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 diseases[3] 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 synthesis[6] 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 heart[31] 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 modest[19] or requires co-stimulation (e.g. p38 down-regulation in the case of FGF1[24]) 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 neuregulin[32]).

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 3β 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 D1[59] 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 D1[62]], 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

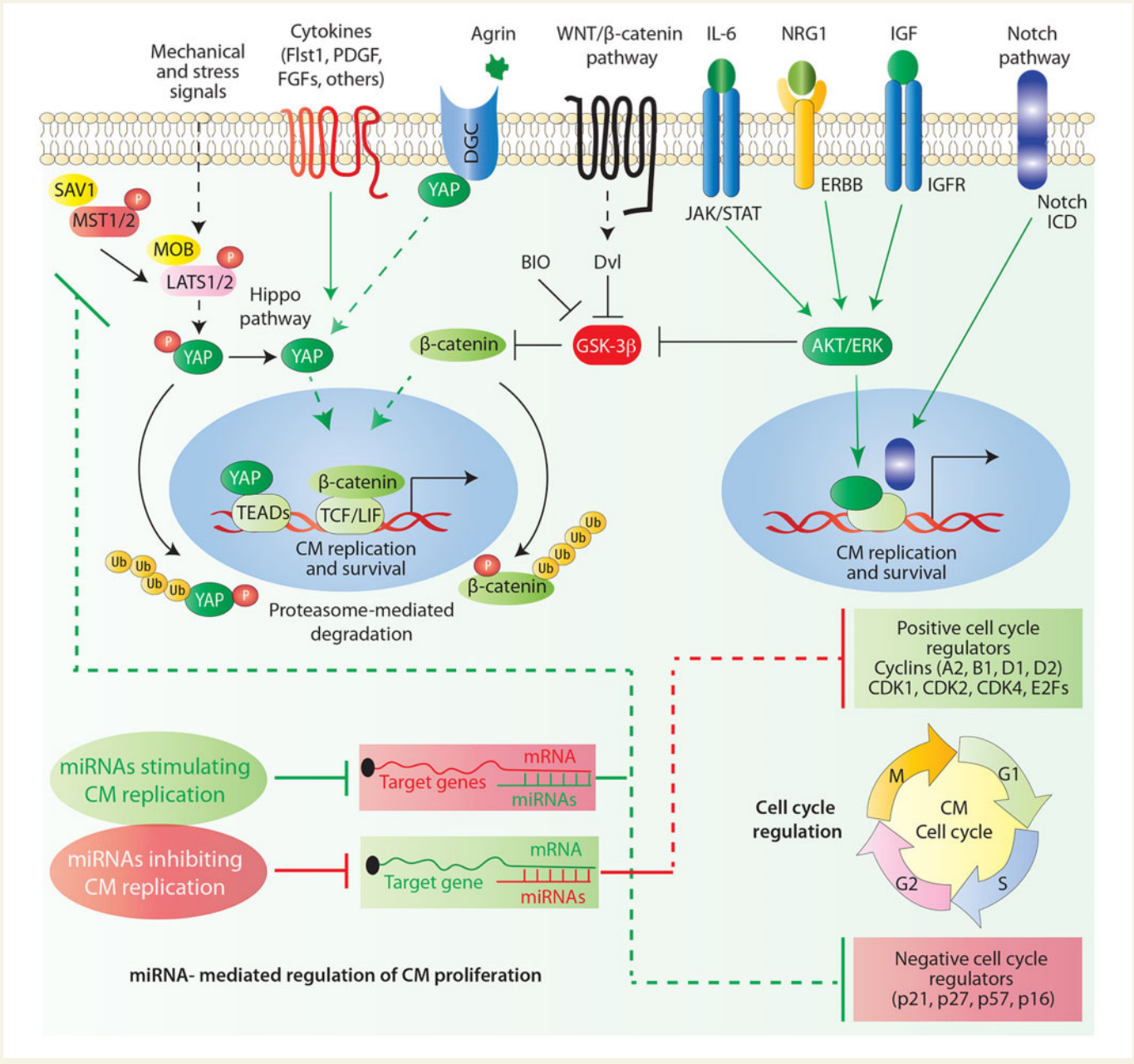

Figure 1 Extra-cellular and intra-cellular signalling regulating cardiomyocyte proliferation and cardiac regeneration. The cardiomyocyte cell cycle is under the control of several molecular regulators. Growth factors and cytokines acting on cell-membrane receptors, including IL-6, neuregulin (NRG1), Fslt1, 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-3β, 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.

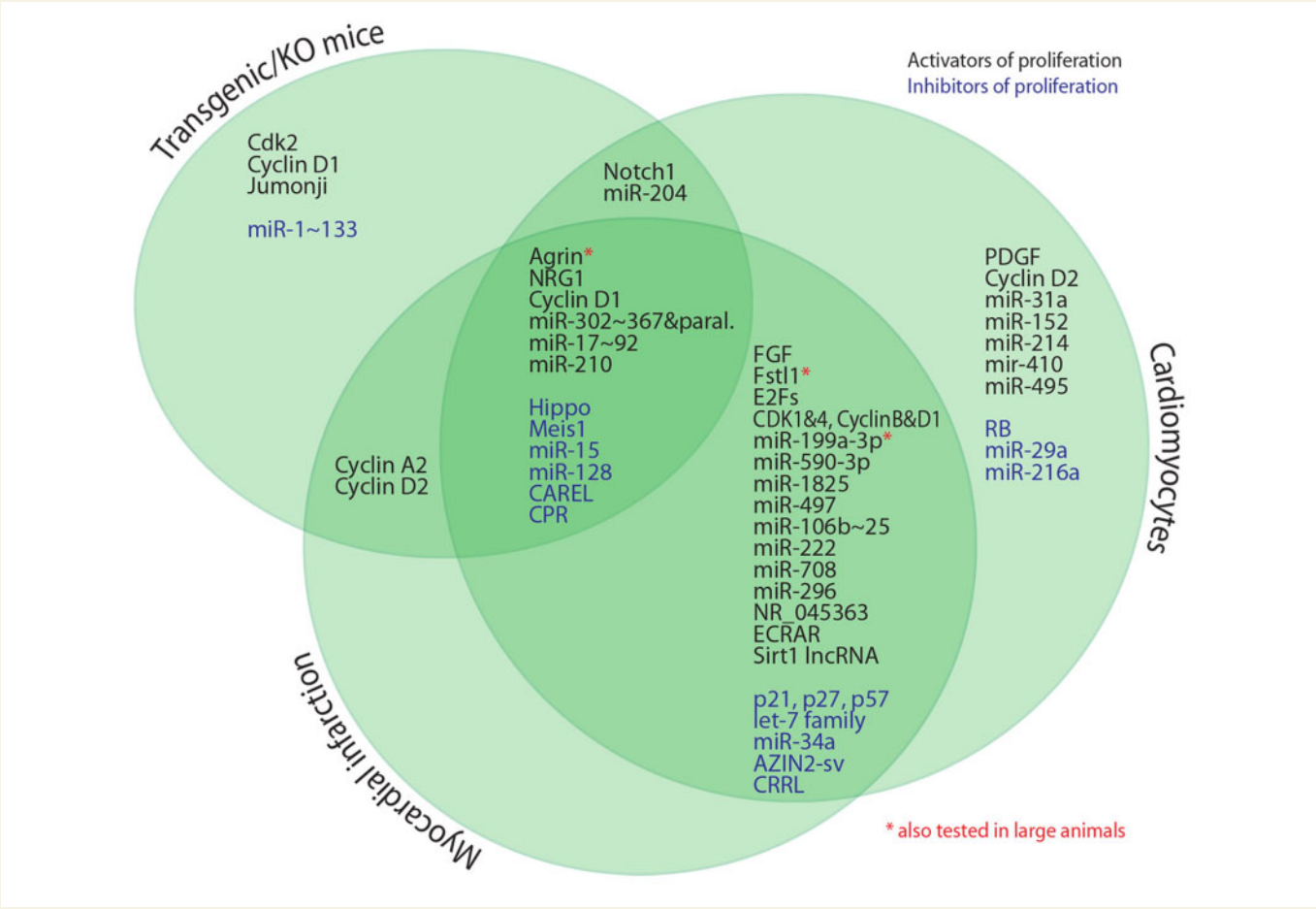

Figure 2 Venn diagram reporting the proteins and nucleic acids shown to stimulate (black) or inhibit (blue) cardiomyocyte proliferation and cardiac repair after myocardial infarction. Factors are grouped according to their action: (i) in transgenic or knock-out mice; (ii) in cultured cardiomyocytes from 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 bioRxiv[63] (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 rodent[64] 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, Itm2a	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

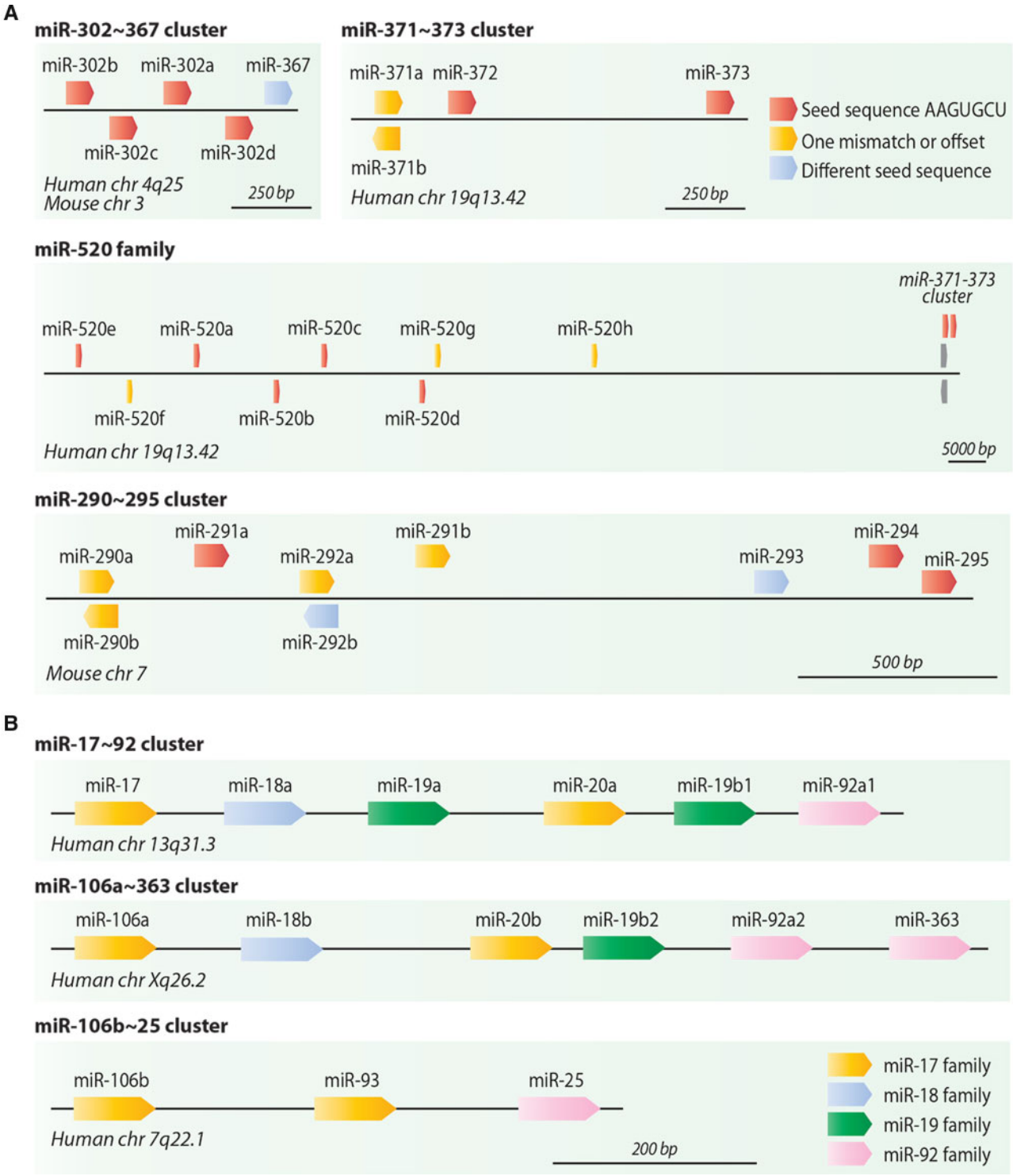

Figure 3 Conserved miRNA families known to regulate cardiomyocyte proliferation. The individual miRNAs are indicated by arrowed boxes in the direction of transcription. The different colours identify specific seed sequences. (*A*) miRNAs active in embryonic stem cells. Human miRNAs include those in the 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 blastocyst[94]) 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 rodent[64] and human[65] cells, both identified several members of the miR-302/290/371/520 group of miRNAs and of the mi-17~92 cluster and its paralogs. 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 (mTOR[113]), 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 neonatal[64] and adult[73] 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 Chk1[119] 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 group[92]) or of the tumour suppressor kinase PTEN (for the miR-17∼92 cluster[69] and miR-486[136]). 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]).

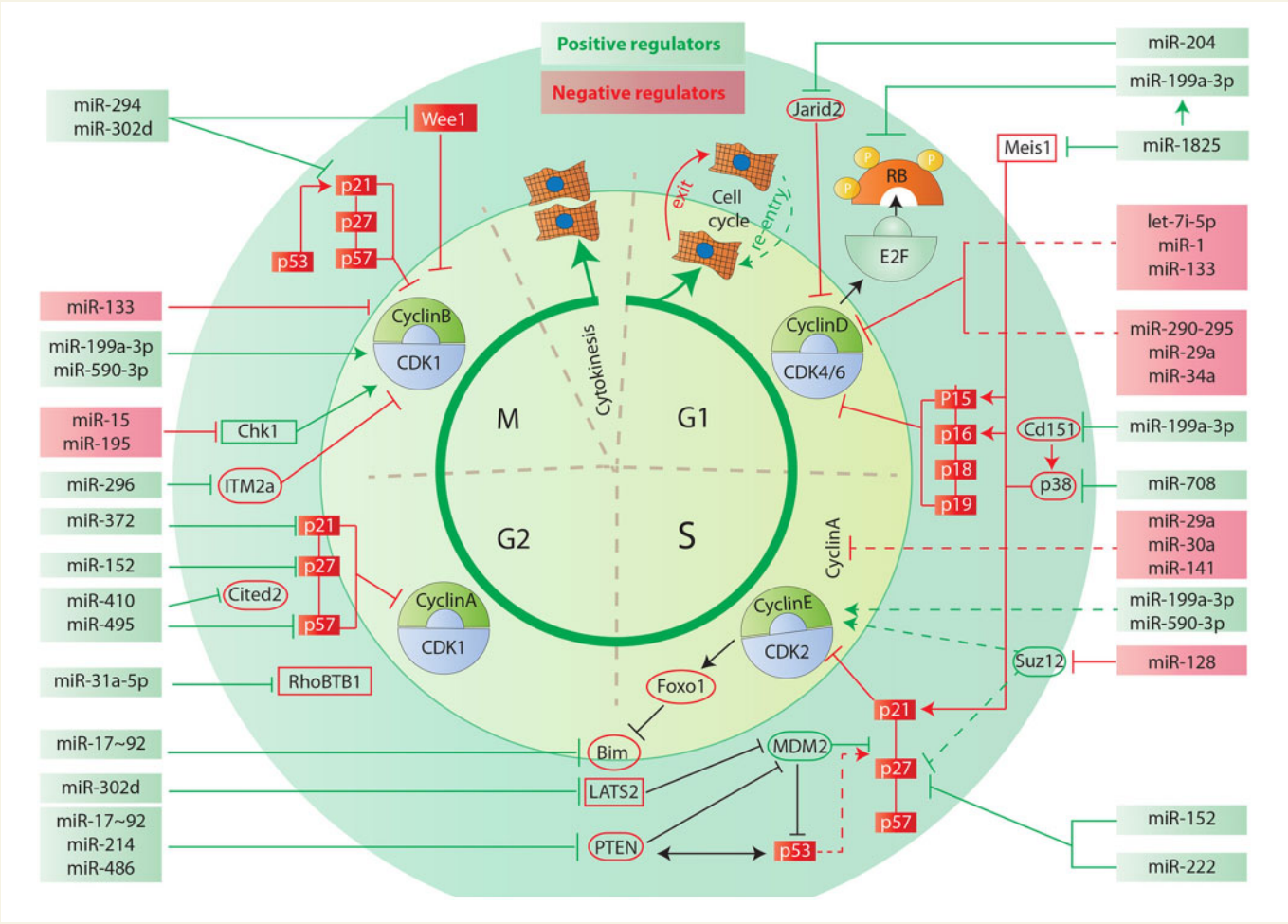

Figure 4 Effect of miRNAs stimulating cardiomyocyte proliferation on the cardiomyocyte cell cycle. Similar to other cell types, the cardiomyocyte cell cycle is governed by cyclin/CDKs, which in turn respond to a number of activator and repressor proteins (in green and red, respectively). The cartoon shows the main direct or indirect effect on these factors of the pro-proliferative miRNAs (in green) and of miRNAs inhibiting proliferation (in red). Further details are reported in the text and in *Tables 1* and *2*.

6. lncRNAs that regulate CM proliferation

Not dissimilar to other cell types, proliferation of CMs is also controlled by various long ncRNAs (lncRNAs), a class of functional non-translated RNA molecules typically more than 200-nt long. In several cases, these were shown to act as sponges for inhibitory miRNAs (*Table 3*). The AZIN2-sv lncRNA, transcribed and processed as a splicing variant of the AZIN2 gene, directly binds miR-214 and blocks its inhibitory function on PTEN, which in turn is an inhibitor of the PI3K/AKT pathway.[88] As a consequence, loss of this lncRNA promotes cellular survival and proliferation. The CRRL lncRNA binds miR-199a-3p and antagonizes its pro-proliferative effect.[141] CAREL instead binds and blocks miR-296, excluding this miRNA from accessing its mRNA targets Trp53inp1 and Itm2a[87]; the former is known to induce cell cycle arrest in G1 and to enhance p53-mediated apoptosis,[144] while Itm2a codes for a transmembrane protein of unknown function, which was is associated to G2/M cell cycle arrest as a tumour suppressor.[145] Transgenic mice overexpressing CAREL in CMs show limited cell cycle activity and impaired cardiac regeneration after apical resection in the perinatal period. The lncRNA NR_045363 also acts as a sponge for another miRNA, in this case, the inhibitory miR-216a.[131] This lncRNA prevents the down-regulation of JAK2, being this a main post-transcriptional activator of the STAT3 pathway.

Other lncRNAs interact with mRNAs and proteins or act as guides for chromatin modifiers with an effect in modulating proliferation. Knock-out mice for CPR display increased CM renewal, in both postnatal and adult life, and after MI. A relevant downstream target of this lncRNA is the MCM3 protein, an initiator of DNA replication.[140]

The antisense Sirt1 lncRNA binds and stabilizes the 3′-UTR of the Sirt1 mRNA, thus enhancing expression of the encoded proteins.[143] This lncRNA was previously described to induce myoblast[146] and endothelial progenitor cell[147] proliferation. Finally, the lncRNA ECRAR promotes cardiomyogenesis and angiogenesis in rats after infarction. This lncRNA is transcribed by E2F1 and binds to and promotes phosphorylation of ERK1/2, which in turn induces G1-phase genes, including E2F1, creating a positive feedback loop.[142]

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 CCRL 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-15b[123] and miR34a[129] 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 mammals[118] 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 superfamily[67] 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 heart[9] 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 mice[64] 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 reagent[70] or by using the same formulation for the daily intravenous administration for 3–7 days of miR302b/c,[66] miR-19a/19b[70] or miR-708[85] mimics, or by the intracardial delivery of cholesterol-modified miR-302b/c mimics using a hydrogel.[68] Of note, however, while AAV9 vector transduction is selective for

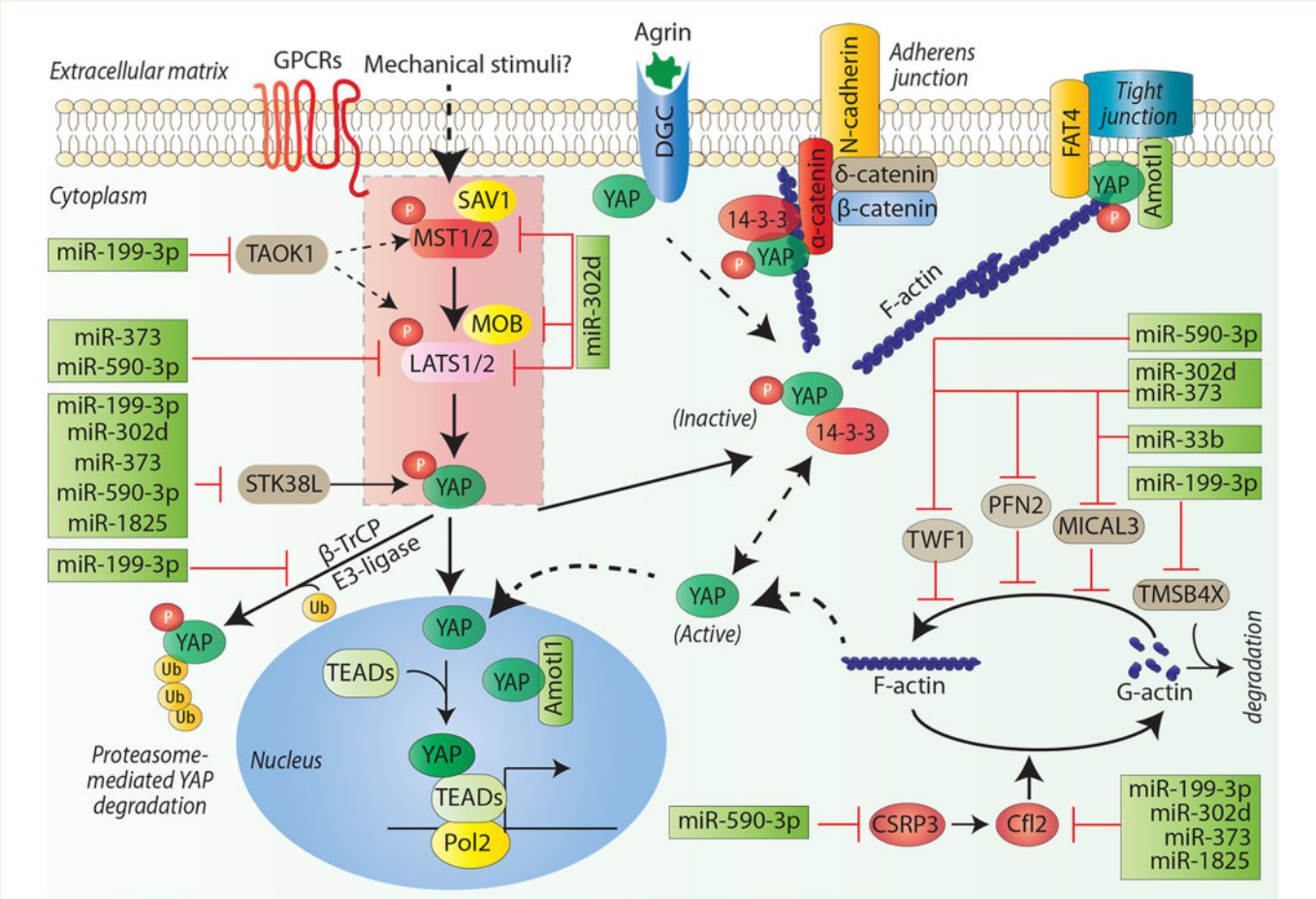

Figure 5 Effect of miRNAs on the Hippo pathway and the actin cytoskeleton. The cardiomyocyte cell cycle is controlled by the YAP transcription co-factor, which, when de-phosphorylated, translocates to the nucleus and interacts with the TEAD family of transcription factor to drive expression of pro-proliferative 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, TWF, 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) systems[169] 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 Drosha 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 injection[148] or miR-19a/19b after intravenous injection[70] using the former reagent, or miR-19a/19b[70] and miR302b/c[66] 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 antagomiR-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 antagomiR 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 exosomes[197] 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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