J Physiol 588.21 (2010) pp 4075–4087

The Journal of Physiology

TOPICAL REVIEW

MicroRNAs in skeletal muscle: their role and regulation in development, disease and function

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Maintaining skeletal muscle function throughout the lifespan is a prerequisite for good health and independent living. For skeletal muscle to consistently function at optimal levels, the efficient activation of processes that regulate muscle development, growth, regeneration and metabolism is required. Numerous conditions including neuromuscular disorders, physical inactivity, chronic disease and ageing are associated with perturbations in skeletal muscle function. A loss or reduction in skeletal muscle function often leads to increased morbidity and mortality either directly, or indirectly, via the development of secondary diseases such as diabetes, obesity, cardiovascular and respiratory disease. Identifying mechanisms which influence the processes regulating skeletal muscle function is a key priority. The discovery of microRNAs (miRNAs) provides a new avenue that will extend our knowledge of factors controlling skeletal muscle function. miRNAs may also improve our understanding and application of current therapeutic approaches as well as enable the identification of new therapeutic strategies and targets aimed at maintaining and/or improving skeletal muscle health. This review brings together the latest developments in skeletal muscle miRNA biology and focuses on their role and regulation under physiological and patho-physiological conditions with an emphasis on: myogenesis, hypertrophy, atrophy and regeneration; exercise and nutrition; muscle disease, ageing, diabetes and obesity.

(Received 4 June 2010; accepted after revision 16 August 2010; first published online 19 August 2010)
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Abbreviations DMD, Duchene muscular dystrophy; EAAs, essential amino acids; FoXO, Forkhead transcription factor; HS, hind limb suspension; MEF2, myocyte enhancer factor 2; MHC, myosin heavy chain; miRNAs, microRNAs; MRFs, myogenic regulatory factors; MyoD, myogenic differentiation 1; SRF, serum response factor; T2D, type 2 diabetes; TWEAK, TNF-related weak inducer of apoptosis.

Introduction

Skeletal muscle makes up approximately 40% of the body's mass. The principal function of skeletal muscle is contraction-related in order to control movement and posture, a process generally requiring the oxidation of nutrients delivered to the body through the diet. Skeletal muscle has a unique ability to adapt to changes in

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DOI: 10.1113/jphysiol.2010.194175

its environment. In response to increased use through regular exercise training, skeletal muscle can increase its size and capacity to produce force (Fry, 2004), improve its resistance to fatigue and enhance its oxidation of carbohydrates and fats (Coyle, 2000). In contrast, ageing, sedentary lifestyles and immobilization, as well as neuromuscular disorders (Lynch, 2001) and chronic disease conditions (Jagoe & Goldberg, 2001; Di Giovanni *et al.* 2004; Tisdale, 2004; Doucet *et al.* 2007) are associated with the loss of skeletal muscle mass and function. Reduced muscle size and contraction have negative consequences for skeletal muscle carbohydrate and fat metabolism (Stein & Wade, 2005) which increases the risk of metabolic disorders (Corpeleijn *et al.* 2009). The important role of muscle mass and function in numerous disease conditions underpins the necessity of understanding the molecular factors which control skeletal muscle development over the entire lifespan, from embryonic, postnatal and adult development, through to the changes which occur in the elderly. The recent identification of microRNAs (miRNAs) has opened up a new field of investigation to understand the molecular processes which control numerous disease states (Croce & Calin, 2005; Pandey *et al.* 2009; Pauley *et al.* 2009; Saal & Harvey, 2009). This review will highlight our present understanding of skeletal muscle miRNA regulation and how miRNAs may control skeletal muscle development, function and adaptation.

What are miRNAs?

miRNAs are small (∼20–30 nucleotides (nt)) non-coding ribonucleic acids (RNAs) which are highly conserved from plants to mammals (reviewed previously in Bartel, 2004). Presently their known functions are to inhibit protein translation or enhance messenger RNA degradation (Hamilton & Baulcombe, 1999; Reinhart *et al.* 2000). miRNAs are initially transcribed in the nucleus as long primary transcripts up to several kilobases long which are known as primary miRNA (pri-miRNA) (Bartel, 2004). The ribonuclease (RNase) III endonuclease Drosha associated with Pasha (also known as DGCR8) cleaves the pri-miRNA into a ∼60–70 nt intermediate referred to as the miRNA precursor, or pre-miRNA (Lee *et al.* 2003). Exportin-5 (XPO5) transports the pre-miRNA from the nucleus to the cytoplasm (Lund *et al.* 2004) where a second RNase III endonuclease, Dicer1, cuts the pre-miRNA into a ∼22 nt miRNA duplex (Lee *et al.* 2003). One strand is degraded while the other confers the mature miRNA. The latter is then incorporated into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC) (Schwarz *et al.* 2002). This complex enables the identification and binding to the target mRNA resulting in its degradation or repression of protein translation (Bartel, 2004). TRBP (human immunodeficiency virus transactivating response RNA-binding protein) forms part of the RISC complex (Chendrimada *et al.* 2005). Phosphorylation of TRBP by the mitogen-activated protein kinase Erk increases the stability of the RISC complex and enhances miRNA production (Paroo *et al.* 2009). Individual miRNAs do not act alone nor do they target only a unique gene. Indeed, miRNAs have multiple gene targets and each target may be regulated by multiple miRNAs (Lewis *et al.* 2003).

As most of this review paper discusses the roles and regulation of the mature miRNA transcripts, 'miRNA' will always refer to the mature form. When referring to the primary miRNA transcript 'pri-miRNA' will be written, while 'pre-miRNA' will be used when discussing a miRNA precursor.

miRNAs highly enriched in muscle

A suite of miRNAs, highly enriched in cardiac and/or skeletal muscle (referred to as myomiRs), has recently been identified and include miR-1, miR-133a, miR-133b, miR-206, miR-208, miR-208b, miR-486 and miR-499 (McCarthy & Esser, 2007; Callis *et al.* 2008; van Rooij *et al.* 2008, 2009; Small *et al.* 2010). Several of these miRNAs are organized under bicistronic clusters on the same chromosome (i.e. miR-1-1/133a-2, miR-1-2/133a-1 and miR-206/133b) and are transcribed together (Chen *et al.* 2009; Liu & Olson, 2010). Regulation of these myomiRs is controlled by key myogenic regulatory factors (MRFs), including myogenic differentiation 1 (MyoD) and myogenin (Rao *et al.* 2006; Rosenberg *et al.* 2006) as well as myocyte enhancer factor 2 (MEF2) (Liu *et al.* 2007), serum response factor (SRF) (Chen *et al.* 2006) and myocardin-related transcription factor-A (MRTF-A) (Small *et al.* 2010). MyomiRs influence multiple facets of muscle development and function through their regulation of key genes controlling myogenesis (Chen *et al.* 2006; Kim *et al.* 2006; Rao *et al.* 2006). miR-1 and miR-133a also regulate SRF and MEF2 demonstrating the existence of negative feedback loops (Chen *et al.* 2006). Aberrant regulation of some of these muscle-enriched miRNAs can disrupt intracellular signalling networks (Elia *et al.* 2009; Small *et al.* 2010) which may result in pathological conditions (Eisenberg *et al.* 2009). Identifying the role and regulation of skeletal muscle miRNAs during various phases of muscle development, as well as in healthy and diseased conditions, will significantly enhance our understanding of skeletal muscle biology and may result in new therapies to target muscle diseases or chronic diseases associated with impaired muscle growth, regeneration or function. Additionally, understanding miRNA biology and function will enhance our understanding and application of current therapies.

miRNAs and myocyte proliferation and differentiation

Skeletal muscle development is a complex process requiring coordination of multiple factors which govern the proliferation of myoblasts, their exit from the cell cycle and subsequent differentiation into multinucleated myotubes (Buckingham, 2006). Myogenesis is mainly controlled by several key transcription factors, including the basic helix–loop–helix MRFs, myogenic factor 5 (Myf5), MyoD, myogenin and MRF4 (Berkes & Tapscott, 2005), as well MEF2 and SRF (Duprey & Lesens, 1994). miRNAs have been shown to be involved in myogenesis (Zhao & Srivastava, 2007) through their regulatory relationship with MRFs (Fig. 1).

miR-133a increases myoblast proliferation, via its repression of SRF (Chen *et al.* 2006), while miR-1 stimulates myoblast differentiation via its inhibition of histone deacetylase 4 (HDAC4) (Chen *et al.* 2006). Along similar lines, miR-206 further influences the differentiation programme via an indirect down-regulation of the helix–loop–helix protein Id, a repressor of MyoD (Kim *et al.* 2006). miR-24 induces cardiomyocyte hypertrophy *in vitro* and is up-regulated during cardiac hypertrophy (van Rooij *et al.* 2006). While miR-24 levels are maintained in adult terminally differentiated cardiac and skeletal muscle, its precise functional role in these tissues is unknown (Sun *et al.* 2008). The early stages of differentiation show an up-regulation of miR-24 and its regulatory pattern mimics that of differentiation-specific markers, such as myosin heavy chain (MHC). Transforming growth factor β (TGF-β)/Smad3 signalling inhibits miR-24, resulting in the inhibition of differentiation (Sun *et al.* 2008). miR-181 is up-regulated at the onset of myoblast terminal differentiation with expression profiles mimicking differentiation-specific genes such as creatine kinase (Naguibneva *et al.* 2006). It was observed that miR-181 may exert its pro-differentiation effects via its inhibition of the homeobox protein Hox-A1; the latter a protein which can inhibit MyoD expression (Yamamoto & Kuroiwa, 2003). miR-27b targets the 3′-UTR of Pax3 leading to its down-regulation and early differentiation (Crist *et al.* 2009). The inhibition of miR-27b maintains

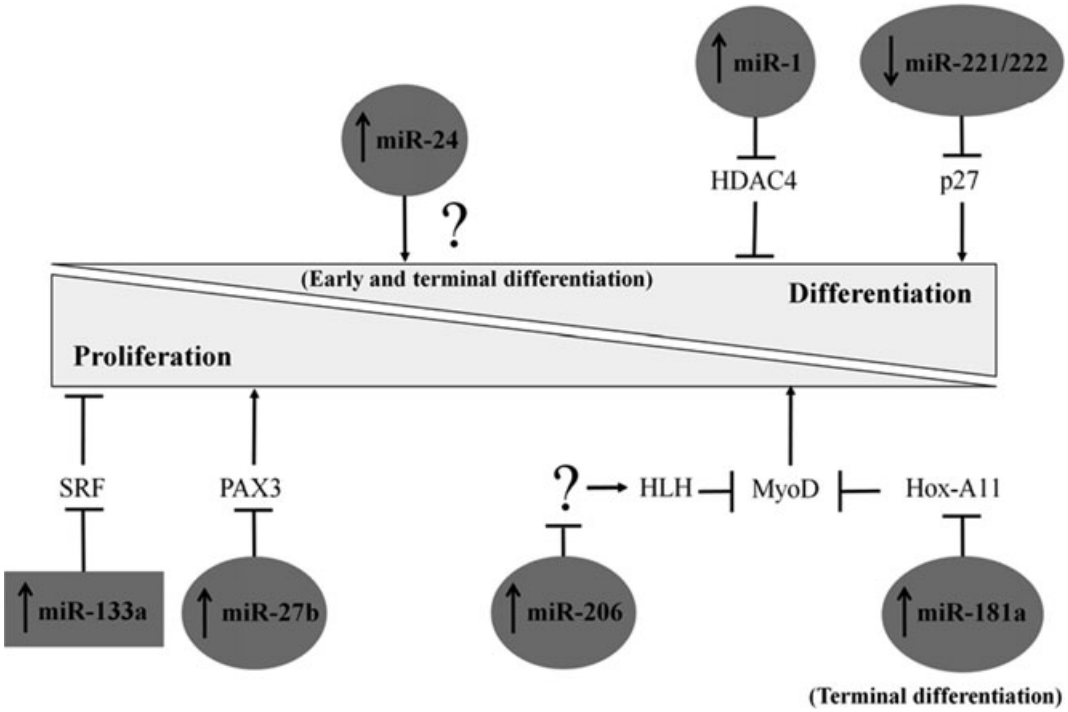

Figure 1. microRNA regulation of muscle proliferation and differentiation
This figure shows the miRNAs and their targets regulated during proliferation (rectangle) and differentiation (circles). miR-133a is increased during proliferation. It reduces serum response factor (SRF), which is known to inhibit proliferation. miR-24 is up-regulated during early differentiation, and maintained during the latter stages of differentiation. Its regulated targets are presently unknown. miR-27b enhances differentiation by targeting Pax3 which is known to stimulate muscle proliferation. miR-1 stimulates differentiation through its direct inhibition of HDAC4, an inhibitor of differentiation, while miR-206 indirectly inhibits helix–loop–helix, the latter, a repressor of MyoD. At the late stage of differentiation, miR-181a is increased and targets Hox-A11, a known inhibitor of MyoD. miR-221/222 are decreased in differentiated myotubes. This removes its repression of p27 which is required to inhibit proliferation. Outside symbols: → activates; —| inhibits; ? unknown target. Within symbols: ↑ increased; ↓ decreased.

Pax3 levels, leads to more proliferation and delays the onset of differentiation. While some miRNAs are up-regulated during the transition from proliferation to differentiation, others, such as miR-221 and miR-222, are down-regulated (Cardinali *et al.* 2009). Decreases in miR-221 and miR-222 are associated with increased expression of the cell cycle inhibitor p27; the latter a direct target of both miR-221 and miR-222. Overexpression of miR-221 and miR-222 in differentiating myotubes delays cell cycle withdrawal and differentiation, a response associated with a reduction in sarcomeric proteins (Cardinali *et al.* 2009).

The regulation between myogenic transcription factors and various miRNAs is complex and appears to depend on the cell cycle and fusion stages. Establishing the role and regulation of muscle miRNAs will enhance our understanding of embryonic and adult muscle development and regeneration.

miRNAs and muscle fibre type

Human skeletal muscle consists of predominantly three muscle fibre types: I, IIa and IIx (also known as IIb). The fibre types are generally classified as slow or fast, depending on their contractile characteristics, a reflection of the expression of either the slow or fast contractile protein isoforms of MHC (Schiaffino & Reggiani, 1996), and metabolic characteristics, a reflection of mitochondrial enzyme activities (Essen *et al.* 1975). Type I fibres (slow) are recruited at low stimulation frequencies and function oxidatively. In contrast, type IIx fibres (fast) require high stimulation frequencies and function glycolytically, while type IIa fibres (fast) possess intermediate characteristics between type I and IIx fibres. miR-208b and miR-499 are a part of a myomiR network that regulates MHC expression, fibre type and muscle performance (van Rooij *et al.* 2007, 2009). miR-208b and miR-499 are encoded in the introns of the mouse slow type I β-MHC (Myh7) gene on chromosome 14 and mouse slow type I MHC7b (My7b) gene positioned on chromosome 2. As such these miRNA/myosin networks share a positive regulatory circuit which promotes a slow skeletal muscle gene programme. This occurs, in part, through the miR-208b and miR-499 targeting of several transcriptional repressors known to regulate muscle gene expression and function including Sox6, Pur β, Sp3 (repressors of slow muscle genes), HP-1 β and Thrap1 (McCarthy *et al.* 2009; van Rooij *et al.* 2009; Bell *et al.* 2010).

miRNAs and skeletal muscle hypertrophy and atrophy

Synergistic ablation, an intervention which increases functional overload (FO), is often used to develop hypertrophy of the plantaris muscle. Following 7 days of FO, mouse plantaris muscle mass increased by 45% with an associated change in several primary miRNAs (pri-miR) and their corresponding mature miRNA transcripts (McCarthy & Esser, 2007). While pri-miR-1-2 and pri-miR-133a2 were increased 2-fold, their mature transcripts, miR-1 and miR-133a, were decreased by 50%. pri-miR-206 was increased 18.3-fold; however, its mature transcript did not change (Fig. 2*A*). The reason for this discordant regulation between the pri-miRNAs and their mature miRNA transcripts is not clear. However, these observations may have been influenced by the time of muscle sampling and future time course experiments are required to determine this. Alternatively, the functional-overload model may have influenced mechanisms that control pri-mRNA processing, such as RNA editing by ADAR (adenosine deaminase, RNA specific) enzymes; a reaction known to suppress pri-mRNA processing by Drosha, resulting in a loss of the mature miRNA transcript (Yang *et al.* 2006). The decrease in mature miR-1 and mature miR-133a expression with FO may contribute to muscle hypertrophy. This suggests an additional function to their previously identified roles in muscle differentiation and proliferation, respectively (Chen *et al.* 2006). The potential of miR-1 and miR-133a to regulate multiple facets of muscle biology may depend upon the stimulus controlling their activation. miR-1 and mature miR-133a may play a role in muscle hypertrophy by the removal of their transcriptional inhibition of growth factor gene targets, including hepatocyte growth factor receptor (c-MET), hepatocyte growth factor (HGF), leukaemia inhibitor factor (LIF), insulin-like growth factor 1 (IGF-1) and SRF (McCarthy & Esser, 2007) (Fig. 2*A*). Indeed, IGF-1 levels are increased in rat plantaris following overload (Adams *et al.* 1999) while work from our laboratory has shown that SRF levels are increased in human skeletal muscle following hypertrophy-inducing resistance exercise (Lamon *et al.* 2009). Further evidence that a regulatory loop between miR-1 and IGF-1 may regulate muscle growth comes from recent observations made in C2C12 myotubes and cardiomyocytes (Elia *et al.* 2009). Increased levels of endogenous miR-1 in differentiating C2C12 myotubes resulted in a reduction in the activation of an IGF-1 luciferase reporter construct and a decrease in IGF-1 protein levels. Conversely, IGF-1 treatment reduced miR-1 levels in C2C12 myotubes. IGF-1 stimulates C2C12 myotube hypertrophy via the activation of Akt signalling and the inhibition of Forkhead (FoXO) transcription factors (Rommel *et al.* 2001). Hypertrophied human skeletal muscle also presents an increase in active Akt and a reduction in FoXO levels (Leger *et al.* 2006*a*). miR-1 levels are reduced by active Akt and increased by active FoXO3a (Elia *et al.* 2009) demonstrating a regulatory loop whereby IGF-1 regulates miR-1 via an Akt/FoXO3a pathway (Fig. 2*B*); a pathway known to increase muscle cell size (Latres *et al.* 2005). Furthermore, miR-1 levels can be increased via FoXO3a which results in reduced

IGF-1 protein levels and reduced Akt activity; a response seen during myotube atrophy (Sandri *et al.* 2004; Latres *et al.* 2005).

A direct genetic link has been observed between miRNAs and muscle hypertrophy (Clop *et al.* 2006). The enhanced muscular development of the Texel sheep has been mapped to a single G-A mutation in the 3′-UTR of the myostatin gene, a gene involved in the repression of muscle growth (Thomas *et al.* 2000; McFarlane *et al.* 2006). The mutation provides a novel binding site for miR-1 and miR-206 which inhibits the translation of the myostatin protein. The existence of polymorphic miRNA–target interactions is of considerable interest and may help to underpin mechanisms involved in congenital and acquired myopathies.

Unloading of skeletal muscle, as caused by the microgravity during spaceflight or via hind limb suspension (HS), decreases muscle mass and force production and shifts the muscle towards a glycolytic phenotype. Following 11 days of spaceflight, miR-206 was significantly decreased (Fig. 3*A*) while miR-1 and miR-133a showed a trend towards a reduction in mouse gastrocnemius muscle (Allen *et al.* 2009). This was paralleled by an increase in the muscle atrophy F-box (MAFbx; also known as atrogin-1) and myostatin, genes that are involved in muscle atrophy (Bodine *et al.* 2001) and inhibition of muscle growth (Morissette *et al.* 2009), respectively, and increased in numerous models of muscle wasting (Glass, 2005; Leger *et al.* 2006*b*; Doucet *et al.* 2007; Leger *et al.* 2008). Whether miR-206 plays a direct or indirect role in repressing these atrophy genes is unknown. miR-206 is up-regulated via MyoD (Chen *et al.* 2006), a protein which can be degraded by MAFbx/atrogin-1 (Tintignac *et al.* 2005). The possibility that a miR-206/MyoD/MAFbx regulatory loop exists which influences skeletal muscle growth requires investigation.

miR-107, miR-208b, miR-221 and miR-499 were significantly down-regulated in rat soleus muscle following 7 days of HS-induced muscle atrophy; miR-23b showed a tendency to be reduced ($P = 0.054$) (McCarthy *et al.* 2009) (Fig. 3*B*). Unlike the space flight model of muscle atrophy, miR-206 was not decreased following HS which may be due to methodological differences including species, muscle analysed and/or experimental duration.

Cytokines are known to increase protein degradation and reduce protein synthesis (Menconi *et al.* 2007; Tisdale, 2007). The pro-inflammatory cytokine TNF-like weak inducer of apoptosis (TWEAK) has been shown to cause muscle atrophy (Dogra *et al.* 2007). TWEAK down-regulated several growth-related myomiRs, including miR-1, miR-23, miR-133a, miR-133b and miR-206 in C2C12 myotubes; however, it only reduced miR-1, miR-133a and miR-133b in mouse skeletal muscle *in vivo* (Panguluri *et al.* 2010) (Fig. 3*C*). There was also an increase in miR-455 which is also elevated in several muscle dystrophies (Eisenberg *et al.* 2007). While treatment with TWEAK regulates several muscle miRNAs involved in muscle growth, it is not known whether their regulation is a cause of muscle wasting or a response to prevent further muscle wasting.

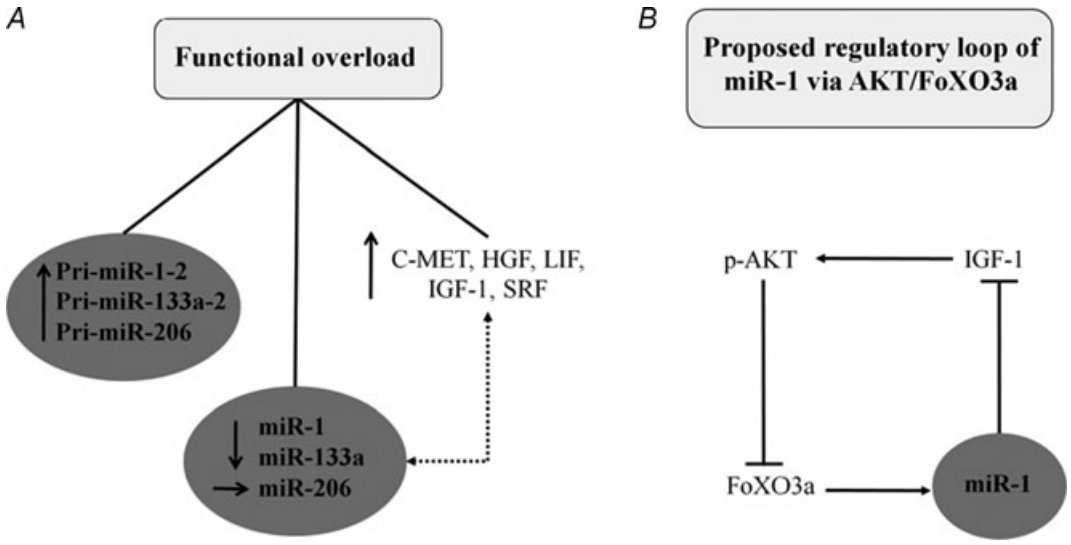

Figure 2. microRNA regulation of skeletal muscle hypertrophy
A, following functional overload-induced hypertrophy in mice, primary miR-1-2, -133a-2 and -206 are increased. In contrast, the mature forms of miR-1 and miR-133a are decreased while miR-206 is unchanged. There is also an increase in several growth-related genes that are predicted targets of these miRNAs; a cause and effect relationship has not been confirmed. ↑ increased; ↓ decreased; → unchanged; ← ·· → cause and effect unknown. *B*, a regulatory loop exists in which Akt activation inhibits the FoXO3a transcriptional activation of miR-1. This could remove the miR-1 repression of IGF-1 and potentially assist with muscle hypertrophy. → activates; —| inhibits.

miRNAs and muscle regeneration

Skeletal muscle regeneration is a vital process required to maintain healthy muscle mass, structure and function throughout the adult life. While few studies have investigated the miRNAs involved in regeneration, miR-27, miR-1, miR-133, miR-206 and miR-188 are potential regulators. In regenerating adult muscle, miR-27 is required to down-regulate Pax3 in order to promote myogenesis, while its inhibition in injured muscle *in vivo* delays muscle regeneration (Crist *et al.* 2009). Following the laceration of rat tibialis anterior, an injection of a cocktail of miRNAs including miR-1, -133 and -206 into the injured site enhanced regeneration and prevented fibrosis (Nakasa *et al.* 2009). This was also associated with increases in the myogenic markers MyoD, myogenin and Pax7; however, no cause and effect relationship between these miRNAs, target genes and muscle regeneration was determined. miR-181 increases during muscle cell differentiation; however, it is expressed at low levels in adult tissue except under conditions of regeneration (Naguibneva *et al.* 2006). Presently the role of miR-181 during regeneration has yet to be determined. Identifying the miRNAs which enhance and/or inhibit muscle regeneration has implications for the development of potential therapeutic strategies for conditions with compromised regeneration as seen in various muscle dystrophies and in sarcopenia.

Influence of exercise on miRNAs

Exercise can reduce morbidity associated with chronic disease, such as type 2 diabetes (T2D) (Lanza *et al.* 2008), cancer (Newton & Galvao, 2008) and cardiovascular disease (Wisloff *et al.* 2005). At one end of the exercise continuum is endurance exercise, commonly performed at a moderate intensity with continuous or interval-based repetitions. Repeated bouts of endurance exercise results in increased mitochondrial content, capillary density and enhanced capacity of the oxidation of carbohydrates and lipids (Fluck, 2006; Joseph *et al.* 2006; Coffey & Hawley,

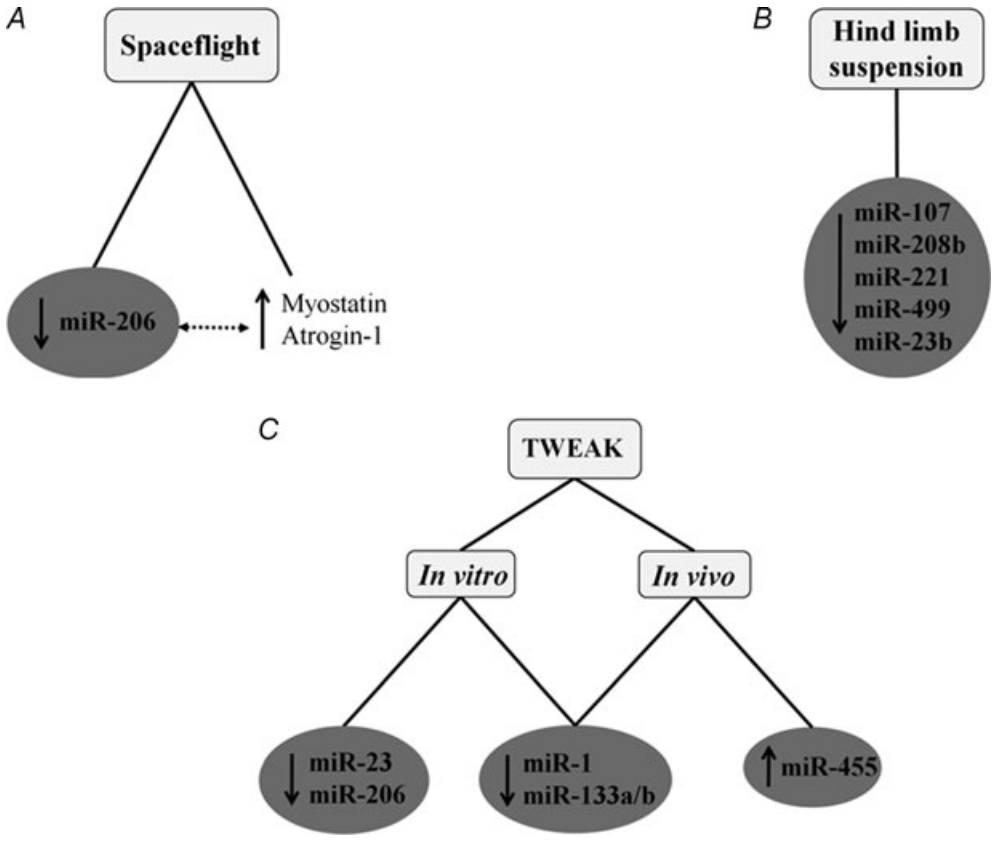

Figure 3. microRNAs and skeletal muscle atrophy
A, following spaceflight-induced atrophy in mice, a decrease in miR-206 has been observed as well as an increase in myostatin and atrogin-1. A cause and effect relation between miR-206 and these targets has not been determined. *B*, hind limb suspension decreases miR-107, -208b, -221, -499 and -23b in rat skeletal muscle; for miR-23, $P = 0.054$. *C*, TWEAK increases protein degradation with an associated decrease in miR-23, miR-206, miR-1 and miR-133a/b in C2C12 myotubes. TWEAK also reduces miR-1 and miR-133a/b and increases miR-455 in mouse muscle *in vivo*. ↑ increased; ↓ decreased; ← ·· → cause and effect unknown.

2007). Following 90 min of exhaustive endurance exercise (forced treadmill running) in mice, miR-1 and miR-181, both thought to increase muscle differentiation and development, as well as miR-107, were increased (Safdar *et al.* 2009). miR-23 was decreased which correlated with an increase in peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (Safdar *et al.* 2009) (Fig. 4*A*), the latter a predicted target of this miRNA (Wilfred *et al.* 2007). PGC-1α, a transcriptional co-activator, regulates numerous skeletal muscle functions including mitochondrial biogenesis, substrate oxidation, muscle fibre type (Russell, 2005) and recently, the protection against muscle wasting (Sandri *et al.* 2006).

Four weeks of treadmill running endurance training in mice resulted in an increase in miR-21 and a decrease in miR-696, -709 and -720 (Aoi *et al.* 2010). The decrease in miR-696 was negatively correlated with PGC-1α protein levels. The transient elevation of miR-696 in C2C12 myotubes did not influence PGC-1α mRNA levels but did result in the suppression of PGC-1α protein levels. Conversely, transfection of the myotubes with a miR-696 inhibitor elevated PGC-1α protein levels, without changing PGC-1α mRNA. While these observations suggest that miR-696 may regulate PGC-1α translation, no cause and effect has been established. To date miR-696 and -709 have not been identified in humans so the relevance of these observations to human skeletal muscle development and function is unknown.

At the other end of the continuum is resistance exercise which is commonly performed at high intensities for shorter durations of time. This type of exercise has an anabolic stimulus and it enhances muscle size by increasing the synthesis of contractile and structural proteins; as a result the muscle is often larger and also more powerful (Fry, 2004; Leger *et al.* 2006*a*). Following an acute bout of resistance exercise, performed by both young and older men, pri-miR-1-2 and pri-miR-133a-1 were reduced in muscle biopsy samples taken from the young subjects 6 h post exercise (Drummond *et al.* 2008). Pri-miR-133a-2 was reduced at 3 and 6 h post exercise in the older and young subjects, respectively. In contrast, pri-miR-206 was increased at 3 and 6 h post exercise in the older and young subjects, respectively (Drummond *et al.* 2008). Of the mature miRNAs, only miR-1 was reduced at 3 and 6 h post exercise in the older and young subjects, respectively; no changes in miR-133a or miR-206 were observed (Fig. 4*B*).

These studies show that exercise is capable of regulating miRNA levels. It will be important to identify which exercise-induced pathways alter miRNA expression and how miRNA regulation contributes to the physiological adaptations to exercise. Investigations that identify the miRNAs responsible for exercise-induced adaptations, or which can mimic some exercise-induced adaptations, will significantly advance the miRNA–muscle field.

Influence of nutrition on miRNAs

The impact of ingesting essential amino acids (EAAs) on skeletal muscle miRNA expression has also been

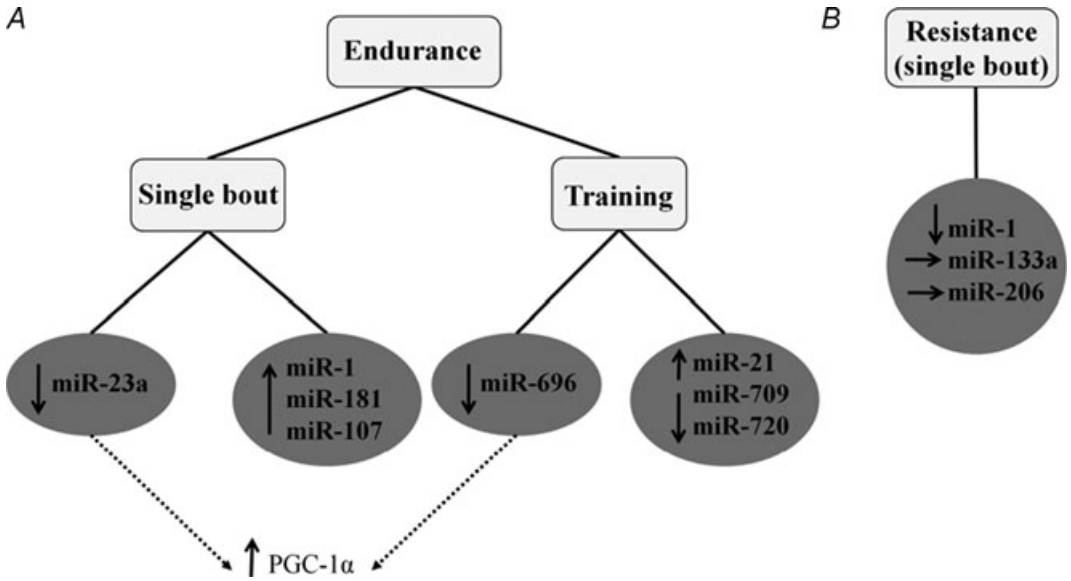

Figure 4. Influence of exercise on microRNAs
A, a single bout of endurance exercise in mice reduces miR-23a and increases miR-1, -181, -107. Endurance training increases miR-21 expression and decreases miR-696, -709, -720 expression in mice. A commonality between acute endurance exercise and endurance exercise training is an increase in PGC-1α. PGC-1α is a predicted target for miR-23a and miR-696. *B*, following a single bout of resistance exercise in men, miR-1 decreases with no changes observed in miR-133a or miR-206. ↑ increased; ↓ decreased; → unchanged; ·· → cause and effect unknown.

investigated (Drummond *et al.* 2009). Healthy males ingested 10 g of EAAs with muscle biopsies taken before and 3 h following. Analyses of microRNAs and muscle-growth-related genes in the skeletal muscle biopsies revealed an increase in miR-499, -208b, -23a, -1 and pri-miR-206, as well as MyoD1 and follistatin-like 1 (FSTL1) mRNA, and a decrease in myostatin and MEF2C expression (Fig. 5). While EAAs can regulate muscle miRNA and mRNA, no causal observations have been determined.

The influence of elevated glucose levels on skeletal muscle miR-1 and miR-133a was investigated in humans following a 3 h hyperglycaemic–euinsulinaemic clamp (Granjon *et al.* 2009). Hyperglycaemia alone, in the absence of hyperinsulinaemia, did not have an impact on the expression levels of these two myomiRs. While no study has investigated the effect of oral glucose ingestion on skeletal muscle miRNA levels, the results from Granjon *et al.* (2009) suggest that any consequence of glucose ingestion would be via an indirect effect of insulin.

miRNAs and skeletal muscle disease and dysfunction

Primary skeletal muscle disorders involve several groups of diseases including muscular dystrophies, inflammatory myopathies and congenital myopathies. While these diseases can be defined by their clinical and pathological traits the molecular pathways involved in these conditions are not well understood. With the evolution of miRNA biology, studies have now begun to identify miRNA signatures which are dysregulated in patient muscle biopsies. Eisenberg and colleagues identified altered regulation of 185 miRNAs from 10 different muscle disorders (Eisenberg *et al.* 2007). Of special interest were miR-146b, miR-155, miR-214, miR-221 and miR-222, which were increased in almost all of the samples analysed. Of further interest will be identifying if, and how, the regulation of these miRNAs contributes to the pathophysiology of these muscle diseases.

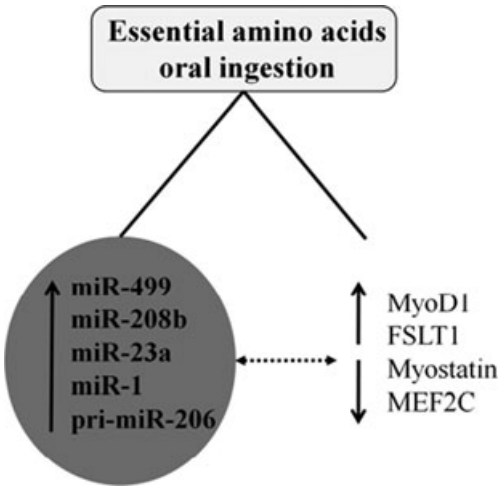

Figure 5. Influence of EAAs on microRNAs
Essential amino acids increased miR-499, -208b, -23a, -1 and pri-miR-206 levels in healthy males. This is associated with an increase in MyoD1 and FSLT1 and a decrease in myostatin and MEF2C. A causal link between these miRNAs and mRNAs has not been determined. ↑ increased; ↓ decreased; ← ·· → cause and effect unknown.

Several studies have investigated the regulation of miRNAs in patients with Duchene muscular dystrophy (DMD), in the *mdx* mouse and the $CXMD_J$ dog, two animal models of muscular dystrophy, as well as in myotonic dystrophy type 1 (MD1) and type 2 (MD2). Greco *et al.* observed significant increases in miR-31, -34c, -206, -222, -223, -335, -449 and -494 and decreases in miR-1, -29c and miR-135a in the quadriceps femoris muscles of DMD patients and adductor muscles of *mdx* mice (Greco *et al.* 2002). Similarly, Yuasa *et al.* observed that, miR-206, but not miR-1, was increased in the tibialis anterior (TA) muscle of *mdx* mice; however, both were reduced in the TA muscle of the $CXMD_J$ dog (Yuasa *et al.* 2008). In vastus lateralis muscle biopsies from patients with MD1 an increase in miR-206, but not miR-1, -133a/b or -181a/b/c, has been observed (Gambardella *et al.* 2010). However, the suite of miRNAs measured by Greco *et al.*, which included miR-206, were unchanged in biceps brachii biopsies from patients with MD2 (Greco *et al.* 2002). The increase in miR-206 in MD1, but not MD2, may be due to the different muscles analysed or may be a characteristic of the diseases.

In the *mdx* mouse there is muscle-specific regulation of various miRNAs. When compared with control animals, miR-206 expression is dramatically increased 4.5-fold in the *mdx* diaphragm, decreased by 29% in the plantaris muscle but unchanged in the soleus muscle (McCarthy *et al.* 2007). miR-133a is modestly decreased by 23% in the soleus muscle, but not in the plantaris, diaphragm (McCarthy *et al.* 2007) or TA muscles (Yuasa *et al.* 2008). The dramatic increase in miR-206 in *mdx* diaphragm is of interest as this is the only muscle in the *mdx* mouse which exhibits extensive and progressive degeneration, fibrosis and functional deficits comparable to the limb muscles of boys with DMD (Stedman *et al.* 1991; Lynch *et al.* 1997). The reason for the different regulatory pattern of these miRNAs from one muscle group to another is unclear; however, dystrophin deficiency is known to cause muscle-specific responses in mice (Moens *et al.* 1993) and humans (Webster *et al.* 1988; Khurana *et al.* 1995). Differences in miRNA regulation across species may relate to the severity of their respective dystrophic phenotypes and relevance to the human DMD. For example, it is generally agreed that the dystrophic phenotype of limb muscles from the $CXMD_J$ dog is a very good representation of human DMD, while the *mdx* mouse presents a much milder phenotype (Shimatsu *et al.* 2005; Banks & Chamberlain, 2008). However, within the *mdx* mouse, the diaphragm muscle does have an evolving dystrophic

phenotype similar to human DMD limb muscle (Stedman *et al.* 1991).

miRNAs and age-related muscle wasting

The age-related loss of skeletal mass and function, known as sarcopenia, is a key factor increasing falls and fractures and reducing independent living. Elevated levels of pri-miRNA-1-1, -1-2, -133a-1 and -133a-2, with no change in pri-miR206, were observed in skeletal muscle biopsies taken from six elderly (70 ± 2 years) when compared with six younger (29 ± 2 years) men (Drummond *et al.* 2008). However, there were no differences in the mature miR-1 or miR-133a species. Whether age-related alterations in miRNAs are a cause or effect in humans *in vivo* is unclear. More studies are required with larger sample sizes to establish if the age-related changes in skeletal muscle are influenced by aberrant miRNA expression and activity. Additionally, it will be important to consider the potential influence of physical activity levels and nutritional status, factors which can influence muscle miRNA levels, when comparing young and older subjects.

miRNA and diabetes

Obesity and the metabolic syndrome are serious public health problems, generating life-threatening diseases such as atherosclerosis, cancer and type 2 diabetes (T2D). The involvement of microRNAs in obesity and T2D has recently been reviewed with a focus on non-muscle tissues such as pancreas, adipose tissue and liver (Lynn, 2009; Pandey *et al.* 2009; Heneghan *et al.* 2010). While microRNAs play important roles in the regulation of glucose and lipid metabolisms through the control of pancreatic islet cell function, adipocyte proliferation and differentiation and cholesterol biosynthesis, their role in skeletal muscle function in diabetes and obesity is not well established. To date only four papers have looked at the consequences of insulin resistance on miRNA expression in skeletal muscle of Goto-Kakizaki (GK) rats (a rodent model of diabetes) (He *et al.* 2007; Huang *et al.* 2009) as well as in T2D patients (Granjon *et al.* 2009; Gallagher *et al.* 2010). miRNA expression profiles, analysed and compared from skeletal muscle of GK and control rats, revealed the differential expression of 15 microRNAs (4 up- and 11 down-regulated; fold change ≥ 1.5). Interestingly, three out of the four up-regulated miRNAs were paralogues of the miR-29 family. Northern blot analyses confirmed the increase in their expression in three insulin-dependent tissues (i.e. skeletal muscle, liver and fat) from diabetic GK rats, suggesting an association between this microRNA family and insulin sensitivity (He *et al.* 2007). A similar study determined the regulation of miRNA expression in GK rats but observed slightly different results (Huang *et al.* 2009). In this study nine microRNAs were differentially expressed (2 up- and 7 down-regulated; fold change ≥ 2.0), with only miR-24 and miR-126 down-regulation confirmed by Northern blot analysis. Disparity in these results may have been due to different microRNA extraction methods, probe preparation and software used to identify the regulated miRNAs in the microarrays. It should also be noted that Huang *et al.* (2009) also observed a 3-fold increase in p38 mitogen-activated protein kinase (MAPK) protein levels. p38 MAPK is known to be a direct target of miR-24 (Kiriakidou *et al.* 2004). Further studies are necessary to analyse the regulation of the insulin-responsive miR-29 family, the relationship between miR-24 and p38 MAPK in diabetic skeletal muscle, as well as define their precise role in the insulin signalling pathway.

To observe the impact of insulin on skeletal muscle microRNA levels, vastus lateralis muscle biopsies were taken from healthy subjects before and after a 3 h euglycaemic–hyperinsulinaemic clamp (Granjon *et al.* 2009). microRNA expression profiling showed that 39 microRNAs were down-regulated by insulin, including the traditional myomiRs miR-1, miR-133a and miR-206. Additionally, miR-29a and miR-29c, microRNAs enriched to insulin-sensitive tissues, such as muscle, liver and adipose tissue, were also down-regulated. In contrast, Drosha and Dicer1 as well as the several Argonaute proteins were increased. The increase in these components of the miRNA synthesis and RISC pathways may be a failed attempt to maintain miRNA levels. Insulin down-regulates miR-1 and miR-133a, via the activation of sterol regulatory element binding protein 1c (SREBP-1c) and its inhibition of MEF2C, in human muscle cells *in vitro* (Granjon *et al.* 2009). Furthermore, the expression levels of miR-1 and miR-133a are increased in an insulin-deficient mouse model induced by streptozotocin injection (Granjon *et al.* 2009). In human skeletal muscle the basal expression levels of miR-1 and miR-133a were not different when compared between five healthy controls and five insulin-resistant type 2 diabetics (T2D) (Granjon *et al.* 2009). However, insulin reduced miR-1 and miR-133a expression in the healthy, but not in the T2D subjects. This response was also associated with the inability of insulin to reduce MEF2C expression in the diabetic patients. Along similar lines, the expression levels of miR-1 were not different when compared between 10 T2D patients, 10 impaired glucose-tolerant patients (IGT) and 10 healthy controls (Gallagher *et al.* 2010). However, this study did observe a decrease in miR-133a and miR-206 in the T2D patients when compared with the IGT and healthy subjects. Of clinical interest was the significant positive correlation between miR-133a and fasting glucose levels and 2 h glucose tolerance, when all 30 subjects were analysed. While a discrepancy with respect to the regulation of

miR-133a between these studies is evident, the reason for this is not clear and highlights the need for further research in this area.

The role of miRNAs in obesity and diabetes has only started to be explored and future investigation will unravel their roles in metabolism and energy expenditure. Based on recent observations the role and regulation of miRNAs in rodent models of diabetes and obesity should be considered with caution when potentially extrapolating to human obesity and diabetes.

Conclusion

The emergence of the miRNA field provides an exciting opportunity to further understand the molecular factors which control skeletal muscle development, regeneration and function. miRNA biology also provides an avenue to dissect the mechanisms which may contribute to genetic and acquired muscle disorders and related complications. The impact of behavioural choices influencing physical activity and nutrition has an import role in determining our longevity and quality of life. The observations that exercise and nutrition may influence miRNA levels has important implications for understanding how to maintain health throughout the lifespan; an issue of great relevance considering our ageing and sedentary communities. Future studies will focus more on the possibility of using miRNA as a therapeutic tool via gain of function and loss of function experiments in muscle cell models and in rodents. As with all models of genetic manipulation issues such as target and tissue specificity will be of major importance. Nevertheless, the potential of miRNA-based therapies offers an exciting and powerful alternative to attenuate and hopefully cure debilitating muscle diseases.

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Acknowledgements

I.G. is supported by a Deakin University International Higher Degree by Research Scholarship. A.P.R. is supported by an NH&MRC Biomedical Career Development Award (479536).