# **ARTICLE IN PRESS**

ADR-12771; No of Pages 16

Advanced Drug Delivery Reviews xxx (2015) xxx-xxx



Contents lists available at ScienceDirect

# Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/addr



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#### ARTICLE INFO

#### Available online xxxx

Keywords: microRNAs Cardiac muscle Skeletal muscle Tissue engineering Regenerative medicine Exosomes Circulating microRNAs

Chemical compounds studied in this article:
Locked adenosine
Locked cytosine
Locked guanosine
Locked uracyl
Polyethylene glycol
Fibrinogen
Collagen
Phenylephrine
Bromo-deoxy-uridine

#### ABSTRACT

microRNAs (miRs) are small non-protein-coding RNAs, able to post-transcriptionally regulate many genes and exert pleiotropic effects. Alteration of miR levels in tissues and in the circulation has been associated with various pathological and regenerative conditions. In this regard, tissue engineering of cardiac and skeletal muscles is a fascinating context for harnessing the complexity of miR-based circuitries and signals. In this review, we will focus on miR-driven regulation of cardiac and skeletal myogenic routes in homeostatic and challenging states. Furthermore, we will survey the intriguing perspective of exosomal and circulating miRs as novel paracrine players, potentially useful for current and future approaches of regenerative medicine for the striated muscles.

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Abbreviations: ACS, acute coronary syndrome; Ago-2, Argonaute 2; Akt, protein kinase B; AMI, acute myocardial infarction; anti-miR, antagomiRs against microRNA; Bcl-2, B cell leukemia/ lymphoma 2; CAD, coronary artery disease; Camk-llγ, calcium/calmodulin-dependent protein kinase II gamma; CDC, cardiosphere-derived cell; Cfc-25a, cell division cycle 25A; circ-miR, circulating microRNA; cFos, cellular FBJ osteosarcoma oncogene; cJun, cellular jun proto-oncogene; CK, creatine kinase; CPC, cardiac progenitor cell; cTnI, cardiac troponin-l; Cx-43, connexin 43; Cypd, peptidylprolyl isomerase D; Dgcr-8, DiGeorge Syndrome Chromosome Region 8; Ezh-2, enhancer of zeste 2 polycomb repressive complex 2 subunit; FoxO-1a, forkhead box O1 a; Gata-4, GATA binding protein 4; Gw-182, Gw bodies protein (trinucleotide repeat coronal ing 6A, Trc-6a); Hand-2, heart and neural crest derivatives expressed transcript 2; Hdac-4, histone deacetylase 4; HDI, high-density lipoproteins; Hif-1, hypoxia inducible factor 1; HoxA-11, homeobox A11; Hsp-60/70, heat-shock protein 60/70; Ip3r-III, inositol 1,4,5-trisphosphate receptor, type 3; Jnk, c-Jun N-terminal kinase; LNA, locked nucleic acid; Maml 1, mastermind-like factor 1; MD, muscular dystrophies; Mecp-2, methyl CpG binding protein 2; Mef-2, myocyte enhancer factor 2; miR, microRNA; Mlk-3, mixed lineage kinase 3 (mitogen-activated protein kinase 11, Map3k-11); Murf-1, tripartite motif containing 63, E3 ubiquitin protein ligase; Myh/MyHC, myosin heavy chain; MyoD, myoblast determination protein; myomiR, myogenic microRNA; Ncx-1, solute carrier family 8 (sodium/calcium exchanger), member 1; Nkx2-5, NK2 homeobox 5; nt, nucleotide; Pax-3, paired box 3; Pdcd-4, programmed cell death 4; Pdlim-5, PDZ and LIM domain 5; pre-miR, precursor of microRNA; pri-miR, primary transcript of microRNA; Pten, phosphatase and tensin homolog; Pur-β, purine rich element binding protein β; RISC, RNA-induced silencing complex; Rybp, RING1 and YY1 binding protein; Sca-1, stem cell antigen 1; Sdn-1/3, small RNA de

- This review is part of the Advanced Drug Delivery Reviews theme issue on "MicroRNAs in tissue engineering and regenerative medicine".
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# http://dx.doi.org/10.1016/j.addr.2015.04.011

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Please cite this article as: M. Quattrocelli, M. Sampaolesi, The mesmiRizing complexity of microRNAs for striated muscle tissue engineering, Adv. Drug Deliv. Rev. (2015), http://dx.doi.org/10.1016/j.addr.2015.04.011

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# 1. Introduction

Striated muscles constitute approximately 40% of our body mass and are responsible for constant blood pumping, i.e. the cardiac muscle, and for posture and movements, i.e. the skeletal muscles. Several diseases chronically affect both cardiac and skeletal muscles, including muscular dystrophies (MDs) [1], cancer cachexia [2] and metabolic disorders [3]. At present, regenerative treatments are not yet available for the clinical care of striated muscle disorders, in spite of increasing demand and global burden [4]. Therefore, the field would benefit from a comprehensive understanding of cellular and molecular mechanisms governing generation and regeneration of both muscle types.

Over the last years, the static notion of post-mitotic tissues is under continuous re-shaping for striated muscles. Many findings have disclosed the possibility of (re)generating adult myocytes by means of stem cells or progenitors with different origins and potency [5]. Although the relevance for steady and pathological states is still fiercely debated [6,7], it is noteworthy that the study of (re)generation mechanisms has fueled a deeper venture into the complexity of muscle formation.

In this context, the post-transcriptional control exerted by microRNAs (miRs) constitutes one of the most remarkable layers of complexity [8]. MiR-gene feedback circuitries finely orchestrate cell-based engineering for striated muscle repair [9]. Also, it has recently become evident that miRs can be released in the circulation, probably cross-signaling on both short-range and long-range [10]. Thus, this review will focus on the miR-mediated complex regulation of myogenic routes in cardiac and skeletal muscles. In addition, it will report on the intriguing perspective of vesicular and non-vesicular circulating miRs (circ-miRs) with respect to challenging conditions of striated muscles.

# 2. Biogenesis of miRs

MiRs are conserved, ~22 nt-long, non protein-coding RNA molecules regulating gene expression at post-transcriptional stage. MiR-based gene regulation is intrinsically complex, considering that one transcript is targetable by different miRs and one miR can target different transcripts [11]. Currently, 1920 murine and 2603 human mature miR entries are reported in the miRbase sequence repository (mirbase.org; March 2015). MiR-encoding genes are present in the genome as intergenic clusters or individual transcriptional units. In both cases, their expression is under the control of promoters and enhancers with similar characteristics and regulation to those for protein-coding genes [12]. Several miRs, however, are embedded in the intronic sequences of other genes (miRtrons) and are then co-transcribed accordingly with the encompassing genes [13]. Following the canonical pathway, miR genes are generally transcribed by RNA polymerase II into primary transcripts of several hundred nucleotides (pri-miRs),

bearing secondary hairpin structures and undergoing 5' capping and 3' polyadenylation [14]. In few cases, miR genes are transcribed by RNA polymerase III [15]. While still inside the nucleus, the microprocessor complex, formed by the RNase-III Drosha and its co-factor Dgcr-8, cleaves the pri-miRs into ~70 nt-long precursor molecules (pre-miRs) [16]. Exportin-5, a Ran-GTP-dependent nuclear export protein, shuttles the pre-miRs into the cytosol [17]. Once in the cytosol, pre-miRs are further cleaved in ~22 nt-long double-stranded molecules by a complex that includes another RNase-III, Dicer, in combination with its RNAbinding cofactor, Tbrp [18]. After Dicer-mediated maturation, miRs are loaded on the RNA-induced silencing complex (RISC) as single strands. Mature miR duplexes are in fact separated in guide and passenger strands. The guide strand (miR) usually presents the weakest base pair at 5' and is preferentially loaded on the RISC complex, whereas the complementary passenger strand (miR\*) is preferentially degraded [19,20]. The RISC complex includes Argonaute proteins, such as Ago-2, and targets the miR to its messenger RNA (mRNA) targets. Also, Ago-2binding protein, Gw-182, shuttles the miR-loaded, active RISC complex to discrete foci in the cytoplasm, called processing bodies (P-bodies), where several enzymes are available for mRNA decapping, deadenylation and degradation [21]. Although the exact mechanisms are still unclear, it seems that miRs generally recognize the 3' untranslated region (3'-UTR) of target mRNAs. According to the binding complementarity of the seed sequence (generally comprised between nt1 and nt9 at miR 5' end), miRs repress gene expression by targeting the mRNA for degradation (complete match) or by mediating translation inhibition (incomplete match). The latter is achieved through various mechanisms, including RISC-mediated destabilization of ribosomal assembly or translation continuation [22] (Fig. 1).

Non-canonical processing of miR transcripts, typical for miRtrons, relies on direct processing of the pre-miRs during intron splicing of the harboring gene mRNA. Other microprocessor-independent sources of pre-miRs encompass small nucleolar RNAs (snoRNAs), transfer RNA precursors (tRNAs) and short hairpin RNAs (shRNAs) [23].

# 2.1. Modulation of miRs

Many factors regulate miR biogenesis and hence affect downstream miR-mediated gene repression. RNA editing of pri-miRs or pre-miRs by deaminases [24], or through 3' uridylation, can influence Drosha- or Dicer-dependent processing steps. Also, abundance of Ago-2 or agonist proteins directly affects RISC activity levels. Another important aspect of intrinsic miR regulation, albeit still rather obscure, consists of turnover and degradation. After mRNA targeting and in the presence of still unknown signals, the *miR* guide strand is released and degraded. The turnover process of miRs still remains largely unknown. Average half-life of miRs has been estimated to ~119 h [25] and exoribonucleases,

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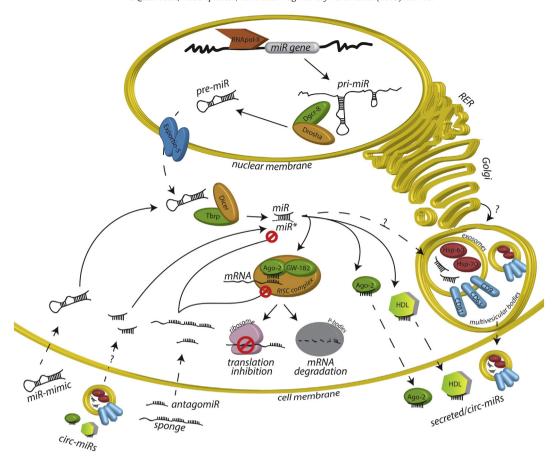


Fig. 1. Biogenesis and trafficking of miRs. Once processed by microprocessor and Dicer complexes, miRs are loaded on the RISC complex, or are shuttled to exosomes or non-vesicular carriers for paracrine signaling, or shed into the circulation. MiRs are modulated through miR-mimics, antagomiRs and miRs that are shuttled by exosomes, protein- and HDL-carriers.

such as Xrn-2 and Sdn-1/3, take part in the process [26]. However, more systematic studies are needed to shed light on the selective mechanisms regulating miR turnover.

#### 2.2. Vesicular and non-vesicular trafficking of miRs

Besides operating intracellularly, miRs can be released from the producing cells in the surrounding areas or in the circulation. Intriguingly, circulating miRs (circ-miRs) are traceable in plasma or serum and appear resistant to harsh conditions such as RNase activity, pH changes, boiling, freeze-thawing and storage at room temperature [27]. Carriers or membranous vesicles generally protect the circ-miRs in the bloodstream. Non-vesicle carriers encompass protein complexes, including Ago-2 and Nucleophosmine-1, and lipoprotein complexes, such as high-density lipoproteins (HDLs) [28]. Among the vesicle-based carriers, exosomes are emerging as important regulators of long-range miR shuttling [29]. Exosomes are small vesicles (40–100 nm diameter) that enclose their cargo with a lipidic bi-layer and are generated from intracellular multivesicular bodies [30]. Exosomes mature through a still unknown process, most likely at the interface with the Golgi reticulum, and then shed from the plasma membrane. Exosomes typically carry heat-shock proteins Hsp-60/70 in the lumen, and present tetraspanins, such as CD9/63/81, and tissue-specific membrane proteins on the surface [31]. At present, the relative abundance of vesicular or carrier-based formulations for circ-miRs in the bloodstream is still debated. Some reports suggest that the majority of circ-miRs are exosome-borne [32], whereas others suggest that circ-miRs are predominantly vesicle-free and carried in protein- [33] or lipoproteinbased complexes [34]. These discrepancies are probably due to still different and rather incomparable methodologies of investigation. However, following the notion of exosomes as miR carriers, a novel layer of miR-based cross-communication emerges on top of the conventional intracellular regulations. Notwithstanding the lack of details concerning putative receptors and intracellular processing, it has been in fact reported that pre- or mature miRs are delivered to other cells [35], where they exert their specific regulation (Fig. 1).

# 2.3. Artificial tools to regulate miRs

Oligonucleotides, i.e. miR-mimics and antagomiRs, can enhance or repress endogenous miRs, respectively. MiR-mimics are artificial oligonucleotides that are similar in sequence and possibly in secondary structure to target pre-miRs. Once processed by the Dicer complex, miR-mimics load the RISC complex with the query mature miR [36]. MiR-mimics are deliverable in vivo through lipid-based preparations, which can however lead to unspecific uptake. Uptake specificity and transduction efficiency are increased with viral vector formulations, yet the issues of biodistribution and genome integration remain [37]. AntagomiRs are modified antisense oligonucleotides, competing with the endogenous miR for matching the target sequences. Also, antagomiRs can interfere with pri-miR biogenesis, nuclear export, pre-miR maturation and RISC loading. AntagomiRs are chemically enhanced with 2'-Omethyl, 2'-O-methoxyethil or locked nucleic acid (LNA) nucleotides, in order to improve stability and hence duration of the inhibitory effect. AntagomiRs are deliverable in physiological-grade solutions, liposomeor nanoparticle-based formulations, or as cholesterol-conjugated oligonucleotides [38]. Noteworthy, an antagomiR has successfully passed a phase-II clinical trial, in which the interaction between hepatitis virus C and liver-expressed miR-122 was targeted. In this clinical study, patients received 5 weekly subcutaneous injections of the LNA-based antagomiR in concentrations ranging from 3 to 7 mg per body weight kg. Beneficial effects on the viral RNA load appeared dose-dependent

during 14 weeks of follow-up after treatment, without apparent doselimiting adverse effects [39].

Another experimental tool of miR inhibition is the sponge, which is an artificially overexpressed gene carrying multiple miR-binding sites in tandem in its expanded 3'-UTR. Interestingly, sponges are particularly useful as they can inhibit up to a whole miR family with similar seed sequences [40]. Despite being highly variable in uptake and modulation efficiency, miR-mimics and antagomiRs/sponges represent attractive candidates to timely manipulate determined miRs during the formation of cardiac and skeletal muscle both in vitro and in vivo, at least in experimental and translational setups (Fig. 1).

# 3. Tissue engineering for striated muscles: room for miRs?

Striated muscle injury and degeneration account for significant fractions of adult mortality and disease burden worldwide. A notable example thereof is the ischemic heart disease, which constituted the first cause of both mortality and burden over the last decade (WHO Global Health Observatory, statistics related to 2000–2012). In addition, genetically inherited chronic myopathies still cause a conspicuous, yet unmet need for muscle tissue regeneration. In fact, the most severe form of MD, the Duchenne MD, afflicts approximately 1 in 3000 young males. Importantly, most MD forms feature chronic degeneration in both striated muscle compartments, i.e. heart and skeletal muscles. Therefore, the need for regenerative strategies for both muscle types is still highly compelling.

At present, in order to reach this ambitious goal, several fields are promisingly explored, including tissue engineering and stem cell-based treatments.

Tissue engineering aims at supporting functional regeneration of damaged striated muscles by means of generally three-dimensional implants that combine biocompatible scaffolds with bioactive molecules and/or stem cells [41,42]. Noteworthy for improving cardiac functionality is the application of cell sheets or tissue slices on the pericardium of damaged hearts. Myoblast cell sheets of ~100 µm thickness have been applied on the left ventricle of hamsters with dilated cardiomyopathy, resulting in maintenance of pre-operative values of fractional shortening [43]. Furthermore, cardiac slices obtained from adult or neonatal murine hearts have also shown promising results. Indeed, application of 200/400 µm-thick cardiac slices on the left ventricle of murine infarcted hearts resulted in improved ejection fraction at one month post-transplantation [44]. In both approaches, host-driven revascularization of the implant supports the hypothesis that paracrine signals play a major role in the functional amelioration. However, the implant-host cross exchange of resident progenitor cells and their fate regulation thereafter still remain largely unaddressed. In this regard, it has been recently reported that an acellular matrix scaffold from porcine urinary bladder extensively mobilized and recruited perivascular progenitors in injured skeletal muscles of animal models and human patients. The colonized scaffold then counteracted the volumetric loss of skeletal muscle with novel functional fibers [45]. Albeit encouraging, it is still unknown whether such approach can cope with the major challenges of the upscaling and the hostile environment of chronically myopathic muscles.

Tissue engineering for striated muscle repair often entwines with stem cell-based treatments. In principle, transplantation of stem cells aims at providing the degenerating muscle tissue with progenitors able to engraft, to reconstitute genetically suitable myocytes and to restore, at least partially, the functionality. Main examples in such context are cardiac stem cells for the heart and satellite cells for the skeletal muscles. Cardiac stem cells, identified as c-Kit<sup>+</sup> resident progenitors, are clonally expandable and induce functional regeneration of ischemic myocardium in animal models [46]. Moreover, autologous c-Kit<sup>+</sup> cells have shown safety and partial efficacy outcome in a phase-I, randomized clinical trial [47,48]. In the skeletal muscle, satellite cells reside as quiescent cells under the basal lamina and, in case of injury, proliferate and potently regenerate damaged fibers [49]. However, satellite cells present a poor migratory potential when injected in the circulation, therefore blunting their clinical translation at present. Although intrinsically less committed, mesoangioblasts, i.e. resident myogenic pericytes, appear a translational alternative for skeletal muscle repair. Mesoangioblasts present, indeed, a high migration ability and have been shown as promising candidates in small and large animal models of MD [50,51]. Also, HLA-matched mesoangioblasts are currently under phase-I clinical study in MD patients (EudraCT #2011-000176-33). Recently, encouraging results for skeletal muscle engineering have been obtained through implantation of mesoangioblasts embedded in a polyethylene glycol-fibrinogen hydrogel. The implant was able to mature into artificial, functional skeletal muscle fibers in mice. Importantly, the cells were engineered to overexpress placental-derived growth factor, and the implant was successful with both murine and human mesoangioblasts [52]. With regard to the heart, myogenic mesoangioblasts [53] or pericytes [54] have been recently isolated also from the cardiac muscle, although their bench-to-bedside progression appears slower as compared to aforementioned cell systems.

Lastly, it is important to mention the growing interest around pluripotent stem cells, both embryonic and induced, for striated muscle (re)generation. Diverse approaches of differentiation and progenitor cell sorting are currently available in literature [55,56], also in combination with genetic engineering [57] or epigenetically biased systems [58].

**Table 1**MiR manipulation strategies relevant for cardiac muscle repair/protection.

miR	Target	Means of manipulation	Effects	Ref
miR-1/-133	Murine embryonic stem cells	Lentiviral transduction	cardiomyogenic potential	[66]
miR-1	Rat neonatal cardiomyocytes/murine adult heart	Adenoviral transduction	cardiac hypertrophic remodeling	[67]
miR-499	Human CPCs	Lentiviral transduction	cardiomyogenic potential	[70]
miR-1/-499	Human fetal cardiomyocyte progenitors	miR-mimic transfection	cardiomyogenic potential	[71]
miR-1/-499	Human embryonic stem cells	Lentiviral transduction	cardiomyogenic potential	[72]
miR-1/-133a/-208a/-499	Murine cardiac fibroblasts	miR-mimic transfection	cardiomyogenic conversion	[74]
miR-499	Rat bone marrow mesenchymal stem cells	Lentiviral transduction	cardiomyogenic conversion	[75]
miR-208a	Rat adult heart (hypertension-induced hypertrophy)	AntagomiR systemic delivery	cardiac hypertrophic remodeling	[76]
miR-21/-129/-212	Rat neonatal cardiomyocytes	miR-mimic transfection	cardiac hypertrophic remodeling	[80]
miR-21	Rat adult heart (acute injury)	Intracardiac adenoviral delivery	cardioprotection	[81]
miR-138	Rat CPCs	miR-mimic transfection	survival	[88]
miR-199a/-590	Rat/murine neonatal heart	miR-mimic transfection/AAV systemic delivery	cardiomyocyte proliferation	[90]
miR-15 family	Murine adult heart (acute injury)	AntagomiR systemic delivery	cardioprotection	[94]
miR-24	Rat cardiomyocytes (ischemia-like conditions)	miR-mimic transfection	survival (in vitro)	[95]
miR-21/-24/-221	Murine CPCs	Lentiviral transduction	survival (in vivo)	[96]
miR-22	Rat neonatal cardiomyocytes	miR-mimic transfection	cardiac hypertrophic remodeling	[101]
miR-34a	Human bone marrow mesenchymal stem cells	AntagomiR transfection	survival (in vivo)	[102]

The table concisely reports several experiments, and the related effects, of miR manipulation in cardiac cells or in the myocardium, with potential relevance to cardiac regeneration strategies.

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Although a comprehensive dissertation on these cell systems would defeat the purpose of this review, it is of note for this work to highlight the theoretical potential of pluripotent cells to give rise to different striated muscle progenies [59]. However, issues such as myogenic efficiency and appropriate lineage commitment still currently hamper this potential, particularly for in vivo applications [60].

It is thus imperative for present and future strategies of striated muscle tissue engineering to potentially overcome the hurdles imposed by myogenic fate efficiency, topic survival rate and adverse signals from the diseased niche. MiRs are emerging as key players in all three aspects and, particularly, as tunable modulators of the myogenic program and the adaptability of both exogenous and resident stem cells [11]. However, factual progress in miR-based strategies still relies on unraveling the complexity of miRs in normal and pathologic contexts of myogenesis. This review will therefore focus on the role of miRs in experimental and translational settings of striated muscle de-/regeneration, and on novel insights from the largely unexplored field of circulating miRs.

#### 3.1. Cardiac myogenesis and miRs

Diverse miRs finely modulate cardiac myogenesis (Table 1). The paramount role of miRs during cardiac muscle generation was firstly highlighted by the severe cardiac defects that, together with other major developmental aberrations, induced fetal lethality in *Dicer-null* mice [61]. The most abundant and investigated miRs involved in cardiac myogenesis are *miR-1*, *miR-133*, *miR-208*, and *miR-499*, members of the

"myomiR" group [62]. These miRs have complex pleiotropic effects on many aspects of cardiac myogenesis and muscle homeostasis (Fig. 2).

During cardiac development and maturation, miR-1 and miR-133 exert entwined spatiotemporal effects on expansion and terminal differentiation of cardiac progenitor cells (CPCs). Excess of miR-1 limits the expansion of cardiac progenitors by repressing Hand-2, while miR-1-lacking murine embryos die at E10.5 because of severe cardiac malformations [63]. MiR-133a is fundamental for cardiac development and CPC proliferation by targeting Cyclin-D2 and Srf in a negative feedback control [64,65]. Importantly, miR-1 and miR-133a/b have also been shown to enhance the differentiation of pluripotent cells towards the mesodermal lineage. This has been assessed in murine embryonic stem cells transduced with a lentiviral vector that induced expression of both miRs at levels comparable to the endogenous miRs in the murine heart. The resulting increase of cardiomyogenic differentiation was due to two parallel mechanisms. MiR-1 translationally repressed the Notch ligand Dll-1, while miR-133 increased the proliferation of CPCs [66]. Therefore, sustained levels of miR-1/-133 appear beneficial to the myogenic differentiation, albeit their modulation must be timely appropriate and stage-specific. Also, miR-1 is downregulated in the diseased heart and adenoviral-mediated overexpression of miR-1 (up to 5 fold endogenous levels) conversely attenuated hypertrophic remodeling. This evidence was gathered in rat neonatal cardiomyocytes and in adult murine hearts, where miR-1 represses Calmodulin and Mef-2a. MiR-1-mediated effects were accordingly reverted in vitro in the presence of 1 pmol cholesterol-linked anti-miR-1 [67]. Interestingly, the mature forms of miR-1 and miR-133a are encoded from two

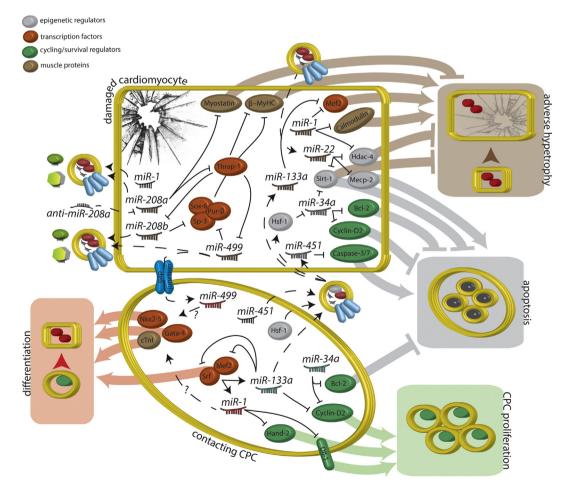


Fig. 2. Complexity of miR-based regulations in cardiac myogenesis. This diagram depicts a fraction of the miR pathways commented on this review, to synthesize the information regarding progenitor commitment and cardiomyocyte damage response. Genes are pseudocolored according to their function (legend), whereas miRs are pseudocolored according to the cognate process (arrows and squares). The same miRs can have different colors in accordance with the different contexts in which they are depicted. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

conserved bicistronic loci, *miR-1-1/miR-133a-2* on chromosome 2 and *miR-1-2/miR-133a-1* on chromosome 18 [68]. *Srf* and *Mef-2* transcription factors, both MADS-box transcription factors regulating cardiomyogenic regeneration, activate both miRs by recognizing specific enhancers [69].

Another important regulator of CPC maturation is miR-499. In human CPCs, lentiviral-mediated overexpression of miR-499 by ~4000 fold endogenous levels depleted ~75% protein levels of targets Sox6 and Rod1, enhancing the cardiomyogenic progression. Transduced, miR-499-overexpressing CPCs presented superior regenerative potential in vivo, once injected in infarcted hearts [70]. Moreover, particularly in combination with miR-1, miR-499 was reported as direct regulator of cardiomyogenic differentiation. Transfection of up to 100 nmol synthetic pre-miR-1/-499 in human fetal cardiomyocyte progenitors resulted in dose-dependent increase of differentiation efficiency at 12 days posttreatment [71]. Furthermore, overexpression of miR-1/-499 precursors by lentiviral transduction in human embryonic stem cells increased target miR levels by ~30 fold and ~130 fold, respectively, across the embryoid body stage and ameliorated in vitro cardiomyogenesis [72]. Remarkably, miR-499 has been implicated in junction-dependent miR trafficking, called "miRcrine mechanism", between cardiomyocytes and regenerating CPCs, both in vitro and in vivo [73]. In addition, together with the other myomiRs, miR-499 improves the direct conversion of cardiac fibroblasts into cardiomyocytes. Transfection of 50 nmol miRmimics of miR-1/-133a/-208a/-499 in murine cardiac fibroblasts was sufficient to exert significant changes on early cardiomyogenic markers at 3 days post-treatment. Analogous effects were obtained on mature differentiation markers at 7 days post-transfection. Accordingly, this defined miR signature is amenable also for conversion of endogenous fibroblasts in vivo. Indeed, direct intramyocardial injection of  $2 \times 10^6$ lentiviral units at two sites downstream of the coronary artery ligation induced resident fibroblasts to convert to cardiac cells and to contribute to regenerated myocardium. In this study, the lentiviral vectors bore the miR/reporter cassette under a promoter prominently active in fibroblasts but not in other cardiac cells, strongly suggesting that the cardiomyogenic conversion was fibroblast-restricted [74]. Noteworthy, miR-499 appeared sufficient to stimulate the expression of cardiac factors, e.g. Nkx2-5, Gata-4 and cTnI, in rat bone marrow mesenchymal stem cells through a positive loop with the canonical Wnt signaling. This study relied on lentiviral-mediated miR-499 overexpression at ~16.5 fold levels vs control, and target gene levels were quantified at 3 days post-transduction [75]. However, these results were obtained in vitro and further proofs are needed to establish their relevance

Genetically clustering with miR-499, miR-208a and miR-208b have gained much attention as regulators of hypertrophic remodeling and therefore as potential therapeutic targets. Remarkable for such perspective is that systemic delivery of LNA-enhanced anti-miR-208a counteracted cardiac dysfunction in response to hypertension-induced hypertrophy in rats. Administration of 25 mg/kg anti-miR-208a appeared sufficient to reduce miR-208a levels in the cardiac tissue by ~70% at 14 days post-injection. Intriguingly, analogous effects were obtained through either intravenous, or intraperitoneal, or subcutaneous delivery [76]. MiR-208a is embedded in one intron of Myh6, encoding for  $\alpha$ -Myosin heavy chain ( $\alpha$ -MyHC), whereas miR-208b and miR-499 are embedded in introns of Myh7 and Myh7b, both encoding for β-MyHC. β-MyHC, characterized by a slower ATPase activity, is mainly expressed in the fetal heart and upon hypertrophic adverse remodeling. Conversely,  $\alpha$ -MyHC is upregulated early after birth and accounts for >90% of cardiac MyHC content in the adult heart. T3 signaling promotes the expression of Myh6 and miR-208a through a positive response element, while inhibiting the expression of MyH7/7b and miR-208b/-499 through a negative element [77]. Upon cardiac stress, miR-208a binds and represses T3 co-regulator Thrap-1, removing the transcriptional block from Myh7/7b loci, thereby promoting the switch to β-MyHC. Hypertrophic remodeling is reinforced by miR-208a-mediated repression of Myostatin [78]. Furthermore, once expressed together with Myh7/7b in the damaged myocardium, miR-208b and miR-499 target Sox-6, Pur- $\beta$  and Sp-3, known repressors of Myh7, thus reinforcing the pathological switch to the slow MyHC isoform [79]. Notable for translational approaches relying on these miRs is that miR-208a-null mice did not upregulate  $\beta$ -MyHC under stress, thus suggesting that miR-208a is hierarchically upstream of miR-208b and miR-499 [78]. Therefore, the molecular switch between miR-208a and miR-208b/miR-499 appears a therapeutic target for the treatment of cardiac muscle hypertrophy. Sustained inhibition of this switch is indeed a novel way to counteract the adverse hypertrophic remodeling, which is a prominent complication in many chronic conditions of the heart.

### 3.2. MiRroring cardiac muscle diseases

Besides the miR-208a/miR-208b/miR-499 circuitry, other miRs have been implicated in the aberrant switch to fetal programs in response to cardiac stress. Transient pulse of 100 nmol pre-miR-21/-129/-212 in rat neonatal cardiomyocytes caused hypertrophy and re-activation of a fetal cardiac gene program at 48 h post-transfection. Moreover, human fetal cardiac tissues and biopsies from end-stage heart failure patients presented upregulation of these miRs, among others [80]. MiR-21, -129 and -212 hence appear part of a fetal miR signature that is reactivated in the failing ventricular myocardium. These miRs share many direct and indirect targets among cardiac differentiation or remodeling genes [80]. Several studies have especially focused on miR-21, reporting contrasting effects on cardiomyocyte survival and cardiac fibrosis. MiR-21 expression was found downregulated in the myocardial infarct and upregulated in the border zone. Temporary (10 s) perfusion of infarcted hearts with 200 µl miR-21-bearing adenoviral units resulted in reduction of infarct size by 29% at 24 h post-insult. Moreover, the cardioprotective effect of miR-21 overexpression has been linked to direct inhibition of one of its targets, *Pdcd-4*, which triggers cardiomyocyte apoptosis [81]. However, a reciprocal loop between miR-21 and  $Tgf\beta r$ -III, a negative regulator of  $Tgf\beta$  signaling and collagen deposition, has been proposed as possible infarct-triggered mechanism for cardiac fibrosis [82]. Other miRs potentially involved in cardiac fibrosis are miR-29 and miR-101 [83]. The miR-29 family is reduced after acute myocardial infarction (AMI) and targets multiple factors involved in the fibrotic matrix, such as collagens, fibrillins and elastin [84,85]. MiR-101a/b are also down-regulated after AMI, and their overexpression inhibited cardiac fibroblast proliferation and collagen deposition, by repressing cFos and Tgf\beta1 pathways [86]. Manipulation of fibrosisspecific miRs is obviously palatable for post-AMI care. However, a more refined knowledge of the cell types interested by these miR networks will be fundamental to advance translational research on this aspect.

A still puzzling example of fetal cardiac miRs playing a role in adult cardiac stress is miR-138 [87]. It has been recently demonstrated that miR-138 stimulates survival of a rat CPC cell line in conditions of hypoxia. The study compared the effects on rat CPCs treated either with 50 nmol pre-miR-138, or 100 nmol anti-miR-138, at 72 h post-transfection. MiR-138-driven amelioration of survival appeared dependent on Mlk-3 targeting and the consequent repression of its downstream targets Ink/cJun [88]. To date, miR-138 is the only miR directly linked to cardiac patterning and ventricle chamber formation. The developmental role of miR-138 resides, at least partially, in the negative regulation of the retinoic acid pathway. This negative loop leads to repression of Versican, a cell adhesion molecule usually restricted to the atrioventricular canal [89]. However, this evidence was gathered in zebrafish and the function of miR-138 during mammalian development remains unknown. Although miR-138 was also found upregulated in myocardial samples from patients exhibiting congenital heart disease, further molecular links are required in order to properly address the relevance of miR-138 cardioprotective role in vivo.

A fetal trait potentially useful to counteract post-ischemic dysfunctions is cardiomyocyte proliferation, which is also partly regulated by miRs. A wide adeno-associated virus-based screen of human miRs identified several miRs, including miR-199a and miR-590, as robust inducers of cardiomyocyte proliferation. These miRs induced ventricle hypertrophy and long-term resistance to permanent ligation of the coronary artery. Intriguingly, intracardiac delivery of 2.8 µg mimics of human miR-199a/590 in rat neonatal hearts after birth resulted in enlarged ventricular wall without fibrosis at 3 weeks of age. According experiments were performed in neonatal mice after birth, through intraperitoneal delivery of miR-bearing adeno-associated viruses (10<sup>11</sup> AAV-9 units). MiR-590 and -199a resulted overexpressed by ~250 fold and ~5 fold, respectively, and significant rates of proliferation of post-natal cardiomyocytes, but not fibroblasts, at 12 days of age were observed [90]. Similarly, miR-17-92 family members mediate a similar phenotype by targeting Pten [91]. These studies provide a promising genetic evidence for putative applications, although a viral-free system of miR delivery with sufficient efficiency in vivo is still missing for human cardiomyocytes.

## 3.3. MiRs involved in heart failure

Many miRs have been reported altered in conditions of heart failure (Fig. 2). One common cause of heart failure is dilated cardiomyopathy, which is common for the dystrophic myocardium [92]. Comparing patients with dilated and ischemic cardiomyopathies, common trends emerged, such as upregulation of miR-100 and miR-195, and downregulation of miR-92 and miR-133b [93]. MiR-195 is of particular interest in this context, as miR-195-overexpressing mice display adverse hypertrophic remodeling leading to heart failure [77]. MiR-195 is part of the broadly expressed miR-15 family, whose members are variably involved in cell proliferation regulation. Recently, LNA-enhanced antagomiRs inhibiting miR-15 family components have been proven effective in protecting against cardiac ischemic injury. Intravenous delivery of 0.5 mg/kg and 1 mg/kg antagomiRs in mice and pigs appeared sufficient to reduce the cardiac levels of miR-15 by ~72% and ~90%, respectively, in conditions of miR-15-inducing cardiac stress. Interestingly, despite rapid clearance from the blood stream after 12 h, antagomiR levels remained detectable and effective for inhibition till 1 week post-injection. Also, systemic delivery of 0.5 mg/kg anti-miR-15 oligonucleotides in mice was associated with reduced infarct size and elevated cardioprotection, and alleviated cardiac dysfunction after ischemia-reperfusion injury [94]. Another non-muscle-specific miR involved in cardioprotection against ischemia is miR-24, which targets the pro-apoptotic factor Bcl2l-11. Immediately after experimental ischemia, miR-24 was upregulated in injured rat hearts. Also, miR-24 was interrogated in rat cardiomyocytes cultured in ischemic-mimicking conditions (serum starvation, hypoxia) by means of 100 nmol miR-mimic. Treated cardiomyocytes presented an ~6 fold increase in miR-24 levels, and reduced rates of in vitro apoptosis and necrosis. These findings and the evidence that hypoxia-triggered Hif-1 activates miR-24 suggest that miR-24 is part of a defective endogenous response enabled by hypoxic cardiomyocytes, and that it is potentially targetable to improve survival [95]. In addition, miR-24, miR-21 and miR-221 constitute a minimal, defined miR cocktail, amenable to improve survival in murine CPCs both in vitro and in vivo. Combined transduction with single miRbearing lentiviruses, in fact, resulted in sustained CPC survival after transplantation in both striated muscle compartments, according to bioluminescence-based, non-invasive imaging. Consequently to the longer survival in vivo, transduced CPCs showed also enhanced effects on the functional outcome of injured hearts [96]. Furthermore, an intriguing miR-regulated pathway in different heart failure etiologies is the calcium flux regulation. An example of such context is miR-214, which was found upregulated in several conditions of heart failure and hypertrophy. MiR-214 protects against excessive calcium uptake by repressing a Na<sup>2+</sup>/Ca<sup>2+</sup> exchanger (Ncx-1), a calmodulin-dependent kinase (Camk-IIy), a mitochondrial permeability modulator (Cypd), and, once again, the pro-apoptotic Bcl2l-11 [97]. In the same context, miR-133a has been recently linked to the calcium channel Ip3r-II in a mutual repressive loop, which regulates calcium signaling and pathological cardiac remodeling [98].

MiRs can also target the myogenic remodeling by affecting key epigenetic regulators [99]. An example consists of miR-22, which promotes hypertrophic remodeling in cardiomyocytes [100]. Transfection of rat neonatal cardiomyocytes with 50 nmol miR-22-mimic resulted in upregulated hypertrophic markers in both presence and absence of phenylephrine, a hypertrophic agonist. Accordingly, genetic ablation of miR-22 decreased cardiac hypertrophic remodeling in vivo. Targets of miR-22 include the deacetylases Sirt-1 and Hdac-4, both involved in the myogenic progression [101]. Sirt-1 is also targeted by another miR involved in cardiac senescence and failure, miR-34a. Pre-treatment of bone marrow mononuclear cells with 500 nmol LNA-enhanced antimiR-34a improved cell survival and engrafting capacity in vivo, in experimental conditions of AMI [102]. Besides its effects on epigenetic regulators, miR-34a targets anti-apoptotic Bcl-2 and cell cycle promoters Cyclin-D2 and related kinases, thus promoting apoptosis and senescence [103]. Considering the theoretical concerted repression of Cyclin-D2 exerted by miR-133a and miR-34a, it would be interesting to know whether this holds true in cardiac regeneration and remodeling.

#### 3.4. Skeletal myogenesis and miRs

A wide range of miRs tightly control skeletal muscle formation and regeneration (Table 2). Similarly to the cardiac muscle, myomiRs play a pivotal role in regulating myoblast commitment and skeletal muscle formation [104]. *MiR-1* promotes differentiation of cultured myoblasts through translational repression of *Hdac-4*, which represses *Mef-2*-dependent expression of myogenic factors [105,106]. Conversely, *miR-133* stimulates myoblast proliferation mostly through negative regulation of *Srf* [107], thus creating a similar negative feedback loop as

**Table 2**MiR manipulation strategies relevant for normal or aberrant skeletal myogenesis.

miR	Target	Means of manipulation	Effects	Ref
miR-1/-206	Murine satellite cells	Adenoviral transduction	skeletal myogenic differentiation	[110]
miR-1/-206	Murine neonatal skeletal muscles	AntagomiR delivery (systemic/intramuscular)	proliferation of myogenic cells	[110]
miR-1/-206	Murine pre-segmented embryos	AntagomiR delivery	skeletal myogenic differentiation	[111]
miR-27b	Murine adult muscle (acute injury)	AntagomiR delivery (intramuscular)	proliferation of myogenic cells	[115]
miR-181	Murine myoblasts	AntagomiR transfection	skeletal myogenic differentiation	[116]
miR-214	Murine satellite cells	Lentiviral transduction	skeletal myogenic differentiation	[120]
miR-29	Murine myoblasts/rhabdomyosarcoma cell line	miR-mimic transfection	skeletal myogenic differentiation	[122]
miR-23a	Murine adult muscle (experimental atrophy)	Plasmid transfection	skeletal muscle atrophy	[136]
miR-669a	Murine neonatal cardiac/skeletal muscles	AAV2/9 delivery (intracardiac/intramuscular)	skeletal myogenic aberrant conversion/differentiation	[139,140]

The table presents several cases and effects of miR manipulation in myogenic cells or skeletal muscle, with potential relevance to applications involving myogenic regeneration in normal, aberrant or chronic disease conditions.

described for CPCs. During skeletal myogenesis, together with *Srf* and *Mef-2*, *MyoD* and *Myogenin* co-regulate *miR-1* and *miR-133* transcription (Fig. 3). Importantly, in combination with *Srf*, *MyoD* activates *miR-486*, which in turn represses *Pten* and *FoxO-1a*, reinforcing the Akt signaling and enhancing muscle growth. Transgenic fate tracking showed that *miR-486* is expressed throughout somite-to-muscle formation stages [108]. Hence, *miR-486* appears an important link between *MyoD* and the Akt signaling during skeletal muscle formation. This notion has been recently explored through a *miR-486*-based transgenic system to counteract muscle wastage in a murine model of Duchenne MD [109].

A specific myomiR for the skeletal muscle is miR-206. Overexpression of miR-206 in combination with miR-1, by means of adenoviral transduction, induced premature differentiation of primary murine satellite cells, by targeting Pax7 3'-UTR. Conversely, administration of 80 mg/kg anti-miR-1/-206 in neonatal mice induced a significant increase of Pax7<sup>+</sup>/Brdu<sup>+</sup> proliferating cells in the skeletal muscle at 24 h post-injection. Noteworthy, this effect was obtained at comparable levels through both intramuscular and intraperitoneal delivery [110]. Furthermore, injection of antagomiRs targeting miR-1/-206 in presegmented murine embryos resulted in dramatically decreased Myogenin expression and perturbed myogenic development. This effect was traced to the ability of miR-1 and miR-206 to bind two conserved regions on the 3'-UTR of Pax3, a myogenic factor sustaining the myoblasts in an immature state [111]. MyoD and Myogenin recognize miR-206 upstream enhancer and activate its expression. MiR-206 promotes myoblast differentiation by repressing many targets, including Pola-1, the largest DNA polymerase subunit, thereby halting the proliferation machinery in favor of differentiation [112]. In addition, miR-206 targets gap-junction

*Cx43* [113], hence putatively promoting the terminal maturation of skeletal fibers. Also, *miR-206* has been implicated as regulator of a spontaneously mutated *Myostatin* 3′-UTR, likely causing muscle mass overgrowth in the Belgian Texel sheep [114].

Analogously to the cardiac muscle, myomiRs miR-208b and miR-499 are upregulated also in slow skeletal muscle fibers following Myh7/7b expression, and reinforce the switch to  $\beta$ -MyHC in the presence of calcium-related fiber stress [79].

Subsequently to the exit from the proliferative state, miR-27b and miR-181 contribute to prime the differentiating cells along later stages of skeletal myogenesis. MiR-27b further promotes progression of the differentiation by translationally repressing Pax-3. Lentiviral-mediated miR-27 overexpression in somite explant cultures drastically reduced Pax3<sup>+</sup> and increased Myogenin<sup>+</sup>, committed progenitors. Accordingly, intramuscular injection of 10 µmol anti-miR-27b upon acute muscle injury resulted in increased number of Pax3<sup>+</sup> satellite cells. As a consequence, the muscle regeneration appeared delayed, although the effects were quantified at a rather early stage (6 days) post-injury [115]. Similarly, miR-181 indirectly activates MyoD by targeting one of its negative regulators, HoxA-11. Transfection of murine myoblasts with miR-181-targeting antagomiR, in fact, affected in vitro myotube formation. However, it is intriguing to note that, in the same experimental model, miR-181-mimic was not altering myoblast differentiation. This suggests that the endogenous levels of miR-181 are normally sufficient to reach effect-exerting plateau [116]. Other regulators of the balance between proliferation and differentiation in myogenic cells are miR-322 and miR-503, which target cell cycle promoter Cdc-25a, although the evidence is at present limited to one myoblast cell line in vitro [117,118].

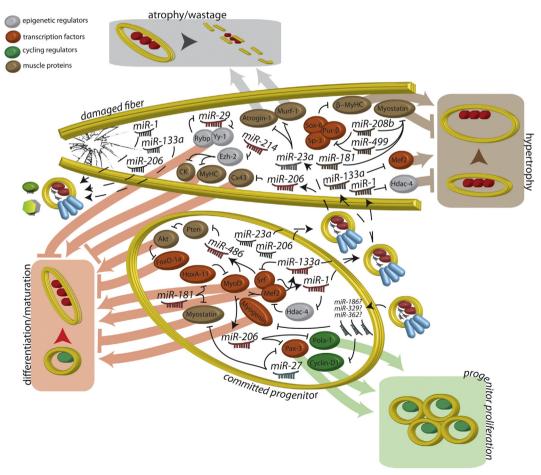


Fig. 3. Complexity of miR-based regulations in skeletal myogenesis. This diagram depicts several pathways presented in this review, in order to synthesize the information gathered from differentiating muscle progenitors and diseased skeletal fibers. Genes are pseudocolored according to their function (legend), whereas miRs are pseudocolored according to the cognate process (arrows and squares). The same miRs can have different colors in accordance with the different contexts in which they are depicted. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As in cardiac myogenesis, epigenetic and post-transcriptional controls are complexly entwined with miRs in skeletal muscle formation [119]. An example of miRs driving indirect epigenetic regulation is miR-214. Lentiviral-mediated overexpression of miR-214 up to ~5 fold endogenous levels increments the number of Myogenin<sup>+</sup> differentiating cells in primary cultures of myofiber-derived satellite cells. MiR-214 positively regulates Myogenin, Ckm (creatine kinase, CK) and Myh (myosin heavy chain) by targeting their negative epigenetic regulator Ezh-2, member of the Polycomb repressive complex [120]. This regulation seems important for normal skeletal muscle development, as transgenic ablation of miR-214 results in embryonic lethality and severe skeletal muscle defects [121]. Yet an example of miR-epigenetics feedback loop is highlighted by the case of the pro-myogenic miR-29. Transfection of 50 μmol pre-miR-29 oligonucleotides, but not the mutated control, increased expression up to ~3000 fold basal levels and associated with higher expression of terminal markers of skeletal myogenesis in murine myoblasts. Intriguingly, the same treatment appeared effective also on rhabdomyosarcoma cell lines, which consequently displayed slower rates of growth. Downstream of NF-kB circuitry, miR-29 is repressed by, and in turn represses, chromatin remodelers Rybp and Yy-1 [122,123]. Conversely, alternative polyadenylation and long-noncoding RNAs constitute two possibilities of refined post-transcriptional control on myogenic miR activity. In the first case, a reported example is the alternative polyadenylation of Pax-3, which presents miR-206 target site at its 3'-UTR only after the onset of differentiation [124]. In the second case, linc-MD1 serves as competing sponge to titrate miR-133 and miR-135 away from their targets, Maml-1 and Mef-2C, further allowing myoblast differentiation [125]. Intriguingly, Maml-1 and Mef-2C have been reported as important fate regulators also in human mesoangioblasts [126]. This notion opens the enticing question whether linc-MD1 might represent a novel target to enhance the potential of myogenic cells amenable for systemic delivery. However, to address this translational perspective, molecular tools to modulate long-noncoding RNAs must first be refined and upscaled.

# 3.5. MiRroring skeletal muscle diseases and fate switch

Chronic muscle disorders disrupt miR circuitries, raising the intriguing possibility of disease-specific miR signatures traceable in muscle biopsies [127]. This point would be particularly useful to help physicians and biologists in discriminating between the diverse forms of chronic myopathies and dystrophies, often mistaken without the support of a complete genetic screen. In this regard, miR-299-5p, -487b, -362 have been reported as specifically upregulated in Duchenne MD, whereas inflammatory miR-155, -146b would characterize inclusion body- or dermato-myositis. Moreover, miR-381, -382 would mark the facioscapulohumeral MD, and miR-100, -103, -107 would hallmark limb-girdle MDs when compared to Duchenne MD [128,129]. Analogously, miR signatures potentially characterizing sarcopenia are intensively investigated, for instance during aging [130, 131]. In mice, it has been reported that *miR-7/-206/-468/-542/-698* are upregulated in aged muscles, whereas miR-124a/-181a/-221/-382/-434/-455 are upregulated in adult control muscles [132]. When comparing muscle miR signatures between old and young individuals in primates, it has been reported that aged muscles presented downregulation of miR-181a and upregulation of miR-15a/-18a/-144/-451 [133]. Despite the quantitative differences, further experiments are required to pinpoint the downstream targets and the biological role of such signatures. Interestingly, in human muscles, not only did mature miRs exhibit differential expression upon aging, but also pri-myomiRs, e.g. pri-miR-1 and pri-miR-133a, presented higher levels [134,135]. This evidence opens the question whether the pri-miR accumulation in elderly muscles is due to deficiencies in the maturing machinery or to aging-specific cues.

In addition to aging, muscle atrophy also causes muscle mass loss, often as a consequence of chronic diseases. In this context, miR-23a

plays an important role, as dexamethasone-induced atrophy of murine adult skeletal fibers is attenuated after transient pulse of a *miR-23a*-expressing plasmid. Consistently, *miR-23a*-overexpressing mice displayed resistance to dexamethasone-induced atrophy. It has been shown that *miR-23a* targets the 3'-UTR of both *Atrogin-1* and *Murf-1*, which mediate atrophy-associated protein degradation [136].

A fascinating perspective for miRs in striated muscle control resides in their role in myogenic fate switches. Murine dystrophic cardiac mesoangioblasts presented the aberrant propensity to differentiate into skeletal muscle fibers, which were functionally uncoupled from the surrounding myocardium and contributed to progressive exacerbation of the dilated cardiomyopathy [137,138]. The pathological myogenic switch has been imputed to miR-669a/q. Indeed, transduction of the neonatal murine myocardium with miR-699a-expressing AAV2/9, but not with scramble-bearing AAV, resulted in traceable, life-long miR overexpression (~6 fold endogenous levels at 18 months of age) and partially alleviated end-stage dystrophic cardiomyopathy [139]. MiR-669a/q are negative regulators of MyoD and are repressed in Sgcb-null murine cardiac mesoangioblasts, owing to two concerted mechanisms. MiR-669a lies within the intron 10 of Sfmbt gene, whereas intron 1 of Sgcb harbors miR-669q. Transgenic deletion of Sgcb expression confers the dystrophic phenotype and ablates expression of miR-669q. In addition, intracellular calcium leakage, typical for dystrophic myocytes, causes degradation of Yv1, activator of Sfmbt, and downregulation of miR-669a [140]. Albeit a direct human ortholog of miR-669a/q is still unidentified, MYOD re-expression in cardiac cells was reported for two subjects with oncocytic cardiomyopathy [141], thus opening the question of which miRs can be targeted to counteract pathological myogenic switches in patients.

#### 3.6. MiRs, not miRacles: hints from and for regenerative medicine

In order to translate the findings about miRs and related targets (Table 3) into clinical-grade solutions for striated muscles, it will be

**Table 3**Relevant miRs and targets in cardiac and skeletal myogenesis.

miR	Reported target genes in cardiac myogenesis	Reported target genes in skeletal myogenesis	Ref.
miR-1	Calmodulin; Dll-1; Hand-2; Mef-2a	Hdac-4; Pax-3;	[63,66,67, 105,111]
miR-17-92	Pten		[91]
miR-21	Pdcd-4; Tgfßr-III		[81,82]
miR-22	Hdac-4; Sirt-1		[101]
miR-23a		Atrogin-1; Murf-1	[136]
miR-24	Bcl2l-11		[95]
miR-27		Pax-3	[115]
miR-29	Col1-a1; Col1-a2; Col3-a1;	Rybp	[84,122,
	Eln-1; Fbn-1;	Yy-1	123]
miR-34a	Bcl-2; Cyclin-D2; Sirt-1	•	[102,103]
miR-101	cFos; Tgfβ1		[86]
miR-133	Cyclin-D2; Ip3r-II; Srf	Maml-1; Mef-2c; Srf	[64,65,98,
			107,125]
miR-135		Maml-1; Mef-2c	[125]
miR-138	Aldh1-a2; Cspg-2; Mlk-3		[88,89]
miR-181		HoxA-11	[116]
miR-199a	Clic-5; Homer-1; Hopx		[90]
miR-206		Cx43; Myostatin	[110-114,
		(mutated);	124]
		Pax-3; Pax-7; Pola-1	
miR-208a	Myostatin; Thrap-1		[77,78]
miR-208b	Pur-β; Sox-6; Sp-3	<i>Pur-β</i> ; <i>Sox-6</i> ; <i>Sp-3</i>	[79]
miR-214	Bcl2l-11; Camk-IIγ; Cypd; Ncx-1	Ezh-2	[97,120]
miR-486		FoxO-1a; Pten	[108]
miR-499	Pur-β; Rod1; Sox6; Sp-3	Pur-β; Sox-6; Sp-3	[70,79]
miR-590	Clic-5; Homer-1; Hopx	-	[90]
miR-669a/q	MyoD (aberrant switch)	MyoD	[139,140]

The table summarizes several miRs and related target genes as reported throughout the review.

fundamental to address the issues of specific tissue targeting and timely in vivo effects of miR-based strategies.

The aforementioned systemic delivery of anti-miR-208a is a brilliant example of a therapeutic approach with beneficial effects restricted to the heart, without reported side effects in the skeletal muscle or in other districts [76]. Although it remains unknown whether such approach is upscalable to humans, its successful outcome is likely due to the natural properties of miR-208a and to the intrinsic characteristics of the disease model. MiR-208a is cardiac-specific and prominently down-regulated after birth, and it is generally re-activated only under pathological remodeling [77]. Moreover, hypertension-prone Dahl rats do not present evident damage in the skeletal muscles [142]. Conversely, when miRs can act in both striated muscle types, their modulation must be confined to the desired district. An example is miR-669a manipulation, which induced beneficial effects in the degenerating heart, but also a potentially negative response in regenerating skeletal fibers [140]. In that respect, miR-206 could be a candidate miRNA for a therapeutic approach focused on the skeletal muscle compartment, owing to its rather specific targets. However, before approaching strategies of miR-206 modulation by means of systemic delivery, it will be primarily important to exclude that miR-206 can induce any aberrant fate switches in resident cardiomyogenic cells.

Other approaches that should be considered for therapeutic advances, possibly in combination, are the multimodal miR modulation and the timely delivery. Multimodal miR modulation consists of combining up- and downregulation of different miRs in the same regenerative strategy. Although obviously more difficult to optimize, owing to often divergent miR-specific requirements, such approach would theoretically increase specificity and efficacy of systemic deliveries. In that respect, it might be interesting to combine the modulation of myomiRs with survival-enhancing miRs in cardiac-oriented strategies, and with epigenetics-regulating miRs in skeletal-oriented strategies. Particularly with regard to myomiR modulation, time-specific dosages will probably increase the applicative potential of translational strategies. Consistently with this notion, it will be useful to determine relative time sequences for the modulation of miR-1/-133/-499, in combination with either miR-208a, as cardiac-specific, or miR-206, as skeletal muscle-specific. Moreover, timely modulation of different myomiRs might be applied to systemic approaches in vivo, considering the transient effects and the rapid clearance of miR-mimics or antagomiRs. Such in vivo strategies will be particularly relevant when addressed in appropriate animal models of acute or chronic muscle damage. Refinement of these studies will likely enable us to better articulate the myomiR-driven regenerative response in the two striated muscle types.

A critical point for progressing miRs as pharmaceutical tools in the regenerative medicine for striated muscle is the means of miR modulation. Notwithstanding recent advances in conjugation and chemistries, oligonucleotides still appear mainly ideal for applications limited in time, owing to short half-life after systemic delivery and often problematic upscaling. AAVs, especially considering the tropism of serotypes 2 and 9 for striated muscles, are experimentally rather potent and ensure miR-modulation on the long term. However, their clinical application appears presently blunted, due to the risks posed by random genomic insertions [143]. In this regard, a putative alternative could be the combination of tissue engineering and miR modulation technologies. Appropriate stem cell pools, possibly with scaffolds enhancing their survival and commitment, might be engineered as in vivo paracrine hubs of miR modulation. The cell implant would then provide the host tissue with specific beneficial miRs or anti-miRs. Importantly, the choice of the stem cell pool will potentially influence specificity and directionality of miR modulation in host cell types. This has indeed been reported in the opposite direction for the miRcrine cross-talk between cardiomyocytes and cardiac stem cells [73].

Lastly, translational research on miRs governing striated muscle regeneration will definitely take advantage of a deeper understanding of the cell-to-cell patterns and the causal relationships between miRs

and downstream biological effects. In this perspective, a necessary path will consist of interrogating the complex regulation/effects of circulating miRs in experimental models and clinical cases of striated muscle regeneration. Therefore, the last part of this review will focus on the rapidly expanding field of exosome-borne and circulating miRs (circ-miRs).

### 4. Exosomal and circulating miRs in striated muscle regulation

4.1. Exosomes: a novel route of miR-based signals for cardiac muscle regulation

The role of exosomes as signaling shuttles for short- and long-range communication between cells and striated muscle tissues has recently emerged (Figs. 2–3). In the plasma of healthy individuals, exosomes are present in a surprisingly high concentration ( $\sim 10^{10}/\text{ml}$ ) [144], suggesting a physiological signaling role beyond the pathological shedding of cell debris. Exosomal cargo may contain proteins, mRNAs and miRs, and accumulating evidence is implicating exosomal miRs in a variety of cardiac and skeletal muscle conditions.

Since the first ultrastructural evidence of exosome secretion by CPCs and cardiosphere-derived cells (CDCs) [145], several other studies have reported on exosomal miRs influencing cardiomyogenic cell behavior. In vitro, rat CPCs have been shown to secrete exosomes enriched in miR-133a after cell death induced by calcium ionophore treatment. Exosomal miR-133a resulted functionally transferred to non-cardiac cells [146]. In vivo, CPC-derived exosomes, enriched in miR-451, significantly decreased cardiomyocyte apoptosis in AMI, likely through repression of Caspase3/7 [147]. In addition, exosomes have been shown to mediate CDC-driven regenerative effects in the heart, partly by shuttling miR-146a [148]. Furthermore, exosomes have been implicated in an experimental model of cardioprotective ischemic preconditioning. This model consists of brief insults in remote myocardial areas to ameliorate the outcome of subsequent sustained ischemia [149]. Moreover, exosome-borne miR-22 has been demonstrated to partly mediate the cardioprotective effect of ischemic preconditioning by targeting the epigenetic regulator Mecp-2 [150]. Mecp-2 exerts pleiotropic effects on several factors regulating survival or apoptosis, although this has currently been demonstrated only in non-muscle contexts [151–153]. These observations partially match previous findings showing the exosomemediated beneficial effects of human mesenchymal cells on ischemia/ reperfusion injured myocardium [154]. Conversely, resident miRs can also be regulated by exosomal proteins/transcripts, as it has been reported for Sca-1<sup>+</sup> CPCs shuttling Hsf-1 into ischemic cardiomyocytes, leading to epigenetic repression of miR-34a and improved survival [155].

Exosomes are probably involved in shuttling circ-miRs between different tissues in pathological conditions. Increased levels of *circ-miR-1* and *-miR-208* were found in the urine of AMI-affected rats. These circ-miRs have been linked to exosome shuttling from the injured heart through the kidney [156], although the observation was gathered from re-injection of exosomes in the animals. Recently, fibroblast-derived exosomal *miR-21\** has been identified as a pro-hypertrophic factor in cardiomyocytes, where it represses *Sorbs-2* and *Pdlim-5* [157]. Furthermore, *circ-miR-214* was found altered in CAD patients [158]. It has also recently been shown that *miR-214* constitutes a fundamental part of the cargo of pro-angiogenic exosomes secreted from endothelial cells [159]. However, a direct proof of functional uptake of endothelial-borne exosomal miRs by injured myocardial cells in vivo is still missing.

# 4.2. Exosome-borne miRs for skeletal myogenesis regulation

Exosomal miRs are attracting increasing levels of attention within the context of skeletal myogenesis. In fact, engineering pharmacological or stem cell-mediated formulations of targeted exosome-borne miRs constitutes an enticing translational perspective for enhancing myogenic regeneration [160]. In support of this speculation, CD34<sup>+</sup> stem cells

were engineered to release exosomes enriched in the Hedgehog signaling agonist Shh, which has pro-angiogenic effects. Functional transfer of Shh was evident in vitro. Moreover, after injection of the engineered CD34<sup>+</sup> cells in the border zone, the myocardial infarct size was reduced, the capillary density was increased, and the functionality resulted improved on the long term [161]. Furthermore, artificial formulations of exosomes carrying specific miRs have already been successfully tested to target the murine brain after systemic delivery [162]. A fascinating putative path will consist of combining specific exosomal-miR formulations with transmembrane signaling proteins able to interact with the endogenous myogenic stem cells, such as Notch ligands [126, 163]. In line with this idea, a feedback circuitry involving *miR-126* and *Dll-4* in mesenchymal stem cells has already been linked to proangiogenic remodeling in the ischemic myocardium [164].

Of potential significance for the translational concept of stimulating resident progenitors is the notion that myoblasts secrete exosomes [165], although with different content types and effects during the proliferation and the differentiation stages in vitro [166]. Myotube-derived exosomes, in fact, promoted differentiation of target myoblasts by down-regulating *Cyclin-D1* and *Myogenin* [167]. Yet the question remains of whether *miR-186*, -329 and -362 are involved, as these are predicted binders of the 3'-UTRs of both genes. Intriguingly, using the same myoblast model, atrophic myotubes presented decreased intracellular levels, but increased exosomal fractions of *miR-23a* [168] and *miR-182* [169]. This observation suggests a selective exosome-loading of miRs under stress conditions. Despite being promising, the field of exosomal miRs in skeletal myogenesis will definitely need more exhaustive investigation in a wider range of muscular disorders and regenerative strategies in vivo.

# 4.3. Circ-miRs: biomarkers and/or effectors in the context of cardiac damage?

During the last decade, evidence has accumulated from animal models and clinical studies, pointing at circ-miRs as novel biomarkers of the striated muscle condition. Circ-miR levels in serum and plasma are indeed readily detectable and appear altered in a variety of conditions. Although many issues must still be overcome, particularly in terms of sensitivity and feasibility [170,171], circ-miR analysis could in principle improve the presently used diagnostic or prognostic tools. Indeed, circ-miRs, especially as signatures, might help in focusing the current diagnostic tools, such as circulating CK, myoglobin or troponins, often altered by issues unrelated to the striated muscle pathology [172,173]. When approaching this exciting body of literature, however, an important caveat to consider is the still substantial lack of experimental evidence discriminating between the circ-miRs effecting a signal and the circ-miRs derived from an affected muscle.

Several studies have linked the circulating levels of *miR-1*, the most abundant miR in striated muscle, with acute or chronic conditions of muscle damage [174]. Indeed, the first evidence of increased plasma levels of *circ-miR-1* in AMI patients [175] was consolidated by preclinical studies, documenting *circ-miR-1* time peaks in AMI animal models. Serum levels of *circ-miR-1* correlated with release of the cardiac-specific CK isoform and with infarct size [176].

Circulating levels of myomiRs were found significantly increased in the aortic flow and correlated with serum levels of troponin-T (current standard of indirect measurement of myocardial damage) in acute coronary syndrome (ACS) patients, when compared to troponin-negative patients with coronary artery disease (CAD). Intriguingly, this study also reported that endothelium-enriched *miR-126* inversely correlated with the extent of myocardial injury, suggesting that *miR-126* is consumed during the trans-coronary passage through either uptake or turnover [177].

Further possible implications of these findings relate to the role of *miR-499* in hypertrophic cardiac remodeling [178] and on the complex vascular regulations exerted by *miR-126*. Indeed, *miR-126* stimulates

angiogenesis by repressing Spred-1 and R2-p58\beta, negative regulators of the Vegf signaling [179]. MiR-126 also decreases inflammatory cell recruitment and extravasation by repressing *Vcam-1* [180]. Accordingly, plasma levels of circ-miR-1 and -miR-126 have been reported as increased and decreased, respectively, at 4 h after the onset of symptoms, when comparing AMI patients to healthy control subjects [181]. The paracrine effects of miR-126 have also been documented in CD34<sup>+</sup> cell-derived exosomes, which improved post-ischemic conditions by stimulating angiogenesis [182,183]. In this regard, it is intriguing to note that diabetes mellitus patients displayed CD34<sup>+</sup> cells with impaired paracrine functions and that miR-126 overexpression was able to revert their dysfunctionality [184]. Thus, this evidence links the known neovascularization defects of diabetes mellitus to cardiac cells by means of miR-bearing exosomes. Another recent study of miRbased trans-coronary biomarkers identified miR-423-5p as a cardiacspecific heart failure plasma marker, positively correlating with the btype natriuretic peptide [185]. Interestingly, miR-423-5p has also been reported, in combination with miR-499, as a putative biomarker discriminating congestive heart failure from AMI [186].

Members of the miR-133 and miR-208 families have also been investigated as circulating biomarkers with potential diagnostic and prognostic value. Circ-miR-133a/b and -miR-208a/b were increased in the plasma of a large cohort of ACS patients. Intriguingly, higher levels of miR-133b and miR-208b apparently correlated with adverse prognosis [187]. In another study conducted on myocardial infarct patients undergoing primary angioplasty, circ-miR-133a levels associated with larger infarcts, higher reperfusion injury and decreased myocardial recovery [188]. Plasma concentration of circ-miR-208a resulted augmented in rats after isoproterenol-induced myocardial injury, but not in rats with cardiac hypertensive hypertrophy. This suggests that circmiR-208a is probably linked directly to the cardiomyocyte wastage and that hypertrophy is not per se sufficient to promote *circ-miR-208a* leakage [189]. In addition, circ-miR-208a appears to be a more sensitive biomarker of the early phase of AMI, particularly within the first 4 h after onset [190].

# 4.4. Circ-miRs in the context of skeletal muscle damage and physical exercise

Circ-miRs are currently also being scrutinized in relation to skeletal muscle disorders [191]. Serum levels of circ-myomiRs (*miR-1*, -133a, -206) were found to be increased in both murine and canine models of Duchenne MD [192]. Interestingly, circ-myomiR levels appeared to correlate with disease progression and were poorly influenced by physical or muscle-unrelated stress [193]. Upregulation of these circ-miRs has also been documented in Duchenne MD patients, when compared to age-matched subjects [194,195]. *Circ-miR-206* has been proposed as a biomarker for amyotrophic lateral sclerosis following plasma measurements in a murine model of the disease [196]. Furthermore, circ-myomiR perturbations have been reported for patients with chronic obstructive pulmonary disease [197], which often display muscle atrophy, and in patients bearing rhabdomyosarcoma tumors [198]. In addition, increased *circ-miR-144* has been associated with insulin-resistance in skeletal muscles of diabetic animals and patients [199].

Muscle-related circ-miRs seem appear to be affected not only by the pathological state, but also by physiological activities such as physical exercise. Time-dependent changes in several circ-myomiRs have been observed during recovery from exercise [200]. The type of exercise influences the entity of fiber damage and muscle response, and circ-miR levels might reflect these changes. Plasma myomiR levels indeed increased after muscle-damaging eccentric exercise, i.e. marathon running, but not after concentric exercise, i.e. prolonged biking [201,202]. In addition, a growing body of literature is dedicated to the differences in circ-miRs with respect to the training status preceding diverse exercise modalities [203]. These studies are apparently pointing at circ-miRs as fine diagnostic biomarkers of pathological remodeling

and disease/exercise-induced fiber wastage. However, one cannot exclude the possibility of a defective circ-miR-based signaling, aimed at eliciting survival and adaptation in non-degenerating fibers. Importantly, both contribution and response are still rather poorly distinguishable between cardiac and skeletal muscles in these studies. Thus, further steps towards a systematic evaluation of causality relations are required to disentangle the complex web of circ-miRs.

# 5. Future perspectives and conclusions

In conclusion, investigation into miR-based feedback circuitries has clearly enriched our current understanding on the myogenic processes during striated muscle specification. Both for cardiac and skeletal myogenic routes, miRs have emerged as refined and potent tools to direct many gene pathways towards a detrimental or beneficial outcome [204]. Still, more work is required to determine how the miRNA interplay critically affects different facets of the myogenic lineage commitment and the adult muscle remodeling. Studying miRNA fluctuations in biomaterial-embedded stem cells would certainly be useful in refining our tools for ex-vivo myogenesis, and in boosting clinical applications. Indeed, the combination of cell-based tissue engineering with dedicated miRNA modulation could be an avenue with potential therapeutic value for striated muscle repair.

Furthermore, through either exosomes or protein carriers, circ-miRs are emerging as possible mediators of long-range biological effects, and as putative biomarkers for more comprehensive diagnoses and prognoses. However, more efforts are needed to shed light on the still numerous open questions. In particular, it will be mandatory to address the directionalities and the causal relationships of miR-based circuitries on a cellular level. Following this perspective, it will be intriguing to decipher how miRs affect the interplay between different cell and stem cell types involved in striated muscle (re)generation. In this context, the combination of induced pluripotent stem cells and nextgeneration sequencing will be of great benefit in modeling the diverse myogenic cell players, and in unraveling the cross-signaling miR cascades.

#### Acknowledgments

We would like to apologize to all authors whose work has not been reported here because of space limitations. We would like to thank Dr Andrew J Smith (Centre of Human & Aerospace Physiological Sciences, School of Biomedical Sciences, King's College London, London, UK) for helping us in revising the text and improving readability. This work has been supported by the contributions of "Opening The Future" Campaign (EJJ-OPTFUT-02010), CARE-MI FP7 (#QPG-394601), AFM Telethon (#18073), Programmafinanciering KUL PF/10/00300, CARIPLO (2007.5639; 2008.2005), FWO (#G060612N, #G0A8813N, #G088715N), GOA (EJJ-C2161-GOA/11/012), IUAP-VII/07 (EJJ-C4851-17/07-P) and OT#09-053 (EJJ-C0420-OT/09/053) grants. MQ is supported by FWO (postdoctoral fellowship) and AFM (trampoline grant #18373). We thank Christina Vochten and Vicky Raets for professional administrative assistance. We would also like to thank Paolo Luban and Rondoufonds voor Duchenne Onderzoek for their kind donations.

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