Non-coding RNAs regulating cardiac muscle mass

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ABSTRACT

Non-coding RNAs, including miRNAs, lncRNAs and circRNAs play roles in the development and homeostasis of nearly every tissue of the body, including the regulation of processes underlying heart growth. Cardiac hypertrophy can be classified as either physiological (beneficial heart growth) or pathological (detrimental heart growth), the latter which results in impaired cardiac function, heart failure and is predictive of a higher incidence of death due to cardiovascular disease. Several miRNAs have a functional role in exercise-induced cardiac hypertrophy, whilst both miRNAs and lncRNAs are heavily involved in pathological heart growth and heart failure. The latter have the potential to act as an endogenous sponge RNA and interact with specific miRNAs to control cardiac hypertrophy, adding another level of complexity to our understanding of the regulation of cardiac muscle mass. In addition to tissue-specific effects, ncRNA-mediated tissue cross talk occurs via exosomes. In particular, miRNAs can be internalized in exosomes and secreted from various cardiac and vascular cell types to promote angiogenesis, as well as protection and repair of ischemic tissues. NcRNAs hold promising therapeutic potential to protect the heart against ischemic injury and aid in regeneration. Numerous preclinical studies have demonstrated the therapeutic potential of ncRNAs, specifically miRNAs, for the treatment of cardiovascular disease. Most of these studies employ antisense oligonucleotides to inhibit miRNAs of interest; however, off-target effects often limit their potential to be translated to the clinic. In this context, approaches using viral and non-viral delivery tools are promising means to provide targeted delivery *in vivo*.

INTRODUCTION

Non-coding RNAs (ncRNAs) are RNA molecules that do not encode for a protein; rather, ncRNAs contribute to the maintenance of cell and tissue homeostasis through a variety of regulatory processes. The role and regulation of non-coding RNAs in mammalian cells have received considerable attention over the past decades. For example, transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) are essential to the production of functional proteins (61). Of particular interest are the more recently described classes of regulatory ncRNAs, comprising small interfering (siRNAs), microRNAs (miRNAs), piwi-associated RNAs (piRNAs), circular RNAs (circRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs) and long non-coding RNAs (lncRNAs). NcRNA molecules can directly or indirectly regulate gene expression (34), process other RNA molecules (84), or act as regulators for other RNA species (127). Through their various functions, ncRNA molecules play an essential role in the development and homeostasis of nearly every tissue of the body.

Cardiac pathologies and cardiovascular diseases are among the most common causes of morbidity and mortality worldwide (91). The increasing burden these conditions place on health systems has prompted countless investigations into the physiological and pathological processes regulating cardiac muscle mass. Cardiac muscle mass in adults is largely altered by changes in cardiac myocyte size because it represents 70-80% of the hearts volume; however, in certain settings, myocyte proliferation, myocyte death and fibrosis can also influence muscle mass (15, 16). Over the past several years, ncRNA molecules have been the focus of an increasing number of studies that, in certain cases, have led to the identification of novel therapeutic targets (24). This mini-review aims to summarize our current understanding of ncRNA-mediated regulation of cardiac muscle mass. It will highlight the most important ncRNAs regulating cardiac hypertrophy, with a focus on the widely studied miRNAs and lncRNAs. NcRNA-mediated tissue crosstalk will be briefly discussed. Finally, it will provide

an insight into the current knowledge and challenges associated to the therapeutic potential of ncRNAs in the context of cardiac medicine.

CLASSES OF NON-CODING RNAs

NcRNAs play a central role in regulating the pathophysiological processes underlying heart growth. Amongst the most vastly studied regulatory ncRNA species are miRNAs, lncRNAs and circRNAs. CircRNAs were first observed in viruses in the 1970's (107), while the existence of miRNAs and lncRNAs was suggested in the early 1990's in non-mammalian and mammalian cells (18, 72, 105). The field has been ever expanding since then.

MiRNAs are 20-22nt single stranded RNA molecules originating from coding and non-coding parts of the nuclear genome (7, 13). They directly and indirectly regulate gene expression in the cytoplasm (53) and, in some cases, in the mitochondria (32). MiRNAs silence protein expression either by degrading specific target mRNA molecules or by directly inhibiting protein translation (7, 13) (Figure 1). In some cases, they may also stabilize mRNA molecules (120). Each miRNA has the potential to target multiple mRNA transcripts via interactions based on Watson-Crick recognition of a 6-8 nucleotide sequence localized at their 5' end, the 'seed' sequence (19). However, it is becoming clearer that non-canonical rules may govern close to 60% of all miRNAs/mRNAs interactions (28, 58). To date, over 4000 miRNAs have been described in human tissue (miRBase database v.22, (49)).

LncRNAs are a more heterogeneous class of single- or double-stranded RNA molecules that are arbitrarily defined as longer than 200nt and shorter than 10,000nt (127). The presence or absence of a poly-A tail determines lncRNA stability (139). More than 17,000 lncRNA molecules may be encoded by the human genome (134), including the mitochondrial genome (43, 103). LncRNA have various functions, and can act as signal, sensor, stabilization and decoy molecules for other ncRNAs to regulate gene expression in the cell (127). This class of

ncRNAs also directly regulates protein expression (Figure 1) and activities by providing a scaffold for regulatory proteins, driving allosteric modifications and facilitating histone methylation (34).

Finally, circRNAs constitute an abundant and conserved class of RNA molecules that was originally considered non-coding (102). Recent studies however showed that some circRNAs can be translated into protein *in vitro* and *in vivo* (99, 135). CircRNAs are covalently closed single-stranded RNA molecules where the 3' and 5' ends have been joined together. Bioinformatics coupled to next-generation RNA sequencing tools predict the existence of thousands of circRNAs in the human genome (102). CircRNA can regulate nuclear gene expression (89), regulate alternative splicing (4), or act as miRNA sponges by competing against endogenous miRNAs for binding (55) (Figure 1). However, their biological functions, as well as their localization and degradation remain mostly unclear.

MiRNAs, lncRNAs and more recently circRNAs are involved in the regulation of nearly every aspect of cellular function (see reviews (53, 89, 127, 140)). Aberrant expression of these ncRNA molecules has been consistently linked to disease initiation and progression, including cardiac and cardiovascular conditions (for reviews see (13, 56, 88, 97, 140)). As such, the role and regulation of these regulatory RNA species constitute one of the most dynamic research topics in the field of molecular medicine.

MOST PROMINENT ncRNAs IN REGULATING CARDIAC MUSCLE MASS

Numerous studies have demonstrated a role of ncRNAs in pathological and physiological cardiac hypertrophy. Of the ncRNAs, the role of miRNAs in regulating cardiac hypertrophy has been extensively studied, whereas much less is known about the role and mechanisms of lncRNAs and circRNAs. Below we summarize the most prominent ncRNAs in regulating cardiac hypertrophy, which are also summarized in Figure 2.

Physiological cardiac hypertrophy – when big is beautiful

Increased heart mass (cardiac hypertrophy) occurs following endurance training and is commonly referred to as physiological cardiac hypertrophy (also called the athlete's heart in humans) and under these circumstances, resting (non-exercise) cardiac function is either normal or enhanced (100). This is in stark contrast to the bigger heart observed under pathological conditions (termed pathological cardiac hypertrophy) that is most commonly associated with impaired cardiac function, heart failure and predicts a higher incidence of death due to cardiovascular disease (74). There are striking molecular and structural differences between physiological cardiac hypertrophy following endurance exercise-training and pathological hypertrophy induced by chronic pressure (i.e. hypertension) or volume overload (16). Unlike physiological hypertrophy, pathological hypertrophy is commonly associated with increased fibrosis, reduced cardiac function, heart failure and increased mortality (16). The insulin-like growth factor 1 (IGF1)–phosphoinositide 3-kinase (PI3K)-Akt signaling pathway is necessary for physiological cardiac hypertrophy, whereas pathological cardiac hypertrophy is regulated in a large part by Gαq signaling (reviewed in (16)).

MiRNAs regulated by exercise and settings of physiological cardiac growth

Endurance exercise in rats and mice alters the expression of many miRNA species in the heart that are implicated in cardiac development and growth. Indeed, over 200 miRNA species are differentially expressed in the hearts of rodents following several weeks of endurance training, with ~60% of these species having their expression downregulated (42, 79, 104, 109). Cardiac (and skeletal) muscle is also enriched in several miRNA species, termed myomiRs, and comprise miR-1, miR-133a, miR133b, miR-206, miR-208a, miR-208b, miR-499, and miR-486, with most being downregulated with physiological cardiac

hypertrophy. miR-1, miR-133a and miR-208a levels are downregulated following exercise-induced cardiac hypertrophy (23, 104, 110, 111) and previous studies have shown miR-1 and miR-133a induction acts to repress cardiac hypertrophy and growth (23, 38). There is conflicting reports of miR-208b and miR-133b being upregulated (104) or downregulated (110, 111) following exercise-induced cardiac hypertrophy. The reason for these conflicting reports for miR-208b are unclear since both studies used similar swim training protocols of several weeks duration in the same strain of rat (104, 110).

miRNAs also play a role in regulating molecular pathways related to exercise-induced heart adaptations (see reviews (15, 45, 95, 128)). Members of the miR-29 family were increased in hypertrophic hearts of swim-trained rats, which was associated with downregulation of collagen gene expression, which may be associated with decreased cardiac fibrosis and improved heart function (111). Enhanced angiogenesis is associated with exercise-induced heart growth and is regulated by the vascular endothelial growth factor (VEGF) pathway. The expression of miR-126 was elevated in swim-trained rodent hearts which facilitated angiogenic signaling by inhibiting its target genes, Spred1 and PI3KR2, negative regulators of the VEGF pathway (31). Microarray profiling identified 62 miRNAs that were regulated by PI3K(p110α), a gene essential for exercise-induced heart growth (77). Collectively, these miRNAs target a wide range of genes, some of which are involved in the regulation of fibrosis, apoptosis, autophagy, angiogenesis and cardiac contraction. Therapeutic inhibition of PI3K-regulated miRNAs using antisense oligonucleotides in cardiac disease mouse models provides therapeutic benefit (9, 11, 12).

Beyond expression profiling, the functional significance of some miRNAs has been established in regulating physiological cardiac hypertrophy. In particular, miR-222 and miR-17-3p were both upregulated in the heart following endurance training and were required for

exercise-induced cardiac growth (79, 109, 124). Inhibition of miR-222 prevented the increase in heart mass, cardiomyocyte hypertrophy and proliferation in mice following endurance training (79, 124). Inhibition of miR-17-3p prevented exercise-induced cardiac hypertrophy, cardiomyocyte hypertrophy and attenuated cardiomyocyte proliferation in mice following endurance training via TIMP3 and PTEN/Akt signaling pathway. However, overexpressing miR-222 or miR-17-3p to levels normally seen following exercise training in the rodent heart did not stimulate the phenotype observed by exercise-induced cardiac hypertrophy (79, 109). Nevertheless, miR-222 and miR-17-3p hold promising therapeutic potential to protect the heart against ischemic injury and aid in regeneration. Indeed, transgenic mice that overexpress miR-222 or agomiR overexpression of miR-17-3p in mice both protected against ischemic injury *in vivo* (79, 109).

In summary, miRNA species are well established to play a regulatory role in cardiomyocyte proliferation, hypertrophy and developmental and adult cardiac enlargement. Much less is known about the role of lncRNAs and circRNAs in the regulation of exercise-induced physiological adaptations. miRNAs also hold promising therapeutic potential for the treatment of cardiac conditions. A large number of miRNA species are known to be differentially expressed in the heart following endurance training and some, such as miR-222 and miR-17-3p are necessary for the induction of cardiac hypertrophy following endurance training. Understanding the molecular mechanisms as to how a short-term physiological stress can induce such long-term benefits for the heart will provide new targets for the treatment of ischemic injury to the heart and its regeneration.

ncRNAs in cardiac hypertrophy and heart failure

miRNAs - A number of miRNAs have pro- or anti-hypertrophic roles in the heart and have been recently reviewed in detail (76, 97). miR-208a is a heart enriched miRNA involved in

196 cardiomyocyte hypertrophy, fibrosis and regulating the shift in myosin heavy chain isoform
197 content during cardiac development and in the adult heart in response to a cardiac stress (21,
198 119). Further, miR-208a controls the expression of hypertrophy-related signaling
199 components, thyroid hormone activity and the cardiac conduction system during adaptation to
200 pathological signaling (21, 119). The expression of miR-499 is upregulated in human failing
201 and hypertrophied hearts, and in mouse models of pathological hypertrophy. Overexpression
202 of miR-499 in the murine heart accelerated heart failure progression and exacerbated the
203 response to pressure overload, through direct and indirect effects on cardiac protein kinases
204 and alterations in protein phosphorylation of proteins in the heart (87). miR-1 is not only
205 downregulated in physiological hypertrophy, but also in heart failure. miR-1 regulates
206 cardiomyocyte hypertrophy by negatively regulating genes in the calcium/calmodulin
207 signaling pathway, which controls cardiomyocyte function and growth (65). In a number of
208 cardiac hypertrophy rodent models (pressure overload, transgenic mice with selective cardiac
209 overexpression of a constitutively active mutant of the Akt kinase, and an exercise model) the
210 expression of miR-133a was downregulated (23). MiR-133a regulates the hypertrophic gene
211 program by targeting multiple genes including RhoA (an exchange protein regulating cardiac
212 hypertrophy), Cdc42 (a signal transduction kinase implicated in hypertrophy) and Nelf-
213 A/WHSC2 (nuclear factor involved in cardiogenesis; although its role in hypertrophy remains
214 unclear) (23).

215 ***LncRNAs*** **-** Two lncRNAs that play a significant role in mouse heart development are
216 Braveheart *(Bvht)* (68) and Foxof1 adjacent non-coding developmental regulatory RNA
217 (*Fendrr)* (50). *Bvht* has an important role in the establishment of the cardiovascular lineage
218 by interacting with SUZ12, suggesting a role in the epigenetic regulation of gene expression
219 programs, and also by activating a number of transcription factors necessary for promoting
220 the cardiac gene expression program in cardiomyocytes (68). Loss of *Fendrr* during the

embryonic stage in mice results in severe cardiac defects and dysfunction (50). Matkovich and colleagues (85) used genome-wide sequencing and bioinformatics to characterize cardiac-enriched lncRNA expression in mouse embryo and adult hearts, where 157 lncRNAs were differentially expressed in embryonic hearts compared to adult hearts. Network analysis revealed a role of these lncRNAs in major cardiac development and metabolic pathways (85).

A number of reports describe a role of lncRNAs in pathological cardiac hypertrophy (78, 123, 136). The cardiac specific lncRNA myosin heavy-chain-associated RNA transcripts *(Mhrt)* consists of a cluster of RNAs (*Mhrt* RNAs) which are abundant in adult mouse hearts (54). *Mhrt* expression was induced during cardiomyocyte maturation and decreased in pressure overload-induced cardiac hypertrophy and heart failure in mice, a profile that correlates with the myosin heavy chain isoform shift during postnatal development and progression to heart failure (54). Re-expression of *Mhrt* in the heart protected mice against cardiac hypertrophy and heart failure by binding and antagonizing Brahma-related gene 1, suggesting a protective role of *Mhrt* in cardiovascular disease (54). Transcriptomic analysis of pressure-overload-induced failing hearts in mice identified a lncRNA highly enriched in the heart which the authors called cardiac-hypertrophy-associated epigenetic regulator *(Chaer)* (136). *Chaer* is specifically expressed in cardiomyocytes, and *Chaer* knockout mice displayed a significantly attenuated cardiac hypertrophic response to pressure overload (136). This was associated with less fibrosis and preserved cardiac function, suggesting a role of *Chaer* in controlling cardiac remodeling. *Chaer* controlled cardiac hypertrophy by its direct interaction with polycomb repressor complex 2 (PRC2), preventing histone methylation at the promoter regions of genes implicated in cardiac hypertrophy (136).

Further adding to the complexity surrounding the molecular mechanisms of cardiac hypertrophy, recent studies demonstrate that lncRNAs may act as an endogenous sponge RNA to interact with miRNAs (Figure 1) to control cardiac hypertrophy (81, 126). Cardiac

hypertrophy related factor, CHRF, was increased in the hearts of mice following pressure overload, and in patients with heart failure (126). *Chrf* acts as a sponge of miR-489, reducing the targeting activity of miRNA-489 on its target gene, myeloid differentiation factor 88 (*Myd88*), an important component in the Toll-like receptor-4-mediated nuclear factor-κB activation pathway, contributing to the development of cardiac hypertrophy (126). The lncRNA Plscr4 was upregulated in hypertrophic mouse hearts, and overexpression of Plscr4 blunted the hypertrophic response in mice following pressure overload (81). Plscr4 exerts its anti-hypertrophic effects by sequestering the pro-hypertrophic miRNA, miR-214, which in turn allowed expression of Mitofusin 2 and alleviated hypertrophic growth (81).

A recent study demonstrated that lncRNAs could represent targets for therapeutic intervention in heart failure (123). Cardiac hypertrophy-associated transcript (*Chast*) was elevated in both mouse and human hypertrophied hearts, and overexpression of *Chast* induced cardiomyocyte hypertrophy *in vitro* and *in vivo* (123). Antisense technology to inhibit Chast (using "GapmeRs") attenuated cardiac remodeling after pressure overload-induced hypertrophy in mice (123), by targeting pleckstrin homology domain-containing protein family M member 1, which inhibits autophagy and influences hypertrophy (123). The use of GapMers (without any signs of toxicology side effects (123)) highlights the therapeutic potential of lncRNAs.

circRNAs – Unlike miRNAs and lncRNAs, the role of circRNAs in regulating cardiac muscle mass has been less extensively studied, with most research focusing on identifying those circRNAs associated with heart disease (see review (129)). Although deep sequencing studies have identified a number of cardiac-expressed circRNAs in diseased human and rodent hearts (114, 137), only a limited number were differentially expressed. CircRNAs were found to be significantly differentially expressed in the rat heart during the

developmental transition from embryo to adult (137), but the functional significance of circRNAs in regulating muscle mass remains unclear.

The ncRNA landscape in human cardiac pathology

Advances in RNA sequencing and genome-wide association studies (GWAS) has allowed the discovery of ncRNAs in human heart disease, and has highlighted their potential as cardiac biomarkers. Here we present an overview of ncRNAs implicated in human cardiac pathology.

miRNAS – Many miRNAs are implicated in human heart disease, often discovered by gene expression microarray profiling. Thum and colleagues (115) profiled the cardiac miRNA and mRNA gene signature in left ventricular tissue from patients with end-stage heart failure, and compared it to that of tissue from healthy adult and fetal human hearts. They found that the miRNA expression patterns in fetal and failing human cardiac tissue was similar, suggesting that these miRNAs contribute to the reactivation of the fetal gene program in human heart failure (115). Using a deep sequencing approach, Lepitidis and colleagues (73) identified >250 differential and etiology-specific miRNAs in patients with dilated cardiomyopathy and in hearts from patients with familial hypertrophic cardiomyopathy (73). Other studies have explored the potential of miRNAs as clinical biomarkers for heart disease (47, 98, 117, 122). Several groups have characterized the levels of miRNAs in the circulation of patients with cardiovascular disease [(47, 98, 117, 122); also see reviews (29, 101, 148)]. In some instances, studies suggest that measurement of a panel of circulating miRNAs may be an alternative approach to conventional biomarkers for early detection in acute myocardial infarction (125, 131). Whilst circulating miRNAs may be useful for diagnostics and monitoring purposes, large-scales studies are still required before circulating miRNAs can be successfully used as biomarkers for cardiovascular pathologies.

lncRNAs – The expression of lncRNAs is altered in a number of human diseases, including cardiovascular disease, and is associated with disease development and progression (see review (75)). Using single nucleotide polymorphism (SNP) arrays and GWAS, the lncRNA Myocardial Infarction Associated Transcript (MIAT) was identified at a susceptible chromosomal location in over 3000 patients with myocardial infarction (compared to control patients) (66). GWAS have identified novel disease-associated DNA regions, including a "hotspot" on human chromosome 9p21, the strongest genetic susceptibility locus for cardiovascular disease (20). The lncRNA gene, Antisense non-coding RNA in the INK4 locus (ANRIL) resides in this hotspot and is associated with atherosclerosis in patients with suspected coronary artery disease, although its role is yet to be understood (59). The lncRNA long intergenic non-coding RNA (LIPCAR) was identified as a potential biomarker for heart failure from transcriptomic analyses of plasma from patients with cardiac pathology post myocardial infarction (69). Increased levels of LIPCAR in the plasma of patients with chronic heart failure was also associated with increased risk of future cardiovascular death (69). However, there are currently too few studies to know whether lncRNAs will be suitable biomarkers for cardiovascular disease.

circRNAs – circRNAs have been less extensively studied in human hearts, with the majority of studies focusing on their potential as biomarkers (see reviews (129, 147)). Due to their closed loop structure, they may represent more stable biomarkers than their linear ncRNA counterparts. Patients with high circular ANRIL expression developed less coronary artery disease, suggesting that circANRIL has an atheroprotective role (which is opposite to that of linear ANRIL as described above) (60). In order to predict patient outcomes following myocardial infarction, Vausort and colleagues identified that patients who had low levels of the circRNA myocardial infarction-associated circular RNA (MICRA) were at higher risk to develop left ventricular cardiac dysfunction (121). Due to their biological properties,

circRNAs have emerged as novel biomarkers, however more research is needed to elucidate their biological function. It is hoped that identification of circRNAs as biomarkers will aid in the diagnosis of cardiovascular disease and personalized medicine in the future.

ncRNAS IN EXOSOMES

Exosomes are small (30-140 nm), membranous vesicles that are ubiquitously expressed by cells *in vivo* (51) and play an important role in tissue crosstalk (Figure 3). Their ability to transport peptides, lipids and genetic material results in functional changes in the recipient tissue or organ (51). Regulatory proteins, mRNAs and miRNAs transported within the exosomal lumen elicit some of these changes (51, 90, 138). MiRNAs comprise up to 76.2% of exosomal RNA species (63), along with lncRNAs (3.36%), piRNA (1.31%), snoRNA (0.18%) and snRNA (0.01%) (63). The remaining exosomal RNA includes coding, ribosomal (9.16%) and transfer (1.24%) RNA (63). The role of exosomal lncRNA, piRNA, snoRNA and snRNA in cardiac hypertrophy has not been investigated thus far. However, the role of exosomal miRNA, which represents a more highly concentrated bioactive pool of circulating miRNAs (ci-miRNAs) than riboprotein-bound miRNAs (2, 27), has been extensively researched in heart failure (see review (29)).

Emerging evidence suggests that cardiac and vascular cells, including cardiac fibroblasts (5, 17, 82), cardiomyocytes (17, 132) and H9c2 cells (17, 44) secrete and internalize exosomes (5, 39). These exosomes can influence cardiac function, inducing proliferation, angiogenesis, autophagy and importantly, cardiac hypertrophy. Exosome release may be constitutive, or induced by various stimuli including calcium concentration, mitogens, cytokines or stress (39). In humans and murine models, both healthy and ischemic cardiomyocytes release exosomes *in vivo* (106). Exosomes secreted from various cardiac and vascular cell types promote angiogenesis, tissue protection and repair post-ischemia in animal models (3, 22,

26). Of particular interest, cardiac-derived exosomes can play a positive role in cases of ischemic tissue repair and regeneration (64, 70).

Exosomal content plays an important role in cardiomyocyte health. The exosomal cargo is not merely a reflection of the relative abundance of compounds on the parental cell. Rather, exosomal cargo is selectively secreted or conserved in various pathophysiological conditions (144). By regulating cell proliferation, apoptosis, cytokine production, immune modulation and metastasis (62), exosomal miRNAs play an integral role in cross-talk between cells and tissues in many cardiac pathologies. For example, hypoxia stimulated C2C12 cells (mouse skeletal myoblasts) secreted miR-29a-enriched exosomes, which were then endocytosed into cardiomyocytes (142).

MiR-21-3p provides an example of exosome-derived miRNA species involved in cardiac hypertrophy. MiR-21-3p was differentially expressed in aging murine hearts (146) and highly expressed in failing human hearts when compared to non-failing human hearts (143). Exosomes enriched in miR-21-3p were released from neonatal rat cardiac fibroblasts and internalized by neonatal cardiomyocytes, leading to an increase in cardiomyocyte size *in vitro* (5), via the regulation of *Sorbs2* and *Pdlim5*. Of interest, pharmacological inhibition of miR-21-3p attenuated pressure overload induced cardiac hypertrophy in a murine model (5), suggesting that manipulation of exosomal cargo may represent a possible treatment pathway.

The miR-212/-132 family has been implicated in pathological cardiac hypertrophy (40) and cardiomyocyte autophagy (118) and is regulated by Angiotensin-II (Ang-II) (40), a peptide hormone playing an important role in cardiovascular disease (82). Ang-II stimulated exosome release from cardiac fibroblasts (82) increased activation of the Ang-II-AT_1R axis and

pathological cardiac hypertrophy (82), possibly via exosomal proteins (141) and miR-132 (82).

Peroxisome proliferator-activated receptor gamma (PPARγ) and miR-200a are suggested to control cross-talk between adipose tissue and cardiac tissue. PPARγ is a master regulator of adipose tissue signalling (1), and its activation induces cardiac hypertrophy in murine cardiomyocytes (112). Cardiac-specific activation of PPARγ in rat and in mouse models induced cardiac hypertrophy (35, 80). There is evidence to suggest that systemic activation of PPARγ may also induce cardiac hypertrophy (35, 57), suggesting a role for exosome-mediated crosstalk. Activation of PPARγ in murine adipocytes stimulated the release of exosomes enriched in miR-200a (41). These exosomes targeted cardiomyocytes and induced cardiac hypertrophy via activation of the mammalian target of rapamycin (mTOR) signaling pathway (41). Treatment with an antagomir for miR-200a blunted cardiomyocyte hypertrophy *in vitro* (41). This phenomenon was maintained in a mouse model of hypertrophy induced by rosiglitazone treatment (a PPARγ agonist), where specific ablation of PPARγ in adipocytes blunted cardiac hypertrophy (41). These data provide a possible pathway by which adipocyte-derived exosomes mediate cardiac health; and suggests a possible role for exosome-mediated crosstalk.

Exosomes released from skeletal muscle also contain many miRNA species that are implicated in cardiac hypertrophy, including miR-1, -133a/b, -222 and -208a (30, 52). In humans, the expression of exosomal miR-1, -222 and -208a was significantly upregulated (30), and miR-133b tended to be upregulated (52) after an acute bout of exercise *in vivo*. It is well established that cardiomyocytes internalize exosomes, and that the species present in exercise-induced exosomes derived from skeletal muscle regulate cardiac hypertrophy. Thus, while there is no direct evidence that these exosomes were internalized by cardiac cells, it is possible these miRNAs were involved in the regulation of cardiac hypertrophy, *via* exosome-

mediated crosstalk. However, this is yet to be elucidated and provides an intriguing pathway for future research.

Exosomes are attractive therapeutic targets for many chronic diseases, including cardiac hypertrophy (39). Due to their biophysical properties, the isolation and manipulation of exosomal contents is relatively straightforward (37). In addition, exosomes have a natural ability to cross biological barriers, such as the blood brain barrier (71). These vesicles therefore constitute possible 'trojan horses' to deliver drugs and other therapeutic substances (39), which would otherwise be free in the circulation and susceptible to damage and degradation. Several exosomal miRNA species may play a role in either the attenuation, or the pathogenesis of cardiac hypertrophy. By manipulating the expression of these species in exosomes, practitioners may have the potential to treat such pathologies in a targeted and effective manner.

ncRNAs as POTENTIAL THERAPEUTIC TARGETS FOR CARDIOVASCULAR DISEASE

Of the ncRNAs, the ability of miRNAs to target several genes within a similar signaling network or pathway may give them an advantage as potential therapeutic targets over other ncRNAs. Although, this can also be considered a drawback as it may cause undesirable responses. As miRNAs control pathophysiological processes of the heart, including cardiomyocyte cell death, autophagy, contractility, fibrosis and hypertrophy, investigators have performed numerous preclinical studies demonstrating the therapeutic potential of miRNAs for the treatment of cardiovascular disease, which have been extensively reviewed elsewhere (13, 56).

Despite promising preclinical studies demonstrating therapeutic efficacy of miRNA inhibitors there are currently no clinical trials for cardiovascular disease. However, there are positive advancements of RNA-based therapeutics (siRNAs, miRNAs) in other areas of disease (24).

Translational hurdles

miRNAs **-** The majority of preclinical and clinical studies employ antisense oligonucleotides that can inhibit the miRNA of interest. These inhibitors are non-tissue specific and are taken up by several organs (namely the liver and kidney) upon systemic administration. This may be problematic given the ubiquitous expression of some miRNAs, and the different functions of miRNAs in different tissues and/or oncogenic effects. In these instances, a targeted-tissue specific approach may be preferred. Further, with advances in next generation sequencing and systems biology, it has become apparent that cardiac miRNAs that regulate transcription can indirectly regulate other cardiac miRNAs (86, 96). This can lead to unexpected effects of antisense oligonucleotide therapies due to the regulation of the mRNA targets of the secondary miRNAs. Thus, a better understanding of precisely how miRNAs molecules interact with one another and regulate complex signaling networks is important for the successful design of miRNA-based therapies.

Other important aspects to consider when considering miRNA-based therapies for the clinic is i) the potential that miRNA inhibitors may affect RNA species beyond the intended target (96, 113), ii) whether targeting an individual miRNA or miRNA family will confer more therapeutic benefit (9), iii) the use of inhibitors that are specific to the seed region which can block the expression of entire miRNA families (94), and iv) that disease severity and sex can influence therapeutic outcome (8, 14, 36).

LncRNAs **-** There are a number of limitations that need to be resolved before lncRNAs can be taken to the clinic (see comprehensive reviews (46, 48, 88)). The lack of

conservation/homology of lncRNAs between different species makes both identification and clinical testing of human lncRNAs challenging, and translation from bench to clinic more difficult. The cellular location of lncRNAs needs to be considered before they can be developed as therapeutic targets. lncRNAs reside in multiple cellular extracellular locations, and some pharmacological agents may not penetrate the intracellular compartment of interest (6). Finally, the relationship between lncRNA structure and function is well not well understood and needs to be further elucidated.

Other ncRNAs- The therapeutic potential of targeting other ncRNAs in the heart including piRNAs, circRNAs and snoRNAs is largely unknown. However, in the cancer field this is being actively explored (83, 92, 145). It is therefore likely that these ncRNAs will also be targeted in the heart in the coming years.

Emerging technologies to deliver ncRNAs to the heart

The most common approach for targeted delivery is to use adeno-associated viral vectors (AAVs), and human clinical trials targeting mRNAs have been undertaken without any adverse side effects (149). There are limited reports of using AAVs for miRNA inhibition in the heart (67) and developing a cardiac-specific approach may prove to be more challenging (10). Other non-viral methods have recently been developed that have the potential to deliver miRNA therapeutics to the heart, including ultrasound microbubbles (133), light-induced miRNA inhibitor activation (a technique which facilitates local delivery) (108), unlockable core-shell nanocomplexes (93), hydrogels (cross-linked polymers capable of carrying and releasing therapeutics after injection in tissues) (130), neutral lipid emulsions (116) and negatively-charged calcium phosphate nanoparticles (33). Finally, CRISPR/cas9 technology could be used to edit ncRNAs and has been shown to be an efficient and stable technology

for inhibiting miRNAs *in vitro* and *in vivo* (25). The emergence of these new technologies may make a cardiac-specific ncRNA drug a reality.

CONCLUSION

It is clear from the studies conducted in the last two decades that ncRNAs have an important role in physiological and pathological cardiac hypertrophy. miRNAs are able to regulate cardiac hypertrophy by targeting genes in multiple hypertrophic-related signaling pathways, whereas the mechanism of lncRNAs are more complex – not only can they directly interact with genes, they can act on hypertrophy-related genes by acting as RNA sponges of miRNAs. The role of circRNAs in cardiac hypertrophy remain poorly understood. Preclinical studies suggest that miRNAs and lncRNAs can be promising therapeutic targets for the treatment of cardiovascular disease, and technologies to deliver ncRNAs to the heart are being developed. Future studies are warranted to further understand the molecular mechanisms of ncRNA and their regulatory networks in cardiac hypertrophy and disease.

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FIGURE LEGENDS

Figure 1: Functional interactions between the different classes of ncRNAs. LncRNAs, mRNAs, miRNAs and circRNAs are all transcribed from genomic DNA. LncRNAs and mRNAs can be translated into polypeptides. The green arrows indicate 'activation'. The blue lines indicate 'inhibition'. Dashed lines indicate that the nature of the interaction is still unknown.

Figure 2: Schematic of the most prominent and extensively studied ncRNAs in physiological and pathological hypertrophy. Increased workload on the heart leads to heart enlargement that is either physiological (due to exercise) or pathological (due to cardiac disease). A number of ncRNAs (miRNAs and lncRNAs) have been identified to play important roles in physiological and pathological hypertrophy, of which the most extensively studied are presented. Little is known about the role of lncRNAs and circRNAs in physiological hypertrophy, and the role of circRNAs in pathological hypertrophy requires further investigation.

Figure 3: Exosomal secretion mediates cross-talk between different tissues. Exosomes contain miRNAs, lncRNAs and potentially piRNAs. When released in cardiomyocytes, these ncRNAs have the potential to promote or protect against cardiac hypertrophy.

Figure 1

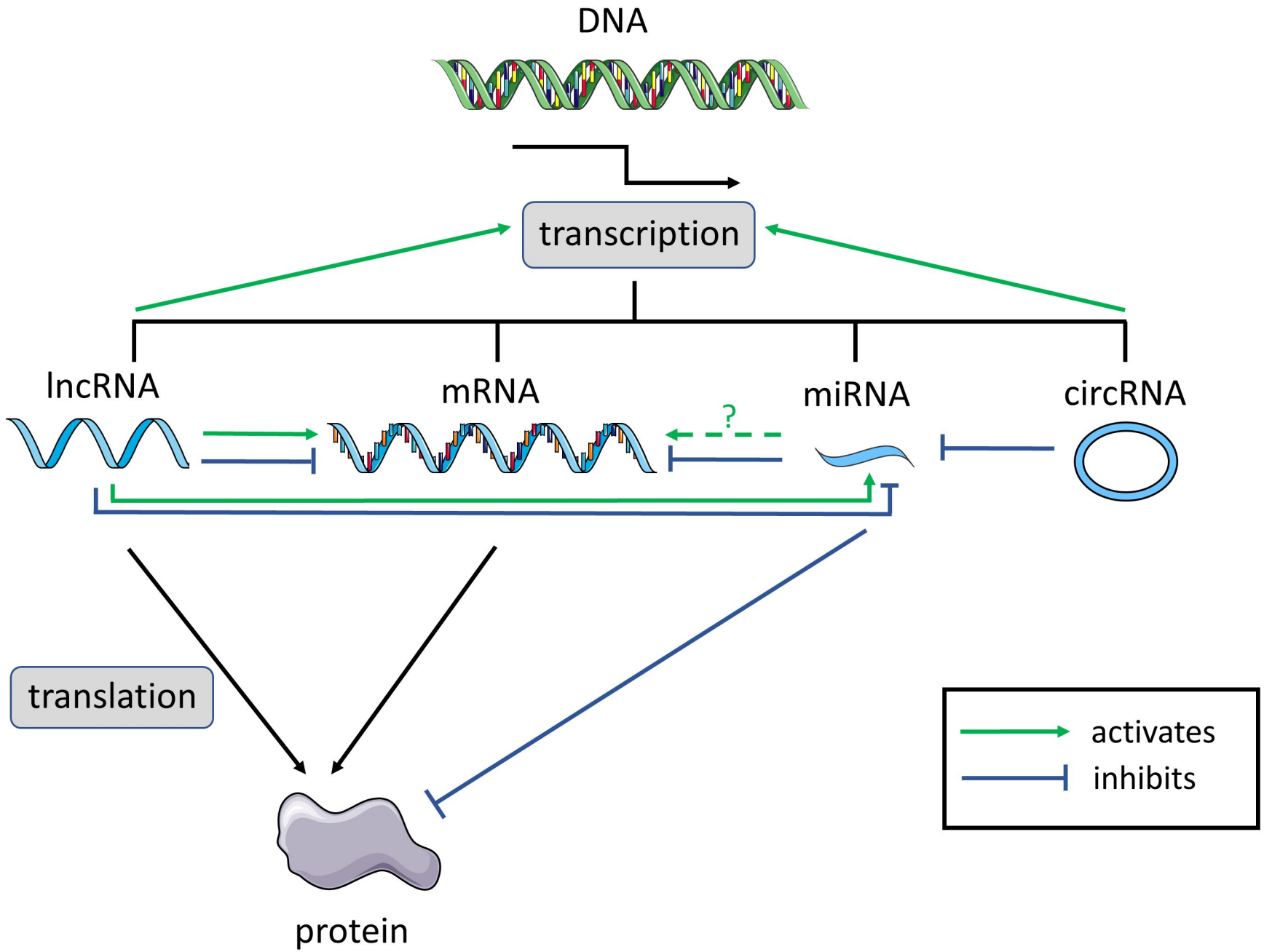

DNA
transcription
lncRNA
mRNA
?
miRNA
circRNA
translation
protein
activates
inhibits

Figure 2

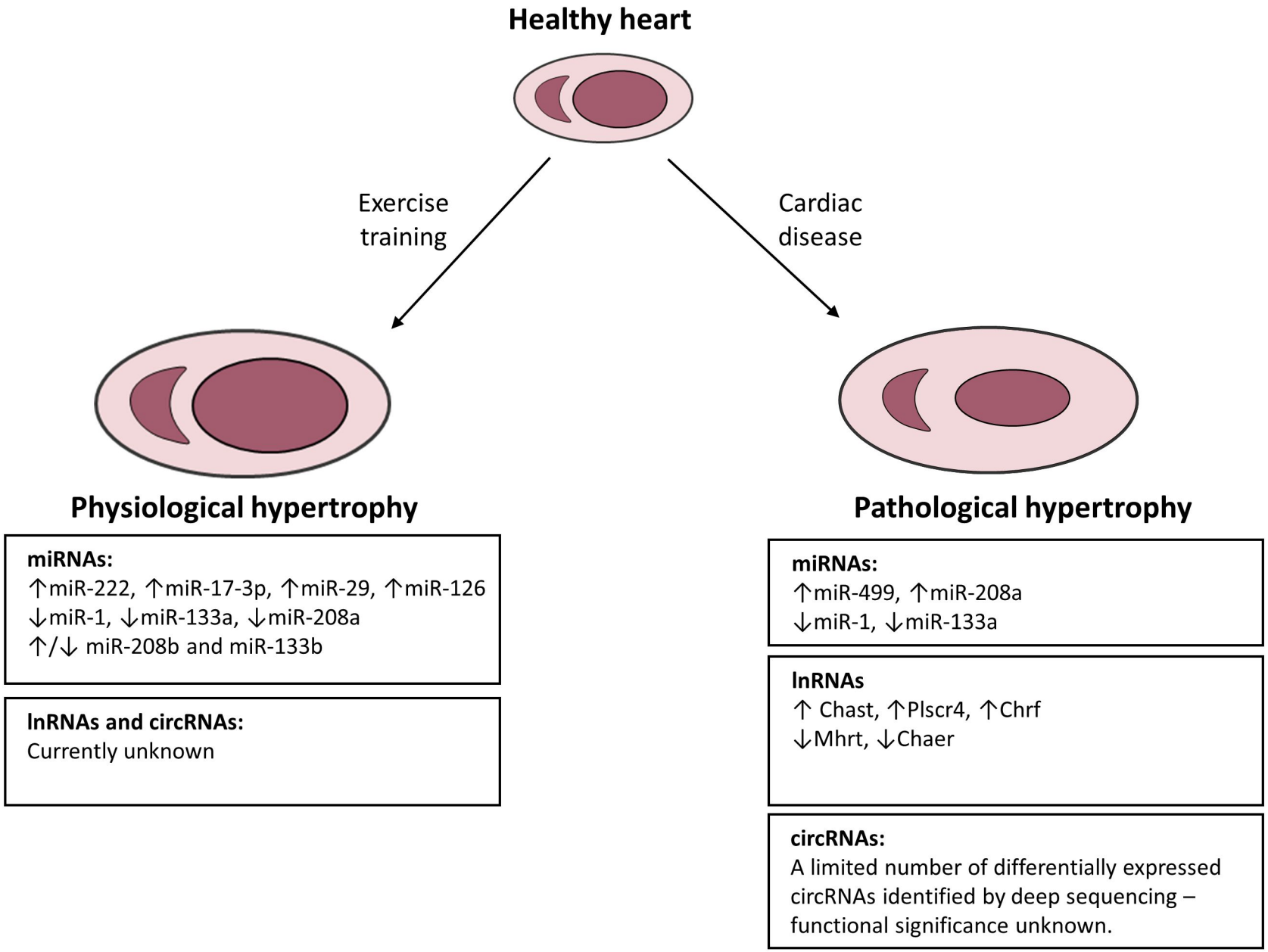
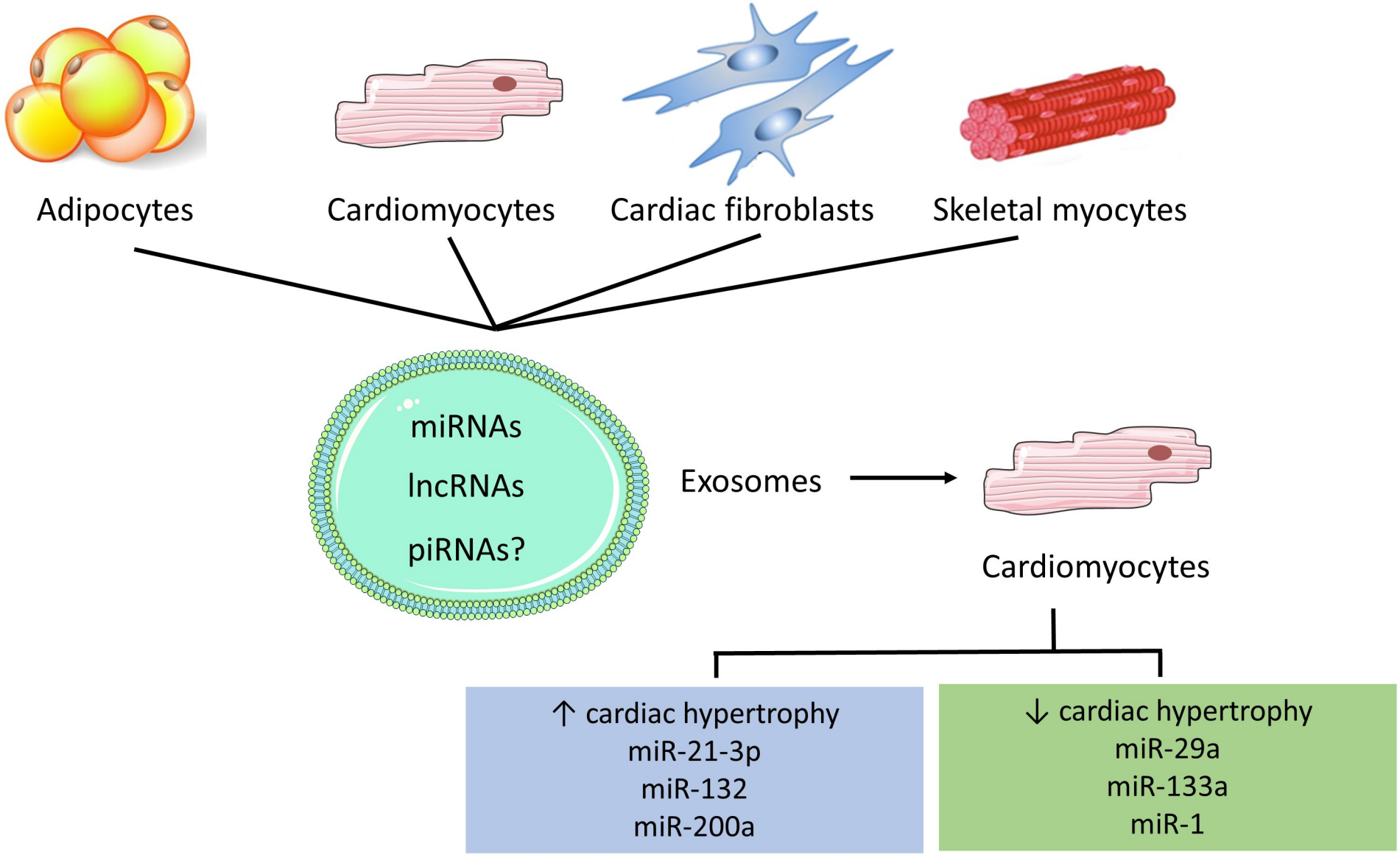

Healthy heart
Exercise training
Cardiac disease
Physiological hypertrophy
miRNAs:
↑miR-222, ↑miR-17-3p, ↑miR-29, ↑miR-126
↓miR-1, ↓miR-133a, ↓miR-208a
↑/↓ miR-208b and miR-133b
lnRNAs and circRNAs:
Currently unknown
Pathological hypertrophy
miRNAs:
↑miR-499, ↑miR-208a
↓miR-1, ↓miR-133a
lnRNAs
↑ Chast, ↑Plscr4, ↑Chrf
↓Mhrt, ↓Chaer
circRNAs:
A limited number of differentially expressed circRNAs identified by deep sequencing – functional significance unknown.

Figure 3


Adipocytes
Cardiomyocytes
Cardiac fibroblasts
Skeletal myocytes
miRNAs
lncRNAs
piRNAs?
Exosomes
Cardiomyocytes
↑ cardiac hypertrophy
miR-21-3p
miR-132
miR-200a
↓ cardiac hypertrophy
miR-29a
miR-133a
miR-1