)*	92.4 ± 7.9	97.3 ± 12.9	75.3 ± 8.4	-
BMI *(kg/m^2)*	28.9 ± 1.9	31.6 ± 3.6	23.6 ± 2.6	-
Fasting glucose *(mmol/L)*	5.4 ± 0.3	8.6 ± 1.7	*Not determined*	
2-hour glucose (*mmol/L)*	6.7 ± 1.0	16.3 ± 3.3 ¤	*Not determined*	
Fasting insulin *(pmol/L)*	61.2 ± 21.3	76.8 ± 33.7 ¤	*Not determined*	
HbA1c *(%)*	4.6 ± 0.2	6.0 ± 0.9 ¤	*Not determined*	
VO_{2peak} *(L/min)*	2.6 ± 0.4	2.2 ± 0.6	2.8 ± 0.4	3.3 ± 0.3 ****
VO_{2peak} *(ml/min/kg)*	28.0 ± 5.8	23.6 ± 7.6	38.3 ± 10.1	44.6 ± 8.4 *****
Triglycerides *(mmol/L)*	1.6 ± 0.6	1.4 ± 0.6	*Not determined*	
Cholesterol *(mmol/L)*	5.8 ± 0.8	4.2 ± 0.6 ¤	*Not determined*	
HDL *(mmol/L)*	1.3 ± 0.5	1.3 ± 0.4	*Not determined*	
LDL *(mmol/L)*	3.8 ± 0.7	2.3 ± 0.6 ¤	*Not determined*	

VO_{2peak}: Peak oxygen consumption. HDL: High-density lipoprotein. LDL: Low-density lipoprotein. Data are mean ± SD. ¤p<0.001 comparing NGT to T2D by Student's t-test in Study II. **p<0.01 and ***p<0.001 comparing pre-training to post-training by paired Student's t-test in Study III.

In this thesis, three different methods for determination of RNA expression have been utilized: miRNA and gene expression arrays (Affymetrix), short RNA sequencing, and RT-

qPCR. In **Study I**, miRNA expression was determined in primary human skeletal muscle cells by miRNA arrays at 48-hour intervals during differentiation of myoblasts into multinucleated myotubes. Gene expression was also determined by gene arrays before inducing differentiation and after four and ten days of differentiation. In **Study II,** an examination of the literature identified the miRNA-29 family members as candidates for further study, thus no hypothesis generating transcriptome analysis was performed. For **Study III**, alterations in miRNA expression in human skeletal muscle following a 14-day consecutive endurance exercise-training program was determined by short RNA sequencing. In this method, short RNAs are purified by miRNA-specific extractions and polyacrylamide gel electrophoresis (PAGE). To identify target genes of miRNAs, gene expression was determined with gene arrays in human skeletal muscle cells following overexpression of either miR-19b-3p or miR-107. In **Study I-III**, RT-qPCR was used for validation and quantification of gene and miRNA expression.

Other potential methods to assess RNA abundance are Northern Blot and *in situ* hybridization. Northern blot is a semi quantitative methodology that requires a large amount of input material. Nevertheless, the method has several advantages including validation of correct size of the RNA molecule being assessed and high specificity of probes. *In situ* hybridization is a method to determine presence and localization of RNAs in tissues. However, this methodology can be time consuming in terms of optimization. Neither of these methods was used for data generation for studies reported in this thesis.

3.5 TRANSLATION OF RODENT RESEARCH TO HUMAN PHYSIOLOGY

Since results generated from *in vitro* models, such as cell cultures, cannot be directly translated into human physiology, other models are warranted. To generate results from an *in vivo* setting, biologists often turn to rodents. Some advantages of murine research include the facts that mice are mammals, there are several mouse models for human diseases, the mouse genome is sequenced, and mice have a fast reproduction time. All work with animals presented in this thesis was approved by the Regional Animal Ethics Committee (Stockholm, Sweden). Animals were housed on a 12 hour light/12 hour dark cycle and received ad libitum access to water and standard rodent chow.

3.5.1 Functional experiments in rodents

Animal models are frequently used in study of metabolic disease. Examples of animal models used for studies of metabolic disorders are genetic models (such as *ob/ob* and *db/db* mice) or animals with high fat diet-induced obesity and glucose intolerance [165]. In **Study II**, expression of the miR-29 family members was assessed in *gastrocnemius* skeletal muscle from wild type and *ob/ob* mice. The *ob/ob* mice develop severe obesity, hyperinsulinemia, and hyperglycemia due to lack of circulating leptin arising from a mutation in the gene coding for leptin [166]. Expression of miR-29 family members was also assessed in rat skeletal muscle of Wistar rats that were randomized to either a sedentary control group or an endurance swimming program. The swimming program consisted of two three-hour bouts of

swimming per day for five consecutive days and *gastrocnemius* skeletal muscle was collected 16 hours after the last swimming bout.

Expression of genes or miRNAs can be altered transiently in rodent skeletal muscle by electroporation of DNA vectors. Pre-treatment of *tibialis anterior* (TA) skeletal muscle for two hours with hyaluronidase (30 µl of 1 unit/µl) improves transfection efficiency while reducing muscle damage [167]. Thereafter, 30 µg of DNA vector was injected directly into the skeletal muscle tissue and electroporation was performed by delivering 220 V/cm as eight pulses of 20 ms using an electroporator. *Flexor digitorum brevis* (FDB) skeletal muscle were also pretreated with hyaluronidase and injected subcutaneously with 20 µg of DNA vector and thereafter electroporated. The contralateral respective skeletal muscles were electroporated with a control vector. TA or FDB skeletal muscles were collected one week after electroporation following a four-hour fast. In **Study II** and **Study III**, electroporation was used for overexpression of pri-miR-29a or pri-miR-29c, and pri-miR-19b-3p or pri-miR-107, respectively, in TA skeletal muscle. In both studies, muscle tissue was used for assessment of miRNA-induced alteration in gene expression and protein abundance. In **Study II**, a modified glucose tolerance test was performed to assess glucose uptake into electroporated TA muscles. A bolus of glucose was administered by gavage, while 2-[^{3}H]-deoxy-D-glucose was given intraperitoneally to assess tissue-specific glucose uptake. In **Study III**, FDB skeletal muscle was electroporated with either miR-19b-3p or miR-107 encoding DNA vectors. One week after electroporation, FDB skeletal muscles were carefully dissected out and subjected to contraction *ex vivo* followed by glucose transport assay to determine basal and contraction-induced glucose uptake.

In **Study III**, expression of endurance exercise-regulated miRNAs was assessed in mouse skeletal muscle collected from wild-type animals following cardiac perfusion. Cardiac perfusion was performed to remove blood from tissues, thereby removing potential contaminating miRNAs from serum or various blood cells. Expression of endurance exercise training-induced miR-19b-3p and miR-107 was also determined in *soleus* and *extensor digitorum longus* (EDL) skeletal muscle. *Soleus* skeletal muscle is predominantly constituted of type I oxidative fibers and EDL is enriched with type II glycolytic fibers.

3.6 IDENTIFICATION OF miRNA TARGETS

Approximately 60% of all human protein-coding genes contains miRNA binding sites that are believed to be under selective pressure [26]. Identification of direct targets of a miRNA is therefore a challenging task. The dominant motif of a miRNA, its seed region, is used by different target identification algorithms (e.g., TargetScan [168] and mirSVR [169]) to predict direct targets of miRNAs. These algorithms often scan 3'UTRs for conserved complementary seeds, but all algorithms have their own models to determine predicted targets. Factors that are given different weight for the final results of such prediction algorithms include complementary binding outside of the seed, location within the 3'UTR, binding outside of the 3'UTR, strength of binding (8mer>7mer>6mer), secondary structure, and number of predicted binding sites in a gene [170]. Unfortunately prediction algorithms

produce many false positives [171], and target recognition can be improved using experimental data where the activity of a miRNA is altered. Since most mammalian miRNAs reduce mRNA abundance [25], determining mRNA abundance using gene arrays or RNA-seq following miRNA inhibition or overexpression is a common approach to identify regulated targets. Proteomics analysis is also a useful tool for determination of miRNA-induced changes in protein abundance, although current techniques are only able to detect a relatively small fraction of all proteins in a biological sample. In **Study II**, algorithms were used for identification of predicted targets of studied miRNAs, while in **Study I and III** a combination of transcriptome data with prediction algorithms was used to identify miRNA targets with altered expression.

Biochemical approaches to infer direct regulation of target expression by miRNAs have been developed. High-throughput sequencing (HITS) of AGO-associated miRNAs and mRNAs can be performed following crosslinking and immunoprecipitation (CLIP) [172]. Several different methods for crosslinking of RNAs to AGO have been developed, as reviewed elsewhere [173]. Recently, AGO CLIP was employed following C2C12 differentiation, providing novel data regarding skeletal muscle-enriched miRNAs and their endogenous targets during skeletal muscle development [174]. Luciferase assays are the 'gold standard' for validation of direct binding of a miRNA to a target's 3'UTR. Using this technique, as employed in **Study I**, the predicted target genes 3'UTRs are cloned downstream of the luciferase gene. If the miRNA binds directly to the 3'UTR, luciferase expression is expected to be reduced following miRNA overexpression.

Directly linking a miRNA-regulated target to cellular effects of that miRNA is challenging. miRNAs can regulate multiple genes, and thus cellular effects induced by a specific miRNA are difficult to tie to a single target gene. miRNA target protectors are tools developed for directly linking miRNA-induced effects on a single target [175]. These target protectors are RNA sequences directly complementary to the sequence where the miRNA:mRNA interaction occurs in the 3'UTR of the target gene. Thus, miRNA will be blocked from binding to the target gene, allowing the mRNA to be translated by the ribosome without miRNA interference. Recently, CRISPR-based methods to alter cellular DNA have revolutionized the field of DNA editing and are now cost-effective alternatives to other gene-editing methods [176]. CRISPR can be utilized in numerous ways to study the effects of miRNAs on cellular function and target gene expression regulation. For example, miRNA binding sites in a target gene's 3'UTR could be specifically edited and direct effects on phenotypic alterations induced by a miRNA could consequently be linked to that target gene.

4 RESULTS AND DISCUSSION

4.1 STUDY I: ASSESSMENT OF miRNA EXPRESSION DURING HUMAN SKELETAL MUSCLE CELL DIFFERENTIATION

Determination of altered expression of miRNAs and genes can provide information on skeletal muscle development in healthy and diseased states. For example, the miRNA maturation machinery is needed for proper skeletal muscle miRNA biogenesis, regulation of muscle mass, and healing in response to injury [54, 55]. miRNAs enriched in muscle are also regulators of functional development and muscle remodeling [43, 66, 67]. The expression of five miRNAs is consistently dysregulated in ten different major human muscular disorders [177]. Moreover, in mice, miR-133 delays progression of Duchenne muscular dystrophy [66]. Given the apparent links between miRNA function and skeletal muscle development, an aim of this thesis was to determine miRNA expression in a human model of muscle differentiation.

4.1.1 Numerous miRNAs are altered during human skeletal muscle cell differentiation

Skeletal muscle precursor cells can readily differentiate *in vitro* into fused myotubes. This can be achieved by reducing growth factor concentrations in the culture media. In this study, human progenitor cells were isolated from skeletal muscle biopsies from healthy volunteers. Thereafter, RNA was collected from cells before the induction of differentiation and at 48-hour intervals during ten days of differentiation. By using miRNA arrays, the expression of 1,276 putative miRNAs was monitored during the differentiation process. Following exclusion of non-expressed miRNAs, 206 were detected in either proliferative or differentiated skeletal muscle cells of which 44 were altered during the differentiation process (**Fig. 4A**). When comparing expression profiles of differentiating cells at any time point during the differentiation process to proliferate cells, 103 miRNAs exhibited altered expression in at least one time point. In comparison, another study, using miRNA arrays that covered fewer miRNAs (365), identified altered expression of 60 miRNAs following differentiation of human skeletal muscle cells [71]. Out of the 103 miRNAs identified in Study I, about 1/3 had not been previously linked to myogenesis in human or mouse. The expression of ten altered miRNAs was validated by RT-qPCR, and eight of these have increased expression following differentiation, as highlighted by increased miR-1, miR-30b and -30c, and miR-206 expression (**Fig. 4B**). The largest relative fold changes upon differentiation were seen for canonical myomiRs, confirming results from several other studies [59, 60, 71]. Thus, 103 miRNAs with altered expression during human skeletal muscle cell differentiation were identified, including novel myogenesis-associated miRNAs.

4.1.2 Identification of biologically relevant targets of altered miRNAs

There are several potential approaches to identify targets of miRNAs. As mammalian miRNAs predominantly alter mRNA levels of target genes [25], gene arrays were utilized prior to and following ten days of differentiation to identify candidate targets of the miRNAs.

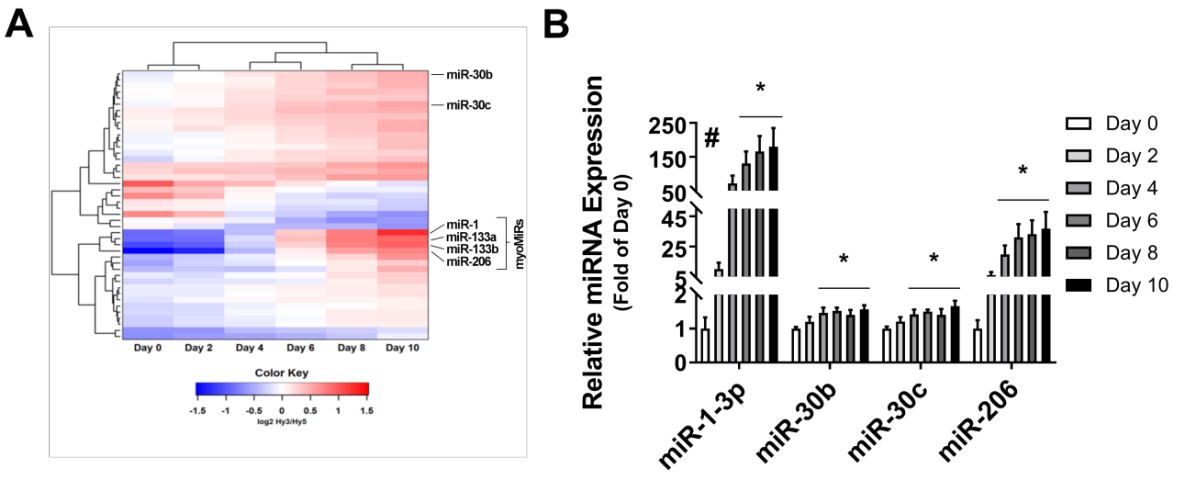

Figure 4. miRNA expression during skeletal muscle cell differentiation. Primary human skeletal muscle cells were cultured and RNA harvested during proliferation and at 48h intervals during differentiation. **A)** Heatmap of 44 miRNAs altered during the time course of differentiation. **B)** RT-qPCR validation of selected miRNAs focused upon in this thesis. Data are mean ± SEM, n = 6. # $p < 0.05$ as determined by repeated-measures ANOVA. *$p < 0.05$ by Dunnett's post hoc test.

Unfortunately, due to limited RNA amounts, expression was only determined in three out of the six subjects initially used for determination of miRNA expression. Nevertheless, 949 genes with altered expression were identified following ten days of differentiation. Using a bioinformatic tool (Ingenuity Pathway Analysis) to overlap altered miRNAs with predicted target genes that were differentially expressed during differentiation, predicted networks with miRNA-centered regulatory nodes were assembled. For example, several downregulated genes predicted as targets of miR-1/206 (which share similar seed regions) and miR-30b/c are highlighted (**Fig. 5A-B**). Using this approach known and novel myogenesis-associated predicted targets of miR-1 and miR-206 with altered expression during differentiation were identified (**Fig. 5A**).

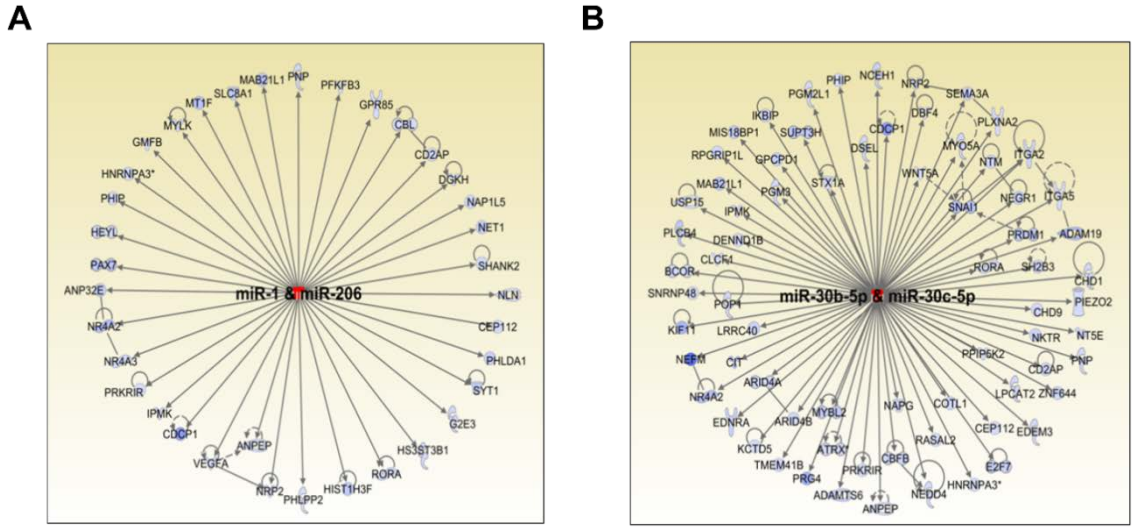

Figure 5. miRNA-centered regulatory networks of human skeletal muscle differentiation. Expression of differentially regulated miRNAs and mRNAs were determined by transcriptomic analysis of cultured primary human skeletal muscle cells. miRNAs (central node) are connected to reciprocally regulated predicted target genes (peripheral nodes) by edges. Predicted regulatory networks of **A)** miR-1 and miR-206, and **B)** miR-30b and miR-30c.

An example of previously validated miR-1 and miR-206 target is *PAX7* [61]. Novel target genes that are linked to satellite cell senescence, skeletal muscle proliferation, differentiation, or regeneration include *HEYL* [178], *PFKFB3* [179], and *PHLDA1* [180]. The relative importance of these genes for miR-1/206-regulated processes during skeletal muscle cell differentiation requires further studies.

4.1.3 miR-30b and miR-30c-regulated genes during human skeletal muscle cell differentiation

The miR-30b and miR-30c-centered gene regulatory network (**Fig. 5B**) identified 76 predicted target genes with decreased expression upon skeletal muscle cell differentiation. Among these targets, several known transcriptional regulators of cell cycle progression, including *E2F7* [181], and skeletal muscle maturation and development, such as *PRDM1* [182] and *SNAI1* [183] were identified. These findings are interesting in regards to the finding that inhibition of miR-30 abrogates differentiation in C2C12 skeletal muscle cells [74]. Further investigation is needed to clarify whether these 76 reciprocally regulated and predicted target genes are involved in the regulatory role of miR-30 during skeletal muscle differentiation.

The roles of miR-30b and miR-30c in the regulation of selected candidate genes from the miRNA-centered network were further investigated. Correlation analysis of upstream miRNAs to downstream target genes was performed and 14 out of the 76 predicted target genes were negatively correlated to miR-30b or miR-30c expression during differentiation (p-values were < 0.05 after Benjamini-Hochberg *post hoc* correction). Among these 14 genes, *RASAL2* and *WNT5A* showed strong reciprocal expression levels to miR-30b (**Fig. 6A-B**) and miR-30c (**Fig. 6C-D**). When miR-30b or miR-30c was overexpressed in human skeletal muscle cells, the mRNA abundance of *RASAL2* and *WNT5A* decreased (**Fig. 6E**). To determine if *RASAL2* and *WNT5A* are direct targets of miR-30b or miR-30c, luciferase reporter plasmids were constructed (**Fig. 6F**). When these constructs were introduced into HEK293 cells that were transfected with either a negative control miRNA precursor or precursors for miR-30b or miR-30c, only *RASAL2* was identified as a direct target of these miRNAs (**Fig. 6G-H**). These findings indicate that, while miR-30 members directly target *RASAL2*, reductions in *WNT5A* expression following miR-30 overexpression is likely achieved through either indirect effects of miR-30 or miR-30 interaction with an unidentified miR-30 binding sequence within the *WNT5A* transcript. RASAL2 is a Ras-specific GTPase-activating protein [184], and has been linked to both promote and inhibit proliferation in different models [185, 186]. However, the effects of RASAL2 on skeletal muscle cell proliferation and differentiation remains to be elucidated.

In conclusion, through integration of simultaneous miRNA and mRNA expression data miRNA-centred regulatory networks of gene expression during differentiation of human skeletal muscle cells were identified.

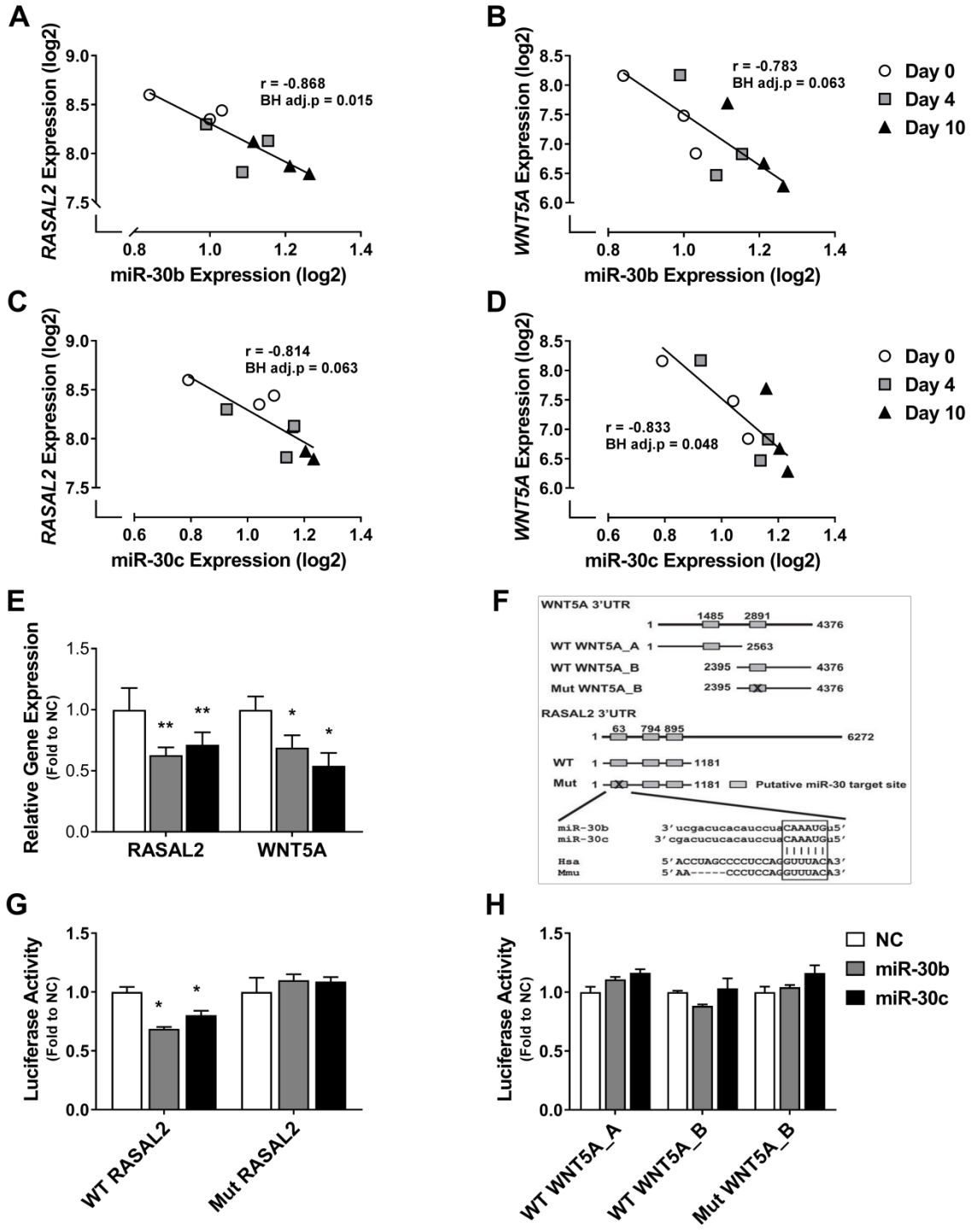

Figure 6. miR-30b and miR-30c are regulators of RASAL2 expression in human skeletal muscle cells. Primary human skeletal muscle cells were cultured and expression of differentially regulated miRNAs and mRNAs was determined by transcriptomic analysis. **A-D**) Expression of miR-30b (**A-B**) and miR-30c (**C-D**) was correlated (Pearson's correlation) to the expression of predicted target genes *RASAL2* (**A** and **C**) and *WNT5A* (**B** and **D**) during the differentiation process (n = 9). **E)** Gene expression of *RASAL2* and *WNT5A* following overexpression of miR-30b or miR-30c was compared to transfection with a negative control (NC) in human skeletal muscle cells (n = 3). **F)** Constructs used for luciferase assays. **G)** Luciferase activity in HEK293 cells overexpressing the *RASAL2* constructs shown in **F** following transfection with negative control (NC), miR-30b, or miR-30c (n = 6). **H)** Luciferase activity in HEK293 cells overexpressing the *WNT5A* constructs shown in **F** following transfection with negative control (NC), miR-30b, or miR-30c (n = 6). Data are mean ± SEM. *p < 0.05 and **p < 0.01 as determined by paired Student's t-test.

4.2 STUDY II: EFFECTS OF miR-29 FAMILY MEMBERS ON GLUCOSE AND LIPID METABOLISM IN SKELETAL MUSCLE

miRNAs are potent regulators of insulin action in peripheral tissues including liver and adipose [109]. miRNAs have been implicated as regulators of skeletal muscle protein abundance in insulin resistant states, such as in T2D [111]. A meta-analysis of miRNA expression in insulin-responsive tissues identified several dysregulated miRNAs in conditions associated with insulin resistance in animal models and humans [187]. miR-29 family members were identified, and miR-29a was the most frequently reported upregulated miRNA. The miR-29 family members consist of three miRNAs: miR-29a, miR-29b, and miR-29c. Increased miR-29 expression was initially identified in skeletal muscle of rats that spontaneously develop insulin resistance and diabetes [113, 188]. However, expression of miR-29 members had not been determined in skeletal muscle of humans with insulin resistance. To address this, the expression of these miRNAs in human skeletal muscle from NGT controls and T2D patients was catalogued and the role of these miRNAs in metabolism was determined.

4.2.1 miR-29a and miR-29c are increased in skeletal muscle from individuals with type 2 diabetes

To determine the expression of miR-29 members in diabetic skeletal muscle, RNA was extracted from skeletal muscle biopsies from a cohort of control subjects with NGT and T2D patients (anthropometric data in **Table 2**). The expression of miR-29a and miR-29c was found to be increased in skeletal muscle from T2D patients, while miR-29b expression was not different between the T2D and NGT cohorts (**Fig. 7A-C**). These results corroborate previous findings of dysregulated miR-29 expression in insulin resistant tissues [187]. Additionally, miR-29 expression has been reported to be increased in skeletal muscle of a separate cohort of T2D patients compared to obese controls [189]. Disturbed lipid metabolism is a well-characterized defect in insulin resistant skeletal muscle [112, 153, 154]. Therefore, to elucidate the effects of lipid overload on miR-29 expression, human myotubes were exposed to palmitate and miR-29 expression was measured following 24 or 96 hours of treatment. Palmitate exposure of myotubes for 96 hours increased expression of miR-29a and miR-29c, reproducing the effects of palmitate on miR-29a expression reported in rat L6 cell cultures [190]. Thus, dysregulated expression of miR-29a and miR-29c in skeletal muscle from T2D patients could be linked to disturbed lipid metabolism. Since mitochondrial biogenesis and improved lipid metabolism is observed following endurance exercise training [157], the expression of miR-29 members was determined in human volunteers undergoing a 14-day endurance training program (anthropometric data in **Table 2**). This training program resulted in increased expression of several mitochondrial proteins [191]. While miR-29a and miR-29b expression was unaltered following endurance training (**Fig. 7D-E**), abundance of miR-29c was decreased in skeletal muscle (**Fig. 7F**). Thus, altered miR-29c expression following endurance training and in T2D could be involved in regulation of skeletal muscle oxidative capacity.

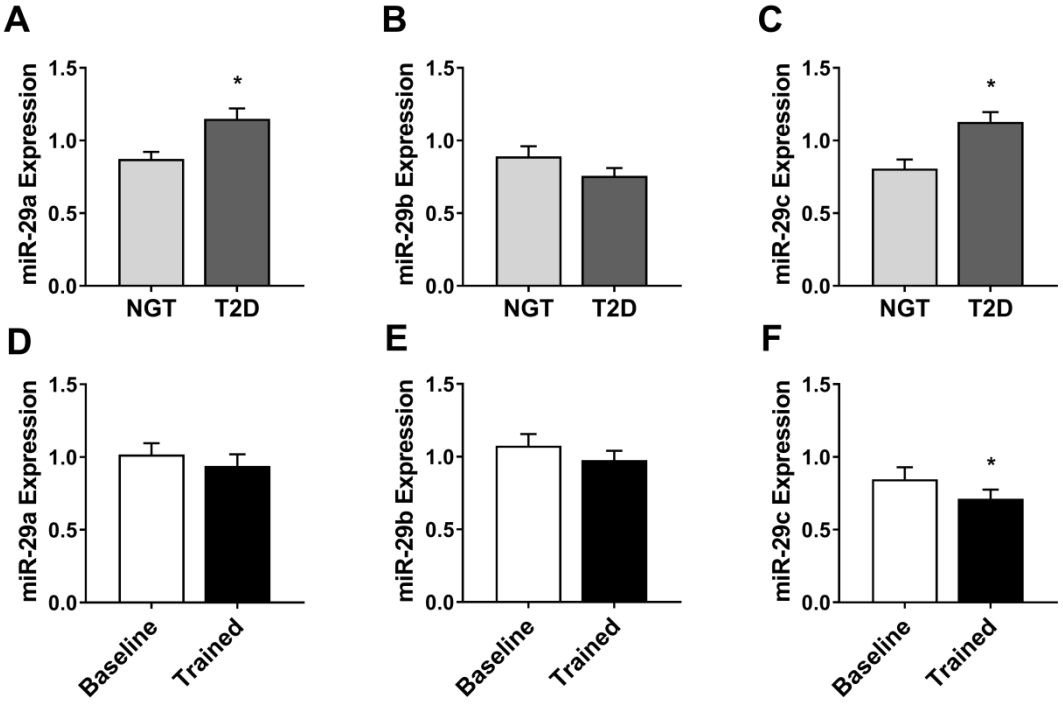

Figure 7. Expression of miR-29 members in skeletal muscle of T2D patients and in healthy volunteers following 14 days of endurance exercise training. Expression of miR-29 members was determined by RT-qPCR in skeletal muscle from **A-C)** healthy volunteers with normal glucose tolerance (NGT) and type 2 diabetes patients (T2D) (n = 10–12). **D-F)** Healthy volunteers before (Baseline) and after completion of 14 consecutive days of endurance exercise training (Trained) (n = 8). Data are mean ± SEM. *p < 0.05 as determined by paired or non-paired Student's t-test.

4.2.2 miR-29a and miR-29c are regulators of glucose metabolism in skeletal muscle

Following the identification of altered miR-29 expression in skeletal muscle with exercise training and T2D, the metabolic effects of miR-29 members were determined. Overexpression of miR-29a and miR-29c decreased glucose uptake and insulin-stimulated glycogen synthesis in human skeletal muscle cells (**Fig. 8A-B**). Glucose uptake and total glycogen levels were reduced by overexpression of miR-29a and miR-29c in intact skeletal muscle of mice (**Fig. 8C-D**). Additionally, inhibition of miR-29c in human skeletal muscle cells enhanced basal and insulin-stimulated glucose uptake. These data support previous findings of decreased glucose uptake in rodent cell lines following miR-29 overexpression [113, 190, 192]. Associated with the miR-29-induced reduction in insulin responsiveness was reduced phosphorylation of Akt^{Ser473} and $GSK3\alpha/\beta^{Ser21/9}$ in response to insulin stimulation of human skeletal muscle cells. Similarly, reduced phosphorylation of Akt^{Ser473} was also observed in mouse skeletal muscle overexpressing miR-29a and miR-29c following a two-hour glucose tolerance test in mice. miR-29a and miR-29c were identified as regulators of lipid oxidation in human skeletal muscle cells. Overexpression of miR-29 members reduced palmitate oxidation, while inhibition of these miRNAs improved the cellular capacity to oxidize lipids. Thus, miR-29 members are potent and endogenous regulators of glucose and lipid metabolism and insulin responsiveness in skeletal muscle.

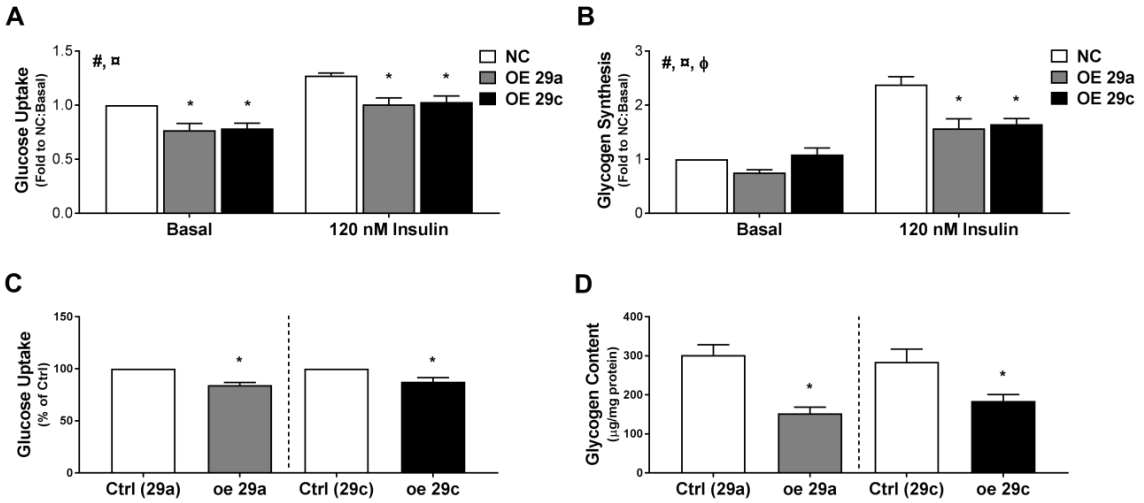

Figure 8. Overexpression of miR-29 members in skeletal muscle reduce insulin-responsive glucose metabolism. miR-29a, miR-29c, or a negative control (NC) was overexpressed in cultured human myotubes. Basal and insulin-stimulated (120 nM) **A)** glucose uptake and **B)** glucose incorporation into glycogen was assessed (n = 5). Intact *tibialis anterior* skeletal muscles of mice were electroporated with plasmids encoding either miR-29a, miR-29c, or a control (Ctrl) sequence. One week thereafter **C)** glucose uptake and **D)** total glycogen in skeletal muscle was assessed following a two-hour glucose tolerance test (n = 10). Data are mean ± SEM. # miRNA effect; ¤ insulin effect; ϕ interaction; determined by 2-way ANOVA.*p < 0.05 as determined by paired Student's t-test or Bonferroni *post hoc* testing.

4.2.3 Linking miR-29-induced metabolic alterations to regulation of gene expression

To identify miR-29-targets that could explain the metabolic effects following overexpression or inhibition in skeletal muscle, conserved predicted miR-29-target genes implicated in insulin signaling and glucose metabolism were identified. The putative miR-29 targets *IRS1*, *PIK3R3*, and *AKT2* were decreased following miR-29a and miR-29c overexpression (**Fig. 9A**). Additionally, miR-29 also reduced expression of non-predicted target genes involved in regulation of glucose metabolism, such as hexokinase 2 (*HK2*) (**Fig. 9A**). These genes were also decreased in mouse skeletal muscle following overexpression of either miR-29a or miR-29c, with miR-29c being more potent to regulate target gene expression *in vivo*. Additionally, miR-29 regulates endogenous expression of *PGC1α* and *PDK4*, genes controlling mitochondrial biogenesis and the cellular ability to switch between glucose or lipid energy sources, respectively. *PDK4* is a predicted target (TargetScan) and *PGC1α* is a luciferase validated target of miR-29 members [193]. Thus, miR-29 family members control the expression of several regulators of skeletal muscle glucose and lipid metabolism.

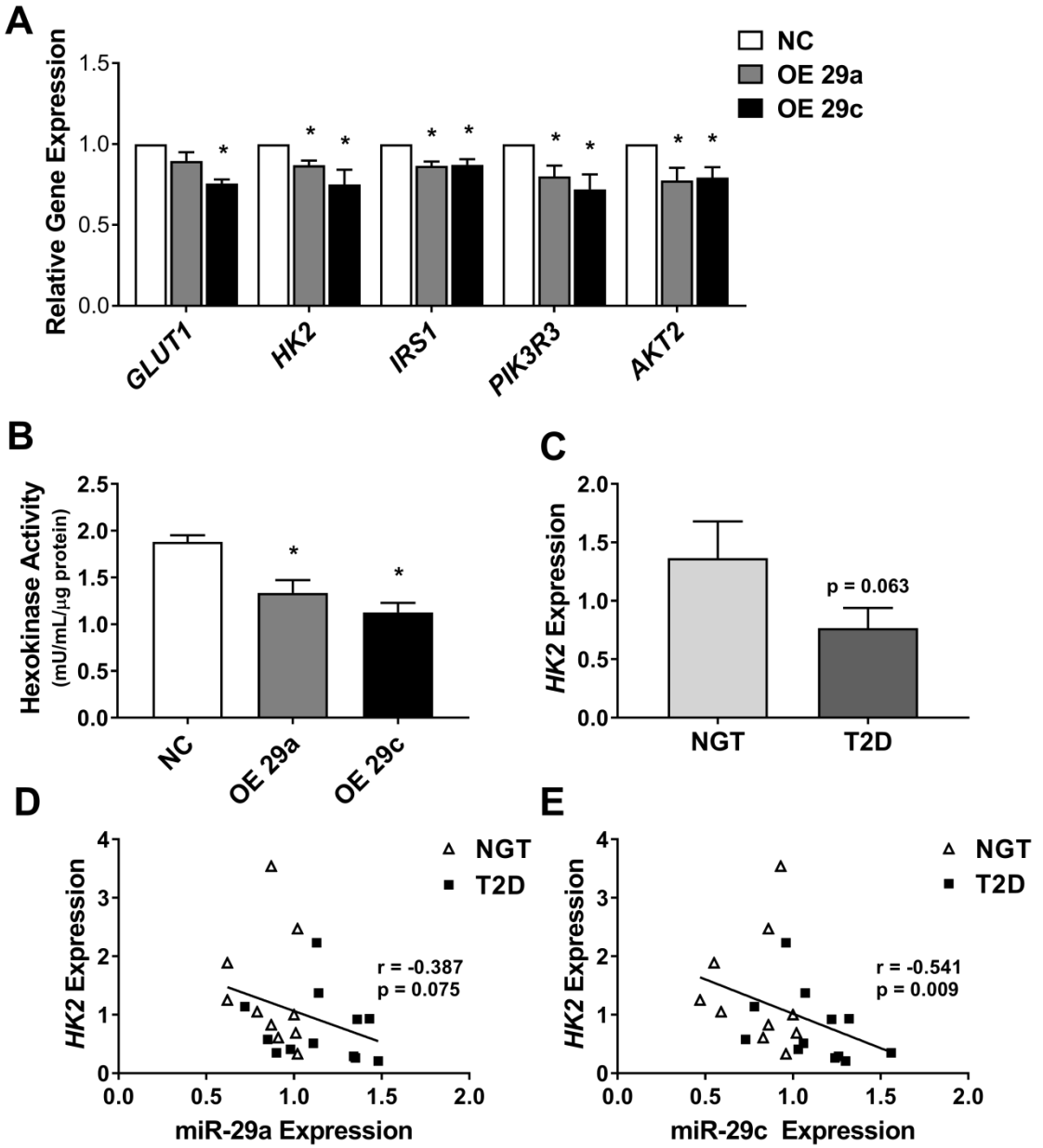

Figure 9. miR-29 overexpression alters several genes involved in glucose metabolism. miR-29a, miR-29c, or a negative control (NC) was overexpressed in cultured human myotubes. **A)** Expression levels of putative targets and genes important for glucose metabolism were determined by RT-qPCR (n = 6), and **B)** hexokinase activity was assessed (n = 10). **C)** Expression of *HK2* in healthy volunteers with normal glucose tolerance (NGT) or type 2 diabetes patients (T2D) (n = 10–12). **D-E)** Spearman correlation of **D)** miR-29a expression and **E)** miR-29c expression to expression of *HK2* in NGT and T2D subjects (n = 10–12). Data are mean ± SEM. *p < 0.05 as determined by paired Student's t-test.

HK2 is a rate-limiting kinase in glycolysis given that it phosphorylates glucose to glucose-6-phosphate and thereby traps glucose metabolites in the cell. *HK2* expression was reduced following miR-29 overexpression (**Fig. 9A**) and increased following inhibition. Consequently, human skeletal muscle cells overexpressing miR-29a or -29c exhibited reduced hexokinase activity (**Fig. 9B**). Inhibition of miR-29a increased hexokinase activity, while a trend was observed for miR-29c inhibition in cells, although no effect on hexokinase activity was found by miR-29 members in mouse skeletal muscle (data not shown).

Previously unpublished data show a trend for decreased *HK2* expression skeletal muscle from T2D patients (**Fig. 9C**). In a larger cohort of NGT and T2D individuals *HK2* mRNA abundance is significantly decreased in skeletal muscle of T2D patients [194]. Correlation analysis of *HK2* expression to miR-29a expression was not significant in skeletal muscle of NGT and T2D individuals (**Fig. 9D**). However, there is a significant inverse relationship between *HK2* and miR-29c expression (**Fig. 9E**). These findings indicate that miR-29c could influence *HK2* expression in human skeletal muscle and the elevated levels of miR-29c may be partially responsible for decreased *HK2* transcript abundance observed in the T2D patients [194]. Nevertheless, *HK2* is not a target gene of miR-29 as there are no miR-29-binding sites in the *HK2* transcript. Because miR-29a and -29c reduced expression of *PIK3R3,* a regulatory subunit of PI3K, and *HK2* expression is regulated through PI3K-dependent mechanisms [195], a miR-29/PI3K-pathway could explain the reduced expression and activity of hexokinase following overexpression pf miR-29a or -29c. Interestingly, insulin-stimulated PI3K activity is negatively regulated in skeletal muscle of insulin resistant individuals [100, 104]. Additionally, endurance exercise training increases insulin-stimulated PI3K activity [196]. Thus, in conditions where PI3K activity and *HK2* expression are known to be differentially regulated, expression of miR-29 members was found to be inversely expressed.

In conclusion, expression of miR-29 members is decreased in skeletal muscle of T2D patients. miR-29a and miR-29c regulates glucose and lipid metabolism through regulation of important mediators of glucose and lipid metabolism, including *HK2* and *PGC1α*. Additionally, miR-29 family members directly regulate the expression of canonical genes involved in the insulin-signaling cascade.

4.3 STUDY III: ENDURANCE EXERCISE TRAINING-REGULATED miRNAS POTENTIATE GLUCOSE METABOLISM AND INSULIN SINGALING IN SKELETAL MUSCLE

Acute exercise and regular exercise training are effective strategies to improve whole-body glucose metabolism and insulin resistance [128, 131]. Skeletal muscle is a plastic tissue that can adapt to increased physical activity by increasing insulin sensitivity [197], remodeling its extracellular matrix [138], and increasing the abundance of proteins involved in glucose metabolism [136]. Epigenetic regulation is associated with exercise training-induced adaptations [198], including alterations of skeletal muscle miRNA expression [137]. Here the aim was to determine the human skeletal muscle miRNA expression profile following 14 consecutive days of endurance exercise. In addition, the functional roles of training-responsive miRNAs in the regulation of training-associated adaptive traits in skeletal muscle were determined.

4.3.1 miRNA expression is altered in human skeletal muscle following endurance exercise training

Eight healthy young men underwent an exercise training program consisting of 14 consecutive days of endurance exercise for one hour per day at 80% VO_{2peak} (anthropometric data in **Table 2).** Skeletal muscle biopsies were taken from *vastus lateralis* skeletal muscle

prior to exercise (baseline) and in the mornings following one, ten, and fourteen days of training. To identify miRNAs with altered expression following exercise training short RNA-seq was performed on RNA collected from biopsies at baseline and after 14 days of training (n = 3). This approach identified 12 differentially regulated miRNAs following training. In comparison to a previous study, this analysis identified fewer miRNAs to be altered following exercise training in skeletal muscle (12 in this study versus 21 in [137]). The duration of exercise training (2 versus 6 weeks) and differences in samples size (3 versus 8 individuals) are likely to explain these differences. RT-qPCR was used to validate 11 of these miRNAs, of which six miRNAs, including miR-19b-3p and miR-107, were altered following exercise training in eight subjects (**Fig. 10A**). Expression of miR-19b-3p and miR-107 was also found to be increased after a single bout of exercise (**Fig. 10B**). The expression of these miRNAs continued to increase throughout the exercise training program and reached maximal levels by the end of the two-week training period (**Fig. 10C-D**). Although miR-107 has previously been found to be induced following acute exercise in mice [199], this is the first report of miR-19b-3p and miR-107 responding to exercise training in humans.

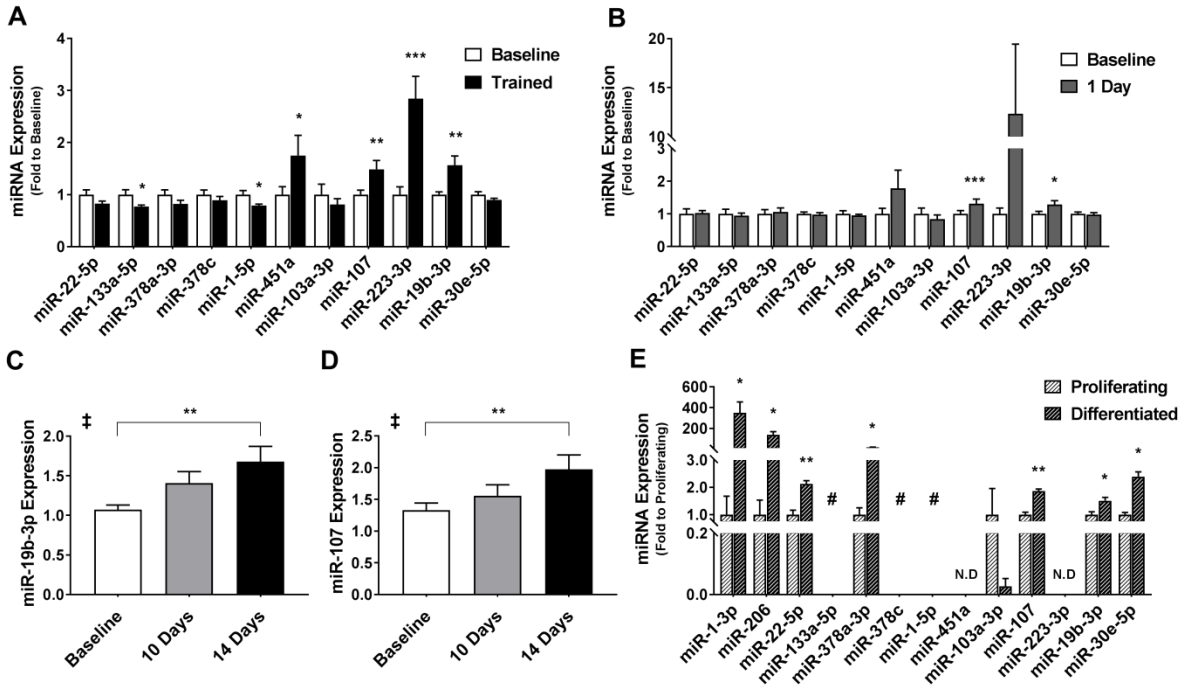

Figure 10. miRNA expression following endurance exercise and training. Healthy young males underwent a training program consisting of 1 hour of exercise at 80% VO_{2peak} per day for 14 consecutive days. *Vastus lateralis* skeletal muscle biopsies were collected prior to and following 1 day, 10 days, and 14 days of exercise. **A)** Expression of miRNAs prior to (Baseline) and following exercise training (Trained) (n = 8). **B)** Expression of miRNAs prior to (Baseline) and after one bout of exercise (1 Day) (n = 5). **C-D)** Expression of **C)** miR-19b-3p and **D)** miR-107 prior to (Baseline) and following 10 and 14 days of exercise (n = 8). **E)** Expression of miRNAs in proliferating and differentiated human skeletal muscle cells (n = 3), # not detected in proliferating cells. Data are mean ± SEM, determination performed by RT-qPCR. ‡ $p < 0.05$ determined by 2-way repeated measures ANOVA.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as determined by paired Student's t-test or Sidak's *post hoc* testing.

To determine if training-associated miRNAs were expressed in human skeletal muscle cultures *in vitro*, the expression of these miRNAs were profiled during human skeletal muscle cell differentiation. Nine out of the 11 miRNAs were expressed in human skeletal muscle cultures (**Fig. 10E**), and eight of these had increased expression in differentiated myotubes compared to proliferative myoblasts (miR-1-5p, miR-133-5p, and miR-378c were not detected in proliferative cells, and thus no fold change could be determined). Out of interest, these results were compared to data acquired in **Study I**. Expression of miR-103 and miR-107 was increased in both studies, while differences were observed for miR-19b and miR-30e (increased in **Study III** but unaltered in **Study I**). miR-22-5p was increased in **Study III,** but did only showed a trend for increased expression in **Study I** ($p = 0.07$). miR-1-5p, miR-133a-5p, miR-378c, miR-223, and miR-451 were below the cut off of intensity levels used on the array data in **Study I**, in partial agreement with the results from **Study III**. Differences in methodology employed in the studies (i.e., RT-qPCR in Study III and miRNA arrays in Study I) could explain these divergent results.

4.3.2 Functional effects of miR-19b-3p and miR-107 on glucose metabolism in human skeletal muscle cells

To determine the functional effects of miR-19b-3p and miR-107 on exercise training-associated adaptations in skeletal muscle, these miRNAs were overexpressed in human skeletal muscle cells. miR-19b-3p and miR-107 were chosen for functional characterization since expression was 1) altered by endurance training in human skeletal muscle, 2) detected in human cell cultures *in vitro*, and 3) still detected in mouse skeletal muscle following cardiac perfusion to remove blood-associated miRNA contamination from muscle. Overexpression of both miR-19b-3p and miR-107 increased basal and insulin-stimulated glucose uptake in cells (**Fig. 11A-B**). There is a significant interaction between miR-19b-3p overexpression and insulin treatment on glucose uptake (**Fig. 11A**), indicating potentiation of insulin sensitivity. These miRNAs also increased insulin-stimulated glucose incorporation into glycogen at two different insulin doses (**Fig. 11C-D**). Additionally, inhibition of these miRNAs in human skeletal muscle cells caused modest reductions in glucose uptake. Thus, miR-19b-3p and miR-107 improves glucose metabolism in human skeletal muscle cells.

Overexpression of miR-19b-3p increased insulin-sensitive signaling as evidenced by increased phosphorylation of Akt^{Thr308} and Akt^{Ser473} under submaximal insulin stimulation. miR-19b-3p also increased phosphorylation at $AS160^{Thr642}$ at baseline and following 10 nM insulin stimulation. This phosphorylation site in AS160 is insulin-responsive and required for insulin-stimulated glucose transport [200]. Our findings of enhanced insulin-stimulated Akt phosphorylation following miR-19b-3p overexpression are supported by findings highlighting miR-19b-3p as a positive regulator of Akt phosphorylation [201-203]. Findings of increased phosphorylation of Akt by miR-19b are associated with decreased expression of *PTEN*, a negative regulator of PI3K-mediated Akt activation [204]. miR-19b-3p decreased *PTEN* expression also in the current study.

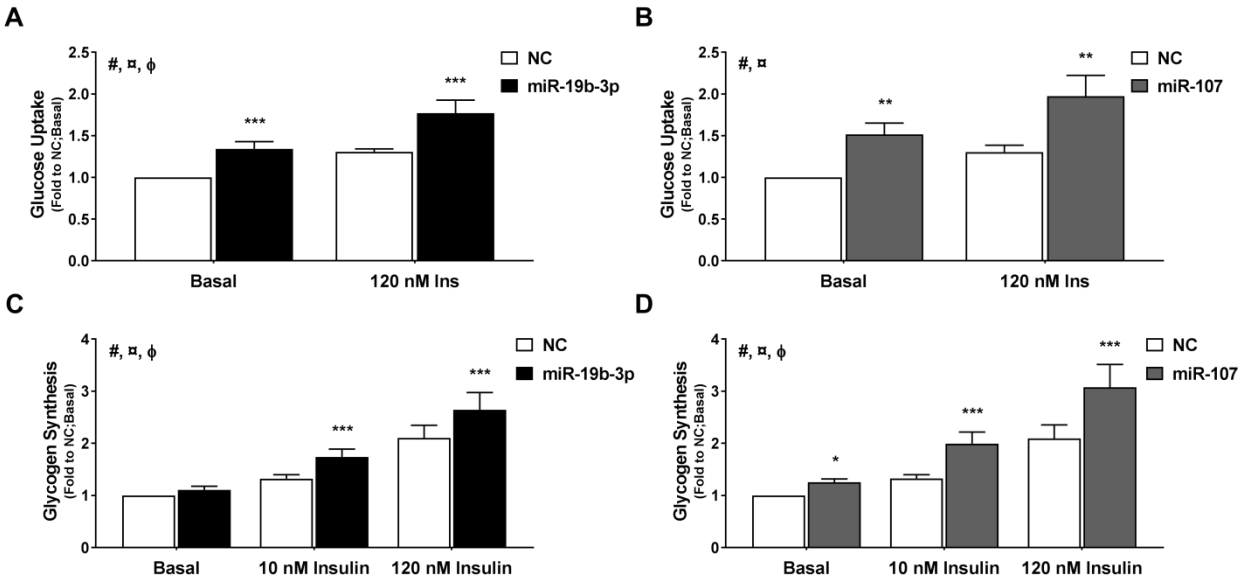

Figure 11. miR-19b-3p and miR-107 regulate glucose metabolism in human skeletal muscle cells. Human skeletal muscle cells were differentiated and transfected with a negative control (NC) miRNA precursor or miRNA precursors for miR-19b-3p and miR-107. **A-B)** Glucose uptake was determined at basal or 120 nM insulin following **A)** miR-19b-3p or **B)** miR-107 overexpression. **C-D)** Glucose incorporation into glycogen was determined Basal or 120 nM insulin following **C)** miR-19b-3p or **D)** miR-107 overexpression. Data are mean ± SEM (n = 6). # miRNA effect; ¤ insulin effect; ϕ interaction; determined by 2-way ANOVA.*p < 0.05, **p < 0.01, and ***p < 0.001 as determined by Bonferroni *post hoc* testing.

Similar to miR-19b-3p, miR-107 overexpression in human myotubes enhanced phosphorylation at Akt^{Thr308}, Akt^{Ser473}, and $AS160^{Thr642}$ during treatment with 10 nM of insulin. Phosphorylation of Akt was also enhanced by 120 nM of insulin stimulation after miR-107 overexpression. These results are in line with a previous publication showing decreased phosphorylation of Akt following miR-107 inhibition in human cells [205]. Thus, increased glucose metabolism following miR-19b-3p and miR-107 overexpression is associated with enhanced insulin signaling.

In mouse skeletal muscle cells, the effects on glucose metabolism of miR-19b-3p were conserved. In contrast, miR-107 failed to potentiate glucose uptake or glycogen synthesis in either primary mouse or C2C12 skeletal muscle cells. miR-107 negatively regulates insulin sensitivity in adipose tissue and liver in mice [109]. Divergent results of miR-107-regulated glucose uptake in human and mouse skeletal muscle cell could be due to differences in miRNA target conservation between human and mouse, or due to the decrease in myogenic markers associated with miR-107 overexpression in human skeletal muscle cells observed in the current study. Additionally, preliminary data from intact mouse FDB skeletal muscle indicate that overexpression of miR-19b-3p (but not miR-107) for one week potentiates contraction-induced glucose transport (glucose transport was enhanced in four out of five animals). In summary, miR-19b-3p, an endurance exercise training-induced miRNA, is a potent inducer of insulin and contraction-stimulated skeletal muscle glucose metabolism in both human and mouse tissues.

4.3.3 miR-19b-3p and miR-107-regulated target genes in human skeletal muscle following endurance exercise training

Endurance exercise training potently alters the skeletal muscle transcriptome [136, 137]. Thus, studies were identified that reported transcriptomic responses in skeletal muscle from healthy humans to either training (paired analysis) [137, 206] or between subjects with different self-reported physical activity levels [207]. Overlapping gene expression signatures from these studies identified 5,817 genes that were regulated ($p < 0.05$) in at least two of the studies. Transcriptome analysis by gene arrays of human skeletal muscle cells subjected to either miR-19b-3p or miR-107 overexpression indicated regulation of 679 and 476 genes (fold-change cut off 1.5, $p < 0.01$), respectively. TargetScan and microRNA.org algorithms were used to identify predicted target genes of miR-19b-3p or miR-107. The identified predicted target genes were overlapped with the 5,817 genes altered by exercise training. This approached identified 26 genes which were downregulated due to miR-19b-3p overexpression and following exercise training. For miR-107, 18 predicted target genes met the same criteria employed for identification of miR-19b-3p-regulated genes. Selected predicted targets were validated to be downregulated following miR-19b-3p (**Fig. 12A**) or miR-107 (**Fig. 12B**) overexpression in human myotubes. All miR-19b-3p-regulated genes (except for *Zbtb4*) were found to be downregulated following overexpression in mouse skeletal muscle cells or tissue. Nevertheless, none of the miR-107 target genes altered in human cells was changed in mouse. Since these genes were indicated to be regulated by endurance training, the expression of miR-19b-3p and miR-107 predicted targets were measured genes in skeletal muscle following 14 consecutive days of exercise by RT-qPCR. Expression of *CAV3*, *KIF13A*, *MAPK6*, *RNF11*, and *VPS37A* were downregulated following exercise training (**Fig. 12C**).

To determine if miR-19b-3p or miR-107 expression was correlated to expression of predicted target genes following exercise training, correlation analysis was performed. Expression levels of miR-19b-3p were negatively correlated to the expression of several target genes, including *MAPK6* and *RNF11* (**Table 3** and **Fig. 12D-E**). miR-107 showed a significant negative correlation with the expression of three out of four target genes (**Table 3**). Thus, expression levels of several genes are inversely correlated with miR-19b-3p or miR-107 expression during skeletal muscle adaptation following exercise training, suggesting a role for miRNA-mediated regulation during this process.

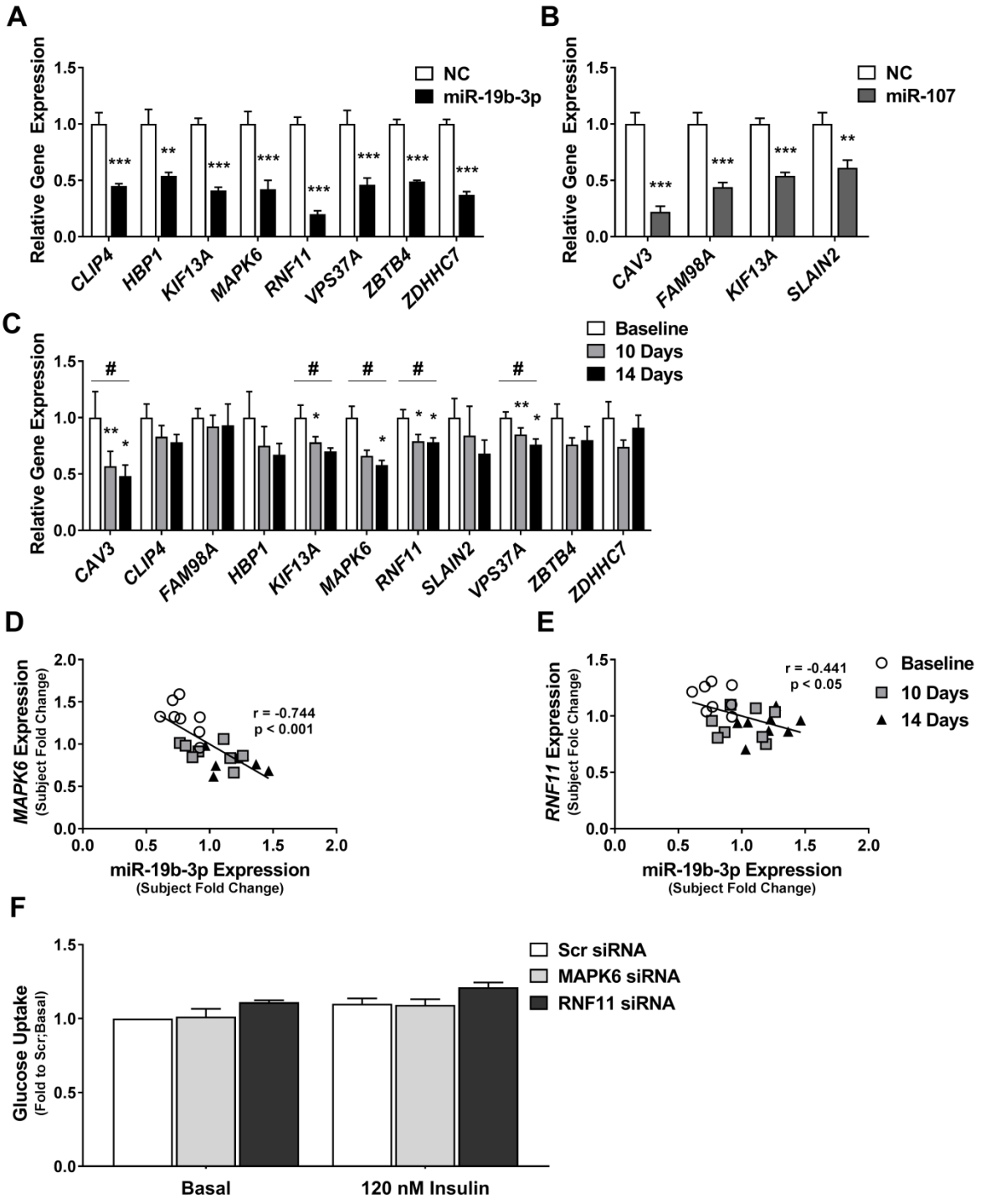

Figure 12. Expression of miR-19b-3p and miR-107 predicted target genes in human skeletal muscle following endurance exercise training. Human skeletal muscle cells were differentiated and transfected with a negative control (NC) miRNA precursor or miRNA precursors for miR-19b-3p or miR-107. **A-B)** Expression of predicted target genes determined by RT-qPCR in human skeletal muscle cells following overexpression of either **A)** miR-19b-3p or **B)** miR-107 (n = 6). **C)** Expression of miR-19b-3p and miR-107 predicted target genes before (Baseline) or after 10 or 14 days of exercise (n = 8). **D-E)** Pearson correlation of miR-19b-3p expression to either **D)** *MAPK6* or **E)** *RNF11* expression (n = 24). **F)** Human skeletal muscle cells were transfected either with a Scr control sequence or with siRNAs directed against MAPK6 or RNF11. Effects on basal and insulin-stimulated (120 nM Insulin) glucose uptake were determined (n = 3). Data are mean ± SEM. #p < 0.05 determined by 2-way repeated measures ANOVA. *p < 0.05, **p < 0.01, and ***p < 0.001 following paired Student's t-test or Sidak's *post hoc* testing.

The functions of several of the genes with decreased expression following exercise training in skeletal muscle are unknown. MAPK6 is an atypical MAP kinase that exhibits increasing protein abundance during skeletal muscle cell differentiation [208]. RNF11 is essential to editing of ubiquitination in cells and has an Akt phosphorylation site [209], suggesting it might be responsive to Akt activation. Nevertheless, the function of these genes in regulation of insulin sensitivity or glucose metabolism is unknown. To determine the functional effect of *MAPK6* and *RNF11* on glucose metabolism, these genes were silenced by siRNA transfection in human skeletal muscle cells. Preliminary data indicate that *RNF11* silencing increased basal and insulin-stimulated glucose transport in all three subjects studied, while *MAPK6* silencing was without any effect (**Fig. 12F**). Thus, decreased expression of *RNF11* following exercise training is associated with increased glucose transport, one of the characteristic adaptations observed following endurance exercise training in skeletal muscle.

Table 3. Correlation of miR-19b-3p or miR-107 to predicted targets in skeletal muscle.

Gene symbol	miR-19b-3p		miR-107	
	r	***p-value***	***r***	***p-value***
CAV3	-	-	-0.572	**
CLIP4	-0.738	***	-	-
FAM98A	-	-	-0.244	n.s
HBP1	-0.709	***	-	-
KIF13A	-0.715	***	0.631	***
MAPK6	-0.744	***	-	-
RNF11	-0.441	*	-	-
SLAIN2	-	-	-0.540	**
VPS37A	-0.628	**	-	-
ZBTB4	-0.636	***	-	-
ZDHHC7	-0.373	n.s	-	-

Pearson correlation of miRNA and gene expression in human skeletal muscle before or after ten or fourteen days of endurance exercise training. Gene expression was determined by RT-qPCR. Individual fold changes were calculated for each miRNA and gene, defined as expression level divided by average expression levels from Baseline, 10 days, and 14 days of training for the individual subject. *r*: Pearson's correlation coefficient. n.s: non-significant. - : correlation was not performed since the gene is not a predicted target of the miRNA. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

In conclusion, miR-19b-3p and miR-107 are induced by endurance exercise training in skeletal muscle. miR-19b-3p increases glucose metabolism and insulin sensitivity in both human and mouse skeletal muscle, indicating conservation of its functions. Silencing of *RNF11*, a gene decreased by miR-19b-3p overexpression and training, enhanced glucose uptake in human skeletal muscle cells. Thus, miR-19b-3p-dependent regulation of RNF11 in human skeletal muscle could be an adaptive response of skeletal muscle to promote glucose uptake following endurance exercise training.

5 CONCLUSION AND FUTURE PERSPECTIVES

5.1 CONCLUDING REMARKS

The three studies included in this thesis have focused on the roles of miRNAs as post-transcriptional regulators in skeletal muscle. See **Figure 13** for a summarizing overview of findings presented in this thesis. In all studies, expression of miRNAs has been determined in skeletal muscle biopsies or cells of human origin. This led to the identification of several novel miRNAs that are altered during the process of cell differentiation, dysregulated with T2D (miR-29 family members), and induced by endurance exercise training (miR-19b-3p and miR-107). Nevertheless, because changes of miRNAs expression do not directly imply functional alterations [210], an important aspect of miRNA research is the identification of their target genes.

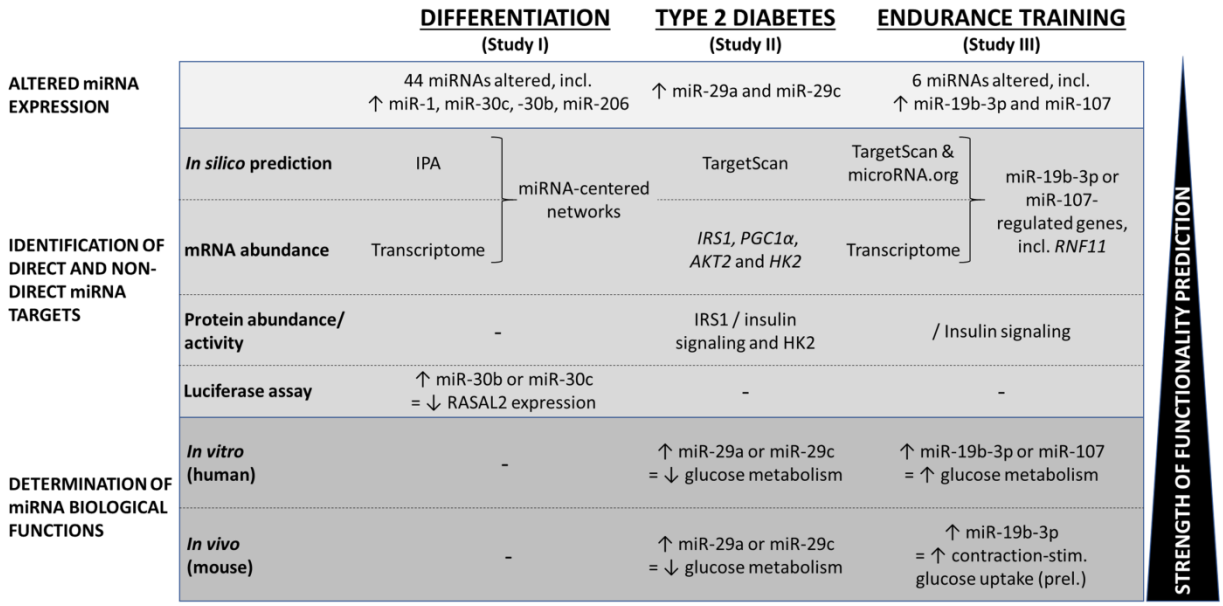

Figure 13. Highlighted findings reported on in this thesis. In this thesis, altered expression of miRNAs was identified in three different biological conditions or disease states. Target genes of differentially regulated miRNAs were predicted *in silico* and changes in mRNA or protein abundance was determined. Also, direct regulation of target genes was inferred by luciferase assay. Direct regulation of biological processes by altered miRNAs was studied *in vitro* and *in vivo*.

All studies reported herein aimed to identify targets of the differentially regulated miRNAs. In **Study I**, mRNA and miRNA expression in skeletal muscle cells during differentiation were measured with transcriptomic resolution to develop *in silico*-based models of miRNA-centered regulatory networks. In **Study II**, predicted miR-29 targets were identified using *in silico* algorithms. In **Study III**, transcriptomic data following overexpression of miR-19b-3p or miR-107 was overlapped with publicly available transcriptomic data sets to identify miRNA-predicted targets that are differentially expressed according to aerobic fitness. miRNA target prediction algorithms are useful tools to predict potential targets, but unfortunately *in silico* predictions typically generate large numbers of

false positives. Thus, experimentally elucidating functional effects of differentially regulated miRNAs in relevant models is necessary to augment the validity of *in silico* prediction of miRNA targets and functions.

To this end, in addition to predicting targets of miRNA that were differentially regulated in skeletal muscle, this thesis also aimed to determine direct functional effects of these miRNAs. Targets of miRNAs are generally well conserved between species [211]. Nevertheless, a majority of functional perturbation experiments presented herein originated from skeletal muscle cell cultures of human origin that increase the translational impact of our data. The altered miRNA expression was connected to functional aspects in each condition being studied, including identification of potential miRNA-centered regulatory networks during differentiation, and alterations of insulin sensitivity and glucose metabolism with training and in T2D. In **Study I**, direct regulation of miR-30b and miR-30c on expression of *RASAL2* was identified, with a reciprocal relationship between miR-30 expression found during differentiation. In **Study II**, impaired glucose uptake following miR-29a or miR-29c overexpression was associated with decreased *HK2* expression and hexokinase activity. *HK2* expression tended to be decreased in skeletal muscle of T2D patients, and *HK2* expression in skeletal muscle of healthy NGT and diabetic subjects was associated with increased miR-29c expression. Additionally, in **Study III**, miR-19b-3p and miR-107 regulate some of the endurance training-associated adaptations in skeletal muscle, including improved insulin sensitivity and glucose transport. Interestingly, an endurance training-regulated gene, *RNF11*, altered glucose transport similarly to miR-19b-3p and mimicked the response of training on glucose metabolism. Thus, in this study, I propose miR-19b-3p to be a potential regulator of endurance training-mediated potentiation of glucose transport.

The use of human *in vitro* models is essential to study direct effects of miRNAs in the context of human genetics. Yet, results generated from *in vitro* experiments are not directly translatable to clinical practice. To address this limitation, in **Study II–III**, the effects of the studied miRNAs on target regulation and metabolism were determined in intact mouse skeletal muscle. In **Study II**, skeletal muscle glucose metabolism was decreased *in vivo* following overexpression of miR-29a or -29c. In **Study III**, miR-19b-3p effects were conserved between human and mouse, and preliminary data indicate that overexpression of this miRNA in intact skeletal muscle of mice increased contraction-induced glucose uptake in 4 out of 5 subjects studied. The approach of combining human *in vitro* and mouse *in vivo* models increases the strength of our findings. Nevertheless, further additional experiments to those presented herein could bolster the translatability of our findings to human physiology. For example, in **Study I**, experiments designed to inhibit endogenous miR-30b or miR-30c to determine the regulation or the function of these miRNAs and their targets during skeletal muscle cell differentiation were not performed. Conclusions related to the miR-29-mediated effects on metabolism in **Study II** could have been strengthened by determining whether normalization of increased expression levels of miR-29, due to palmitate treatment in cells or in *ob/ob* skeletal muscle tissue of mice, could enhance insulin sensitivity in these conditions.

Additionally, in **Study III**, determining all relevant endogenous targets of miR-19b-3p or miR-107 could yield further insights. Since these miRNAs were induced following endurance training, determining their direct roles *in vivo* on endurance training adaptation-associated traits would be of interest. Our preliminary data indicates increased *ex vivo* contraction-stimulated glucose uptake following miR-19b-3p overexpression in intact skeletal muscle of mice, however additional experiments are needed to support these conclusions.

To advance the field of miRNA research in skeletal muscle, the focus of future research should shift from determining changed abundance of miRNAs toward resolving alterations in miRNA activities, which could be inferred, for example, by HITS-CLIP. Advances in genetic editing, such as CRISPR [176], could be used to directly determine roles of miRNAs on functional aspects of biology both *in vitro* and *in vivo*.

In conclusion, results presented in this thesis identified novel miRNAs altered during skeletal muscle maturation, insulin resistance and exercise training adaptation. Altogether, this work identifies miRNAs as potent regulators of functions important for skeletal muscle adaptability and disease.

5.2 miRNA AS THERAPEUTICS IN SKELETAL MUSCLE

As the abundance of various miRNAs are altered in skeletal muscle from several disease states, including cancer cachexia [212], atrophy [177], and diabetes (as shown in this thesis), pharmacological interference of dysfunctional miRNA expression could have attractive therapeutic potential. Initial trials with deliveries of miRNA mimics were ineffective due to degradation and inefficient delivery to targeted tissues [213]. Improved stability of miRNAs by chemical modifications, for example by using locked nucleic acids (LNA), has been achieved [214]. Targeting of skeletal muscle is complicated by the fact that it has a relatively large volume and low muscle selectivity was achieved with earlier delivery methods. High pharmacological doses might result in off-site effects, and safety of therapies using miRNA inhibitors or mimics have to be demonstrated: adverse events terminated a phase I clinical trial investigating the effects of a miR-34 mimic (MRX34) in cancer treatment. In mice, oligonucleotide-based delivery resulted in successful gene suppression in skeletal muscle [215]. In addition, chemical modifications are being tested to improve the selectivity of these oligonucleotides for delivery into skeletal muscle. For example, a recent study reported that oligonucleotide modifications using specific RNA aptamers improved muscle selectivity *in vivo* in mice [216]. Nevertheless, such treatment strategies demand validation in humans, particularly if the goal is to treat T2D given the chronic condition of this disease. Adeno-associated viruses of different serotypes can also be used to achieve tissue enrichment, and applications using tissue-specific promotors can reduce expression in non-targeted tissues. Adeno-associated viruses are employed in clinical trials for intramuscular injection and targeting of delivery to skeletal muscle [217]. Thus, specific targeting of skeletal muscle tissue can be achieved. In perspective of findings reported in this thesis and previous publications [187], miR-29 members, that are increased in insulin resistant tissues, could be attractive pharmaceutical targets.

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7 REFERENCES

1. Crick FHC: **On Protein Synthesis**: In F.K. Sanders. Symposia of the Society for Experimental Biology, Number XII: The Biological Replication of Macromolecules. Cambridge University Press; 1958.

2. Chen Z, Li S, Subramaniam S, Shyy JY, Chien S: **Epigenetic Regulation: A New Frontier for Biomedical Engineers**. *Annu Rev Biomed Eng* 2017, **19**:195-219.

3. Strahl BD, Allis CD: **The language of covalent histone modifications**. *Nature* 2000, **403**(6765):41-45.

4. The ENCODE Project Consortium: **An integrated encyclopedia of DNA elements in the human genome**. *Nature* 2012, **489**(7414):57-74.

5. Hombach S, Kretz M: **Non-coding RNAs: Classification, Biology and Functioning**. *Adv Exp Med Biol* 2016, **937**:3-17.

6. Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS: **Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells**. *Genes Dev* 2002, **16**(8):948-958.

7. Ambros V: **The functions of animal microRNAs**. *Nature* 2004, **431**(7006):350-355.

8. Lee RC, Feinbaum RL, Ambros V: **The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14**. *Cell* 1993, **75**(5):843-854.

9. Wightman B, Ha I, Ruvkun G: **Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans**. *Cell* 1993, **75**(5):855-862.

10. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T: **Identification of novel genes coding for small expressed RNAs**. *Science* 2001, **294**(5543):853-858.

11. Lau NC, Lim LP, Weinstein EG, Bartel DP: **An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans**. *Science* 2001, **294**(5543):858-862.

12. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC: **Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans**. *Nature* 1998, **391**(6669):806-811.

13. Bartel DP: **MicroRNAs: genomics, biogenesis, mechanism, and function**. *Cell* 2004, **116**(2):281-297.

14. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Rådmark O, Kim S *et al*: **The nuclear RNase III Drosha initiates microRNA processing**. *Nature* 2003, **425**(6956):415-419.

15. Gregory RI, Chendrimada TP, Shiekhattar R: **MicroRNA biogenesis: isolation and characterization of the microprocessor complex**. *Methods Mol Biol* 2006, **342**:33-47.

16. Yi R, Qin Y, Macara IG, Cullen BR: **Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs**. *Genes Dev* 2003, **17**(24):3011-3016.

17. Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G, Mello CC: **Genes and mechanisms related to RNA interference**

regulate expression of the small temporal RNAs that control C. elegans developmental timing. *Cell* 2001, **106**(1):23-34.

18. Hammond SM, Bernstein E, Beach D, Hannon GJ: **An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells**. *Nature* 2000, **404**(6775):293-296.

19. Meijer HA, Smith EM, Bushell M: **Regulation of miRNA strand selection: follow the leader?** *Biochem Soc Trans* 2014, **42**(4):1135-1140.

20. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB: **Prediction of mammalian microRNA targets**. *Cell* 2003, **115**(7):787-798.

21. Hutvagner G, Simard MJ: **Argonaute proteins: key players in RNA silencing**. *Nat Rev Mol Cell Biol* 2008, **9**(1):22-32.

22. Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshua-Tor L, Hannon GJ: **Argonaute2 is the catalytic engine of mammalian RNAi**. *Science* 2004, **305**(5689):1437-1441.

23. Chendrimada TP, Finn KJ, Ji X, Baillat D, Gregory RI, Liebhaber SA, Pasquinelli AE, Shiekhattar R: **MicroRNA silencing through RISC recruitment of eIF6**. *Nature* 2007, **447**(7146):823-828.

24. Petersen CP, Bordeleau ME, Pelletier J, Sharp PA: **Short RNAs repress translation after initiation in mammalian cells**. *Mol Cell* 2006, **21**(4):533-542.

25. Guo H, Ingolia NT, Weissman JS, Bartel DP: **Mammalian microRNAs predominantly act to decrease target mRNA levels**. *Nature* 2010, **466**(7308):835-840.

26. Friedman RC, Farh KK, Burge CB, Bartel DP: **Most mammalian mRNAs are conserved targets of microRNAs**. *Genome Res* 2009, **19**(1):92-105.

27. Shabalina SA, Koonin EV: **Origins and evolution of eukaryotic RNA interference**. *Trends Ecol Evol* 2008, **23**(10):578-587.

28. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Müller P *et al*: **Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA**. *Nature* 2000, **408**(6808):86-89.

29. Meunier J, Lemoine F, Soumillon M, Liechti A, Weier M, Guschanski K, Hu H, Khaitovich P, Kaessmann H: **Birth and expression evolution of mammalian microRNA genes**. *Genome Res* 2013, **23**(1):34-45.

30. Berezikov E: **Evolution of microRNA diversity and regulation in animals**. *Nat Rev Genet* 2011, **12**(12):846-860.

31. Zierath JR, Hawley JA: **Skeletal muscle fiber type: influence on contractile and metabolic properties**. *PLoS biology* 2004, **2**(10):e348.

32. Szent-Gyorgyi AG: **Calcium regulation of muscle contraction**. *Biophysical journal* 1975, **15**(7):707-723.

33. Rassier DE: **Sarcomere mechanics in striated muscles: from molecules to sarcomeres to cells**. *American journal of physiology Cell physiology* 2017, **313**(2):C134-C145.

34. Rolfe DF, Brown GC: **Cellular energy utilization and molecular origin of standard metabolic rate in mammals**. *Physiological reviews* 1997, **77**(3):731-758.

35. Zurlo F, Larson K, Bogardus C, Ravussin E: **Skeletal muscle metabolism is a major determinant of resting energy expenditure**. *The Journal of clinical investigation* 1990, **86**(5):1423-1427.

36. Wilson JM, Loenneke JP, Jo E, Wilson GJ, Zourdos MC, Kim JS: **The effects of endurance, strength, and power training on muscle fiber type shifting**. *Journal of strength and conditioning research* 2012, **26**(6):1724-1729.

37. Simoneau JA, Lortie G, Boulay MR, Marcotte M, Thibault MC, Bouchard C: **Human skeletal muscle fiber type alteration with high-intensity intermittent training**. *European journal of applied physiology and occupational physiology* 1985, **54**(3):250-253.

38. Russell AP, Feilchenfeldt J, Schreiber S, Praz M, Crettenand A, Gobelet C, Meier CA, Bell DR, Kralli A, Giacobino JP *et al*: **Endurance training in humans leads to fiber type-specific increases in levels of peroxisome proliferator-activated receptor-gamma coactivator-1 and peroxisome proliferator-activated receptor-alpha in skeletal muscle**. *Diabetes* 2003, **52**(12):2874-2881.

39. Lillioja S, Young AA, Culter CL, Ivy JL, Abbott WG, Zawadzki JK, Yki-Jarvinen H, Christin L, Secomb TW, Bogardus C: **Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man**. *The Journal of clinical investigation* 1987, **80**(2):415-424.

40. Oberbach A, Bossenz Y, Lehmann S, Niebauer J, Adams V, Paschke R, Schon MR, Bluher M, Punkt K: **Altered fiber distribution and fiber-specific glycolytic and oxidative enzyme activity in skeletal muscle of patients with type 2 diabetes**. *Diabetes care* 2006, **29**(4):895-900.

41. Henriksen EJ, Bourey RE, Rodnick KJ, Koranyi L, Permutt MA, Holloszy JO: **Glucose transporter protein content and glucose transport capacity in rat skeletal muscles**. *The American journal of physiology* 1990, **259**(4 Pt 1):E593-598.

42. Daugaard JR, Nielsen JN, Kristiansen S, Andersen JL, Hargreaves M, Richter EA: **Fiber type-specific expression of GLUT4 in human skeletal muscle: influence of exercise training**. *Diabetes* 2000, **49**(7):1092-1095.

43. van Rooij E, Quiat D, Johnson BA, Sutherland LB, Qi X, Richardson JA, Kelm RJ, Olson EN: **A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance**. *Dev Cell* 2009, **17**(5):662-673.

44. Gan Z, Rumsey J, Hazen BC, Lai L, Leone TC, Vega RB, Xie H, Conley KE, Auwerx J, Smith SR *et al*: **Nuclear receptor/microRNA circuitry links muscle fiber type to energy metabolism**. *The Journal of clinical investigation* 2013, **123**(6):2564-2575.

45. Dumont NA, Bentzinger CF, Sincennes MC, Rudnicki MA: **Satellite Cells and Skeletal Muscle Regeneration**. *Comprehensive Physiology* 2015, **5**(3):1027-1059.

46. Petrella JK, Kim JS, Cross JM, Kosek DJ, Bamman MM: **Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women**. *American journal of physiology Endocrinology and metabolism* 2006, **291**(5):E937-946.

47. Petrella JK, Kim JS, Mayhew DL, Cross JM, Bamman MM: **Potent myofiber hypertrophy during resistance training in humans is associated with satellite cell-mediated myonuclear addition: a cluster analysis**. *J Appl Physiol (1985)* 2008, **104**(6):1736-1742.

48. Lepper C, Partridge TA, Fan CM: **An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration**. *Development* 2011, **138**(17):3639-3646.

49. Braun T, Gautel M: **Transcriptional mechanisms regulating skeletal muscle differentiation, growth and homeostasis**. *Nat Rev Mol Cell Biol* 2011, **12**(6):349-361.

50. Bober E, Franz T, Arnold HH, Gruss P, Tremblay P: **Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells**. *Development* 1994, **120**(3):603-612.

51. Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA: **Pax7 is required for the specification of myogenic satellite cells**. *Cell* 2000, **102**(6):777-786.

52. Rudnicki MA, Schnegelsberg PN, Stead RH, Braun T, Arnold HH, Jaenisch R: **MyoD or Myf-5 is required for the formation of skeletal muscle**. *Cell* 1993, **75**(7):1351-1359.

53. Hasty P, Bradley A, Morris JH, Edmondson DG, Venuti JM, Olson EN, Klein WH: **Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene**. *Nature* 1993, **364**(6437):501-506.

54. O'Rourke JR, Georges SA, Seay HR, Tapscott SJ, McManus MT, Goldhamer DJ, Swanson MS, Harfe BD: **Essential role for Dicer during skeletal muscle development**. *Developmental biology* 2007, **311**(2):359-368.

55. Cheung TH, Quach NL, Charville GW, Liu L, Park L, Edalati A, Yoo B, Hoang P, Rando TA: **Maintenance of muscle stem-cell quiescence by microRNA-489**. *Nature* 2012, **482**(7386):524-528.

56. Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V: **Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation**. *Genome Biol* 2004, **5**(3):R13.

57. McCarthy JJ: **MicroRNA-206: the skeletal muscle-specific myomiR**. *Biochimica et biophysica acta* 2008, **1779**(11):682-691.

58. Rao PK, Kumar RM, Farkhondeh M, Baskerville S, Lodish HF: **Myogenic factors that regulate expression of muscle-specific microRNAs**. *Proceedings of the National Academy of Sciences of the United States of America* 2006, **103**(23):8721-8726.

59. Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL, Wang DZ: **The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation**. *Nature genetics* 2006, **38**(2):228-233.

60. Kim HK, Lee YS, Sivaprasad U, Malhotra A, Dutta A: **Muscle-specific microRNA miR-206 promotes muscle differentiation**. *The Journal of cell biology* 2006, **174**(5):677-687.

61. Chen JF, Tao Y, Li J, Deng Z, Yan Z, Xiao X, Wang DZ: **microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7**. *The Journal of cell biology* 2010, **190**(5):867-879.

62. Goljanek-Whysall K, Sweetman D, Abu-Elmagd M, Chapnik E, Dalmay T, Hornstein E, Munsterberg A: **MicroRNA regulation of the paired-box transcription factor Pax3 confers robustness to developmental timing of myogenesis**. *Proceedings of the National Academy of Sciences of the United States of America* 2011, **108**(29):11936-11941.

63. Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, Tsuchihashi T, McManus MT, Schwartz RJ, Srivastava D: **Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2**. *Cell* 2007, **129**(2):303-317.

64. Williams AH, Valdez G, Moresi V, Qi X, McAnally J, Elliott JL, Bassel-Duby R, Sanes JR, Olson EN: **MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice**. *Science* 2009, **326**(5959):1549-1554.

65. Sokol NS, Ambros V: **Mesodermally expressed Drosophila microRNA-1 is regulated by Twist and is required in muscles during larval growth**. *Genes Dev* 2005, **19**(19):2343-2354.

66. Liu N, Williams AH, Maxeiner JM, Bezprozvannaya S, Shelton JM, Richardson JA, Bassel-Duby R, Olson EN: **microRNA-206 promotes skeletal muscle regeneration and delays progression of Duchenne muscular dystrophy in mice**. *The Journal of clinical investigation* 2012, **122**(6):2054-2065.

67. Wüst S, Dröse S, Heidler J, Wittig I, Klockner I, Franko A, Bonke E, Günther S, Gärtner U, Boettger T *et al*: **Metabolic Maturation during Muscle Stem Cell Differentiation Is Achieved by miR-1/133a-Mediated Inhibition of the Dlk1-Dio3 Mega Gene Cluster**. *Cell Metab* 2018.

68. Koning M, Werker PM, van Luyn MJ, Krenning G, Harmsen MC: **A global downregulation of microRNAs occurs in human quiescent satellite cells during myogenesis**. *Differentiation* 2012, **84**(4):314-321.

69. Chen Y, Gelfond J, McManus LM, Shireman PK: **Temporal microRNA expression during in vitro myogenic progenitor cell proliferation and differentiation: regulation of proliferation by miR-682**. *Physiol Genomics* 2011, **43**(10):621-630.

70. Wong CF, Tellam RL: **MicroRNA-26a targets the histone methyltransferase Enhancer of Zeste homolog 2 during myogenesis**. *J Biol Chem* 2008, **283**(15):9836-9843.

71. Dmitriev P, Barat A, Polesskaya A, O'Connell MJ, Robert T, Dessen P, Walsh TA, Lazar V, Turki A, Carnac G *et al*: **Simultaneous miRNA and mRNA transcriptome profiling of human myoblasts reveals a novel set of myogenic differentiation-associated miRNAs and their target genes**. *BMC Genomics* 2013, **14**:265.

72. Castel D, Baghdadi MB, Mella S, Gayraud-Morel B, Marty V, Cavaillé J, Antoniewski C, Tajbakhsh S: **Small-RNA sequencing identifies dynamic microRNA deregulation during skeletal muscle lineage progression**. *Sci Rep* 2018, **8**(1):4208.

73. Qiu H, Liu N, Luo L, Zhong J, Tang Z, Kang K, Qu J, Peng W, Liu L, Li L *et al*: **MicroRNA-17-92 regulates myoblast proliferation and differentiation by targeting the ENH1/Id1 signaling axis**. *Cell Death Differ* 2016, **23**(10):1658-1669.

74. Guess MG, Barthel KK, Harrison BC, Leinwand LA: **miR-30 family microRNAs regulate myogenic differentiation and provide negative feedback on the microRNA pathway**. *PLoS One* 2015, **10**(2):e0118229.

75. Gong C, Li Z, Ramanujan K, Clay I, Zhang Y, Lemire-Brachat S, Glass DJ: **A long non-coding RNA, LncMyoD, regulates skeletal muscle differentiation by blocking IMP2-mediated mRNA translation**. *Dev Cell* 2015, **34**(2):181-191.

76. Yu X, Zhang Y, Li T, Ma Z, Jia H, Chen Q, Zhao Y, Zhai L, Zhong R, Li C *et al*: **Long non-coding RNA Linc-RAM enhances myogenic differentiation by interacting with MyoD**. *Nat Commun* 2017, **8**:14016.

77. Jin CF, Li Y, Ding XB, Li X, Zhang LL, Liu XF, Guo H: **lnc133b, a novel, long non-coding RNA, regulates bovine skeletal muscle satellite cell proliferation and differentiation by mediating miR-133b**. *Gene* 2017, **630**:35-43.

78. Zhu M, Liu J, Xiao J, Yang L, Cai M, Shen H, Chen X, Ma Y, Hu S, Wang Z *et al*: **Lnc-mg is a long non-coding RNA that promotes myogenesis**. *Nat Commun* 2017, **8**:14718.

79. Han X, Yang F, Cao H, Liang Z: **Malat1 regulates serum response factor through miR-133 as a competing endogenous RNA in myogenesis**. *FASEB J* 2015, **29**(7):3054-3064.

80. Roh E, Song DK, Kim MS: **Emerging role of the brain in the homeostatic regulation of energy and glucose metabolism**. *Exp Mol Med* 2016, **48**:e216.

81. Holst JJ, Gribble F, Horowitz M, Rayner CK: **Roles of the Gut in Glucose Homeostasis**. *Diabetes care* 2016, **39**(6):884-892.

82. Röder PV, Wu B, Liu Y, Han W: **Pancreatic regulation of glucose homeostasis**. *Exp Mol Med* 2016, **48**:e219.

83. Boucher J, Kleinridders A, Kahn CR: **Insulin receptor signaling in normal and insulin-resistant states**. *Cold Spring Harb Perspect Biol* 2014, **6**(1).

84. Raju TN: **The Nobel Chronicles. 1947: Carl Ferdinand Cori (1896-1984); Gerty Theresa Radnitz Cori (1896-1957); and Bernardo Alberto Houssay (1887-1971)**. *Lancet* 1999, **353**(9158):1108.

85. Kelley D, Mitrakou A, Marsh H, Schwenk F, Benn J, Sonnenberg G, Arcangeli M, Aoki T, Sorensen J, Berger M: **Skeletal muscle glycolysis, oxidation, and storage of an oral glucose load**. *The Journal of clinical investigation* 1988, **81**(5):1563-1571.

86. DeFronzo RA, Jacot E, Jequier E, Maeder E, Wahren J, Felber JP: **The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization**. *Diabetes* 1981, **30**(12):1000-1007.

87. WHO: **Obesity and Overweight.** 2017. http://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight.

88. Eckel RH, Kahn SE, Ferrannini E, Goldfine AB, Nathan DM, Schwartz MW, Smith RJ, Smith SR: **Obesity and type 2 diabetes: what can be unified and what needs to be individualized?** *J Clin Endocrinol Metab* 2011, **96**(6):1654-1663.

89. WHO: **Diabetes.** 2017. http://www.who.int/en/news-room/fact-sheets/detail/diabetes.

90. Cho NH, Shaw JE, Karuranga S, Huang Y, da Rocha Fernandes JD, Ohlrogge AW, Malanda B: **IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045**. *Diabetes Res Clin Pract* 2018, **138**:271-281.

91. Sarwar N, Gao P, Seshasai SR, Gobin R, Kaptoge S, Di Angelantonio E, Ingelsson E, Lawlor DA, Selvin E, Stampfer M *et al*: **Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies**. *Lancet* 2010, **375**(9733):2215-2222.

92. WHO: **The top 10 causes of death.** 2017. http://www.who.int/en/news-room/fact-sheets/detail/the-top-10-causes-of-death.

93. WHO.: **Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia**. 2006. http://www.who.int/diabetes/publications/Definition%20and-%20diagnosis%20of%20diabetes_new.pdf.

94. Halban PA, Polonsky KS, Bowden DW, Hawkins MA, Ling C, Mather KJ, Powers AC, Rhodes CJ, Sussel L, Weir GC: **β-cell failure in type 2 diabetes: postulated mechanisms and prospects for prevention and treatment**. *Diabetes care* 2014, **37**(6):1751-1758.

95. Nauck M, Stöckmann F, Ebert R, Creutzfeldt W: **Reduced incretin effect in type 2 (non-insulin-dependent) diabetes**. *Diabetologia* 1986, **29**(1):46-52.

96. Hardy OT, Czech MP, Corvera S: **What causes the insulin resistance underlying obesity?** *Curr Opin Endocrinol Diabetes Obes* 2012, **19**(2):81-87.

97. Cusi K: **The role of adipose tissue and lipotoxicity in the pathogenesis of type 2 diabetes**. *Curr Diab Rep* 2010, **10**(4):306-315.

98. Sharabi K, Tavares CD, Rines AK, Puigserver P: **Molecular pathophysiology of hepatic glucose production**. *Mol Aspects Med* 2015, **46**:21-33.

99. DeFronzo RA, Tripathy D: **Skeletal muscle insulin resistance is the primary defect in type 2 diabetes**. *Diabetes care* 2009, **32 Suppl 2**:S157-163.

100. Björnholm M, Kawano Y, Lehtihet M, Zierath JR: **Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity in skeletal muscle from NIDDM subjects after in vivo insulin stimulation**. *Diabetes* 1997, **46**(3):524-527.

101. Karlsson HK, Zierath JR, Kane S, Krook A, Lienhard GE, Wallberg-Henriksson H: **Insulin-stimulated phosphorylation of the Akt substrate AS160 is impaired in skeletal muscle of type 2 diabetic subjects**. *Diabetes* 2005, **54**(6):1692-1697.

102. Ryder JW, Yang J, Galuska D, Rincón J, Björnholm M, Krook A, Lund S, Pedersen O, Wallberg-Henriksson H, Zierath JR *et al*: **Use of a novel impermeable biotinylated photolabeling reagent to assess insulin- and hypoxia-stimulated cell surface GLUT4 content in skeletal muscle from type 2 diabetic patients**. *Diabetes* 2000, **49**(4):647-654.

103. Garvey WT, Maianu L, Zhu JH, Brechtel-Hook G, Wallace P, Baron AD: **Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance**. *The Journal of clinical investigation* 1998, **101**(11):2377-2386.

104. Krook A, Björnholm M, Galuska D, Jiang XJ, Fahlman R, Myers MG, Wallberg-Henriksson H, Zierath JR: **Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients**. *Diabetes* 2000, **49**(2):284-292.

105. Arner E, Mejhert N, Kulyté A, Balwierz PJ, Pachkov M, Cormont M, Lorente-Cebrián S, Ehrlund A, Laurencikiene J, Hedén P *et al*: **Adipose tissue microRNAs as regulators of CCL2 production in human obesity**. *Diabetes* 2012, **61**(8):1986-1993.

106. Thomou T, Mori MA, Dreyfuss JM, Konishi M, Sakaguchi M, Wolfrum C, Rao TN, Winnay JN, Garcia-Martin R, Grinspoon SK *et al*: **Adipose-derived circulating miRNAs regulate gene expression in other tissues**. *Nature* 2017, **542**(7642):450-455.

107. Krützfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M: **Silencing of microRNAs in vivo with 'antagomirs'**. *Nature* 2005, **438**(7068):685-689.

108. Hsu SH, Wang B, Kota J, Yu J, Costinean S, Kutay H, Yu L, Bai S, La Perle K, Chivukula RR *et al*: **Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver**. *The Journal of clinical investigation* 2012, **122**(8):2871-2883.

109. Trajkovski M, Hausser J, Soutschek J, Bhat B, Akin A, Zavolan M, Heim MH, Stoffel M: **MicroRNAs 103 and 107 regulate insulin sensitivity**. *Nature* 2011, **474**(7353):649-653.

110. Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, Pfeffer S, Tuschl T, Rajewsky N, Rorsman P *et al*: **A pancreatic islet-specific microRNA regulates insulin secretion**. *Nature* 2004, **432**(7014):226-230.

111. Gallagher IJ, Scheele C, Keller P, Nielsen AR, Remenyi J, Fischer CP, Roder K, Babraj J, Wahlestedt C, Hutvagner G *et al*: **Integration of microRNA changes in vivo identifies novel molecular features of muscle insulin resistance in type 2 diabetes**. *Genome Med* 2010, **2**(2):9.

112. Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, Puigserver P, Carlsson E, Ridderstråle M, Laurila E *et al*: **PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes**. *Nature genetics* 2003, **34**(3):267-273.

113. He A, Zhu L, Gupta N, Chang Y, Fang F: **Overexpression of micro ribonucleic acid 29, highly up-regulated in diabetic rats, leads to insulin resistance in 3T3-L1 adipocytes**. *Mol Endocrinol* 2007, **21**(11):2785-2794.

114. Mohamed JS, Hajira A, Pardo PS, Boriek AM: **MicroRNA-149 inhibits PARP-2 and promotes mitochondrial biogenesis via SIRT-1/PGC-1α network in skeletal muscle**. *Diabetes* 2014, **63**(5):1546-1559.

115. Chen GQ, Lian WJ, Wang GM, Wang S, Yang YQ, Zhao ZW: **Altered microRNA expression in skeletal muscle results from high-fat diet-induced insulin resistance in mice**. *Mol Med Rep* 2012, **5**(5):1362-1368.

116. Granjon A, Gustin MP, Rieusset J, Lefai E, Meugnier E, Güller I, Cerutti C, Paultre C, Disse E, Rabasa-Lhoret R *et al*: **The microRNA signature in response to insulin reveals its implication in the transcriptional action of insulin in human skeletal**

muscle and the role of a sterol regulatory element-binding protein-1c/myocyte enhancer factor 2C pathway. *Diabetes* 2009, **58**(11):2555-2564.

117. Booth FW, Roberts CK, Laye MJ: **Lack of exercise is a major cause of chronic diseases**. *Comprehensive Physiology* 2012, **2**(2):1143-1211.

118. Reimers CD, Knapp G, Reimers AK: **Does physical activity increase life expectancy? A review of the literature**. *J Aging Res* 2012, **2012**:243958.

119. Bize R, Johnson JA, Plotnikoff RC: **Physical activity level and health-related quality of life in the general adult population: a systematic review**. *Prev Med* 2007, **45**(6):401-415.

120. Lieberman DE: **Is Exercise Really Medicine? An Evolutionary Perspective**. *Curr Sports Med Rep* 2015, **14**(4):313-319.

121. Matheson GO, Klügl M, Engebretsen L, Bendiksen F, Blair SN, Börjesson M, Budgett R, Derman W, Erdener U, Ioannidis JP *et al*: **Prevention and management of non-communicable disease: the IOC consensus statement, Lausanne 2013**. *Sports Med* 2013, **43**(11):1075-1088.

122. Thompson D, Karpe F, Lafontan M, Frayn K: **Physical activity and exercise in the regulation of human adipose tissue physiology**. *Physiological reviews* 2012, **92**(1):157-191.

123. Berzigotti A, Saran U, Dufour JF: **Physical activity and liver diseases**. *Hepatology* 2016, **63**(3):1026-1040.

124. Slentz CA, Tanner CJ, Bateman LA, Durheim MT, Huffman KM, Houmard JA, Kraus WE: **Effects of exercise training intensity on pancreatic beta-cell function**. *Diabetes care* 2009, **32**(10):1807-1811.

125. Schwingshackl L, Dias S, Hoffmann G: **Impact of long-term lifestyle programmes on weight loss and cardiovascular risk factors in overweight/obese participants: a systematic review and network meta-analysis**. *Syst Rev* 2014, **3**:130.

126. Gill JM, Cooper AR: **Physical activity and prevention of type 2 diabetes mellitus**. *Sports Med* 2008, **38**(10):807-824.

127. Batacan RB, Duncan MJ, Dalbo VJ, Tucker PS, Fenning AS: **Effects of high-intensity interval training on cardiometabolic health: a systematic review and meta-analysis of intervention studies**. *Br J Sports Med* 2017, **51**(6):494-503.

128. Church TS, Blair SN, Cocreham S, Johannsen N, Johnson W, Kramer K, Mikus CR, Myers V, Nauta M, Rodarte RQ *et al*: **Effects of aerobic and resistance training on hemoglobin A1c levels in patients with type 2 diabetes: a randomized controlled trial**. *JAMA* 2010, **304**(20):2253-2262.

129. Colberg SR, Sigal RJ, Yardley JE, Riddell MC, Dunstan DW, Dempsey PC, Horton ES, Castorino K, Tate DF: **Physical Activity/Exercise and Diabetes: A Position Statement of the American Diabetes Association**. *Diabetes care* 2016, **39**(11):2065-2079.

130. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM, Group DPPR: **Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin**. *N Engl J Med* 2002, **346**(6):393-403.

131. Morrison DJ, Kowalski GM, Grespan E, Mari A, Bruce CR, Wadley GD: **Measurement of postprandial glucose fluxes in response to acute and chronic**

endurance exercise in healthy humans. *American journal of physiology Endocrinology and metabolism* 2017.

132. Babraj JA, Vollaard NB, Keast C, Guppy FM, Cottrell G, Timmons JA: **Extremely short duration high intensity interval training substantially improves insulin action in young healthy males**. *BMC Endocr Disord* 2009, **9**:3.

133. Rogers MA, King DS, Hagberg JM, Ehsani AA, Holloszy JO: **Effect of 10 days of physical inactivity on glucose tolerance in master athletes**. *J Appl Physiol (1985)* 1990, **68**(5):1833-1837.

134. Egan B, Zierath JR: **Exercise metabolism and the molecular regulation of skeletal muscle adaptation**. *Cell Metab* 2013, **17**(2):162-184.

135. Schmutz S, Däpp C, Wittwer M, Vogt M, Hoppeler H, Flück M: **Endurance training modulates the muscular transcriptome response to acute exercise**. *Pflugers Arch* 2006, **451**(5):678-687.

136. Egan B, O'Connor PL, Zierath JR, O'Gorman DJ: **Time course analysis reveals gene-specific transcript and protein kinetics of adaptation to short-term aerobic exercise training in human skeletal muscle**. *PLoS One* 2013, **8**(9):e74098.

137. Keller P, Vollaard NB, Gustafsson T, Gallagher IJ, Sundberg CJ, Rankinen T, Britton SL, Bouchard C, Koch LG, Timmons JA: **A transcriptional map of the impact of endurance exercise training on skeletal muscle phenotype**. *J Appl Physiol (1985)* 2011, **110**(1):46-59.

138. Timmons JA, Jansson E, Fischer H, Gustafsson T, Greenhaff PL, Ridden J, Rachman J, Sundberg CJ: **Modulation of extracellular matrix genes reflects the magnitude of physiological adaptation to aerobic exercise training in humans**. *BMC Biol* 2005, **3**:19.

139. Neubauer O, Sabapathy S, Ashton KJ, Desbrow B, Peake JM, Lazarus R, Wessner B, Cameron-Smith D, Wagner KH, Haseler LJ *et al*: **Time course-dependent changes in the transcriptome of human skeletal muscle during recovery from endurance exercise: from inflammation to adaptive remodeling**. *J Appl Physiol (1985)* 2014, **116**(3):274-287.

140. Wagner PD: **Skeletal muscle angiogenesis. A possible role for hypoxia**. *Adv Exp Med Biol* 2001, **502**:21-38.

141. Lee AD, Hansen PA, Holloszy JO: **Wortmannin inhibits insulin-stimulated but not contraction-stimulated glucose transport activity in skeletal muscle**. *FEBS Lett* 1995, **361**(1):51-54.

142. Hayashi T, Hirshman MF, Kurth EJ, Winder WW, Goodyear LJ: **Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport**. *Diabetes* 1998, **47**(8):1369-1373.

143. Wojtaszewski JF, Higaki Y, Hirshman MF, Michael MD, Dufresne SD, Kahn CR, Goodyear LJ: **Exercise modulates postreceptor insulin signaling and glucose transport in muscle-specific insulin receptor knockout mice**. *The Journal of clinical investigation* 1999, **104**(9):1257-1264.

144. Cartee GD: **Roles of TBC1D1 and TBC1D4 in insulin- and exercise-stimulated glucose transport of skeletal muscle**. *Diabetologia* 2015, **58**(1):19-30.

145. Frosig C, Rose AJ, Treebak JT, Kiens B, Richter EA, Wojtaszewski JF: **Effects of endurance exercise training on insulin signaling in human skeletal muscle: interactions at the level of phosphatidylinositol 3-kinase, Akt, and AS160**. *Diabetes* 2007, **56**(8):2093-2102.

146. Deshmukh A, Coffey VG, Zhong Z, Chibalin AV, Hawley JA, Zierath JR: **Exercise-induced phosphorylation of the novel Akt substrates AS160 and filamin A in human skeletal muscle**. *Diabetes* 2006, **55**(6):1776-1782.

147. Kraniou GN, Cameron-Smith D, Hargreaves M: **Acute exercise and GLUT4 expression in human skeletal muscle: influence of exercise intensity**. *J Appl Physiol (1985)* 2006, **101**(3):934-937.

148. O'Gorman DJ, Karlsson HK, McQuaid S, Yousif O, Rahman Y, Gasparro D, Glund S, Chibalin AV, Zierath JR, Nolan JJ: **Exercise training increases insulin-stimulated glucose disposal and GLUT4 (SLC2A4) protein content in patients with type 2 diabetes**. *Diabetologia* 2006, **49**(12):2983-2992.

149. Vind BF, Pehmøller C, Treebak JT, Birk JB, Hey-Mogensen M, Beck-Nielsen H, Zierath JR, Wojtaszewski JF, Højlund K: **Impaired insulin-induced site-specific phosphorylation of TBC1 domain family, member 4 (TBC1D4) in skeletal muscle of type 2 diabetes patients is restored by endurance exercise-training**. *Diabetologia* 2011, **54**(1):157-167.

150. Pilegaard H, Saltin B, Neufer PD: **Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle**. *J Physiol* 2003, **546**(Pt 3):851-858.

151. Correia JC, Ferreira DM, Ruas JL: **Intercellular: local and systemic actions of skeletal muscle PGC-1s**. *Trends Endocrinol Metab* 2015, **26**(6):305-314.

152. Kelley DE, Mandarino LJ: **Fuel selection in human skeletal muscle in insulin resistance: a reexamination**. *Diabetes* 2000, **49**(5):677-683.

153. Kelley DE, He J, Menshikova EV, Ritov VB: **Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes**. *Diabetes* 2002, **51**(10):2944-2950.

154. Kelley DE, Simoneau JA: **Impaired free fatty acid utilization by skeletal muscle in non-insulin-dependent diabetes mellitus**. *The Journal of clinical investigation* 1994, **94**(6):2349-2356.

155. Goodpaster BH, Katsiaras A, Kelley DE: **Enhanced fat oxidation through physical activity is associated with improvements in insulin sensitivity in obesity**. *Diabetes* 2003, **52**(9):2191-2197.

156. Bruce CR, Anderson MJ, Carey AL, Newman DG, Bonen A, Kriketos AD, Cooney GJ, Hawley JA: **Muscle oxidative capacity is a better predictor of insulin sensitivity than lipid status**. *J Clin Endocrinol Metab* 2003, **88**(11):5444-5451.

157. Holloszy JO, Coyle EF: **Adaptations of skeletal muscle to endurance exercise and their metabolic consequences**. *Journal of applied physiology: respiratory, environmental and exercise physiology* 1984, **56**(4):831-838.

158. Russell AP, Lamon S, Boon H, Wada S, Guller I, Brown EL, Chibalin AV, Zierath JR, Snow RJ, Stepto N *et al*: **Regulation of miRNAs in human skeletal muscle following acute endurance exercise and short-term endurance training**. *J Physiol* 2013, **591**(18):4637-4653.

159. Nielsen S, Scheele C, Yfanti C, Akerström T, Nielsen AR, Pedersen BK, Laye MJ, Laye M: **Muscle specific microRNAs are regulated by endurance exercise in human skeletal muscle**. *J Physiol* 2010, **588**(Pt 20):4029-4037.

160. Davidsen PK, Gallagher IJ, Hartman JW, Tarnopolsky MA, Dela F, Helge JW, Timmons JA, Phillips SM: **High responders to resistance exercise training demonstrate differential regulation of skeletal muscle microRNA expression**. *J Appl Physiol (1985)* 2011, **110**(2):309-317.

161. Ringholm S, Biensø RS, Kiilerich K, Guadalupe-Grau A, Aachmann-Andersen NJ, Saltin B, Plomgaard P, Lundby C, Wojtaszewski JF, Calbet JA *et al*: **Bed rest reduces metabolic protein content and abolishes exercise-induced mRNA responses in human skeletal muscle**. *American journal of physiology Endocrinology and metabolism* 2011, **301**(4):E649-658.

162. Režen T, Kovanda A, Eiken O, Mekjavic IB, Rogelj B: **Expression changes in human skeletal muscle miRNAs following 10 days of bed rest in young healthy males**. *Acta Physiol (Oxf)* 2014, **210**(3):655-666.

163. Al-Khalili L, Kramer D, Wretenberg P, Krook A: **Human skeletal muscle cell differentiation is associated with changes in myogenic markers and enhanced insulin-mediated MAPK and PKB phosphorylation**. *Acta physiologica Scandinavica* 2004, **180**(4):395-403.

164. Rando TA, Blau HM: **Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy**. *The Journal of cell biology* 1994, **125**(6):1275-1287.

165. Wong SK, Chin KY, Suhaimi FH, Fairus A, Ima-Nirwana S: **Animal models of metabolic syndrome: a review**. *Nutr Metab (Lond)* 2016, **13**:65.

166. Dubuc PU: **The development of obesity, hyperinsulinemia, and hyperglycemia in ob/ob mice**. *Metabolism* 1976, **25**(12):1567-1574.

167. McMahon JM, Signori E, Wells KE, Fazio VM, Wells DJ: **Optimisation of electrotransfer of plasmid into skeletal muscle by pretreatment with hyaluronidase -- increased expression with reduced muscle damage**. *Gene Ther* 2001, **8**(16):1264-1270.

168. Lewis BP, Burge CB, Bartel DP: **Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets**. *Cell* 2005, **120**(1):15-20.

169. Betel D, Wilson M, Gabow A, Marks DS, Sander C: **The microRNA.org resource: targets and expression**. *Nucleic Acids Res* 2008, **36**(Database issue):D149-153.

170. Agarwal V, Bell GW, Nam JW, Bartel DP: **Predicting effective microRNA target sites in mammalian mRNAs**. *Elife* 2015, **4**.

171. Pinzón N, Li B, Martinez L, Sergeeva A, Presumey J, Apparailly F, Seitz H: **microRNA target prediction programs predict many false positives**. *Genome Res* 2017, **27**(2):234-245.

172. Chi SW, Zang JB, Mele A, Darnell RB: **Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps**. *Nature* 2009, **460**(7254):479-486.

173. Hausser J, Zavolan M: **Identification and consequences of miRNA-target interactions--beyond repression of gene expression**. *Nat Rev Genet* 2014, **15**(9):599-612.

174. Xie SJ, Li JH, Chen HF, Tan YY, Liu SR, Zhang Y, Xu H, Yang JH, Liu S, Zheng LL *et al*: **Inhibition of the JNK/MAPK signaling pathway by myogenesis-associated miRNAs is required for skeletal muscle development**. *Cell Death Differ* 2018.

175. Choi WY, Giraldez AJ, Schier AF: **Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430**. *Science* 2007, **318**(5848):271-274.

176. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E: **A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity**. *Science* 2012, **337**(6096):816-821.

177. Eisenberg I, Eran A, Nishino I, Moggio M, Lamperti C, Amato AA, Lidov HG, Kang PB, North KN, Mitrani-Rosenbaum S *et al*: **Distinctive patterns of microRNA expression in primary muscular disorders**. *Proceedings of the National Academy of Sciences of the United States of America* 2007, **104**(43):17016-17021.

178. Fukada S, Yamaguchi M, Kokubo H, Ogawa R, Uezumi A, Yoneda T, Matev MM, Motohashi N, Ito T, Zolkiewska A *et al*: **Hesr1 and Hesr3 are essential to generate undifferentiated quiescent satellite cells and to maintain satellite cell numbers**. *Development* 2011, **138**(21):4609-4619.

179. Repele A, Lupi R, Eaton S, Urbani L, De Coppi P, Campanella M: **Cell metabolism sets the differences between subpopulations of satellite cells (SCs)**. *BMC cell biology* 2013, **14**:24.

180. Coleman SK, Cao AW, Rebalka IA, Gyulay G, Chambers PJ, Tupling AR, Austin RC, Hawke TJ: **The Pleckstrin homology like domain family member, TDAG51, is temporally regulated during skeletal muscle regeneration**. *Biochemical and biophysical research communications* 2018, **495**(1):499-505.

181. Lammens T, Li J, Leone G, De Veylder L: **Atypical E2Fs: new players in the E2F transcription factor family**. *Trends in cell biology* 2009, **19**(3):111-118.

182. von Hofsten J, Elworthy S, Gilchrist MJ, Smith JC, Wardle FC, Ingham PW: **Prdm1- and Sox6-mediated transcriptional repression specifies muscle fibre type in the zebrafish embryo**. *EMBO reports* 2008, **9**(7):683-689.

183. Soleimani VD, Yin H, Jahani-Asl A, Ming H, Kockx CE, van Ijcken WF, Grosveld F, Rudnicki MA: **Snail regulates MyoD binding-site occupancy to direct enhancer switching and differentiation-specific transcription in myogenesis**. *Mol Cell* 2012, **47**(3):457-468.

184. Noto S, Maeda T, Hattori S, Inazawa J, Imamura M, Asaka M, Hatakeyama M: **A novel human RasGAP-like gene that maps within the prostate cancer susceptibility locus at chromosome 1q25**. *FEBS Lett* 1998, **441**(1):127-131.

185. Jia Z, Liu W, Gong L, Xiao Z: **Downregulation of RASAL2 promotes the proliferation, epithelial-mesenchymal transition and metastasis of colorectal cancer cells**. *Oncology letters* 2017, **13**(3):1379-1385.

186. Fang JF, Zhao HP, Wang ZF, Zheng SS: **Upregulation of RASAL2 promotes proliferation and metastasis, and is targeted by miR-203 in hepatocellular carcinoma**. *Mol Med Rep* 2017, **15**(5):2720-2726.

187. Zhu H, Leung SW: **Identification of microRNA biomarkers in type 2 diabetes: a meta-analysis of controlled profiling studies**. *Diabetologia* 2015, **58**(5):900-911.

188. Portha B, Giroix MH, Tourrel-Cuzin C, Le-Stunff H, Movassat J: **The GK rat: a prototype for the study of non-overweight type 2 diabetes**. *Methods Mol Biol* 2012, **933**:125-159.

189. Dahlmans D, Houzelle A, Jorgensen JA, Phielix E, Lindeboom L, Hesselink MKC, Schrauwen P, Hoeks J: **Evaluation of Muscle microRNA Expression in Relation to Human Peripheral Insulin Sensitivity: A Cross-Sectional Study in Metabolically Distinct Subject Groups**. *Frontiers in physiology* 2017, **8**:711.

190. Yang WM, Jeong HJ, Park SY, Lee W: **Induction of miR-29a by saturated fatty acids impairs insulin signaling and glucose uptake through translational repression of IRS-1 in myocytes**. *FEBS Lett* 2014, **588**(13):2170-2176.

191. Egan B, Dowling P, O'Connor PL, Henry M, Meleady P, Zierath JR, O'Gorman DJ: **2-D DIGE analysis of the mitochondrial proteome from human skeletal muscle reveals time course-dependent remodelling in response to 14 consecutive days of endurance exercise training**. *Proteomics* 2011, **11**(8):1413-1428.

192. Zhou Y, Gu P, Shi W, Li J, Hao Q, Cao X, Lu Q, Zeng Y: **MicroRNA-29a induces insulin resistance by targeting PPARdelta in skeletal muscle cells**. *International journal of molecular medicine* 2016, **37**(4):931-938.

193. Liang J, Liu C, Qiao A, Cui Y, Zhang H, Cui A, Zhang S, Yang Y, Xiao X, Chen Y *et al*: **MicroRNA-29a-c decrease fasting blood glucose levels by negatively regulating hepatic gluconeogenesis**. *Journal of hepatology* 2013, **58**(3):535-542.

194. Vestergaard H, Bjorbaek C, Hansen T, Larsen FS, Granner DK, Pedersen O: **Impaired activity and gene expression of hexokinase II in muscle from non-insulin-dependent diabetes mellitus patients**. *The Journal of clinical investigation* 1995, **96**(6):2639-2645.

195. Osawa H, Sutherland C, Robey RB, Printz RL, Granner DK: **Analysis of the signaling pathway involved in the regulation of hexokinase II gene transcription by insulin**. *J Biol Chem* 1996, **271**(28):16690-16694.

196. Houmard JA, Shaw CD, Hickey MS, Tanner CJ: **Effect of short-term exercise training on insulin-stimulated PI 3-kinase activity in human skeletal muscle**. *The American journal of physiology* 1999, **277**(6 Pt 1):E1055-1060.

197. Frøsig C, Rose AJ, Treebak JT, Kiens B, Richter EA, Wojtaszewski JF: **Effects of endurance exercise training on insulin signaling in human skeletal muscle: interactions at the level of phosphatidylinositol 3-kinase, Akt, and AS160**. *Diabetes* 2007, **56**(8):2093-2102.

198. Rasmussen M, Zierath JR, Barres R: **Dynamic epigenetic responses to muscle contraction**. *Drug discovery today* 2014, **19**(7):1010-1014.

199. Safdar A, Abadi A, Akhtar M, Hettinga BP, Tarnopolsky MA: **miRNA in the regulation of skeletal muscle adaptation to acute endurance exercise in C57Bl/6J male mice**. *PLoS One* 2009, **4**(5):e5610.

200. Sano H, Kane S, Sano E, Miinea CP, Asara JM, Lane WS, Garner CW, Lienhard GE: **Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation**. *J Biol Chem* 2003, **278**(17):14599-14602.

201. Liu GL, Yang HJ, Liu B, Liu T: **Effects of MicroRNA-19b on the Proliferation, Apoptosis, and Migration of Wilms' Tumor Cells Via the PTEN/PI3K/AKT Signaling Pathway**. *Journal of cellular biochemistry* 2017, **118**(10):3424-3434.

202. Li RK, Gao J, Guo LH, Huang GQ, Luo WH: **PTENP1 acts as a ceRNA to regulate PTEN by sponging miR-19b and explores the biological role of PTENP1 in breast cancer**. *Cancer gene therapy* 2017, **24**(7):309-315.

203. Li X, Xie W, Xie C, Huang C, Zhu J, Liang Z, Deng F, Zhu M, Zhu W, Wu R *et al*: **Curcumin modulates miR-19/PTEN/AKT/p53 axis to suppress bisphenol A-induced MCF-7 breast cancer cell proliferation**. *Phytotherapy research : PTR* 2014, **28**(10):1553-1560.

204. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW: **Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN**. *Cell* 1998, **95**(1):29-39.

205. Xiong J, Wang D, Wei A, Lu H, Tan C, Li A, Tang J, Wang Y, He S, Liu X *et al*: **Deregulated expression of miR-107 inhibits metastasis of PDAC through inhibition PI3K/Akt signaling via caveolin-1 and PTEN**. *Experimental cell research* 2017, **361**(2):316-323.

206. Turan N, Kalko S, Stincone A, Clarke K, Sabah A, Howlett K, Curnow SJ, Rodriguez DA, Cascante M, O'Neill L *et al*: **A systems biology approach identifies molecular networks defining skeletal muscle abnormalities in chronic obstructive pulmonary disease**. *PLoS computational biology* 2011, **7**(9):e1002129.

207. Lanza IR, Short DK, Short KR, Raghavakaimal S, Basu R, Joyner MJ, McConnell JP, Nair KS: **Endurance exercise as a countermeasure for aging**. *Diabetes* 2008, **57**(11):2933-2942.

208. Coulombe P, Rodier G, Pelletier S, Pellerin J, Meloche S: **Rapid turnover of extracellular signal-regulated kinase 3 by the ubiquitin-proteasome pathway defines a novel paradigm of mitogen-activated protein kinase regulation during cellular differentiation**. *Molecular and cellular biology* 2003, **23**(13):4542-4558.

209. Azmi P, Seth A: **RNF11 is a multifunctional modulator of growth factor receptor signalling and transcriptional regulation**. *Eur J Cancer* 2005, **41**(16):2549-2560.

210. Mullokandov G, Baccarini A, Ruzo A, Jayaprakash AD, Tung N, Israelow B, Evans MJ, Sachidanandam R, Brown BD: **High-throughput assessment of microRNA activity and function using microRNA sensor and decoy libraries**. *Nature methods* 2012, **9**(8):840-846.

211. Chen K, Rajewsky N: **Deep conservation of microRNA-target relationships and 3'UTR motifs in vertebrates, flies, and nematodes**. *Cold Spring Harbor symposia on quantitative biology* 2006, **71**:149-156.

212. Narasimhan A, Ghosh S, Stretch C, Greiner R, Bathe OF, Baracos V, Damaraju S: **Small RNAome profiling from human skeletal muscle: novel miRNAs and their targets associated with cancer cachexia**. *Journal of cachexia, sarcopenia and muscle* 2017, **8**(3):405-416.

213. Rupaimoole R, Slack FJ: **MicroRNA therapeutics: towards a new era for the management of cancer and other diseases**. *Nature reviews Drug discovery* 2017, **16**(3):203-222.

214. Elmen J, Thonberg H, Ljungberg K, Frieden M, Westergaard M, Xu Y, Wahren B, Liang Z, Orum H, Koch T *et al*: **Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality**. *Nucleic Acids Res* 2005, **33**(1):439-447.

215. Lieberman AP, Yu Z, Murray S, Peralta R, Low A, Guo S, Yu XX, Cortes CJ, Bennett CF, Monia BP *et al*: **Peripheral androgen receptor gene suppression rescues disease in mouse models of spinal and bulbar muscular atrophy**. *Cell reports* 2014, **7**(3):774-784.

216. Philippou S, Mastroyiannopoulos NP, Makrides N, Lederer CW, Kleanthous M, Phylactou LA: **Selection and Identification of Skeletal-Muscle-Targeted RNA Aptamers**. *Molecular therapy Nucleic acids* 2018, **10**:199-214.

217. Bengtsson NE, Seto JT, Hall JK, Chamberlain JS, Odom GL: **Progress and prospects of gene therapy clinical trials for the muscular dystrophies**. *Human molecular genetics* 2016, **25**(R1):R9-17.