microRNAs: modulators of the underlying pathophysiology of sarcopenia?

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Abstract
Skeletal muscle homeostasis depends on an intricate balance between muscle hypertrophy, atrophy and regeneration. As we age, maintenance of muscle homeostasis is perturbed, resulting in a loss of muscle mass and function, termed sarcopenia. Individuals with sarcopenia exhibit impaired balance, increased falls (leading to subsequent injury) and an overall decline in quality of life. The mechanisms mediating sarcopenia are still not fully understood but clarity in our understanding of the precise pathophysiological changes occurring during skeletal muscle ageing has improved dramatically. Advances in transcriptomics has highlighted significant deregulation in skeletal muscle gene expression with ageing, suggesting epigenetic alterations may play a crucial and potentially causative role in the skeletal muscle ageing process. microRNAs (miRNAs, miRs), novel regulators of gene expression, can modulate many processes in skeletal muscle, including myogenesis, tissue regeneration and cellular programming. Expression of numerous evolutionary conserved miRNAs is disrupted in skeletal muscle with age. Given that a single miRNA can simultaneously affect the functionality of multiple signaling pathways, miRNAs are potent modulators of pathophysiological changes. miRNA-based interventions provide a promising new therapeutic strategy against alterations in muscle homeostasis. The aim of this review is two-fold; firstly to outline the latest understanding of the pathophysiological alterations impacting the deregulation of skeletal muscle mass and function with ageing, and secondly, to highlight the mounting evidence for a role of miRNAs in modulating muscle mass, and the need to explore their specific role in sarcopenia.

Keywords
Sarcopenia, skeletal muscle, ageing, microRNA

Contents

1. Introduction

As we age, our ability to maintain muscle homeostasis is impaired and results in a progressive loss of muscle mass and function (Cruz-Jentoft et al., 2010; Goodpaster et al., 2008; Nilwik et al., 2013; Snijders et al., 2014; Visser et al., 2005). This phenomenon has been termed "sarcopenia" and has been associated with impaired balance, increased prevalence of falls, elevated sense of frailty, and reduced independence (Cruz-Jentoft et al., 2010; Rhodes et al., 1999; Visser et al., 2005). Maintenance of healthy, functional skeletal muscle mass as we age is fundamental to maintaining quality of life and increasing our "health-span" (duration of life spent in a healthy state). At the molecular level, there is a significant deregulation in skeletal muscle gene expression with ageing (Sifakis et al., 2013; Welle et al., 2003), suggesting epigenetic alterations may play a crucial and potentially causative role in the skeletal muscle ageing process. This review outlines the latest understanding of the pathophysiological alterations underlying the deregulation of skeletal muscle mass and function with ageing. Furthermore, we aim to highlight the mounting evidence for a role of miRNAs in modulating muscle mass and the need to explore their specific role in sarcopenia.

2. Sarcopenia: Diagnostic criterion, prevalence and impact

Sarcopenia comprises a combined loss of muscle mass and strength or performance associated with ageing (Cruz-Jentoft et al., 2010) which ultimately impacts mobility and all-cause mortality (Landi et al., 2013; Visser et al., 2005). The European Working Group on Sarcopenia in Older People (EWGSOP) recently provided a consensus clinical diagnostic criterion for the assessment of sarcopenia (Cruz-Jentoft et al., 2010), detailing a loss of muscle mass and/or function, largely defined by a cut-off criterion of two standard deviations below the mean value of a young adult reference population. Using this criterion, it was reported that that between 6-8% of community dwelling men (n = 103; mean age of 73 years) have sarcopenia (Patel et al., 2013). The assessment of an older cohort of males and females (n = 364; mean age of 82 years), revealed 22% of individuals had sarcopenia (Landi et al., 2013), suggesting the prevalence of sarcopenia may increase substantially between the ages of 70-80 years. Moreover, Lauretani et al. (2003) examined a large population cohort (1,030 people aged 20-102 years) to reveal that with increasing age, both men and women exhibit a progressive decline in muscle strength/power and a reduction in their ability to walk 1km. Walking related assessments (either distance covered or maximal walking speed) have also proven excellent markers of health related events in older populations (Cesari et al., 2009; Lauretani et al., 2003; Yazdanyar et al., 2014), suggesting the capacity of skeletal muscle to function within the context of the individuals cardiovascular and respiratory systems is of particular prognostic value. Recent evidence also implicates that a "muscle mass index" (relative muscle mass) is a good predictor of longevity in humans (Srikanthan and Karlamangla, 2014), demonstrating the importance of maintaining muscle mass with ageing. Ultimately, skeletal muscle forms a fundamental component of the human locomotive system and thus understanding age-associated alterations in muscle pathophysiology and examining interventions to ameliorate sarcopenia is of international importance to increase the health span of an increasingly longer living population.

3. Pathophysiological alterations in skeletal muscle during ageing

The multifactorial and progressive nature of sarcopenia makes a single definition or diagnostic criterion difficult and perhaps unrealistic. Alchin (2014) recently discussed

that despite the efforts of the EWGSOP, the current definition of sarcopenia is somewhat incomplete and perhaps presents a description rather than a definition *per se*. Although recognition of the symptoms of sarcopenia has been broadly understood for a while, it's only recently that the multifactorial pathophysiological alterations underlying sarcopenia have really come to light and will be reviewed in this section. A graphical summary of alterations occurring during muscle ageing is displayed in Figure 1.

3.1 Targeted atrophy of type II muscle fibres

There is a characteristic and well-documented decline in muscle mass or muscle cross-sectional area (CSA) with ageing, reported in both humans (Goodpaster et al., 2008; Nilwik et al., 2013; Snijders et al., 2014) and rodent models (Akasaki et al., 2014; Houtkooper et al., 2011). A recent series of studies in humans, however, have consistently identified a specific reduction in type II, but not type I, muscle fibre CSA in elderly men (Gueugneau et al., 2014; Hvid et al., 2014; Nilwik et al., 2013; Snijders et al., 2014), suggesting age-associated muscle atrophy is strongly dependent on muscle fibre type. The CSA of type II muscle fibres can have functional consequences, impacting overall muscle strength (Akasaki et al., 2014). The targeted atrophy of type II muscle fibres in elderly men can be so severe that type II fibre CSA can become significantly smaller than type I fibre CSA (Gueugneau et al., 2014; Snijders et al., 2014), thus deregulating the normal fibre-type fibre-size relationship. By comparing shifts in muscle fibre CSA and whole muscle CSA in young and old men prior to and following a 6 month resistance training program, Nilwik et al. (2013) suggested that the alterations in type II muscle fibre CSA account for the changes in whole muscle CSA observed with ageing. The authors therefore dismissed the loss/death of muscle fibres in mediating the reduction in whole muscle CSA with ageing in humans. The involvement of muscle fibre loss/death in sarcopenia remains an unresolved point of controversy amongst the literature (Brown, 1987; Deschenes, 2004; Eddinger et al., 1985; Faulkner et al., 2007; Lushaj et al., 2008). Reasons for this controversy are currently unknown but may reflect difficulties in accessing whole muscle samples from human subjects, relying primarily on small muscle biopsy sampling. Recent evidence has also proposed that muscle fibre loss may be an artifact of muscle ageing in rodent models (Sheard and Anderson, 2012) and may not occur in humans (Nilwik et al., 2013). The existence of species-specific differences in muscle fibre loss/death with ageing warrants investigation as it may elucidate a role for anatomical and postural differences in regulating the maintenance of muscle fibre number. The loss of muscle fibres in aged mouse models has been shown to vary, with fibre loss occurring in loaded limb muscles but not in muscles supporting the neck (Sheard and Anderson, 2012), highlighting that muscle fibre loss with ageing may occur in a muscle-specific and activity-dependent manner.

3.2 Muscle fibre type dependent alterations in satellite cell biology

Skeletal muscle contains its own resident population of self-renewing stem cells, commonly termed satellite cells due to their location under the basal laminar on the muscle fibre periphery, which contribute to postnatal muscle fibre repair and regeneration (Relaix and Zammit, 2012; Zammit et al., 2006). Muscle fibres of rodents show a significantly reduced satellite cell content with ageing (Bernet et al., 2014) and demonstrate abnormalities such as an increased susceptibility to apoptosis (Jejurikar et al., 2006) and an impaired ability to proliferate *ex vivo* (Bernet et al., 2014). Using human muscle biopsy samples, Snijders et al. (2014) and Verdijk et al.

(2014) have recently shown that age associated alterations in satellite cell content is specific to muscle fibre type; type II, but not type I, muscle fibres from older people contain fewer satellite cells per fibre compared to that of their young counterparts. In addition, satellite cells associated with type II, but not type I, muscle fibres were shown to exhibit a delayed increase in cell number following a single bout of resistance exercise (Snijders et al., 2014). Taken together, these data suggest fibre type specific differences in satellite cell content and behaviour may differ between old and young individuals, a phenotype that may exacerbate the reduced ability to maintain type II muscle fibre mass during ageing. However, although partial depletion of satellite cells impairs the regenerative capacity of skeletal muscle, it does not worsen the muscle fibre atrophy associated with the development of sarcopenia in aged mice (Fry et al., 2014).

3.3 Role of the neuromuscular junction in maintaining muscle mass

The role of motor neurons and the neuromuscular junction (NMJ) in maintaining muscle homeostasis during ageing is difficult to examine in humans but has been highlighted by a collection of recent studies in rodent models (Butikofer et al., 2011; Hettwer et al., 2014; Sakellariou et al., 2014; Valdez et al., 2010). A time-course analysis of the NMJ in ageing mice (between 1 and 24 months of age) clearly highlights progressive detrimental alterations in NMJ morphology with ageing (Valdez et al., 2010), leading to destabilization of the NMJ. Using neurotrypsin over-expressing mice to sporadically destabilize NMJs in skeletal muscle, Butikofer et al. (2011) demonstrated a critical role of NMJ stability in regulating muscle fibre mass and function. Although neurotrypsin does not mediate sarcopenia, this work demonstrated that the loss of muscle mass and function can be mediated by NMJ destabilization (Butikofer et al., 2011). Hettwer et al. (2014) recently reported that administration of an Agrin fragment, resistant to neurotrypsin cleavage, re-stabilized the NMJ in neurotrypsin over-expressing mice and subsequently maintained body mass during early postnatal ageing and improved early age-associated decrements in muscle function. A study by Sakellariou et al. (2014) elegantly demonstrated a significant role of the motor neurons in regulating muscle homeostasis in a mouse model of oxidative damage induced accelerated muscle ageing (*Sod1*$^{-/-}$ mice; Muller et al. (2006)). Using a transgenic approach to specifically re-express *Sod1* in neuronal cells of *Sod1*$^{-/-}$ mice, the authors demonstrated a substantial recovery in the loss of muscle mass and function normally exhibited by the *Sod1*$^{-/-}$ mice. Taken together, oxidative damage of neuronal cells and alterations in NMJ morphology play a critical role in mediating the loss of muscle mass and function during ageing. As such, it is imperative that the interplay between muscle and nerve are considered when investigating age-associated alterations in muscle homeostasis.

3.4 Muscle fibre type switching

Adult muscle is composed of a heterogeneous population of muscle fibre types, displaying a broad range of contractile characteristics (Gundersen, 2011). Different muscle fibre types express distinct isoforms of the sarcomeric myosin heavy chain (MyHC; types I, IIa, IIx and IIb), which elicit different ATPase activities and therefore dictate the contractile capacity of the fibre (Weiss and Leinwand, 1996). Klitgaard et al. (1990b) revealed an increase in co-expression of multiple MyHC isoforms in single muscle fibres with ageing, which may represent ongoing transitions in muscle fibre type. The general consensus is of a shift towards slower muscle fibre types with ageing (Gannon et al., 2009; Nilwik et al., 2013; Ohlendieck, 2011), thus

reducing the capacity to perform fast, powerful contractions, like those often required to prevent a fall. However, some have shown either no change in muscle fibre type (Gueugneau et al., 2014; Klitgaard et al., 1990b) or even an increase in fast fibres (Frontera et al., 2000) with ageing. Muscle fibre type is largely influenced by the innervating neuron (Buller et al., 1960) and thus alterations in NMJ morphology and extensive re-innervation may influence muscle fibre type with ageing. Furthermore, reduced mechanical loading of muscle induces a transition from slow-to-fast muscle fibre types (Caiozzo et al., 1996; Loughna et al., 1990) and thus lower levels of physical activity in elderly populations may also impact fibre type with ageing. It is noteworthy that inference of muscle fibre type transitions occurring in rodent models should be interpreted with caution as, unlike rodents, humans only express three MyHC isoforms, lacking the fast contracting type IIB MyHC isoform (Pellegrino et al., 2003; Smerdu et al., 1994), due to a genetic difference in the promoter of this gene (Brown et al., 2014; Harrison et al., 2011).

3.5 Nutrient repartitioning and disrupted lipid deposition

The age-associated loss of muscle mass and function is often accompanied by an increase in whole body fat mass (Akasaki et al., 2014; Houtkooper et al., 2011). Furthermore, there is significant accumulation of fat mass in non-adipose tissues and accompanying metabolic consequences of such nutrient repartitioning with ageing. Inter- and intra- muscular fat content increases with ageing in humans (Conte et al., 2013; Goodpaster et al., 2008; Gueugneau et al., 2014; Marcus et al., 2010) and the inter-muscular fat infiltration is associated with a loss of muscle function (Marcus et al., 2012) and presents a good predictor for loss of mobility in aged populations (Visser et al., 2005). Using a rodent model, Tardif et al. (2014) recently demonstrated that aged rats, challenged with a high fat diet, display an inability to uptake lipids into adipose tissue and show an increased lipid deposition in skeletal muscle, compared to young rats. The accumulation of lipids in non-adipose tissue is considered a strong contributor to insulin resistance (Slawik and Vidal-Puig, 2007), linking age-associated alterations in muscle composition with whole body metabolism in aged individuals. Tardif et al. (2014) also demonstrated that ectopic lipid deposition in skeletal muscles of old rats reduced protein synthetic rate, suggesting a “toxicity” effect of lipid accumulation in muscle cells, which may subsequently accelerate the symptoms of sarcopenia. Thus, altered homeostasic control in adipose tissue and subsequent fat infiltration of skeletal muscle may present detrimental effects on muscle function, dysregulation of whole body metabolism and resistance to anabolic signals in muscles of older individuals.

Taken together, the underlying pathophysiology of sarcopenia is multifactorial and progressive, resulting in an impaired ability to maintain healthy muscle mass with age. Interventions to ameliorate these alterations would improve the quality of life, independence and health care costs of an increasingly longer living population.

4. Exercise as a therapeutic intervention against sarcoponia

There is currently no optimal treatment for sarcopenia. Exercise interventions improve quality of life in older adults (Napoli et al., 2014) and some forms of exercise, particularly resistance exercise, effectively modulate muscle mass and function in older populations (Klitgaard et al., 1990a; Nilwik et al., 2013). Akasaki et al. (2014) recently demonstrated that targeted restoration of type II muscle fibre mass in middle aged mouse models (using muscle specific over-expression of constitutively

active Akt1; Izumiya et al. (2008)) effectively reduced the age-associated increases in whole body fat mass, circulating leptin and insulin levels and hepatic steatosis. This highlights that exercise interventions that maintain type II muscle fibre mass positively impact muscle function and whole body physiology with ageing (LeBrasseur et al., 2011). However, simple dissemination of this information has not resulted in adherence to such lifestyle modifications by the general population, including older adults (Biedenweg et al., 2014; Dunn, 2009). An elevated perception of frailty and poor health are considered major barriers to exercise adherence in older adults (Rhodes et al., 1999), thus limiting the effectiveness of exercise prescription as a therapeutic strategy against sarcopenia. With an increasingly longer living population, it is important to explore alternative therapeutic options that perhaps mimic the effects of lifestyle modifications to improve the quality of life and independence that is lost with the development of sarcopenia in old age.

Resistance exercise or increased muscle loading induces a myriad of intracellular signaling events in muscle cells, such as activation of the Akt/mTOR (Bolster et al., 2003; Dreyer et al., 2008; Sakamoto et al., 2003) and myostatin pathways (Louis et al., 2007; Matsakas et al., 2005). Experimental manipulation of "exercise-inducible" factors such as Akt and myostatin demonstrate a powerful role for these factors, and their associated signaling pathways, to regulate muscle size (Akasaki et al., 2014; Lee and McPherron, 2001; McPherron et al., 1997; Schiaffino and Mammucari, 2011). We discuss herein how miRNAs, novel regulators of gene expression (detailed below), are capable of modulating "exercise-inducible" signaling pathways that determine muscle size, thus presenting exciting alternative therapeutic avenues to maintain muscle homeostasis in elderly populations.

5. microRNAs, potent regulators of gene expression

microRNAs (miRNAs; miRs) are short, non-coding RNAs that regulate gene expression at the post-transcriptional level. miRNAs are predicted to regulate two-thirds of all protein-coding genes in the human genome, suggesting that miRNAs modulate many physiologically relevant processes (Friedman et al., 2009). miRNAs have been strongly implicated in regulating muscle development and maintaining muscle homeostasis (Goljanek-Whysall et al., 2012c). Although the fundamental biogenesis of miRNAs is broadly understood (described below), it is noteworthy that the intricate pre- and post-transcriptional mechanisms regulating miRNA abundance are multifactorial and still poorly understood (Ha and Kim, 2014). Mature miRNAs are generated from primary-miRNA (pri-miRNA) precursors, which are cleaved in the nucleus by the enzyme Drosha to form the pre-miRNA transcript. The pre-miRNA, containing a hallmark stem-loop, is transported into the cytoplasm, whereby it is cleaved by the enzyme Dicer to generate a 19–24 base pairs long miRNA duplex (Bartel, 2004). This duplex is unwound and the mature miRNA strand is incorporated into a protein complex called RISC (RNA Induced Silencing Complex). The non-incorporated strand is often degraded however in some cases it may also be incorporated into the RISC complex. miRNAs function to guide the RISC to partially complementary sequences, usually contained within the 3' UTR of target mRNA transcripts. The result of miRNA interaction with its target(s) is a translational block and often degradation of the transcript. miRNAs operate on a "many-to-many" relationship, whereby a single miRNA can regulate many target genes, and a single gene can be regulated by many miRNAs. In addition, most mammalian miRNAs only have partially complementary sequences to their target mRNAs (Bartel, 2004)

resulting in difficult bioinformatic-based prediction of the, sometimes hundreds of, microRNA target genes. Several algorithms exist to predict target gene(s) for known miRNAs; for example, microcosm targets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), TargetScan (http://www.targetscan.org/), miRanda (http://www.microrna.org/microrna/home.do), miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/), however the distinct lack of consensus overlap between the predictions of miRNA targets (Goljanek-Whysall et al., 2012b) makes interpretation difficult. miRNAs are also not restricted to the cell in which they were transcribed and can be released into extracellular spaces and systemic circulation, packaged in microvesicles/exosomes or complexed with Argonaute2 (Ago2) or high-density lipoproteins (HDL) (Arroyo et al., 2011; Camussi et al., 2011; Guescini et al., 2015; He et al., 2014; Vickers et al., 2011). Secretion of miRNAs permits systemic transfer of genetic material, enabling miRNA mediated epigenetic cross-talk between tissues (Camussi et al., 2011).

By influencing the mRNA and protein expression of multiple target genes, miRNAs provide a potent and highly responsive mechanism that enables cells to react to changes within their immediate or surrounding cellular context. Given their role as novel regulators of gene expression and their vast deregulation in a myriad of pathophysiological conditions, functional experiments are beginning to improve our understanding of these powerful regulatory molecules and exposing their potential as novel therapeutic agents (Van Rooij and Kauppinen, 2014).

6. The potential role of miRNAs in modulating muscle homeostasis during ageing

6.1 miRNAs are an important component of muscle cell biology

By manipulating expression levels of the miRNA biogenesis machinery, a significant role of miRNAs in muscle cell biology has been exposed (Cheung et al., 2012; Hitachi et al., 2014). Using inducible, satellite cell specific knock out mice for the gene, Dicer, effectively ablating satellite cells of miRNAs, Cheung et al. (2012) demonstrated an impaired maintenance of satellite cell quiescence and reduced survival of proliferating satellite cell progeny. Ablation of miRNAs in satellite cells (due to the absence of Dicer) resulted in a reduced number of satellite cells in the muscle, mild muscle atrophy with ageing and an impaired ability to regenerate muscle fibres following muscle injury. Using siRNAs specific to the Dicer1 gene in post-mitotic, 5-day differentiated myotubes, Hitachi et al. (2014) showed an induction of myotube atrophy within 48 hours, revealing the dependence on miRNAs to maintain myotube size, at least in culture. Expression levels of the miRNA machinery also appear to be dynamic in response to mechanical stress (McCarthy and Esser, 2007). Analysis of 7-day functionally overloaded mouse muscle revealed an up-regulation of transcripts encoding both Drosha and Exportin-5, but no change in Dicer expression (McCarthy and Esser, 2007). Taken together, miRNAs are a critical component capable of modulating muscle cell biology, both *in vitro* and *in vivo*.

6.2 Muscle enriched miRNAs regulate myogenic processes

Muscle contains its own set of muscle-enriched miRNAs, which include, but are not restricted to, miR-1, miR-206, miR-208, miR-208b, miR-133a, miR-133b, miR-486, and miR-499, and have appropriately been termed “myomiRs” (Sharma et al., 2014). MyomiRs exhibit significant and dynamic roles in myogenic processes during embryonic development and in adults by regulating quiescence and myogenic

differentiation of satellite cells (Chen et al., 2009; Cheung et al., 2012; Crist et al., 2012; Crist et al., 2009; Goljanek-Whysall et al., 2012a; Goljanek-Whysall et al., 2011; Nakasa et al., 2010). In order to promote myogenesis, miR-1/206 and miR-27 are required to down-regulate Pax3 expression during embryonic development and during muscle regeneration in adults, respectively (Crist et al., 2009; Goljanek-Whysall et al., 2011). Following injury of rat skeletal muscle, injection of miR-1, miR-133 or miR-206 into the injured muscle enhanced regeneration (Nakasa et al., 2010). This was associated with increased expression of myogenic markers, such as MyoD, myogenin and Pax7. miR-489 and miR-31 have also been shown to control muscle regeneration via regulation of satellite cell quiescence (Cheung et al., 2012; Crist et al., 2012). Crist et al. (2012) demonstrated that miR-31 regulates translation of Myf5 by sequestration of Myf5 mRNA in mRNP granules. mRNP granules are dissociated upon the activation of satellite cells resulting in accumulation of Myf5 protein. The study demonstrated that manipulation of miR-31 expression could affect satellite cell differentiation and muscle regeneration. These data strongly implicate miRNAs in holding quiescent stem cells poised to enter the myogenic differentiation program and thereby influencing myogenesis in both embryonic development and adult myogenesis.

miRNAs have also been shown to control muscle fibre type during development (Van Rooij et al., 2009). Intronic regions of myosin heavy chain genes, *Myh6, Myh7*, and *Myh7b*, contain muscle-enriched miRNAs, miR-208b and miR-499 (Van Rooij et al., 2009). The authors generated miR-208b and miR-499 double knock-out mice and revealed a substantial loss of type I muscle fibres in the soleus muscle as well as a reduced expression of slow beta-myosin heavy chain and increase in the expression of type IIx/d and IIb (fast) myosin isoforms (Van Rooij et al., 2009). Conversely, overexpression of miR-499 only was sufficient to induce conversion of all fast muscle fibres in the soleus muscle to a slow phenotype. To conclude, miR-208b and miR-499 redundantly program skeletal muscle fibres to a slow (type I) phenotype at the expense of the fast phenotype.

6.3 Non-consensus deregulation of miRNAs during muscle ageing

The skeletal muscles of young and old animals display substantially different gene (Sifakis et al., 2013; Welle et al., 2003) and protein (McDonagh et al., 2014) expression profiles. Given that miRNAs elicit their effects by post-transcriptional regulation of mRNA transcripts, it is perhaps unsurprising that miRNAs in muscle also elicit differential expression with ageing. Recent evidence has highlighted significant deregulation of many muscle enriched as well as non-muscle specific miRNAs in skeletal muscle during ageing in multiple species, including mice (Kim et al., 2014), rats (Hu et al., 2014), rhesus monkeys (Mercken et al., 2013) and humans (Drummond et al., 2011; Rivas et al., 2014; Zacharewicz et al., 2014). Furthermore, Zacharewicz et al. (2014) and Rivas et al. (2014) showed differential expression of miRNAs in skeletal muscle of young and old humans in response to an acute bout of resistance exercise, suggesting an altered capacity to regulate miRNA expression in muscles of the elderly. However, it is imperative to note that there is a considerable lack of overlap in deregulated miRNA expression in skeletal muscles of old/ageing animals amongst the available literature and an explanation as to why is of critical importance to the progress of this field. This may be due to the use of different transcriptomic platforms between studies or perhaps due to differential ageing processes in anatomically distinct muscle types.

Potential miRNAs capable of modulating age-associated changes in muscle homeostasis are summarized in Figure 1.

The levels of circulating miRNAs are also altered with ageing (Jung and Suh, 2014). Recent evidence has highlighted that muscle cells can modulate intracellular miRNA content during dexamethasone induced atrophy by extracellular release of specific miRNAs (Hudson et al., 2014a; Hudson et al., 2014b). It is therefore possible that systemically released miRNAs, packaged in exosomes/microvesicles or complexed with Ago2 or HDL, may provide useful biomarkers of intracellular events occurring during muscle ageing. Secreted miRNAs may also mediate epigenetic mediated cross talk between tissues (Camussi et al., 2011) during the ageing process.

6.4 A unique "miRNA signature" during muscle catabolism

Soares et al. (2014) recently demonstrated differential miRNA expression profiles in murine skeletal muscle during several catabolic conditions induced by a variety of different stimuli, such as denervation, starvation, diabetes and cancer cachexia. These data demonstrated that a unique and dynamic "miRNA signature" may exist for specific catabolic conditions in skeletal muscle, and thus likely modulate muscle atrophy in a condition specific manner. By conducting an intricate time course analysis following denervation induced muscle atrophy, Soares et al. (2014) showed that maximal changes in miRNA expression were delayed in comparison to maximal changes in mRNA transcript abundance, with peak miRNA changes occurring 7 days post-denervation compared to only 3 days for mRNA transcripts. The authors thus implicate that miRNAs may serve a modulatory or fine tuning role on an already initiated muscle atrophy program rather than causatively initiating muscle atrophy *per se*.

Elucidating causative and/or compensatory roles of specific miRNAs during muscle catabolism with ageing is a challenging and complex task. Although, to our knowledge, there are few studies examining the causal effects of deregulated miRNA expression in ageing muscle (Hu et al., 2014), recent studies have implicated miRNAs in modulating multiple signaling pathways regulating muscle size, as detailed below.

7. miRNAs modulate signaling pathways that regulate muscle mass

7.1 miR-23a modulates expression of MuRF1 and MAFbx atrophy genes

Molecular analysis of skeletal muscle atrophy revealed an up-regulation of two ubiquitin ligases, Muscle RING Finger 1 (MuRF1) and Muscle Atrophy F-box (MAFbx; Bodine et al. (2001)). Mice lacking MuRF1 or MAFbx are resistant to muscle atrophy (Bodine et al., 2001), demonstrating a causative role of these factors in mediating muscle atrophy. These genes have appropriately been termed "atrogenes" and are considered a critical component of the muscle atrophy program. Due to the powerful role of these genes in regulating muscle atrophy, Wada et al. (2011) conducted bioinformatic analysis to predict miRNAs targeting the 3' UTRs of both MuRF1 and MAFbx. The authors revealed only two miRNAs, miR-23a and miR-23b, showed complementarity to the 3' UTRs of both atrogenes. Interestingly, the miR-23a binding site in the 3' UTRs of MuRF1 and MAFbx 3' is highly conserved across mice, rats and humans. Using 3' UTR reporter plasmids, the authors demonstrated a direct interaction of miR-23a with MuRF1 and MAFbx. Over-expression of miR-23a in cultured myotubes was capable of inhibiting dexamethasone

induced myotube atrophy (Wada et al., 2011). Accordingly, transgenic mice over-expressing miR-23a were partially protected from dexamethasone induced muscle atrophy (Wada et al., 2011), revealing miR-23a as a molecule with therapeutic potential for perturbing atrogene (MAFbx/MuRF1) mediated muscle atrophy. Recent findings from Hudson et al. (2014b) also show reduced intracellular expression of miR-23a and increased exosomal release of miR-23a from cultured myotubes, suggesting altered miR-23a expression/release may be a useful diagnostic biomarker in conditions causing muscle atrophy.

7.2 miR-182 regulates expression of atrophy genes by modulation of Foxo3

The family of Forkhead (FoxO) transcription factors, particularly FoxO1 and FoxO3, have been implicated in regulating muscle atrophy by modulating expression of several atrogenes involved in the ubiquitous protesome system (Sandri et al., 2004). During muscle atrophy, FoxO3 binds the MAFbx promoter to elevate MAFbx transcription (Sandri et al., 2004). Consequently, over-expression of FoxO3 in murine skeletal muscle *in vivo* upregulates MAFbx promoter activity and induces myofibre atrophy (Sandri et al., 2004). Pharmacological interventions to inhibit FoxO3 are attractive anti-atrophy therapeutics. Hudson et al. (2014a) investigated whether miRNAs could interact with the FoxO3 transcript to post-transcriptionally modulate levels of FoxO3 expression in muscle. The authors demonstrated that miR-182 directly interacts with the FoxO3 3' UTR and that over-expression of miR-182 in cultured C2C12 muscle cells is sufficient to reduce FoxO3 protein expression. Treatment of cultured C2C12 myotubes with atrophy inducing drugs, dexamethasone or streptozotocin, resulted in an increase in FoxO3 mRNA expression with a concomitant reduction in miR-182. Over-expression of miR-182 was capable of blunting the dexamathosone induced increases in expression of FoxO3 target genes, including MAFbx, microtubule-associated protein light chain 3 (LC3), autophagy-related protein 12 (ATG-12) and Cathepsin L (Hudson et al., 2014a). Therefore, miR-182 modulates expression of genes involved in the autophagy/lysosome system via post-transcriptional regulation FoxO3 expression in muscle (Hudson et al., 2014a).

7.3 miR-486 targets FoxO1 and PTEN to modulate muscle size

Another member of the Forkhead family, FoxO1, is a potent regulator of muscle atrophy and induces atrogene expression when de-phosphorylated (Sandri et al., 2004). Recent studies show miR-486 can target FoxO1 to effectively modulate muscle size (Hitachi et al., 2014; Xu et al., 2012). Over-expression of miR-486 in C2C12 myotubes causes a modest increase in myotube diameters, whilst inhibition of miR-486 induces mild myotube atrophy (Hitachi et al., 2014). Inhibition of miR-486 in mouse skeletal muscle reduces muscle fibre cross sectional area, confirming the role of miR-486 both *in vitro* and *in vivo* (Hitachi et al., 2014). The work of Xu et al. (2012) demonstrated that miR-486 blunts dexamethasone-induced atrophy in primary myotubes by ablating the activity of FoxO1. Interestingly, over-expression of miR-486 not only inhibited expression of FoxO1 and its target atrogenes, MuRF1 and MAFbx, it also inhibited the expression of Phosphatase and Tensin Homolog (PTEN; Xu et al. (2012)). The inhibition of PTEN by miR-486 caused an increase in phosphorylation of the Serine/Threonine Protein Kinase, Akt, which subsequently phosphorylates and sequesters FoxO1 into the cytoplasm, perturbing its ability to up-regulate atrogene expression (Xu et al., 2012). Thus, miR-486 represses FoxO1 expression via direct post-transcriptional regulation and also indirectly regulates FoxO1 nuclear localization via inhibition of PTEN expression. Over-expression of

miR-486 significantly blunted dexamethasone induced increases in protein degradation, but interestingly had no impact on protein synthesis (Xu et al., 2012). During muscle atrophy, miR-486 is an exciting therapeutic candidate that regulates the ubiquitous proteasome by modulating Foxo1 activity.

7.4 Context dependency of miR-21 and miR-206 in muscle atrophy

Recent evidence has revealed that the cellular environment is of critical importance in determining the function of miRNAs (Carroll et al., 2013; Erhard et al., 2014) and examples of miRNA context specificity have been observed in skeletal muscle (Soares et al., 2014; Williams et al., 2009; Winbanks et al., 2013). Denervation induced muscle atrophy is associated with an increase in muscle enriched miRNAs, miR-21 and miR-206 (Soares et al., 2014). Although over-expression of miR-21 in skeletal muscle of healthy mice induced no change in muscle fibre diameters, over-expression of miR-21 in denervated muscle exacerbated muscle atrophy (Soares et al., 2014). The authors suggest that these results imply an already initiated atrophy program is required for miR-21 to modulate muscle atrophy and that miR-21 may "fine-tune" the muscle atrophy process. Similarly, although miR-206 over-expression induced mild muscle atrophy in skeletal muscles of healthy mice, over-expression of miR-206 in denervated muscles capable of exacerbating muscle atrophy (Soares et al., 2014). Both miR-21 and miR-206 were shown to inhibit expression of translation initiation factor, EIF4E3, and programmed cell death factor, PDCD10, which were both reduced during denervation-induced muscle atrophy (Soares et al., 2014). It is noteworthy that others have shown no effect of miR-206 over-expression on muscle fibre diameters in healthy mice (Winbanks et al., 2013), but this may be due to differences in the magnitude of miRNA over-expression (Soares et al., 2014). Expression levels of experimental manipulated miRNAs must be closely monitored as saturation of, or competition for, miRNA processing machinery has practical implications for interpreting results (Grimm et al., 2006; Khan et al., 2009). Furthermore, both Soares et al. (2014) and Winbanks et al. (2013) prompted the hypothesis that muscle enriched miRNAs may also interact or work in conjunction with other miRNAs, thus mediating context dependent effects on target gene expression and physiological outcomes. These data reveal that the functions of miRNAs in regulating muscle atrophy display a dependency on the cellular environment within which they reside.

7.5 miR-27a/b modulates muscle size by inhibiting myostatin

The transforming growth factor family member, Myostatin, is a well-characterized negative regulator of muscle size (McPherron et al., 1997; Trendelenburg et al., 2009), making it an attractive candidate for therapeutic interventions to modulate muscle mass. The Myostatin 3' UTR contains a highly conserved binding site for miR-27a/b (Allen and Loh, 2011; McFarlane et al., 2014), a miRNA highly enriched in skeletal muscles of mice (Allen and Loh, 2011). Over-expression of miR-27a in cultured primary myotubes caused an increase in myotube size, a response that was not observed in primary myotubes lacking myostatin (McFarlane et al., 2014), confirming that miR-27a modulates myotube size via myostatin. Similarly, over-expression of miR-27a in mouse skeletal muscle caused a substantial reduction in myostatin mRNA expression and a modest increase muscle fibre cross sectional area (McFarlane et al., 2014). Interestingly, myostatin and miR-27 show opposing fibre type dependent expression profiles (Allen and Loh, 2011). It has been postulated that miR-27 may therefore regulate the fibre type dependent expression profile of

myostatin (Allen and Loh, 2011). It is also noteworthy that manipulation of myostatin signaling results in differential expression of miRNAs involved in myogenic and growth process (Javed et al., 2014; Rachagani et al., 2010).

7.6 miR-128 is a modulator of the insulin/IGF1 signaling pathway

Insulin like growth factor 1 (IGF1) demonstrates a potent ability to modulate muscle mass (Adams and McCue, 1998; Lee et al., 2004). IGF1 signals predominantly via the IGF1 receptor and to a lesser extent, the insulin receptor (Pandini et al., 2002), to activate a myriad of intracellular signaling events, via the well-described Akt/PKB pathway (Schiaffino and Mammucari, 2011). Elevated expression of IGF1 in old mice is protective against the age-associated decline in muscle mass and function (Barton-Davis et al., 1998). Motohashi et al. (2013) recently demonstrated that miR-128 (a microRNA highly enriched in skeletal muscle and brain) directly targets the 3' UTRs of multiple genes involved in the insulin/IGF1 signaling pathway, including the insulin receptor (INSR), insulin receptor substrate 1 (IRS1) and phosphatidylinositol 3-kinases regulatory 1 (PIK3R1). Inhibition of miR-128 in cultured muscle cells revealed an increase in muscle cell proliferation and a dramatic induction of myotube hypertrophy, independent of alterations in differentiation (Motohashi et al., 2013). Confirmation of a role for miR-128 in negatively regulating muscle cell size was confirmed *in vivo*, with several muscles exhibiting an increase in mass following 35 days intravenous delivery of vectors over-expressing an antagonist against miR-128 (Motohashi et al., 2013). Therefore, interventions that inhibit miR-128 can act as an "enhancer" of IGF-Akt signaling in skeletal muscle.

7.7 miR-206 modulates "muscle-nerve communication"

Stability of the NMJ is progressively perturbed with ageing (Valdez et al., 2010) and is considered a critical stimulus for maintaining muscle mass with ageing (Butikofer et al., 2011; Sakellariou et al., 2014). A role for miRNAs in mediating homeostatic control of the NMJ was highlighted by Valdez et al. (2014) who used a muscle-specific conditional Dicer knockout mouse, effectively ablating the muscle of miRNAs, to reveal impaired re-innervation of muscle fibres following nerve injury. Using a mouse model of Amyotrophic lateral sclerosis (ALS), whereby mice show considerable NMJ destabilization, Williams et al. (2009) reported that an absence of miR-206 was sufficient to accelerate muscle fibre atrophy and ultimately exacerbated disease progression. The authors demonstrated that miR-206 targeted histone deacetylase 4 (HDAC4) and was required for fibroblast growth factor (FGF) signaling-induced compensatory neuronal re-innervation following nerve injury. Interestingly, miR-206 expression is also specifically enriched in synaptic regions of muscle fibres (Williams et al., 2009), suggesting miRNAs can display heterogeneity in expression levels within a single muscle fibre. Therefore, localization of miRNAs in muscle to within close proximity of NMJs may impact muscle-nerve communication and indirectly regulate muscle mass and function by modulating innervation.

A summary of miRNAs that target genes involved in signaling pathways that regulate muscle size is detailed in Table 1.

8. Conclusions and future work

Sarcopenia is underpinned by a myriad of progressive pathophysiological alterations, which ultimately perturb the maintenance of muscle homeostasis with ageing. Type II

muscle fibres appear to be more susceptible to atrophy than their type I counterparts, which is also consistent with fibre type dependent alterations in satellite cell biology and behaviour. Skeletal muscle is increasingly infiltrated with lipid during ageing, ultimately impacting muscle function, anabolic resistance and whole body metabolism. Finally, neuromuscular junction morphology becomes progressively disrupted with age and the subsequent alterations in interplay between muscle and nerve may be critical for the maintenance of healthy, functioning muscle mass.

Interestingly, it has become clear that miRNAs modulate many signaling pathways associated with the regulation of muscle mass. The manipulation of miRNA expression offers exciting therapeutic potential for correcting detrimental reductions in muscle mass with sarcopenia. However, with regard to restoring perturbed miRNA expression with ageing, it is imperative to explore whether the deregulation of endogenous miRNAs changes progressively, chronically, or dynamically with age or whether there is a "trigger point" of vast and chronic deregulation in miRNA expression. Furthermore, the field of miRNA therapeutics requires functional studies to establish which differentially expressed miRNAs initiate age-associated alterations in muscle homeostasis, those which fine-tune an already initiated pathophysiological process, and those which compensate for perturbed muscle mass and function in older individuals.

Conflict of interests

The authors declare no conflict of interest regarding the publication of this article.

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