

Non-coding RNA therapeutics for cardiac regeneration

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Abstract

A growing body of evidence indicates that cardiac regeneration after myocardial infarction can be achieved by stimulating the endogenous capacity of cardiomyocytes (CMs) to replicate. This process is controlled, both positively and negatively, by a large set of non-coding RNAs (ncRNAs). Some of the microRNAs (miRNAs) that can stimulate CM proliferation is expressed in embryonic stem cells and is required to maintain pluripotency (e.g. the miR-302~367 cluster). Others also govern the proliferation of different cell types, including cancer cells (e.g. the miR-17~92 cluster). Additional miRNAs were discovered through systematic screenings (e.g. miR-199a-3p and miR-590-3p). Several miRNAs instead suppress CM proliferation and are involved in the withdrawal of CMs from the cell cycle after birth (e.g. the let-7 and miR-15 families). Similar regulatory roles on CM proliferation are also exerted by a few long ncRNAs. This body of information has obvious therapeutic implications, as miRNAs with activator function or short antisense oligonucleotides against inhibitory miRNAs or lncRNAs can be administered to stimulate cardiac regeneration. Expression of miRNAs can be achieved by gene therapy using adeno-associated vectors, which transduce CMs with high efficiency. More effective and safer for therapeutic purposes, small nucleic acid therapeutics can be obtained as chemically modified, synthetic molecules, which can be administered through lipofection or inclusion in lipid or polymer nanoparticles for efficient cardiac delivery. The notion that it is possible to reprogramme CMs into a regenerative state and that this property can be enhanced by ncRNA therapeutics remains exciting, however extensive experimentation in large mammals and rigorous assessment of safety are required to advance towards clinical application.

Keywords

AAV vectors • Cardiomyocyte • Gene therapy • Heart • Infarction • lncRNA • MicroRNA • Nanoparticle • Regeneration • YAP

1. Introduction

The burden of cardiovascular disease is enormous. Current estimates indicate that, in particular, heart failure has reached epidemic proportions, affecting ~38 million people and standing as the main cause of death, disability, and financial burden worldwide.¹

Multiple lines of evidence indicate that cardiomyocyte (CM) loss is a major correlate of cardiovascular disease and heart failure, in most instances playing a causative role. This is obvious after myocardial infarction (MI), as the human left ventricle has 2–4 billion CMs, and MI can kill as many as 25% of these cells.² In addition to this acute loss of cells, persistent levels of CM death accompany most forms of chronic cardiac diseases³ and is one of the general hallmarks of normal cardiac ageing.⁴ Eventually, the loss of functional contractile mass in the heart leads to

decreased cardiac output, with consequently impaired capability of the organ to face haemodynamic challenges, thus leading to heart failure.

Physiological or pathological CM loss in adult life in mammals is not balanced by significant new CM generation. The regenerative capacity of the normal adult human heart was estimated, through ¹⁴C-carbon dating, to be less than 50% renewal in a 70-year lifetime.⁵ This estimate is consistent with measurements in mice obtained by analysing DNA synthesis⁶ or using imaging mass spectrometry.⁷

Concordant evidence indicates that the lack of cardiac renewal parallels a loss in CM replicative activity. CM proliferation is robust during embryonic, foetal, and immediate post-natal life, to then stop permanently later.⁸ In a consistent manner, 1-day-old neonatal mice can regenerate after myocardial damage; however, this capacity is lost by day 7 of age.⁹ Analogous findings hold true in swine, in which MI-induced CM loss was

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restored in 2-day-old piglets with minimal scarring and complete functional recovery, in contrast to adult animals.¹⁰ Although no cellular evidence of neonatal CM proliferation is available in humans, a case report provided evidence of complete recovery of cardiac function after an ischaemic insult in a newborn child.¹¹ In contrast to mammals, CM regenerative capacity remains robust throughout life in urodeles and fish, which can completely regenerate significant portions of lost myocardium in adulthood after myocardial injury.^{12,13} In these animals, regeneration occurs thanks to the proliferation of already differentiated CMs.^{14,15} The replicative activity of CMs also increases after damage in mice,^{6,7} however, it remains well below the threshold required to provide significant clinical benefit while scarring predominates.

The reason why CM replication irreversibly stops after birth in mammals while it remains possible throughout life in lower vertebrates still escapes our full understanding. Birth coincides with sudden oxidative stress to the myocardium,¹⁶ increased ventricular load,¹⁷ metabolic switch from glycolysis to oxidative phosphorylation,¹⁸ lack of exposure to maternal factors,¹⁹ and sudden change in hormone stimulation.²⁰ One of these reasons, or most likely a combination of them, is responsible for blocking the CM cell cycle and initiating a gene programme leading to hypertrophy.

Of note, when cardiac regeneration occurs in the hearts of neonate mammals or lower vertebrates, the regenerative process not only entails the expansion of resident CMs but also induces their integration and vascularization to generate a properly formed myocardial tissue, with eventual normal cardiac function.

2. Molecules and pathways controlling CM proliferation: setting the stage for non-coding RNA activity

Taken together, the observations reported so far hint at the possibility of achieving cardiac regeneration in adult, damaged hearts by stimulating the endogenous capacity of CMs to proliferate, as opposed to alternative strategies based on the transplantation of CMs obtained *in vitro* by various means (for recent reviews on the possible approaches for cardiac regeneration, cf. Refs^{21–23}).

CM proliferation is under the control of both extracellular and intracellular cues (Figure 1). A number of cytokines and growth factors are known to stimulate the proliferation of embryonic and neonatal CMs, several of which are active during development. These include members of the fibroblast growth factor (FGF) family,²⁴ IL-6,²⁵ platelet-derived growth factor,²⁶ follistatin-like 1,²⁷ and neuregulin-1 (NRG1).^{28,29} Recent evidence also indicates that CM replication responds to changes in the extracellular matrix, which are mediated by the protein agrin.³⁰ Finally, other cell types, in particular, mononuclear cells resident in the heart³¹ and T-regulatory cells during pregnancy,¹⁹ exert paracrine control of CM replication. In some instances, the pro-proliferative effect of each individual cytokine or cell is relatively modest¹⁹ or requires co-stimulation (e.g. p38 down-regulation in the case of FGF1²⁴) or depends on the presence of receptors that are expressed during development but no longer in mature CMs (e.g. the c-ErbB2 receptor activated by neuregulin³²).

Not unlikely other cell types, these extracellular regulators act through well-known signal transduction pathways. In cultured neonatal

CMs, activation of AKT by platelet-derived growth factor,²⁶ peroxisome proliferator-activated receptor δ by a small molecule,³³ Notch1 by its ligand Jagged1,^{34–37} and β -catenin upon glycogen synthase kinase 3 β inhibition^{38,39} are all able to stimulate proliferation.

A very relevant signal transduction pathway that regulates the rate of CM replication during embryonic development and can potentially be exploited for regenerative purposes in adult animals is the Hippo pathway. Originally discovered through genetic screens in *Drosophila*, this pathway is also a broad and essential regulator of cellular proliferation and organ size in mammals.^{40,41} The final positive effectors of this pathway are the transcriptional co-activators yes-associated protein (YAP) and the transcriptional coactivator with PDZ-binding motif (TAZ), which, when not phosphorylated, enter the nucleus and associate with the transcriptional enhancer factor domain family members (TEAD1–4) transcription factors to drive transcription of genes involved in cell proliferation.⁴² Evidence in genetic mouse models shows that knock-down of YAP determines myocardial hypoplasia and early embryonic lethality,^{43,44} while transgenic overexpression of constitutively active YAP causes CM hyper-proliferation.^{43,45} Activation of YAP is a critical component of CM mechanosensing, namely the set of mechanisms that transduce stretch and tension signals from the extracellular environment into activation of nuclear gene expression programmes.⁴² The pathway will be further discussed later, being YAP activation a crucial mechanism of action of most pro-proliferative miRNAs.

Finally, regulation of CM proliferation, similar to other cycling cells, is governed by a series of positive and negative regulators of the cell cycle. Past work has shown that overexpression of E2F family members,^{46–48} cyclin D1,^{49,50} cyclin D2,⁵¹ or silencing of the cyclin-dependent kinase inhibitors p21^{WAF1/CIP1}, p27^{KIP1}, and p57^{KIP2}⁵² can stimulate CM entry into the cell cycle. Analogous conclusions were drawn from studies in transgenic animals overexpressing cyclin A2,^{53,54} cdk2,⁵⁵ cyclin D1,⁴⁹ and cyclin D2.^{56,57} Most of the extracellular or intracellular controllers of CM proliferation also converge on regulating the levels of cell cycle proteins. For example, Meis1, a member of the TALE homoeodomain transcription factors that suppresses proliferation, activates expression of p16^{INK4a} and p21^{WAF1/CIP1}⁵⁸; activated Notch1 interferes with the levels of both G1 and G2/M cyclin/CDKs^{34,35}; the epigenetic regulator Jumonji blocks CM proliferation by repressing expression of cyclin D1⁵⁹ and enhancing the repressive function of the RB protein.⁶⁰

Several of the studies employing overexpression of cell cycle proteins, however, have failed in observing actual mitosis and cell division following DNA duplication. In addition, in some instances, for example, following overexpression of E2F1 or E2F3, reactivation of the cell cycle led to apoptosis.⁶¹ This indicates that targeting a single-cell cycle factor, either positively or negatively, is not sufficient to achieve complete cell replication. Consistent with this conclusion, a recent study indicates that CM division can be achieved by the simultaneous delivery of genes coding for multiple cell cycle activators [in the specific case, cyclin-dependent kinase 1 (CDK1), CDK4, cyclin B1, and cyclin D1⁶²], which together are required to override the multiple, intrinsic mechanisms that block cell cycle entry, and progression in CMs. The simultaneous delivery of four factors to the heart *in vivo*, obviously, poses demanding problems in terms of gene transfer.

When all the studies that have investigated the regulation of CM proliferation are considered collectively, caution must be exerted in concluding that cardiac regeneration might be achieved quite easily by stimulating the re-entry of CM into the cell cycle. Indeed, the vast

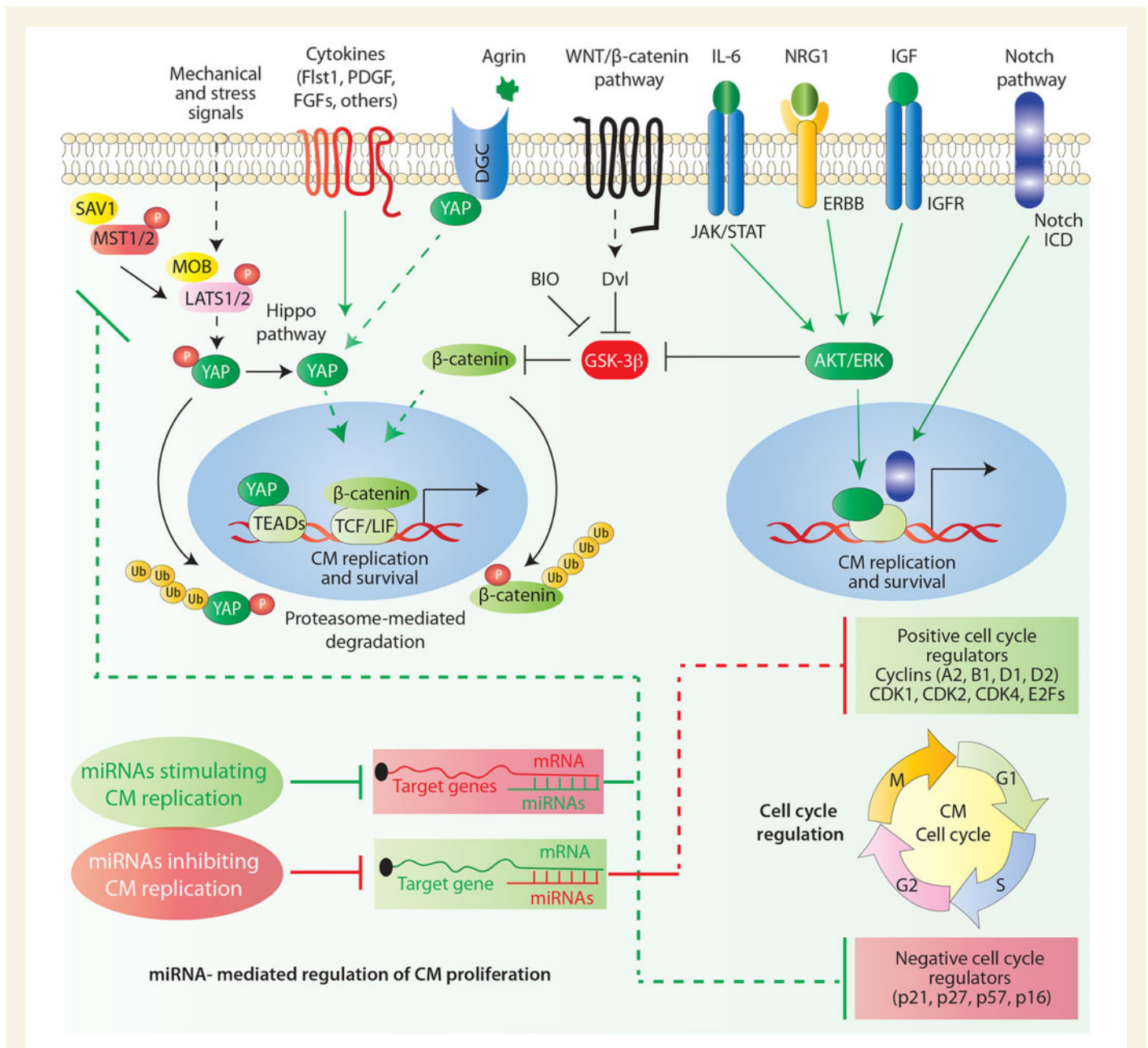


Figure 1 Extra-cellular and intra-cellular signalling regulating cardiomyocyte proliferation and cardiac regeneration. The cardiomyocyte cell cycle is under the control of several molecular regulators. Growth factors and cytokines acting on cell-membrane receptors, including IL-6, neuregulin (NRG1), Fst1, mechanical stress signals, and modification in the extracellular matrix, mediated by the protein agrin (which acts through the dystrophin glycoprotein complex, DGC), can all stimulate entry of cardiomyocytes into the cell cycle. Three main intracellular signal transduction pathways end up in the translocation of positive transcriptional co-activators into the nucleus. These are YAP, β -catenin, and Notch intracellular domain (ICD). In particular, YAP is maintained inactive through phosphorylation by the Hippo kinase cascade and degraded through the ubiquitin-proteasome pathway. In the absence of Wnt ligands, β -catenin is also degraded, in non-proliferative conditions, by a destruction complex including GSK-3 β , which can be inhibited by the small molecule BIO or by the Dishevelled (Dvl) protein. Notch is a cell-membrane receptor that, upon binding to ligands expressed by neighbouring cells (in particular Jagged1 in the heart) releases its intracellular domain that translocates into the nucleus. Similar to all cell types, the cardiomyocyte cell cycle is regulated by a number of positive activators (cyclins/CDKs, E2F transcriptional factors) and inhibitors (e.g. the cyclin-dependent kinase inhibitors p21 and p27). Several microRNAs interfere with these pathways by modulating the levels of critical regulators in the signal transduction pathways or at the level of cell cycle regulation. See text for further details.

majority of investigations has been performed with embryonic or neonatal rodent CMs *ex vivo*, which are at a developmental stage at which these cells are naturally permissive for proliferation. Fewer studies have instead convincingly met the goal of stimulating proliferation of adult CMs *ex vivo*

or in the hearts of adult or ageing animals. Similar considerations obviously apply to the stimulation of CM proliferation by manipulating the non-coding RNA (ncRNA) network, which will be the subject of the following chapters.

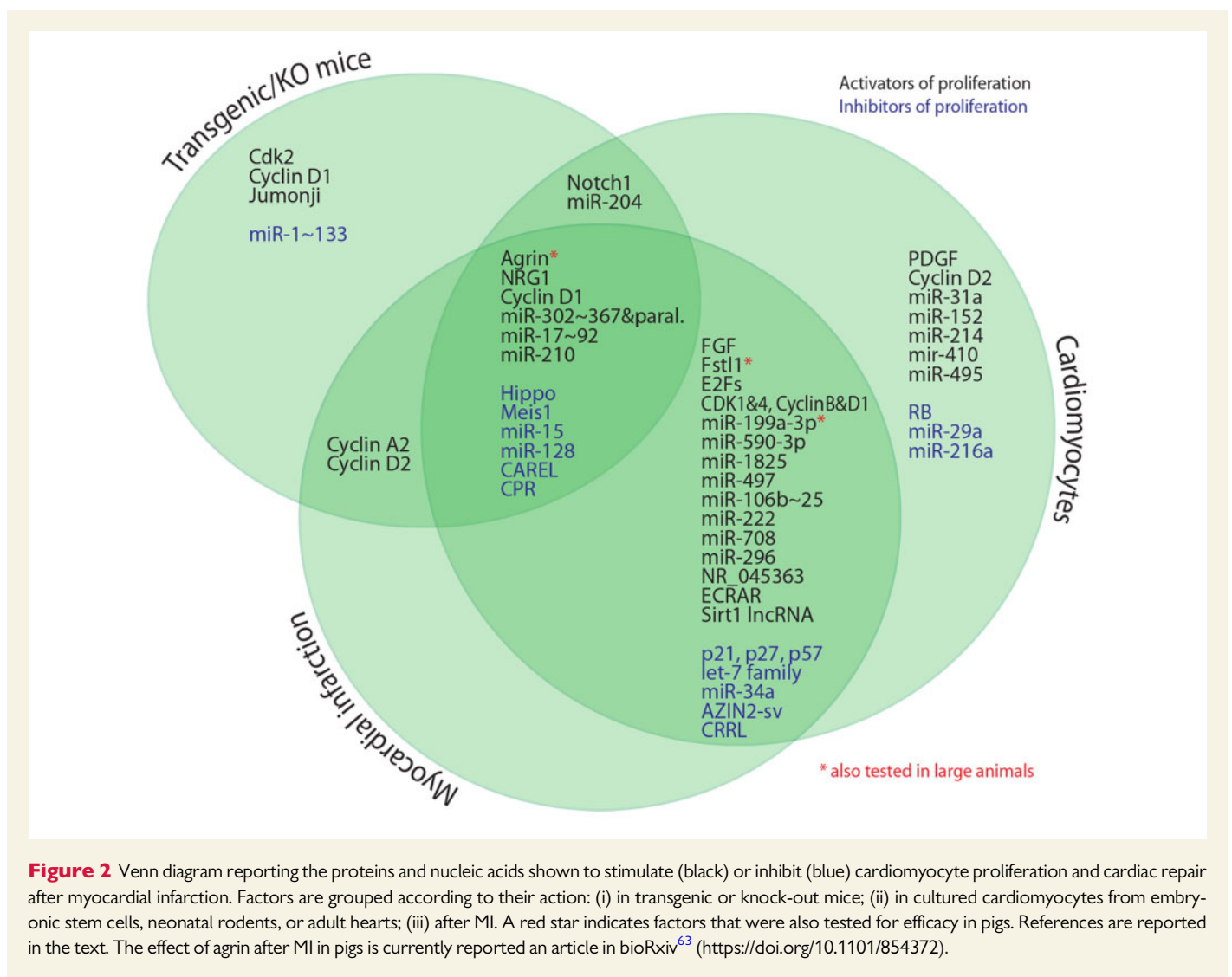


Figure 2 Venn diagram reporting the proteins and nucleic acids shown to stimulate (black) or inhibit (blue) cardiomyocyte proliferation and cardiac repair after myocardial infarction. Factors are grouped according to their action: (i) in transgenic or knock-out mice; (ii) in cultured cardiomyocytes from embryonic stem cells, neonatal rodents, or adult hearts; (iii) after MI. A red star indicates factors that were also tested for efficacy in pigs. References are reported in the text. The effect of agrin after MI in pigs is currently reported an article in bioRxiv⁶³ (<https://doi.org/10.1101/854372>).

The Venn diagram in Figure 2 reports the effect of the so far investigated molecules and treatments in cultured CMs, transgenic animals, or adult animals after MI.

3. Identification of microRNAs that stimulate CM proliferation

A growing body of evidence now indicates that CM proliferation is essentially regulated by a large set of ncRNAs. In particular, several microRNAs (miRNAs) have been identified that exert a positive effect on cell cycle entry and progression during embryonic and foetal development while others are involved in the withdrawal of CMs from the cell cycle after birth. Several other miRNAs, although not normally expressed in the heart, can still impact on cell proliferation once delivered to CMs exogenously, as a pharmacological application. As each miRNA has evolved to target tens or hundreds of different mRNAs, this class of molecules appears suited to fulfil the role of broad regulators of complex biological functions, as is the control of replication of highly structured cells, such as CMs.

Several miRNAs capable of stimulating CM proliferation were identified in two large screenings, both carried out with human miRNAs, the first in neonatal rodent⁶⁴ and the second in human iPS-derived CMs.⁶⁵ The first screening was based on the incorporation of the thymidine analogue 5-ethynyl-2'-deoxyuridine and on the decrease of p21 levels and led to the identification of 204 miRNAs increasing neonatal CM proliferation in rat cells; of these, 40 were also active in mouse cells. The second screening, which used both 5-ethynyl-2'-deoxyuridine incorporation and cytokinesis as readouts, identified 96 miRNAs active in iPS-derived, more immature, human CMs. Of these, 30 overlapped with the 204 originally identified in rat cells.⁶⁵ Other studies reported additional miRNAs that were individually found to regulate CM proliferation. A summary of this information is shown in Table 1.

The miRNAs exerting a positive effect on CM proliferation can be broadly classified into one of three categories.

3.1 miRNAs expressed in embryonic stem cells and active during the early stages of embryonic development

Several of the miRNAs reported to stimulate proliferation of neonatal CM belong to a few miRNA families highly active in embryonic stem cells

Table 1 MicroRNAs that stimulate cardiomyocyte proliferation and cardiac regeneration

MicroRNA	Mechanism related to proliferation	Relevant target gene(s)	Main observations	References
miR-302~367 cluster miR-290~295 cluster miR-371~373 cluster miR-520 family	Cell cycle regulation, Hippo inhibition	p21, Rbl2, LATS, Wee1, and other negative cell cycle regulators	miRNAs sharing a common seed sequence; constitute vast majority of ES cell miRNA content; maintain stem cell identity; miR-294, miR-302/367 beneficial after MI; TG expression of miR-302/367 induces hyperplasia and dysfunction	66–68
miR-17~92 cluster	Cell cycle regulation	PTEN, E2F, Bim, others	Involved in tumorigenesis (OncomiR1); TG mice show increased CM proliferation; miR19a/19b mimics beneficial after MI	69,70
miR-106b~25 cluster miR-106a~363 cluster	Cell cycle regulation	Cyclin E2, Bim, others	Paralogues of miR-17-92 cluster; oncogenic in human cancer; miR-25 down-regulated after MI	71
miR-199a-3p	Cell cycle regulation, regulation of cytoskeleton	Homer1, Hopx, TAOK1, β -TrCP, Cofilin2, c-Met, mTOR, Meis2, Rb1, others	AAV9 vectors induce regeneration in mice and pigs; long-term expression causes arrhythmia; single injection of mimic induces regeneration in mice; tumour suppressor for several human cancers	64,72–74
miR-590-3p		Homer1, Hopx, Clic5	Intracardiac injection of mimic or systemic administration of targeted exosomes induce regeneration in rodents	64,75
miR-1825		NDUFA10 and other genes involved in mitochondrial oxidative phosphorylation; miR-199a	Increases levels of miR-199a; beneficial after MI	64,73,76
miR-497		Mfn2	miR-15 family member; beneficial after I/R, prevents CM apoptosis	77
miR-31a	Signal transduction (Rho GTPases)	RhoBTB1	Down-regulated after birth	78
miR-152	Cell cycle regulation	DNMT1, p27kip1	Levels decrease after birth; regulated by YAP1 in turn activated by TLR3	79
miR-204	Regulation of transcription	Jarid2	TG mice show cardiac hyperplasia	80
miR-210	Signal transduction (β -catenin)	APC	Regulates proliferation of various cell types; regulated by hypoxia; beneficial after MI	81
miR-222	Regulation of transcription	HIPK1	Up-regulated by exercise	82,83
miR-410, miR-495	Regulation of transcription	Cited2 (p300/CBP binding factor)	Regulated by MEF2; Cited2 inhibits transactivation of HIF1a-induced genes by competing with binding of HIF1a to p300	84
miR-486		FOXO1, PTEN (in other cell types)	Increased by stretch; improves CM contractility; induces CM proliferation in newborn hearts	85
miR-708	Activation of signal transduction (MAPK)	Mapk14	More abundant in embryonic and foetal than in adult CMs; beneficial after MI	86
miR-296	Regulation of cell cycle	Trp53inp1, ltm2a	Decreased after birth; antagonized by lncRNA CAREL; mimic beneficial after MI	87
miR-214	Signal transduction (PTEN-AKT)	PTEN	Decreased in adult hearts; antagonized by lncRNA AZIN2-sv; transcribed as a cluster with miR-199a-3p	88

ES, embryonic stem; TG, transgenic; CM, cardiomyocyte; MI, myocardial infarction.

and involved in the maintenance of stemness. Of note, several of these miRNAs share an identical or almost identical seed sequence, likely mediating recognition of a largely overlapping set of target mRNAs.

A first of these families includes miRNAs from the miR-302~367 cluster. In both humans and mice, this is a set of miRNAs encoded by a single

polycistronic transcript that produces five precursor miRNAs (miR-302b, miR-302c, miR-302a, miR-302d, and the unrelated miR-367; see Ref.⁸⁹; Figure 3A). The first four of these miRNAs share the common seed sequence AAGUGCU. Another cluster of mouse miRNAs related to miR-302 is the miR-290~295 cluster. The 3p strand of three miRNAs

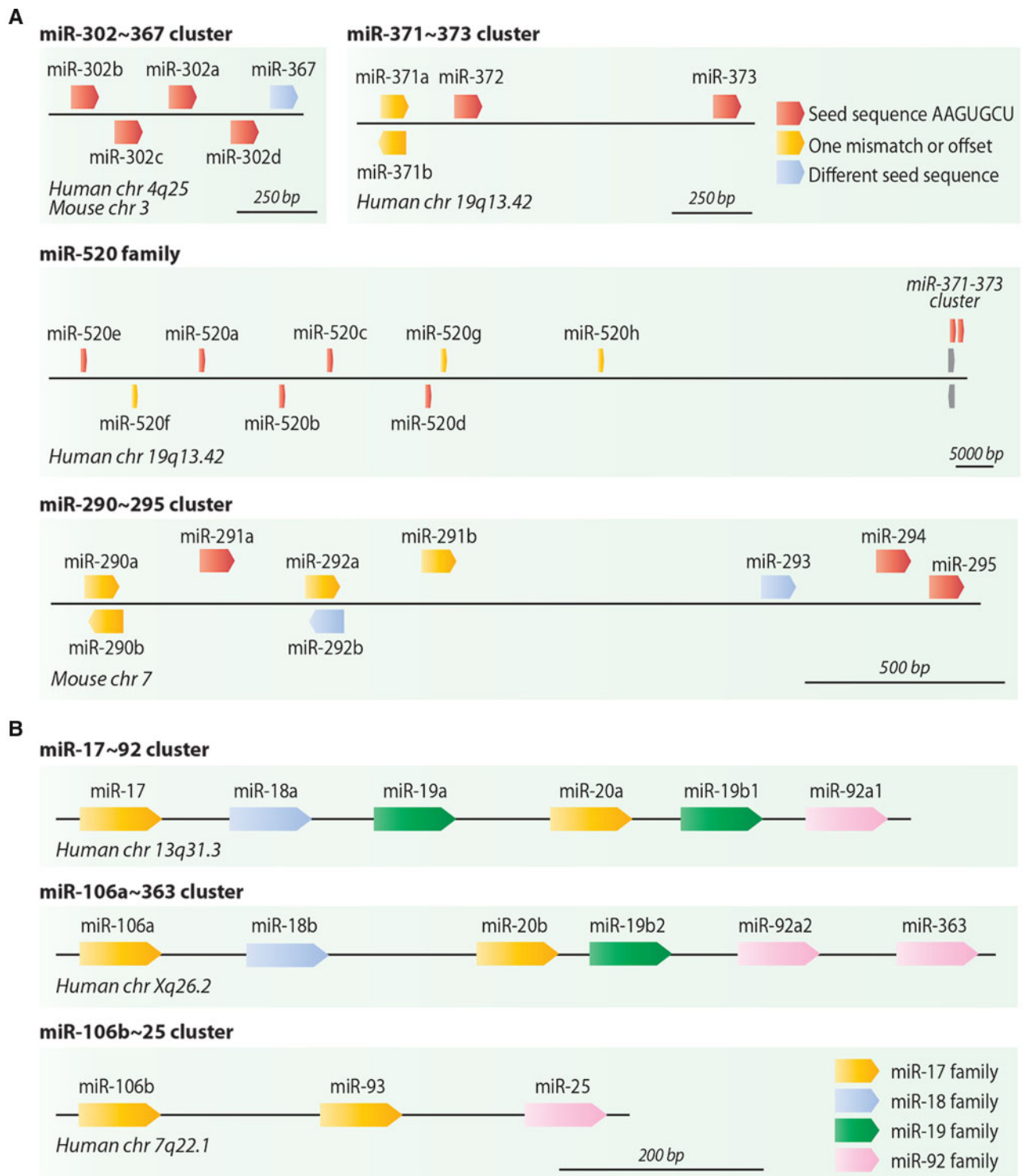


Figure 3 Conserved miRNA families known to regulate cardiomyocyte proliferation. The individual miRNAs are indicated by arrowed boxes in the direction of transcription. The different colours identify specific seed sequences. (A) miRNAs active in embryonic stem cells. Human miRNAs include those in the miR-302~367 and miR-371~373 clusters together with several miRNAs of the miR-520 family scattered along chromosome 19. Mouse miRNAs include those in the miR-302~367 cluster together with miRNAs belonging to the miR-290~295 cluster, which is the functional homologue of the miR-371~373 in humans. (B) miRNAs that also regulate proliferation of cancer cells. These belong to three clusters (the miR-17~92 cluster and its paralogues miR-106b~25 and miR-106a~363 clusters). Together, these three clusters encode 15 miRNAs, which can be grouped according to their seed sequence into four families, as indicated by the different colours.

of this cluster (miR-291a, miR-294, and miR-294) carries exactly the same seed sequence as miR-302, while other four miRNAs in the cluster (miR-290a, miR-290b, miR-292a, and miR-291b) have a closely related sequence with only one nucleotide mismatch (in an offset position for miR-290b-3p; see [Supplementary material online, Figure S1](#)).

The miR-302/miR-290 members are involved in the specification and maintenance of pluripotency of murine embryonic stem cells, in which the miR-290 cluster alone constitutes ~70% of the entire miRNAs content.⁹⁰ Members of these families promote the abbreviated G1 phase of these cells and suppress their differentiation.^{91–93} In particular, the miR-302~367 cluster is a downstream transcriptional target of the pluripotency transcription factors Oct3/4, Sox2, and Nanog.

Most members of the miR-302 family were found to stimulate neonatal CM proliferation in a screening involving 988 mature human miRNAs.⁶⁴ In mice, loss of the miR-302~367 cluster leads to decreased CM proliferation during development while its reactivation after MI-induced cardiac regeneration.⁶⁶ In a consistent manner, miR-294 was shown to decrease after birth, concomitant with the withdrawal of CMs from the cell cycle.⁶⁷

In humans, other miRNAs also share the same seed sequence as miR-302/290. These are miR-372-3p and miR-373-3p of the miR-371~373 cluster (this cluster is the human homologue of the miR-290~295 cluster in mice and is the most up-regulated miRNA group in the human blastocyst⁹⁴) and various members of the miR-520 family, including miR-520e, miR-520a, miR-520b, miR-520c, and miR-520d ([Figure 3A](#)); in other three miRNAs of the same family, the same seed sequence is an offset position (see [Supplementary material online, Figure S1](#)). Most of these miRNAs can also induce neonatal mouse and rat CM proliferation.⁶⁴

3.2 miRNAs regulating the cell cycle in different cell types and in cancer cells

Other miRNA families that control proliferation of other cell types, in particular of cancer cells, are also active in CMs once delivered exogenously. This is the case of the miR-17~92 cluster, which is included in a precursor transcript containing six tandem stem-loop hairpin structures that result in six mature miRNAs (miR-17-5p, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1; see Ref.⁹⁵; [Figure 3B](#)). Overexpression of this cluster, which is activated by c-Myc, is associated with a number of malignancies, hence the name Oncomir1.^{96,97} A main function for some members of this cluster is to inhibit expression of the transcription factor E2F1, which may result in resistance to apoptosis, a characteristic of cancer cells.⁹⁸ Transgenic overexpression of the miR-17~92 cluster in CMs induces their proliferation in embryonic, postnatal, and adult hearts and exerts a therapeutic effect after MI.⁶⁹

The miR-17~92 cluster has two paralogues, the miR-106b~25 and the miR-106a~363 clusters, located on two different chromosomes in humans. The miR-106b~25 cluster contains three miRNAs (miR-106b, miR-93, and miR-25) while the miR-106a~363 cluster encodes six miRNAs (miR-106a, miR-18b, miR-20b, miR-19b, miR-92a-2, and miR-363; [Figure 3B](#)). Both clusters are overexpressed in numerous human malignancies (reviewed in Refs^{99,100}).

Considering all the 15 miRNAs encoded by the three clusters together, these can be grouped into four families according to their seed sequence (the miR-17, miR-18, miR-19, and miR-92 families). Remarkably, the miR-17 family shares the same AAGUGCU sequence as the miR-302/371/520/290 families overlapping with its seed sequence at a +1-offset position (see [Supplementary material online, Figure S1](#)), suggestive of the existence of common mRNA targets for these miRNA

groups. Recent work has indicated that the pro-proliferative and pro-regenerative effect of the miR-17~92 cluster in the heart can be recapitulated by expressing two members of the miR-19 family.⁷⁰ Other recent work in rat neonatal CMs indicates that miR-25, which shares seed identity with miR-92a-3p, also stimulates CM proliferation and prevents apoptosis by targeting the Bcl2-like protein Bim.⁷¹

Of note, the above-mentioned, high-throughput miRNA screenings for CM proliferation performed with human miRNAs in rodent⁶⁴ and human⁶⁵ cells, both identified several members of the miR-302/290/371/520 group of miRNAs and of the mi-17~92 cluster and its paralogues. This indicates that these miRNAs are likely to exert conserved functions in evolution.

3.3 Other miRNAs exerting specific functions

One of the most effective pro-proliferative miRNAs is miR-199a-3p. The human miR-199a gene is duplicated on chromosomes 19 and 1 (miR-199a-1 and miR-199a-2, respectively). From the miR-199a hairpin, two miRNAs are generated, miR-199a-5p and miR-199a-3p; the sequence of the latter is identical across species.⁷² Transcription of the miR-199a/214 cluster is under the control of p53.^{101,102}

MicroRNA-199a-3p is known to act as a tumour suppressor in several human cancers, including papillary carcinoma,¹⁰³ ovarian carcinoma,¹⁰⁴ hepatocellular carcinoma,^{105,106} prostate cancer,^{107,108} lung cancer,¹⁰⁹ and osteosarcoma.^{110,111} This tumour suppressive activity can be explained by the direct targeting of factors regulating cancer cell proliferation and metastasis, including c-Met,¹¹² mammalian target of rapamycin (mTOR¹¹³), and CD44.¹¹⁰ In CMs, this miRNA is expressed at relatively low levels after birth, however, its exogenous transfection remarkably increases CM proliferation of both neonatal⁶⁴ and adult⁷³ CMs.

[Table 1](#) reports a list of other miRNAs that various studies have shown to stimulate CM proliferation. A few of these deserve specific mention for either mechanism of regulation or efficacy. Among the miRNAs physiologically expressed in the heart, miR-222 was shown to mediate the cardiac hyperplastic response to physical exercise,^{82,83} while miR-486 was recently reported to be induced in both patients and large animals in response to cyclic stretch.⁸⁵ Transfection studies with exogenously delivered miRNAs have instead revealed that miR-590-3p and miR-1825 are among the most effective exogenous miRNA in stimulating neonatal CM proliferation.^{64,76} Both miR-199a-3p and miR-590-3p share common target genes, including Hopx and Homer1.⁶⁴ The significance of targeting these genes in mediating the pro-proliferative effect of these miRNAs is still unexplored.

4. Endogenous miRNAs that inhibit CM proliferation

Several miRNAs that are endogenously expressed in CMs, in particular in the transition from foetal to neonatal life when CM proliferation ceases, specifically inhibit the CM cell cycle. A prominent group of these inhibitory miRNAs belongs to the let-7 family. Of interest, part of the aforementioned pro-proliferative effect of the miR-302/miR-290 miRNAs is exerted through the inhibition of these suppressive miRNAs.¹¹⁴

Caenorhabditis elegans let-7 was one of the first miRNAs to be discovered.¹¹⁵ It rapidly turned out that it belongs to a highly conserved family of miRNAs, which act as suppressors of cell cycle progression during

Table 2 MicroRNAs that inhibit cardiomyocyte proliferation and cardiac regeneration

MicroRNA	Mechanism related to proliferation	Relevant target gene(s)	Main observations	References
let-7 family	Cell cycle inhibition	E2F2, Cyclin D2	Regulators of cell proliferation in ES cells; up-regulated after birth; inhibition beneficial after MI	64,118,119
miR-1~133 cluster	Regulation of CM differentiation during cardiogenesis	Hand2, SRF, Cyclin D2, IGF1-R, others	Transcriptional target of muscle differentiation regulators, including serum response factor and Mef2	124–126
miR-15 family	Cell cycle inhibition	Chk1, others	Up-regulated after birth, dysregulated after MI; inhibition beneficial after MI	120,122,123
miR-29a	Cell cycle inhibition	Cyclin D2	Up-regulated after birth	121,130
miR-34a	Cell cycle inhibition	Sirt1, Cyclin D1, Bcl2	Suppressor of cell proliferation; major regulatory of ageing; overexpressed after MI; inhibition beneficial after MI	129
miR-128	Epigenetic control of cell cycle regulators	Suz12	Up-regulated after birth; knock-out beneficial after MI	128
miR-216a	Suppression of signal transduction (JAK/STAT)	JAK2	Overexpression blocks CM proliferation; competed by lncRNA NR_045363	131

ES, embryonic stem; CM, cardiomyocyte; MI, myocardial infarction.

development and stem cell differentiation.^{116,117} In CMs, expression of let-7 miRNAs increases as these cells exit the cell cycle. Increased expression of two conserved miRNA clusters, both including one member of the family (the miR-99/let-7c and miR-100/let-7a clusters) is also observed in adult zebrafish hearts. Of interest, during heart regeneration in fish, the levels of these clusters decrease, which fails to occur in mammals in which regeneration does not occur.¹¹⁸ Consistently, the forced inhibition of these miRNAs after MI exerts regenerative effects in mice.^{118,119}

Exit of CMs from the cell cycle is also causally correlated with increased levels of another miRNA family, composed of miR-15a, miR-15b, miR-16-1, miR-16-2, miR-195, and miR-497 (the miR-15 family; see Ref.¹²⁰) as well as of miR-29a.¹²¹ The miR-15 family miRNAs target various components of the cell cycle and DNA damage response machineries, including the checkpoint kinase Chk1¹¹⁹ and act as broad suppressors of cell proliferation. Their inhibition using antisense molecules leads to improved cardiac repair after MI.^{122,123}

Other inhibitory miRNAs comprise miRNAs that are involved in the regulation of CM proliferation during cardiogenesis. These include the muscle tissue-specific miR-1~133 cluster,^{124–126} of which miR-133 also inhibits cardiac regeneration in zebrafish,¹²⁷ and miR-128, which regulates expression of cell cycle controllers through the Polycomb complex-associated Suz12 protein.¹²⁸ Finally, miR-34a, a regulator of cell aging, also suppresses CM proliferation and its inhibition exerts beneficial effects after MI.¹²⁹ Table 2 lists the main miRNAs with inhibitory function on CM replication and their characteristics.

5. Mechanism of action of miRNAs that induce CM proliferation

Work carried out in the last few years has explored the main molecular pathways by which the miRNAs that induce CM proliferation exert their effect. A common characteristic of most of these miRNAs is the activation of the already mentioned YAP transcriptional co-factor. In

mammalian cells, YAP is kept inactive through phosphorylation by a kinase cascade that includes TAOK1, MST1/2 (Hippo in *Drosophila*, acting in complex with SAV1), and LATS1/2 (which associates with MOB1); phosphorylated YAP is then recognized by the β -TrCP E3 ubiquitin ligase and destined to degradation through the ubiquitin-proteasome pathway.^{132–135}

All the investigated pro-proliferative miRNAs target one or more components of this pathway. In particular, miR-302/367 down-regulates MST1, LATS2, and MOB1,⁶⁶ while miR-199a-3p down-regulates TAOK1 and β -TrCP (see Ref.⁷²; Figure 4). A high-throughput screening performed on hiPSC-derived CMs has revealed that the vast majority of the identified miRNAs that increases CM proliferation converge on the Hippo pathway.⁶⁵ Not only YAP activation is a consistent feature of all these miRNAs but it also appears essential to mediate their effect. In a recent study, the down-regulation of YAP in CMs blunted the pro-proliferative effect of any of 10 tested pro-proliferative miRNAs, including miR-199a-3p and three members of the miR-302 family.⁷²

Besides YAP activation, function of the pro-proliferative miRNAs appears to require additional effects on other cell machineries that are necessary for cell cycle progression (Figure 5). This includes, for example, down-regulation of the CDK inhibitor p21 (for the miR-302/290 miRNA group⁹²) or of the tumour suppressor kinase PTEN (for the miR-17~92 cluster⁶⁹ and miR-486¹³⁶). Another common characteristic of these miRNAs is to regulate the rate of actin polymerization. In particular, several of them, including miR-199a-3p, miR-1825, and miRNAs in the miR-302 family, down-regulate Cofilin2, a muscle-specific member of the ADF/Cofilins family of proteins,^{137,138} which prevents assembly of actin monomers and causes depolymerization of acting filaments.⁷² This observation is in keeping with the notion that the rearrangement of the actin cytoskeleton, which links the extracellular matrix to the CM nucleus, is intimately associated with the regulation of CM proliferation and mediates the withdrawal of these cells from the cell cycle at birth (reviewed in Ref.¹³⁹).

Table 3 lncRNAs that regulate cardiomyocyte proliferation and cardiac regeneration

lncRNA	Effect on CM proliferation	Relevant factors involved	Mechanism	Main findings	References
CAREL	Inhibitor	Sponge for miR-296	Increases Trp53inp1 and Itm2a, two miR-296 targets	Up-regulated after birth; inhibition promotes CM proliferation and cardiac regeneration after MI	⁸⁷
CPR	Inhibitor	Silencing of MCM3 expression	Recruits DNMT3A to the MCM3 promoter	Up-regulated in adult hearts; negative regulator of CM proliferation; knock-out beneficial after MI	¹⁴⁰
CRRL	Inhibitor	Sponge for miR-199a-3p	Increases expression of Hopx, a miR-199a-3p target gene acting as an inhibitor of CM proliferation	Up-regulated in human adult heart; negative regulator of CM proliferation; inhibition improves cardiac function after MI	¹⁴¹
AZIN2-sv	Inhibitor	Acts as sponge for miR-214 and stabilizes PTEN mRNA	Binds PTEN mRNA and increases its stability; acts as a miR-214 sponge to release PTEN, which down-regulates AKT	Splice variant of the AZIN2 gene (unknown if also coding for a protein); up-regulated in human adult heart; negatively regulates CM proliferation; knock-down improves cardiac function after MI	⁸⁸
ECRAR	Activator	Activates ERK1/2	Binds and promotes phosphorylation of ERK1/2, resulting in downstream activation of Cyclin D1 and Cyclin E1, which in turn activate E2F1	Up-regulated in foetal hearts; promotes CM proliferation and regeneration after MI; up-regulated by E2F1	¹⁴²
NR_045363	Activator	Sponge for miR-216a	Increases JAK2 of the JAK2/STAT3 pathway, a miR-216a target	Overexpression increases CM proliferation and improves cardiac function after MI; knock-down blocks proliferation	¹³¹
Sirt1 antisense lncRNA	Activator	Stabilizes Sirt1 mRNA	Binds Sirt1 mRNA 3'-UTR	Elevated during embryonic development, decreases after birth; promotes CM proliferation and repair after MI	¹⁴³

CM, cardiomyocyte; MI, myocardial infarction.

Unfortunately, it is still unclear what is the actual relevance, for cardiac biology, of the lncRNAs so far described to regulate neonatal CM proliferation. This will require proper analysis of the stoichiometry of the interaction between each individual lncRNA with its target miRNA or protein and the demonstration that this interaction really occurs *in vivo*. For example, the proposed mechanism for the anti-proliferative action of CRRL was proposed to be the antagonism with pro-proliferative miR-199a-3p.¹⁴¹ However, this miRNA is expressed at very low levels in differentiated cardiac cells and most likely does not take part in normal CM function. Even most notably, it remains largely unclear for most of these lncRNAs whether their inhibition is active in fully differentiated CMs of adult hearts.

7. Harnessing ncRNAs to achieve cardiac regeneration

The notion that some ncRNAs physiologically regulate CM proliferation during development and in neonates while some others can do so once delivered pharmacologically prompts the development of innovative therapeutic strategies based on these molecules. Table 4 reports the main successfully approaches to achieve cardiac repair so far attempted.

Suppression of inhibitory miRNAs or lncRNAs can be achieved through the use of antisense oligonucleotides that are modified to enhance RNase protection and cellular uptake. The design of these molecules commonly includes 2-O-methylation of the sugar, inclusion of phosphorothioate bonds and the possible addition of a cholesterol moiety to the 5' end of the molecule (antagomiRs); reviewed in Ref.¹⁵⁰. The inclusion of locked nucleic acid (LNA) nucleotides further increases the stability and thermodynamic strength of duplex formation with complementary target RNAs.¹⁵¹ These LNA-containing oligonucleotides are often designed using a steric block approach, in which a central LNA segment is flanked by non-LNA gaps, as this design still permits recruitment of RNase H to the duplex (LNA gapmers; see Ref.¹⁵²). AntimiR LNAs against miR-15b¹²³ and miR34a¹²⁹ were shown to exert a beneficial effect after MI in rodents. In both cases, the inhibitory oligonucleotides were administered systemically, which would represent a significant advantage in terms of therapeutic application.

An alternative manner to inhibit endogenously expressed ncRNA molecules is to take advantage of the intramyocardial administration of viral vectors expressing inhibitory short-hairpin RNAs (shRNAs) targeting ncRNAs. This was originally reported for the let-7 and miR-99/100 miRNAs, which are normally down-regulated during heart regeneration in zebrafish but fail to be de-repressed in mammals¹¹⁸ and later by the

Table 4 Small nucleic acid therapies for cardiac regeneration in animal models

miRNA/lncRNA	Method for delivery or overexpression	Delivery route	Main findings	References
miR-199a	AAV9 (mice) AAV6 (pigs)	Intramyocardial	Reduces infarct size and improves cardiac function after MI in both mice and pigs; in pigs, arrhythmias after 7 weeks from administration	64,74
miR-199a-3p	Mimic lipofection using RNAiMAX	Intramyocardial	Increases CM proliferation and cardiac function after MI	148
	Hydrogel-encapsulated nanoparticles	Intramyocardial	Increases CM proliferation and cardiac function after MI	149
miR-590	AAV9	Intramyocardial	Reduces infarct size and improves cardiac function after MI	64
miR-590-3p	Mimic lipofection using RNAiMAX	Intramyocardial	Increases CM proliferation and cardiac function after MI	148
	Targeted exosomes	Intravenous	Increases CM proliferation and cardiac function after MI	75
miR-1825	AAV plus hydrogel	Intramyocardial	Increases CM proliferation and cardiac function after MI	76
miR-294	Inducible AAV9	MI, vector intravenous 2 weeks before MI; activation at MI time	Increases CM proliferation and cardiac function after MI	67
miR-294-3p	LNA mimic	Intramyocardial	Improves cardiac function at 2 weeks after MI, lost at later times	67
miR302b/c	Mimic lipofection using neutral lipid emulsion (MaxSuppressor in vivo RNA-LANCEr II)	Intravenous	Increases CM proliferation and cardiac function after MI	66
	Cholesterol-modified mimic using hydrogel	Intramyocardial	Increases CM proliferation and cardiac function after MI	68
miR-302~367	Cardiac inducible transgenic		Regeneration at 3 weeks after MI; dilatation and cardiac dysfunction later	66
miR-708	Mimic lipofection using neutral lipid emulsion (MaxSuppressor in vivo RNA-LANCEr II)	Isoproterenol damage, intravenous	Increases CM proliferation and cardiac function after isoproterenol-induced cardiac damage	86
miR-17~92	Cardiac-specific transgenic		Increases CM proliferation and cardiac function	69
miR-19a/19b	Mimic lipofection using neutral lipid emulsion (MaxSuppressor in vivo RNA-LANCEr II)	Intramyocardial	Increases CM proliferation and cardiac function	70
	Mimic lipofection using neutral lipid emulsion (MaxSuppressor in vivo RNA-LANCEr II) or RNAiMAX	Intravenous	Increases CM proliferation and cardiac function	70
	AAV9	Intramyocardial	Increases CM proliferation and cardiac function	70
miR-210	Transgenic		Increased CM proliferation and cardiac function in TG animals	81
miR-296	Agonist	Intramyocardial	Increases CM proliferation and cardiac function	87
ECRAR	Adenovirus	Intramyocardial	Increases CM proliferation and cardiac function	142
NR_045363	AAV9	Intramyocardial at P1 followed by MI at P7	Increases CM proliferation and cardiac function	131
Sirt1 antisense lncRNA	AAV9	Intramyocardial	Increases CM proliferation and cardiac function	143
miR-128 KO	Conditional knock-out		Increased CM proliferation and cardiac function in knock-out animals	128

Continued

Table 4 Continued

miRNA/lncRNA	Method for delivery or overexpression	Delivery route	Main findings	References
Anti-miR-15 family	LNA anti-miR-15b; cholesterol-modified antagomir-15b	Intravenous	Reduces infarct size and improves cardiac function	123
Anti-miR-34a	LNA antimiR	Intravenous	Increases CM proliferation and cardiac function	129
Anti-let-7a/c and anti-miR-99/10	AAV9-shRNA	Intramyocardial	Increases CM proliferation and cardiac function	118
Anti-let-7i-5p	AAV9-anti-let-7i-5p	Intramyocardial	Increases CM proliferation and cardiac function	119
Anti-CAREL	Ad-shRNA	Intramyocardial	Increases CM proliferation and cardiac function	87
CPR KO	Knock-out		Increases CM proliferation and cardiac function in knock-out animals	140
Anti-CRRL	AAV-siRNA	Intramyocardial	Increases CM proliferation and cardiac function in knock-out animals; activation of endogenous miR-199a	141
Anti-AZIN2v	Ad-shRNA	Intramyocardial	Increases CM proliferation and cardiac function	88

CM, cardiomyocyte; MI, myocardial infarction; TG, transgenic.

specific inhibition of the let-7i-5p family member.¹¹⁹ Viral vectors expressing inhibitory shRNAs were also reported for the CAREL, CRRL, and AZIN2sv lncRNAs.^{87,88,141}

The efficiency of molecules that suppress endogenously expressed inhibitory ncRNAs strictly depends on relative relevance of these molecules in controlling CM proliferation. To date, most of the identified inhibitor miRNAs or lncRNAs do not appear to qualify as master regulators of cell proliferation or they are also involved in the control of proliferation of other cell types, thus raising safety issues. A more tempting approach is thus to impart a new, regenerative function to the heart by the exogenous delivery of ncRNAs, irrespective whether these take part in normal CM biology. In this respect, a number of miRNAs, and especially those selected through comparative high-throughput screenings for efficacy in stimulating CM replication, are appealing for therapeutic purposes.

Prolonged expression of exogenous miRNAs in the heart can be achieved using AAV9 vectors, which transduce CMs at high efficiency, after either intracardiac or systemic delivery (reviewed in Ref.¹⁵³). Expression of miR-199a or miR-590a in mice resulted remarkable formation of new cardiac mass at 1 and 2 months after MI, paralleled by CM proliferation and consequent restoration of cardiac function.⁶⁴ Analogous findings were reported for AAV vectors expressing miR-294, a member of the miR-302 superfamily⁶⁷ and the miR-17~92 cluster member miR-19a/19b.⁷⁰

Expression of miRNAs using AAV vectors, however, is not devoid of problems. First, these vectors persist in an episomal form indefinitely, virtually for the whole life of the organism (or at least of the transduced cells).¹⁵⁴ This property is not necessarily desirable for a pro-proliferative molecule with pleiotropic functions, such as most miRNAs are, especially because work in zebrafish^{15,118} and in the neonatal heart⁹ indicates that the stimulation of CM proliferation requires partial cell de-differentiation to permit proliferation. Accordingly, transgenic overexpression of the miR-302/367 cluster in mice permitted cardiac

regeneration early after MI, however, led to cardiac dysfunction due to CM de-differentiation and hyper-proliferation at later time points.⁶⁶ Second, AAV vectors contain a gene coding for the pri-miRNA, which is then processed to eventually give rise to the mature miRNA duplex. From this, both strands can be used by the expressing cells, depending on the endogenous miRNA processing machinery. Thus, not only the desired miRNA is produced but often also the miRNA encoded by the complementary pre-miRNA stem-loop strand, which might exert unwanted effects. In agreement with these concerns, the permanent expression of the miR-199a pri-miRNA after MI in pigs led to remarkable regeneration at 1 month after treatment, as assessed by cardiac MRI, however, determined fatal arrhythmias in a few animals while approaching 2 months.⁷⁴ In evaluating these arrhythmias, however, it should be noted that such events were never observed in mice⁶⁴ and that cardiac electrophysiology in pigs is remarkably different from humans and other mammals in the early repolarization period.¹⁵⁵ Thus, to what extent the arrhythmic phenotype observed in pigs might be peculiar to this species remains a matter open to investigation. Finally, AAV9 vectors can also transduce other organs, in particular, liver and skeletal muscle,^{156,157} and can cross the blood-brain barrier,¹⁵⁸ raising additional safety caveats.

Despite the permanent expression of pro-proliferative miRNAs using AAV9 vectors should be considered with caution in translational terms, still, the transient delivery of miRNA mimics appears to have the potential of solving these issues. The intracardiac injection of miR-199a-3p or miR-590-3p mimics using cationic lipid formulations in mice led to the persistence of these miRNAs for over 10 days inside the heart and this was sufficient to stimulate cardiac repair.¹⁴⁸ Analogous functional results were obtained, after MI in mice, by the intracardiac injection of miR-19a/19b mimics using a neutral lipid delivery reagent⁷⁰ or by using the same formulation for the daily intravenous administration for 3–7 days of miR302b/c,⁶⁶ miR-19a/19b⁷⁰ or miR-708⁸⁵ mimics, or by the intracardiac delivery of cholesterol-modified miR-302b/c mimics using a hydrogel.⁶⁸ Of note, however, while AAV9 vector transduction is selective for

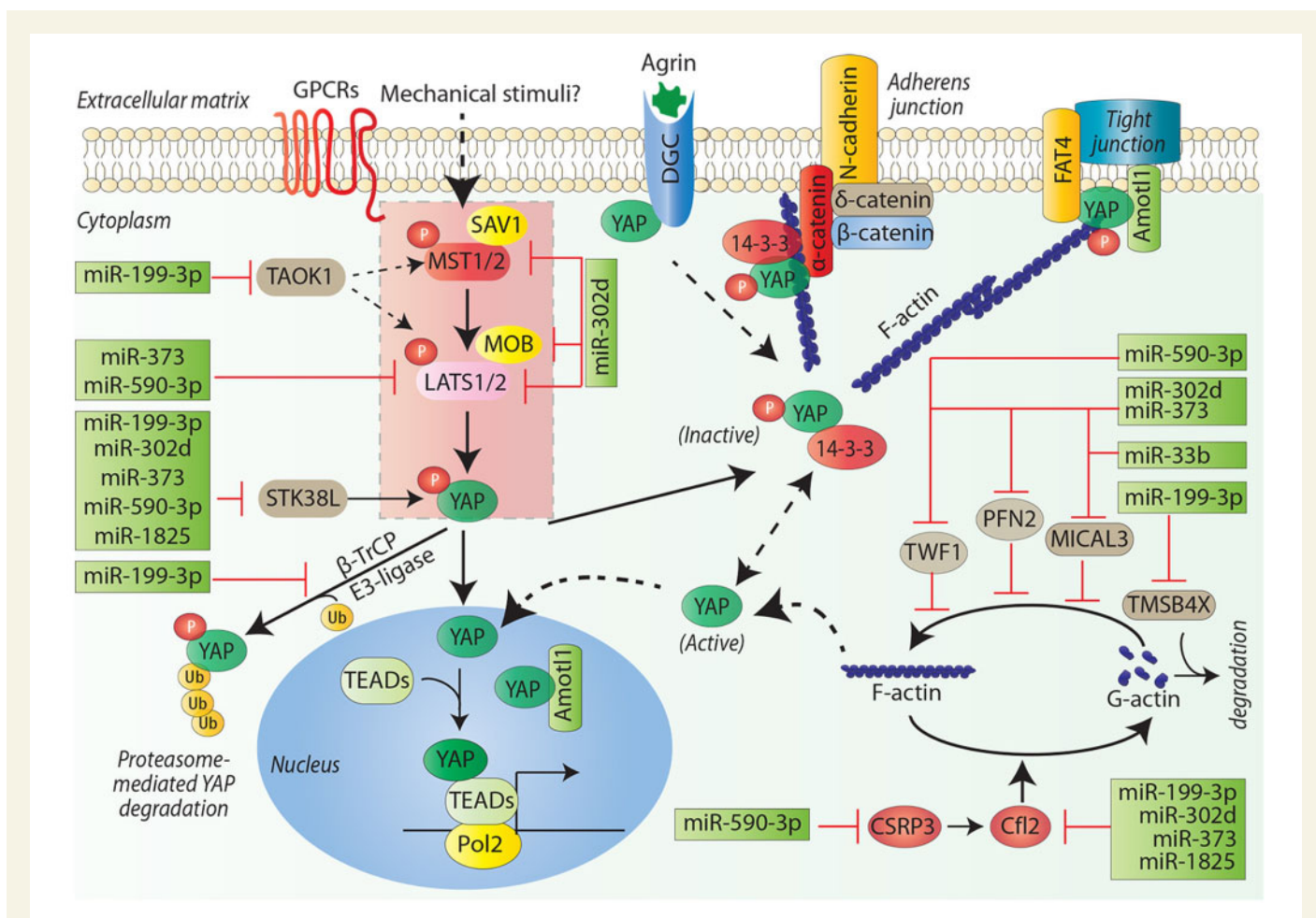


Figure 5 Effect of miRNAs on the Hippo pathway and the actin cytoskeleton. The cardiomyocyte cell cycle is controlled by the YAP transcription co-factor, which, when de-phosphorylated, translocates to the nucleus and interacts with the TEAD family of transcription factor to drive expression of pro-proliferative genes. YAP phosphorylation is controlled by various protein kinases, including TAOK1, STK38L, LATS, and MST1. These are targeted by different pro-proliferative miRNAs (in green). Proliferation is also linked to the cardiomyocyte actin cytoskeleton, which connects to cell-membrane dystrophin glycoprotein complex (DGC) and cell-to-cell junctions. Different pro-proliferative miRNAs regulate the rate of polymerized vs. globular actin (F- and G-actin, respectively) by targeting one or more of the cellular factors controlling the rate of actin polymerization (including Twinfilin, TWV, Profilin, PFN2, thymosin-b4, TMSB4X and Cofilin2, and CFL2). For further description, cf. text and references therein.

CMs,¹⁵³ mimic lipofection can affect all other cell types in the heart (fibroblasts, endothelial cells, and macrophages). This might have consequences on the efficacy and safety of each specific treatment.

8. Future perspectives

The notion that CMs are endowed with an intrinsic capacity to proliferate and that this property might be enhanced by ncRNA therapeutics is exciting. It reverses a long-standing paradigm in cardiology, namely that cardiac regeneration might only be attainable through the transplantation of exogenously cultured cells. However, the perspective of using short nucleic acids as therapeutics for cardiac regeneration poses a series of novel challenges, in terms of both biological understanding and clinical application. In this session, we try to anticipate some of the future developments that are expected in this area and the challenges posed by these new nucleic acid-based medicines.

8.1 Novel ncRNAs for cardiac regeneration

The number of ncRNAs capable to regulate endogenous CM proliferation is certainly destined to increase in the near future. The latest release of the miRBase database (miRBase 22.1, released at the end of 2018; <http://www.mirbase.org/index.shtml>) reports information for 1917 annotated precursors, and 2654 mature miRNAs encoded by the human genome.¹⁵⁹ However, the two largest high-throughputs screenings that so far systematically analysed these molecules for CM proliferation^{64,65} were both based on a library of <1000 miRNA mimics from miRBase release 13.0. Thus, it is very likely that additional pro-proliferative human miRNAs still await identification from the sequences discovered after 2009.

As far as lncRNAs are concerned, there is a broad range of estimates for their number in humans, ranging from less than 20 000 to over 100 000.¹⁶⁰ Thus, in any case, the potential for finding new lncRNAs involved in the regulation of CM proliferation is very high. Unfortunately, the identification of these molecules is more limited due to the lack of

extensive libraries to perform systematic screenings. As a matter of fact, all the current lncRNAs that regulate CM proliferation were identified in the last couple of years starting from RNA sequencing experiments that compared lncRNA datasets in embryonic or foetal tissue vs. adult heart, with the purpose of identifying RNAs the levels of which drastically varies at birth. This is in contrast with miRNA identification, in which some of the most effective miRNAs were identified from systematic screenings and do not take part in normal cardiac physiology. Thus, while deeper NGS keeps providing novel ncRNA molecules and our knowledge on lncRNAs expands, methods should be devised to systematically couple sequencing with high-throughput, functional screenings of the identified molecules (or their LNA inhibitors) for their effect on cell proliferation.

In terms of comparing lncRNAs with miRNAs, it appears worth considering that all the lncRNAs so far reported to regulate CM proliferation exert a punctual effect on one specific cellular regulator, while miRNAs, for their intrinsic nature, are by far more pleiotropic in their mechanism of action. Since CM replication is a complex process that requires not only cell cycle control but also coping with the complex sarcomeric machinery and with electrical coupling of CMs, miRNAs thus appear more suited to exert such a complex effect. In this respect, it remains possible that more effective lncRNAs than those currently identified will be found in the category of lncRNAs that regulate enhancers or super-enhancers, which are more likely to exert broader functions.¹⁶¹

8.2 Cardiac expression of small RNAs using AAV vectors

AAV vectors are the current viral system of choice for efficient gene transfer into CMs. Their relative genetic simplicity renders these vectors also amenable for the expression of short ncRNAs. The AAV vector backbone can accommodate multiple shRNA cassettes, in which expression of short (60–70 bp long) hairpins against a lncRNA of choice can be driven by polymerase III using the H1 or U6 promoters.¹⁶² In the case of miRNAs, these can be delivered as pri-miRNA genes using polymerase II promoters. In both the shRNA and miRNA cases, the primary transcript enters the endogenous miRNA pathway and is eventually processed by Dicer to form a mature, 21- to 23-bp double-stranded RNA molecule.¹⁶³ In the case of anti-lncRNA shRNAs, these molecules can also be expressed using polymerase II promoters.¹⁶⁴ This offers a dual advantage, as expression can be driven from cardiac-specific promoters and the levels of shRNA expression are lower than those derived from polymerase III transcription. This avoids the risk of saturating the RNAi pathway, which was shown to exert possible cytotoxicity (e.g. cf. Refs^{165–167}). Finally, both Polymerase II and III promoters can be used to express anti-miRNA sponges, namely antisense RNA molecules carrying multiple complementary sites to a given miRNA and acting as decoy for it. These artificial sponges mimic the regulatory activity of competing endogenous RNAs, such as naturally expressed lncRNAs, RNAs encoded by pseudogenes and circular RNAs, all of which can sequester miRNAs and buffer their activities.¹⁶⁸

As already discussed, a major problem of expressing an ncRNA exerting a pro-proliferative effect using an AAV vector is that transgene delivery with these vectors is permanent, hence the need to regulate their promoters. In past years, a few inducible transcription systems have been engineered, in which transgene expression is controlled by small molecules. The two most commonly used systems for experimental purposes are the tetracycline-inducible (TetOn) or the repressible (TetOff)

systems¹⁶⁹ and a method based on rapamycin-induced dimerization of FKBP12- and mTOR-derived proteins.¹⁷⁰ Both systems require multiple expression cassettes to achieve transgene regulation, which is problematic given the limited cloning capacity of the AAV vector backbone (detailed in Ref.¹⁵³). This becomes cumbersome in light of translational applications. Additionally, in contrast to rodents, the prokaryotic Tet regulator has proved immunogenic in non-human primates, thus posing an important limitation to its clinical use.¹⁷¹

In light of these issues, novel tools for the control of small RNAs are definitely required. These might be based on post-translational, as opposed to translational, control of RNA stability or processing using RNA elements collectively termed riboswitches. For ncRNAs, this can be achieved by taking advantage of an aptazyme (a small molecule-specific RNA aptamer inserted within a ribozyme) positioned at the 5' end of the transcript. In the presence of the small molecule ligand, the ribozyme self-cleaves, and the RNA gets degraded. Alternatively, for both shRNAs and miRNAs, ligand-dependent aptamers or aptazymes can be inserted at sites of the RNA where their presence can interfere by processing by either Droscha or Dicer (reviewed in Refs^{172–174}).

It has to be expected that these molecular strategies to control the levels of regulatory ncRNAs will become further developed and increasingly applied for cardiac applications over the coming years.

8.3 Methods for the efficient delivery of synthetic small nucleic acid therapeutics

In alternative to the use of viral vectors, small nucleic acid therapy of the heart will take enormous advantage from the development of innovative delivery methods for synthetic molecules. An ideal system should provide stability to the payload by protecting it against nuclease degradation, cross the membrane of endothelial cells to reach CMs when administered systemically, provide target cell specificity, elicit minimal or no toxicity and inflammatory or immune response, and be scalable for clinical applications. No such system exists yet, however, remarkable progress has been made over the last few years in this area.

The original lipofection technology has taken advantage of small nucleic acid entrapment by mixtures of cationic lipid molecules (such as DOTMA or DOTAP) and neutral lipids (such as DOPE).¹⁷⁵ An example of these lipoplexes are those formed by the commercial reagent lipofectamine and its derivatives, which are very efficient in transfecting CMs and several other cell types *in vitro*. Alternatively, to improve biodistribution and avoid rapid clearance from the circulation, neutral lipids can be used. Both cationic lipoplexes based on Lipofectamine RNAiMAX and neutral lipid emulsions based on MaxSuppressor In Vivo RNA-LANCER II have been successfully used to deliver the pro-regenerative miRNAs miR-199a-3p and miR-590-3p upon intramyocardial injection¹⁴⁸ or miR-19a/19b after intravenous injection⁷⁰ using the former reagent, or miR-19a/19b⁷⁰ and miR302b/c⁶⁶ upon systemic injection using the latter preparation.

These lipoplexes, however, have usually large particle size (often > 1 µm) and, in the case of cationic lipids, the positive charge results in rapid plasma clearance, toxicity, and inflammation once administered systemically.^{176–178} To overcome these issues, significant progress was permitted by the use of ionizable cationic lipids, such as DODAP, which are positively charged at low pH and are thus efficient in encapsulating negatively charged nucleic acids, however, become neutral when the pH is raised to physiological values. These properties, coupled with the possibility of loading the lipid–nucleic acid complexes into small (<100 nm diameter) particles, permits the generation of neutral lipid nanoparticles

(LNPs) that are devoid of significant toxicity.¹⁷⁵ The first RNA interference therapeutics approved in USA and Europe in 2018 was patisiran, an siRNA LNP targeting transthyretin in the liver for the therapy of transthyretin-induced amyloidosis, a rapidly progressive, and often fatal disease.¹⁷⁹ At least other eight LNP formulations of siRNAs and miRNAs are currently undergoing clinical experimentation for the treatment of a number of other disorders. In the cardiovascular field, one of these is inclisiran, which includes an siRNA against PCSK9 for gene therapy of primary hyperlipidaemias.¹⁸⁰

LNPs can further be modified to improve their biodistribution and are amenable to inclusion of targeting molecules, such as cell-specific peptides and scFv antibodies, to specifically address these molecules to desired body districts.¹⁸¹ The identification of specific molecular targets in the infarcted myocardium would permit specific accumulation of LNPs carrying one of the pro-regenerative miRNAs to the heart after their systemic administration. In this respect, it is worth noting that, while the endothelial barrier poses a limit to the passive diffusion of 100-nm-sized particles such as LNPs, regions with inflammation show highly increased vascular permeability, allowing penetration of small particles into the tissue. This is not dissimilar from tumour tissues, in which the preferential accumulation of LNPs was termed the 'enhanced penetration and retention' effect.¹⁸²

As an alternative to lipids, natural, or synthetic polymers can also be used for the delivery of small nucleic acid therapeutics.¹⁸³ Among the synthetic polymers, poly(ethylenimine) is a cationic polymer that is very efficient in complexing to negatively charged DNA. Being the resulting polyplexes still positively charged, it is efficiently internalized by the cells and exits readily from the endosomal compartment. Poly(ethylenimine) has been used to deliver different miRNAs upon local or systemic administration.^{184,185} Unfortunately, however, it is rather cytotoxic for clinical development. Another synthetic polymer that can be used for miRNA and anti-miR delivery is poly(lactic-co-glycolic acid).^{186–188} In this case, the large size of the delivery particle can be suitable for gene transfer into the vasculature. For example, the intracoronary administration of an antagonistic miR-92 encapsulated in poly(lactic-co-glycolic acid) microspheres of >9 µm diameter was shown to stimulate angiogenesis and improve cardiac function after MI in pigs.¹⁸⁹

Small RNAs also complex readily to dendrimers, highly branched cationic polymers that can mediate their intracellular uptake and release from endocytosis vesicles. PAMAM dendrimers were successful in delivering an RNA-triple-helix structure comprising two miRNAs and an antagonistic miR to breast cancer cells in mice.¹⁹⁰ However, again due to their cationic nature, a major limitation of these molecules remains their toxicity *in vivo*.

Additional molecules that are considered for small nucleic acid delivery are the natural cationic polymer chitosan, which is composed of glucosamine and N-acetylglucosamine and appears attractive in light of its biocompatibility and biodegradability,^{191,192} or a few inorganic systems, such as gold, magnetic nanoparticles, carbon nanotubes, and silica- and calcium-based nanoparticles (reviewed in Ref.¹⁹³). These systems, however, have lower cargo capability, are less efficient compared to lipoplexes or LNPs and, most relevant, they lack sufficient pharmacological experimentation and extensive proof of efficacy in animals.

Finally, exciting tools for miRNA delivery are represented by biological carriers. A clinical experimentation for pleural mesothelioma is currently based on bacterial minicells having 400 nm diameter filled with miR-16 mimic and targeted to EGFR using a specific antibody.^{194,195} Another interesting possibility is to take advantage of exosomes. These are 30–100 nm extracellular vesicles released by the cells and involved in

cell-to-cell communication through the transfer of bioactive material, including nucleic acids.¹⁹⁶ Initial evidence already indicates that selected miRNAs can be preferentially loaded into exosomes¹⁹⁷ and that these vesicles can be targeted to specific molecules through the inclusion of specific ligands within the exosome membrane.¹⁹⁸ While this technology appears immature, still, it represents an interesting avenue for further development.

9. Conclusion

The possibility of regenerating the heart through the stimulation of endogenous CM proliferation using small nucleic acid drugs was unthinkable until 10 years ago, however, it appears at reach now. Rigorous assessment of the safety, improvement in delivery methods, precise understanding of the mechanisms of action are, however, required before setting these RNA-based therapeutics as an innovative, reliable tools to promote cardiac regeneration in patients.

At least two challenges lie immediately ahead for future studies, both of which prompt extensive experimentation in large animals as opposed to small rodents. First, the rodent heart is markedly smaller than the human heart. Past experience in various gene therapy applications has taught us that gene transfer efficiency is usually inversely related to host size, because of limited tissue diffusion of the gene delivery vector and larger volumes of the treated tissues (discussed in Ref.¹⁹⁹). For cardiac regeneration specifically, the number of cells that need to be produced is in the order of 1 billion in large animals, while of few hundred thousand in the mouse. Despite the very many studies that have addressed the issue of CM proliferation in small animals, to the best of our knowledge only three studies have so far attempted cardiac regeneration through the stimulation of CM proliferation in large animals to date (Figure 2).

Second, it also remains unclear whether we should aim at achieving true CM duplication, including cell division and formation of new daughter cells, or, alternatively, whether the stimulation of multinucleation would be sufficient to increase cardiac mass and function. In swine, for example, CM multinucleation is very frequent, leading to large number of CMs with up to eight or more nuclei.²⁰⁰ Multinucleation is a much easier endpoint compared to cell division for a therapy aimed at generating new cardiac mass, especially because CM duplication requires disassembly of the cardiac sarcomere, which can be achieved during development and in young cells,²⁰¹ however, might be much more difficult in old, already hypertrophic cells. Our own recent experience in pigs indeed showed that the remarkable cardiac repair we observed upon miR-199a overexpression was paralleled by nuclear mitosis and nuclear duplication, while actual cell division was less prominent.⁷⁴ Thus, the stimulation of CM multinucleation might be an equally effective manner of increasing cardiac mass and thus sustaining cardiac function.

In conclusion and despite these unknowns, the perspective of achieving cardiac repair through the stimulation of endogenous CM proliferation by small nucleic acids remains notable. This is especially in light of the enormous burden of cardiovascular disease and the possibility, for these gene therapy medicines, to be formulated off-the-shelf to virtually fit every patient.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Authors' contributions

H.A., L.B., I.S., and M.G. conceived the article and wrote the text.

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References

- Roth GA, Johnson C, Abajobir A, Abd-Allah F, Abera SF, Abyu G, Ahmed M, Aksut B, Alam T, Alam K, Alla F, Alvis-Guzman N, Amrock S, Ansari H, Arnlov J, Asayesh H, Atey TM, Avila-Burgos L, Awasthi A, Banerjee A, Barac A, Barnighausen T, Barregard L, Bedi N, Belay Ketema E, Bennett D, Berhe G, Bhutta Z, Bitew S, Carapetis J, Carrero JJ, Malta DC, Castaneda-Orjuela CA, Castillo-Rivas J, Catala-Lopez F, Choi JY, Christensen H, Cirillo M, Cooper L Jr, Criqui M, Cundiff D, Damasceno A, Dandona L, Dandona R, Davletov K, Dharmaratne S, Dorairaj P, Dube M, Ehrenkrantz R, El Sayed Zaki M, Faraon EJA, Esteghamati A, Farid T, Farvid M, Feigin V, Ding EL, Fowkes G, Gebrehiwot T, Gillum R, Gold A, Gona P, Gupta R, Habtewold TD, Hafezi-Nejad N, Hailu T, Hailu GB, Hankey G, Hassen HY, Abate KH, Havmoeller R, Hay SI, Horino M, Hotez PJ, Jacobsen K, James S, Javanbakht M, Jeemon P, John D, Jonas J, Kalkonde Y, Karimkhani C, Kasaeian A, Khader Y, Khan A, Khang YH, Khara S, Khoja AT, Khubchandani J, Kim D, Kolte D, Kosen S, Krohn KJ, Kumar GA, Kwan GF, Lal DK, Larsson A, Linn S, Lopez A, Lotufo PA, El Razek HMA, Malekzadeh R, Mazidi M, Meier T, Meles KG, Mensah G, Meretoja A, Mezgebe H, Miller T, Mirzakhimov E, Mohammed S, Moran AE, Musa KI, Narula J, Neal B, Ngalesoni F, Nguyen G, Obermeyer CM, Owolabi M, Patton G, Pedro J, Qato D, Qorbani M, Rahimi K, Rai RK, Rawaf S, Ribeiro A, Safiri S, Salomon JA, Santos I, Santric Milicevic M, Sartorius B, Schutte A, Sepanlou S, Shaikh MA, Shin MJ, Shishehbor M, Shore H, Silva DAS, Sobngwi E, Stranges S, Swaminathan S, Tabares-Seisdedos R, Tadele Atnafu N, Tesfay F, Thakur JS, Thrift A, Topor-Madry R, Truelsen T, Tyrovolas S, Ukwaja KN, Uthman O, Vasankari T, Vlassov V, Vollset SE, Wakayo T, Watkins D, Weintraub R, Werdecker A, Westerman R, Wuyisong CS, Wolfe C, Workicho A, Xu G, Yano Y, Yip P, Yonemoto N, Younis M, Yu C, Vos T, Naghavi M, Murray C. Global, regional, and national burden of cardiovascular diseases for 10 causes, 1990 to 2015. *J Am Coll Cardiol* 2017;**70**:1–25.
- Murry CE, Reinecke H, Pabon LM. Regeneration gaps: observations on stem cells and cardiac repair. *J Am Coll Cardiol* 2006;**47**:1777–1785.
- Whelan RS, Kaplinsky V, Kitsis RN. Cell death in the pathogenesis of heart disease: mechanisms and significance. *Annu Rev Physiol* 2010;**72**:19–44.
- Olivetti G, Giordano G, Corradi D, Melissari M, Lagrasta C, Gambert SR, Anversa P. Gender differences and aging: effects on the human heart. *J Am Coll Cardiol* 1995;**26**:1068–1079.
- Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabé-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisén J. Evidence for cardiomyocyte renewal in humans. *Science* 2009;**324**:98–102.
- Soonpaa MH, Field LJ. Assessment of cardiomyocyte DNA synthesis in normal and injured adult mouse hearts. *Am J Physiol* 1997;**272**:H220–H226.
- Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M, Wu TD, Guerin-Kern JL, Lechene CP, Lee RT. Mammalian heart renewal by pre-existing cardiomyocytes. *Nature* 2013;**493**:433–436.
- Sedmera D, Reckova M, DeAlmeida A, Coppen SR, Kubalak SW, Gourdie RG, Thompson RP. Spatiotemporal pattern of commitment to slowed proliferation in the embryonic mouse heart indicates progressive differentiation of the cardiac conduction system. *Anat Rec* 2003;**274A**:773–777.
- Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, Sadek HA. Transient regenerative potential of the neonatal mouse heart. *Science* 2011;**331**:1078–1080.
- Ye L, D'Agostino G, Loo SJ, Wang CX, Su LP, Tan SH, Tee GZ, Pua CJ, Pena EM, Cheng RB, Chen WC, Abdurrahim D, Lalic J, Tan RS, Lee TH, Zhang J, Cook SA. Early regenerative capacity in the porcine heart. *Circulation* 2018;**138**:2798–2808.
- Haubner BJ, Schneider J, Schweigmann U, Schuetz T, Dichtl W, Velik-Salchner C, Stein JJ, Penninger JM. Functional recovery of a human neonatal heart after severe myocardial infarction. *Circ Res* 2016;**118**:216–221.
- Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. *Science* 2002;**298**:2188–2190.
- Oberpriller JO, Oberpriller JC. Response of the adult newt ventricle to injury. *J Exp Zool* 1974;**187**:249–253.
- Kikuchi K, Holdway JE, Werdich AA, Anderson RM, Fang Y, Egnaczyk GF, Evans T, Macrae CA, Stainier DY, Poss KD. Primary contribution to zebrafish heart regeneration by GATA4(+) cardiomyocytes. *Nature* 2010;**464**:601–605.
- Jopling C, Sleep E, Raya M, Marti M, Raya A, Izpisua Belmonte JC. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* 2010;**464**:606–609.
- Puente BN, Kimura W, Muralidhar SA, Moon J, Amatruda JF, Phelps KL, Grinsfelder D, Rothermel BA, Chen R, Garcia JA, Santos CX, Thet S, Mori E, Kinter MT, Rindler PM, Zacchigna S, Mukherjee S, Chen DJ, Mahmoud AI, Giacca M, Rabinovitch PS, Aroumougame A, Shah AM, Szewda LI, Sadek HA. The oxygen-rich postnatal environment induces cardiomyocyte cell-cycle arrest through DNA damage response. *Cell* 2014;**157**:565–579.
- Canseco DC, Kimura W, Garg S, Mukherjee S, Bhattacharya S, Abdisalaam S, Das S, Asaithamby A, Mammen PP, Sadek HA. Human ventricular unloading induces cardiomyocyte proliferation. *J Am Coll Cardiol* 2015;**65**:892–900.
- Lopaschuk GD, Jaswal JS. Energy metabolic phenotype of the cardiomyocyte during development, differentiation, and postnatal maturation. *J Cardiovasc Pharmacol* 2010;**56**:130–140.
- Zacchigna S, Martinelli V, Moimas S, Colliva A, Anzini M, Nordio A, Costa A, Rehman M, Vodret S, Pierro C, Colussi G, Zentilin L, Gutierrez MI, Dirx E, Long C, Sinagra G, Klatzmann D, Giacca M. Paracrine effect of regulatory T cells promotes cardiomyocyte proliferation during pregnancy and after myocardial infarction. *Nat Commun* 2018;**9**:2432.
- Hirose K, Payumo AY, Cutie S, Hoang A, Zhang H, Guyot R, Lunn D, Bigley RB, Yu H, Wang J, Smith M, Gillett E, Muroy SE, Schmid T, Wilson E, Field KA, Reeder DM, Maden M, Yartsev MM, Wolfgang MJ, Grutzner F, Scanlan TS, Szewda LI, Buffenstein R, Hu G, Flamant F, Olgin JE, Huang GN. Evidence for hormonal control of heart regenerative capacity during endothermy acquisition. *Science* 2019;**364**:184–188.
- Hashimoto H, Olson EN, Bassel-Duby R. Therapeutic approaches for cardiac regeneration and repair. *Nat Rev Cardiol* 2018;**15**:585–600.
- Tzahor E, Poss KD. Cardiac regeneration strategies: staying young at heart. *Science* 2017;**356**:1035–1039.
- Uygun A, Lee RT. Mechanisms of cardiac regeneration. *Dev Cell* 2016;**36**:362–374.
- Engel FB, Hsieh PC, Lee RT, Keating MT. FGF1/p38 MAP kinase inhibitor therapy induces cardiomyocyte mitosis, reduces scarring, and rescues function after myocardial infarction. *Proc Natl Acad Sci U S A* 2006;**103**:15546–15551.
- Przybyl E, Krenning G, Brinker MG, Harmsen MC. Adipose stromal cells primed with hypoxia and inflammation enhance cardiomyocyte proliferation rate in vitro through STAT3 and ERK1/2. *J Struct Med* 2013;**11**:39.
- Hinrichsen R, Haunsø S, Hinrichsen R, Haunsø S, Busk PK, Hinrichsen R, Haunsø S, Busk PK. Different regulation of p27 and Akt during cardiomyocyte proliferation and hypertrophy. *Growth Factors* 2007;**25**:132–140.
- Wei K, Serpooshan V, Hurtado C, Diez-Cunado M, Zhao M, Maruyama S, Zhu W, Fajardo G, Nosedá M, Nakamura K, Tian X, Liu Q, Wang A, Matsuura Y, Bushway P, Cai W, Savchenko A, Mahmoudi M, Schneider MD, van den Hoff MJ, Butte MJ, Yang PC, Walsh K, Zhou B, Bernstein D, Mercola M, Ruiz-Lozano P. Epicardial FSTL1 reconstitution regenerates the adult mammalian heart. *Nature* 2015;**525**:479–485.
- Zhao YY, Sawyer DR, Baliga RR, Opel DJ, Han X, Marchionni MA, Kelly RA. Neuregulins promote survival and growth of cardiac myocytes. Persistence of ErbB2 and ErbB4 expression in neonatal and adult ventricular myocytes. *J Biol Chem* 1998;**273**:10261–10269.
- Bersell K, Arab S, Haring B, Kuhn B. Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell* 2009;**138**:257–270.
- Bassat E, Mutlak YE, Genzelinakh A, Shadrin IY, Baruch Umansky K, Yifa O, Kain D, Rajchman D, Leach J, Riabov Bassat D, Udi Y, Sarig R, Sagi I, Martin JF, Bursac N, Cohen S, Tzahor E. The extracellular matrix protein agrin promotes heart regeneration in mice. *Nature* 2017;**547**:179–184.
- Lavine KJ, Epelman S, Uchida K, Weber KJ, Nichols CG, Schilling JD, Ornitz DM, Randolph GJ, Mann DL. Distinct macrophage lineages contribute to disparate patterns of cardiac recovery and remodeling in the neonatal and adult heart. *Proc Natl Acad Sci U S A* 2014;**111**:16029–16034.
- D'Uva G, Aharonov A, Lauriola M, Kain D, Yahalom-Ronen Y, Carvalho S, Weisinger K, Bassat E, Rajchman D, Yifa O, Lysenko M, Konfino T, Hegesh J, Brenner O, Neeman M, Yarden Y, Leor J, Sarig R, Harvey RP, Tzahor E. ERBB2 triggers mammalian heart regeneration by promoting cardiomyocyte dedifferentiation and proliferation. *Nat Cell Biol* 2015;**17**:627–638.
- Magadam A, Ding Y, He L, Kim T, Vasudevarao MD, Long Q, Yang K, Wickramasinghe N, Renikunta HV, Dubois N, Weidinger G, Yang Q, Engel FB. Live cell screening platform identifies PPARdelta as a regulator of cardiomyocyte proliferation and cardiac repair. *Cell Res* 2017;**27**:1002–1019.
- Campa VM, Gutiérrez-Lanza R, Cerignoli F, Díaz-Trelles R, Nelson B, Tsujii T, Barcova M, Jiang W, Mercola M. Notch activates cell cycle reentry and progression in quiescent cardiomyocytes. *J Cell Biol* 2008;**183**:129–141.
- Collesi C, Zentilin L, Sinagra G, Giacca M. Notch1 signaling stimulates proliferation of immature cardiomyocytes. *J Cell Biol* 2008;**183**:117–128.

36. Croquelois A, Domenighetti AA, Nemir M, Lepore M, Rosenblatt-Velin N, Radtke F, Pedrazzini T. Control of the adaptive response of the heart to stress via the Notch1 receptor pathway. *J Exp Med* 2008;**205**:3173–3185.
37. Collesi C, Felician G, Secco I, Gutierrez MI, Martelletti E, Ali H, Zentilin L, Myers MP, Giacca M. Reversible Notch1 acetylation tunes proliferative signalling in cardiomyocytes. *Cardiovasc Res* 2018;**114**:103–122.
38. Wang S, Ye L, Li M, Liu J, Jiang C, Hong H, Zhu H, Sun Y. GSK-3beta inhibitor CHIR-99021 promotes proliferation through upregulating beta-catenin in neonatal atrial human cardiomyocytes. *J Cardiovasc Pharmacol* 2016;**68**:425–432.
39. Tseng AS, Engel FB, Keating MT. The GSK-3 inhibitor BIO promotes proliferation in mammalian cardiomyocytes. *Chem Biol* 2006;**13**:957–963.
40. Saucedo LJ, Edgar BA. Filling out the Hippo pathway. *Nat Rev Mol Cell Biol* 2007;**8**:613–621.
41. Pan D. The hippo signaling pathway in development and cancer. *Dev Cell* 2010;**19**:491–505.
42. Panciera T, Azzolin L, Cordenonsi M, Piccolo S. Mechanobiology of YAP and TAZ in physiology and disease. *Nat Rev Mol Cell Biol* 2017;**18**:758–770.
43. Xin M, Kim Y, Sutherland LB, Qi X, McAnally J, Schwartz RJ, Richardson JA, Bassel-Duby R, Olson EN. Regulation of insulin-like growth factor signaling by Yap governs cardiomyocyte proliferation and embryonic heart size. *Sci Signal* 2011;**4**:ra70.
44. von Gise A, Lin Z, Schlegelmilch K, Honor LB, Pan GM, Buck JN, Ma Q, Ishiwata T, Zhou B, Camargo FD, Pu WT. YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. *Proc Natl Acad Sci U S A* 2012;**109**:2394–2399.
45. Xin M, Kim Y, Sutherland LB, Murakami M, Qi X, McAnally J, Porrello ER, Mahmoud AI, Tan W, Shelton JM, Richardson JA, Sadek HA, Bassel-Duby R, Olson EN. Hippo pathway effector Yap promotes cardiac regeneration. *Proc Natl Acad Sci U S A* 2013;**110**:13839–13844.
46. Kirshenbaum LA, Abdellatif M, Chakraborty S, Schneider MD. Human E2F-1 reactivates cell cycle progression in ventricular myocytes and represses cardiac gene transcription. *Dev Biol* 1996;**179**:402–411.
47. Agah R, Kirshenbaum LA, Abdellatif M, Truong LD, Chakraborty S, Michael LH, Schneider MD. Adenoviral delivery of E2F-1 directs cell cycle reentry and p53-independent apoptosis in postmitotic adult myocardium in vivo. *J Clin Invest* 1997;**100**:2722–2728.
48. van Amerongen MJ, Diehl F, Novoyatleva T, Patra C, Engel FB. E2F4 is required for cardiomyocyte proliferation. *Cardiovasc Res* 2010;**86**:92–102.
49. Soonpaa MH, Koh GY, Pajak L, Jing S, Wang H, Franklin MT, Kim KK, Field LJ. Cyclin D1 overexpression promotes cardiomyocyte DNA synthesis and multinucleation in transgenic mice. *J Clin Invest* 1997;**99**:2644–2654.
50. Tamamori-Adachi M, Ito H, Sumrejkanchanakij P, Adachi S, Hiroe M, Shimizu M, Kawauchi J, Sunamori M, Marumo F, Kitajima S, Ikeda MA. Critical role of cyclin D1 nuclear import in cardiomyocyte proliferation. *Circ Res* 2003;**92**:e12–e19.
51. Busk PK, Hinrichsen R, Bartkova J, Hansen AH, Christoffersen TE, Bartek J, Haunso S. Cyclin D2 induces proliferation of cardiac myocytes and represses hypertrophy. *Exp Cell Res* 2005;**304**:149–161.
52. Di Stefano V, Giacca M, Capogrossi MC, Crescenzi M, Martelli F. Knockdown of cyclin-dependent kinase inhibitors induces cardiomyocyte re-entry in the cell cycle. *J Biol Chem* 2011;**286**:8644–8654.
53. Chaudhry HW, Dashoush NH, Tang H, Zhang L, Wang X, Wu EX, Wolgemuth DJ. Cyclin A2 mediates cardiomyocyte mitosis in the postmitotic myocardium. *J Biol Chem* 2004;**279**:35858–35866.
54. Woo YJ, Panlilio CM, Cheng RK, Liao GP, Atluri P, Hsu VM, Cohen JE, Chaudhry HW. Therapeutic delivery of cyclin A2 induces myocardial regeneration and enhances cardiac function in ischemic heart failure. *Circulation* 2006;**114**:1206–1213.
55. Liao HS, Kang PM, Nagashima H, Yamasaki N, Usheva A, Ding B, Lorell BH, Izumo S. Cardiac-specific overexpression of cyclin-dependent kinase 2 increases smaller mononuclear cardiomyocytes. *Circ Res* 2001;**88**:443–450.
56. Pasumarthi KB, Nakajima H, Nakajima HO, Soonpaa MH, Field LJ. Targeted expression of cyclin D2 results in cardiomyocyte DNA synthesis and infarct regression in transgenic mice. *Circ Res* 2005;**96**:110–118.
57. Hassink RJ, Pasumarthi KB, Nakajima H, Rubart M, Soonpaa MH, de la Riviere AB, Doevendans PA, Field LJ. Cardiomyocyte cell cycle activation improves cardiac function after myocardial infarction. *Cardiovasc Res* 2008;**78**:18–25.
58. Mahmoud AI, Kocabas F, Muralidhar SA, Kimura W, Koura AS, Thet S, Porrello ER, Sadek HA. Meis1 regulates postnatal cardiomyocyte cell cycle arrest. *Nature* 2013;**497**:249–253.
59. Toyoda M, Shirato H, Nakajima K, Kojima M, Takahashi M, Kubota M, Suzuki-Migishima R, Motegi Y, Yokoyama M, Takeuchi T. Jumoni downregulates cardiac cell proliferation by repressing cyclin D1 expression. *Dev Cell* 2003;**5**:85–97.
60. Jung J, Kim TG, Lyons GE, Kim HR, Lee Y. Jumoni regulates cardiomyocyte proliferation via interaction with retinoblastoma protein. *J Biol Chem* 2005;**280**:30916–30923.
61. Ebelt H, Hufnagel N, Neuhaus P, Neuhaus H, Gajawada P, Simm A, Müller-Werdan U, Werdan K, Braun T. Divergent siblings: E2F2 and E2F4 but not E2F1 and E2F3 induce DNA synthesis in cardiomyocytes without activation of apoptosis. *Circ Res* 2005;**96**:509–517.
62. Mohamed TMA, Ang YS, Radzinsky E, Zhou P, Huang Y, Effenbein A, Foley A, Magnitsky S, Srivastava D. Regulation of cell cycle to stimulate adult cardiomyocyte proliferation and cardiac regeneration. *Cell* 2018;**173**:104–116.e112.
63. Baehr A, Baruch Umansky K, Bassat E, Klett K, Jurisch V, Bozdoglu T, Hornaschewitz N, Solyanik O, Kain D, Ferrero B, Cohen-Rabi R, Krane M, Cyran C, Soehnlein O, Laugwitz KL, Kupatt C, Tzahor E. Agrin promotes coordinated therapeutic processes leading to improved cardiac repair in pigs. *BioRxiv* 2020:https://doi.org/10.1101/854372.
64. Eulalio A, Mano M, Dal Ferro M, Zentilin L, Sinagra G, Zacchigna S, Giacca M. Functional screening identifies miRNAs inducing cardiac regeneration. *Nature* 2012;**492**:376–381.
65. Diez-Cunado M, Wei K, Bushway PJ, Maurya MR, Perera R, Subramaniam S, Ruiz-Lozano P, Mercola M. miRNAs that induce human cardiomyocyte proliferation converge on the Hippo pathway. *Cell Rep* 2018;**23**:2168–2174.
66. Tian Y, Liu Y, Wang T, Zhou N, Kong J, Chen L, Stitow M, Morley M, Li D, Petrenko N, Zhou S, Lu M, Gao E, Koch WJ, Stewart KM, Morrissey EE. A microRNA-Hippo pathway that promotes cardiomyocyte proliferation and cardiac regeneration in mice. *Sci Transl Med* 2015;**7**:279–238.
67. Borden A, Kurian J, Nickoloff E, Yang Y, Troupes CD, Ibeti J, Lucchese AM, Gao E, Mohsin S, Koch WJ, Houser SR, Kishore R, Khan M. Transient introduction of miR-294 in the heart promotes cardiomyocyte cell cycle reentry after injury. *Circ Res* 2019;**125**:14–25.
68. Wang LL, Liu Y, Chung JJ, Wang T, Gaffey AC, Lu M, Cavanaugh CA, Zhou S, Kanade R, Atluri P, Morrissey EE, Burdick JA. Local and sustained miRNA delivery from an injectable hydrogel promotes cardiomyocyte proliferation and functional regeneration after ischemic injury. *Nat Biomed Eng* 2017;**1**:983–992.
69. Chen J, Huang ZP, Seok HY, Ding J, Kataoka M, Zhang Z, Hu X, Wang G, Lin Z, Wang S, Pu WT, Liao R, Wang DZ. miR-17-92 cluster is required for and sufficient to induce cardiomyocyte proliferation in postnatal and adult hearts. *Circ Res* 2013;**112**:1557–1566.
70. Gao F, Kataoka M, Liu N, Liang T, Huang ZP, Gu F, Ding J, Liu J, Zhang F, Ma Q, Wang Y, Zhang M, Hu X, Kyselovic J, Hu X, Pu WT, Wang J, Chen J, Wang DZ. Therapeutic role of miR-19a/19b in cardiac regeneration and protection from myocardial infarction. *Nat Commun* 2019;**10**:1802.
71. Qin X, Gao S, Yang Y, Wu L, Wang L. microRNA-25 promotes cardiomyocytes proliferation and migration via targeting Bim. *J Cell Physiol* 2019;**234**:22103–22115.
72. Torrini C, Cubero RJ, Dirx E, Braga L, Ali H, Prosdocimo G, Gutierrez MI, Collesi C, Licastro D, Zentilin L, Mano M, Zacchigna S, Vendruscolo M, Marsili M, Samal A, Giacca M. Common regulatory pathways mediate activity of microRNAs inducing cardiomyocyte proliferation. *Cell Reports* 2019;**27**:2759–2771.e2755.
73. Pandey R, Ahmed RP. MicroRNAs inducing proliferation of quiescent adult cardiomyocytes. *Cardiovasc Regen Med* 2015;**2**: pii: e519.
74. Gabisonia G, Prosdocimo G, Aquaro GD, Carlucci L, Zentilin L, Secco I, Ali H, Braga L, Gorgodez N, Bernini F, Burchielli S, Collesi C, Zandona L, Sinagra G, Piacenti M, Zacchigna S, Bussani R, Recchia FA, Giacca M. MicroRNA therapy stimulates uncontrolled cardiac repair after myocardial infarction in pigs. *Nature* 2019;**569**:418–422.
75. Wang Y, Ding N, Guan G, Liu G, Huo D, Li Y, Wei K, Yang J, Cheng P, Zhu C. Rapid delivery of Hsa-miR-590-3p using targeted exosomes to treat acute myocardial infarction through regulation of the cell cycle. *J Biomed Nanotechnol* 2018;**14**:968–977.
76. Pandey R, Velasquez S, Durrani S, Jiang M, Neiman M, Crocker JS, Benoit JB, Rubinstein J, Paul A, Ahmed RP. MicroRNA-1825 induces proliferation of adult cardiomyocytes and promotes cardiac regeneration post ischemic injury. *Am J Transl Res* 2017;**9**:3120–3137.
77. Qin L, Yang W, Wang YX, Wang ZJ, Li CC, Li M, Liu JY. MicroRNA-497 promotes proliferation and inhibits apoptosis of cardiomyocytes through the downregulation of MFN2 in a mouse model of myocardial ischemia-reperfusion injury. *Biomed Pharmacother* 2018;**105**:103–114.
78. Xiao J, Liu H, Cretoiu D, Toader DO, Suci N, Shi J, Shen S, Bei Y, Sluijter JP, Das S, Kong X, Li X. miR-31a-5p promotes postnatal cardiomyocyte proliferation by targeting RhoBTB1. *Exp Mol Med* 2017;**49**:e386–e386.
79. Wang X, Ha T, Liu L, Hu Y, Kao R, Kalbfleisch J, Williams D, Li C. TLR3 mediates repair and regeneration of damaged neonatal heart through glycolysis dependent YAP1 regulated miR-152 EXPRESSION. *Cell Death Differ* 2018;**25**:966–982.
80. Liang D, Li J, Wu Y, Zhen L, Li C, Qi M, Wang L, Deng F, Huang J, Lv F, Liu Y, Ma X, Yu Z, Zhang Y, Chen YH. miRNA-204 drives cardiomyocyte proliferation via targeting Jarid2. *Int J Cardiol* 2015;**201**:38–48.
81. Arif M, Pandey R, Alam P, Jiang S, Sadayappan S, Paul A, Ahmed R. MicroRNA-210-mediated proliferation, survival, and angiogenesis promote cardiac repair post myocardial infarction in rodents. *J Mol Med* 2017;**95**:1369–1385.
82. Liu X, Xiao J, Zhu H, Wei X, Platt C, Damilano F, Xiao C, Bezzerides V, Boström P, Che L, Zhang C, Spiegelman BM, Rosenzweig A. miR-222 is necessary for exercise-induced cardiac growth and protects against pathological cardiac remodeling. *Cell Metab* 2015;**21**:584–595.
83. Vujic A, Lerchenmuller C, Wu TD, Guillemier C, Rabolli CP, Gonzalez E, Senyo SE, Liu X, Guerin-Kern JL, Steinhilber ML, Lee RT, Rosenzweig A. Exercise induces new cardiomyocyte generation in the adult mammalian heart. *Nat Commun* 2018;**9**:1659.

84. Clark AL, Naya FJ. MicroRNAs in the myocyte enhancer factor 2 (MEF2)-regulated Gtl2-Dio3 noncoding RNA locus promote cardiomyocyte proliferation by targeting the transcriptional coactivator cited2. *J Biol Chem* 2015;**290**:23162–23172.
85. Lange S, Banerjee I, Carrion K, Serrano R, Habich L, Kameny R, Lengsfelder L, Dalton N, Meili R, Borgeson E, Peterson K, Ricci M, Lincoln J, Ghassemian M, Fineman J, Del Alamo JC, Nigam V. miR-486 is modulated by stretch and increases ventricular growth. *JCI Insight* 2019;**4**:e125507.
86. Deng S, Zhao Q, Zhen L, Zhang C, Liu C, Wang G, Zhang L, Bao L, Lu Y, Meng L, Lu J, Yu P, Lin X, Zhang Y, Chen YH, Fan H, Cho WC, Liu Z, Yu Z. Neonatal heart-enriched miR-708 promotes proliferation and stress resistance of cardiomyocytes in rodents. *Theranostics* 2017;**7**:1953–1965.
87. Cai B, Ma W, Ding F, Zhang L, Huang Q, Wang X, Hua B, Xu J, Li J, Bi C, Guo S, Yang F, Han Z, Li Y, Yan G, Yu Y, Bao Z, Yu M, Li F, Tian Y, Pan Z, Yang B. The long noncoding RNA CAREL controls cardiac regeneration. *J Am Coll Cardiol* 2018;**72**:534–550.
88. Li X, He X, Wang H, Li M, Huang S, Chen G, Jing Y, Wang S, Chen Y, Liao W, Liao Y, Bin J. Loss of AZIN2 splice variant facilitates endogenous cardiac regeneration. *Cardiovasc Res* 2018;**114**:1642–1655.
89. Barroso-del Jesus A, Lucena-Aguilar G, Menendez P. The miR-302-367 cluster as a potential stemness regulator in ESCs. *Cell Cycle* 2009;**8**:394–398.
90. Marson A, Levine SS, Cole MF, Frampton GM, Brambrink T, Johnstone S, Guenther MG, Johnston WK, Wernig M, Newman J, Calabrese JM, Dennis LM, Volkert TL, Gupta S, Love J, Hannett N, Sharp PA, Bartel DP, Jaenisch R, Young RA. Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell* 2008;**134**:521–533.
91. Melton C, Judson RL, Belloch R. Opposing microRNA families regulate self-renewal in mouse embryonic stem cells. *Nature* 2010;**463**:621–626.
92. Wang Y, Baskerville S, Shenoy A, Babiarz JE, Baehner L, Belloch R. Embryonic stem cell-specific microRNAs regulate the G1-S transition and promote rapid proliferation. *Nat Genet* 2008;**40**:1478–1483.
93. Judson RL, Babiarz JE, Venere M, Belloch R. Embryonic stem cell-specific microRNAs promote induced pluripotency. *Nat Biotechnol* 2009;**27**:459–461.
94. Zhang Z, Zhuang L, Lin CP. Roles of microRNAs in establishing and modulating stem cell potential. *Int J Mol Sci* 2019;**20**:2643.
95. Mogilyansky E, Rigoutsos I. The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. *Cell Death Differ* 2013;**20**:1603–1614.
96. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A* 2006;**103**:2257–2261.
97. He L, Thomson JM, Hemann MT, Hernandez-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, Hammond SM. A microRNA polycistron as a potential human oncogene. *Nature* 2005;**435**:828–833.
98. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* 2005;**435**:839–843.
99. Gruszka R, Zakrzewska M. The oncogenic relevance of miR-17-92 cluster and its paralogous miR-106b-25 and miR-106a-363 clusters in brain tumors. *Int J Mol Sci* 2018;**19**:879.
100. Mehlich D, Garbicz F, Włodarski PK. The emerging roles of the polycistronic miR-106b approximately 25 cluster in cancer – a comprehensive review. *Biomed Pharmacother* 2018;**107**:1183–1195.
101. Wang J, He Q, Han C, Gu H, Jin L, Li Q, Mei Y, Wu M. p53-facilitated miR-199a-3p regulates somatic cell reprogramming. *Stem Cells* 2012;**30**:1405–1413.
102. Yang R, Xu X, Li H, Chen J, Xiang X, Dong Z, Zhang D. p53 induces miR199a-3p to suppress SOCS7 for STAT3 activation and renal fibrosis in UUO. *Sci Rep* 2017;**7**:43409.
103. Minna E, Romeo P, De Cecco L, Dugo M, Cassinelli G, Pilotti S, Degl'Innocenti D, Lanzi C, Casalini P, Pierotti MA, Greco A, Borrello MG. miR-199a-3p displays tumor suppressor functions in papillary thyroid carcinoma. *Oncotarget* 2014;**5**:2513–2528.
104. Kinose Y, Sawada K, Nakamura K, Sawada I, Toda A, Nakatsuka E, Hashimoto K, Mabuchi S, Takahashi K, Kurachi H, Lengyel E, Kimura T. The hypoxia-related microRNA miR-199a-3p displays tumor suppressor functions in ovarian carcinoma. *Oncotarget* 2015;**6**:11342–11356.
105. Ghosh A, Dasgupta D, Ghosh A, Roychoudhury S, Kumar D, Gorain M, Butti R, Datta S, Agarwal S, Gupta S, Krishna Dhali G, Chowdhury A, Schmittgen TD, Kundu GC, Banerjee S. MiRNA199a-3p suppresses tumor growth, migration, invasion and angiogenesis in hepatocellular carcinoma by targeting VEGFA, VEGFR1, VEGFR2, HGF and MMP2. *Cell Death Dis* 2017;**8**:e2706.
106. Henry JC, Park JK, Jiang J, Kim JH, Nagorney DM, Roberts LR, Banerjee S, Schmittgen TD. miR-199a-3p targets CD44 and reduces proliferation of CD44 positive hepatocellular carcinoma cell lines. *Biochem Biophys Res Commun* 2010;**403**:120–125.
107. Liu R, Liu C, Zhang D, Liu B, Chen X, Rycak J, Jeter C, Calhoun-Davis T, Li Y, Yang T, Wang J, Tang DG. miR-199a-3p targets stemness-related and mitogenic signaling pathways to suppress the expansion and tumorigenic capabilities of prostate cancer stem cells. *Oncotarget* 2016;**7**:56628–56642.
108. Qu F, Zheng J, Gan W, Lian H, He H, Li W, Yuan T, Yang Y, Li X, Ji C, Yan X, Xu L, Guo H. MiR-199a-3p suppresses proliferation and invasion of prostate cancer cells by targeting Smad1. *Oncotarget* 2017;**8**:52465–52473.
109. Kim S, Lee UJ, Kim MN, Lee EJ, Kim JY, Lee MY, Choung S, Kim YJ, Choi YC. MicroRNA miR-199a* regulates the MET proto-oncogene and the downstream extracellular signal-regulated kinase 2 (ERK2). *J Biol Chem* 2008;**283**:18158–18166.
110. Gao Y, Feng Y, Shen JK, Lin M, Choy E, Cote GM, Harmon DC, Mankin HJ, Hornicek FJ, Duan Z. CD44 is a direct target of miR-199a-3p and contributes to aggressive progression in osteosarcoma. *Sci Rep* 2015;**5**:11365.
111. Zhang L, Lyer AK, Yang X, Kobayashi E, Guo Y, Mankin H, Hornicek FJ, Amiji MM, Duan Z. Polymeric nanoparticle-based delivery of microRNA-199a-3p inhibits proliferation and growth of osteosarcoma cells. *Int J Nanomed* 2015;**10**:2913–2924.
112. Migliore C, Petrelli A, Ghiso E, Corso S, Capparuccia L, Eramo A, Comoglio PM, Giordano S. MicroRNAs impair MET-mediated invasive growth. *Cancer Res* 2008;**68**:10128–10136.
113. Callegari E, D'Abundo L, Guerriero P, Simioni C, Elamin BK, Russo M, Cani A, Bassi C, Zagatti B, Giacomelli L, Blandamura S, Moshiri F, Ultimo S, Frassoldati A, Altavilla G, Gramantieri L, Neri LM, Sabbioni S, Negrini M. miR-199a-3p modulates MTOR and PAK4 pathways and inhibits tumor growth in a hepatocellular carcinoma transgenic mouse model. *Mol Ther Nucleic Acids* 2018;**11**:485–493.
114. Johnson CD, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, Ovcharenko D, Wilson M, Wang X, Shelton J, Shingara J, Chin L, Brown D, Slack FJ. The let-7 microRNA represses cell proliferation pathways in human cells. *Cancer Res* 2007;**67**:7713–7722.
115. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvi AE, Horvitz HR, Ruvkun G. The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 2000;**403**:901–906.
116. Roush S, Slack FJ. The let-7 family of microRNAs. *Trends Cell Biol* 2008;**18**:505–516.
117. Coppola A, Romito A, Borel C, Gehrig C, Gagnebin M, Falconnet E, Izzo A, Altucci L, Banfi S, Antonarakis SE, Minchiotti G, Cobellis G. Cardiomyogenesis is controlled by the miR-99a/let-7c cluster and epigenetic modifications. *Stem Cell Research* 2014;**12**:323–337.
118. Aguirre A, Montserrat N, Zacchigna S, Nivet E, Hishida T, Krause MN, Kurian L, Ocampo A, Vazquez-Ferrer E, Rodriguez-Esteban C, Kumar S, Moresco JJ, Yates JR III, Campistol JM, Sancho-Martinez I, Giacca M, Izpisua Belmonte JC. In vivo activation of a conserved microRNA program induces mammalian heart regeneration. *Cell Stem Cell* 2014;**15**:589–604.
119. Hu Y, Jin G, Li B, Chen Y, Zhong L, Chen G, Chen X, Zhong J, Liao W, Liao Y, Wang Y, Bin J. Suppression of miRNA let-7i-5p promotes cardiomyocyte proliferation and repairs heart function post injury by targeting CCND2 and E2F2. *Clin Sci* 2019;**133**:425–441.
120. Porrello ER, Johnson BA, Aurora AB, Simpson E, Nam YJ, Matkovich SJ, Dorn GW II, van Rooij E, Olson EN. MiR-15 family regulates postnatal mitotic arrest of cardiomyocytes. *Circ Res* 2011;**109**:670–679.
121. Cao X, Wang J, Wang Z, Du J, Yuan X, Huang W, Meng J, Gu H, Nie Y, Ji B, Hu S, Zheng Z. MicroRNA profiling during rat ventricular maturation: a role for miR-29a in regulating cardiomyocyte cell cycle re-entry. *FEBS Lett* 2013;**587**:1548–1555.
122. Porrello ER, Mahmoud AI, Simpson E, Johnson BA, Grinsfelder D, Canseco D, Mammen PP, Rothermel BA, Olson EN, Sadek HA. Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family. *Proc Natl Acad Sci U S A* 2013;**110**:187–192.
123. Hullinger TG, Montgomery RL, Seto AG, Dickinson BA, Semus HM, Lynch JM, Dalby CM, Robinson K, Stack C, Latimer PA, Hare JM, Olson EN, van Rooij E. Inhibition of miR-15 protects against cardiac ischemic injury. *Circ Res* 2012;**110**:71–81.
124. Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, Tsuchihashi T, McManus MT, Schwartz RJ, Srivastava D. Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell* 2007;**129**:303–317.
125. Zhao Y, Samal E, Srivastava D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature* 2005;**436**:214–220.
126. Liu N, Bezprozvannaya S, Williams AH, Qi X, Richardson JA, Bassel-Duby R, Olson EN. microRNA-133a regulates cardiomyocyte proliferation and suppresses smooth muscle gene expression in the heart. *Genes Dev* 2008;**22**:3242–3254.
127. Yin VP, Lepilina A, Smith A, Poss KD. Regulation of zebrafish heart regeneration by miR-133. *Dev Biol* 2012;**365**:319–327.
128. Huang W, Feng Y, Liang J, Yu H, Wang C, Wang B, Wang M, Jiang L, Meng W, Cai W, Medvedovic M, Chen J, Paul C, Davidson WS, Sadayappan S, Stambrook PJ, Yu XY, Wang Y. Loss of microRNA-128 promotes cardiomyocyte proliferation and heart regeneration. *Nat Commun* 2018;**9**:700.
129. Yang Y, Cheng HW, Qiu Y, Dupee D, Noonan M, Lin YD, Fisch S, Unno K, Sereti KI, Liao R. MicroRNA-34a plays a key role in cardiac repair and regeneration following myocardial infarction. *Circ Res* 2015;**117**:450–459.
130. Zhang Y, Matsushita N, Eigler T, Marban E. Targeted microRNA interference promotes postnatal cardiac cell cycle re-entry. *J Regen Med* 2013;**2**:2.
131. Wang J, Chen X, Shen D, Ge D, Chen J, Pei J, Li Y, Yue Z, Feng J, Chu M, Nie Y. A long noncoding RNA NR_045363 controls cardiomyocyte proliferation and cardiac repair. *J Mol Cell Cardiol* 2019;**127**:105–114.

132. Boggiano JC, Vanderzalm PJ, Fehon RG. Tao-1 phosphorylates Hippo/MST kinases to regulate the Hippo-Salvador-Warts tumor suppressor pathway. *Dev Cell* 2011; **21**:888–895.
133. Plouffe SW, Meng Z, Lin KC, Lin B, Hong AW, Chun JV, Guan KL. Characterization of Hippo pathway components by gene inactivation. *Mol Cell* 2016; **64**:993–1008.
134. Poon CL, Lin JJ, Zhang X, Harvey KF. The sterile 20-like kinase Tao-1 controls tissue growth by regulating the Salvador-Warts-Hippo pathway. *Dev Cell* 2011; **21**:896–906.
135. Zhao B, Li L, Tumaneng K, Wang CY, Guan KL. A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP). *Genes Dev* 2010; **24**:72–85.
136. Alexander MS, Casar JC, Motohashi N, Vieira NM, Eisenberg I, Marshall JL, Gasperini MJ, Lek A, Myers JA, Estrella EA, Kang PB, Shapiro F, Rahimov F, Kawahara G, Widrick JJ, Kunkel LM. MicroRNA-486-dependent modulation of DOCK3/PTEN/AKT signaling pathways improves muscular dystrophy-associated symptoms. *J Clin Invest* 2014; **124**:2651–2667.
137. Bernstein BW, Bamburg JR. ADF/Cofilin: a functional node in cell biology. *Trends Cell Biol* 2010; **20**:187–195.
138. Bravo-Cordero JJ, Magalhaes MA, Eddy RJ, Hodgson L, Condeelis J. Functions of Cofilin in cell locomotion and invasion. *Nat Rev Mol Cell Biol* 2013; **14**:405–415.
139. Ali H, Braga L, Giacca M. Cardiac regeneration and remodelling of the cardiomyocyte cytoarchitecture. *FEBS J* 2020; **287**:417–438.
140. Ponnusamy M, Liu F, Zhang YH, Li RB, Zhai M, Liu F, Zhou LY, Liu CY, Yan KW, Dong YH, Wang M, Qian LL, Shan C, Xu S, Wang Q, Zhang YH, Li PF, Zhang J, Wang K. Long noncoding RNA CPR (cardiomyocyte proliferation regulator) regulates cardiomyocyte proliferation and cardiac repair. *Circulation* 2019; **139**:2668–2684.
141. Chen G, Li H, Li X, Li B, Zhong L, Huang S, Zheng H, Li M, Jin G, Liao W, Liao Y, Chen Y, Bin J. Loss of long non-coding RNA CRR1 promotes cardiomyocyte regeneration and improves cardiac repair by functioning as a competing endogenous RNA. *J Mol Cell Cardiol* 2018; **122**:152–164.
142. Chen Y, Li X, Li B, Wang H, Li M, Huang S, Sun Y, Chen G, Si X, Huang C, Liao W, Liao Y, Bin J. Long non-coding RNA ECRAR triggers post-natal myocardial regeneration by activating ERK1/2 signaling. *Mol Ther* 2019; **27**:29–45.
143. Li B, Hu Y, Li X, Jin G, Chen X, Chen G, Chen Y, Huang S, Liao W, Liao Y, Teng Z, Bin J. SIRT1 antisense long noncoding RNA promotes cardiomyocyte proliferation by enhancing the stability of SIRT1. *J Am Heart Assoc* 2018; **7**:e009700.
144. Tomasini R, Seux M, Nowak J, Bontemps C, Carrier A, Dagorn JC, Pebusque MJ, Iovanna JL, Dusetti NJ. TP53INP1 is a novel p73 target gene that induces cell cycle arrest and cell death by modulating p73 transcriptional activity. *Oncogene* 2005; **24**:8093–8104.
145. Nguyen TM, Shin IW, Lee TJ, Park J, Kim JH, Park MS, Lee EJ. Loss of ITM2A, a novel tumor suppressor of ovarian cancer through G2/M cell cycle arrest, is a poor prognostic factor of epithelial ovarian cancer. *Gynecol Oncol* 2016; **140**:545–553.
146. Wang GQ, Wang Y, Xiong Y, Chen XC, Ma ML, Cai R, Gao Y, Sun YM, Yang GS, Pang WJ. SIRT1 AS lncRNA interacts with its mRNA to inhibit muscle formation by attenuating function of miR-34a. *Sci Rep* 2016; **6**:21865.
147. Ming GF, Wu K, Hu K, Chen Y, Xiao J. NAMPT regulates senescence, proliferation, and migration of endothelial progenitor cells through the SIRT1 AS lncRNA/miR-22/SIRT1 pathway. *Biochem Biophys Res Commun* 2016; **478**:1382–1388.
148. Lesizza P, Prosdocimo G, Martinelli V, Sinagra G, Zacchigna S, Giacca M. Single-dose intracardiac injection of pro-regenerative microRNAs improves cardiac function after myocardial infarction. *Circ Res* 2017; **120**:1298–1304.
149. Yang H, Qin X, Wang H, Zhao X, Liu Y, Wo HT, Liu C, Nishiga M, Chen H, Ge J, Sayed N, Abilez OJ, Ding D, Heilshorn SC, Li K. An in vivo miRNA delivery system for restoring infarcted myocardium. *ACS Nano* 2019; **13**:9880–9894.
150. van Rooij E, Olson EN. MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles. *Nat Rev Drug Discov* 2012; **11**:860–872.
151. Elmen J, Lindow M, Schutz S, Lawrence M, Petri A, Obad S, Lindholm M, Hedtjarn M, Hansen HF, Berger U, Gullans S, Kearney P, Sarnow P, Straarup EM, Kauppinen S. LNA-mediated microRNA silencing in non-human primates. *Nature* 2008; **452**:896–899.
152. Fluiter K, Mook ORF, Vreijling J, Langkjær N, Højland T, Wengel J, Baas F. Filling the gap in LNA antisense oligo gapmers: the effects of unlocked nucleic acid (UNA) and 4'-C-hydroxymethyl-DNA modifications on RNase H recruitment and efficacy of an LNA gapmer. *Mol Biosyst* 2009; **5**:838–843.
153. Zacchigna S, Zentilin L, Giacca M. Adeno-associated virus vectors as therapeutic and investigational tools in the cardiovascular system. *Circ Res* 2014; **114**:1827–1846.
154. Buchlis G, Podsakoff GM, Radu A, Hawk SM, Flake AV, Mingozzi F, High KA. Factor IX expression in skeletal muscle of a severe hemophilia B patient 10 years after AAV-mediated gene transfer. *Blood* 2012; **119**:3038–3041.
155. Antzelevitch C, Yan GX, Ackerman MJ, Borggrefe M, Corrado D, Guo J, Gussak I, Hasdemir C, Horie M, Huikuri H, Ma C, Morita H, Nam GB, Sacher F, Shimizu W, Viskin S, Wilde AA. J-Wave syndromes expert consensus conference report: emerging concepts and gaps in knowledge. *Heart Rhythm* 2016; **13**:e295–e324.
156. Rabinowitz JE, Rolling F, Li C, Conrath H, Xiao W, Xiao X, Samulski RJ. Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J Virol* 2002; **76**:791–801.
157. Lovric J, Mano M, Zentilin L, Eulalio A, Zacchigna S, Giacca M. Terminal differentiation of cardiac and skeletal myocytes induces permissivity to AAV transduction by relieving inhibition imposed by DNA damage response proteins. *Mol Ther* 2012; **20**:2087–2097.
158. Saraiva J, Nobre RJ, Pereira de Almeida L. Gene therapy for the CNS using AAVs: the impact of systemic delivery by AAV9. *J Control Release* 2016; **241**:94–109.
159. Kozomara A, Birgaoanu M, Griffiths-Jones S. miRBase: from microRNA sequences to function. *Nucleic Acids Res* 2019; **47**:D155–D162.
160. Kopp F, Mendell JT. Functional classification and experimental dissection of long noncoding RNAs. *Cell* 2018; **172**:393–407.
161. Ounzain S, Pedrazzini T. The promise of enhancer-associated long noncoding RNAs in cardiac regeneration. *Trends Cardiovasc Med* 2015; **25**:592–602.
162. Grimm D, Kay MA. Therapeutic short hairpin RNA expression in the liver: viral targets and vectors. *Gene Ther* 2006; **13**:563–575.
163. Ku G, McManus MT. Behind the scenes of a small RNA gene-silencing pathway. *Hum Gene Ther* 2008; **19**:17–26.
164. Giering JC, Grimm D, Storm TA, Kay MA. Expression of shRNA from a tissue-specific pol II promoter is an effective and safe RNAi therapeutic. *Mol Ther* 2008; **16**:1630–1636.
165. van Gestel MA, van Erp S, Sanders LE, Brans MA, Luijckendijk MC, Merksteijn M, Pasterkamp RJ, Adan RA. shRNA-induced saturation of the microRNA pathway in the rat brain. *Gene Ther* 2014; **21**:205–211.
166. Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marioni P, Salazar F, Kay MA. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 2006; **441**:537–541.
167. Martin JN, Wolken N, Brown T, Dauer WT, Ehrlich ME, Gonzalez-Alegre P. Lethal toxicity caused by expression of shRNA in the mouse striatum: implications for therapeutic design. *Gene Ther* 2011; **18**:666–673.
168. Bak RO, Mikkelsen JG. miRNA sponges: soaking up miRNAs for regulation of gene expression. *Wires RNA* 2014; **5**:317–333.
169. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. *Science* 1995; **268**:1766–1769.
170. Rivera VM, Clackson T, Natesan S, Pollock R, Amara JF, Keenan T, Magari SR, Phillips T, Courage NL, Cerasoli F Jr, Holt DA, Gilman M. A humanized system for pharmacologic control of gene expression. *Nat Med* 1996; **2**:1028–1032.
171. Guiner C, Stieger K, Snyder R, Rolling F, Moullet P. Immune responses to gene product of inducible promoters. *Curr Gene Ther* 2007; **7**:334–346.
172. Berens C, Groher F, Suess B. RNA aptamers as genetic control devices: the potential of riboswitches as synthetic elements for regulating gene expression. *Biotechnol J* 2015; **10**:246–257.
173. Hallberg ZF, Su Y, Kitto RZ, Hammond MC. Engineering and in vivo applications of riboswitches. *Annu Rev Biochem* 2017; **86**:515–539.
174. Yokobayashi Y. Aptamer-based and aptzyme-based riboswitches in mammalian cells. *Curr Opin Chem Biol* 2019; **52**:72–78.
175. Kulkarni JA, Cullis PR, van der Meel R. Lipid nanoparticles enabling gene therapies: from concepts to clinical utility. *Nucleic Acid Ther* 2018; **28**:146–157.
176. Filion MC, Phillips NC. Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. *Biochim Biophys Acta* 1997; **1329**:345–356.
177. Ito Y, Kawakami S, Charoensit P, Higuchi Y, Hashida M. Evaluation of proinflammatory cytokine production and liver injury induced by plasmid DNA/cationic liposome complexes with various mixing ratios in mice. *Eur J Pharm Biopharm* 2009; **71**:303–309.
178. Litzinger DC. Limitations of cationic liposomes for antisense oligonucleotide delivery in vivo. *J Liposome Res* 1997; **7**:51–61.
179. Adams D, Gonzalez-Duarte A, O'Riordan WD, Yang CC, Ueda M, Kristen AV, Tourneville I, Schmidt HH, Coelho T, Berk JL, Lin KP, Vita G, Attarian S, Plante-Bordeneuve V, Mezei MM, Campistol JM, Buades J, Brannagan TH III, Kim BJ, Oh J, Parman Y, Sekijima Y, Hawkins PN, Solomon SD, Polydefkis M, Dyck PJ, Gandhi PJ, Goyal S, Chen J, Strahs AL, Nochur SV, Sweetser MT, Garg PP, Vaishnav AK, Gollob JA, Suhr OB. Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N Engl J Med* 2018; **379**:11–21.
180. Ray KK, Landmesser U, Leiter LA, Kallend D, Dufour R, Karakas M, Hall T, Troquay RP, Turner T, Visseren FL, Wijnjaard P, Wright RS, Kastelein JJ. Inclisiran in patients at high cardiovascular risk with elevated LDL cholesterol. *N Engl J Med* 2017; **376**:1430–1440.
181. Tam C, Wong JH, Cheung RCF, Zuo T, Ng TB. Therapeutic potentials of short interfering RNAs. *Appl Microbiol Biotechnol* 2017; **101**:7091–7111.
182. Maeda H. Macromolecular therapeutics in cancer treatment: the EPR effect and beyond. *J Control Release* 2012; **164**:138–144.
183. Christian DA, Cai S, Bowen DM, Kim Y, Pajeroski JD, Discher DE. Polymersome carriers: from self-assembly to siRNA and protein therapeutics. *Eur J Pharm Biopharm* 2009; **71**:463–474.

184. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* 1995;**92**:7297–7301.
185. Zintchenko A, Philipp A, Dehshahri A, Wagner E. Simple modifications of branched PEI lead to highly efficient siRNA carriers with low toxicity. *Bioconjug Chem* 2008;**19**:1448–1455.
186. Babar IA, Cheng CJ, Booth CJ, Liang X, Weidhaas JB, Saltzman WM, Slack FJ. Nanoparticle-based therapy in an in vivo microRNA-155 (miR-155)-dependent mouse model of lymphoma. *Proc Natl Acad Sci U S A* 2012;**109**:E1695–E1704.
187. Devulapally R, Sekar NM, Sekar TV, Foygel K, Massoud TF, Willmann JK, Paulmurugan R. Polymer nanoparticles mediated codelivery of anti-miR-10b and anti-miR-21 for achieving triple negative breast cancer therapy. *ACS Nano* 2015;**9**:2290–2302.
188. Wang S, Zhang J, Wang Y, Chen M. Hyaluronic acid-coated PEI-PLGA nanoparticles mediated co-delivery of doxorubicin and miR-542-3p for triple negative breast cancer therapy. *Nanomedicine* 2016;**12**:411–420.
189. Bellera N, Barba I, Rodriguez-Sinovas A, Ferret E, Asin MA, Gonzalez-Alujas MT, Perez-Rodon J, Esteves M, Fonseca C, Toran N, Garcia Del Blanco B, Perez A, Garcia-Dorado D. Single intracoronary injection of encapsulated antagomir-92a promotes angiogenesis and prevents adverse infarct remodeling. *J Am Heart Assoc* 2014;**3**:e000946.
190. Conde J, Oliva N, Atilano M, Song HS, Artzi N. Self-assembled RNA-triple-helix hydrogel scaffold for microRNA modulation in the tumour microenvironment. *Nat Mater* 2016;**15**:353–363.
191. Ragelle H, Vandermeulen G, Preat V. Chitosan-based siRNA delivery systems. *J Control Release* 2013;**172**:207–218.
192. Kaban K, Salva E, Akbuga J. In vitro dose studies on chitosan nanoplexes for microRNA delivery in breast cancer cells. *Nucleic Acid Ther* 2017;**27**:45–55.
193. Boca S, Gulei D, Zimta AA, Onaciu A, Magdo L, Tigu AB, Ionescu C, Irimie A, Buiga R, Berindan-Neagoe I. Nanoscale delivery systems for microRNAs in cancer therapy. *Cell Mol Life Sci* 2019;**77**:1059–1086.
194. MacDiarmid JA, Brahmbhatt H. Minicells: versatile vectors for targeted drug or si/shRNA cancer therapy. *Curr Opin Biotechnol* 2011;**22**:909–916.
195. van Zandwijk N, Pavlakis N, Kao SC, Linton A, Boyer MJ, Clarke S, Huynh Y, Chrzanowska A, Fulham MJ, Bailey DL, Cooper WA, Kritharides L, Ridley L, Pattison ST, MacDiarmid J, Brahmbhatt H, Reid G. Safety and activity of microRNA-loaded minicells in patients with recurrent malignant pleural mesothelioma: a first-in-man, phase 1, open-label, dose-escalation study. *Lancet Oncol* 2017;**18**:1386–1396.
196. Jiang L, Vader P, Schifflers RM. Extracellular vesicles for nucleic acid delivery: progress and prospects for safe RNA-based gene therapy. *Gene Ther* 2017;**24**:157–166.
197. Mathiyalagan P, Sahoo S. Exosomes-based gene therapy for microRNA delivery. *Methods Mol Biol* 2017;**1521**:139–152.
198. Ohno S, Kuroda M. Exosome-mediated targeted delivery of miRNAs. *Methods Mol Biol* 2016;**1448**:261–270.
199. Yla-Herttuala S, Alitalo K. Gene transfer as a tool to induce therapeutic vascular growth. *Nat Med* 2003;**9**:694–701.
200. Grabner W, Pfitzer P. Number of nuclei in isolated myocardial cells of pigs. *Virchows Arch B Cell Pathol* 1974;**15**:279–294.
201. Ahuja P, Perriard E, Perriard JC, Ehler E. Sequential myofibrillar breakdown accompanies mitotic division of mammalian cardiomyocytes. *J Cell Sci* 2004;**117**:3295–3306.