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microRNAs in skeletal muscle development

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Abstract

A fundamental process during both embryo development and stem cell differentiation is the control of cell lineage determination. In developing skeletal muscle, many of the diffusible signaling molecules, transcription factors and more recently non-coding RNAs that contribute to this process have been identified. This has facilitated advances in our understanding of the molecular mechanisms underlying the control of cell fate choice. Here we will review the role of non-coding RNAs, in particular microRNAs (miRNAs), in embryonic muscle development and differentiation, and in satellite cells of adult muscle, which are essential for muscle growth and regeneration. Some of these short post-transcriptional regulators of gene expression are restricted to skeletal muscle, but their expression can also be more widespread. In addition, we discuss a few examples of long non-coding RNAs, which are numerous but much less well understood.

Keywords

Non-coding RNA; microRNA; lncRNA; myogenesis; skeletal muscle; satellite cell

1. Introduction

MicroRNAs (miRs or miRNAs) are short non-coding RNAs, a sub-group of a growing number of RNAs with regulatory functions, which also includes long non-coding RNAs (lncRNAs). miRNAs were first discovered in the nematode *C. elegans* where the miRNA, let-7, controls the timing of cell differentiation during development. Subsequently, miRNAs have been found to be widespread in both animals and plants and have revolutionised our appreciation of the complexities underlying the regulation of gene expression [1].

MiRNAs are involved in post-transcriptional regulation of gene expression [2] and have been implicated in many biological processes, including development and adult tissue maintenance. Because they are often mis-regulated in pathophysiological conditions, miRNAs serve as biomarkers and potential therapeutic targets [3, 4]. Mature miRNAs are generated from longer primary transcripts, further processed to yield the short mature miRNA, which is then incorporated into RISC (RNA Induced Silencing Complex) [5]. RISC guides miRNAs to partially complementary sequences usually found in the 3'-UTR (untranslated region) of target mRNAs, although target sequences can be located in 5' -UTR and even within the open reading frame. Interaction between miRNAs and their targets leads to translational block and often, but not always, the degradation of the transcript. A high degree of complementarity between the miRNA 'seed' sequence (nucleotides 2-8 at the 5'-end) and its target favours target recognition [6].

Several algorithms predict putative target genes for a known miRNA in the genome, but experimental validation of targets is critical in order to evaluate functional implications of any given miRNA:target gene interaction. Mathematical modelling of experiments with synthetic reporters suggests that miRNAs confer precision to protein expression by reducing noise. Noise reduction is enhanced for genes targeted by multiple miRNAs and for genes expressed at low levels [7]. Therefore, although miRNA action often leads to only a moderate reduction of target gene expression, this can confer robustness to biological processes [7-9].

1.1 MiRNA biogenesis

MiRNAs are small (~18 to 22nt) non-coding RNAs, which are present in different genomic contexts (Fig. 1). In animals, many of the known miRNAs are located within a protein-coding host gene, usually within an intron but in some cases within an exon [10]. Both 'exonic' and 'intronic' miRNAs are transcribed along with the host gene by RNA polymerase II. In addition, miRNAs can be produced from within long non-coding transcripts [10] (see section 4.). Alternatively, miRNAs can be generated independent from a host gene using their own regulatory sequences and promoter, so-called intergenic miRNAs. Often, several miRNAs are encoded in close proximity of each other, constituting a polycistronic transcription unit [11]. Polycistronic miRNAs from the same cluster generally have comparable tissue expression profiles. However, sometimes not all members from a cluster share the same expression profile, this is likely due to further regulation of the primary transcript to allow differential processing and expression of the miRNAs within the cluster.

Biogenesis (Fig. 1) begins with the synthesis of the primary miRNA (pri-miRNA) by RNA polymerase II [12]. The pri-miRNA has a characteristic hairpin stem-loop structure with imperfect complementarity in the stem region, resulting in mismatches and bulges. The pri-miRNA is cleaved by the Microprocessor complex, which comprises Drosha, a ribonuclease III enzyme, and DGCR8 (DiGeorge syndrome critical region 8 in human) or Pasha in flies and worms, a double-stranded RNA binding protein [13-18].

The basal junction between single-stranded RNA and double-stranded RNA, and the apical junction, which links to the terminal loop of the pre-miRNA, are two important regions for determining accurate and efficient cleavage by Drosha [19, 20]. The liberated stem-loop containing RNA (~60 to 110nt) is known as precursor miRNA (pre-miRNA) and is exported to the cytoplasm by Exportin-5, a RanGTP-dependent double-stranded RNA-binding protein located on the nuclear membrane [21-23].

Further aspects of regulation of pri-miRNA processing include transcription factors that interact with Drosha and/or certain pri-miRNAs [12, 24]. For example SMAD proteins,

transcriptional effectors of TGFβ/BMP signaling, can bind to pri-miRNAs to control their processing by Drosha [25, 26] (reviewed in [27]). The efficiency of Drosha-mediated processing is important for determining miRNA abundance. Thus, several mechanisms exist to control Drosha expression, activity and specificity, including the reciprocal regulation between Drosha and DGCR8. Post-translational modification of the Microprocessor complex regulates stability, nuclear localisation and processing activity [28].

In the cytoplasm, the pre-miRNA undergoes further processing by Dicer, a Ribonuclease III enzyme, and TRBP complex (or Loqs in *Drosophila*), which removes the terminal loop, yielding a short double-stranded RNA molecule (~22nt) with a two-base overhang on the 3' end [29-31]. Dicer/TRBP facilitates activation of the RNA-induced silencing complex (RISC). The miRNA duplex is incorporated into RISC, unwound and eventually one strand remains as the mature miRNA and the other strand is excluded. The relative thermodynamic stability of the two ends of the duplex determines which strand is incorporated; the strand whose 5' terminal nucleotides are less stable is usually preferred and is incorporated more efficiently [32, 33]. However, in some cases, sequences derived from both arms of the hairpin can have biological function [34, 35]. Moreover, the dominant miRNA produced may switch between strands in different developmental stages, tissues or during evolution [36]. Therefore, a previous nomenclature where the less predominant form was denoted by an asterisk, as miRNA*, has been altered. Instead, miRNAs originating from either the 5' or 3' ends of the pre-miRNA hairpin are now annotated as the -5p and -3p miRNAs [37].

An alternative and less common pathway for miRNA biogenesis bypasses cleavage by the Microprocessor complex containing Drosha/DGCR8. Instead these Mirtrons, which have been discovered in mammals, *C. elegans* and *Drosophila*, are located within introns of protein coding host genes and use the splicing machinery to generate miRNAs [38-40].

1.2 Mechanism of action / function

The small RNA duplex generated by Dicer/TRBP is loaded onto an Argonaute (Ago) protein to form the RISC [41, 42]. There are four closely-related Ago proteins (Ago1-4) that

form RISC (Fig. 1) [43], which also includes the GW repeat-containing protein GW182, known as TNRC6A-C in human [44]. Ago proteins determine which of the miRNA strands, -5p or -3p, will be incorporated, as indicated above the thermodynamic stability of the duplex ends is important. Once the mature miRNA has been incorporated, it guides RISC to target complementary sequences usually present in the 3'-UTR of mRNAs [45]. The interaction of the miRNA with its target site, mediated by Watson-Crick base-pairing, requires thermodynamic stability (i.e. minimal folding free energy) and site accessibility [6, 46]. Animal miRNAs typically have imperfect complementarity with their target site, and the degree of complementarity influences the outcome of the interaction between the miRNA and its target sites. Usually protein translation is repressed and the mRNA is subsequently de-capped, de-adenylated and degraded. Although the relative contribution of inhibition of translation and degradation of mRNA varies [47], it appears that these processes are linked [48-51]. Overall, miRNAs cause post-transcriptional repression of gene expression, with mRNA destabilisation the dominant effect of miRNAs [52].

To understand the role of miRNAs in different biological systems and contexts, many approaches aim to either inhibit or enhance miRNA function (Fig. 1). In zebrafish embryos, inhibition of miRNA function is achieved by microinjection of synthetic oligonucleotides, such as morpholinos. These can either target Drosha-cleavage sites or target the hairpin loops of pre-miRNAs to inhibit Dicer interactions, or they hybridize with target sites in the 3'-UTR to prevent interactions with specific miRNAs [53, 54]. Morpholinos that directly target the mature miRNA prior to Ago-incorporation have also been used, for example to study muscle development in zebrafish [55]. In chick embryos, synthetic miRNA inhibitors, known as antagomiRs can be delivered by targeted microinjection to investigate the role of miRNAs in a particular tissue. Target-protector morpholinos have also been used to interfere with specific miRNA:target interactions in chick somites [56]. In mice, miRNA function can be assessed through genetic knockout (examples in section 2.3), or by over-expression of pre-miRNAs, or the introduction of synthetic miRNA mimics that resemble the mature miRNA

duplex [57, 58]. These tools have improved our understanding of the biological function of miRNAs *in vivo*.

1.3 Target identification

Target recognition by RISC is largely determined by the miRNA 'seed'-sequence, six to seven nucleotides spanning nucleotides 2-7/8, at the 5'-end of the miRNA [59]. Therefore, targets can be predicted by algorithms, which locate sequences complimentary to the seed in the 3'-UTR of mRNAs. Complementary sites at the 3' end of the miRNA, usually nucleotides 12-17, are also important [60, 61]. However, there are exceptions and targeting of miRNA to non-canonical sites with seed-mismatches has also been reported [62, 63]. Thus, understanding miRNA targeting remains a challenge needing further investigation.

Computational target prediction can identify potential miRNA:mRNA interactions, although different databases, such as TargetScan [64], miRanda [65], Pictar [66], miRmap [67], utilise different algorithms and produce different lists of predicted targets. These can be narrowed down by comparing outputs of different search algorithms and/or using GO-term analysis, but ultimately predicted targets include false positives and have to be validated experimentally and examined for their biological function. Validation often uses luciferase assays to confirm miRNA target interactions *in vitro*: candidate 3'-UTR sequences are placed downstream of a firefly luciferase gene and co-transfection with miRNA mimics leads to reporter gene repression [68, 69]. Confirmation of effects on candidate target genes *in vivo*, in a tissue of interest, involves the analysis of endogenous gene expression levels after miRNA inhibition or overexpression.

Experimental approaches to identify targets typically quantify effects on gene expression after miRNA manipulation, either enhancing or blocking function (indicated in green or red in Fig. 1) (for example [69]. After such manipulations, differential down regulation or de-repression of candidate target mRNAs or proteins can be assessed by microarrays, RNA sequencing or proteomics. Such genome-wide analysis has the potential to capture many targets for any given miRNA, although some may be missed if they fall below a threshold of

sensitivity (false negatives). In addition, any candidates have to be validated as described above.

Alternatively, miRNA:mRNA target interactions can be identified by isolating mRNAs associated with RISC. Several approaches capture miRNA:mRNA complexes by immunoprecipitation or pull-down methods followed by sequencing. Co-precipitation with Ago identifies the bound miRNA and mRNAs [70]. In addition, UV crosslinking of RNA-protein complexes combined with an RNase step, so-called HITS-CLIP, accurately identifies miRNA binding sites [71]. Similar techniques, such as PAR-CLIP and CLASH, either use photo-reactive 4-thiouridine or introduce a ligase step to increase the efficiency of capturing interactions [72-74]. Through direct sequencing these approaches identify specific miRNAs together with a target mRNA for any given site of Ago binding [73]. Furthermore, miRNA:mRNA target interactions can be identified by streptavidin pull-down of biotinylated synthetic miRNAs [75]. Unbiased identification of miRNA:mRNA target interactions on a genome-wide scale offers major advantages for capturing non-canonical interactions. The next challenge will be to utilise the data to confirm the functional significance of specific interactions in a biological context.

2. miRNAs in vertebrate muscle development

2.1 Myogenesis in vertebrate embryos

Skeletal muscle serves as a paradigm to investigate molecular mechanisms that determine cell fate decisions and subsequent cellular differentiation programmes. Genetic approaches in mice and zebrafish, together with classic embryology and gene function analyses in chick have all contributed to our current understanding of the signals and gene networks that are crucial for vertebrate myogenesis [76].

In vertebrate embryos, skeletal muscle of the trunk and limbs are derived from somites, transient paired segments that form in a regular sequence along the axis, on either side of the neural tube [77]. In response to extrinsic signaling molecules, including Wnt, Shh and Notch, the initially epithelial somite undergoes morphogenetic changes and differentiates

[78-80]. On the ventral side, cells dissociate to form the sclerotome, whilst the dermomyotome on the dorsal side remains epithelial and contributes myocyte progenitors to the myotome. These progenitors express the paired-box transcription factor Pax3, which is important for the entry into the myogenic programme [81]. Myotome formation is initiated at the epaxial lip of the dermomyotome, abutting the neural tube [82]. Interactions with migrating neural crest cells triggers translocation of dermomyotomal lip progenitors into the myotome [80], where they upregulate myogenic regulatory factors (MRFs). The MRFs initiate the myogenic differentiation programme, of which activation of miRNA expression by Myf5 and MyoD is an intricate part [83] (see below).

Whole mount *in situ* hybridization identified numerous miRNAs expressed in developing somites of zebrafish, *Xenopus* and chick embryos [84-86]. Only a subset of these has been investigated in more detail. Interestingly, despite a high degree of sequence conservation, the timing and location of miRNA expression is not always conserved across vertebrates [87]. For example, miR-1 is expressed in skeletal muscles of zebrafish, medaka, *Xenopus*, chick and mouse; however, its additional expression in cardiomyocytes is seen only in amniotes: chick and mouse [56, 83, 87].

2.2 Cell based models to study the role of miRNAs in myogenesis

C2C12 cells are a widely used model for skeletal muscle cell differentiation and they have been useful to characterize candidate miRNAs and their targets. Undifferentiated C2C12 cells express Myf5 and MyoD, however the ability of these MRFs to induce differentiation is blocked under growth conditions. The removal of growth factors, either through depletion or by switching cells into low serum containing medium leads to myoblast differentiation.

This cell-based system has been used extensively to study the role of myomiRs, a group of miRNAs enriched in, or restricted, to striated muscles [88, 89] (Fig. 2). MyomiRs include the miR-1 and miR-133 families, comprising miR-1-1/miR-1-2/miR-206 and miR-133a/miR-133b. These families are encoded by three loci in mouse and human, and by four loci in chicken. The members of each family have identical seed sequences and because they

differ in only a few nucleotides outside the seed region, they are assumed to be functionally redundant. One member of the miR-1 family is usually co-expressed with one member of the miR-133 family, from the same primary bi-cistronic transcript [83]. MRFs regulate expression of miR-1, miR-206 and miR-133 in C2C12 myoblasts [90, 91], where they affect the balance between differentiation and proliferation through interactions with multiple targets.

Early work showed that miR-1, miR-206 and miR-133 expression increases during C2C12 myoblast differentiation [92-94]. The gap junction protein, connexin 43 (Cx43), and histone deacetylase 4 (HDAC4), a repressor of muscle gene expression, were identified as direct targets for miR-1 [92, 93]. Similarly, miR-1 and miR-206 have been shown to downregulate Pax7, in both C2C12 cells and in primary myoblasts, by directly targeting its 3'-UTR. Pax7 is co-regulated by miR-486 during myoblast differentiation [95, 96]. Although expression is not completely abolished but reduced by approximately 30-60% by individual miRNAs, interactions with multiple target genes, some of which have multiple target sites and can sometimes respond to multiple miRNAs, provide a mechanism through which miRNAs become functionally significant. The example above illustrates how miR-1, miR-206 and miR-486 promote myogenic differentiation.

In addition, miR-133 was found to promote C2C12 proliferation by targeting Serum Response Factor (SRF) [93]. More recent reports showed that miR-133 inhibits myoblast proliferation by repressing Cyclin D1 (CCND1) and inducing G1 phase arrest [97]. Furthermore, proliferation is negatively regulated by miR-133 targeting of FGFR1 and PP2AC, which regulate phosphorylated ERK MAP kinase levels [98]. Thus, the roles of miR-133 with respect to myoblast proliferation may be context dependent; moreover, these regulatory interactions need to be confirmed *in vivo*.

Differential microarray analyses of C2C12 cells, in which miR-206 was overexpressed or inhibited revealed many potential direct targets including a subunit of DNA polymerase alpha [94] as well as Meox2, RARB, Fzd7, MAP4K3, CLCN3, NFAT5, and the chromatin remodelling factors Smarcd2 and Smarcb1 [69]. The sustained expression of some of these validated miR-1 and/or miR-206 targets inhibits C2C12 cell myogenesis and leads to

aberrant expression of genes related to alternative cell fates, such as chondrogenesis [69]. This is similar to findings in embryonic stem (ES) cells where miR-1 and miR-133 promote mesoderm formation and repress non-muscle cell fates, in part mediated by repression of Delta-like 1 (Dll-1), a Notch ligand [99].

Microarray analysis identified miRNAs differentially expressed during C2C12 myogenesis [100]. Many of the genes encoding these miRNAs contained binding sites for YY1, a ubiquitously expressed transcription factor. Further analysis revealed that YY1 inhibits miR-1/miR-133 expression in C2C12 myoblasts; YY1 itself is a miR-1 target, thus forming a feed-back circuit. Taqman low density arrays (TLDA) of human affinity purified myoblasts identified additional miRNAs, that are either up- or down-regulated during myogenic differentiation. Transcriptomics performed in parallel was combined with bioinformatics to identify a number of putative targets [101].

2.3 The role of miRNAs in myogenic development and tissue maintenance

Genetic approaches in mice first suggested the importance of miRNAs in vertebrate muscle development (Fig. 3). The conditional deletion of Dicer in muscle causes perinatal lethality with reduced skeletal muscle mass and abnormalities in muscle fibre morphology [102].

Because of their striking expression, much attention has focused on the miR-1 and miR-133 families. In both mouse and chick embryos the MRFs regulate their expression, together with myocyte enhancer factor-2 (MEF2) [83, 103]. Surprisingly, despite sequence conservation and conservation of myomiR expression across vertebrates, including human [104], the double-knockout of the miR-1 and miR-133 families has no overt effect on skeletal muscle development [105] and muscle is grossly normal in a KO of the miR-206/miR-133b cluster [106]. However, mice lacking miR-133a develop an adult onset centronuclear myopathy [107]. Furthermore, miR-133 mediated repression of Prdm16 regulates cell fate choice between muscle progenitors and brown adipocytes, two related lineages [108, 109]. In miR-133a1$^{-/-}$/miR-133a2$^{-/+}$ mice, the brown and thermogenic gene programs are enhanced

in white adipose tissue, leading to increased insulin sensitivity and glucose tolerance [110]. In these mice, skeletal and cardiac muscle is not affected, due to the remaining miR-133 function. More generally, the dysfunction of brown fat in ageing and obesity coincides with reduced expression of Dicer leading to global miRNA downregulation. This suggests fine-tuning gene expression is important for healthy tissue maintenance [111].

In zebrafish, morpholino mediated knock-down of miR-1 and miR-133 disrupts actin organization and sarcomere assembly during muscle differentiation [55]. The activity of miR-1 and miR-206 in muscle also affects angiogenesis by modulating the levels of Vascular endothelial growth factor A (VegfA), which signals to adjacent endothelial cells [112]. Thus, miRNAs can regulate cross-tissue signaling. In somites of chick embryos, the targeted microinjection of antagomiRs revealed that miR-206 modulates the progenitor to committed myoblast transition by negatively regulating Pax3 during early myogenesis [56]. AntagomiR-mediated inhibition also shows that miR-1/miR-206 and miR-133 orchestrate the down-regulation of BAF60A and BAF60B, enabling preferential incorporation of BAF60C into the Brg1/BAF chromatin remodelling complex during myoblasts differentiation [113]. The phenotypes resulting from genetic or transient manipulations of the myomiR families in different species and tissues highlight their varied and context dependent functions.

In addition to regulation of VegfA mentioned above, miRNAs have been found to regulate other signaling molecules and pathways and may act to refine morphogen gradients (reviewed in [114]). For example, interference with miR-30 and miR-214 function in zebrafish leads to mis-regulation of the Hedgehog pathway and affects the specification of superficial slow-muscle fibres. This results from targeting the transmembrane receptor smoothened [115] or su(fu) [116], a negative regulator of Hedgehog signaling. In mouse primary myoblasts, polycomb group proteins repress transcription of miR-214, an intronic miRNA. Upon differentiation and recruitment of MRFs, miR-214 transcription is activated. miR-214 targets Ezh2, the catalytic subunit of PRC2 polycomb complex, thus promoting differentiation through negative feed-back [117].

The Bone Morphogenetic Protein (BMP) signalling pathway, which inhibits myogenic

differentiation, is negatively regulated by miR-26a [118]. Exogenous miR-26a promotes myoblast differentiation, while miR-26a inhibition delays both, differentiation in neonatal mice and regeneration after injury, due to de-repression of Smad1 and Smad4 expression.

In the zebrafish Myf5 locus an intronic miRNA, miR-3906 or miR-In300, was identified to silence dickkopf-3-related gene (DKK3a). DKK3a in turn interacts with the membrane receptor integrin alpha 6b (ITGA6b) to activate Myf5 promoter activity via p38 signaling [119, 120]. In addition, both miR-3906 and miR-203a impair fast muscle differentiation by targeting Homer-1b or Dmrt2a respectively [121, 122].

Myotube maturation and myofiber hypertrophy is modulated by miR-128a, which is highly expressed in developing somites, brain and differentiating skeletal muscle [84] and regulates genes involved in insulin signaling, including Insulin receptor (Insr), Insulin receptor substrate 1 (Irs1) and Phosphatidylinositol 3-kinases regulatory 1 (Pik3r1) [123]. In muscle side population cells, mesenchymal cells with myogenic potential, miR-128a regulates genes involved in adipogenic, osteogenic and myogenic fates, including PPARγ, Runx1 and Pax3 [124]. Thus, it was suggested that miR-128a maintains quiescence in muscle side population cells by inhibiting their differentiation into multiple cell types.

3. miRNAs in adult muscle stem cells

3.1 Introduction to Satellite cells

Satellite cells (SCs), a self-renewing population of adult stem cells, facilitate the long-term regenerative capacity of skeletal muscle [125]. (See review by Zammit in this issue.) SCs were initially identified based on their morphology and position using electron microscopy. They are located outside the plasma membrane of multinucleated muscle cells and beneath the basal lamina surrounding each myofiber [126]. In adult muscle, SCs are normally quiescent and characterized by expression of the paired box transcription factor Pax7 [127]; although the SC population is heterogeneous and in different anatomical locations, i.e. trunk muscles and diaphragm, some of the cells express Pax3 [128-130].

In response to stimuli, such as muscle injury, SCs leave their quiescent state, become activated and contribute to the muscle repair process. SC activation is accompanied by proliferation, expression of MRFs and migration. Some SCs undergo asymmetric cell division to generate another stem cell, which re-enters the niche, as well as a committed myogenic cell [131, 132]. In ageing or diseased muscle, SC activity is reduced, decreasing the regenerative potential of skeletal muscles. This can lead to loss of skeletal muscle mass, quality and strength. MiRNAs have been identified as potential therapeutics or therapeutic targets which could alleviate some of these effects [3, 4].

3.2 microRNAs in Satellite Cells

MiRNAs are important for skeletal muscle regeneration as they help to maintain SC quiescence, but are also involved in SC proliferation and differentiation [133] (Fig. 4). The myomiRs, miR-1, miR-206 and miR-133 are up-regulated in SCs activated *in vitro*. This correlates with enhanced SC differentiation, potentially mediated through direct targeting of *Pax7* and *Pax3* mRNA, as indicated by a 50% reduction in expression of 3'-UTR reporter constructs [95, 134]. Interestingly, MyoD$^{-/-}$ SC-derived myoblasts display enhanced survival and accelerated growth rates, correlating with delayed terminal differentiation and muscle regeneration. The resistance of MyoD$^{-/-}$ SCs, which can self-renew, to apoptosis is at least in part due to the impaired activation of miR-1 and miR-206 expression in these cells. As a result Pax3, a direct miR-1/miR-206 target, is de-repressed and activates anti-apoptotic genes, including Bcl2 [134].

SC-derived myoblasts lacking the miRNA biogenesis enzyme, Dicer, have also been examined. Not unexpectedly, they display reduced expression levels for several miRNAs: miR-1, miR-206, miR-133, miR-22 and miR-24, and their growth *in vitro* is affected [134]. It has also been demonstrated that SCs lacking Dicer escape quiescence and become activated precociously [135]. Loss of Dicer affects miR-489 expression, which is highly enriched in quiescent SCs; its main function is to maintain the quiescent state by targeting the oncogene Dek, thus preventing cell cycle entry [135]. Similarly, miR-195 and miR-497

are involved in the maintenance of quiescence and induce cell cycle arrest by targeting cell cycle genes, Cdc25 and Ccnd [136].

Some quiescent SCs express high levels of Pax3, which becomes resistant to miR-206 regulation due to alternative polyadenylation and a shorter 3'-UTR [137]. Indeed, SC-specific knock-out in mice suggest that miR-1 and miR-206 expression is repressed by TRAF6/c-JUN signaling to maintain SCs quiescence [138]. Another miRNA important for maintaining quiescence is miR-31, which is sequestered in mRNP granules together with mRNAs. The storage of miR-31 together with Myf5 mRNA prevents its translation, thus halting myogenic commitment but rendering SCs poised for activation [139]. Interestingly, abnormal levels of miR-31 are present in activated Mdx-derived SCs [140]. In dystrophic muscle of zebrafish, mice and human the SRF-dependent intronic miR-199a-5p is elevated. Effects on cell size, proliferation and differentiation are mediated by miR-199a-5p targeting of WNT pathway components, including FZD4, JAG1 and WNT2 [141].

At the onset of SC differentiation, Pax3 is negatively regulated by miR-27b [142]. Expression of miR-27b in turn is inhibited by Pitx2c, the most highly expressed isoform of the transcription factor Pitx2 in myoblasts and a mediator of canonical Wnt signaling [143-145]. Interference with miR-27b function results in continued Pax3 expression, more proliferation and delayed onset of differentiation [142]. The exquisite regulation of Pax3 in SCs mirrors the fine-tuning of this key myogenic regulator during the progenitor to myoblast transition by miR-206 or miR-27b in chick and mouse embryos respectively [56, 142].

Additional miRNAs that are negatively regulated by Pitx2c include miR-15b, miR-23b, miR-106b and miR-503. This promotes cell proliferation in early-activated SCs as it allows the expression of key cell cycle regulators, including CyclinD1 and CyclinD2, in myogenic cells. Moreover, Pitx2c enhances Myf5 expression in SCs by regulating miR-106b, promoting the commitment to a myogenic cell fate [146]. In addition, the mTOR-regulated miR-125b, which declines during myogenesis has been shown to negatively modulate muscle regeneration in mice by targeting IGF-II (insulin-like growth factor 2) [147].

Muscle regeneration is impaired in MEF2A knockout mice due to mis-regulation of the largest known mammalian miRNA cluster, the Gtl2-Dio3 locus. Two miRNAs in this locus, miR-410 and miR-433, enhance skeletal muscle regeneration by targeting secreted Frizzled-related proteins (sFRPs), inhibitors of WNT signalling [148]. In injured Mef2a knockout muscle sFRP expression is upregulated and Wnt activity is attenuated. Subsequent conditional knockouts of three of the four MEF2 genes in SCs [149] showed that MEF2A is not absolutely required for regeneration, although its role in SCs is revealed by the observation that a double knockout of C and D does not have a regenerative phenotype, whereas a triple knockout of A, C and D does. In addition, MEF2A may be important in non-SCs where it could elicit non-cell autonomous effects during the regeneration process, including modulation of Wnt signaling.

In differentiated myofibres two intronic miRNAs, miR-208b and miR-499, control the slow myofiber gene programme (Fig. 4) at the expense of fast muscle gene expression by inhibiting Sox6, a transcriptional repressor. Double knockout mice, miR-208b$^{-/-}$; miR-499$^{-/-}$, display substantial loss of β-MHC and type I (slow) myofibers and a concomitant increase of fast type myosin isoforms [89]. In zebrafish, miR-499 mediated repression of Sox6 also governs the slow muscle programme. Deletion of target sites in the Sox6 3'-UTR in the context of a transgenic EGFP reporter gene led to its ectopic expression in slow-twitch fibres. Conversely ectopic expression of miR-499 resulted in reduced Sox6 protein in fast-twitch fibres, although the consequence for fast/slow specific gene expression, or a loss-of-function phenotype, was not investigated [150].

The myogenic capacity of myoblasts decreases with age and expression of numerous miRNAs is disrupted in ageing muscle [151, 152]. Comparative analysis of miRNA expression profiles identified reduced abundance of miR-431 in aged myoblasts. Smad4, a downstream effector of TGFβ signalling, which inhibits myogenesis, is a direct target. Injections of miR-431 improved regeneration in a muscle injury model in mice, whereas miR-431 inhibition impaired the myogenic capacity of human skeletal myoblasts [151]. Thus, miR-

431 acts alongside miR26a, which promotes differentiation of myoblasts, both *in vitro* and *in vivo*, by targeting the transcription factors Smad1 and Smad4 [118]. In addition, expression of miR-181a and its target gene, sirtuin1 (Sirt1), are disrupted in skeletal muscle from old mice [152]. Similarly, expression of miR-143, a regulator of the insulin growth factor-binding protein 5 (Igfbp5), is reduced in SCs from old mice. It is proposed that downregulation of miR-143 may act as a compensatory mechanism during aging to improve myogenesis [153]. Overall it seems that the dysregulation of miRNA:mRNA target interactions contributes to the age-related changes in SC function.

4. Long noncoding RNAs in skeletal muscle

Long non-coding RNAs (lncRNAs) have emerged as key regulators of gene expression, they are numerous but are often poorly conserved at sequence level [154]. They have different modes of action and their functions in muscle development, differentiation and disease are currently emerging (reviewed in [155, 156]).

The classic lncRNA, H19, which is imprinted and expressed exclusively from the maternally inherited allele, is strongly expressed in mesoderm and endoderm during embryogenesis. After birth, H19 is maintained in skeletal muscle, where it generates the conserved miR-675-3p and miR-675-5p. These target Smad transcription factors, mediators of BMP signaling and inhibitors of muscle differentiation [157, 158].

Another muscle-specific long noncoding RNA, linc-MD1, acts as a competing endogenous RNA (ceRNA) by sequestering miR-133; thereby it regulates muscle-specific gene expression during myoblast differentiation [159]. An intergenic lncRNA encoded next to the MyoD gene, LncMyoD, is directly activated by MyoD and plays a role in myoblast differentiation. LncMyoD binds to IGF2-mRNA-binding protein 2 (IMP2) and negatively regulates IMP2-mediated translation of proliferation genes [160]. Similarly, MUNC (MyoD upstream noncoding) is located upstream of MyoD and specifically expressed in skeletal muscle. MUNC acts in *trans* on multiple promoters to increase myogenic gene expression during differentiation [161]. Another MyoD induced lncRNA is Dum, which promotes

myoblast differentiation and muscle regeneration by silencing its neighbouring gene, Dppa2, in *cis*, via intrachromosomal looping between the Dum locus and the Dppa2 promoter [162].

It turns out that some RNAs originally categorised as non-coding actually encode short peptides. For example, myoregulin (MLN) is a micropeptide encoded by a skeletal muscle-specific, putative long noncoding RNA. MLN interacts directly with SERCA, a pump that regulates $Ca^{(2+)}$ uptake into the sarcoplasmic reticulum, and inhibits its function [163]. Another peptide called DWORF (dwarf open reading frame), also encoded by a long noncoding RNA, enhances SERCA activity by displacing inhibitors, including myoregulin. Together, these two antagonistic peptides are crucial for the regulation of skeletal muscle contractility [164].

Overall, regulatory RNA molecules, whether short, long, non-coding or producing short peptides, are certain to have more surprises in store and their importance for development, differentiation and regeneration should not be underestimated.

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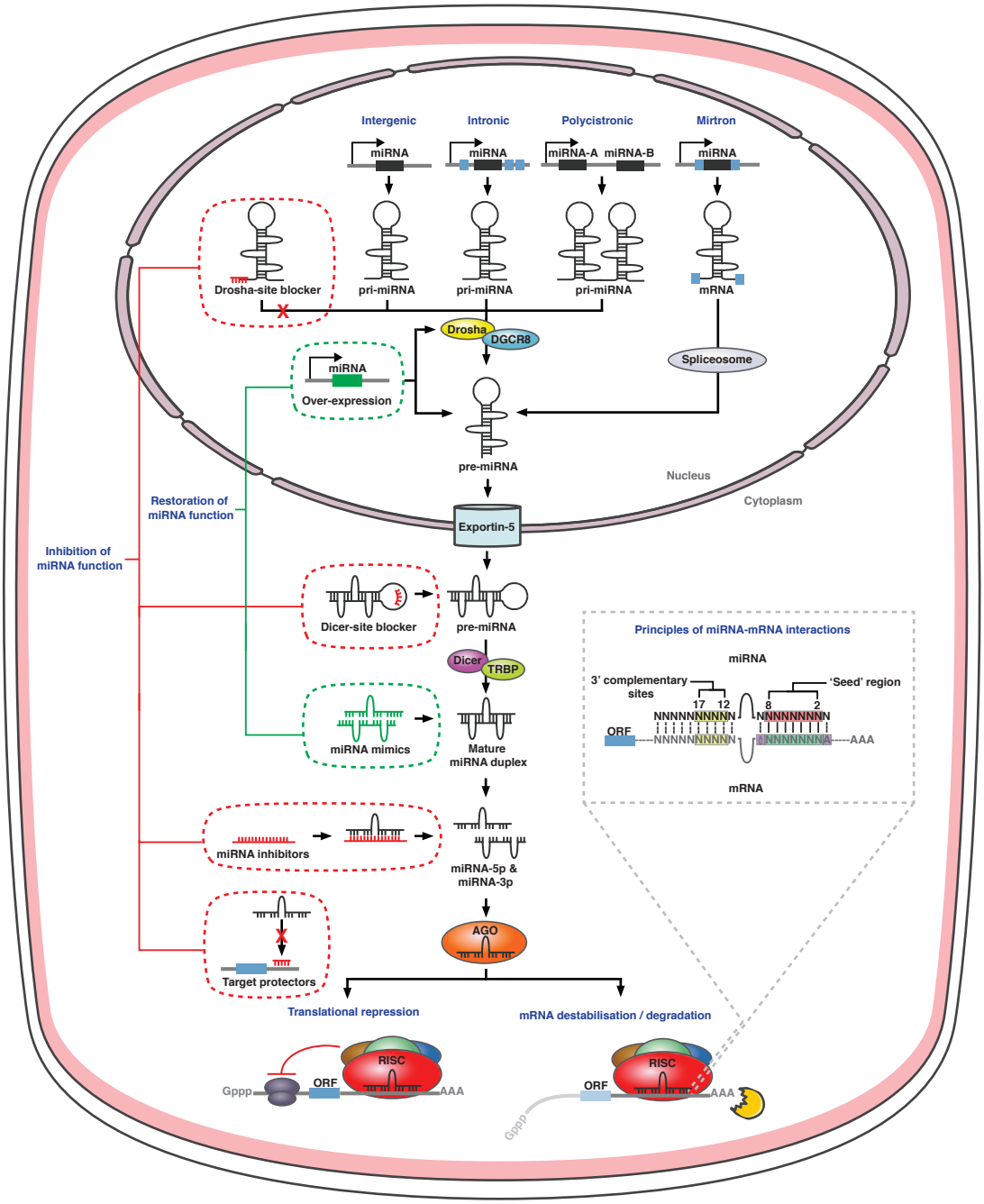
Figures and legends

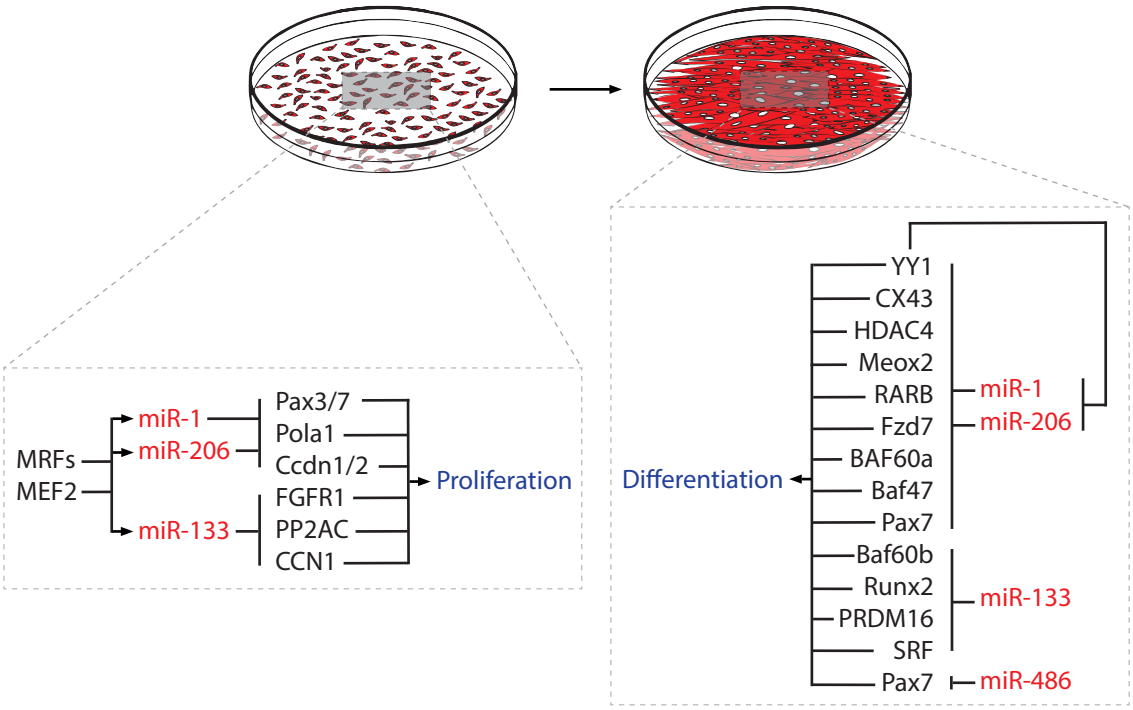
Figure 1. miRNA biogenesis, experimental approaches to modulate miRNA activity and principles of miRNA-mRNA interactions. MiRNAs are transcribed by RNA polymerase II from intergenic, intronic or polycistronic loci to form primary miRNAs (pri-miRNAs) which are cleaved by Drosha/DGCR8 to form precursor miRNAs (pre-miRNAs). Mirtrons by-pass Drosha/DGCR8 and are spliced to form pre-miRNAs. Pre-miRNAs are exported by Exportin-5 out of the nucleus into the cytoplasm and cleaved by Dicer/TRBP to form mature miRNA duplexes. These are incorporated into Argonaute (Ago) protein to form an RNA-induced silencing complex (RISC). The duplex is unwound and a single miRNA strand is retained, which guides RISC to target mRNAs, resulting in translational repression and/or mRNA destabilisation/degradation. Multiple approaches are used to modulate miRNA activity: inhibition of miRNA production or function with Drosha-site blockers, Dicer-site blockers, target-site blockers or anti-sense miRNA olionucleotides. miRNA activity can be enhanced by over-expression of pre-miRNA or by introducing miRNA mimics. Metazoan miRNAs interact with mRNA targets by imperfect complimentary base pairing usually to the mRNA 3'-UTR. Perfect pairing of nucleotides 2-8 in the 5' of the miRNA, the 'seed' region, is important for miRNA-mRNA interaction. Bulges in the central region, mismatches in the seed region, A or AU nucleotides at positions 1 or 9 and 3' complimentary sites at 12-17 nucleotides can greatly affect miRNA function. For more details see references cited in section 1.

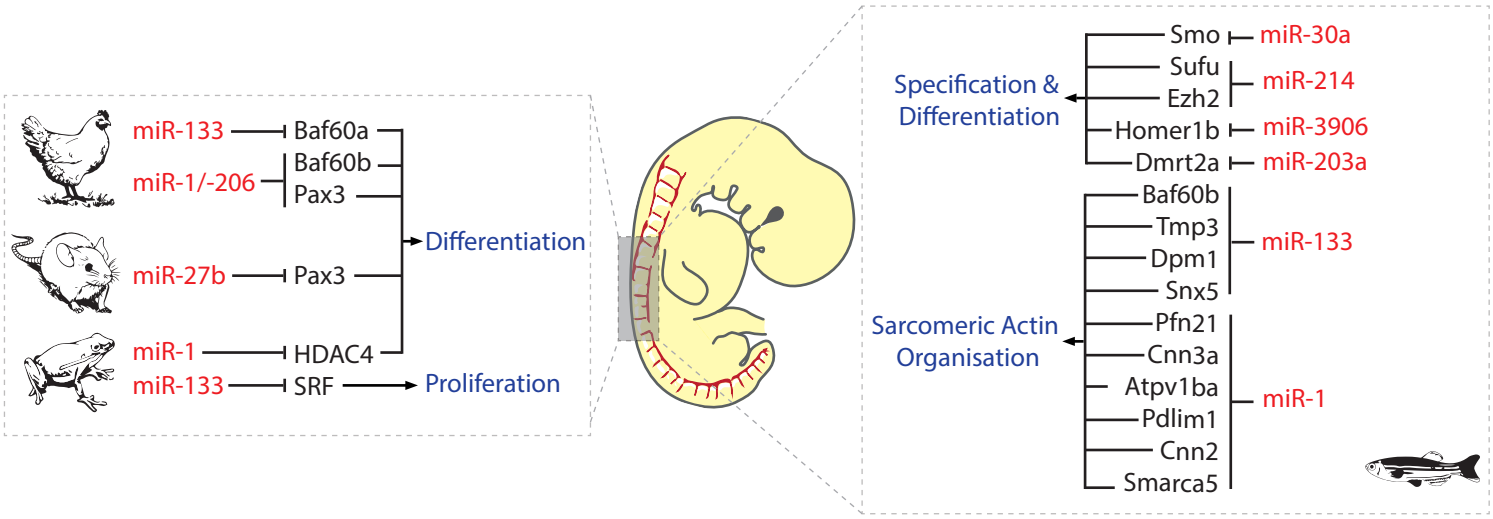
Figure 2. Schematic of miRNAs and their targets in C2C12 myoblasts, with a focus on the myomiRs. Target genes that enhance proliferation in absence of miR-1, miR-206 and miR-133, which are not expressed in growing C2C12 cells, are indicated in the left panel. After cells reach a high density, growth factors are depleted and the MRFs become activated. This leads to expression of miR-1, miR-206, miR-133 and miR-486, suppression of the target genes indicated and to differentiation. See section 2.2 for references and more details.

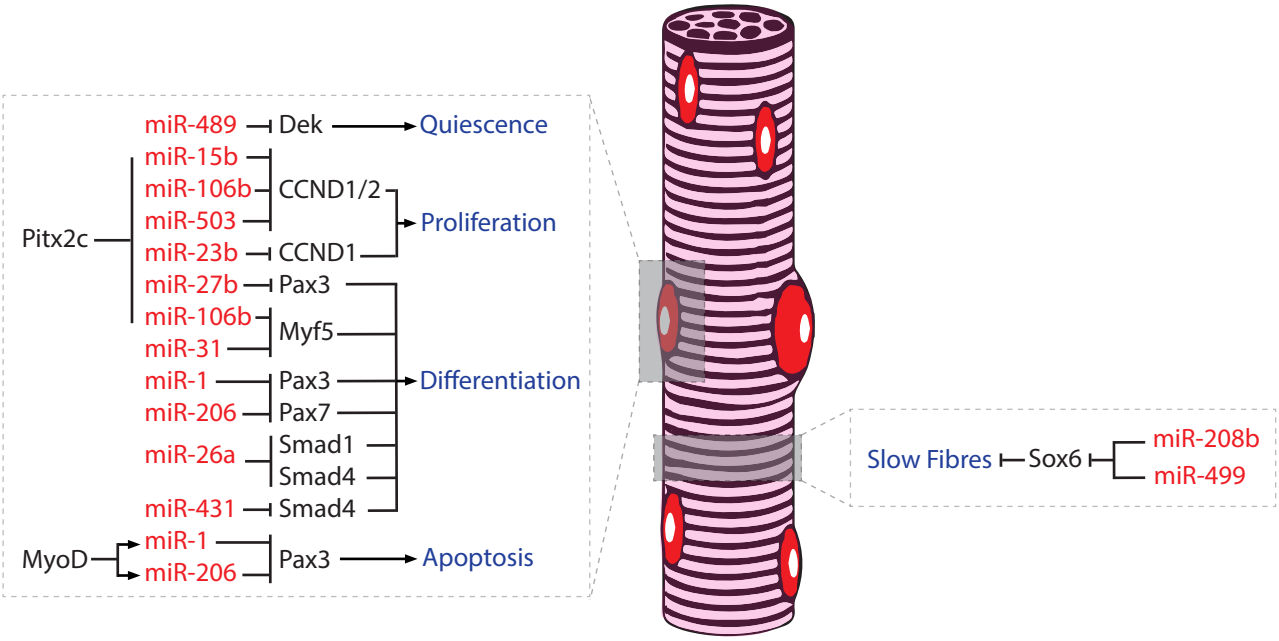
Figure 3. miRNAs in embryonic myogenesis, four vertebrate model species, mouse, chicken, frog and fish, have been used to study miRNAs in muscle development. miRNAs and their validated targets are indicated, together with the biological effect. Pointed arrows represent activation and blunt arrows represent inhibition. See section 2.3 for references and more details.

Figure 4. miRNAs in SCs and myofibre differentiation. Muscle homeostasis and regeneration depends on SCs whose quiescence and activation is tightly controlled. miRNAs and their respective targets are shown, as well as some upstream transcriptional regulators. Sharp arrows represent activation and blunt arrows represent inhibition. See section 3.2 for references and more details.


Intergenic
Intronic
Polycistronic
Mirtron
miRNA
miRNA
miRNA-A miRNA-B
miRNA
Drosha-site blocker
pri-miRNA
pri-miRNA
pri-miRNA
mRNA
Drosha
DGCR8
Spliceosome
miRNA
Over-expression
pre-miRNA
Nucleus
Cytoplasm
Restoration of miRNA function
Exportin-5
Inhibition of miRNA function
Dicer-site blocker
pre-miRNA
Dicer
TRBP
miRNA mimics
Mature miRNA duplex
Principles of miRNA-mRNA interactions
miRNA
3' complementary sites
'Seed' region
17 12
8 2
NNNNNNNNNN
NNNNNNNNNN
ORF
NNNNNNNNNN
ANNNNNNNA
AAA
mRNA
miRNA inhibitors
miRNA-5p & miRNA-3p
AGO
Target protectors
Translational repression
mRNA destabilisation / degradation
RISC
Gppp
ORF
AAA
RISC
ORF
AAA
Gppp


MRFs
MEF2
miR-1
miR-206
miR-133
Pax3/7
Pola1
Ccdn1/2
FGFR1
PP2AC
CCN1
Proliferation
Differentiation
YY1
CX43
HDAC4
Meox2
RARB
Fzd7
BAF60a
Baf47
Pax7
Baf60b
Runx2
PRDM16
SRF
Pax7
miR-1
miR-206
miR-133
miR-486


miR-133
Baf60a
miR-1/-206
Baf60b
Pax3
miR-27b
Pax3
Differentiation
miR-1
HDAC4
miR-133
SRF
Proliferation
Specification &
Differentiation
Smo
miR-30a
Sufu
Ezh2
miR-214
Homer1b
miR-3906
Dmrt2a
miR-203a
Baf60b
Tmp3
Dpm1
Snx5
miR-133
Sarcomeric Actin
Organisation
Pfn21
Cnn3a
Atpv1ba
Pdlim1
Cnn2
Smarca5
miR-1


miR-489
Dek
Quiescence
miR-15b
miR-106b
miR-503
CCND1/2
Proliferation
Pitx2c
miR-23b
CCND1
miR-27b
Pax3
miR-106b
Myf5
miR-31
miR-1
Pax3
Differentiation
miR-206
Pax7
miR-26a
Smad1
Smad4
miR-431
Smad4
MyoD
miR-1
miR-206
Pax3
Apoptosis
Slow Fibres
Sox6
miR-208b
miR-499