MicroRNA therapeutics in cardiovascular medicine

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Cardiovascular diseases are the most common causes of human morbidity and mortality despite significant therapeutic improvements by surgical, interventional and pharmacological approaches in the last decade. MicroRNAs (miRNAs) are important and powerful mediators in a wide range of diseases and thus emerged as interesting new drug targets. An array of animal and even human miRNA-based therapeutic studies has been performed, which validate miRNAs as being successfully targetable to treat a wide range of diseases. Here, the current knowledge about miRNAs therapeutics in cardiovascular diseases on their way to clinical use are reviewed and discussed.

Introduction

Diseases of the cardiovascular system are the most common cause of human morbidity and mortality in the industrialized nations (European World Health Organisation, WHO/Europe). The costs of cardiovascular diseases to the European health care system are estimated to be as high as 200 billion Euros per year (according to www.cordis.europa.eu). Although classical pharmacologic treatment strategies have improved cardiovascular outcome and survival of heart failure patients [*e.g.* using beta-blockers and ACE (angiotensin (Ang)-converting enzyme) inhibitors], the prognosis of affected individuals remains poor with about 2 million cardiovascular deaths in the European Union per year. It is estimated that up to 50 million people are currently suffering from heart failure in the European Union.

This highlights the need for further understanding of underlying mechanisms and development of innovative effective therapies for heart diseases. A variety of cardiovascular diseases, such as coronary artery disease, hypertension, myocardial infarction, valvular heart diseases, myocarditis and genetic forms of cardiomyopathies often result in a phenotypically similar endpoint, which is heart failure (Hill & Olson, 2008). Exposure of the heart to different stressors leads to cardiac remodelling with finally detrimental outcomes (Fig 1). At the cellular level, there is fibroblast activation and proliferation with subsequently increased growth factor secre-

tion and extracellular matrix (ECM) production leading to fibrosis and further decline in cardiac function (Hill & Olson, 2008). Impaired vascularization and low capillary density as well as inflammatory processes further contribute to the development of heart failure (Fiedler et al, 2011; Heymans et al, 2009). Indeed, alterations of non-cardiomyocytes such as cardiac fibroblasts and endothelial cells strongly impact on cardiomyocyte and thus on general cardiac function.

Recent studies have uncovered important and unexpected roles for a family of small regulatory RNA molecules, known as microRNAs (miR; miRNAs) in the regulation of diverse aspects of cardiac function (Bonauer et al, 2009; Care et al, 2007; da Costa Martins et al, 2010; Fiedler et al, 2011; Thum et al, 2008b; van Rooij et al, 2007). MiRNAs are non-protein-coding, small RNAs of 20–23 nucleotides (nt) that exist in virtually all organisms and are highly evolutionary conserved (Ambros, 2001) suggesting a superior role in essential biological processes. Initially, primary miRNAs (pri-miRNA) are generated in the cellular nucleus by the transcription machinery and are then processed by the RNase-III-type enzyme Drosha to form so-called precursor miRNAs (pre-miRNAs; Lee et al, 2003; Thum et al, 2008a). Following exportation into the cytoplasm, miRNAs are processed by the ribonuclease Dicer into small 20–23 nt long miRNA duplexes. Finally, miRNAs are incorporated into RNA-induced silencing complexes (RISC) to silence gene expression at the post-transcriptional level by targeting messenger RNAs

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(mRNAs) with the result of mRNA degradation or by translational inhibition finally leading to target protein repression. Details about the biogenesis and regulation of cardiovascular miRNAs have recently been reviewed (Bauersachs & Thum, 2011). The miRNA expression patterns change in various cardiovascular diseases, such as myocardial infarction, cardiac hypertrophy and heart failure (Bonauer et al, 2009; Care et al, 2007; da Costa Martins et al, 2010; Fiedler et al, 2011; Thum et al, 2007, 2008b; van Rooij et al, 2007, 2008).

Surprisingly, also circulating extracellular miRNAs are present in body fluids of cardiovascular-diseased patients (Fichtlscherer et al, 2010; Gupta et al, 2010; Widera et al, 2011; Zampetaki et al, 2010). Despite the existence of ribonucleases, miRNAs remain stable in serum and other body fluids due to loading of the miRNAs into proteins, lipids or lipoprotein complexes such as exosomes or microvesicles. Thus, they may be used as biomarkers but may also function as mediators of disease (Gupta et al, 2010; Hunter et al, 2008; Valadi et al, 2007). As miRNAs target not only single mRNAs, but complete networks of often functionally related transcripts, they emerged as interesting novel candidates for the development of miRNA-based therapeutic strategies in cardiovascular disease. In the following, the current knowledge about the use of miRNA modulators as cardiovascular therapeutics is reviewed and discussed.

Historical perspective and chemical structures of miRNA modulators

To inhibit miRNAs *in vivo*, synthetic complementary oligonucleotides of 8–25 nt of length against the seed sequence and adjacent nucleotides of the miRNA of interest have been synthesized. Normally, nucleic acids are quickly degraded in biological compartments; thus various chemical modifications have been tested to stabilize their structure and to increase binding efficacy and cellular uptake. Chemical modifications mainly include 2-*O*-methyl-modified oligonucleotides and locked nucleic acid (LNA)-modified oligonucleotides, where the 2-oxygen is connected to the 4-position by a methylene linker to result in a tight bicycle and is locked into a C3-endo (RNA) sugar conformation (Elmen et al, 2008a; Krutzfeldt et al, 2007, 2005). In addition, the balance between phosphodiester- and phosphorothioate linkages between the nucleotides is important for the oligonucleotide stability as phosphorothioates show improved stability (Faria & Ulrich, 2008).

As shown in Table 1, the 2-*O*-methyl modification has been used very often to achieve high oligonucleotide stability and efficacy. A cholesterol construct has been linked to improve cellular uptake (Krutzfeldt et al, 2005; Thum et al, 2008b) and this approach has successfully been used to target cardiac tissue *in vivo* (Table 1). Recently, an excellent historical description of miRNA therapeutic development was provided (van Rooij, 2011). The group of Stoffel was the first to report mammalian miRNA knockdown using cholesterol-conjugated antagomirs to inhibit a liver-specific miRNA, miR-122 (Krutzfeldt et al, 2005). In addition, this group investigated the knockdown efficacy of many other antagomirs and showed for the first time that the cholesterol-based chemistry was also able to knockdown miRNA expression in cardiac tissue after intravenous injection (Krutzfeldt et al, 2005). After this landmark study, Care et al employed a cholesterol-based antagomir against miR-133, which resulted in cardiac hypertrophy of mice (Care et al,

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Glossary

AAVs
Adeno-associated viruses, whose various serotypes have some cell-type tropism. Thus, AAVs may be used to improve cell-type enriched delivery of miRNAs.

Angiogenesis
Development of blood vessels in the embryo or in an adult tissue.

Antagomirs/antimirs
Different names for chemistries used for miRNA inhibitors that lead to miRNA silencing *in vitro* or *in vivo*.

Arrhythmogenesis
Development of arrhythmia, an irregular heartbeat or abnormal rhythm.

Atherosclerosis
A condition in which the artery wall thickens due to accumulation of fatty material, which can restrict blood flow.

Cardiac hypertrophy
Thickening of the heart muscle, which results in increased heart size and decreased chamber size.

Cardiomyocyte
Muscle cell of the heart.

Cardiovascular disease
A class of diseases that involves the heart and/or blood vessels.

Fibrosis
Development of excessive fibrous connective tissue in an organ.

microRNAs
Small RNA molecules, that regulate a large fraction of the genome by binding to complementary mRNA sequences leading to post-transcriptional gene silencing.

miRNA therapeutics
A new form of drugs leading to miRNA silencing (see antagomirs/antimirs description) or miRNA enrichment in individual cell types to counteract derailed gene expression and impaired function in cardiovascular and other diseases.

Morpholino
25-mer antisense oligonucleotide with modified bases containing morpholine ring, making them very stable.

Myocardial infarction
Commonly known as heart attack. Irreversible necrosis of the heart muscle caused by prolonged ischemia.

Neointima
New layer of arterial intima (inner lining of the artery) formed especially on a prosthesis and in atherosclerosis.

Vascular smooth muscle cell
The particular type of smooth muscle cell composing the majority of the wall of blood vessels.
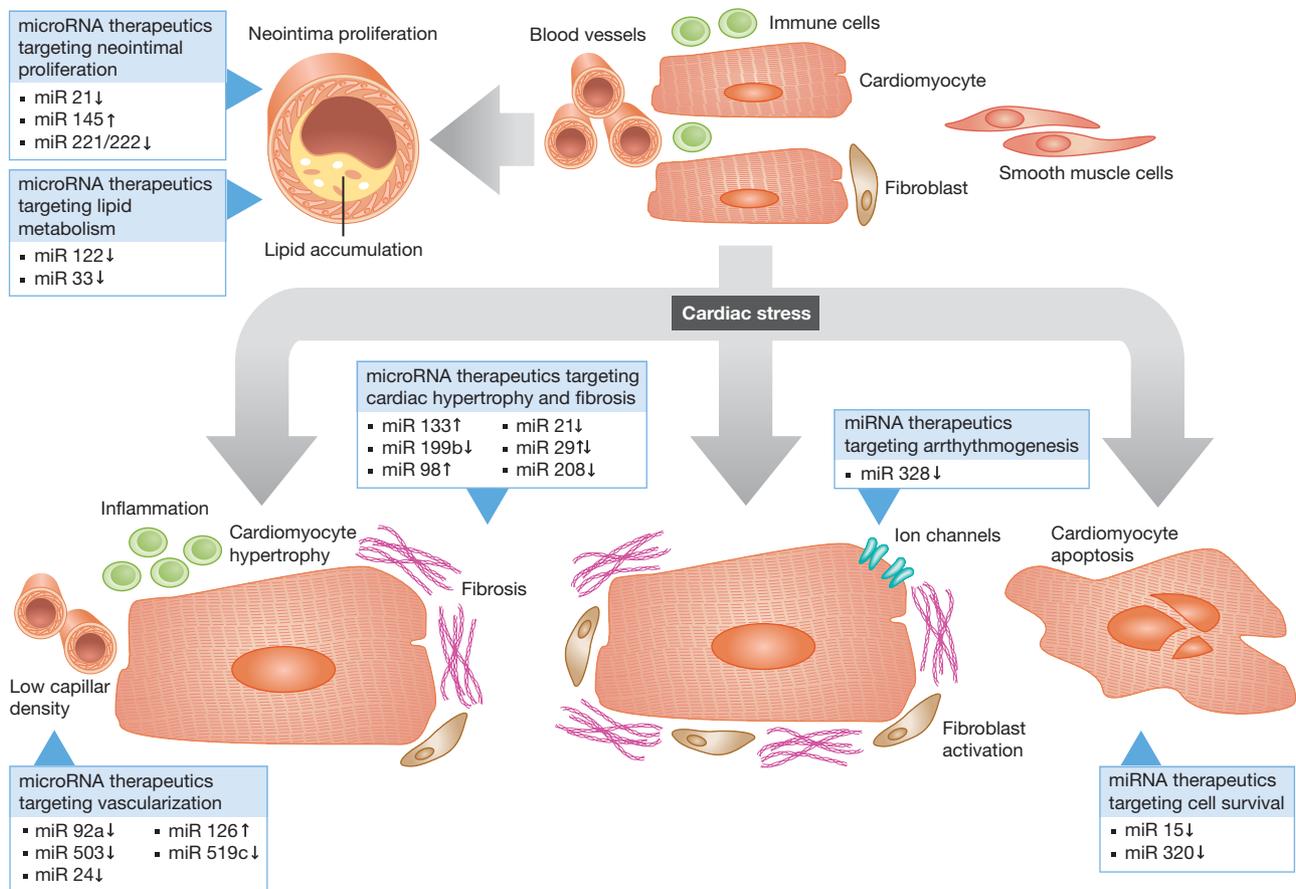
Figure 1. Identified miRNAs to be of therapeutic interest during cardiovascular disease.

2007). Another group showed the first successful therapeutic approach using an antagomir against fibroblast-enriched miR-21 to prevent cardiac fibrosis (Thum et al, 2008b). This was followed by many other studies successfully using miRNA inhibitors to beneficially effect cardiovascular function (Table 1).

Alternative modification strategies include use of LNA chemistries, which were shown to effectively knockdown target miRNA levels. LNA modification of oligonucleotides results in a thermodynamically very strong duplex formation with the complementary RNA (Grunweller & Hartmann, 2007) and thus is biologically highly effective as shown in many studies (Table 1). For instance, systemic delivery of unconjugated LNA-antimiR significantly silenced miR-122 both in mice and non-human primates (Elmen et al, 2008a, 2008b) and these antimiRs were successfully used in a disease model of hepatitis C in primates (Lanford et al, 2010). Importantly, use of this technique is currently under investigation in first phase I and II clinical trials (Santaris Pharma, www.ClinicalTrials.gov). Specifically, a clinical phase II trial treating patients with hepatitis C with an LNA-based specific inhibitor of miR-122 is ongoing and first results are expected soon.

Thus, both antagomir and LNA-modified oligonucleotides can effectively target miRNAs *in vivo*, although LNA-modified

chemistries appear to require lower doses based on their high binding affinity. Recently, very short 8-mer fully modified LNA oligomers only directed against the seed region of a miRNA were introduced (Obad et al, 2011; Patrick et al, 2010). This approach might be helpful when targeting of multiple miRNA family members with the same seed sequence is desired. However, the pharmacokinetics of so-called tiny miRs appear to differ in various organ types; for instance, whereas, long-lasting high concentrations after application of tiny miR-21 were seen in organs such as liver and kidney, an extremely fast drop in tissue availability was seen in lung and heart (Obad et al, 2011) indicating that optimal targeting of different organs may need different chemistries. This may explain why treatment with 22 nt-long cholesterol- or (fluoro)*O*-methoxyethylphosphorothio-ate (F/MOE)-based antagomirs against miR-21 was successful to prevent pressure-overload-induced cardiac fibrosis in a mouse model (Thum et al, 2011, 2008b), whereas, application of LNA-based 8 nt-long antimiRs did not (Patrick et al, 2010).

Additional strategies to lower miRNA expression is to use miRNA erasers or sponges (Wang, 2011). The principle is to employ vector constructs that harbour multiple miRNA binding sites where the miRNA of interest can bind to and thus is not any more available to bind its targets.

Table 1. Cardiovascular therapeutic miRNA modulation *in vivo*

Field of studies	Reference	miRNA	miRNA modulator chemistry	Company	Dose	Timing	Species/ organ	Outcome
Early studies and hypertrophy and cardiac fibrosis								
	Krutzfeldt et al (2005)	let7, 22 16, 122 192.194	2′-O-methyl + 3′cholesterol	Done by authors	80 mg/kg body weight	Up to 22 days	Mice	Administration of antagomirs against miR-16, miR-122, miR-192 and miR-194 resulted in a marked reduction of corresponding miRNA levels. In mice treated with antagomir-16, miR-16 was efficiently silenced in all tissues tested (including heart) except brain
	Care et al (2007)	133	2′-O-methyl + 3′cholesterol	Dharmacon	80 mg/kg body weight continously via osmotic minipumps	1 month	Mice	*In vivo* inhibition of miR-133 caused cardiac hypertrophy
	da Costa Martins et al (2010)	199b	2′-O-methyl + 3′cholesterol	n.a.	80 mg kg body weight/ day, i.p.	Three daily injections	Mice, heart	*In vivo* inhibition of miR-199b normalized significantly attenuated cardiac functional impairment, fibrosis and NFAT activity; Dyrk1 is a miR199b target
	Yang et al (2011)	98	Adenoviral miR-98 or anti-miR-98 contructs	Done by authors	10^9 pfu, intracardial (LV) injection	14 days	Mice	MiR-98 overexpression reduced AngII-mediated fibrosis and amount of apoptotic myocytes
	Thum et al (2008b)	21	2′-O-methyl + 3′cholesterol	Regulus Therapeutics	80 mg/kg body weight, jugular vein	Three daily injections	Mice	Antagomir-21 decreased development of cardiac fibrosis and improved cardiac function
	Thum et al (2011)	21	2′-O-methyl + 3′cholesterol, F/MOE Modification, 8 mer LNAs	Regulus Therapeutics	10–80mg/kg body weight	Two injections	Mice	Differential effects of miRNA inhibitor chemistries on cardiac fibrosis and heart function
	Patrick et al (2010)	21	LNA-modified, unconjugated and fully phosphorothiolated oligonucleotides, 8-mer	Santaris Pharma	25 mg/kg	Three daily injections	Mice	No effect on cardiac remodeling and fibrosis
	van Rooij et al (2007)	29b	2′-O-methyl + 3′cholesterol, miR mimics	n.a.	80 mg/kg body weight	Two injections, tail-vein	Mice	MiR-29 downregulation increases fibrosis
	Boon et al (2011)	29	LNA-modified oligonucleutides, 16-mer	Exiqon	20 mg/kg	Intravenous injection	Mice	MiR-29 downregulation prevents aortic dilatation
Neointima formation								
	Cheng et al (2009)	145	Adenoviral miR-145 construct	Done by authors	5×10^9 pfu/ml	One local injection	Mice	Inhibition of neointima formation
	Cordes et al (2009)	145	Pre-miR-145 lentivirus	System Biosciences	1×10^7 infectious units	External gel application	Mice	Inhibition of neointima formation
	Ji et al (2007)	21	2′-O-methyl	Integrated DNA Technologies	30 µg/carotid artery	local Delivery at days 3 and 7	Mice	Inhibition of neointima formation
	Liu et al (2010)	221/222	2′-O-methyl	Integrated DNA Technologies	10 µg/carotid artery	Local delivery at do + gel	Mice	Inhibition of neointima formation

(Continued)

Table 1. (Continued)

Field of studies	Reference	miRNA	miRNA modulator chemistry	Company	Dose	Timing	Species/ organ	Outcome
Lipid metabolism								
	Krutzfeldt et al (2005)	122	2′-O-methyl + 3′cholesterol	Done by authors	80 mg/kg body weight	Three daily injections	Mice	Cholesterol reduction
	Marquart et al (2010)	33	MiR-33 inhibitor (no chemistry mentioned)	miRagen Therapeutics	5 mg/kg/day	Three consecutive days	Mice	Increase in plasma HDL levels
	Najafi-Shoushtari et al (2010)	33a	LNA-modified oligonucleutides	n.a.	20 mg/kg/day i.v.	Three consecutive days	Mice	Increase in plasma HDL levels
	Rayner et al (2010)	33	Lentiviral anti-miR-33 construct	System Biosciences	2×10^9 pfu/mouse retroorbitally	Single injection	Mice	Increase in plasma HDL levels
	Rayner et al (2011)	33a/b	2′-F/MOE modification	Regulus Therapeutics	5 mg/kg s.c.	Twice weekly for 2 weeks, then weekly	Monkeys	Increase in plasma HDL levels Decrease in VLDL triglycerides
Endothelial biology								
	Bonauer et al (2009)	92a	2′-O-methyl + 3′cholesterol	VBC Biotech	8 mg/kg body weight (5 injections)	14 days	Mice	Inhibition of miR-92a led to enhanced blood vessel growth and functional recovery of damaged tissue
	Caporali et al (2011)	503	Adenoviral decoy-miR-503	Invitrogen	10^9 pfu, one muscular injection	3–21 days	Mice	Inhibition of miR-503 normalizes post-ischemic flow
	van Solingen et al (2009)	126	2′-O-methyl + 3′cholesterol	Dharmacon	1.0 mg/ mouse i.v.	10 days	Mice	MiR-126 inhibition reduced angiogenic response
	McArthur et al (2011)	200b	n.a.	Dharmacon	1.4 µg/week	4 weeks	Rat retina	Increased VEGF production
	Fiedler et al (2011)	24	2′-O-methyl + 3′cholesterol	Regulus Therapeutics	80 mg/kg body weight, retroorbital injection	14 days	Mice	Endothelial miR-24 blockade improves capillary density, limits infarct size and improves heart function
Cell survival								
	Porrello et al (2011)	15b,16	Locked nucleic acid-modified anti-miRNAs	miRagen Therapeutics	25 mg/kg body weight	Three injections	Mice	Knockdown of miR-15 family was associated with an increased number of mitotic cardiomyocytes
	Ren et al (2009)	320	2′-O-methyl + 3′cholesterol	Dharmacon	80 mg/kg body weight	Single tail vein injection, 3 days	Mice	*In vivo* treatment with antagomir-320 reduced infarction size and increased Hsp20 in the heart
Cardiac rhythm								
	Lu et al (2010)	328	Adenoviral miR-328 precursor; antagomir: 2′-O-methyl + 3′cholesterol	Over-expression GenScript and by authors Antagomir: Ribobio Co.	Overexpression: 10^9 pfu, multiple Intraatrial injections; antagomir 80 mg/kg/d i.v. for 3 days	Dogs: 12 h Mice: 14 days	Dogs (over-expression) Mice (antagomir)	Antagomir-328 abrogated or stopped atrial fibrillation and improved cardiac function post-myocardial infarction

n.a., not available.

In addition to silencing of miRNAs, their upregulation and/or enhancement may be of therapeutic interest. However, the approaches to optimize miR mimics in terms of stabilization and efficacy of miRNA overexpression have been rather disappointing so far. A miR-mimic consists of a double-stranded oligonucleotide including the mature miRNA sequence and the complementary passenger strand. In a first attempt, van Rooij and colleagues locally injected miR-29 mimics into the heart after myocardial infarction and observed modest miR upregulation by this approach (van Rooij et al, 2008). Here, clearly improved chemical stabilization and/or conjugation to delivery molecules is needed to result in superior biological effects. A further strategy to augment miRNA levels is to use miRNA delivery through adeno-associated viruses (AAVs; Hinkel et al, 2011). Certain AAV serotypes have the unique feature of cell-type favouring tropism without leading to

significant inflammatory responses (Wang et al, 2011). An example is AAV9, which is enriched in the heart after administration (Bish et al, 2008). Thus, upon viral delivery via specific AAV serotypes selected miRNAs can be enriched in target tissues, which may be important for such miRNAs that are downregulated during cardiovascular disease. A cell-type specific homing of such miRNA delivery tools may be achieved by use of tissue-specific promoters for expression.

Delivery of miRNA therapeutics, pharmacokinetics and costs

Most oligonucleotide chemistries are soluble in water and/or saline and have been injected into animals by various approaches and application routes (see Table 1). Intravenous delivery including the tail vein, jugular vein or the retro-orbital vein plexus has been the most commonly used route of administration. However, recent studies showed that also either intraperitoneal injections of cholesterol-based antagomirs (da Costa Martins et al, 2010) or LNA-based chemistries can be used to silence miRNAs within the heart (Table 1). A recent study compared intravenous, intraperitoneal or subcutaneous delivery routes and here 25 mg/kg antimiR-208a were injected (Montgomery et al, 2011). All three administration routes of showed robust inhibition of miR-208a at days 1, 4, 7 and 14 with no significant differences in antimiR-208a detection between the different delivery methods for plasma, heart, liver or kidney. Less experiences to target, for instance, the aorta are available but it seems that higher concentrations are needed (Boon et al, 2011). Another study compared the effects of different doses of cholesterol-based antagomirs on cell-type specific uptake and silencing of miR-24 (Fiedler et al, 2011). In this study, dosing with 80 mg/kg of an antagomir against miR-24 led to the silencing of miR-24 expression in both cardiomyocytes and cardiac endothelial cells, whereas, lower dosing with 5 mg/kg reduced miR-24 expression selectively in endothelial cells but not cardiomyocytes based on Cy3-labelling and cell fractionation studies (Fiedler et al, 2011). As endothelial cells that form cardiac capillaries are the first cells where antagomirs are delivered via the blood stream, low doses may be preferably used to target this cell compartment, whereas, higher concentrations subsequently affect capillary surrounding cells. An alternative explanation is altered cholesterol- and/or oligonucleotide uptake mechanisms within different cardiovascular cells, but scientific insight into this issue is limited so far.

Dosing is obviously dependent on the used chemistry and the abundance of the miRNA in the target organ. Thus, the optimal concentrations need to be determined individually for various miRNAs. Cell-type specific delivery may also be achievable in the future by linking the miRNA inhibitors and/or miR-mimics to cell-type specific antibodies, peptides, lipids or other cell-type-specific pro-trophic molecules.

Information about tissue distribution and pharmacokinetics of miRNA modulators are presently scarce. As mentioned above, Obad et al compared tissue distribution and effects of various doses of 8 nt-long LNA-based antimiR-21 (Obad et al,

2011). Another study showed significant differences in cell-type specific silencing based on the used dose of cholesterol-based antagomirs, with endothelial cells more specifically targeted by lower doses (Fiedler et al, 2011). Using an LNA-based antagonist of miR-208a, van Rooij's group recently showed progressive reduction in rat cardiac miR-208a levels using concentrations ranging from 0.0625 to 4 mg/kg body weight with only minor further miR reductions at higher doses (Montgomery et al, 2011). Thus, there are (probably chemistry-depended) dose/effect-relationships that need to be addressed with high accuracy in future studies in the field, as currently the range of used doses of miRNA modulators is large and only little direct comparisons have been made (see Table 1).

Initial studies employing miRNA modulating substances such as antimiRs or antagomirs were only possible by interaction of university-based laboratories with pharmaceutical industry and miRNA start-up companies and in 2005/2006, the costs per injection per mouse were in the several thousand Euros (personal experience). Today, many groups still have cooperations with miRNA companies and thus receive miRNA inhibitors for free pending on material transfer agreements and intellectual property discussions. However, it is nowadays also possible to purchase LNA-based antimiRs, cholesterol-based antagomirs or others (although the used detailed chemistry from such companies may differ from that of big miRNA therapeutic companies) and costs differ between 100 and 600 Euros per injection per mouse pending on the used dose (personal experience). Thus, as frequently observed for successful compounds in pharmaceutical development, the costs for miRNA-modulating chemistries have been reduced significantly in the past (which probably will continue) allowing more research group to enter this exciting field and paving the way for large animal and finally clinical studies where higher amounts of miRNA chemistries will be needed.

miRNA modulation by oligonucleotides in zebrafish

As described above, delivery of oligonucleotide chemistries to target miRNAs in organisms results in a better understanding of miRNA function and potential therapeutic effects. The zebrafish model has been used in the past to test effects of various miRNA modulators (Table 2). Modulation of miRNAs in zebrafish either by downregulation using morpholinos or by upregulation through injection of specific pre-miRNAs is an elegant approach to identify miRNAs with importance for the cardiovascular system.

The injection of miR-218 morpholinos resulted in cardiac oedema at 48 h post-fertilization, although gross vascular patterning appeared normal (Fish et al, 2011). The observation of a dysmorphic heart and pericardial oedema as well as the absence of severe vascular defects suggested that miR-218 might be directly required for cardiogenesis. Indeed, the authors showed that miR-218 and multiple Slit/Robo signalling components are required for heart tube formation in zebrafish and that this network modulates the previously unappreciated function of Vegf signalling in this process.

Overexpression of miR-92a leads to disturbed vessel growth and patterning in zebrafish (Bonauer et al, 2009). Quantification

Table 2. microRNA modulation in zebrafish leading to cardiovascular phenotypes

Field of studies	Reference	miRNA	miRNA modulator chemistry	Company	Dose	Time	Species	Results
Zebrafish								
	Bonauer et al (2009)	92a	miR mimics	Ambion	2 nl of 20 μM stock solution	30–48 hpf	Zebrafish	Overexpression of miR-92a induced defects in vessel formation
	Lagendijk et al (2011)	23	Morpholinos, miR mimics	Gene Tools	1 nl of 0.5 mmol/L; 200 ng/μl	2 days 2 days	Zebrafish	Loss of miR-23 results in endocardial defects
	Fish et al (2011)	218	Morpholinos	Gene Tools/ Open Biosystems	morpholino 8–12 ng	2 days	Zebrafish	Loss of miR-218 results in cardiovascular defects and impaired circulation
	Fiedler et al (2011)	24	miR mimics	Ambion	2 nl of 25 μM stock solution	2 days	Zebrafish	miR-24 overexpression leads to blood accumulation and abnormal intersegmental vessel formation

of the number of intersegmental vessels, which are properly connected to the aorta or cardinal vein on the ventral side and to the dorsal longitudinal anastomotic vessel on the dorsal side, revealed a significant reduction in vessel growth after miR-92a overexpression.

A significant contribution to our understanding of miRNAs involved in valve formation using the zebrafish as a model organism was made recently. Dicer null mutants showed excessive endocardial cushion formation accompanied by a strong increase in cardiac jelly (Lagendijk et al, 2011). This was mimicked by silencing miR-23 using morpholino injection. *In vitro* miR-23 upregulation prevented TGF-beta induced endothelial–mesenchymal transition. These studies suggest a dominant role of miR-23 during valve formation (see Table 2). Whether this mechanism is involved in other animal models and humans and may help to develop therapeutic strategies to target valve diseases such as development of aortic valve stenosis remains to be determined.

miR-24 is also enriched in cardiac endothelial cells and its overexpression results in endothelial apoptosis and impaired angiogenesis (Fiedler et al, 2011). Overexpression of miR-24 in zebrafish embryos resulted in pericardial oedema as well as blood accumulation. Studies in transgenic zebrafish that overexpress GFP in the vasculature showed that miR-24 overexpression impaired intersegmental vessel formation and resulted in impaired blood transportation (Fiedler et al, 2011). Morpholino-mediated silencing of the miR-24 targets gata2 and pak4 in zebrafish mimicked the effects of miR-24 overexpression showing an involvement and potential therapeutic importance of those targets in angiogenesis (Fiedler et al, 2011).

miRNA modulation by oligonucleotides in mice and large animals

Modulation of cardiovascular miRNA expression was achieved by many different approaches (see Fig 1 and Table 1). Most of the studies used pharmacological interventions such as antisense oligonucleotide-mediated knockdown or miR-mimic based overexpression techniques. Although mechanistic details are not clear, many different miRNA therapeutics can be taken up by cardiovascular cells including cardiomyocytes, fibroblasts, endothelial cells, smooth muscle cells and inflammatory cells. Upon cellular uptake such miRNA inhibitors bind to their target miRNAs resulting in a functional deactivation, which also can be seen by upregulation of the respective miRNA targets. In the following, miRNA therapeutics targeting different features of cardiovascular disease are summarized.

miRNA therapeutics targeting cardiac hypertrophy and fibrosis

One of the first studies that used an antagomir to inhibit a miRNA involved in cardiac hypertrophy was performed by Care and coworkers (Care et al, 2007). This group implanted mice subcutaneously with osmotic minipumps for a continuous delivery of a cholesterol-based antagomir targeting miR-133. After 1 month of infusion, echocardiographic analysis showed a marked increase in cardiac hypertrophy suggesting that miR-133 mimics may be of therapeutic relevance.

Another group showed involvement of miRNAs in cardiac hypertrophy; miR-199b is a direct calcineurin/nuclear factor of activated T cells (NFAT) target that increases in expression during mouse and human heart failure thus being a potential interesting target for therapies (da Costa Martins et al, 2010). Indeed, *in vivo* inhibition of miR-199b by a specific intraperitoneally administered antagomir normalized reduced nuclear NFAT activity and caused inhibition and even reversal of cardiac hypertrophy and fibrosis in mouse models of heart failure.

Thioredoxin, which regulates in part cardiomyocyte hypertrophy, leads to an increase in expression of the let-7 family including miR-98 (Yang et al, 2011). To evaluate the function of miR-98 in the heart *in vivo*, adenoviral miR-98 or antimiR-98 was injected into mouse hearts. Ang II-induced increases in hypertrophy, cardiomyoctye apoptosis and fibrosis were attenuated by Ad-miR-98 and significantly enhanced by Ad-antimiR-98. MiR-98 negatively regulates Ang II-induced cardiac

hypertrophy and the accompanying histopathologic changes *in vivo* and thus is an attractive target for the treatment of cardiac remodelling.

Cardiac fibrosis development is often linked to cardiac stress including pathological cardiomyocyte hypertrophy. Cardiac fibroblasts are enriched with certain miRNAs, such as miR-21 (Liu et al, 2010; Roy et al, 2009; Thum et al, 2011, 2008b), which regulates the ERK–MAP kinase signalling pathway via targeting sprouty-1. By this mechanism, fibroblast survival and growth factor secretion, apparently regulating the extent of interstitial fibrosis and cardiac hypertrophy, is controlled. *In vivo* silencing of miR-21 by a specific cholesterol-based antagomir in a mouse pressure-overload-induced disease model reduced cardiac ERK–MAP kinase activity, inhibited interstitial fibrosis and attenuated cardiac dysfunction. In addition, recently it has been shown that miR-21 antagonism blocks endothelial–mesenchymal transition in TGF-beta-treated endothelial cells as well as *in vivo* in a cardiac hypertrophy model resulting in reduced fibrosis development (Kumarswamy et al, 2011). Strikingly, the effects of miR-21 inhibition on fibrosis prevention were validated in other organs often affected by pathological fibrosis during diseases such as the lung (Liu et al, 2010) and the kidney (Zhong et al, 2011). Thus, miR-21 inhibition may represent a general approach to inhibit exaggerated organ fibrosis.

Another miRNA implicated in fibrosis development is miR-29. Van Rooij et al found that members of the miR-29 family are downregulated in the region of the heart adjacent to the infarct. Interestingly, the miR-29 family targets mRNAs that encode proteins involved in fibrosis, such as multiple collagens, fibrillins and elastin. Indeed, overexpression of miR-29 in fibroblasts reduced collagen expression (van Rooij et al, 2008). In contrast to a downregulation, during aging, an upregulation of miR-29 was seen in the aorta of mice and the increased expression of miR-29 family members was associated with a profound downregulation of numerous ECM components in aortas of aged mice and during aneurysm formation (Boon et al, 2011). Of clinical relevance, miR-29b levels were also profoundly increased in human thoracic aneurysms (Boon et al, 2011). The same group showed that LNA-modified antisense oligonucleotide-mediated silencing of miR-29 led to induction of ECM expression and inhibition of Ang II-induced dilation of the aorta in mice. This could be a therapeutic entry point for aneurysm treatment and/or stabilization also for clinical applications.

miRNA therapeutics targeting angiogenesis and vascularization

The Dimmeler group was the first to employ miRNA inhibitors to increase neovascularization after hindlimb ischemia and myocardial infarction (Bonauer et al, 2009). Indeed, hypoxia and tissue ischemia lead to upregulation of several miR-17-92 cluster members including miR-92a although the underlying detailed regulatory mechanisms are not entirely clear (Bonauer et al, 2009). Mice systemically injected with a cholesterol-based antagomir-92a exhibited a significant reduction in toe necrosis in comparison with mice treated with a control antagomir. Recovery of blood flow was increased, as was the number of

capillaries and smooth muscle actin-positive arterioles after antagomir-92a treatment. To test the effects after myocardial infarction, antagomir-92a or control was intravenously injected at days 0, 2, 4, 7 and 9 after occlusion of the left coronary artery. Antagomir-92a treatment improved left ventricle systolic and diastolic function, reduced the infarct size, suppressed the number of apoptotic cells and augmented the number of *in vivo* perfused lectin-positive vessels, particularly in the infarct border zone. Whether the positive effects were mediated only by inhibition of miR-92a in endothelial cells remains to be determined, as it may be that there are additional, direct effects on cardiomyocytes or smooth muscle cells.

Another group found miR-503 expression to be enriched in endothelial cells isolated from ischemic hind limbs especially in a diabetic setting (Caporali et al, 2011). They tested whether local miR-503 inhibition would improve post-ischemic reparative neovascularization and blood flow recovery in diabetic mice using a decoy strategy to lower miR-503 availability in the muscle during ischemia. Indeed, post-ischemic foot blood flow recovery was impaired in diabetic mice and local injections of Ad decoy-miR-503 completely normalized blood flow recovery in diabetic mice. In addition, this therapy increased capillary and arteriolar densities in ischemic muscles of diabetic mice. Thus, manipulation of miR-503 may represent a novel molecular approach to foster reparative angiogenesis in diabetic patients.

MiR-126 is an endothelial cell-enriched abundant miRNA and miR-126 knockout mice die early due to impaired angiogenesis (Wang et al, 2008). To study the consequences of miR-126 silencing on vascular regeneration, van Solingen and colleagues injected mice with a single dose of antagomir-126 and exposed them to ischemia of the left hindlimb (van Solingen et al, 2009). Quantification of the capillary density in the ischemic hindlimb of mice after miR-126 knockdown revealed that they showed a markedly reduced angiogenic response suggesting that miR-126 mimics may be an appropriate strategy to increase vascularization in ischemic tissues. However, this awaits further experimentation and validation.

Angiogenic events are partly controlled by the hypoxia-inducible factor-1α (HIF-1α), which is regulated by miR-519c through direct binding to the HIF-1α 3′ untranslated region (Cha et al, 2010). Antagomir-mediated inhibition of miR-519c increased HIF-1α protein levels and enhanced angiogenic activity in tumors. This is interesting as blockade of miR-519c may represent an alternative strategy to stimulate angiogenesis in ischemic tissues such as after myocardial infarction although this remains to be tested.

In contrast to ischemic tissues, there is a need to block exaggerated angiogenesis in diabetic retinopathy, which is a leading cause of blindness. In the retina of diabetic rats, downregulation of miR-200b occurs, whereas, levels of its target VEGF are increased (McArthur et al, 2011). Local intravitreal injection of miR-200b mimics prevented diabetes-induced increased VEGF levels and prevented glucose-induced increased permeability and angiogenesis. This may be a very interesting therapeutic approach in affected patients due to its local and easy administration.

miRNA therapeutics targeting cardiovascular cell survival

Mammalian cardiomyocytes are prevented from cell cycling during early post-natal development, which limits the capacity of the adult heart to regenerate after injury. During early post-natal cardiac development, miR-195, which is a member of the miR-15 family, is highly upregulated and drives the expression of a number of cell cycle genes (Porrello et al, 2011). Importantly, knockdown of the miR-15 family in neonatal mice with LNA-modified antimiRNAs was associated with an increased number of mitotic cardiomyocytes and de-repression of the cell cycle protein Chek. Upregulation of the miR-15 family during the neonatal period may be an important regulatory mechanism governing cardiomyocyte cell cycle withdrawal and thus may be an interesting therapeutic entry point to re-stimulate cardiomyocyte division for regenerative purposes (Porrello et al, 2011).

Following ischemia/reperfusion (I/R) injury in mice, miR-320 expression is significantly decreased in the heart (Ren et al, 2009). *In vitro* overexpression of miR-320 enhanced cardiomyocyte apoptosis, whereas, its knockdown appeared cytoprotective. Indeed, *in vivo* treatment with cholesterol-modified antagomir-320 prevented cardiomyocyte apoptosis and reduced infarction size relative to the administration of mutant antagomir-320 and saline controls.

miRNA therapeutics targeting arrhythmogenesis

miRNAs may also regulate ion channels including the L-type calcium current and thus impact on action potential duration (Latronico & Condorelli, 2010; Thum et al, 2008a). miR-328 was upregulated in atria from dogs with induced atrial fibrillation and targets the L-type calcium channel (Lu et al, 2010). Strikingly, normalization of miR-328 levels with an antagomir reversed the conditions, and also a genetic knockdown of endogenous miR-328 dampened atrial fibrillation vulnerability (Lu et al, 2010). This suggests the potential of miR-328 as a target for atrial fibrillation treatment with normalization of miR-328 level for converting fibrillation to sinus rhythm and the prevention of arrhythmogenesis in a clinical setting.

miRNA therapeutics targeting atherosclerosis

Atherosclerosis is a pathological multifactorial process, which very often starts with endothelial dysfunction and at later stages results in the deposition of fatty acid compounds, cholesterol, calcium, waste products and other substances that progressively accumulate within arteries, finally leading to the formation of plaques. In addition, there is often activation of smooth muscle cells, which contribute to the pathologic arterial remodelling processes, for example during neointima formation of injured vessels. The general role of miRNAs in the process of atherosclerosis and inflammation has recently been reviewed (Weber et al, 2010). Accordingly, I only briefly summarize miRNA-based therapeutic strategies successfully affecting smooth muscle cell function, neointima formation and lipid metabolism here (Fig 1 and Table 1).

MiR-21 has been shown to activate and to induce a contractile phenotype in human vascular smooth muscle cells by affecting TGF-beta and bone morphogenic proteins (BMPs; Davis et al, 2008). Indeed, a miR-21 antisense strategy prevented restenosis by blocking smooth muscle cell proliferation in a balloon-injured rat carotid artery model (Ji et al, 2007). MiR-145 and miR-143 are smooth muscle cell-enriched miRNAs that regulate crucial functions in this cell type and miR-143/145-knockout mice show decreased number of contractile smooth muscle cells and increased cell proliferation (Boettger et al, 2009; Cordes et al, 2009; Elia et al, 2009). *In vivo*, virus-mediated upregulation of miR-143/145 in injured rat carotid arteries and other mouse models of vascular injury resulted in a pronounced reduction of neointima lesion formation (Cheng et al, 2009; Elia et al, 2009; Xin et al, 2009) showing the general potential of those miRNAs for many vascular diseases such as restenosis prevention (Table 1). Finally, miR-221 and miR-222 have been described to be activated in vascular smooth muscle cells from injured carotid arteries (Liu et al, 2009). The same authors showed that *in vivo* silencing of miR-221 and miR-222 inhibited smooth muscle cell proliferation and thus neointima thickening after vascular injury (see Fig 1 and Table 1).

A dysbalanced metabolic state facilitates atherosclerosis development and recently, miRNAs have been implicated in the regulation of lipid metabolism [reviewed in (Fernandez-Hernando et al, 2011)]. Here, I briefly summarize the recent miRNA-based therapeutic trials that have been performed to target lipid metabolism both in small and large animals. Until now, several different miRNAs have been studied for their properties to regulate lipid metabolism and these include miR-122, miR-370, miR-378/378*, miR-335, miR-125a-5p and miR-33 [reviewed in (Fernandez-Hernando et al, 2011)]. I focus on therapeutic trials using inhibitors against miR-122 and miR-33.

MiR-122 is enriched in the liver and is involved in regulation of cholesterol metabolism (Krutzfeldt et al, 2005) and hepatitis C infection (Lanford et al, 2010). Indeed, the group of Stoffel was the first showing that plasma cholesterol levels are reduced in antagomir-122-treated mice (Fig 1 and Table 1). MiR-122 modulation *in vitro* and *in vivo* let to alterations of many genes implicated in liver metabolism, fatty acid synthesis and oxidation (Elmen et al, 2008a; Esau et al, 2006; Krutzfeldt et al, 2005).

The other miRNA I would like to mention here because of its intriguing translational potential is miR-33. The intronic miR-33a is located in the human sterol regulatory element-binding protein 2 (SREBF-2), which controls several genes involved in cholesterol uptake and synthesis (Horie et al, 2010). An important miR-33 target gene is the transporter responsible for shifting cholesterol out of the cell, ABCA1 (Marquart et al, 2010; Najafi-Shoushtari et al, 2010). Of great therapeutic relevance was the investigation that inhibition of endogenous miR-33 *in vivo* increased ABCA1 protein expression resulting in increased cholesterol efflux to apoA1 (Marquart et al, 2010; Najafi-Shoushtari et al, 2010; Rayner et al, 2010). There are several other miR-33 targets involved in lipid metabolism, which have been reviewed recently (Fernandez-Hernando et al, 2011) and thus are not focus of this Review. MiR-33 additionally regulates high-density lipoprotein (HDL) levels in *in vivo*-based studies with viral and LNA-stabilized miRNA antagonists (Marquart et al, 2010; Najafi-Shoushtari et al, 2010; Rayner et al, 2010; Table 1). This is important as low HDL levels are a hallmark of patients with coronary artery disease. An exciting

translational study was now performed in non-human primates where therapeutic silencing of miR-33 by 2′F/MOE-modified antagomirs resulted in raised high-density lipoprotein (HDL) and reduced plasma very low-density lipoprotein (VLDL) triglyceride levels (Rayner et al, 2011). It will be interesting to see whether this promising approach will develop soon into first clinical phase I/II trials.

Future developments and concluding remarks

In the next years, we likely will see further breakthroughs in cardiovascular miRNA research. First, large animal data also in the cardiovascular field will probably appear as this is worldwide currently under investigation in several laboratories. This will not only result in more insight into their therapeutic potential in larger species but also lead to important information about pharmacokinetics of such drugs and safety data. Second, we will see first detailed phase I and II data using miRNA therapeutics in non-cardiovascular but also cardiovascular medicine. This may pave the way for large-scale mechanism orientated miRNA-based therapeutic trials in cardiovascular medicine.

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Conflict of interest statement: T. T. has filed and licensed patents concerning the use of miRNAs as cardiovascular diagnostics and therapeutics.

For more information

Clinical trials including miRNA therapeutic studies:
www.Clinicaltrials.com

microRNA data base:
www.mirbase.org

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